The Journal of Neuroscience, February 15, 1997, 17(4):1425–1434

The Expression and Function of *Notch* Pathway Genes in the Developing Rat Eye

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The *Notch* gene plays a role in the development of disparate tissues in multiple organisms. Because the vertebrate eye is an excellent model system for both patterning and cell fate determination, two processes that can involve *Notch*, we examined the expression patterns of *Notch 1* and *Notch 2*, and their ligands *Delta* and *Jagged*, in the developing rat eye. *Notch 1* and *Delta* were found to be expressed in the neural retina during the period of cell fate determination and differentiation. *Notch 2* was found to be expressed in the non-neuronal derivatives of the optic cup, including the pigment epithelium, optic stalk, and ciliary body. *Jagged* was expressed in distinct regions within the optic vesicle, ciliary body, and lens, with patterns that changed over time. The potential function of *Notch 1* in cell-type specification and differentiation was examined by introducing a constitutively active form of Notch 1 *in vivo* using a replication-incompetent retrovirus. This form of Notch 1 was found to cause abnormal growth and interfere with the differentiation of multiple retinal cell types.

Key words: Notch 1; Notch 2; Delta; Jagged; *eye development; neural retina; PE; ciliary body; expression pattern*

The vertebrate eye has been an excellent model system for both neurobiologists and developmental biologists. The areas that have received the most attention are the formation of the various components of the eye through inductive interactions and the determination and differentiation of retinal neurons. For example, the anterior neural plate has been found to be necessary for induction of the lens placode, whereas the optic vesicle and lens are thought to induce overlying surface ectoderm to form the cornea (Duke-Elder and Cook, 1963; Graw, 1996). The formation of the retina occurs after invagination of the optic vesicle, which creates a cup with an inner layer [presumptive neural retina (PNR)] and an outer layer [presumptive pigment epithelium (PPE)]. The peripheral margin of the optic cup differentiates into the ciliary body and iris (Bard and Ross, 1982). During these processes, cells become fated to be neural cell types, epithelial cell types, muscle cells, or other specialized cells peculiar to the eye. Within the neural retina, the cells differentiate into six different types of neurons and one type of glial cell from multipotent progenitor cells (Finlay and Sengelaub, 1987; Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990).

Vertebrate homologs of characterized *Drosophila* genes have proven to be a valuable starting point for studying complex developmental processes in vertebrates. The *Notch* gene is among the best-characterized genes in *Drosophila* (for review, see Artavanis-Tsakonas et al., 1995; Lewis, 1996). First identified as having a role in the patterning of the wing margin, *Notch* was subsequently studied extensively for its role in early neurogenesis, where loss-of-function alleles of *Notch* were found to lead to production of excess neurons. It is now appreciated that *Notch* is widely expressed and functions in many tissues throughout *Drosophila* development, including development of the mesoderm, germ line, sensory bristles, wing structures, and compound eye. *Notch* seems to be able to participate in several key aspects of development, including patterning and lateral inhibition.

The *Notch* gene encodes a large transmembrane receptor. The extracellular domain of Notch interacts with the ligands Delta and Serrate (Vässin et al., 1987; Kopczynski et al., 1988; Fleming et al., 1990; Thomas et al., 1991). Once activated by ligand-binding, Notch signaling occurs via its cytoplasmic domain. Constructs that express only the cytoplasmic domain of Notch signal constitutively, as judged by their ability to cause phenotypes similar to gain-of-function alleles or duplications of the *Notch* locus (Fortini et al., 1993; Lieber et al., 1993).

Vertebrate homologs of *Notch*, *Delta*, and *Serrate* have been identified in several species (Betterhausen et al., 1995; Chitnis et al., 1995; Henrique et al., 1995; Lindsell et al., 1995; Lewis, 1996; Myat et al., 1996). The structure and function of Notch have been found to be remarkably conserved. Each mammalian species, however, has several homologs of *Notch*, e.g., there are at least three murine *Notch* genes (Weinmaster et al., 1991, 1992; Reaume et al., 1992; Kopan and Weintraub, 1993; Lardelli and Lendahl, 1993; Lardelli et al., 1994). The multiplicity of these molecules in vertebrates makes it more difficult to study their roles in development and raises questions concerning their ligand specificity and biological functions.

To begin to examine these issues in a vertebrate system, we set out to study the expression patterns of the genes in the *Notch* pathway, including *Notch 1*, *Notch 2*, *Delta*, and *Jagged* (the rat homolog of the *Serrate* gene) in the developing rat eye. We have further explored the function of *Notch* using a replication-incompetent retroviral vector to deliver a gain-of-function allele of *Notch* to the developing rat retina *in vivo*.

Received July 25, 1996; revised Dec. 5, 1996; accepted Dec. 13, 1996.

This work was supported by funding from the Howard Hughes Medical Institute and the National Institutes of Health (Grant EYO 9676 to C.L.C. and EY 06726-01 to Z.-Z.B.). We thank Dr. Jeffrey S. Nye for his generosity in providing the mNIC and mNEC constructs, and Dr. Gerry Weinmaster for her gift of the Jagged probe. We thank John Lin, Eric Morrow, Michael Belliveau, Xianjie Yang, Malcolm Logan, and Vern Twombly for discussion and helpful comments on this manuscript, and Liz Molinari and Shawn Fields-Berry for excellent technical assistance.

