The Genetic Sequence of Retinal Development in the Ciliary Margin of the Xenopus Eye

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INTRODUCTION

The vertebrate retina, with its limited number of neuronal cell types organized in a stereotypical laminar pattern, is an excellent model system for developmental studies of cellular determination in the central nervous system (CNS). Lineage studies in rodents using retroviral insertion markers (Price et al., 1987; Turner and Cepko, 1987; Turner et al., 1990), and studies in Xenopus using tracer injection (Holt et al., 1988; Wetts and Fraser, 1988), suggest that the dividing neuroepithelial cells in the retina represent a pool of multipotential precursors. In fish and amphibians, the retina grows throughout life by adding new cells of all types from the ciliary marginal zone (CMZ), a region at the peripheral edge of the retina (Straznicky and Gaze, 1971; Reh, 1989; Wetts et al., 1989; Johns, 1977). The CMZ has the exceptional advantage of being spatially ordered with respect to cellular development and differentiation, with the youngest and least determined stem cells closest to the periphery, the proliferative retinoblasts in the middle, and the cells that have stopped dividing at the central edge (Wetts et al., 1989; Dorsky et al., 1995).

Homologues of the Drosophila neurogenic genes Notch, Delta, E(spl), and Su(H) have been identified in several vertebrate species (Coffman et al., 1990; Bettenhausen et al., 1995; Chitnis et al., 1995; Henrique et al., 1995; Lewis, 1996; Myat et al., 1996; D. Turner, unpublished data) and as in flies play a significant role in vertebrate retinogenesis. Transfection of an activated form of Notch in Xenopus retinal cells causes them to retain neuroepithelial characteristics (Dorsky et al., 1995). In rats and chicks, when Notch or Delta activity is increased using retroviral vectors or decreased with antisense, the differentiation of progenitors into ganglion cells is decreased or increased, respectively (Austin et al., 1995; Ahmad et al., 1997). Recent evidence shows that Notch/Delta signaling might control neuronal diversity in Xenopus retina by regulating...
Delta promotes different cell types (Dorsky et al., 1997). However, the molecular mechanisms upstream and downstream of Delta and Notch during retinogenesis are poorly understood. The bHLH (basic helix loop helix) genes related to achaete and scute have proneural functions in Drosophila (Alonso and Cabrera, 1988; Ghysen and Dambly-Chaudiere, 1988; Campuzano and Modolell, 1992), and the bHLH gene atonal is essential for Drosophila eye development (Jarmain et al., 1993, 1994, 1995). In Xenopus, several homologues of these proneural genes have been identified (Xash1: Ferreiro et al., 1992; Xash3: Zimmerman et al., 1993; Turner and Weintraub, 1994; neuroD: Lee et al., 1995; Neurogenin-1: Ma et al., 1996; ATH3: Takebayashi et al., 1997; Xath5: Kanekar et al., 1997). Proneural genes play a role in vertebrate retinal development. For example, in mouse retinal explants, Mash1 promotes neuronal differentiation of retinal cells (Tomita et al., 1996), and recently we have shown that overexpression of Xath5 in retinal precursor cells can promote ganglion cell differentiation in Xenopus (Kanekar et al., 1997).

In Drosophila, proneural and neurogenic genes interact at several different levels. The Notch pathway negatively regulates the expression of proneural genes via the activation of the bHLH proteins belonging to the E(spl) complex (Bailey and Posakony, 1995; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). Conversely, proneural gene activity promotes the expression of the neurogenic gene Delta (Hinz et al., 1994; Kunisch et al., 1994). Though it is clear that there is potential cross-regulation between the neurogenic and proneural genes, it is not clear which gene is upstream of which in the natural genetic cascade during cell differentiation. To understand this hierarchy, knowledge of the relative chronology of the expression of these genes during development is essential. Because spatial cellular organization reflects cellular development in the Xenopus retinal CMZ, this would seem an excellent model to study this issue. If one gene is expressed more peripherally than another, it indicates that it is expressed earlier in the sequence of retinogenesis. We thus determined relative spatial expression in the CMZ of neurogenic and proneural genes during retinogenesis by double in situ hybridizations.

With the aim of better understanding the function of these genes, we took advantage of the CMZ to position these genes during development. We focused on some transcription factors that are known to be involved in early steps of eye development, in the determination of the retina, such as Xrx1 (Casarosa et al., 1997; Mathews et al., 1997), XSIx3 (Oliver et al., 1995, 1996; Zuber et al., 1997), Xotx2 (Matsuo et al., 1995; Pannese et al., 1995; Blitz and Cho, 1995), and Pax6 (Hirsch and Harris, 1997b; Altman et al., 1997). Some are known to be involved in later steps, in the specification of certain retinal cell types, such as Brn-3.0 (Hirsch and Harris, 1997a; Gan et al., 1996) or Pax6 (Hirsch and Harris, 1997b). Although the expression patterns of a few of these genes have already been published, our focus here is to understand the relative position of all the expression patterns of these genes in the CMZ in order to uncover the genetic hierarchy underlying retinogenesis. Indeed, comparison of the expression patterns of the neurogenic and proneural genes to that of these other genes provides a general view of the developmental sequence of gene expression that takes place during retinogenesis. This work shows that there are two waves of transcription which turn on the 10 neurogenic and proneural genes. This neurogenic and proneural activity follows the expression of putative eye formation genes and leads the expression of putative cellular determination genes. Our results allow the subdivision of the CMZ in four subregions and predict a genetic hierarchy. Unexpectedly, our results also highlight the possibility that combinatorial expression patterns of transcription factors might be essential for laminar cell identity in the retina.

