Pax-6, Prox 1, and Chx10 Homeobox Gene Expression Correlates With Phenotypic Fate of Retinal Precursor Cells

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Purpose. To study the expression patterns of the homeobox genes Pax-6, Prox 1, and Chx10 during chick retinal development in vivo and in vitro.

Methods. Sections of paraformaldehyde-fixed, paraffin-embedded eyes were obtained at a range of developmental stages. In situ hybridization was carried out on tissue sections using digoxigenin-labeled sense and antisense RNA probes that recognize chicken Pax-6 and Prox 1 (whose sequences were already available), and chicken Chx10 (which was cloned and sequenced as part of this study). Selected developmental stages were also studied by immunocytochemistry with antibodies against Pax-6 and Prox 1, and by Northern blot analysis using 32P-labeled probes.

Results. Until embryonic day (ED) 5, in situ hybridization shows widespread, diffuse distribution of all three genes. Between ED 6 and ED 8, however, they acquire distinct, topographically specific patterns of expression. The Prox 1 signal is predominantly expressed in the prospective horizontal layer of the neuroepithelium, decreases vitreally, and is absent from ganglion cells and the prospective photoreceptor layer. Pax-6 is strongly expressed only in the prospective ganglion-cell and amacrine-cell regions at the same stages, and is not detected in prospective photoreceptors. Chx10 expression becomes concentrated in the future bipolar-cell region of the inner nuclear layer. Similar patterns are maintained by ED 15 through ED 18, after cell differentiation has taken place. Pax-6 and Prox 1 immunoreactive materials showed nuclear localization and a pattern of laminar distribution equivalent to that seen by in situ hybridization.

Conclusions. These results suggest that the differentiated fate of retinal precursor cells may be influenced by Pax-6, Prox 1, or Chx10; this hypothesis is now being tested using dissociated chick embryo retinal cell cultures. Invest Ophthalmol Vis Sci. 1997;38:1293-1303.

The mature neural retina contains an assortment of postmitotic, differentiated cell types that occupy precise positions within its distinctively laminated structure. At early stages of embryonic development, however, the retina consists of a simple, pseudostratified neuroepithelium, in which all cells are capable of mitotic division. The life history of each mature cell involves a series of divisions, a terminal mitosis, migration of the postmitotic precursor to one of the retinal layers, and the coordinated expression of distinctive differentiated properties. The stage at which precursor cells become committed to specific phenotypic fates is unknown, although findings in lineage-tracing studies have shown that proliferating neuroepithelial cells remain multipotent at least until a time close to their terminal mitosis. Specification at the time of cell birth has been proposed based on the detection of antigenic "markers" in precursor cells shortly after terminal mitosis; conversely, results of in vitro studies have shown that the differentiated fate of postmitotic precursor cells changes as a function of their length of exposure to the retinal microenvironment before their isolation for culture. The possible exist-
tence of “master programs” controlling retinal cell differentiation has been suggested by experiments testing the developmental potential of undifferentiated retinal precursor cells from chick embryo, grown in the absence of contact-mediated cell interactions in low-density cultures. The underlying genetic mechanisms, however, have not been elucidated. Considerable attention has been focused in recent years on genetic mechanisms regulating cell differentiation. Progress has been impressive in the case of Drosophila in general and its compound eye in particular, in which a large number of DNA-binding transcription factors have been shown to control cell differentiation through the coordinated activation of networks of cell-specific genes. The involvement of regulatory genes in the control of retinal cell differentiation has now been conclusively demonstrated in vertebrates, but a variety of candidate genes are expressed in the dividing retinal neuroepithelium, whereas others show a laminar pattern of retinal expression that correlates with the distribution of differentiating cell types. These include Pax-6, expressed in the region occupied by putative ganglion and amacrine cells in the quail, two related homeobox genes, Chxl0 and Vax-1, present in the inner nuclear layer and concentrated in bipolar cells in the mouse, and the goldfish, respectively, and members of the Brn-3 family of POU-domain factors, which are expressed in retinal ganglion cells. Other genes showing topographically restricted retinal expression patterns have been described.