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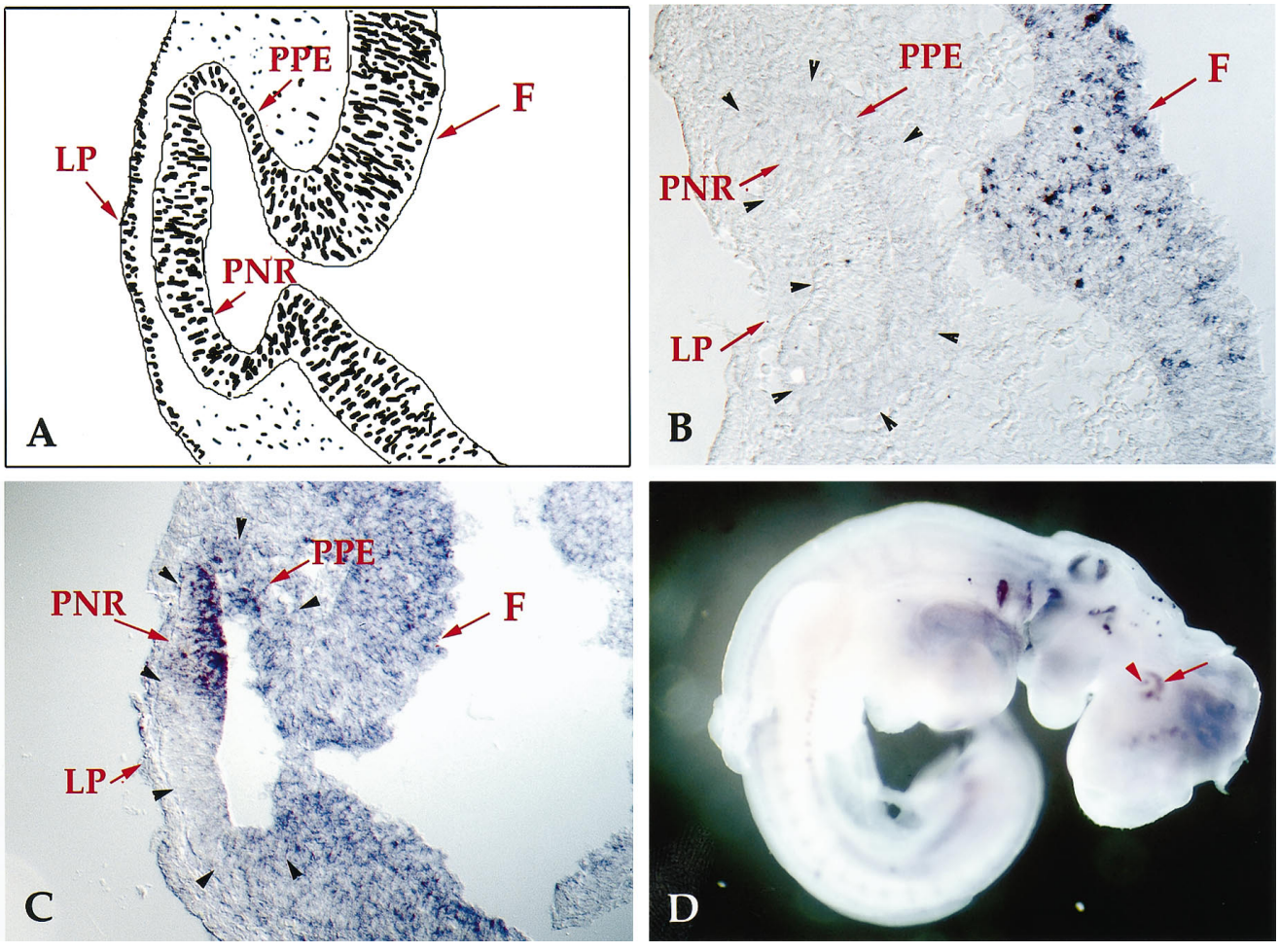

Figure 1. Expression of *Delta* and *Jagged* in the optic vesicle at E12.5. *A*, Diagram of the E12.5 optic vesicle. *B*, *In situ* hybridization of a section with the *Delta* probe. *Delta* was not detected in the optic vesicle region (marked by *arrowheads*). In contrast, *Delta* hybridization was observed in a subset of cells in the VZ of the forebrain (*F*). *C*, *In situ* hybridization with the *Jagged* probe. *Jagged* hybridization was observed in the dorsal region of both PNR and lens placode. It was also expressed in the VZ of the forebrain. *D*, Whole-mount *in situ* hybridization with the *Jagged* probe. *Jagged* hybridization was observed in the dorsal region of the retina (*arrow*) and lens placode (*arrowhead*). *LP*, Lens placode; *PPE*, presumptive pigment epithelium; *PNR*, presumptive neural retina.