The possibility of misexpression in Xenopus blastomeres by RNA injection has allowed us to test some of the predicted gene relationships. We showed that both neuroD and Xath5 are expressed prior to Brn-3.0 in the retina and when overexpressed in the early embryo are able to induce ectopic expression of Brn-3.0. These results support our proposed genetic sequence. The sequential expression and functional interactions of these CMZ genes, when taken together, suggest a genetic pathway of cellular development, from stem cell to differentiated neuron, during retinogenesis.

MATERIALS AND METHODS

In Situ Hybridization on Retinal Cross Sections

Double in situ hybridizations were done on 10-μm paraffin sections of Xenopus embryos essentially as described in Dorsky et al. (1997), except that the PBT (PBS, 0.2% bovine serum albumin, 0.2% Triton X-100) was replaced with MAB (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and the PBT/20% normal goat serum was replaced with MAB/2% BSA (Boehringer-Mannheim blocking reagent). In addition, instead of inactivating the remaining alkaline phosphatase after the first signal is obtained, the anti-fluorescein antibody bound to the fluorescein-labeled probe was removed in glycine HCl, pH 2.2, for 10 min, and sections were then washed five times in PBS. The cDNAs we used were Xath5 (Kanekar et al., 1997), neuroD (Lee et al., 1995), X-Notch-1 (Coffman et al., 1990), X-Delta-1 (Chitnis et al., 1995), X-NGNR-1 (Ma et al., 1996), ESR1 and ESR3 (D. Turner, unpublished data), Xotx2 (Blitz and Cho, 1995), Brn-3.0 (Hirsch and Harris, 1997a), Pax6 (Hirsh and Harris, 1997b), Xash1 (Ferreiro et al., 1992), Xash3 (Zimmerman et al., 1993), Xsx1 (Casarosa et al., 1997), XSIx3 (Zuber et al., 1997), and Ath-3 (Takebayashi et al., 1997). Those cDNAs were used as templates to generate DIG-labeled antisense RNA probes and fluorescein-labeled antisense RNA probes with T7, T3, or SP6 RNA polymerase. DIG-labeled RNA probes were revealed using NBT/BCIP substrate (Boehringer-Mannheim) and fluorescein-labeled RNA probes were revealed using Fast Red substrate (Boehringer-Mannheim). Even when the NBT/BCIP staining is strong, we observed that it does not obscure the Fast Red fluorescence. To ensure the reproducibility of the results, each in situ hybridization
experiment was done on four to six retinas and repeated at least two more times with other clutches of larvae.

**BrdU Staining**

BrdU was injected intraabdominally, and the animals were allowed to recover for 1 h postinjection so as to label cells before they had undergone more than one division. The mRNA was detected with a blue coloration visible in bright field (using NBT/BCIP substrate as above), while BrdU was detected with a fluorescent secondary antibody (Boehringer-Mannheim) after a 45-min treatment in 2 N HCl.

**RNA Injection**

Capped RNA was synthesized in vitro by SP6 transcription from pCS2-Xath5, pCS2MT-NeuroD, or pCS2-nuclear βgalactosidase (nβgal) template DNA using a Message Machine kit from Ambion. For two-cell stage injections, RNA was injected in a volume of 5 nl into one of the two blastomeres in the following amounts: Xath5 (0.8 ng), NeuroD (0.6 ng), and nβgal (0.1 ng). Since the first cleavage plane often defines the plane of bilateral symmetry in the embryo, the cells in one-half of the embryo express the injected RNA and the other half of the embryo serves as an uninjected control. The embryos were allowed to develop and were then staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). The embryos were fixed in MEMFA (Harland, 1991) for 1 h at room temperature and then stored in methanol.

**Whole-Mount in Situ Hybridization and βgal Staining**

Embryos injected with β-galactosidase RNA were stained with Magenta-gal (Biosynth International) using the same protocol as previously described for X-gal staining (Turner and Weintraub, 1994). Digoxigenin (DIG)-labeled antisense RNA probes were generated for Bm3.0, as described in Harland (1991) and whole-mount in situ hybridization was performed on albino Xenopus embryos using the procedure described in Harland (1991), except that BM purple (Boehringer-Mannheim) was used as the substrate for the alkaline phosphatase, since the background was lower than that obtained using NBT/BCIP.

