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Transcriptional Activity of the Paired-like Homeodomain Proteins CHX10 and VSX1*

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CHX10 and VSX1 are homeodomain (HD) proteins essential for normal retinal development. CHX10 is required first for retinal progenitor cell proliferation and later for bipolar cell differentiation, whereas VSX1 is important in the terminal differentiation of a subset of bipolar cells. Elucidating the transcriptional activity of CHX10 and VSX1 is required to understand how these factors control retinal development. We show that CHX10 and Vsx1 can function as transcriptional repressors. When tethered to a promoter by a heterologous LexA DNA-binding domain or its HD, CHX10 repressed multiple classes of activators in different immortalized cell lines. CHX10 blocked TATA-containing and TATA-less promoters, repressed at a distance, and inhibited a complex enhancer positioned upstream or downstream of the reporter gene, whereas retinoblastoma protein (RB) inhibited the downstream enhancer only. Interestingly, CHX10 mildly potentiated a subset of activators in chick neuronal cultures. Thus, CHX10 is both a versatile repressor and a context-specific weak activator. The CHX10 HD and CVC domains were sufficient for DNA binding and repression. VSX1 contains closely related homeo and CVC domains and, like CHX10, also repressed transcription. A VSX1 HD mutation, R166W, that impairs DNA binding and causes keratoconus in humans, hindered repressor function. Therefore, CHX10 and VSX1 may control retinal bipolar cell specification or differentiation by repressing genes required for the development of other cell types.

Chx10 and VS*X*1 are transcription factors that share a homeodomain (HD),[1] and a CVC motif, named after CHX10, VSX1, and ceh-10 (1–3). CHX10 is expressed early in development in dividing retinal progenitor cells (RPCs) and is also expressed in mature bipolar neurons and a subset of Müller glia (2, 4). Null mutations results in microphthalmia in humans (5) and mice (6), and antisense *Chx10* RNA injected into zebrafish embryos impairs retinal development (7). Recessive mutations identified in patients with microphthalmia that modified residues in helix III of the CHX10 HD (R200P and R200Q) ablated DNA binding *in vitro* (5). The ocular retardation (or^J) mouse carries a mutation that generates a premature stop codon in helix I of the HD and loss of Chx10 protein (6). On a 129Sv background, a homozygous or^J mutation causes retinal development to go awry as early as embryonic day 10 (E10). The first detectable problems are a reduction in RPC proliferation and decreased cell death in the optic vesicle, followed by failure to form the optic fissure where the optic nerve normally exits the eye (6). RPCs in the periphery are severely affected by the proliferation defect and fail to differentiate. RPCs in the central retina are less affected and generate a very thin laminated retina that contains all major cell types except for bipolar neurons (6). The more severe effects of Chx10 loss on peripheral RPC proliferation are linked to gradual transdifferentiation of the periphery into retinal pigment epithelium (4). Transdifferentiation is associated with loss of progenitor cell markers and/or genes implicated in promoting division (*e.g. FGF15*, *E2F1*, *cyclin D1*, and *cyclin E1*), and induction of retinal pigment epithelium factors (*e.g. MITF*) and cell cycle inhibitors (*e.g. GAS1* and $p57^{Kip2}$) (4). It is not known whether CHX10 directly regulates the expression of these genes.

When the or^J allele is bred onto a *Mus musculus castaneus* background retinal development is partially rescued (8). Deletion of the cell cycle inhibitor $p27^{Kip1}$ also partially rescues proliferation (9). The number of cells in newborn or^J retina is 19-fold less than wild type, but in mice lacking both *Chx10* and $p27^{Kip1}$ this difference is only 4-fold (9). Therefore, a mechanism linking CHX10 to cell cycle regulators controls the expansion of RPCs. Interestingly, bipolar neurons are absent in $Chx10^{-/-}$, $p27^{Kip1-/-}$ retinas, suggesting that CHX10 plays a role in the development of these cells independent of its role in controlling RPC proliferation (9). In mice carrying a reporter gene driven by Chx10 regulatory regions, expression was observed in a subset of retinal Müller glia as well as RPCs and bipolar cells (4). Therefore CHX10 may affect the development of a subset of Müller cells, in addition to bipolar cells (4).

Unlike CHX10, which is expressed in RPCs as early as E9.5 and is present in all mature bipolar cells (2), *VSX1* is not induced in the retina until postnatal day 5 and is restricted to the outer tier of the inner nuclear layer (10–14). Double labeling experiments showed that VSX1 is absent in PKCα-positive rod bipolar cells, but is present in recoverin-positive OFF cone bipolar cells (15). Analysis of knock-in mice in which the *VSX1* gene was replaced by *lacZ* confirmed this finding and showed that *VSX1* is also expressed in ON cone bipolar cells (16, 17). Inactivation of *VSX1* in the mouse retina leads to incomplete differentiation of OFF-cone bipolar cells (16, 17). In humans, *VSX1* mutations are linked to inherited corneal dystrophies,

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[1] The abbreviations used are: HD, homeodomain; RPC, retinal progenitor cell; CAT, chloramphenicol acetyltransferase; PCNA, proliferating cell nuclear antigen; E, enbryonic day.

with notable defects in bipolar cell function (18, 19).

Given the important roles of CHX10 and VSX1 in retinal development, we sought to determine their transcriptional activity. We found that CHX10 inhibited activated transcription in multiple contexts. The minimal region required for CHX10 repressor function included the HD and CVC domains. However, CHX10 also enhanced the activation of a specific subset of activators in chick neuronal cultures. Interestingly, we found that VSX1 also repressed transcription. The VSX1 HD mutation R166W, linked to keratoconus, impaired VSX1-mediated repression.

EXPERIMENTAL PROCEDURES

Cell Lines and Primary Chick Retinal Cultures—C33A cells or NG108 cells were grown on 100-mm diameter plates in α-minimal essential medium supplemented with 10% fetal bovine serum. Retinal, brain, and liver cells isolated from E8–E9 chicks (staged according to Hamburger and Hamilton (20)) were dissociated by incubating in 0.25% trypsin at 37 °C for 30–40 min. The cells were plated onto 35-mm wells pre-coated with 50–100 μg/ml PORN in Dulbecco's H21 + 10% fetal bovine serum media at an optimal transfection density of 2–3 $\times$ 10^6 cells/well.

CAT and Luciferase Assays—One day before transfection by the calcium phosphate method, cell lines were trypsinized and plated on 60-mm dishes at 70% confluency. E8 chick cultures were transfected using FuGENE (Roche Diagnostics) after 1–3 days *in vitro*. 0.3 μg of cytomegalorivus-β-galactosidase was included in each transfection and β-galactosidase activity used to normalize for transfection efficiency. CHX10, RB, and VSX1 expression vectors were used at 5 μg unless otherwise stated in the figure legends. All activator and reporter plasmids were used at 0.5 μg. CAT assays were performed 24 h post-transfection as described (21). The amount of lysate used and the incubation time for the CAT assay varied with transcriptional activator, such that sample activity was within the linear range of the assay (*i.e.* CAT assay reactions for a given activator were allowed to proceed such that the counts extracted were less than or equal to 20,000 cpm). 100% CAT activity is taken as that obtained in the presence of control effector plasmid.