MATERIALS AND METHODS

In situ hybridization. The cDNA fragments corresponding to nucleotide sequence 6737–7279 of rat *Notch 1* (GenBank number X57405) and 6887–7443 of rat *Notch 2* (GenBank number M 93661) were obtained by RT-PCR and confirmed by sequencing. The cDNA fragments were cloned into pBluescript (Stratagene, La Jolla, CA) and served as templates for *Notch 1* and *Notch 2* probes. This region between the cdc10/ankyrin repeats and PEST was chosen because it is the least conserved region among the mammalian *Notch* genes. Between *Notch 1* and *Notch 2*, there is only 34% similarity in this region. The template for the rat *Delta 1* probe was similarly obtained by RT-PCR on the basis of the nucleotide sequence 320–820 of the mouse *Delta 1* sequence (GenBank number X 80903). The probe for *Jagged* was full length and was kindly provided by G. Weinmaster (University of California Los Angeles School of Medicine, Department of Biological Chemistry) (Lindsell et al., 1995). Plasmids were linearized and transcribed with either T3 or T7 RNA polymerase to obtain the antisense probes. Probes were labeled nonradioactively by digoxigenin-labeled nucleotides according to the instructions of the manufacturer (Boehringer Mannheim, Indianapolis, IN).

Whole-mount and section *in situ* hybridization procedures were essentially as described in Riddle et al. (1993), using the parameters listed below. For whole-mount *in situ* hybridization, embryonic day 12.5 (E12.5) rat embryos were dissected and rinsed in PBS and fixed with 4% paraformaldehyde at 4°C for 12–24 hr. For *in situ* hybridization on sections, 15 μm cryosections were prepared from tissue OCT blocks on a Reichert Jung CM3000 cryostat (Leica, Deerfield, IL) and collected on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). The tissue sections were fixed with 4% paraformaldehyde at room temperature for 15 min. Whole-mount embryos were treated with 10 μg/ml of proteinase K (Boehringer Mannheim) for 15 min, whereas tissue sections were treated with 1 μg/ml of proteinase K for 10 min at room temperature. Hybridization was carried out by incubation with the probes (~1 μg/ml) overnight at 70°C.

Production of retroviruses. A replication-incompetent pLIA viral vector, based on the pBABE vector [derived originally from Moloney Murine Leukemia Virus (MMLV) by Morgenstern, 1990], was constructed previously in our lab (E. Raviola, S. Fields-Berry, L. Lillien, and C. Cepko, unpublished data). In addition to the MMLV long terminal repeats, it also contains an internal ribosomal entry site (IRES) sequence derived from the encephalomyocarditis virus (Jang et al., 1989), in which the eleventh ATG of the IRES was fused in frame to the human placental alkaline phosphatase gene (PLAP) (see Fig. 5*B*). Truncated forms of mouse *Notch 1* gene, mNIC and mNEC, were inserted into pLIA by Jeffrey S. Nye (Northwestern University Medical School, Department of Molecular Pharmacology and Biological Chemistry and Pediatrics). A myc epitope tag was added to the N terminus of the Notch 1 cytoplasmic domain initiating at amino acid 1753 (Nye et al., 1994). mNEC lacks the entire intracellular domain of the Notch 1 protein and has a myc tag at the C terminus (see Fig. 5*A*). Replication-incompetent viral stocks were prepared as described (Ausubel et al., 1996). Briefly, BOSC cells (Pear et al., 1993) were transfected by the $CaPO_4$ method. A glycerol shock was carried out 4–6 hr after transfection, and the culture supernatant of the transfected cells was collected twice, at 24 and 48 hr post-transfection.