To investigate whether distinctive sets of putative regulatory genes are expressed in differentiating retinal cell types in the chick embryo retina, we have used in situ hybridization and immunocytochemistry to compare the temporal and spatial patterns of expression of three candidate genes, Pax-6, Prox 1, and Chxl0. Pax-6 is the vertebrate homologue of the Drosophila eyeless gene, whose ectopic expression leads to the development of histologically normal eyes in the fly. Pax-6 mutations cause the small eye phenotype in mice, and a complex syndrome in humans that includes aniridia and foveal abnormalities in heterozygotes. The chicken homeobox gene Prox 1 is homologous to the mouse Prox 1 and to the Drosophila propero genes; the latter has been shown to be important in neuroblast differentiation in the fly. The third gene, Chxl0, belongs to the paired family of homeobox transcription factors and was initially cloned in the mouse by Liu et al. Abnormalities in mouse Chxl0 are the bases for the ocular retardation mutation (or) that causes microphthalmia in heterozygotes.

We report here that all three genes show a widespread, diffuse distribution in the proliferating retina neuroepithelium in the chicken embryo but acquire distinct, topographically specific patterns of expression as postmitotic cells become organized into layers between ED 6 and ED 8. These results suggest that the differentiated fate of retinal precursor cells may be influenced by Pax-6, Prox 1, and Chxl0, either individually or in combination.

**MATERIALS AND METHODS**

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Materials**

Reagents used were from the following suppliers: protease K; formamide; RNase A; 0.25% trypsin, medium 199 (Gibco BRL, Indianapolis, IN); Vectabond, biotinylated goat antimouse IgG, RTIC Avidin D (Vector Labs, Burlingame, CA); Triton X-100; glycine; heparin; Tween 20; Denhardt’s solution; EDTA; polyvinyl alcohol; acetic anhydride (Sigma, St. Louis, MO); tRNA; CHAPS; Genius kit; triethanolamine; T; RNase (Boehringer Mannheim, Indianapolis, IN); Aquament (Poly sciences, Warrington, PA); PGE2 (Pharmacia, Piscataway, PA); protein A affinity columns (Pierce Chemicals, Rockford, IL) fetal calf serum (Atlanta Biologicals, Norcross, GA); and culture chamber slides (Fisher Scientific, Pittsburgh, PA). Other chemicals were of analytical grade and were purchased from Sigma.

**In Situ Hybridization**

Digoxigenin-labeled sense and antisense RNA probes were synthesized according to Li et al. The Pax-6 probes corresponded to a 512 bp segment extending to the 3’ end of the chicken Pax-6-coding region. Probes for two different regions of the chicken Prox 1 gene were made: Probe A encompassed 838 nucleotides encoding amino acids 458 to 735, and probe B was 661 nucleotides long, and corresponded to amino acids 218 through 438. Chxl0 probes were synthesized from a 1.2 kb segment of the chick Chxl0 3’ untranslated region.

RNase–free solutions and containers were used throughout the in situ hybridization protocol. Tissues were dissected in 0.1 M phosphate buffered saline (PBS), fixed in 4% paraformaldehyde at 4°C, either for 4 hours or overnight, rinsed in PBS at 4°C, and embedded in paraffin according to standard protocols. Twelve micron sections were collected on slides coated with Vectabond, dried at 40°C for 2 to 3 hours, and stored at −70°C until processed. For hybridization, sections were heated to 65°C for 15 minutes, deparaffinized in xylene and absolute EtOH, and al-
allowed to dry for 15 minutes. Sections were then treated with 0.3% Triton X-100 in PBS at 37°C for 15 minutes; digested for 10 minutes with 10 μg/ml proteinase K in PBS at 37°C; rinsed in 100 mM glycine, pH 7.2; dehydrated with ethanol; and air-dried. Sections were then covered with 110 μl of 400-ng/ml probe solution in a hybridization mixture containing 50% formamide, 5 × SSC (1 × SSC: 8.8 g NaCl/1; 4.4 g sodium citrate/1, pH 7); 100 μg/ml heparin; 1% Tween 20; 1 mg/ml tRNA; 1 × Denhardt’s solution; 0.1% CHAPS; and 5 mM EDTA; covered with a coverslip, and incubated overnight at 63°C in a humid chamber. Posthybridization washes were: two in 50% formamide in 2 × SSC for a total of 1 hour at 60°C; two in 2 × SSC at 37°C, for a total of 30 minutes; one in RNase A and T1, RNase in 2 × SSC for 30 minutes at 37°C; one in 50% formamide, 0.1% CHAPS, in 2 × SSC for 15 minutes at 60°C; and one in 0.1% Tween 20 in 0.2 × SSC for 15 minutes at 45°C. Sections were then processed as recommended by the Genius kit manufacturer (Boehringer Mannheim) except that a polyvinyl alcohol-containing solution was incorporated into the alkaline phosphatase development reaction mix according to Barth and Ivarie. After an overnight incubation at 37°C, sections were briefly rinsed in TE (10 mM Tris, 1 mM EDTA, pH 7.5), and distilled water, and mounted with Aquamount.