**RESULTS**

**Mutual Expression of Neurogenic Genes**

It has already been shown that X-Notch-1 and X-Delta-1 are coexpressed in the CMZ (Dorsky et al., 1997). However, the expression of the potential downstream neurogenic components of the Notch pathway, the E(spl) homologues, has not previously been studied in this system. Double in situ hybridizations with a X-Notch-1 and a Xenopus E(spl) homologue, either an ESR1 or ESR3 probe (D. Turner, unpublished data), show that both ESR1 and ESR3 are expressed in the ciliary marginal zone, but not in the most peripheral region containing stem cells nor in the central region where differentiated cells are present (Figs. 1A and 1F). X-Notch-1 is expressed in the same region (Figs. 1B and 1G). The superposition of ESR1 or ESR3 and X-Notch-1 expression patterns shows that they overlap completely in the CMZ (Figs. 1E and 1J) and demonstrates that X-Notch-1 and two members of the E(spl) complex are expressed in the same step of retinal cell development. These results are consistent with a function for ESR1 and ESR3 as transcriptional inhibitors of differentiation downstream of the Notch pathway. Since it has been shown that X-Notch-1 and X-Delta-1 are coexpressed in the CMZ (Dorsky et al., 1997), we can also conclude that the four neurogenic genes, X-Notch-1, X-Delta-1, ESR1, and ESR3, are expressed in the same area of the CMZ in stage 40 embryos.

**Xath5 before Xath**

To determine the relationships between neurogenic and proneural genes in the retina, we analyzed their relative expression patterns (Figs. 2, 3, and 4). Since the four neurogenic genes are expressed in the same area, we chose X-Notch-1 as a representative of this class and compared the expressions of the different proneural genes to X-Notch-1 expression.

On cross-sections of stage 40 retina, Xash1 and Xash3 expressions are restricted to the ciliary marginal zone, with no detectable expression in the most peripheral region of the CMZ nor in the central retina (Figs. 2A and 2F). Confirming the previously reported distribution for Xash1 and Xash3 in stage 41 and 34 embryo retina, respectively (Ferreiro et al., 1992; Turner and Weintraub, 1994). Xash3, however, is expressed at a much lower level than Xash1 and is downregulated in later stages (unpublished data). Double in situ hybridization experiments show that Xash1, Xash3, and X-Notch-1 expressions start at the same level in the CMZ (Figs. 2E and 2J). Xash1 expression extends in the central region of the CMZ as far as X-Notch-1, while Xash3 expression is narrower and stops more peripherally than X-Notch-1. This narrower Xash3 expression pattern has been confirmed by comparing Xash3 and another proneural gene (see Figs. 3K–3O). The downregulation of Xash-3 might be a consequence of an inhibition by ESR function. If so, one might expect a higher level of ESR expression at the point where Xash3 is turned off or just preceding it. However, these in situ experiments do not allow such a quantitative analysis. Translating space into developmental time, the four neurogenic genes and the two proneural achaete-scute homologues are turned on at the same time, while Xash3 is turned off before the others.

Expression in the Xenopus retina of the proneural gene Xath5, a Drosophila achaete homologue, is also restricted to the CMZ in stage 40 embryos (Kanekar et al., 1997). As was observed with the others so far described, Xath5 is not expressed in the most peripheral region of the CMZ (Fig. 3A and Kanekar et al., 1997). Double in situ hybridization shows that X-Notch-1 starts more in the periphery than Xath5 and that both stop at the same central level in the CMZ (Fig. 3E). To confirm this offset between a neurogenic gene and Xath5 expressions, we also performed a double in situ hybridization with X-Delta-1 (Figs. 3F–3J). Again, some
X-Delta-1 positive cells in the periphery of the retina are Xath5 negative but all Xath5 positive cells are X-Delta-1 positive (Fig. 3). These results suggest that Xath5 is expressed later during retinal cell differentiation than the neurogenic genes.

Since Xash3 and Xash1 expression starts at the same level in the CMZ as X-Notch-1, and since Xath5 starts more centrally, both Xash3 and Xash1 should also start more peripherally than Xath5. This suggests that the achaete-scute-like proneural genes Xash1 and Xash3 may be expressed before the aortal-like proneural genes during the differentiation of the retinal cells. For this reason, and because Xash3 expression stops before X-Notch-1 (and thus Xath5) is turned off, it may be turned off before Xath5 starts. To assay this, we compared the expressions of Xash3 and Xath5 (Figs. 3K–3O). This experiment shows that there is a small area in the CMZ where Xash3 and Xath5 expressions overlap, suggesting that Xash3 expression stops just after Xath5 starts to be expressed (Fig. 3O).

neuroD is also expressed in the CMZ of stage 40 embryos (Fig. 4A and Kanekar et al., 1997). However, unlike all the genes cited above, neuroD expression is maintained in the central retina in the outer part of the inner nuclear layer containing horizontal and bipolar cells and in the outer nuclear layer containing the photoreceptor cells. Double in situ hybridization with neuroD and X-Notch-1 probes shows that X-Notch-1 expression begins more in the periphery than neuroD (data not shown). Thus, neuroD, like Xath5, is turned on after the neurogenic and achaete-scute homologues. We compared the expression of Xath5 and neuroD to see whether one starts before the other (Figs. 4A–4E). Double in situ hybridizations show that these two proneural genes are coexpressed in the CMZ (Fig. 4E; Kanekar et al., 1997), indicating that they are turned on at the same developmental step.