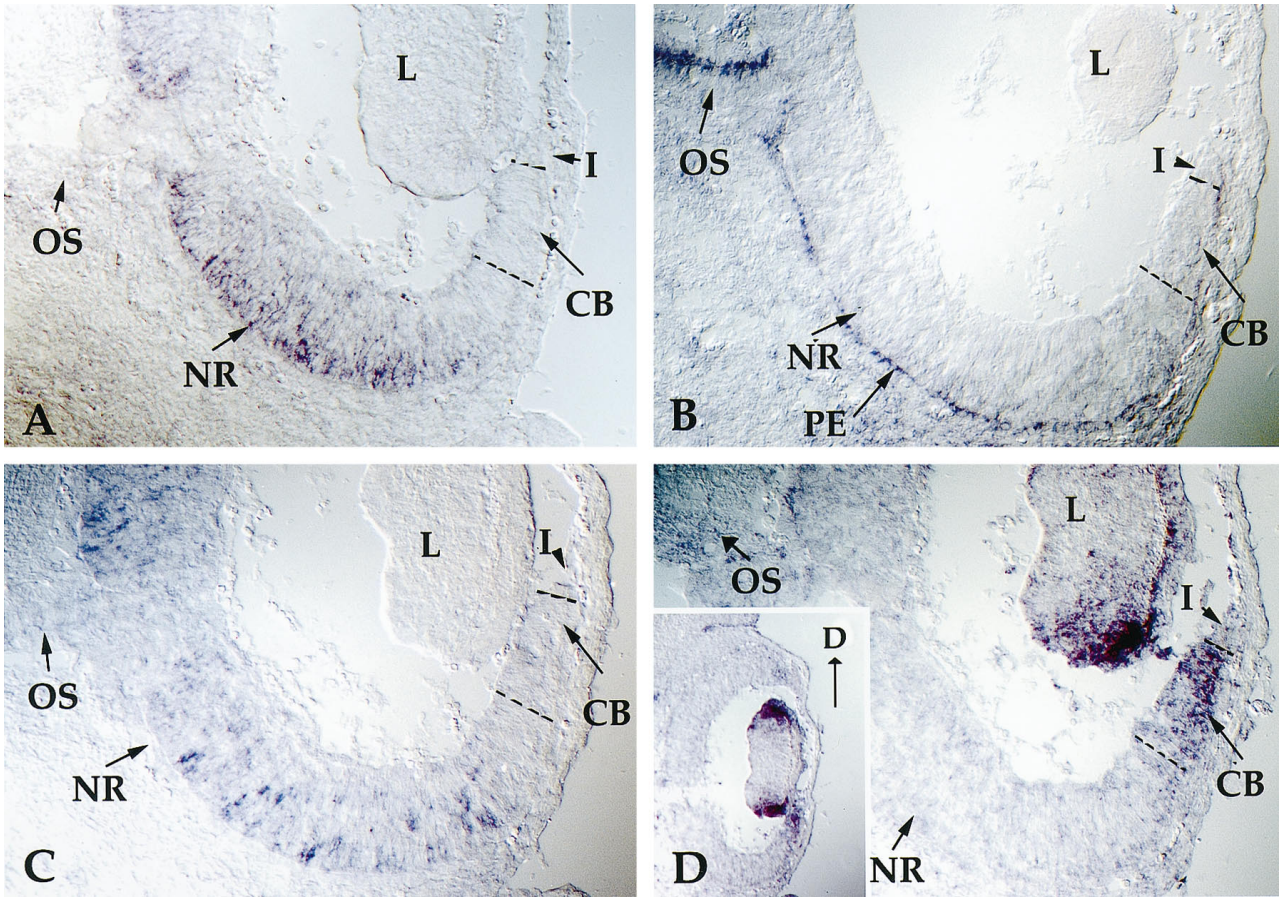

Figure 2. Expression of *Notch 1*, *Notch 2*, *Delta*, and *Jagged* in the E15.5 eye cup. Coronal sections of an E15.5 rat eye (ventral side) are shown. *A*, *Notch 1* expression was observed only in the neural retina. *B*, *Notch 2* expression was observed in the PE and optic stalk. *C*, *Delta* expression was observed in neural retina, similar to that of *Notch 1*. *D*, *Jagged* hybridization signal was observed in the presumptive ciliary body and the equatorial region and anterior of the lens. In contrast to the earlier dorsal expression patterns, the expression of *Jagged* was largely symmetrical in the dorsal and ventral sides of the optic cup and lens (*inset*). *NR*, Neural retina; *CB*, ciliary body region; *I*, iris; *L*, lens; *OS*, optic stalk; *PE*, pigment epithelium.

The supernatant was filtered through 0.45 μm filters and concentrated by ultracentrifugation. The concentrated stocks were aliquoted, titered on NIH 3T3 cells, and stored at −70°C before use. The titers for mNIC, mNEC, and control LIA viruses after concentration were 2×10^6, 1×10^7, and 2×10^7 CFU/ml, respectively.

Infection of retinae. In vivo infection of retinae was carried out by injection of virus into the eyes of postnatal day 0 (P0) rats (C/D, Charles River Laboratories, Wilmington, MA). The subretinal space between the PE and retina was targeted (Turner and Cepko, 1987). Infected retinae were dissected after 3–5 weeks, fixed in 4% paraformaldehyde, and stained for alkaline phosphatase activity (AP) using X-phos/NBT (Fields-Berry et al., 1992). The stained retinae were mounted in OCT (Miles, Elkhart, IN) and cryosectioned at 20 μm thickness to visualize the morphology of infected clones.

E18 rat retinal explants were infected *in vitro* by the following procedures. Retinae were dissected and placed on top of polycarbonate filters with 0.8-μm-diameter pore (Costar, Cambridge, MA). The filters were then floated in medium containing 45% DMEM, 45% F12 medium, and 10% fetal calf serum (FCS), in 12-well culture plates. Viral infection was carried out immediately by adding 1 μl of a viral stock with 8 μg/ml of polybrene in a drop of medium (~50 μl) covering the retina. The retinal explants were cultured at 37°C in a 5% CO_2 humidified incubator and harvested 2 weeks later. For double staining, X-gal histochemistry for β-galactosidase activity was carried out first. The tissues were washed extensively with PBS before the AP staining proceeded.

Immunohistochemical staining. Anti-myc tag antibody (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). NIH 3T3 cells infected with either mNIC or mNEC viruses were fixed with 4% paraformaldehyde at room temperature for 10 min. After they were blocked with 10% FCS in DME plus 0.2% Triton X-100 for 30 min, the cells were incubated with 0.5 μg/ml 9E10 antibody in 1% bovine serum albumin (BSA) in PBS for 1 hr at room temperature. Biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) was used at 1:400 dilution in 1% BSA and 1% normal horse serum. It was followed by incubation with avidin and biotin-conjugated horseradish peroxidase complexes (Elite ABC kit, Vector Laboratories) for 20 min at room temperature. DAB substrate kit (Vector Laboratories) was used to visualize the staining.

