Delayed Expression of the *Crx* Gene and Photoreceptor Development in the *Chx10*-Deficient Retina

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Purpose. The *Chx10* homeobox gene is expressed in neural progenitor cells during retinal development. The absence of Chx10 causes microphthalmia in humans and in the mouse mutant *ocular retardation*. The purpose of this study was to examine how neuronal development is affected by absence of the Chx10 transcription factor in the mouse retina.

Methods. Expression of transcription factor genes, *Crx*, *Pou4f2*, and *Pax6*, that mark specific cell types as they begin to differentiate was analyzed by RNA in situ hybridization of retina from wild-type and Chx10-null *ocular retardation* mice (*Chx10*$^{or\text{-}J/or\text{-}J}$). RT-PCR analysis was used to compare expression of these genes and putative targets of Crx regulation. Photoreceptor development was analyzed by using peanut agglutinin (PNA)-rhodamine and blue cone opsin antibody to label cones and rhodopsin antibody to label rods.

Results. The photoreceptor gene *Crx*, normally expressed during embryonic retinal development, was not detected in the embryonic mutant retina, but was expressed after birth. Expression of the targets of *Crx* regulation, rhodopsin, peripherin, rod phosphodiesterase beta (*Pdeb*), and arrestin, with the exception of interphotoreceptor retinoid binding protein (*Irbp*), was delayed in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina. Rhodopsin localization in rod outer segments was also delayed. By contrast, temporal and spatial expression of *Pou4f2* and *Pax6* in developing ganglion and amacrine cells and PNA and blue opsin in developing cone cells was relatively normal in the mutant.

Conclusions. Delay of the normal temporal expression of genes essential for photoreceptor disc morphogenesis leads to failure of correct rod and cone outer segment formation in the *Chx10*$^{or\text{-}J/or\text{-}J}$ mutant retina. In addition, the absence of Chx10 appears to affect the development of late-born cells more than that of early-born cells, in that a low number of rods develops, whereas formation of ganglion, amacrine, and cone cells is relatively unaffected. (*Invest Ophthalmol Vis Sci.* 2004;45: 375–384) DOI:10.1167/iovs.03-0332

The neural retina is formed when paired evaginations of the forebrain invaginate to give rise to the bilayered optic cups. The inner layer of the optic cup becomes the neural retina and the outer layer becomes the retinal pigmented epithelium. The neural retina initially comprises a layer of pluripotent retinal progenitor cells. Over the subsequent period of development a process of cell proliferation, migration, and differentiation leads to the cellular organization of the mature retina. Adjacent to the pigmented epithelium, the light-sensitive rod and cone photoreceptor cells are aligned in the outer nuclear layer. Photoreceptor cells connect to the ganglion cells that project their axons along the optic nerve to the brain, through interneurons (bipolar, horizontal, and amacrine cells) of the inner nuclear layer. These six distinct types of retinal neuron and Müller glial cells all derive from common retinal progenitors during development.[1–3] The order in which the different cell types are generated is conserved between species, with ganglion cells being born first and rods and bipolar cells being born late.[4] It is proposed that combinations of intrinsic and extrinsic factors regulate the generation of distinct cell types in an orderly sequence during development.[5] Central to this model is the idea that progenitor cells are competent to give rise to distinct types of cells at different stages of development and that cellular competence is an intrinsic property defined in part by a cell's complement of active transcription factors.

A range of transcription factor genes has now been described that are expressed in distinct spatial and temporal patterns during retinal development.[6,7] Several of these genes are essential for normal development of the retina in vivo.[8–10] These genes are expressed in dividing neural progenitor cells and/or in postmitotic and differentiating cells and are implicated in proliferation and fate-determination pathways. Understanding the coordination between these genes is key to understanding the process of retinal development.

The *Chx10* homeobox transcription factor gene is expressed in proliferating retinal progenitor cells throughout retinal development.[11–13] Expression is first detected in the presumptive neural retina of the invaginating optic vesicle. As progenitor cells exit the cell cycle and differentiate, *Chx10* expression is maintained only in the mitotic layer of the retina. By the time the retina is fully postmitotic *Chx10* expression persists only within bipolar cells of the inner nuclear layer. In the absence of *CHX10* in patients, or in the *ocular retardation* (*Chx10*$^{or\text{-}J/or\text{-}J}$) mouse mutant carrying a null allele (Y176stop) of *Chx10*, eye development is severely impaired.[11,12] The phenotype is characterized by microphthalmia, (small eye) and blindness. Histologic analysis of the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina shows it to be thin and hypocellular. The optic nerve does not form, and the photoreceptors are abnormal with truncated outer segments[11,14,15] In the adult *Chx10*$^{or\text{-}J/or\text{-}J}$ eye, Müller glial cells and all types of retinal neurons have been detected, except bipolar cells, which are apparently absent.[11] Bromodeoxyuridine (BrdU) labeling of *Chx10*$^{or\text{-}J/or\text{-}J}$ shows reduced labeling indices in the peripheral retina compared with wild-type, although central retinal proliferation was not affected.[11] Expression of the transcription factors, retinoid-related orphan receptor-β and the forkhead gene *Foxn4*, both normally expressed in retinal progenitors, show decreased expression in the *Chx10*$^{or\text{-}J/or\text{-}J}$ mutant,[16,17] whereas the cyclin-dependent kinase inhibitor, p27^{kip1}which is normally expressed transiently in retinal cells as they exit the cell cycle, is abnormally

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Supported by Fight for Sight, United Kingdom. ADR and ND are Fight for Sight Research Students.

Submitted for publication April 1, 2003; revised August 13, 2003; accepted August 28, 2003.

Disclosure: **A.D. Rutherford**, None; **N. Dhomen**, None; **H.K. Smith**, None; **J.C. Sowden**, None

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present in progenitors of the *Chx10*$^{or\text{-}J/or\text{-}J}$ mutant.[18] Crossing *Chx10*$^{or\text{-}J/or\text{-}J}$ mice with p27^{kip1} null mice rescues much of the retinal size deficit, but bipolar cells are still absent.[18] In vitro, Chx10 protein has been shown to associate with the retinoblastoma protein and other members of the pRB gene family that are known to act as negative regulators of cell cycle progression.[19] Together these data suggest that *Chx10* has at least two essential roles in the developing retina: in retinal progenitor proliferation and in bipolar cell differentiation. The effect of the absence of *Chx10* on the expression of genes that are normally activated as cells leave the cell cycle and commit to a specific pathway of neuronal differentiation is unknown.