Plasmids—SVLex was built by replacing the *N-Myc* insert in SVN-Myc[2] with a ~700-bp HindIII/XbaI fragment, derived from CDMLex (22). SVLex contains the SV40 enhancer/promoter upstream of the gene coding the bacterial transcription factor, LexA (amino acids 1–202).

LexRB encodes full-length human RB and has been previously described (21). LexCHX10 was generated by inserting a 3-kb BspMI/blunt XbaI fragment from pBS-KS$^+$-CHX10(Δ57) (a gift from R. McInnes) into SmaI/XbaI-digested SVLex. LexCHX10 encodes a protein in which LexA is fused to the complete human CHX10 protein (amino acids 1–361). The CHX10 upstream sequence adds 7 codons between the SVLex sequence and the CHX10 ATG. The plasmid used to express *Chx10* alone was pECE-HACHX10, a gift from P. Hamel. Expression in this plasmid is also driven by the SV40 enhancer/promoter. To generate GAL4-CHX10, Gal-hRb (21) was digested with SmaI/RI to remove *RB*, then a blunt SphI/RI fragment (full-length human *Chx10*) from LexCHX10 was cloned in. The Chx-ABCD series of deletion vectors were cloned into a cytomegalorivus-driven vector (pCDNA) that contained two C-terminal tags: SV40NLS (PKKKRKVE) and triple FLAG (DYKDHDG-DYKDHD-IDYKDDDDK) (3.1NF). CHX-ABCD (amino acids 1–361) was generated from pBS-KS$^+$-CHX10(Δ57) and the *Chx10* stop codon was removed by inserting a PCR fragment containing the 3′ sequence downstream of the Cfr10I site. CHX-B (amino acids 147–208), CHX-BC (amino acids 147–264), and CHX-BCD (amino acids 147–361) were engineered by PCR to contain a 5′ Kozak sequence, which generated a methionine residue upstream of the indicated CHX10 coding sequence. Full-length human *VSX1* was subcloned from pBSKS(−)h*VSX1* (kind gift of R. McInnes) into 3.1NF at a BamHI/XhoI junction to generate hV3.1NF. The *VSXI* stop codon was removed by insertion of a synthetic double-stranded oligo containing the *VSX1* sequence 3′ of the BstXI site. The R166W mutation was introduced into Vsx1 using the Promega site-directed mutagenesis kit.

All activator expression plasmids have been described previously. GAL4-activator fusions express the GAL4 DNA-binding domain fused to the following activation domains: VP16 (amino acids 410–490) (23), HSF1 (amino acids 201 to 529) (22), c-JUN (amino acids 5 to 253) (22), c-MYC (amino acids 1 to 262 of c-Myc) (24), and SP1 (amino acids 83 to 621) (25). A control vector, expressing amino acids 1–147 of GAL4 is pBXG1 (M. Ptashne).

The reporter plasmids, X4G2CAT, X6G2CAT, X300G2CAT, and X1700G2CAT have all been described previously (22). Briefly, X4G2CAT contains two GAL4 binding sites placed upstream of the human *hsp70* TATA region (−40 to +160) and the CAT reporter gene. Four LexA binding sites were placed distal to activator binding sites, 20 bp upstream. X6G2CAT is identical to X4G2CAT, except that there are six LexA binding sites distal to the activator binding sites, instead of four. X300G2CAT is based on X6G2CAT and contains a fragment of ~300 bp inserted between the LexA binding sites and the GAL4 binding sites. X1700G2CAT is also based on X6G2CAT and contains an ~1,700-bp fragment inserted between the LexA binding sites and the GAL4 binding sites. X4G2PCNA CAT was built by inserting a BamHI/blunt NcoI 0.7-kb fragment from UAS-PCNA-CAT (provided by M. Sopta) into SalI/blunt NcoI-digested X4G2CAT, which replaces the hsp70 promoter in the latter plasmid with the proliferating cell nuclear antigen (PCNA) promoter. pLD85 and pG5SV40CAT have also been described before (21, 26). HD4pG5EC was built by J. Epstein, and contains four P3 sites (TAATtaaATTA) each separated by 8 bp, and positioned upstream of five GAL4 sites present in the parent vector pG5EC (M. Ptashne).

Gel Shifts—CHX10 plasmids were *in vitro* translated using rabbit reticulocyte lysates (Promega), in the presence of either ^{35}S-labeled or unlabeled methionine. 2 μl of each *in vitro* translated sample was analyzed by 10 or 12% SDS-PAGE for quantification of protein levels by phosphorimager analysis (Bio-Rad Molecular Imager FX) of [^{35}S]methionine signal (adjusted for methionine content of each protein) or chemiluminescent densitometry of anti-FLAG Western-blotted proteins using a Bio-Rad Fluor MultiImager with Quantity One version 4.1.0 software. These two methods of normalizing the amount of *in vitro* translated protein generated similar results in the gel shift assays used to compare different CHX10 proteins, which were carried out a total of six times. 200 ng of double-stranded probe was end-labeled by T4 DNA kinase end labeling with [γ-^{32}P] and polynucleotide kinase. Probe sequences were: HD4, top strand: 5′-CTCTAGCTAATTAAATTAGC-3′; HD4 mutant, top strand: 5′-CTCTAGCTCCTTAAAGGAGC-3′. Probes were purified using G25 spin columns (Amersham Biosciences catalog number 27-5325-01) and 40,000 cpm used per reaction (0.2–2 ng). *In vitro* translated protein was incubated with labeled probe for 15 min at 26–28 °C in a final volume of 15 μl (2 mM Tris, pH 8.0, 0.5 mM EDTA, 10% glycerol, 0.05% Triton, 88 mM KCl, 150 ng/μl poly(dI-dC), 1 mM dithiothreitol, 12.5 ng/μl salmon sperm DNA). For competition reactions, excess unlabeled probe was first incubated with *in vitro* translated protein for 2–3 min at 26–28 °C, then labeled probe was added and the tubes incubated for 15 min at 26–28 °C. Samples were resolved on a 4.5% non-denaturing acrylamide gel using 0.5$\times$ TBE running buffer. Gels were dried and exposed overnight on phosphorimager screens.

Western Blotting—In vitro translated proteins or cell lysates were separated on 12 or 15% SDS-PAGE, transferred to nitrocellulose, and blocked in 1:10 BM Blocking reagent overnight at 4 °C. Blots were washed briefly and incubated with 3 μg/ml mouse anti-FLAG antibody in phosphate-buffered saline (M2-Sigma). Following washing with phosphate-buffered saline, 0.1% Tween, blots were hybridized with rabbit anti-mouse horseradish peroxidase secondary (Jackson Laboratories) at 1:5000 in 1:20 Roche blocking reagent in phosphate-buffered saline. Blots were washed and chemilumiscence was detected with Roche Western blotting kit and captured and quantified using a Bio-Rad Fluor MultiImager with Quantity One version 4.1.0 software.