RESULTS

Expression patterns of *Notch 1, Notch 2, Delta,* and *Jagged* define domains in the embryonic rat eye

To define the expression patterns of genes in the *Notch* pathway, *in situ* hybridization was carried out on intact embryos ("whole mounts") and sections of ocular structures. At rat E12.5, the lens placode has not yet invaginated to form the lens vesicle, and the PNR appears thicker than the PPE (Fig. 1*A*). Sections and whole mounts of E12.5 embryos were hybridized with antisense probes for *Notch 1*, *Notch 2*, *Delta*, and *Jagged*. At this stage, *Delta* RNA was not detectable in the optic vesicle, but was detectable at a high level in scattered cells in the ventricular zone (VZ) of the forebrain (Fig. 1*B*). In contrast, *Jagged* was found to be expressed at a high level in both the PNR and the lens placode. *Jagged* expression was limited to the dorsal half of these areas (Fig. 1*C,D*). *Notch 1* expression was barely detectable in the optic vesicle at this stage, but was detectable at a high level in the forebrain VZ (data

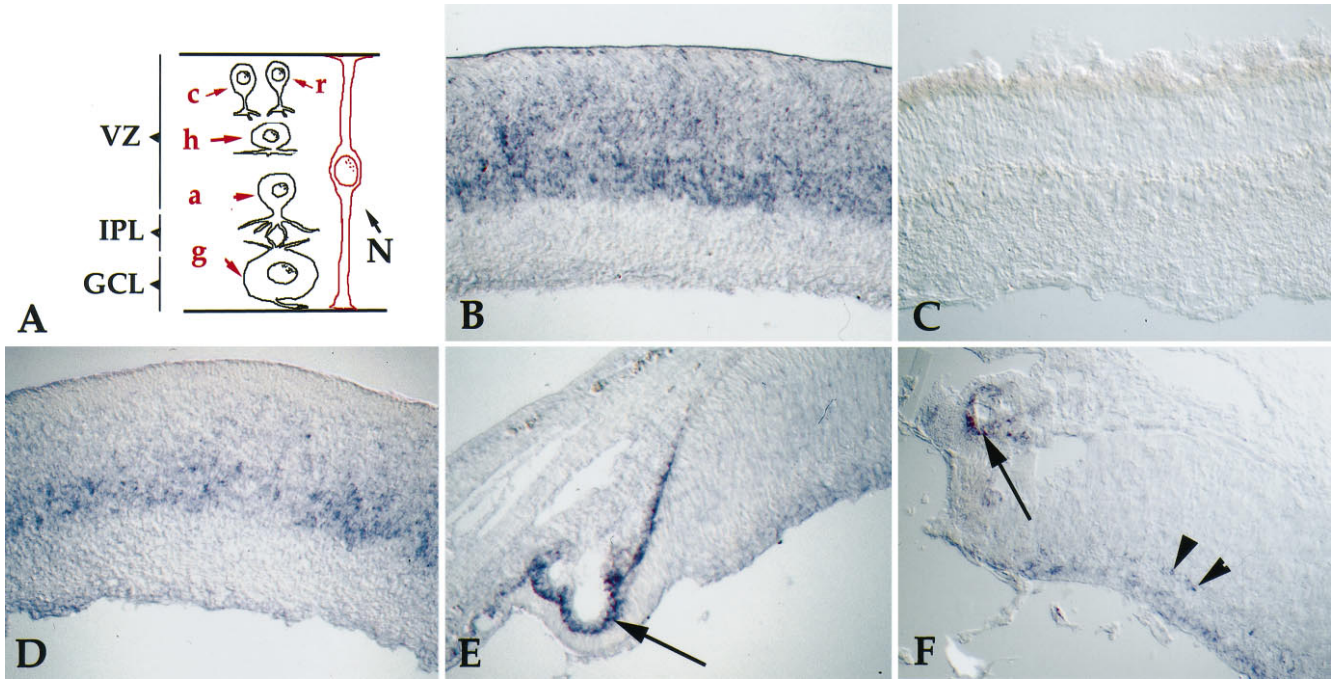

Figure 3. Expression of *Notch 1*, *Notch 2*, *Delta*, and *Jagged* in the postnatal eye. *A*, Diagram of the cellular composition of the P5 retina. At this age, three layers can be distinguished. The ganglion cell layer contains mostly differentiated ganglion cells; the inner plexiform layer consists of dendritic processes of the differentiated amacrine cells and ganglion cells; the *VZ* has both differentiated and undifferentiated cells at this stage. Differentiated cone photoreceptors and some rod photoreceptors are located within the top half of the VZ, whereas horizontal cells are within the center and bottom half of the VZ. *B*, *Notch 1* in P5 retina; *C*, *Notch 1* in adult retina; *D*, *Delta* in P5 retina; *E*, *Notch 2* in P5 eye; *F*, *Jagged* in P5 eye. Note that both *Notch 2* and *Jagged* are expressed in the ciliary body (*arrows*). A subset of cells in the INL and GCL also express *Jagged* (*arrowheads*). *GCL*, Ganglion cell layer; *IPL*, inner plexiform layer; *VZ*, ventricular layer: *N*, undifferentiated neuroblast; *c*, cone photoreceptor; *h*, horizontal cell; *a*, amacrine cell; *g*, ganglion cell; *r*, rod photoreceptor.

not shown). A low level of *Notch 2* expression was seen in the PPE (data not shown).

At E15.5, the lens vesicle and optic cup are completely formed due to the invagination of the lens placode and optic vesicle, respectively. The PE comprises a layer that is one cell thick surrounding the neural retina. Sections of the eye of an E15.5 embryo were hybridized with *Notch 1*, *Notch 2*, *Delta*, and *Jagged* probes. At this stage, *Notch 1* and *Delta* were expressed in many cells in the neural retina (Fig. 2*A,C*). The positive cells, however, appeared to be excluded from the periphery of the retina, the area corresponding to the presumptive ciliary body and iris. Interestingly, *Jagged* expression was limited to the presumptive ciliary body region and was not seen in the neural retina or presumptive iris (Fig. 2*D*). *Jagged* was also expressed at higher levels on the ventral side of the ciliary body region (Fig. 2*D*, *inset*), in contrast to its dorsal-restricted expression pattern at E12.5. *Notch 2* was absent from the neural retina but was expressed in the PE and optic stalk (Fig. 2*B*). Only *Jagged* was expressed in the lens, mostly in the equatorial region, and to a lesser extent in the anterior portion in the area of actively proliferating cells (Fig. 2*D*). At this stage, the posterior of the lens is filled by differentiating lens fiber cells.