Rod photoreceptors are the most abundant cell type in the adult retina, and these are the cell types primarily affected in a variety of retinal dystrophies.[20] In vitro studies have shown the importance of extracellular signaling molecules for photoreceptor development.[21] The significance of these factors in vivo and how they mediate interactions between photoreceptors and other retinal neurons is largely unknown. The identification of the homeobox gene *Crx*, which is expressed in photoreceptors and plays a vital role in photoreceptor differentiation provides a useful marker for investigating photoreceptor development in vivo.[22,23] *Crx* is detected in the outer aspect of the embryonic day (E)12.5 murine retina in the prospective photoreceptor layer. *Crx* is necessary for the formation of outer segments of photoreceptor cells in vivo, and targeted disruption of the *Crx* gene prevents the development of outer segments and leads to degeneration of photoreceptor cells.[9,23] Mutations in the human homologue *CRX* cause cone–rod dystrophy and Leber congenital amaurosis.[24,25] The photoreceptor outer segment is a specialized region of the cell comprising a stack of disc membranes containing the proteins necessary for phototransduction. During development and morphogenesis of the outer segments, proteins of the outer segment plasma membrane and the disc membranes are sorted into their separate domains in a tightly choreographed process.[26] Many of the photoreceptor-specific genes, which are essential for outer segment morphogenesis, contain *Crx*-binding sites in their 5′ promoter regions, and their expression is reduced in *Crx* mutants, indicating that they are likely targets for *Crx* regulation.[22,27]

In the present study we addressed the question of how retinal differentiation is affected by the absence of Chx10 protein. To study the effect on photoreceptor development we analyzed the expression of the *Crx* transcription factor and its putative target genes in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina. To compare photoreceptor genesis with development of other retinal neurons in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina we analyzed expression of *Pax6* and *Pou4f2* (*Brn3b*), as these genes are known to be critical for retinal development and mark specific cell types as they begin to differentiate.[8,10,28] The *Pax6* homeobox gene is expressed throughout the developing optic vesicle and optic cup, and later expression is maintained in amacrine and ganglion cells.[29–31] The POU-domain transcription factor gene *Pou4f2* is expressed early on in ganglion cell differentiation and, although not required for the initial ganglion cell fate decision, is essential for ganglion cell differentiation and survival.[8,32–34]

We report that expression of *Crx*, and several of its downstream targets are severely delayed in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina and that this temporal disturbance of photoreceptor gene expression is likely to be the cause of failure of outer segment formation. By contrast, temporal expression of *Pax6* and *Pou4f2* in developing ganglion and amacrine cells is relatively normal in the mutant retina.

Materials and Methods

RNA In Situ Hybridization

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The *ocular retardation Chx10*$^{or\text{-}J/or\text{-}J}$ mice on the 129/Sv-Sl genetic background and wild-type mice, either C57Bl-6 or 129/Sv-Sl, were kept in a 12-hour light–dark cycle. Mice were paired in the evening, and when vaginal plugs were observed the following morning, 12.00 PM on that day was taken to be E0.5. Embryos were dissected with watchmaker forceps in cold PBS before fixation in 4% wt/vol paraformaldehyde in PBS overnight at 4°C. Tissue was dehydrated in ethanol, cleared in xylene, paraffin embedded, and cut into 6- to 8-μm sections.

In situ hybridization was performed, using a protocol modified from Breitschopf et al.[35] Sections were dewaxed in two changes of xylene and rehydrated through graded alcohols. Sections were postfixed in 4% paraformaldehyde in PBS for 10 minutes, washed for 15 minutes in three changes of Tris-buffered saline pH 7.5 (TBS), and partially denatured in 0.2 M HCl for 10 minutes and washed as before in TBS. Sections were acetylated for 10 minutes in 0.5% acetic anhydride in 0.1 M Tris (pH 8.0) and washed as before in TBS. The tissue was digested with proteinase-K for 10 minutes at a concentration of 50 $\mu g/mL^{-1}$ in a solution of TBS with 2 mM $CaCl_2$ and washed in TBS. Antisense and sense RNA probes were diluted in hybridization buffer consisting of 2× SSC, 10% dextran sulfate, 100 $\mu g/mL^{-1}$ sheared salmon sperm, and 50% formamide, and applied to sections overnight at 65°C. Nonhybridized probe was washed off with 1× SSC, 50% formamide at 60°C for 1 hour and then 20 minutes in 1× SSC and 10 minutes in 0.5× SSC at room temperature. Sections were then washed in 0.1 maleic acid buffer (pH 7.5) with 0.3% Tween 20 for 10 minutes and blocked in 1% blocking reagent (Roche Diagnostics, Lewes, UK) in maleic acid buffer for 30 minutes. Slides were incubated for 1 hour with 400 μL anti-digoxigenin alkaline-phosphatase–conjugated Fab fragments at a 1:500 dilution in 1% blocking solution and washed for 1 hour in maleic acid buffer. RNA probes were visualized by incubating in a 1:1 molar ratio of 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium (BCIP-NBT) in Tris pH 9.5, usually for 2 to 4 hours. Images were digitally captured and processed using image-analysis software (Photoshop 3.0; Adobe, Mountain View, CA). For comparisons between expression in wild-type and *Chx10*$^{or\text{-}J/or\text{-}J}$ retina, consistent results were detected in at least four independent experiments in tissue samples from at least three animals. No signal was detected with sense RNA probes. *Crx* expression was not detected in the embryonic *Chx10*$^{or\text{-}J/or\text{-}J}$ retina, even after extended (up to 24 hours) color development times. No differences in gene expression patterns were observed between the wild-type C57Bl-6 and 129/ Sv-Sl mice used in the study.

Histology

For histologic examination, dissected retinal tissue was fixed in 3% glutaraldehyde (pH 7.4), 0.1 M sodium cacodylate, and 5 mM calcium chloride; rinsed; and transferred to 1% osmium tetroxide for 2 hours before being processed and embedded in resin (Agar 100 Resin; Baltec, Lichtenstein, Austria) using standard procedures. Semithin sections were cut on a microtome (Ultracut E; Leica, Cambridge, UK) microtome with glass knives and stained with 1% toluidine blue in 1% borax. Observations were made in four P12 *Chx10*$^{or\text{-}J/or\text{-}J}$ and four 129/Sv-Sl mice. For each retina, replicate counts of the number of cells in the inner and outer nuclear layers were made from 1-μm semithin sections. For each retina, sections were prepared at 20-μm intervals at four positions across the central retina. Observations were restricted to the central retinal zone, avoiding the peripheral region where the cell layers are thinner. For the mutant, the analysis was performed on the thickest region of central retina in each section.

Reverse Transcription–Polymerase Chain Reaction

RNA was isolated with extraction reagent (RNAzol; Biogenesis, Poole, UK) for cDNA synthesis, according to the manufacturer's instructions. RNA was quantified by spectrophotometry and the presence of equivalent concentrations of intact RNA from all specimens confirmed by electrophoresis in denaturing ethidium-bromide–stained agarose gels. Identical amounts of RNA (1 μg) were used in 25-μL reverse transcription reactions using Moloney murine leukemia virus (MMLV) reverse transcriptase, oligodT, and dNTPs from Promega (Southampton, UK). Identical aliquots of first-strand cDNA were used in gene-specific RT-PCR reactions using *Taq* polymerase (Promega). PCR reactions conditions were typically 5 minutes of denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 60 seconds, and an extension at 72°C for 60 seconds, with a final extension at 72°C for 10 minutes. Thirty-four cycles of amplification were used for *Pou4f2*. Quantification of PCR products at 26, 28, 30, 32, 34, and 36 cycles using digital imaging of ethidium bromide stained gels (Alpha Imager; Alpha Innotech, San Leandro, CA) confirmed linear range of amplification. At each time point, RNA from three separate litters was analyzed in at least three independent RT-PCR amplifications. Sequences of gene-specific primers used in PCR reaction were as follows: Chx10 (343 bp) 5′-ATGACGGGGAAAGCGGGGGAAG/ 5′-CGCTCTGCCTAGTGGCTGCAAG; Crx (636 bp) 5′-TGCTTCAAGAATCGTAGGGC/ 5′-TGAAACTTCCAGGCACTCTG; Pou4f2 (531 bp) 5′-ATCGTCTCCCAGAGCAAGAG/ 5′-GACAGTGTGAGGGACTCGAAA; Irbp (576 bp) 5′-CCTGACAGTAAGTCTGCCTC / 5′-GTCCCAGGGAGCATTTTCTG; Pgm1 (417 bp) 5′-GAAAAATCAAAGCCATTGGTGGG /5′-GGCACCGAGTTCTTCACAGAGGAT; peripherin (644 bp) 5′-CAGATACGGCGGCCTAGATT/ 5′-CGTTGTTCCCACAGCACTTG; Pdeb (512 bp) 5′-GGGAAGAAGTTGAGCCCTGA/ 5′-TCACTGCCATGATCACAGCC; arrestin (523 bp) 5′-AAGACCTGGATGTACTGGGC/ 5′-ACTTGTACTGCGCGGTGCTA; and rhodopsin (490 bp) 5′-CTTTACCTAAGGGCCTCCAC/ 5′-GCAGCTTCTTGTGCTGTACG.