**Immunocytochemistry**

Rabbit antibodies were used against Pax-6 (a kind gift of Dr S. Saule), and Prox 1. To generate the latter, a human Prox 1 cDNA fragment encoding the homeo-domain and C-terminal prospero domain (position 546 to 736; amino acid sequence identical in human and chicken) was subcloned in pGEX-2 and expressed as a fusion protein with glutathione-S-transferase according to standard procedures. The fusion protein, purified by glutathione-affinity chromatography, was used to immunize rabbits (performed as a service by Boehringer Mannheim) except that a polyvinyl alcohol-containing solution was incorporated into the alkaline phosphatase development reaction mix according to Barth and Ivarie. After an overnight incubation at 37°C, sections were briefly rinsed in TE (10 mM Tris, 1 mM EDTA, pH 7.5), and distilled water, and mounted with Aquamount.

**Northern Blot Hybridization**

Ten micrograms of total RNA were separated by electrophoresis on a 1.2% agarose–22 M formaldehyde gel, transferred to a Duralon UV membrane (Strategene, La Jolla, CA), cross-linked by ultraviolet irradiation and hybridized with [32P] probes for Pax-6, Prox 1, or Chxl10 (see above) in QuikHyb solution (Strategene), as recommended by the manufacturer. Filters were stained with 0.02% methylene blue after autoradiography for normalization of the amount of loaded RNA as described.

**Cloning and Sequencing of Chicken Chxl10 cDNA**

An ED 8 chicken eye library prepared in Agt11 (a gift from C. Cepko) was screened according to standard procedures using as a probe a HindIII human Chxl10 cDNA restriction enzyme fragment (2.6 kb) including the entire coding region and most of the 3′ untranslated region. The cDNA fragment was labeled with [32P] dCTP (DuPont–NEN) by the method of Feinberg and Vogelstein. Hybridization was carried out overnight at 42°C at reduced stringency, in 40% formamide, 3 × SSC, 1 × Denhardt’s reagent, 50 mM sodium phosphate (pH 6.8), 200 μg/ml sheared salmon sperm DNA, 10% Dextran sulphate, and 1 × 10⁶ cpm/ml of denatured probe. The filters were washed twice in 2 × SSC, 0.1% SDS at room temperature for 15 minutes and once in 0.2 × SSC, 0.1% SDS at 55°C for 30 minutes.