Another aortal homologue, ATH-3 (Takebayashi et al., 1997), is also expressed in the retina of stage 40 embryos (Fig. 4F). Similar to neuroD, ATH-3 is expressed in both the CMZ and the central retina, but only in the outer part of the inner nuclear layer. Double in situ hybridization shows that ATH-3 also starts to be expressed later than X-Notch-1 (data not shown) and at the same time as Xath5 in the CMZ (Fig. 4J). X-NGNR-1, another aortal related gene, is expressed only faintly in the CMZ (data not shown). Therefore, precise comparisons with other genes are difficult. X-MyT1 is a zinc-finger motif proneural gene expressed in the CMZ and the inner nuclear layer of the central retina (Fig. 4K and Bellefroid et al., 1996). Double in situ hybridization with

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**FIG. 1.** Comparison of X-Notch-1 and E(spl)-related gene expression patterns in stage 40 Xenopus retina. (A–E) Comparison of X-Notch-1 and ESR1 expression patterns. Double in situ hybridizations were performed on sections of stage 40 Xenopus retina using DIG-labeled ESR1 probe and fluorescein-labeled X-Notch-1 probe. ESR1 expression was viewed under visible light (deep purple) and X-Notch-1 expression was viewed under fluorescence (red). A and B show the same section. (A) ESR1 expression is detected in the CMZ except in cells in the most peripheral region. (B) X-Notch-1 labeling is also detected in the CMZ except in cells in the most peripheral region. Occasional labeled cells are seen in the central retina, in late born cells (Dorsky et al., 1995). C and D show a higher magnification of the CMZ from the sections depicted in A and B, respectively. (E) Double labeling demonstrates that ESR1 and X-Notch-1 expressions overlap in the CMZ. CMZ, ciliary marginal zone; CR, central retina; L, lens. Scale bar in A and B, 100 μm; scale bar in C, D, and E, 30 μm. (F–J) Comparison of X-Notch-1 and ESR3 expression patterns. Double in situ hybridizations were performed as above with ESR3 in deep purple and X-Notch-1 in red. (F) ESR3 expression is detected in the CMZ except in cells in the most peripheral region. (G) X-Notch-1 labeling is also detected in the CMZ except in cells in the most peripheral region. (J) Double labeling demonstrates that ESR3 and X-Notch-1 expressions overlap in the CMZ. Scale bar in F and G, 100 μm; scale bar in H, I, and J, 30 μm.
X-MyT1 and X-Notch-1 show that X-MyT1 starts to be expressed more centrally than X-Notch-1 (data not shown) and at the same level as Xath5 (Fig. 40).

Together, these data allow us to position the different proneural and neurogenic genes in a hierarchical pathway in the CMZ. There are two waves of transcription. The first turns on the four neurogenic genes and the two achaete-scute homologues. The second wave turns on the three atonal-like genes and X-MyT1. Xash3 is the first gene to be shut down, and then all the neurogenic genes and Xath5 are turned off at the same stage of differentiation. neuroD, ATH-3, and X-MyT1 show maintained expressions in distinct subsets of neurons in the central retina.

Positioning the Proneural/Neurogenic Genes in the Greater Genetic Cascade

To position neurogenic and proneural gene expression in the greater genetic cascade that leads to eye formation, we have compared their expression patterns in the CMZ to that of genes encoding transcription factors ostensibly involved in early eye development and other genes involved in later cellular determination in the retina. XSix3 and Xrx1, genes expressed very early in the development of the eye (Casarosa et al., 1997; Zuber et al., 1997), are expressed throughout the CMZ, including the most peripheral cells (Figs. 5A and 5F and Mathers et al., 1997) indicating that they are expressed before X-Notch-1, a suggestion that is confirmed by double in situ hybridizations (Figs. 5E and 5J). Both of these genes remain on during development as seen by expression in the central retina. XSix3 is expressed in the ganglion cell layer and the inner nuclear layer (Fig. 5A), while Xrx1 is expressed in a subset of cells of the outer part of the inner nuclear layer and faintly in the outer nuclear layer (Fig. 5F). However, Xrx1 expression in the central retina becomes downregulated in late stage 40 (unpublished data and Mathers et al., 1997). Pax6 is also expressed in an early phase of eye development throughout the forming optic vesicle and in a later phase in amacrine and ganglion cells (Fig. 5K and Hirsch and Harris, 1997b). However, a light staining is often detected throughout the CMZ (Fig. 5K). Double in situ hybridization with X-Notch-1 shows that the strong Pax6 staining in the central retina does not overlap with X-Notch-1 expression (Fig. 5O). However, the light Pax6 staining starts from the most peripheral region of the CMZ. Xotx2, a gene implicated in eye formation and cell determination, is expressed in the CMZ but not in the most peripheral cells. It is also found in the central retina, in the outer part of the inner nuclear layer (Fig. 6A). Double in situ hybridization with Xotx2 and neuroD probes shows that these two genes start to be expressed at the same level in the CMZ (Fig. 6E). Brn-3.0, a transcription factor involved in ganglion cell specification (Gan et al., 1996), is expressed in the ganglion cell layer of Xenopus retina (Hirsch and Harris, 1997a; Fig. 6F). To test whether Brn-3.0 expression overlaps with the neurogenic or proneural genes
in the CMZ, we performed double in situ hybridizations with X-Notch-1 (Fig. 6J). There is no overlapping area in the retina only when the neurogenic genes are already turned off.