Immunohistochemistry

Immunohistochemistry was performed on retinal sections of eyes dissected from ocular retardation *Chx10*$^{or\text{-}J/or\text{-}J}$ and 129Sv-S1 wild-type mouse eyes. The eyes were fixed in 4% wt/vol paraformaldehyde in PBS overnight, placed in a 20% wt/vol sucrose solution overnight, and cryoembedded in optimal cutting temperature (OCT) compound (BDH Chemicals Ltd., Poole, UK). Seven-micrometer sections were cut and stored at −80°C until use. Sections were thawed for 15 minutes, rinsed in PBS for a further 15 minutes, and blocked with 10% normal goat serum and 0.2% Triton X-100 for 2 hours; 10% normal goat serum, 3% BSA and 0.1% Tween for 30 minutes; or 4% normal donkey serum and 1% Triton X-100 for 2 hours for treatment with PNA, anti-blue short-wavelength (S) cone opsin and anti-rhodopsin antibodies, respectively. The sections were incubated with rhodamine-conjugated PNA (1:10; Vector Laboratories, Peterborough, UK), chicken anti-blue cone opsin antibody (1:5000[36]), or goat anti-rhodopsin antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. PNA-treated sections were then incubated with Hoechst nuclear dye 33258 (1:1000 dilution of a 1.2 mg/mL solution; Sigma-Aldrich, St. Louis, MO) for 40 minutes and mounted with antifade mounting medium (Citifluor Ltd., London, UK). Anti-blue cone opsin– and anti-rhodopsin-treated sections were incubated with Alexa594-conjugated goat anti-chicken (1:100; Molecular Probes, Eugene, OR) and FITC-conjugated donkey anti-goat (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) antibodies respectively for 2 hours. The sections were then treated with Hoechst nuclear dye 33258 for 40 minutes and mounted with antifade mounting medium (Citifluor Ltd.). At each time point (postnatal day [P]7, P14–15, P20), results reported were reproduced in experiments on at least eight sections from two eyes. Some variation was observed in the density of blue cone opsin–positive cells across wild-type retinal sections, which is presumed to reflect the fact that S-cones are more abundant in the superior part of the retina.[37] No similar variation was seen in the mutant. Regions of highest densities of immunopositive cone cells are shown from mutant and wild-type retina in Figure 4.

Results

Timing of *Crx* Expression in the *Ocular Retardation* Retina

We compared the expression of *Crx* by using in situ hybridization in the *Chx10*$^{or\text{-}J/or\text{-}J}$ and wild-type retina to establish how the absence of Chx10 protein affects photoreceptor development. At E13.5 the wild-type retina comprises an inner and outer layer (Fig. 1A). The *Chx10*$^{or\text{-}J/or\text{-}J}$ eye at this stage is smaller, and only one retinal layer is visible (Fig. 1E). *Crx* mRNA was detected in the outer layer of the E13.5 wild-type retina (Fig. 1B), whereas *Crx* expression was not detected in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina at the same stage (Fig. 1F). Absence of *Crx* expression continued throughout gestation in the *Chx10*$^{or\text{-}J/or\text{-}J}$ embryos. By E18.5, lamination of the wild-type retina is well underway (Fig. 1I). The ganglion cell layer (gcl) is separated by the presumptive inner plexiform layer (ipl) from the developing inner nuclear layer (inl), which is distinct from the neuroblastic retina (nb). By contrast, the mutant retina appears unstratified (Fig. 1N). At E18.5 in wild-type embryos, *Crx* mRNA was detected in the prospective photoreceptor layer (Fig. 1J), consistent with the previously reported expression pattern,[23] whereas significant levels of expression were not detected in the same stage *Chx10*$^{or\text{-}J/or\text{-}J}$ retina (Fig. 1O).

By P12 in the mouse, the neural retina is mature and is organized into its characteristic three cellular layers (Fig. 1S). Mitosis has ceased, and all retinal neurons have been born.[4,38] The P12 *Chx10*$^{or\text{-}J/or\text{-}J}$ central retina has become partially stratified (Fig. 1W). A plexiform layer (ipl) of variable thickness is visible, separating a ganglion cell layer from a poorly stratified layer containing most of the cells. At this stage (P12) in the wild-type retina, *Crx* expression was limited to cells within the outer nuclear layer (Fig. 1T, onl). In contrast to the embryonic stages examined, *Crx* mRNA was detected in the postnatal *Chx10*$^{or\text{-}J/or\text{-}J}$ retina in a layer of cells on the outer aspect of the retina, adjacent to the retinal pigmented epithelium (Fig. 1X). A previous study reported the presence of rod photoreceptors in the P18 *Chx10*$^{or\text{-}J/or\text{-}J}$ retina, based on detection by rhodopsin immunostaining.[11] The layer of *Crx*-positive cells detected in the P12 mutant retina were presumed to be developing photoreceptors.

The distribution of the Y176stop mutation-carrying *Chx10* mRNA, *Chx10*(−), was also examined and compared with *Chx10* expression in wild-type mice. At E18.5 in the wild-type, *Chx10* and *Crx* were expressed in the outer retina, with *Chx10* mostly in the inner portion and *Crx* mostly in the outer portion (Figs. 1J, 1K). *Chx10* mRNA was not detected within the developing ganglion cell layer (Fig. 1K). By P12, *Chx10* mRNA was restricted to cells within the inner nuclear layer (Fig. 1U), consistent with its proposed role in bipolar cell development.[11,13] Point mutations often do not affect transcript stability. Analysis of expression patterns of *Chx10*(−) mRNA allowed examination of the distribution of *Chx10*(−)-expressing cells in the mutant retina. At E18.5, *Chx10*(−) mRNA was detected throughout the nonlaminated retina, except in a narrow band of cells on the vitreous side of the retina (Fig. 1P, arrows). By P12, *Chx10*(−) mRNA was restricted to a medially located layer of cells (Fig. 1Y). The layer of *Chx10*(−) expression was complementary to and did not overlap with expression of *Crx* on adjacent sections (Fig. 1X).