**RESULTS**

**Comparison of Chicken and Mouse Chxl10 Proteins**

Although the chicken Chxl10 cDNA sequence shown in Figure 1 is incomplete, lacking 29 amino acids toward the amino terminus compared with those in mouse Chxl0, the overall identity (86% of 332 residues) between the two predicted protein sequences, the 100% and 98% identity of the homeodomain and CVC domains, respectively, the conservation of the sequence amino-terminal to the homeodomain (105 to 118 or 89% identity) and the fact that 48 of 59 amino acids immediately C-terminal to the CVC domain are also highly conserved (81% identity) all indicate that this cDNA encodes the chicken orthologue of the mouse Chxl0 cDNA. As in mouse Chxl0, the octapeptide characteristic of many prd–homeodomain proteins is present in the chicken sequence, with an identical composition (FGIQEILG) and location (residues 32 through 39). In addition, a putative nuclear targeting sequence (Thr15Lys16Arg17Lys) is situated immediately N-terminal to the homeodomain. Chicken Chxl0 also has, like the mouse protein, three
FIGURE 1. A partial chicken Chx 10 cDNA sequence and the predicted protein, and an alignment of the predicted sequences of the chicken and mouse Chx10 polypeptides. Dots indicate identical amino acid sequences, and mismatches in the amino acid sequence are indicated by a letter placed under the initial amino acid. The 60-amino acid homeobox is underlined, and the 57-amino acid CVC domain is underlined by a dashed line. The octapeptide (residues 32 through 39) is also underlined. The BamHI site used for subcloning the in situ probe is double underlined.

Potential transactivation domains at virtually identical positions in the predicted polypeptide: a proline-rich sequence (10 of 84 residues from 30 to 114), a serine–threonine-rich region (50% of the residues from 117 to 138) and an acidic sequence in which 6 of 10 residues are aspartic or glutamic acid (residues 335 through 346). Like the mouse Chx10 transcript, the 3'UTR of the chicken message is also long (1016 bp).

Northern Blot Analysis of Pax-6, Prox 1, and Chx10

To assess mRNA levels for Pax-6, Prox 1, and Chx10 during development, Northern blots using [32P]-labeled probes were carried out with total retinal RNA from ED 5 (when most retinal precursor cells are still undergoing mitosis and have not yet differentiated), ED 8 (when most cells have stopped dividing and the laminar organization of the retina is being established), and ED 15 to ED 18 (when cell differentiation is advanced and the retina reaches a degree of cell diversity and organization that resembles that seen in the adult). Chx10 was expressed as a single band, approximately 2.4 kb in size, and its relative abundance remained fairly constant throughout the developmental stages studied. At ED 5, the Pax-6 message appeared to be a single band, but a doublet of approximately 2.4 kb in size appeared at later stages. They are likely to represent alternatively spliced products similar to those seen in other species.45,46 Pax-6 levels appeared to increase between ED 5 and ED 8 but remained essentially unchanged thereafter. Prox 1 mRNA was barely detectable on ED 5 when the filters were overexposed (not shown) but was readily observed by ED 8 under standard conditions (Fig. 2). The Prox 1 probe recognized a major band approximately 8.0 kb in size, but smaller bands, more abundant on ED 15 through ED 18 than on ED 8, were also detectable. This pattern is reminiscent of that...
Homeobox Expression in Chick Retina

FIGURE 2. Northern blot analysis of Pax-6, Prox 1, and Chx10, and expression in chick retinas of ED 5, ED 8, ED 15, and ED 18. Ten micrograms of total retinal RNA were separated by electrophoresis on a 1.2% agarose, 2.2 M formaldehyde gel, transferred to a membrane, and hybridized with [32P]-labeled probes. After autoradiography, filters were stained with 0.02% methylene blue for normalization of the amount of RNA loaded. Although only the methylene blue-stained filter probed for Prox 1 is shown in the figure, similar amounts of total RNA were detected in the other niters as well (not shown). Chx10 and Pax-6 are detected as single bands, approximately 2.4 kb in size. The Prox 1 probe (A) detected a major band of approximately 8 kb, as well as smaller bands that have also been observed in the lens and that represent alternatively spliced products32; Prox 1 signals could be observed on ED 5 if the autoradiograms were overexposed.