Notch pathway genes are expressed in areas of active neurogenesis and morphogenesis

Notch 1 expression correlates well with neurogenesis in the retina, which occurs from E14 to P10, with the peak around P0 (Alexiades and Cepko, 1996). With the progression of neurogenesis, the number of *Notch 1*-positive cells decreased and eventually was reduced to zero when all cells were differentiated (Fig. 3*C*). An example of *Notch 1* expression in the postnatal period is shown in Figure 3. By P5, the majority of retinal neurons, including ganglion cells, cone photoreceptors, amacrine cells, horizontal cells, and some rod photoreceptors have been born and have differentiated to a certain extent. Differentiated cells, such as the ganglion cells in the ganglion cell layer, have turned off *Notch 1* expression completely (Fig. 3*B*). In contrast, Notch 1 was observed to be expressed in a subset of cells in the VZ, most likely the remaining undifferentiated cells at this stage. *Delta* showed a pattern similar to that of *Notch 1* during retinal neurogenesis (Fig. 3*D*). Its expression eventually diminished when the cells had differentiated.

Postnatal *Notch 2* expression appeared to be stronger in the ciliary body region than in the PE, in contrast to the E15 expression pattern (Fig. 3*E*). *Jagged* expression was confined to the ciliary body (Fig. 3*F*). Extending from the periphery of the optic cup, the ciliary body undergoes extensive folding in the neonatal period. At P0 and P5, a subset of inner nuclear layer cells and ganglion layer cells also appeared to express *Jagged* (Fig. 3*F*).

The spatial and temporal expression patterns of *Notch 1*, *Notch 2*, *Delta*, and *Jagged* are summarized in Figure 4. They appear to define different domains in the developing eye. *Notch 2* expression is only in the non-neuronal tissues, including the PE, optic stalk, and ciliary body, whereas *Notch 1* is expressed only in the neural retina. The domain of *Delta* expression is largely overlapping with that of *Notch 1*. The spatial–temporal pattern of expression of *Jagged* is especially dynamic. Its expression is seen in the neural retina, ciliary body, and lens. In each case, it is expressed by a

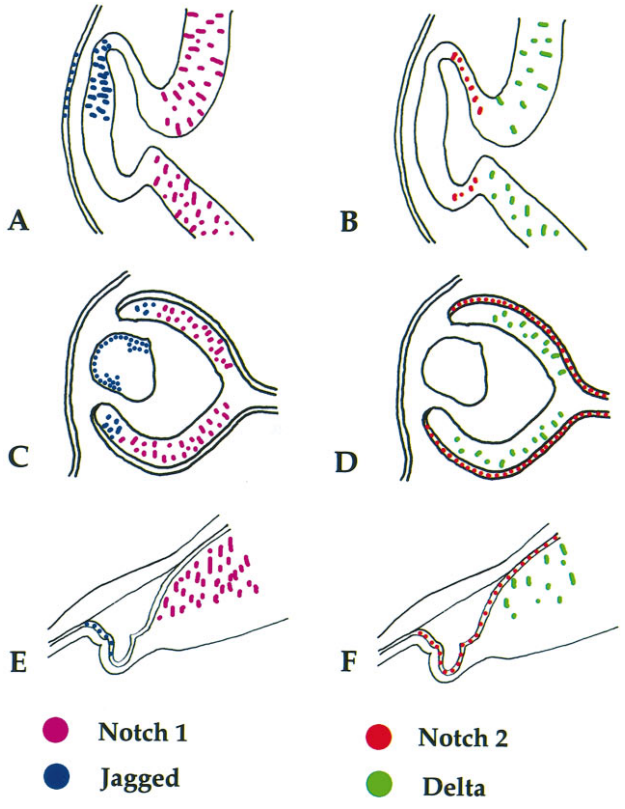

Figure 4. Schematic summary of *Notch 1*, *Notch 2*, *Delta*, and *Jagged* expression patterns in the developing eye, at E12.5 (*A, B*), E15.5 (*C, D*), P0 (*E, F*). *Notch 1* and *Jagged* patterns are shown in *A, C, E*; *Notch 2* and *Delta* patterns are summarized in *B, D, F*.

subset of cells in a particular region (e.g., dorsal retina and lens placode at E12.5).

Persistent *Notch* activity interferes with retinal cell differentiation

The expression patterns of *Notch 1* and *Delta* correlate well with specification and differentiation in the neural retina. To assess the role of *Notch 1* in the developing retina, a replication-incompetent retrovirus was used to express portions of the *Notch* 1 gene. This approach allowed examination of the effects of these genes on small subsets of cells in an otherwise unperturbed retina. The retroviral vector, LIA, was engineered to express a cDNA from the LTR promoter, which also includes an IRES sequence directing the translation of the human PLAP gene (Fig. 5*B*). This enables detection of infected clones by histochemical staining for AP. mNIC encodes the cytoplasmic domain of mouse Notch 1 with a myc tag at the N terminus (Fig. 5*A*). mNEC encodes the extracellular and transmembrane domains of mouse Notch 1 with a myc tag attached at the C terminus (Fig. 5*A*). On the basis of the results obtained with similar truncations in *Drosophila* Notch (Rebay et al., 1993), mNIC is expected to be a constitutively active receptor, whereas mNEC is expected to be a dominant negative form.