Two important points emerge from these observations. The *Chx10*(−) mRNA was correctly localized in the putative inner nuclear layer of the mutant retina, marking either postmitotic bipolar cells blocked in their differentiation pathway or other

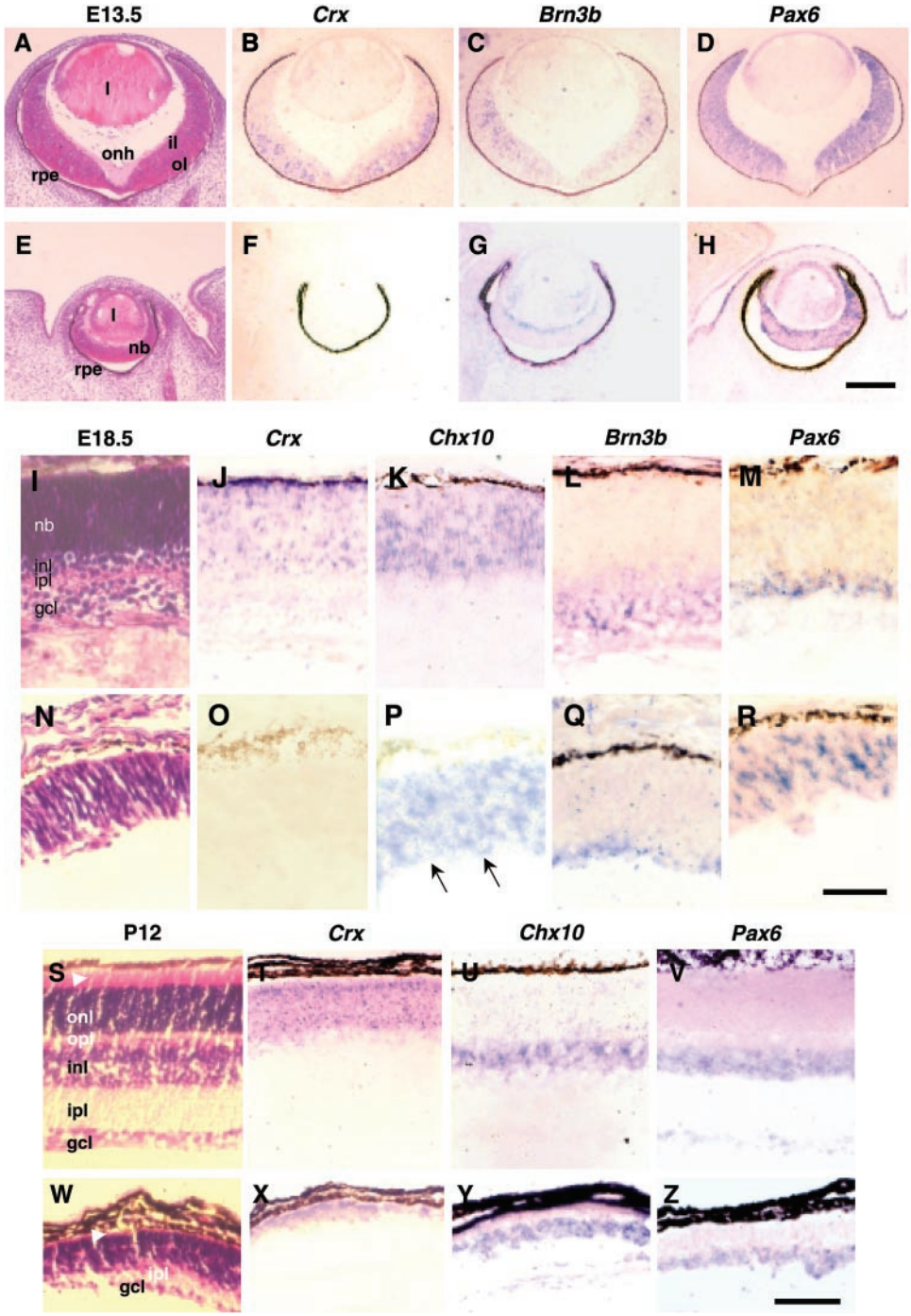


FIGURE 1. Expression of transcription factors in the *ocular retardation* retina at E13.5, E18.5, and P12. In situ hybridization using digoxygenin-labeled RNA probes for *Crx*, *Pou4f2*, and *Pax6*, as indicated. Rows of plates are organized to allow ease of comparison between expression of different genes at the same developmental stage. Midline sections through (**A**–**D**) wild-type and (**E**–**F**) $Chx10^{orJ/orJ}$ central retinas at E13.5. (**A**, **E**) Hematoxylin and eosin stain. (**B**) In the wild-type retina, *Crx* was expressed in the outer neuroblastic layer. (**C**) *Pou4f2* was expressed in the inner neuroblastic layer. (**D**) *Pax6* mRNA was detected throughout the neural retina. (**F**) In the $Chx10^{orJ/orJ}$ retina, *Crx* mRNA was not detected. (**G**) *Pou4f2* is detected in the innermost cells of the neural retina. (**H**) *Pax6* mRNA is detected within the neural retina and in surface ectoderm. Sections through (**I**–**M**) wild-type and (**N**–**R**) $Chx10^{orJ/orJ}$ central retinas at E18.5. (**I**, **N**) Stained with hematoxylin and eosin. (**J**) In the wild-type retina, *Crx* was expressed in the neuroblastic outer retinal layer. (**K**) *Chx10* was similarly expressed in the neuroblastic layer. (**L**) *Pou4f2* was expressed in the developing ganglion cell layer. (**M**) *Pax6* mRNA was detected within the developing inner nuclear layer (inl) and the ganglion cell layer (gcl). (**O**) In the $Chx10^{orJ/orJ}$ retina, *Crx* mRNA was not detected. (**P**) *Chx10*(−) mRNA carrying the Y176stop mutation was detected across the neural retina, except in the innermost vitreally located cells, indicated by *arrows*. (**Q**) *Pou4f2* was detected in the innermost cells of the neural retina. (**R**) *Pax6* expression was punctate throughout the neural retina. Sections through (**S**–**V**) wild-type and (**W**–**Z**) $Chx10^{orJ/orJ}$ central retinas at P12. (**S**, **W**) Stained with hematoxylin and eosin. In the wild-type retina, three cellular and two plexiform layers that are characteristic of the mature retina are visible. (**S**, *arrowhead*) Photoreceptor inner and outer segments. (**T**) *Crx* was expressed in the outer nuclear layer (onl). (**U**) *Chx10* was expressed in medially located cells of the inner nuclear layer. (**V**) *Pax6* mRNA was detected within cells in the inner aspect of the inner nuclear layer and in the ganglion cell layer. In the $Chx10^{orJ/orJ}$ retina, only the inner plexiform layer (ipl) and ganglion cell layer (gcl) are visible (**W**). Normal lamination of the outer retina has not taken place, and the photoreceptor inner and outer segments are shortened compared with the wild-type (**S** and **W**, *arrowheads*). (**X**) *Crx* mRNA was detected in a narrow layer of cells adjacent to the retinal pigmented epithelium. (**Y**) *Chx10*(−) mRNA was detected within the inner half of the outer retinal layer. (**Z**) *Pax6* mRNA was similarly located within cells of the inner retina. l, lens; il, inner neuroblastic layer; ol, outer neuroblastic layer; onh, optic nerve head; nb, neuroblastic layer; ipl, developing inner plexiform layer; opl, outer plexiform layer. Scale bar, 100 μm.

inner nuclear layer cells. The former seems most likely, because previous reports indicate *Chx10* is restricted to bipolar cells and colocalizes with the bipolar marker protein kinase C (PKC) at this stage of retinal development, and PKC staining is absent in the $Chx10^{orJ/orJ}$ retina.[11,13] Localization of *Chx10*(−) RNA to presumptive inner nuclear layer cells suggests that fated bipolar cells are born but do not differentiate in the absence of Chx10 protein. Variation in the level of *Chx10* mRNA (seen as strong blue staining) in the mutant (Figs. 1P, 1Y) compared with the wild-type retina (Figs. 1K, 1U) was not confirmed in RT-PCR experiments (Fig. 2) and is probably caused by variable color reactions. The absence of Chx10(−) mRNA in the outer *Crx*-positive layer of developing photoreceptors indicates that in these cells downregulation of *Chx10* transcription is independent of functional Chx10 protein. It is also notable that the putative photoreceptor (Crx positive/