In Situ Hybridization

The description of the distribution of Pax-6, Prox 1, and Chx10 mRNA is clearer when presented in reverse chronological order, beginning with advanced stages of development when the retina has already achieved its characteristically laminated organization (ED 15 to postnatal day 1). The layer closest to the pigment epithelium and choroid is the outer nuclear layer (ONL), occupied by photoreceptors, whereas ganglion cells (GC) are at the opposite surface of the retina, in the layer closest to the vitreal cavity; between them is the inner nuclear layer (INL), which in turn shows an intermediate region, containing the cell bodies of bipolar neurons and Müller glial cells, sandwiched between the horizontal cell sublayer (separated from the ONL by the outer plexiform layer), and the region of amacrine cells (separated from the ganglion cell layer by the inner plexiform layer; Figs. 3, 4, 5). Labeling seen at advanced stages will be compared with that observed on ED 8, when the precursors of photoreceptor and nonphotoreceptor neurons still remain undifferentiated but are already postmitotic and have reached their definitive positions within the retina, and with those on ED 4 through ED 7, when the retina still contains an active proliferating neuroepithelium as well as cells undergoing terminal mitosis.67

Pax-6. From ED 15 through ED 18 and PI retinas showed strong Pax-6 hybridization signals in the ganglion cell layer and the amacrine cell subregion of the INL; faint labeling was observed in the scleral side of the INL, including scattered cells in the horizontal cell sublayer, but no expression was detected in the ONL (photoreceptor layer), or in the bipolar–Müller glial cell region of the INL (Figs. 3A, 3B, 3C). Pax-6 expression was already confined to the vitreal half of the retina at ED 8, encompassing the region occupied by presumptive ganglion and amacrine cells (Fig. 3D). In the scleral direction there was a band of negative cells, a single cell layer with light (but readily detectable) label, and another negative region corresponding to the prospective ONL.

Examination of the retina at lower magnification showed a relatively diffuse pattern of expression at ED 4 (not shown) and ED 5 (Fig. 3F). This diffuse pattern of mRNA distribution was also detectable at higher magnification (Fig. 3E), but isolated cells located at the vitreal edge of the retina, probably representing ganglion and/or displaced amacrine cells, already showed stronger hybridization signals (Fig. 3E). Their frequency appeared to increase in the ED 7 retina, when strongly positive cells also appeared in the prospective amacrine cell layer (not shown).

Prox 1. Prox 1 expression was not detectable in the photoreceptors, amacrine or ganglion cells on ED 15 through ED 18 retinas (Figs. 4A, 4B). Conversely, hybridization signals, were observed in the scleral region of the INL, that were stronger in horizontal cells, and decreased gradually toward the amacrine cell region (Figs. 4A, 4B). Similar overall patterns of expression were detected at PI (Fig. 4C), but the hybridization signals appeared much weaker at this stage. As with
Pax-6, the distribution of Prox 1 mRNA on ED 8 seemed to predict that seen at more advanced stages (Fig. 4D), with the region that appears to correspond to prospective horizontal cells showing a strong hybridization signal, and the prospective ONL appearing completely negative. Vitreal to the horizontal layer, the hybridization signals were somewhat irregular to prospective horizontal cells showing a strong hybridization signal, and the prospective ONL appearing completely negative. By ED 18, the ganglion cell layer and the outer nuclear layer are negative. By PI the signals are much weaker but show a similar pattern of distribution (C). ED 8 retinas (D) already resembled the more advanced stages of development in the negativity of the ganglion cells and ONL, and the presence of strong signals in the outer part of the INL; however, a region of weaker hybridization signals (probably corresponding to the prospective bipolar–Müller cell region) separates the horizontal cell layer from the remaining cells of the INL. At ED 5 (E,F), hybridization signals in the neural retina are weak and diffuse, some expression can also be seen in the lens and the neural tube. The photomicrographs shown in the figure were obtained from sections hybridized with probe A, corresponding to amino acids 458 through 735; identical expression patterns were obtained at all stages with probe B, corresponding to amino acids 218 through 438 (not shown). Arrowheads (A to D) indicate the vitreal surface of the retina. INL = inner nuclear layer; ONL = outer nuclear layer; GC = ganglion cells; HC = horizontal cells; L = lens; NR = neural retina; NT = neural tube. Magnification bar = 50 μm (A to E); 500 μm (F).