**Transcriptional Relationships with the Mitotic State**

To map the patterns of gene expression onto cell division in the CMZ, we performed double in situ hybridizations with representative genes, combined with anti-BrdU immuno-histochemistry (Fig. 7). Previously, it was shown that dividing cells in the margin do not express X-Notch-1, dividing cells in the middle region express X-Notch-1, and a few postmitotic cells in the most central region of the CMZ express X-Notch-1 (Dorsky et al., 1995). We observed the same result when we compared X-Delta-1 expression and BrdU positive cells (data not shown). Xath5 and neuroD were chosen as representatives of the genes starting to be expressed more centrally in the CMZ than the neurogenic genes (Figs. 7A–7C and 7D–7F). Both start to be expressed in BrdU positive cells (Figs. 7C and 8F). In the central CMZ, there are a few Xath5 positive cells which are no longer BrdU positive (Fig. 7C), while neuroD continues to be expressed in BrdU negative cells in the central retina (Fig. 7F). Brn-3.0 is only expressed in postmitotic cells (Fig. 7I). The strong expression of Pax6 in the ganglion cell layer and the inner part of the inner nuclear layer is restricted to postmitotic cells (Fig. 7L). However, cells in the CMZ which express Pax6 at a very low level are BrdU positive.
(Fig. 7L). Taken collectively, these data show that BrdU staining starts at the periphery of the retina with XSix3, Xrx1, and Pax6 and ends after the start of ATH-3, Xath5, neuroD, Xotx2, and X-MyT1 expressions. Moreover, BrdU staining ends before the end of the four neurogenic genes, Xash1 and Xath5 expressions, and before the start of Brn-3.0 expression. The expression patterns and the mitotic state, taken together, subdivide the CMZ into four zones. Central to the CMZ, certain genes are turned off and others are maintained.

**Combinatorial Coding of Transcription by Layer**

A surprising result of this analysis is that each retinal layer expresses a particular combination of genes that remains on in the central retina (summarized in Fig. 8). The ganglion cell layer expresses XSix3 (Fig. 5A), Pax6 (Fig. 5K), and Brn-3.0 (Fig. 6F); the inner part of the inner nuclear layer expresses XSix3 (Fig. 5A), Pax6 (Fig. 5K), and X-MyT1 (Fig. 4K); the outer part of the inner nuclear layer expresses XSix3 (Fig. 5A), X-MyT1 (Fig. 4K), ATH-3 (Fig. 4F), Xotx2 (Fig. 6A), neuroD (Fig. 4A), and Xrx1 in
some cells (Fig. 5F); and the outer nuclear layer expresses neuroD (Fig. 4A) and Xrx1 in some cells (Fig. 5F).

### Genetic Interactions

The genetic hierarchy we propose here is supported by some genetic interactions that we previously showed between Xash3, neuroD, and Xath5 in the early Xenopus embryo (Kanekar et al., 1997). To further test some relationships between genes of this proposed pathway, we injected RNA of several different genes of this pathway into cleavage-stage embryos and then assayed for the expression of others by whole-mount in situ hybridization in stage 14 embryos.

We observed that Brn-3.0 is only expressed in the central retina, which suggests that it functions at a late step in the proposed pathway, after the proneural and neurogenic genes. Under this model, genes expressed in the CMZ should regulate expression of Brn-3.0. We showed here that injection of neuroD mRNA causes a

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FIG. 5. Comparison in stage 40 Xenopus retina of X-Notch-1 expression with that of genes involved in early eye development. (A–E) Comparison of X-Notch-1 and XSix3 expression patterns. Double in situ hybridizations were performed as in Fig. 1 with XSix3 in deep purple and X-Notch-1 in red. (A) XSix3 is expressed in the CMZ, from the most peripheral cells of the retina, and in the central retina, in the ganglion cell layer and the inner nuclear layer. (B) X-Notch-1 labeling is detected in the CMZ except in cells in the most peripheral region. C and D show a higher magnification of the CMZ from the sections depicted in A and B, respectively. (E) Double labeling demonstrates that XSix3 expression starts more in the periphery than X-Notch-1 expression. Scale bar in A and B, 100 μm; scale bar in C, D, and E, 30 μm. (F–J) Comparison of X-Notch-1 and Xrx1 expression patterns. Double in situ hybridizations were performed as in Fig. 1 with Xrx1 in deep purple and X-Notch-1 in red. (F) Xrx1 is expressed in the CMZ, from the most peripheral cells of the retina, and in the central retina, in a subset of cells in the outer part of the inner nuclear layer and in the outer nuclear layer. (G) X-Notch-1 labeling is detected in the CMZ except in cells in the most peripheral region. H and I show a higher magnification of the CMZ from the sections depicted in F and G, respectively. (J) Double labeling demonstrates that Xrx1 expression starts more in the periphery than X-Notch-1 expression. Scale bar in F and G, 100 μm; scale bar in H, I, and J, 30 μm. (K–O) Comparison of X-Notch-1 and Pax6 expression patterns. Double in situ hybridizations were performed as in Fig. 1 with Pax6 in deep purple and X-Notch-1 in red. (K) Pax6 is strongly expressed in the central retina, in the ganglion cell layer and in the inner part of the inner nuclear layer, and faint staining is also detected throughout the CMZ. (L) X-Notch-1 labeling is detected in the CMZ except in cells in the most peripheral region. M and N show a higher magnification of the CMZ from the sections depicted in K and L, respectively. (O) Double labeling demonstrates that the strong expression of Pax6 and X-Notch-1 expression does not overlap but that the faint expression of Pax6 in the CMZ starts more peripherally than X-Notch-1 expression. Scale bar in K and L, 100 μm; scale bar in M, N, and O, 30 μm.
clear increase in the size of the patch of Brn-3.0 expression in the anterolateral neural plate, the region of the prospective retina (Fig. 9B). When Xath5 is overexpressed there is a similar, but even more widespread increase in Brn-3.0 expression that extends beyond the boundaries of the anterolateral neural plate (Fig. 9C). Together, these results are consistent with the genetic hierarchy suggested by spatially ordered expression of these genes in the CMZ. In addition, they demonstrate the predictive value of understanding the sequential pattern of gene expression for suggesting functional relationships between genes in a differentiation pathway. Further relationships suggested by our analysis remain to be tested.