RESULTS

CHX10 Can Repress Transcription—Because CHX10 contains three regions that loosely resemble sequences in some activators (2), we tested whether it has positive regulatory activity. Transient transfection assays were performed with a LexCHX10 expression vector, encoding the bacterial DNA-binding protein LexA fused to human CHX10, together with a reporter plasmid, X4G2CAT, containing four LexA binding sites, two GAL4 binding sites upstream of the heat shock (*hsp70*) minimal promoter, and the CAT reporter gene.

When a human cervical carcinoma line (C33A (27)) and a neuronal/glial line (NG108, generated by fusion of mouse neuroblastoma and rat glioma cells (28)), were transfected with X4G2CAT and LexCHX10 plasmids, no transcriptional activa-

[2] R. Bremner, unpublished data.

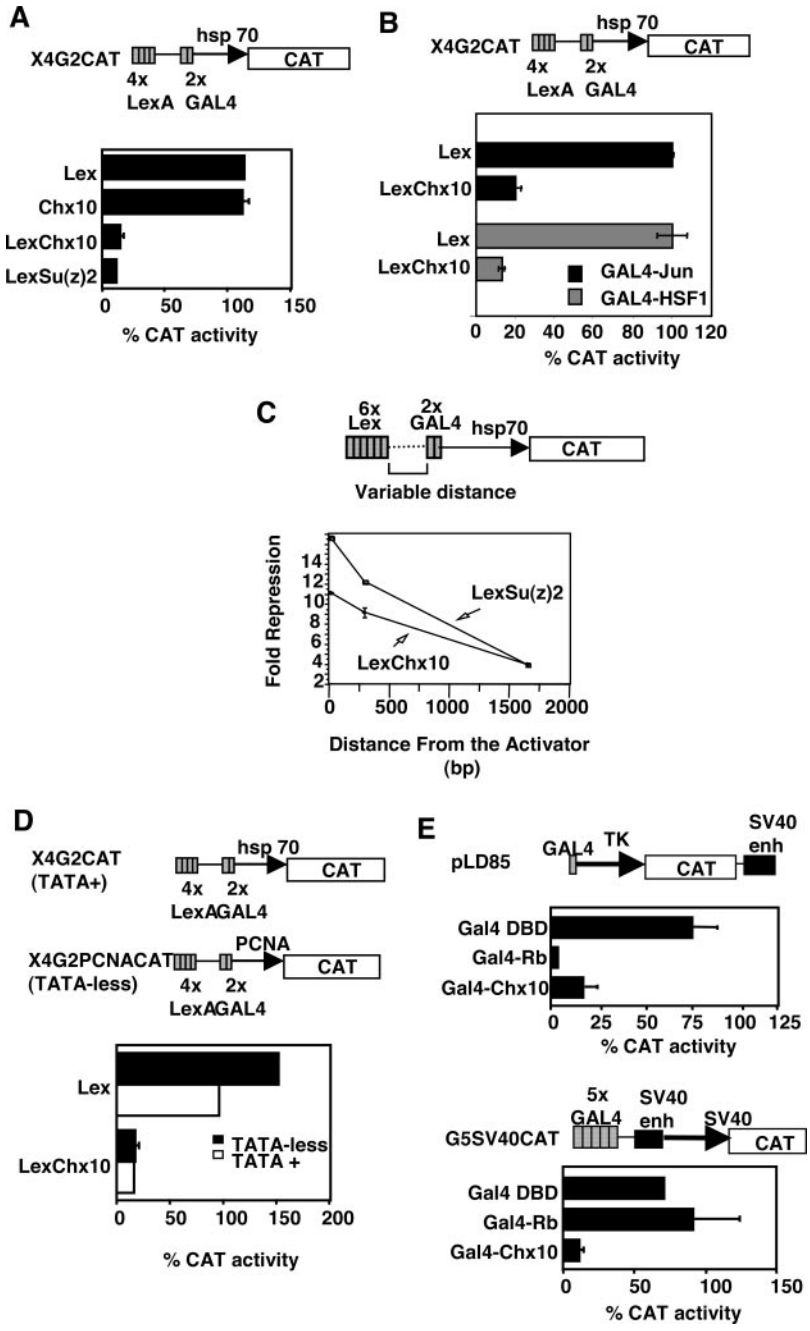

FIG. 1. **CHX10 can repress transcription.** *A*, promoter-bound CHX10 represses activation by GAL4-MYC. C33A cells were transfected with X4G2CAT, GAL4-MYC, and equimolar amounts of plasmids expressing the indicated proteins. *B*, CHX10 represses c-JUN and HSF1. NG108 cells were transfected with GAL4-c-JUN or GAL4-HSF1, equimolar amounts of LexA or LexCHX10, and X4G2CAT. *C*, CHX10 represses at a distance. C33A cells were transfected with equimolar amounts of LexCHX10 or LexSu(z)2, GAL4-MYC, and one of three reporters, in which the distance between the LexA (repressor) and GAL4 (activator) binding sites was 20 (X6G2CAT), 300 (X330G2CAT), or 1660 (X1660G2CAT) base pairs. *D*, CHX10 represses activation of a TATA-less promoter by C-Jun. C33A cells were transfected with TATA-containing (X4G2CAT) or TATA-less (X4G2PCNACAT) reporter plasmids, GAL4-c-JUN, and equimolar amounts of vectors that express LexCHX10 or LexA. *E*, CHX10 represses the SV40 enhancer. C33A cells were transfected with pLD85 or G5SV40CAT together with equimolar amounts of the indicated plasmids. 100% CAT activity is taken as that obtained in the presence of control effector plasmid. CAT activity was corrected for transfection efficiency using a β-galactosidase internal control. *Error bars* represent the S.D. obtained from three independent experiments.

tion was observed (data not shown). A plasmid expressing LexA alone (SVLex) had no effect (data not shown). To explore whether CHX10 might inhibit transcription, we tested the effect of Chx10 on several activators. GAL4 fusions used in these assays contained the ADs of HSF1, which are acidic (22); and c-JUN and c-MYC ADs, which are proline- and glutamine-rich (24, 29). c-MYC has an additional AD that has no preponderance of specific amino acids (24).

As a positive control for repression, cells were transfected with LexSu(z)2, which encoded LexA fused to the *Drosophila* polycomb group protein suppressor two of zeste (Fig. 1*A*), together with X4G2CAT and GAL4-c-MYC (GM1–262). Su(z)2 is a powerful transcriptional repressor responsible for maintaining the proper spatially restricted expression pattern of the homeotic loci during *Drosophila* development (22). LexCHX10 repressed activated transcription as effectively as LexSu(z)2 in C33A cells (Fig. 1*A*). LexA alone (SVLex) did not affect transcription. Repression was dependent on CHX10 being targeted to the promoter by LexA because CHX10 alone failed to repress (Fig. 1*A*). In NG108 cells, LexCHX10 inhibited GAL4-c-JUN and GAL4-HSF1 mediated activation by 80 and 87%, respectively (Fig. 1*B*). Similar results were obtained in C33A cells (data not shown). In NG108 cells, titration of LexCHX10 demonstrated dose-dependent repression of HSF1-mediated activation (data not shown).