Immunocytochemical Analysis

The pattern of expression of Pax-6 and Prox 1 in ED 8 retinas was also investigated at the protein level by...
FIGURE 5. ChxlO expression in the developing chick retina. At advanced developmental stages on ED 15 (A), ED 18 (B) and P1 (C), ChxlO probes show strong hybridization signals in the region of the INL just vitreal to the horizontal cells, which corresponds to the bipolar-Müller cell region. No expression is detectable in the horizontal cells, in the ganglion cell layer, or in the ONL. A fairly similar pattern can already be observed on ED 8 (D), although the hybridization signals appear to extend further and to decrease more gradually in a vitreal direction on ED 8 than those at older stages. Outer nuclear layer, horizontal cells and ganglion cells are already negative at this stage. The distribution is relatively diffuse by ED 5 (E), but some conspicuously negative cells, probably corresponding to putative ganglion cells, can be seen near the vitreal surface (arrows). At ED 4 (F), ChxlO expression is fairly diffuse in the neural retina, where hybridization signals are much stronger than in other regions of the neural tube. Arrowheads (A to D) indicate the vitreal surface of the retina. INL = inner nuclear layer; ONL = outer nuclear layer; GC = ganglion cells; HC = horizontal cells; L = lens; NR = neural retina; NT = neural tube; IPL = inner plexiform layer). Magnification bar = 50 μm (A to E; 500 μm (F).

immunocytochemistry. Immunoreactive materials showed in both cases a pattern of laminar distribution reminiscent of that observed by in situ hybridization, and appear concentrated in the nucleus of the cells. Pax-6 immunoreactivity was observed in the inner part of the INL as well as in the ganglion cell layer, where the immunofluorescence signals appeared particularly strong. In addition, scattered immunoreactive cells were also observed in the region corresponding to future horizontal cells (Fig. 6A). Consistent with the in situ hybridization data, this layer of putative horizontal cells contained most of the Prox 1-immunoreactive nuclei (Fig. 6B). Scattered Prox 1-positive nuclei, usually elongated in shape, were also observed vitreally to the prospective horizontal layer (Fig. 6B), in a region where in situ hybridization showed a much more diffuse pattern on expression (Fig. 4D). Nuclear localization of immunoreactivity has also been observed in the chick embryo retina with an antibody against Chxl0 (RRM, unpublished observations), but the antibody did not react well with sections processed under the conditions used in the current study.

DISCUSSION

The results described above can be summarized as follows: The homeobox-containing genes Pax-6, Prox 1, and Chxl0 are expressed in the chick embryo retina at all developmental stages investigated, between ED 5 and P1. Most cells in the proliferating neuroepithel-
Homeobox Expression in Chick Retina

Putative horizontal layer are also positive for *Pax-6*, *Prox 1*, and *ChxlO* mRNA becomes concentrated in putative horizontal layer (arrowheads), although their immunoreactivity is clearly weaker than that observed in the ganglion cell layer. *Prox 1* immunoreactivity (B) is predominantly localized to cells in the putative horizontal layer (arrowheads), but elongated nuclei scattered through the inner nuclear layer (arrow) also show immunoreactivity. Magnification bar = 50 μm.

**Figure 6.** Distribution of *Pax-6* (A) and *Prox 1* (B) immunoreactivity in ED 8 chick retina, processed for immunofluorescence. *Pax-6* immunoreactivity is observed in the ganglion cell layer (A, short arrow), where cells generally show an intense fluorescent signal, as well as in the inner part of the inner nuclear layer (long arrow); scattered cells in the putative horizontal layer are also positive (arrowheads), although their immunoreactivity is clearly weaker than that observed in the ganglion cell layer. *Prox 1* immunoreactivity (B) is predominantly localized to cells in the putative horizontal layer (arrowheads), but elongated nuclei scattered through the inner nuclear layer (arrow) also show immunoreactivity. Magnification bar = 50 μm.