DISCUSSION

Defining the Genetic Pathway of Retinogenesis

The proposition that the relative peripheral to the central position in the CMZ of the retina reflects development along a spatial dimension is substantiated by the waves of gene expression that we have described, with genes involved in early eye development placed most peripherally and genes involved in cell determination placed most centrally. There is no evidence that cell division is in any way synchronized in the CMZ, such that all cells in a particular region are in the same phase of the cell cycle. Nevertheless, our spatial analysis of gene expression in the CMZ indicates that there is a clear progression in developmental state that proceeds in an orderly way from peripheral to central. Since the CMZ continues to produce retinal neurons throughout the life of lower vertebrates, we suggest that the order of gene expression that we see at stage 40 in Xenopus is preserved at later stages. Though the stability of gene expression in the perpetually mitotic CMZ has not been tested in detail, it has been shown that X-Notch-1 is maintained in a similar region of the CMZ from Xenopus tailbud stage until adult stage (Dorsky et al., 1995), which suggests that molecular events in the CMZ are conserved from tailbud stage onward. However, Xash3 seems to be down-regulated in the CMZ in late stage 40, which may mean that the molecular mechanisms in the CMZ evolve during development. It would thus be interesting to look at the expression of all the genes studied here later in development and compare their expression patterns in the CMZ.

Our aim was to position neurogenic and proneural genes...
relative to each other and relative to other genes involved in early and late steps of retinal development, to highlight a genetic hierarchy during retinogenesis. The relative expression patterns shown by our double in situ hybridization experiments at stage 40 suggest four phases of retinal development that correlate with four zones of the CMZ.

FIG. 7. Double staining for BrdU uptake and gene expression in stage 40 Xenopus CMZ. (A–C) Double staining for BrdU uptake and Xath5 expression. Xath5 staining is shown in A, BrdU immunostaining in B. Double staining in C shows that BrdU+ cells in the peripheral CMZ are Xath5+. In the central CMZ, BrdU+ cells are stained for Xath5. A few cells are BrdU− and stained with Xath5. (D–F) Double staining for BrdU uptake and neuroD expression. neuroD staining is shown in D, BrdU immunostaining in E. Double staining in F shows that BrdU+ cells in the peripheral CMZ are neuroD+. In the central CMZ, BrdU+ cells are stained for neuroD. neuroD+ cells in the central retina are BrdU−. (G–I) Double staining for BrdU uptake and Brn-3.0 expression. Brn-3.0 staining is shown in G, BrdU immunostaining in H. Double staining in I shows that all BrdU+ cells in the CMZ are Brn-3.0+ and all Brn-3.0+ cells are BrdU+. (J–L) Double staining for BrdU uptake and Pax6 expression. Pax6 staining is shown in J, BrdU immunostaining in K. Double staining in L shows that BrdU+ cells in the CMZ express a low level of Pax6 and that cells in the central retina expressing a strong level of Pax6 are BrdU−. Peripheral CMZ is on the left. Scalebar in A–L, 30 μm.
Phase 1/Zone 1

Stem cells, found in the most peripheral part of the CMZ, do not express any of the neurogenic or proneural genes but do express Xrx1, XSix3, and a low level of Pax6. One might imagine that these genes are important for giving the stem cells a retinal identity, while the lack of proneural genes could mean they are pluripotent to produce both neural and nonneural cells. Indeed, injection of the most peripheral...
cells of the CMZ with fluorescent tracers shows that these deep stem cells may give rise to both pigment epithelial cells and neurons (Wetts et al., 1989). Xrx1 belongs to a paired-like class of homeobox genes (Casarosa et al., 1997). It is expressed in neural structures of the developing eye (Casarosa et al., 1997; Mathers et al., 1997). Xenopus embryos injected with synthetic Xrx1 RNA develop an ectopic retinal pigmented epithelium and display hyperproliferation in the neuroretina suggesting that this gene plays an important role in early eye development (Mathers et al., 1997). Six3 is the murine homologue (Oliver et al., 1995) of the homeobox gene sine oculis implicated in Drosophila eye development (Serikaku and O'Tousa, 1994; Cheyette et al., 1994). It has recently been shown that the murine Six3 is able to induce an ectopic lens in fish (Oliver et al., 1996). Two homologues have also been isolated in chick (Bovolenta et al., 1996) and a Xenopus sine oculis homologue, XSix3, has recently been isolated and is specifically expressed in the developing eye (Zuber et al., 1997), supporting the idea that it is involved in early eye development. Our results confirmed that Xrx1 and XSix3 are expressed in early retinogenesis in the CMZ since they are expressed in the most peripheral region of the CMZ and thus before the neurogenic and proneural genes. Pax6 is a homologue of the Drosophila gene eyeless (ey) which encodes a transcription factor with both a paired domain and a homeodomain. Ectopic expression of ey in Drosophila is able to give ectopic eye structures (Halder et al., 1995), and in Xenopus Pax6 misexpression induces lens marker expression (Altmann et al., 1997). Loss-of-function mutations in mammals lead to a reduction or absence of eye structures. In goldfish, Pax6 is highly expressed in the CMZ (Hitchcock et al., 1996), but in Xenopus embryos Hirsh and Harris (1997b) reported that Pax6 was downregulated in the CMZ at stage 33/34. However, the low levels of Pax6 expression we observed throughout the CMZ in this study are consistent with a function, possibly a diminished one, in postembryonic retinogenesis.