CHX10 Represses at a Distance—Some activators and repressors function even when bound far away from the transcription start site. For example, Su(z)2 represses transcription when positioned 1,700 bp away from activator binding sites (22). In contrast, the HD protein Eve does not repress when placed 500 bp away from the activator binding sites (30). To determine whether CHX10 represses at a distance, reporter plasmids were used in which the LexA sites were separated from the GAL4 sites by 300 or 1700 bp (Fig. 1*C*) (22). LexCHX10 repressed most effectively when the LexA sites were directly upstream of the GAL4-activator binding sites. As the

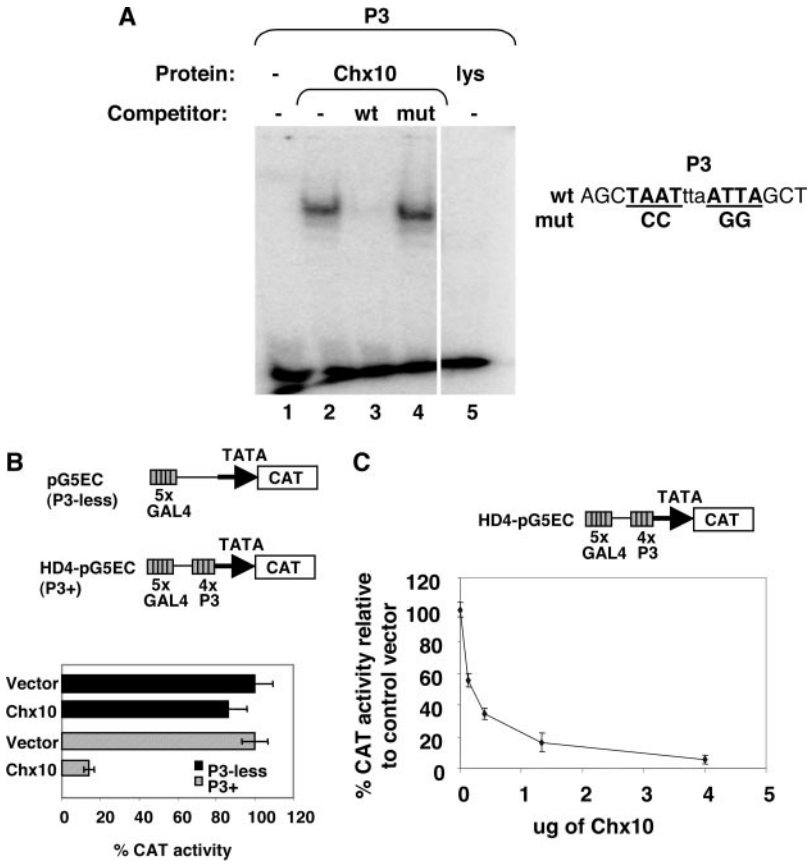

FIG. 2. **CHX10 represses when targeted to promoters by its HD.** *A*, CHX10 binds a P3 sequence. *In vitro* translated Chx10 (*lanes 2–4*) or unprogrammed lysate (*lane 5*) were incubated with end-labeled P3 probe alone, or in the presence of 100 times excess unlabeled wild type (*lane 3*) or mutated (*lane 4*) P3 oligonucleotides. *B*, CHX10 represses a promoter containing P3 sites. NG108 cells were transfected with pG5EC or HD4-pG5EC, GAL4-HSF1, and Chx-ABCD plasmid. *C*, CHX10 repression is dose-dependent. CHX-ABCD (0.13, 0.4, 1.3, or 4 μg), GAL4-HSF1, and HD4-pG5EC were co-transfected into NG108 cells. Equimolar amounts of effector plasmid were achieved by adding appropriate amounts of empty effector plasmid. 100% CAT is taken as that obtained in the presence of control effector plasmid. CAT activity was corrected for transfection efficiency using a β-galactosidase internal control. *Error bars* represent the S.D. obtained from three independent experiments.

distance between these sites was increased, CHX10-mediated repression was compromised, but a similar degree of repression was observed when either LexChx10 or LexSu(z)2 were targeted to sites 1,700 bp upstream of the activator binding sites (Fig. 1*C*). Apart from showing that CHX10 represses from a distance, these data suggest that repression is not because of steric interference.

CHX10 Also Represses TATA-less Promoters—Because TATA-containing and TATA-less promoters sometimes show differences in response to activators and repressors (31–34), we also tested the properties of CHX10 on a TATA-less promoter, X4G2PCNACAT. Here, the TATA-containing hsp70 promoter in X4G2CAT was replaced by the TATA-less core promoter from the gene for PCNA (35). LexChx10 inhibited GAL4-c-JUN-mediated activation of this reporter by 85% (Fig. 1*D*), indicating that Chx10 does not require a TATA box to inhibit gene expression.

CHX10 Represses a Complex Enhancer—The above experiments indicate that CHX10 represses individual activators (c-MYC, HSF1, and c-JUN). *In vivo*, however, these factors operate in the context of multiprotein structures, or enhanceosomes, which form by cooperative binding of activators to enhancers (36). Thus, we assayed the effect of CHX10 on the SV40 enhancer using the reporters pLD85 (21), in which a GAL4 binding site lies upstream of the thymidine kinase promoter, CAT, and the SV40 enhancer, or G5SV40CAT (26), in which five GAL4 sites lie upstream of the SV40 enhancer/promoter (Fig. 1*E*). GAL4-CHX10, which encodes the GAL4 DNA-binding domain fused to CHX10, repressed both reporters by 85–90%, whereas the GAL4 DNA-binding domain alone, encoded by pBXG1, had no effect (Fig. 1*E*). Thus, in addition to discrete activators, CHX10 can repress a complex enhancer. The retinoblastoma protein (RB) is another potent transcriptional repressor (21, 37–42). As observed previously (21), RB repressed the pLD85 reporter (Fig. 1*E*). Surprisingly, however, RB did not inhibit G5SV40CAT, in which the SV40 enhancer is positioned upstream of the CAT gene. These results suggest that RB and CHX10 use different mechanisms to inhibit transcription.

Repression When CHX10 Is Tethered to Promoters by the HD—HDs of the paired or paired-like classes, bind DNA most efficiently as homo- or heterodimers to a palindromic TAAT-$(N)_n$ATTA motif (43). Spacing between the TAAT half-sites is predicted by the nature of the residue at position 50 of the HD. In CHX10 this residue is a glutamine (Gln^{50}) (2) suggesting that CHX10 may bind a palindromic P3 consensus site in which TAAT half-sites are separated by three nucleotides (43). *In vitro* translated CHX10 protein retarded the mobility of a ^{32}P-labeled P3 oligonucleotide in a gel shift assay (Fig. 2*A*, *lane 2*). CHX10 was competed by excess unlabeled wild-type P3 probe, but not mutated P3 probe (Fig. 2*A*, *lanes 3* and *4*). Unprogrammed lysate did not bind the P3 probe (Fig. 2*A*, *lane 5*). Similar results were obtained using the bacterially expressed GSTChx10 fusion protein (data not shown).