To examine the expression of the Notch constructs, NIH 3T3 cells were infected by the viruses, and the expression of the truncated *Notch* genes was examined by immunohistochemical staining with an anti-myc antibody. The mNIC-infected clones demonstrated mostly nuclear staining, whereas mNEC-infected clones displayed staining on the plasma membrane (Fig. 6*A,C*).

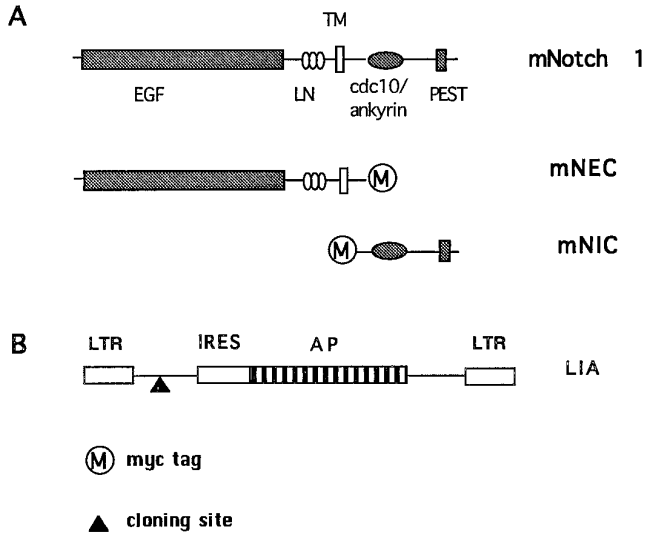

Figure 5. Viral constructs for expressing truncated forms of the *Notch 1* gene. *A*, Full-length Notch 1 protein consists of extracellular EGF repeats, Lin/Notch repeats (*LN*), transmembrane domain (*TM*), cytoplasmic cdc10/ankyrin repeats, and a *PEST* sequence. Truncations of Notch, mNIC, and mNEC, with myc epitope tags, were inserted into the cloning site of pLIA. *B*, pLIA retroviral vector. On the basis of the MMLV backbone, pLIA has a cloning site for expressing exogenous genes under the transcriptional control of the *LTR*. It also contains an *IRES* sequence directing the translation of a marker gene, PLAP.

This is consistent with previous findings that truncated Notch, without the extracellular domain, is translocated to the nucleus, although full-length Notch is localized on the cell membrane (Fortini et al., 1993; Lieber et al., 1993). AP enzyme activity was examined by X-Phos/NBT histochemical staining. A much lower level of histochemical staining was detected within the mNIC-infected cells compared with the mNEC-infected or control LIA-infected cells, suggesting effects of the upstream sequence on the expression of PLAP under the IRES translational control. The number of AP+ clones was much higher than the number of clones detected by anti-myc staining, probably because of the much higher sensitivity of the histochemical staining (data not shown).

Newborn (P0) rat retinae were infected *in vivo* by intraocular injection of virus (Turner and Cepko, 1987). Three weeks later, when retinal cells were fully differentiated, the infected retinae were dissected and stained for AP activity. Cross-sections were observed for the morphology of the infected AP+ clones. Clusters of labeled cells were clearly visible on sections. Each cluster probably represents one clone, because the number of clusters per retina was low. The control LIA virus-infected clones included four cell types: rod photoreceptors, bipolar cells, amacrine cells, and Müller glial cells. The cell types were identified on the basis of their morphology and the locations of the cell bodies (Fig. 7*A–C*). The distribution of the cell types in the clones was consistent with the results derived from previous lineage analyses (Turner and Cepko, 1987). Because only dividing progenitor cells can be infected by this type of retrovirus, only these four cell types would be expected in the clones infected at P0; all other cell types are postmitotic by P0. The size of the control LIA clones also appeared to be normal, with an average clone containing approximately two cells, as described by Turner and Cepko (1987).

The mNIC-infected clones displayed grossly abnormal morphology and were much larger than the control LIA-infected