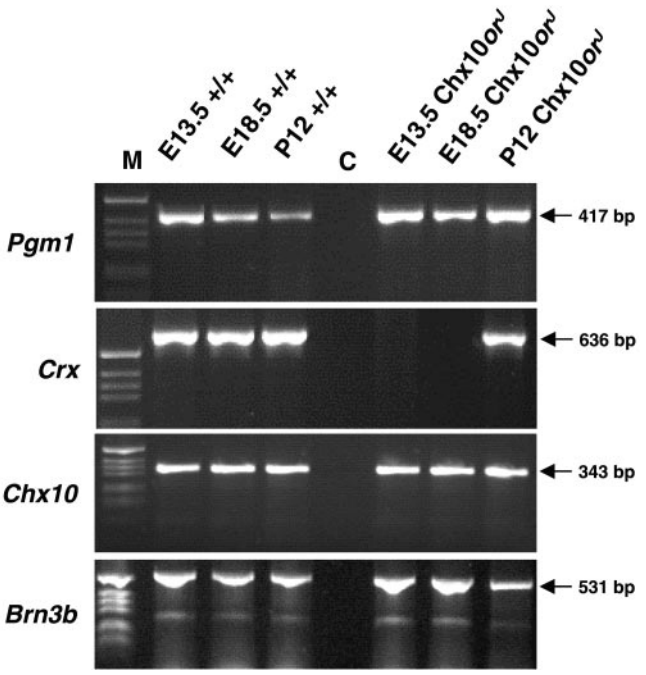


FIGURE 2. RT-PCR analysis of transcription factor gene expression in the *ocular retardation* retina during development. *Pou4f2* and *Chx10* mRNA were detected in wild-type (+/+) and *Chx10*$^{or\text{-}J/or\text{-}J}$ retinas at E13.5, E18.5, and P12, as was the ubiquitously expressed gene *Pgm1*. *Crx* was not detected at E13.5 and E18.5, but was detected at P12 in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina. By contrast, *Crx* expression was detected at E13.5, E18.5, and P12 in the wild-type retina. M, molecular weight standard. C, control without reverse transcriptase.

Chx10 negative) layer in the mutant retina had a considerably reduced thickness compared with that of the wild-type retina (Figs. 1T, 1X).

Early Expression of *Pou4f2* in Developing Ganglion Cells in the *Ocular Retardation* Retina

We compared the expression of *Pou4f2* and *Pax6* by in situ hybridization in the *Chx10*$^{or\text{-}J/or\text{-}J}$ and wild-type retina to establish how the absence of Chx10 protein affects ganglion and amacrine cell development.

Pou4f2 expression was detected in a proportion of cells within the inner layer of the wild-type retina (Fig. 1C), consistent with previous studies describing *Pou4f2* expression in newly developing ganglion cells.[8] *Pou4f2* expression was reduced in the *Chx10*$^{or\text{-}J/or\text{-}J}$ mutant at E13.5. Expression was detected only on the innermost aspect of the retina, indicating the presence of developing ganglion cells within the apparently unstratified mutant retina (Fig. 1G). In wild-type mice, but not in the *Chx10*$^{or\text{-}J/or\text{-}J}$ mice (Fig. 1G) *Pou4f2* expression was detected in presumptive ganglion cells migrating toward the developing ganglion cell layer (Fig. 1C).

By E18.5 in the wild-type retina, *Pou4f2* expression was abundant within the forming ganglion cell layer (Fig. 1L), and both a nerve fiber layer and inner plexiform layer were visible (Fig. 1I). By E18.5 in the mutant *Chx10*$^{or\text{-}J/or\text{-}J}$ retina, *Pou4f2* expression was reduced to a narrow band of presumptive ganglion cells on the inner aspect of the retina (Fig. 1Q). These are cells that did not express the *Chx10*(−) mRNA (Fig. 1P). No lamination or nerve fiber layer is visible in the mutant retina (Fig. 1N). An optic nerve does not form in *Chx10*$^{or\text{-}J/or\text{-}J}$ mice on the 129/Sv-Sl genetic background[14] and was not visible in sections throughout specimens analyzed in this study. Based on the expression of *Pou4f2* in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina, ganglion cell birthing is occurring in a temporally and spatially correct manner in the mutant retina, although the absence of optic nerve formation indicates other aspects of ganglion cell development are impaired. Optic nerve aplasia in the *Chx10*$^{or\text{-}J/or\text{-}J}$ mouse has been ameliorated by crossing with another strain,[39] and the optic nerve is present in two human cases homozygous for *CHX10* mutations.[12] These findings indicate that the optic nerve phenotype is affected by genetic modifiers, and the absence of Chx10 does not cause a primary defect in ganglion cell development.

At E13.5 *Pax6* expression, normally seen throughout the E13.5 retina, was more sparse in the mutant (Figs. 1D, 1H). At E18.5 *Pax6* expression, which normally occurs in early inner nuclear layer cells and the developing ganglion cells, was found scattered throughout the unstratified abnormal retina in the mutant (Figs. 1M, 1R). *Pax6*-expressing cells are presumed to be differentiating ganglion and amacrine cells, based on previous reports,[29–31] and the expression pattern suggests that in the mutant these cells are born independent of the process of lamination. Downregulation of *Pax6* mRNA levels is occurring in the absence of Chx10 protein in approximately 50% of retinal cells.

At P12, normal *Pax6* expression was on the vitreous side of the inner nuclear layer and in the ganglion cell layer (Fig. 1V). *Pax6* expression in the mutant is in the inner most region (Fig. 1Z), previously shown to include ganglion cells as well as amacrine cells.[11] These *Pax6* expressing cells are presumed to be ganglion and amacrine cells that have now migrated to appropriate locations across the laminar axis of the retina. *Pax6* expression overlapped with the *Chx10*(−)-expressing cells in the inner layer of the mutant retina (Figs. 1Y, 1Z, and data not shown), and so the possibility that *Chx10*(−) mRNA is localized within amacrine cells cannot be excluded.

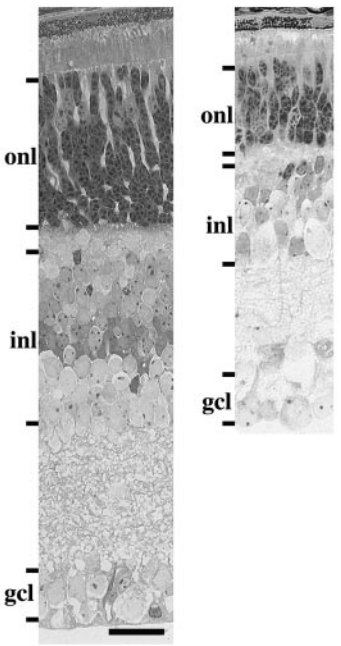
Herein, we report that the temporal profile of early ganglion cell and amacrine development is not significantly affected by the absence of Chx10. Ganglion and amacrine cell birth, as defined in [^{3}H]thymidine labeling studies, spans the period from E10.5 to P3.[4,38] Both cell types are early-born retinal neurons compared with the late-born bipolar cells and rod photoreceptors whose birth dates span the period from E14 to P12 and E12 to P12, respectively.