To determine the effect of CHX10 on a promoter containing P3 binding sites we used the reporter plasmid HD4-pG5EC, which contains four P3 sites, five GAL4 sites, an E1b minimal promoter, and the CAT gene (Fig. 2*B*). CHX10 inhibited GAL4-HSF1-mediated activation of this reporter in NG108 cells (Fig. 2*B*). Repression required DNA binding (Fig. 2*B*, pG5EC) and was dose-dependent (Fig. 2*C*). Finally, CHX10 repressed c-JUN-activated transcription of a reporter vector containing a single P3 site (P3G2CAT, TAATtgaATTA; data not shown). Thus, CHX10 repressed transcription whether bound to promoters via a heterologous DNA-binding domain (Fig. 1) or its own HD (Fig. 2).

Selective Repression in Primary Chick Cultures—Results described above suggest that CHX10 is a global inhibitor, because it repressed c-MYC, c-JUN, HSF1, and the SV40 enhancer. The cell lines used are immortalized and transformed, so our next

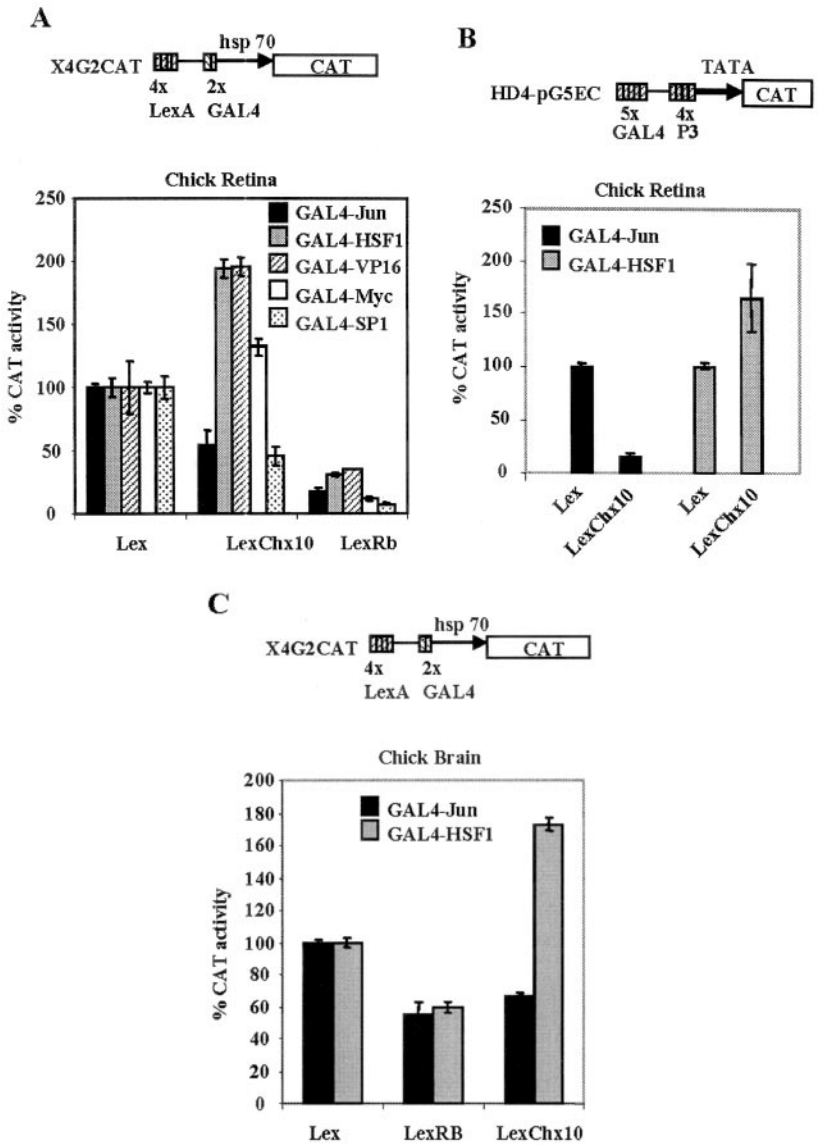

FIG. 3. **CHX10 selectively represses in primary cell cultures.** *A*, CHX10 represses c-JUN and SP1, but potentiates activation of HSF1 and VP16 in primary chick retinal cells. Dissociated E8 chick retinal cells were co-transfected with equimolar amounts of LexA or LexCHX10 or LexRB, together with GAL4 activator and X4G2CAT. *B*, CHX10 represses c-JUN but enhances activation of HSF1 in primary chick retinal cells when targeted through the HD. Dissociated E8 chick retinal cells were co-transfected with equimolar amounts of LexA or LexCHX10, together with GAL4-c-JUN or GAL4-HSF1 and HD4pG5EC. *C*, CHX10 potentiates activation of HSF1 in primary chick brain cells. Dissociated E8 chick brain cells were co-transfected with equimolar amounts of LexA or LexCHX10 or LexRB, together with GAL4-c-JUN or GAL4-HSF1 and X4G2CAT. 100% CAT activity is taken as that obtained in the presence of control effector plasmid. CAT activity was corrected for transfection efficiency using a β-galactosidase internal control. *Error bars* represent the S.D. obtained from three to six independent experiments.

goal was to determine whether CHX10 represses transcription in primary cultures, including retina. Dissociated embryonic day 8 (E8) chick retinal cells were co-transfected with different GAL4 DNA-binding domain activators as well as LexCHX10 or LexRB and X4G2CAT. LexRB repressed all the activators tested, whereas LexCHX10 repressed c-JUN and SP1 but slightly augmented the activity of VP16, HSF1, or c-MYC (1.2–1.9-fold, Fig. 3*A*). We also co-transfected chick retinal cells with LexCHX10, either GAL4-c-JUN or GAL4-HSF1, and HD4-pG5EC, the reporter containing P3 sites (see Fig. 2*B*). Again, CHX10 repressed c-JUN (~6-fold) but weakly augmented HSF1-mediated activation (~1.6-fold, Fig. 3*B*).

To extend these analyses, transient transfections were also performed using primary chick brain and liver cells. CHX10 is expressed in the developing ventral spinal cord and hindbrain in chick, mouse, and goldfish, but is absent from liver (2, 44, 45). In dissociated brain cultures LexCHX10 and LexRB repressed c-JUN with similar potency, but LexRB inhibited while LexCHX10 potentiated HSF1 (Fig. 3*C*). In liver, both LexCHX10 and LexRB repressed c-Jun and HSF1 activity (data not shown). In summary, RB repressed all activators in primary chick retina, brain, and liver. CHX10 repressed c-JUN and SP1 in chick neuronal cultures (retina and brain), c-JUN and HSF1 in liver cells and numerous activators in various mammalian cell lines, but potentiated HSF1 in chick neuronal cultures. These results suggest that CHX10 can function as a context-dependent repressor or weak activator. Both the cell type and nature of the activator influence the ability of CHX10 to repress transcription.