After ED 8, each of the major retinal cell types appears to be denoted by a particular pattern of *Pax-6*, *Prox 1*, and *ChxlO* expression. Thus, photoreceptors are negative for all three genes; horizontal cells are strongly positive for *Prox 1*, weakly positive for *Pax-6* (at least in some cases), and negative for *ChxlO*; cells in the bipolar–Müller region are strongly positive for *ChxlO* and weakly labeled with *Prox 1* (and, in an irregular pattern, also by *Pax-6*); amacrine cells are strongly *Pax-6*–positive and show variable levels of *ChxlO* expression depending on their position in the INL, and cells in the ganglion cell layer are rich in *Pax-6* mRNA but devoid of detectable signals for *Prox 1* and *ChxlO*. Given their structure, and the nuclear localization of immunoreactive materials observed with antibodies against *Pax-6* and *Prox 1*, the proteins encoded by these homeobox genes are likely to function as transcriptional regulators. It is, therefore, tempting to speculate that the differentiated fate of each retinal precursor cell in the chick embryo may be influenced by the presence of the gene products for *Prox 1*, *Pax-6*, and *ChxlO*, acting either individually or in a combinatorial manner. It is also tempting to speculate that the simultaneous absence of *Pax-6*, *Prox 1*, and *ChxlO* gene products may be necessary for the differentiation of precursor cells as photoreceptors. Given that all three genes are expressed in differentiating nonphotoreceptor neurons but are absent from photoreceptors in retinal cell cultures (data not shown), this and other hypothetical roles of *Pax-6*, *Prox 1*, and *ChxlO* in retinal differentiation should be amenable to experimental analysis, using gene transfer, antisense oligonucleotide approaches, or both.

Although all three genes have not been analyzed simultaneously in other species, several similarities have been observed between other species and the chick. *Pax-6*, for example, has also been localized to the amacrine and ganglion cell layers of the developing quail retina. Similarities between *ChxlO* distribution in the chick and its homologue in the mouse include their diffuse distribution in the undifferentiated neuroepithelium and progressive restriction to the bipolar–Müller cell region of the INL and a gradual decrease toward the vitreal aspect of this layer. Its functional importance has been underscored by the analysis of the ocular retardation phenotype in mice, caused by a mutation in the *ChxlO* gene; correlations between the expression pattern of this gene and particular developmental abnormalities were extensively discussed in that study. *Vtx-1*, a goldfish homologue of *ChxlO*, has also been reported to have a similar distribution. *Prox 1* was reported not to be expressed in the mouse retina, but others have since detected it in this tissue (Dow and Doherty, unpublished observations, 1996). To evaluate the possible functional significance of these genes in retinal cell differentiation, suggested by their conserved pattern of expression, it is important to note that they are also expressed in other regions of the nervous system, and that other DNA-binding transcrip-
tion factors are also expressed in developmentally regulated, topographically specific patterns in the embryonic retina. 4,5-7 Evidence from cell lineage studies indicated that, in the chick and other vertebrates, retinal precursor cells remain uncommitted to specific phenotypes while they are mitotically active. 8-10 This agrees with the diffuse patterns of expression reported in the undifferentiated retinal neuroepithelium for such genes as Notch, 11-13 Mash-1, 14 and Cash 15 and mouse Chx10 16 and with our findings with Pax-6, Prox-1, and Chx10 in the chick retina. The transition from the diffuse, low-level pattern of expression detected in the neuroepithelium, to the topographically restricted pattern observed at later stages, may involve the upregulation of expression of each gene in some cells and its downregulation in others. With the exception of putative ganglion cells, which migrate to the vitreal surface of the retina by ED 5 or ED 6, and can already be seen at that time to be Pax-6(+), Chx10(-), and with our findings with Pax-6, Prox-1, and Chx10 in the chick retina. When the generation of neurons and photoreceptors is essentially over and the laminar organization of the retina generally did not become detectable before ED 7 or ED 8, already be seen at that time to be Pax-6(+), Chx10(-), and with our findings with Pax-6, Prox-1, and Chx10 in the chick retina. Further studies will be necessary, however, to determine whether changes in homeobox gene expression take place abruptly after terminal mitosis or only after the postmitotic cells have migrated to their laminar positions within the retina.

Key Words
cell differentiation, cell lineage, homeobox genes, photoreceptors, retina development, transcription factors

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