Comparison of Profiles of Transcription Factor Gene Expression

We used RT-PCR as an independent method for comparing gene expression profiles of *Pou4f2*, *Chx10*(−), and *Crx* in the mutant retina between E13.5 and P12. Approximately equivalent levels of *Pou4f2*, *Chx10,* and the ubiquitously expressed gene phosphoglucomutase 1 (*Pgm1*) cDNA were amplified from equal amounts of RNA extracted from eyes dissected at E13.5, E18.5, and P12 (Fig. 2). Expression of *Pou4f2* in *Chx10*$^{or\text{-}J/or\text{-}J}$ and wild-type retina is consistent with the in situ hybridization results. Amplification of *Chx10*(−) mRNA confirms its stability, despite the mutation. In agreement with the in situ hybridization data, *Crx* mRNA was not detected at E13.5 in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina. By E18.5, *Crx* mRNA was either not detected (Fig. 2; $n = 2$ litters) or a weak amplification product was detected ($n = 1$ litter; data not shown). *Crx* mRNA was readily detected at levels approximately equivalent to those in wild-type eyes from P1 to P12. These data suggest that embryonic expression of *Crx* is largely absent or significantly reduced compared with the wild-type retina. After birth, *Crx* expression is approximately equivalent to that of the wild-type.

Reduced levels of proliferation have been detected in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina as early as E11.5, and the reduced size of the mutant retina compared with wild-type retina is apparent from E11.5.[11] Our data indicate that development of different

A



B

Comparison of retinal tissues from wild-type (+/+) and *Chx10*$^{or\text{-}J/or\text{-}J}$ (-/-) mice

Chx10 status	Nuclear cell layer (no of nuclei)	
	inner	outer
+/+	8.8 +/- 1.2	11.7 +/- 0.8
or-J/or-J	4.0 +/-0.9	4.2 +/- 1.4

Observations were made on four eyes in each category. Numbers refer to the mean values +/- SD.

FIGURE 3. (**A**) Semithin sections of wild-type (*left*) and *Chx10*$^{or\text{-}J/or\text{-}J}$ (*right*) retinas. Toluidine blue–stained sections of central retina showing reduction in thickness of outer and inner nuclear layers. (**B**) Comparison of thickness, by number of nuclei, of retinal tissues from wild-type (+/+) and *Chx10*$^{or\text{-}J/or\text{-}J}$ (−/−) mice. onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 25 μm.

types of retinal neuron in the embryonic retina may be differentially affected by absence of Chx10. Specifically, it appears that the development of photoreceptors is severely disrupted in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina.

We performed histologic analysis using semithin retinal sections to address the question of whether there are proportionally fewer photoreceptors (compared with other cell types) in P12 *Chx10*$^{or\text{-}J/or\text{-}}$ mice. Comparison of the number of cells in the outer and inner nuclear layers confirmed that both layers showed a significant reduction (Fig. 3). The reduction was most marked in the outer nuclear layer.

Analysis of Cone and Rod Photoreceptor Formation in *Ocular Retardation* and Wild-Type Retina

Because our data indicated that the development of photoreceptors is disrupted in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina, we sought to distinguish whether both cones and rods were affected. This point is of interest because cones are generated early along with amacrine and ganglion cells, whereas rods and bipolar cells are born late.[4] We wanted to identify whether the absence of Chx10 and delay of *Crx* expression affects both rods and cones and/or whether late-born cells are more disrupted than early-born cells. To investigate we looked specifically at cone photoreceptor formation. We used cone cell-specific PNA-rhodamine staining (red fluorescence; Figs. 4A–F) and immunostaining for the blue S cone opsin gene (Figs. 4G–L) to compare cone development in the postnatal *Chx10*$^{or\text{-}J/or\text{-}J}$ and wild-type retinas. Immunostaining for rhodopsin was also performed to compare rod development at the same stages (Figs. 4M–R).

PNA labels all developing cone types.[40] By P14 in both the wild-type and the mutant retina distinct cone outer segments were detected with PNA (Figs. 4B, 4E). Blue cone opsin–