The CHX10 Repression Domain Maps to the HD-CVC Region—To examine the minimal region necessary for repression, CHX10 was divided into four sections, A–D, corresponding to the N terminus, HD, CVC motif, and C terminus, respectively (Fig. 4*A*). Vectors were constructed that expressed various combinations of these regions (CHX-ABC, CHX-BCD, CHX-BC, and CHX-B) fused to a nuclear localization signal and triple FLAG tag. Nuclear localization was confirmed by anti-FLAG immunostaining following transfection of three different cell lines (data not shown). An additional vector was generated in which codon 51 was altered from asparagine to alanine (CHX-N51A). Mutation at this invariant amino acid disrupts DNA binding (46). In gel shift assays, *in vitro* translated CHX-ABC, CHX-BCD, and CHX-BC interacted with the P3 probe as well as wild type CHX10 (Fig. 4*B*, *cf. lanes 2–4* with *lanes 8–16*), but CHX-B exhibited reduced binding and, as expected, CHX-N51A did not bind at all (Fig. 4*B*, *lanes 5–7* and *17–19*). A dose-response titration confirmed DNA binding levels of the CHX10 proteins (data not shown). The amount of *in vitro* translated protein used in

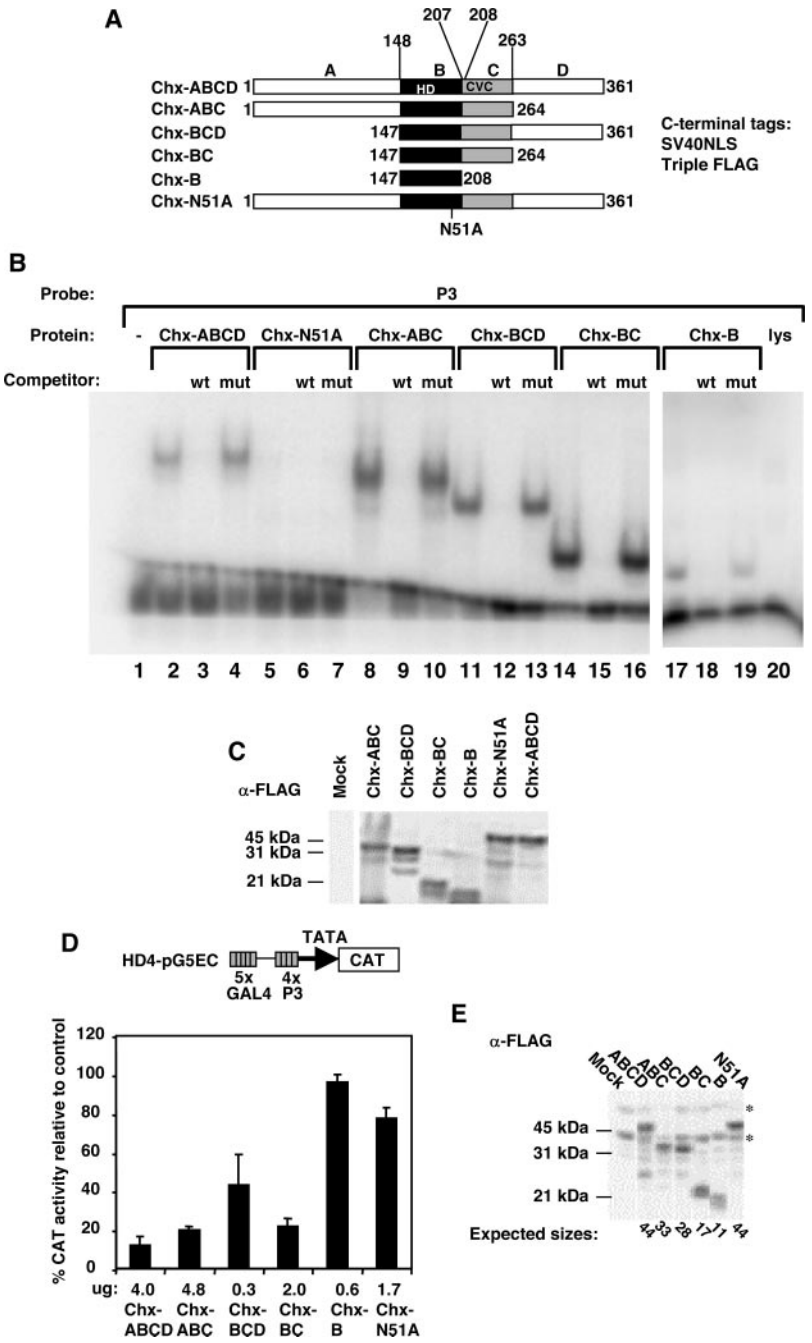

FIG. 4. **The HD and CVC are sufficient for DNA binding and repression.** *A*, schematic diagram illustrating CHX10 deletion vectors. All vectors contain two C-terminal tags: SV40 large T nuclear localization signal and triple FLAG. Nuclear localization was confirmed by anti-FLAG immunostaining following transfection of three different cell lines (data not shown). *B*, gel shift analysis using full-length and mutated *in vitro* translated CHX10 proteins and a ^{32}P-end-labeled P3 probe. CHX10 (*lanes 2–4*) and CHX10 derivatives (*lanes 5–19*) interacted specifically with the P3 sequence except for Chx-Ns1A (lanes 5–7). Competitor reactions contained 100 times excess unlabeled wild type or mutated P3 oligonucleotides. *Lys*, unprogrammed lysate (*lane 20*). *C*, anti-FLAG Western blotting indicating similar expression of all *in vitro* translated CHX10 proteins in *B*. The lower molecular weight minor protein species in each lane is presumably the result of initiation at internal ATGs, a common event in *in vitro* translation. N-terminal truncated proteins would contain the C-terminal FLAG tag. *D*, the CHX10 HD and CVC are sufficient for repression. NG108 cells were transfected with GAL4-HSF1 activator, HD4-pG5EC reporter, and the indicated CHX10 vector. Equimolar amounts of effector plasmid were achieved by adding appropriate amounts of empty effector plasmid. 100% CAT activity is taken as that obtained in the presence of control effector plasmid. CAT activity was corrected for transfection efficiency using a β-galactosidase internal control. *Error bars* represent the S.D. obtained from three independent experiments. *E*, anti-FLAG Western analysis on transfected lysates from *D*. The amount of lysate loaded was normalized for transfection efficiency. The *asterisks* indicate two cross-reacting proteins present in the mock-transfected cells. Other smaller minor species represent truncation products of each specific CHX10 protein.

these gel shift assays was normalized by two methods (see "Experimental Procedures" for details) such that any variance between wild type CHX10 and any mutated protein was less than 2-fold. Anti-FLAG Western analysis of normalized lysates confirmed that adjusted *in vitro* translated protein levels were within this range (Fig. 4*C* and data not shown, see "Experimental Procedures" for quantification method).