**Phase 2/Zone 2**

The neurogenic genes X-Notch-1, X-Delta-1, ESR1, and ESR3 are turned on in the next phase. Since expression of the ESR genes might be indicative of functional inhibition through the neurogenic pathway, the coordinated expression of all these neurogenic genes throughout this zone implies that the cells here are involved in mutual inhibition, rather than directional inhibition in which there would be distinct subdomains of X-Notch-1 and X-Delta-1 expression. However, our data cannot rule out the possibility that occasional cells in this region express only X-Notch-1 or X-Delta-1 and antibodies would be required to test this. According to the general literature about proneural and neurogenic genes in Drosophila, one can have expected to see achaete-scute homologue expression appearing first, followed by neurogenic gene expression. However, that is not what we saw in the CMZ since both achaete-scute-related genes, Xash1 and Xash3, and the neurogenic genes start to be expressed at the same level in the CMZ. A study at the protein level might reveal some subtle offsets, undetectable at the RNA level. On the other hand, the coexpression of these proneural and neurogenic...
Phase 3/Zone 3

In phase 3, the proneural genes ATH-3, Xath5, neuroD, and X-MyT1 are turned on. neuroD is a bHLH protein, related to atonal, that has been identified as a neuronal differentiation factor (Lee et al., 1995). While Xash1 and Xash3 are expressed in the neuroblast of the developing nervous system, neuroD is expressed in differentiating progenitors and neurons (Ferreiro et al., 1993; Zimmerman et al., 1993; Turner and Weintraub, 1994; Lee et al., 1995). Unlike Xash3, neuroD seems competent to bypass the normal inhibitory influences that usually prevent neurogenesis in the ventral and lateral ectoderm and is capable of converting most of the embryonic ectoderm into neurons. These data suggested that neuroD may participate in a later differentiation step during vertebrate neuronal development (Lee et al., 1995). This is consistent with the expression data in the retina which show that Xash3 is expressed more in the periphery of the CMZ than neuroD and that neuroD is still expressed in the central retina. ATH-3 and Xath5 encode bHLH transcription factors related to atonal and are able to promote neural development at the expense of nonneural tissues (Takebayashi et al., 1997; Kanekar et al., 1997). X-MyT1 is a C2HC-type zinc-finger protein which can promote ectopic neuronal differentiation in cooperation with bHLH proteins (Bellefroid et al., 1996). Since ATH-3, Xath5, neuroD, and X-MyT1 genes are turned on more in the central retina than Xash genes, it suggests they are downstream. This leads to predictions that can be functionally tested in vivo. For example, the coexpression of Xath5 and neuroD in the CMZ suggests either a positive cross-regulation in the initiation of their expressions or that they both are common downstream targets of genes expressed in zone 3. It has recently been shown that overexpression of Xath5 is able to induce ectopic neuroD expression and that, conversely, overexpression of neuroD is able to induce ectopic Xath5 expression (Kanekar et al., 1997).

Phase 4/Zone 4

Finally, the cells in the CMZ stop dividing but differentiation of neurons will occur only after neurogenic genes are turned off and some proneural genes maintained, which is consistent with the model that cells must escape the neurogenic signaling to be able to proceed to the next developmental step. In addition, together with the neurogenic genes, some other proneural genes are also turned off, depending on the neural layer where cells will differentiate. Among the transcription factors we studied, only Brn-3.0 is expressed solely in the central retina in stage 40 Xenopus retina. Brn-3.0 is a member of the Brn-3 family of POU domain transcription factors. It is expressed in retina ganglion cells in Xenopus (Hirsch and Harris, 1997a). The disruption of murine Brn-3 family genes leads to a loss of retinal ganglion cells (Gen et al., 1996; Erkman et al., 1996). Our finding that Brn-3.0 is expressed centrally is a strong confirmation of its function in specific cellular determination and also confirms the hypothesis that the peripheral-central gradient in the CMZ recapitulates retinal ontogeny. We show that injection of neuroD and Xath5 mRNA is able to increase the size of the area of the normal expression of Brn3.0. This effect was most pronounced in the anterolateral neural plate suggesting that other factors must be involved. Since the expression of Brn3.0 does not overlap with that of neuroD or Xath5 in the CMZ, this transcription regulation is likely to be indirect. This suggests that these proneural genes are able to turn on the expression of a transcription factor(s) involved in Brn3.0 expression activation and supports our proposed genetic sequence. However, because interactions between the same genes might be different in different tissues, experiments in the CMZ will...
be necessary to establish the proposed hierarchy in the retina.