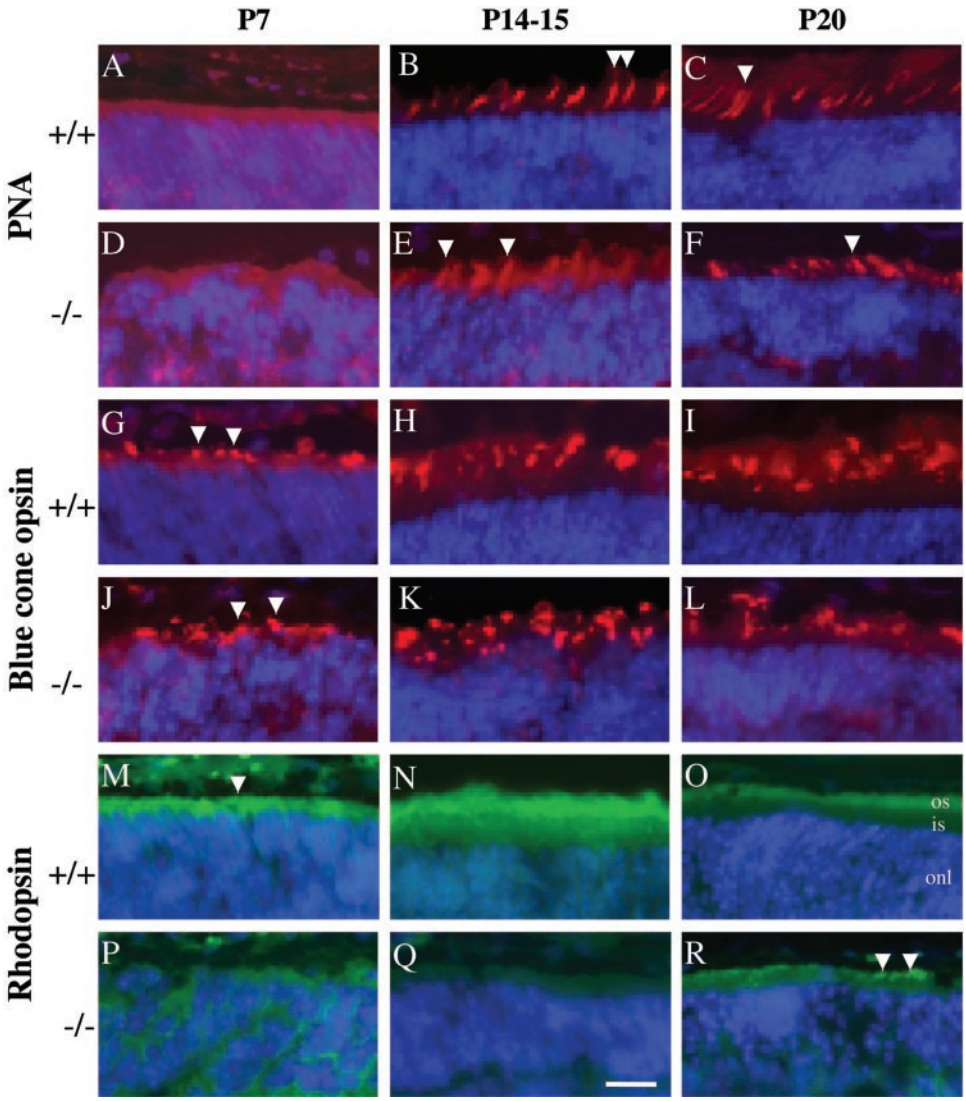


FIGURE 4. Early-born cone photoreceptors are appropriately generated in wild-type and *Chx10*$^{or\text{-}J/or\text{-}J}$ mutant retinas, but show abnormal morphogenesis in the mutant retina. Cone photoreceptors of wild-type (+/+) and *Chx10*$^{or\text{-}J/or\text{-}J}$ mutant (−/−) retinas were labeled with PNA (**A**–**F**, *red*) and blue cone opsin (**G**–**L**, *red*) at P7, P14–15, and P20. PNA staining of cone outer segments was first observed at P14–15 in both the wild-type (**B**, *arrowheads*) and mutant (**E**, *arrowheads*) retinas. By P20, the outer segments of the cone photoreceptors of the mutant retina appeared shorter than the cones of the wild-type retina at the same stage (compare **F** with **C**, *arrowheads*). Blue cone opsin labeled blue cone outer segments as early as P7 in wild-type and mutant retinas (**G**, **J**) and expression persisted at later stages (**H**, **I**, **K**, **L**) in both. By contrast, rhodopsin labeling (**M**–**R**, *green*) was observed at P7 in the developing outer segments of rod photoreceptors (**M**, *arrowhead*) and continued to be in the outer segments of the wild-type retina at later stages (**N**, **O**), but was not expressed in the mutant retina until P20 (**R**), where it was only sparsely distributed (*arrowheads*). Hoechst 33258 nuclear dye was used to label the nuclei of the photoreceptor layer (*blue*) in all sections. os, outer segments; is, inner segments; onl, outer nuclear layer. Scale bar, 20 μm.

positive outer segments were detected in both the mutant (Fig. 4J) and the wild-type retina (Fig. 4G) at P7 and at later stages (Figs. 4H, 4I, 4K, 4L), consistent with previous reports of wild-type expression patterns.[37] The cones were present at similar densities within the wild-type and mutant (Figs. 4B, 4E). At P20 the cone outer segments were reduced in length in the mutant (Fig. 4F) compared with the wild-type (Fig. 4C). By contrast, rhodopsin was localized in the developing outer segments at P7 (Fig. 4M) and P15 (Fig. 4N) in the wild-type but similar expression was markedly absent in the mutant (Figs. 4P, 4Q). At P20 the rhodopsin was specifically localized throughout the rod outer segment layer in the wild-type retina (Fig. 4O). By this stage, the mutant retina showed more sparsely distributed rhodopsin-positive outer segments with an abnormal truncated appearance (Fig. 4R, see also Fig. 3). These data show that the cone photoreceptors are generated relatively normally in the absence of *Chx10*, suggesting that generation of early-born cells is less disrupted than that of late-born cells. It follows that the reduction in cell number in the outer nuclear layer (Fig. 3) is due to a rod deficit. These data also indicate that the absence of Chx10 is affecting the differentiation of photoreceptors as outer segment morphogenesis is abnormal for rods and cones. It seems likely that this phenotype could result from the delay in Crx expression in the mutant.

Analysis of Expression of Putative Targets of Crx in *Ocular Retardation* and Wild-Type Retina

A number of genes that are expressed in photoreceptors are proposed to be targets of transcriptional regulation by Crx protein. These photoreceptor genes contain the *Crx* consensus binding sequence in their 5′ upstream regions and/or are downregulated in the Crx$^{-/-}$ retina.[9,22,27] We examined the expression of several of these genes, rhodopsin, peripherin, interphotoreceptor retinoid-binding protein (*Irbp*), rod phosphodiesterase-β (*Pdeb*), and arrestin, by RT-PCR and found that with the exception of *Irbp*, temporal expression of all these genes was disrupted in the mutant (Fig. 5). Rhodopsin mRNA was readily detectable from E13.5 in the wild-type mouse (Fig. 5). By contrast, rhodopsin transcripts were not detectable at E13.5 and E18.5 in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina. Rhodopsin mRNA was first detected at P2 (data not shown), 1 day later than *Crx* mRNA was first detected in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina. Subsequent postnatal expression of rhodopsin was equivalent to wild-type expression. Expression of *Pdeb* was not detected at all stages examined, compared with increasing levels of expression during wild-type retinal development. Levels of arrestin and peripherin mRNA were reduced compared with expression in the wild-type. By contrast *Irbp* expression appeared equivalent in *Chx10*$^{or\text{-}J/or\text{-}J}$ and wild-type retina.

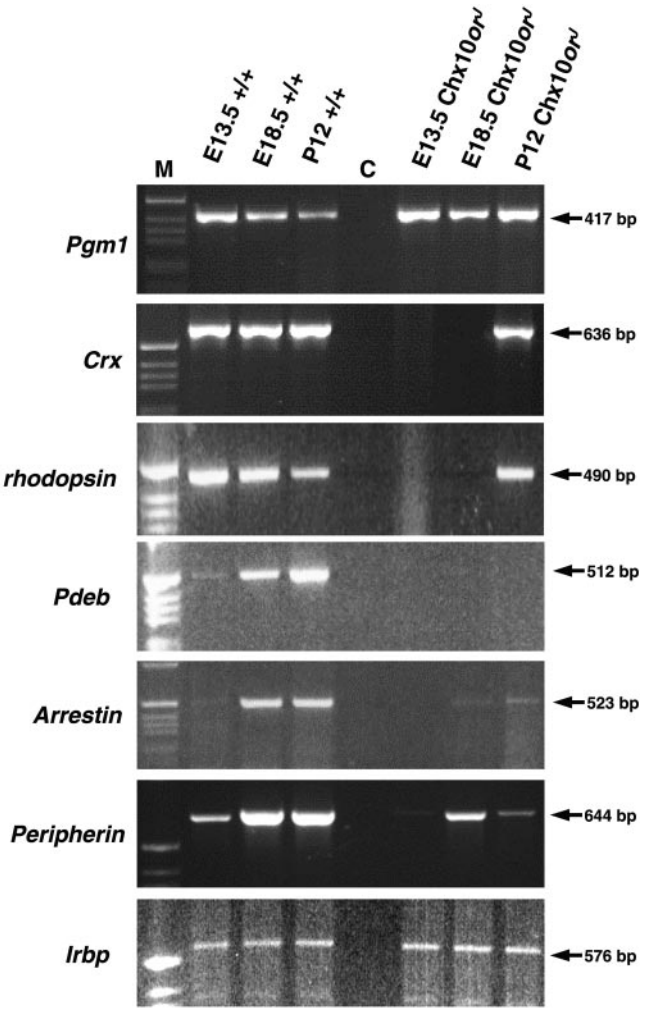


FIGURE 5. RT-PCR analysis of photoreceptor gene expression during development in the *ocular retardation* retina. Expression of photoreceptor-specific genes, with *Crx* consensus binding motifs in their promoter regions, was investigated by RT-PCR in retinas at E13.5, E18.5, and P12. *Left*: wild-type (+/+); *right*: *Chx10*$^{or\text{-}J/or\text{-}J}$. Expression of the ubiquitously expressed gene *Pgm1* was detected in all samples. A relatively high level of rhodopsin signal at E13.5 was matched by a high level of expression of Pgm1 at this stage and was not considered significant. The signal probably reflects small variations in RNA sample quality. Onset of expression of *Crx*, *Pdeb*, and arrestin occurred later in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina than in the wild-type. Peripherin was readily detected by E18.5 in the mutant. Expression of *Irbp* was unaffected by lack of *Crx* in the mutant retina, and expression was detected at all stages examined. M, molecular weight standard; C, control without reverse transcriptase.