Next, NG108 cells were co-transfected with a HD4-pG5EC reporter, a GAL4-HSF1 activator, and the CHX10 plasmids described above. The amount of each CHX10 plasmid was adjusted to achieve levels of expression such that wild type and mutant proteins were within a 2-fold variance (Fig. 4, *D* and *E*, see "Experimental Procedures" for quantification of Western blots). Subsequently, changes of less than 2-fold in repression activity were ignored. Total moles of plasmid were equalized using empty vector. In the case of CHX-BCD and CHX-B, ~10-fold less plasmid was required to generate the same amount of protein observed with other vectors (Fig. 4, *D* and *E*). This result suggests that the N terminus may destabilize CHX10, although a simple correlation was not evident because CHX-BC, which also lacks the N terminus, was required in amounts similar to the full-length vectors (Fig. 4, *D* and *E*). Further studies are required to determine whether the differences in expression are because of an effect on translation and/or stability. CHX-ABC and CHX-BC repressed transcription almost as well as wild type CHX10 (ABCD) (Fig. 4*D*), indicating that neither the N nor C termini were required for repression. CHX-BCD also repressed transcription, albeit to a lesser extent than CHX-ABC or CHX-BC, suggesting that, in the absence of the N terminus, the C terminus slightly inhibits repression (Fig. 4*D*). As expected, the DNA-binding defective protein CHX-N51A did not repress transcription (Fig. 4*D*). CHX-B, which retains significant DNA binding activity also did not repress transcription (Fig. 4*D*). These data indicate that the CHX10 HD and CVC domains are sufficient for DNA binding and repression and that removal of the CVC domain

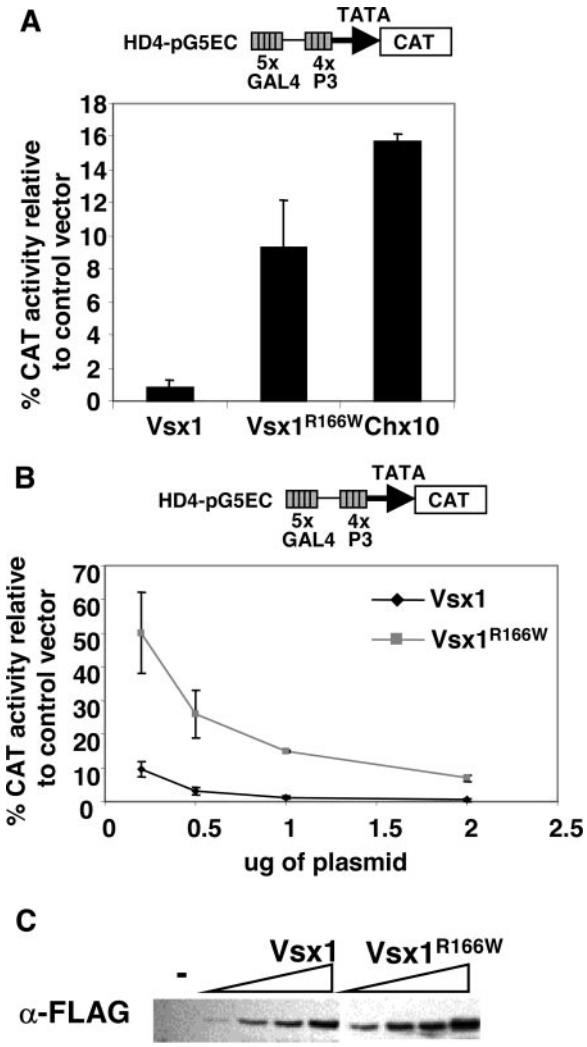

FIG. 5. **An R166W mutation impairs Vsx1 transcriptional repression.** *A*, VSX1 represses more efficiently than CHX10. NG108 cells were co-transfected with 2 μg of CHX10-ABCD, VSX1, or VSX1^{R166W} expression vector, together with GAL4-HSF1 and HD4-pG5EC. *B*, Vsx1 repression is dose-dependent. NG108 cells were co-transfected with increasing amounts of VSX1 or VSX1^{R166W} expression vector (0.25, 0.5, 1, or 2 μg), together with GAL4-HSF1 and HD4-pG5EC. Equimolar amounts of effector plasmid were achieved by adding appropriate amounts of empty effector plasmid. 100% CAT activity is taken as that obtained in the presence of control effector plasmid. CAT activity was corrected for transfection efficiency using a β-galactosidase internal control. *Error bars* represent the S.D. obtained from three independent experiments. *C*, anti-FLAG Western blotting of transfected lysates from *B*.

slightly impairs DNA binding but ablates repression.

VSX1 Can Repress Transcription—It has been suggested that the CHX10-related protein VSX1 may be an activator (12), but our results with CHX10 raised the possibility that VSX1 may repress transcription. Previously we showed that Vsx1 interacts with the P3 motif (18) and that a natural mutation in the VSX1 HD, R166W, which causes keratoconus in humans, impairs binding to this motif (18). To test VSX1 activity, and whether this mutation impairs VSX1 function, NG108 cells were co-transfected with wild type or VSX1^{R166W} expression vectors, together with the HD4pG5EC reporter plasmid and GAL4-HSF1. Vsx1 repressed HSF-1-mediated activation more effectively than CHX10 (Fig. 5*A*), although this may be in part because of slightly lower protein expression level of CHX10 compared with Vsx1 as detected by anti-FLAG Western blotting (data not shown). The R166W mutation significantly impaired repression (Fig. 5*A*). A dose-response curve confirmed that wild type VSX1 repressed transcription more effectively than VSX1^{R166W} over a wide range of concentrations (Fig. 5*B*). Protein expression was assessed by Western blot analysis (Fig. 5*C*) and quantification (see "Experimental Procedures") indicated that the mutated protein was expressed at the same level or slightly higher (up to 1.4-fold) than wild type VSX1.

DISCUSSION

Our data suggest that CHX10 and VSX1 regulate retinal development, at least in part, by inhibiting the expression of downstream target genes. CHX10 inhibited several activators in multiple cell types and blocked expression whether tethered to DNA by a heterologous DNA-binding domain or through interaction of its own HD with one or more P3 sites. CHX10 also repressed at a distance, inhibited TATA-containing and TATA-less promoters, and down-regulated a complex enhancer placed up- or downstream of a reporter gene. In one scenario, CHX10 repressed enhancer activity even when pRB did not. Studies with promoters lacking appropriate binding sites, and with CHX10 variants that do not bind DNA, showed that DNA binding was essential for repression, which distinguishes CHX10 from certain other HD repressors, including the paired-like HD protein rHox (47–50). We also found that CHX10 slightly enhanced transcription of specific activators in primary chick neuronal cultures. Finally, we showed that VSX1, a close relative of CHX10, can also function as a repressor. A naturally occurring disease-causing VSX1 mutation that impairs DNA binding reduced transcriptional activity.