Combinations of Transcription Factors Define the Different Layers of the Central Retina

Among the 10 neurogenic and proneural genes studied, Xash3 is the first to be turned off. Which gene inhibits its transcription and why it is turned off earlier in retinogenesis than the other genes studied remain to be investigated. All of the 4 studied neurogenic genes and 2 of the proneural genes (Xash1 and Xath5) are also turned off in the central retina. However, some proneural genes are still expressed in differentiated cells of the central retina: neuroD, ATH-3, and X-MyT1. It is noticeable that they acquire a particular pattern of expression in the central retina. ATH-3 is only expressed in the outer part of the inner nuclear layer; X-MyT1 is expressed in all the inner nuclear layer; and neuroD is expressed in both the outer part of the inner nuclear layer and the outer nuclear layer. neuroD expression in the outer part of the inner nuclear layer decreases in later stages of development until it becomes restricted to the outer nuclear layer only (unpublished data). One thus must be aware that the expression patterns described in this paper may continue to evolve in later stages of development. Interestingly, the three earliest expressed genes in the CMZ, XSix3, Pax6, and Xrx1, are also expressed in the central retina. XSix3 is expressed in both the ganglion cell layer and the inner nuclear layer but not in the outer nuclear layer. Pax6 is expressed in both the ganglion cell layer and the inner part of the inner nuclear layer. Xrx1 is expressed in a subset of cells in the outer part of the inner nuclear layer and in the outer nuclear layer. The type of neuron that expresses Xrx1 in the outer part of the inner nuclear layer remains to be investigated. Moreover, the expression of Xrx1 in the central retina seems to be progressively downregulated (unpublished data and Mathers et al., 1997). This suggests that Xrx1 is not required for the maintenance of the neurons in the central retina but rather for their first phases of differentiation. In the central retina, Xotx2 also continues to be expressed in the outer part of the inner nuclear layer.

Combining all the data in the central retina, we find that each cell layer maintains a particular combination of the 14 studied genes expressed in the CMZ (Fig. 8). The ganglion cells express XSexi3, in addition to Brn-3.1 and Pax6. Cells of the inner part of the inner nuclear layer, presumably amacrine cells, express XSexi3, X-MyT1, and Pax6. Cells of the outer part of the inner nuclear layer, presumably bipolar and horizontal cells, express XSexi3, neuroD, ATH-3, and Xotx2. Moreover, a subset of cells in this layer express Xrx1. Photoreceptor cells, in the outer nuclear layer, express neuroD and Xrx1. Retinoblasts, when they reach the central edge of the CMZ, may receive different signals from freshly postmitotic differentiating cells. Such signals could induce or maintain the expression of genes specific to the different retinal layers and lead to the differentiation of each type of retinal neuron. This model is consistent with previous results which suggested that CMZ retinoblasts are not committed to a particular cell type (Reh and Tully; 1986; Wetts et al., 1989), yet are responsive to the cellular composition of the more central retina. The other possibility, that subsets of precursors in the CMZ express particular gene combinations before they stop dividing, indicative of diverse populations of committed progenitors, is inconsistent with our results which indicate that the genes expressed in any subdomain of the CMZ are expressed in all the cells of this region. However, because we cannot be certain we see every cell in our in situ material, we do not rule out this possibility completely. Antibodies against the different proteins we have studied might be required to compare cellular distribution of all these proteins in the CMZ.

CONCLUSION

The CMZ of the Xenopus retina is a powerful system to study the genetic pathway of retinogenesis in vertebrates. The spatial ordering of expression of genes, from peripheral to central, reflects a developmental sequence and suggests a developmental genetic cascade governing vertebrate retinogenesis. Such developmental ordering of expression has not been clearly seen in any other system except perhaps the morphogenetic furrow in the eye disc of Drosophila which bears a striking genetic similarity to the CMZ. As in the CMZ, the proneural and neurogenic genes are expressed in stripes that parallel and overlap the morphogenetic furrow in the eye disc. For example, the Drosophila atonal gene is expressed in a stripe in the anterior edge of the morphogenetic furrow and then becomes restricted to small groups of cells and finally becomes confined to isolated R8 photoreceptor cells (Jarman et al., 1995). This can be compared to the expression in the Xenopus retina of ATH-3, neuroD, or X-MyT1, which are first expressed together throughout the third and fourth zone of the CMZ and then their expression becomes refined to subsets of neurons in the central retina. Because of these striking parallels, one can propose that the CMZ is the vertebrate counterpart of the Drosophila morphogenetic furrow. Surely, there are many other genes that turn on and off in the CMZ, and perhaps anatomical and functional analysis of these will lead to further subdivision of the CMZ, illustrating in fine detail the developmental genetics of the vertebrate retina, as other such proliferative zones may in turn shed light on the particular development of other areas of the CNS.

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