The delay in expression of photoreceptor genes observed here in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina is consistent with the hypothesis that delayed onset of *Crx* expression impairs normal photoreceptor differentiation. Mice with homozygous targeted disruption of *Crx* show reductions, but not complete absence, in expression of rhodopsin, *Pdeb*, arrestin, and peripherin, indicating that *Crx* is essential for expression of these photoreceptor specific genes, but is not the sole regulator.[9] Kimura et al.,[41] demonstrated that high-level photoreceptor specific gene expression required the presence of photoreceptor conserved element 1 (PCE1)/Rx interactions as well as OTX element/Crx interactions. Expression of cone opsins, which are also putative targets of Crx regulation were not examined by RT-PCR in this study. However, immunostaining for blue opsin (Fig. 4) suggests that expression is not delayed and this is consistent with previous findings that blue and green cone opsin are first expressed postnatally[42,43] and so are not likely to be affected by the absence of embryonic *Crx* expression.

The finding that *Irbp* expression is not affected in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina is consistent with our previous report that *Irbp* is expressed later than *Crx* during human and mouse development.[42] Levels of expression of *Irbp* in the *Crx*$^{-/-}$ mouse are also normal.[9] Although in vitro, Crx is capable of transactivating the *Irbp* promoter,[22] these in vivo data and evidence that *Irbp* is expressed in dividing retinal cells in culture,[44] strongly indicate that Crx is not the sole activator of *Irbp* expression. Cotransfection experiments show that the human *OTX2* homeobox transcription factor as well as Crx activates the Irbp promoter,[45] and in vivo in the *Chx10*$^{or\text{-}J/or\text{-}J}$ and Crx$^{-/-}$ mice, OTX2 may compensate for the absence of Crx.

The presence of photoreceptor specific genes such as *Irbp* in the embryonic *Chx10*$^{or\text{-}J/or\text{-}J}$ retina before expression of *Crx*, suggests that prospective photoreceptor cells are present, but their Crx-dependent differentiation pathways are severely disrupted. Hence, fated photoreceptor cells are born prenatally, but do not express *Crx*. This disruption of normal differentiation is likely to affect the development of outer segments in photoreceptors in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina. The similarity between the photoreceptor phenotypes of *Chx10*$^{or\text{-}J/or\text{-}J}$ homozygotes and the *Crx* null homozygotes supports the view that *Crx* is essential for the regulation of the cascade of genes required for inner and outer segment formation and maintenance.

DISCUSSION

In this study we examined the effect that the absence of the transcription factor Chx10 has on the coordination of neuronal development in the retina. In particular, we focused on the development of photoreceptors to gain a better understanding of the molecular and cellular mechanisms that cause the low number of photoreceptors and the truncated appearance of the photoreceptor outer segments (Figs. 1S, 1W, 3, 4) in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina.

The study reports two main findings regarding photoreceptor development. The expression of *Crx* is delayed during embryonic development, and the putative targets of Crx regulation are also delayed in their expression. These include proteins that are essential for the structural integrity of rod and cone photoreceptor outer segments. Our analysis suggests that differentiation of rod and cone outer segments is abnormal in the mutant.

Relatively little is known about the molecular mechanisms that mediate outer segment morphogenesis. Homozygous mutations in peripherin, located in disc rims, result in a complete failure of outer segment formation and in heterozygotes, outer segments are reduced and appear abnormal.[46,47] Similarly, mutations in rhodopsin result in failure of outer segment formation. A requirement for expression of rhodopsin in a discrete window of developmental time has been suggested by experiments in *Drosophila ninaE* mutants. The *ninaE* gene encodes rhodopsin in the fly and is expressed in the rhabdomere, a structure equivalent to vertebrate outer segments. Although lack of rhabdomere development in *ninaE* flys can be rescued by appropriate expression of a *ninaE* transgene, delaying expression of the transgene until later than the normal window of expression fails to rescue.[48] It seems likely that the disruption of normal temporal expression of genes essential for photoreceptor disc morphogenesis leads to failure of correct outer segment formation in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina.

In this study, we report that development of specific cell types appear to be more disrupted by absence of Chx10 than

others. Namely, we have shown that photoreceptor development is affected and lack of bipolar cells has previously been reported. By contrast, expression patterns of *Pou4f2* and *Pax6* in differentiating ganglion and amacrine cells are relatively normal within the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina suggesting that these cells are being generated appropriately. Cones also appear to be generated appropriately, consistent with the conclusion that the reduced thickness of the outer nuclear layer is due to a rod deficit. It is striking that the generation of early-born cells appears normal at the expense of the late-born neurons (bipolar cells and rods). Progenitor cells exit the cell cycle in the G1 phase and differentiate. Appropriate terminal exit from the cell cycle is likely to be important for determining the size of the adult retina. In the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina, coordination between cell proliferation and differentiation appears to be disrupted. Why early- and late-born cells are differentially affected in the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina remains to be discovered, but is likely to reflect aspects of the normal role of Chx10.

Recent work has shown that progenitor cells in the developing retina are heterogeneous in terms of the components of the cell cycle machinery that they express.[49,50] Such heterogeneity may result in different consequences of absence of Chx10. Alternatively, Chx10 may play a specific role in bipolar and photoreceptor progenitors. Our findings support a direct role for Chx10 in coordinating Crx activity during the early phase of retinal development (possibly in progenitor cells fated to become bipolar and photoreceptor cells). Indeed, it has been proposed that Chx10 is a repressor of transcription acting to prevent presumptive bipolar cells from adapting a photoreceptor cell fate during development by repressing Crx-dependent activity[51] (Bremner R, Chen S, Zack DJ, unpublished results, 2002). An in vitro study lends support to the existence of a bipolar-photoreceptor progenitor by identifying postmitotic cells that were fated to express the photoreceptor marker opsin, whose fate could be switched by the addition of ciliary neurotropic factor (CNTF) to that of a bipolar cell, expressing PKC.[52] As Crx and Chx10 proteins have not been colocalized so far within the same cell during development, a direct role for Chx10 on *Crx* transcription seems more likely than a protein–protein interaction between these two homeodomain proteins. It is interesting in this context to note that *Crx* expression has been reported in bipolar cells.[42,51] However, an indirect (even non-cell autonomous) mechanism could equally explain our findings possibly with Chx10 regulating expression of secreted factors that promote photoreceptor development.

To date, no downstream targets regulated by *Chx10* have been identified, and their identification is the focus of future work. Components of the cell cycle machinery are possible candidates, particularly those that appear to have specialized roles during retinal development. Mice without cyclin D1 have a hypoplastic retina,[53] and, similar to the *Chx10*$^{or\text{-}J/or\text{-}J}$ retina, the outer nuclear layer is particularly affected, suggesting that these genes may act in a common pathway. Recent work indicates that Chx10 interacts with the p27^{Kip1} cell cycle regulator through a mechanism that is largely dependent on cyclin D1 but both these genes appear to be indirect targets of Chx10 transcriptional regulation.[18]

Acknowledgments

The authors thank Ray Lund for the *ocular retardation* mice, David Latchman and Veronica van Heyningen for *Pou4f2* and *Pax6* cDNAs, and Brian Young for technical assistance.

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