How Do CHX10 and VSX1 Regulate Transcription?—Only a handful of HD proteins have a defined repression domain. Engrailed contains two distinct repression domains, one type is most effective in transient assays, whereas the other potently represses *in vivo* with little effect on episomal templates (51). The repression domain for Even-skipped was mapped to a Ala-Pro-rich region, which included the HD (30, 53). Outside of its function as a DNA-binding motif, the HD has been show to mediate protein-protein interactions for transcriptional repression (53–57). For instance, Msx-1 and Even-skipped interact with the general transcription factors TFIID/TBP, whereas Msx2 interacts with TFIIF (RAP47/RAP30). Msx-1 and -2 require residues from the N-terminal arm for this interaction, whereas this region is not necessary for Even-skipped (53, 55, 58). Alternatively, the HD can mediate interactions with co-repressor molecules, as is the case for NK-2 and Groucho (56, 57).

Whereas a truncated version of CHX10 consisting of the HD and CVC regions bound a P3 site as efficiently as full-length CHX10, removal of the CVC impaired DNA binding. This suggested that the HD alone (Chx-B) may require the CVC domain for effective DNA binding. Alternatively, reduced DNA binding of Chx-B may be a result of changes in conformation or stability, indicating a potential need for additional flanking residues. As CHX10 can repress in several different scenarios, it seems reasonable that it could bind and/or interfere with a basal transcription factor. This action may be mediated through the HD and/or CVC motif of CHX10. Intriguingly, mutations in the CVC domain of ceh10 or human VSX1 cause lethality and eye defects, respectively (1, 18). Also, an isoform of CHX10 has been described that contains a 21-amino acid insert in the CVC domain (7, 44). It will be interesting to determine the effect of these variants on DNA binding or repression.

A number of HD proteins contain eh1-like motifs capable of interaction with members of the Groucho/TLE co-repressor family of proteins (59–62). The N termini of CHX10, VSX1, and the related Prd-like HD protein Rx have an eh1-like octapeptide sequence (63). We found that *in vitro* translated CHX10 did not interact with a subset of mammalian Groucho homo-

logues (data not shown). However, this does not rule out the possibility that Chx10 may interact with other members of the large Groucho/TLE family or utilize the eh1 motif for repression *in vivo* (51). An eh1-like sequence in D-Gsc, FTIDSILG, promotes heterodimerization and "interactive repression" (64). Thus, the role of the CHX10 or VSX1 eh1 or other functional domains, as well as the co-repressors that mediate repression by this or other motifs remain to be clarified.

Certain Prd-like HD proteins, including Chx10, interact with the RB protein family (65). RB, and its relatives, are potent transcriptional repressors (21, 37–41, 66), and could potentially mediate repression by CHX10. In the context of the SV40 enhancer, CHX10 appears to use a Rb-independent mechanism of repression (Fig. 1*E*). In addition, repression by CHX10 seems to be unaffected by overexpression of E1A, which binds and antagonizes the function of the RB protein family.[3]

DNA Binding by Paired-like HD Proteins—DNA binding specificity for different HDs is determined in large part by the two nucleotides downstream of a TAAT core, but the immediate 3′-flanking nucleotide is most important. Several HDs in which the 50th residue of the HD is a Gln interact with a TAATT motif, whereas Lys^{50} HDs bind TAATC (43, 67–71). However, other amino acids in the N-terminal arm of the HD, the DNA binding helix, and downstream amino acids can alter the specificity of Gln^{50} HDs (72, 73). A screen for consensus CHX10 binding sites was performed and revealed the sequence TAATTAGC (5). A thymidine directly 3′ to the TAAT core is in agreement with the P3 sequences we tested for CHX10 (Fig. 2*A*, data not shown) and VSX1 (18). Interestingly, the Lys^{50} HD proteins RX and CRX interact with TAATC sequences and activate transcription (74, 75). The repertoire of TAATT and TAATC sites present on different promoters is likely to have a profound effect on the timing and extent of target gene induction in response to the same pool of Gln^{50} and Lys^{50} HD proteins.

Biological Role of CHX10 and VSX—Chx10 loss impairs the proliferation of RPCs early in retinal development and blocks the differentiation of bipolar cells in the postnatal rodent retina (6, 9). Our data are consistent with the possibility that CHX10 may facilitate proliferation and bipolar cell formation by repressing the expression of genes that promote growth arrest and the formation of cell types other than bipolar neurons, respectively. Indeed, a variety of cell cycle inhibitors are upregulated in the CHX10 null retina (4) and, in support of the idea that CHX10 may repress non-bipolar cell genes, both CHX10 and VSX1 were isolated in a one-hybrid assay that utilized three tandem copies of the cone opsin locus control region (*LCR*) (12), a gene that is silent in bipolar cells. CHX10 affects cell fate (6, 9, 76), whereas VSX1 sculpts the terminal differentiation characteristics of cone bipolar cells (16, 17), thus in addition to terminal differentiation markers like cone opsin, CHX10 may also repress fate determinants. Bipolar cell births parallel those of rod photoreceptors and Müller glia, so CHX10 may repress genes that promote the formation of these cell types (77–80). The observation that ectopic CHX10 expression promotes inner nuclear cell fates at the expense of photoreceptors (76, 81) supports the idea that CHX10 may block the expression of rod rather than Müller determinants. Nevertheless, a *Chx10* reporter gene is expressed in Müller glia in the or^J retina (4), consistent with the idea that CHX10-deficient cells become Müller rather than rod cells. However, to rule out the possibility that the latter phenomenon is not a secondary effect of the dramatic effects on proliferation, it will be important to determine whether inactivating Chx10 late in retinal development switches cells from the bipolar to the rod or Müller cell fate.

In addition to facilitating proliferation and bipolar cell differentiation through repression, CHX10 may also potentiate activators that drive these processes. Indeed, as well as increased levels of cell cycle inhibitors, the or^J retina also contains reduced levels of cell cycle activators, such as cyclin D1 (4, 9) and CHX10 cooperates with the basic helix loop helix activators Mash1 and Math3 to drive bipolar cell genesis (76). Furthermore, the CHX10 HD augments transcriptional activation mediated by PAX6 (52). We found that CHX10 weakly potentiated a subset of activators in certain contexts. For example, CHX10 consistently repressed c-JUN activity in all these cell types but while Chx10 repressed HSF1 activity in mammalian cell lines and in chick primary liver cells, it enhanced HSF1 activation mildly in primary chick retinal or brain neuronal cultures. Thus, depending on the availability of cell-specific cofactors and the activators bound to the same target locus, CHX10 can act as a mild activator. Elucidating the full complement of direct CHX10 target genes *in vivo* will be necessary to clarify targets it represses or potentiates to control retinal cell proliferation and differentiation.

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[3] K. F. Ahmad, K. M. Dorval, and R. Bremner, unpublished data.

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