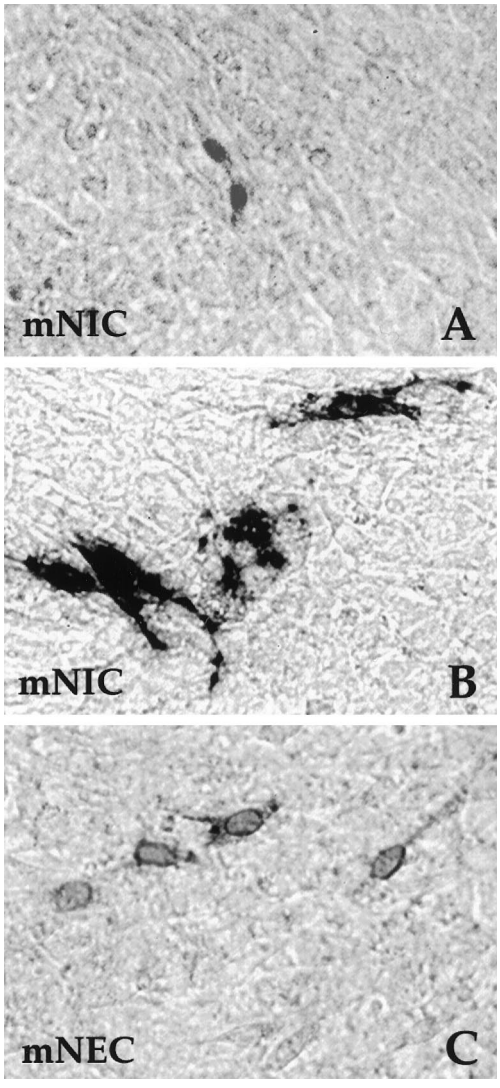

Figure 6. Expression of the truncated *Notch 1* gene in infected 3T3 cells, detected by immunohistochemical staining with an anti-myc antibody, 9E10. *A*, mNIC-infected 3T3 cells stained with the 9E10 monoclonal antibody (mAb). *B*, mNIC-infected 3T3 cells stained with X-Phos/NBT shows the expression of PLAP. *C*, mNEC-infected cells stained with the 9E10 mAb. Note that the Notch 1 intracellular domain was mostly translocated to nuclei, whereas the Notch 1 extracellular domain was localized to the cell membrane.

clones (Fig. 7*D–F*). Although the control clones were usually contained within one 20 μm section, the majority of mNIC-infected clones spanned 5–10 sections. Extensive attempts to quantify the number of cells within the mNIC clones were unsuccessful. The formazan precipitate obscured the position of cell bodies, particularly because the processes were heavily labeled and wrapped around nearby cells. The formazan precipitate also absorbed fluorescence of dyes such as DAPI, making it difficult to count the number of nuclei within a clone. Nonetheless, when a series of sections were made through mNIC-infected clones, it was clear that there were more than two cells within a clone, making these clones larger than the control, LIA-infected clones.

Although clonal morphology varied among the mNIC clones, the most common morphology (35 of 45 clones) resembled a "Christmas tree" with a major "trunk" extending across the retina (Fig. 7*D–F*). Long, horizontal processes ("branches") were found in both the optic fiber layer and the inner plexiform layer. Shorter processes were seen in the outer plexiform layer. Some clones had a more severe phenotype in which the extensive formazan precipitates in the stained cells almost resembled a tumor. The laminated structure of the retina was disturbed around the clones and appeared thicker than in uninfected regions. A few clones (2 of 45 clones) had normal morphology. The effect of the mNEC virus was assayed similarly; however, the mNEC-infected clones appeared to be completely normal, with normal morphology, clonal composition, and size (data not shown).

To determine whether embryonic retinal cells responded similarly to the truncated forms of Notch, E18 retinae were used for infection. Because injection into the subretinal space is much more difficult *in utero*, E18 retinae were dissected, infected, and cultured as explants. Viruses were added to the explant on the first day of culture, and the infected retinae were harvested 2 weeks later. The growth and differentiation of retinal cells in explant cultures are similar to those *in vivo* (Sparrow et al., 1990). Another advantage with infection of explants versus *in vivo* is that a much larger volume of virus can be used. This allows two different viruses to be used to infect each explant: a control BAG virus, which encodes lacZ, in combination with either mNIC or mNEC virus. Two weeks later, the explants were harvested and stained with X-gal to visualize the BAG-infected clones and with X-Phos/NBT to visualize the mNIC or mNEC-infected clones. Similar to the *in vivo* infection results, the mNIC-infected clones appeared much larger in size, when viewed in whole mounts (Fig. 8*A*). The activated form of Notch thus had a similar effect on embryonic retinae. Moreover, the control BAG-infected clones appeared normal in morphology and clone size, even when they were in the immediate vicinity of clones infected by mNIC virus, where the retinal layers were disturbed by the mutant clones. This suggests that the effects of this activated Notch construct were either autonomous or were limited to a very small distance from infected cells. Similar to the results of *in vivo* infection, the mNEC-infected clones within explants appeared to be normal (Fig. 8*B*).

DISCUSSION

In this study, we determined the expression patterns in the developing eye of several genes in the *Notch* pathway: *Notch 1*, *Notch 2*, *Delta*, and *Jagged*. We have also investigated the function of Notch 1 *in vivo* by using a replication-incompetent retrovirus to deliver a constitutively active allele of *Notch 1*. For genes such as *Notch*, which can have broad and complex effects, analysis of gene function within clones in an otherwise normal environment allows one to examine cell autonomous effects. With use of this retroviral strategy, it was found that activated Notch interferes with the normal differentiation of retinal cells, resulting in large aberrant clones with abnormal morphology.

The expression patterns of *Notch* pathway genes suggest a role in the patterning of the eye

The expression patterns of *Notch 2* and *Jagged* suggest a role in the patterning of ocular tissues. *Notch 2* was expressed only in the non-neuronal derivatives of the optic cup, including the PE, optic stalk, and ciliary margin. The onset of *Notch 2* expression in these domains was quite early. Therefore, *Notch 2* will be useful as an early marker for these regions. Because very little is known about the mechanisms that define these nonretinal tissues as distinct from the contiguous retina, it will be of interest to investigate whether *Notch 2* plays an active role in patterning this region of the optic cup.

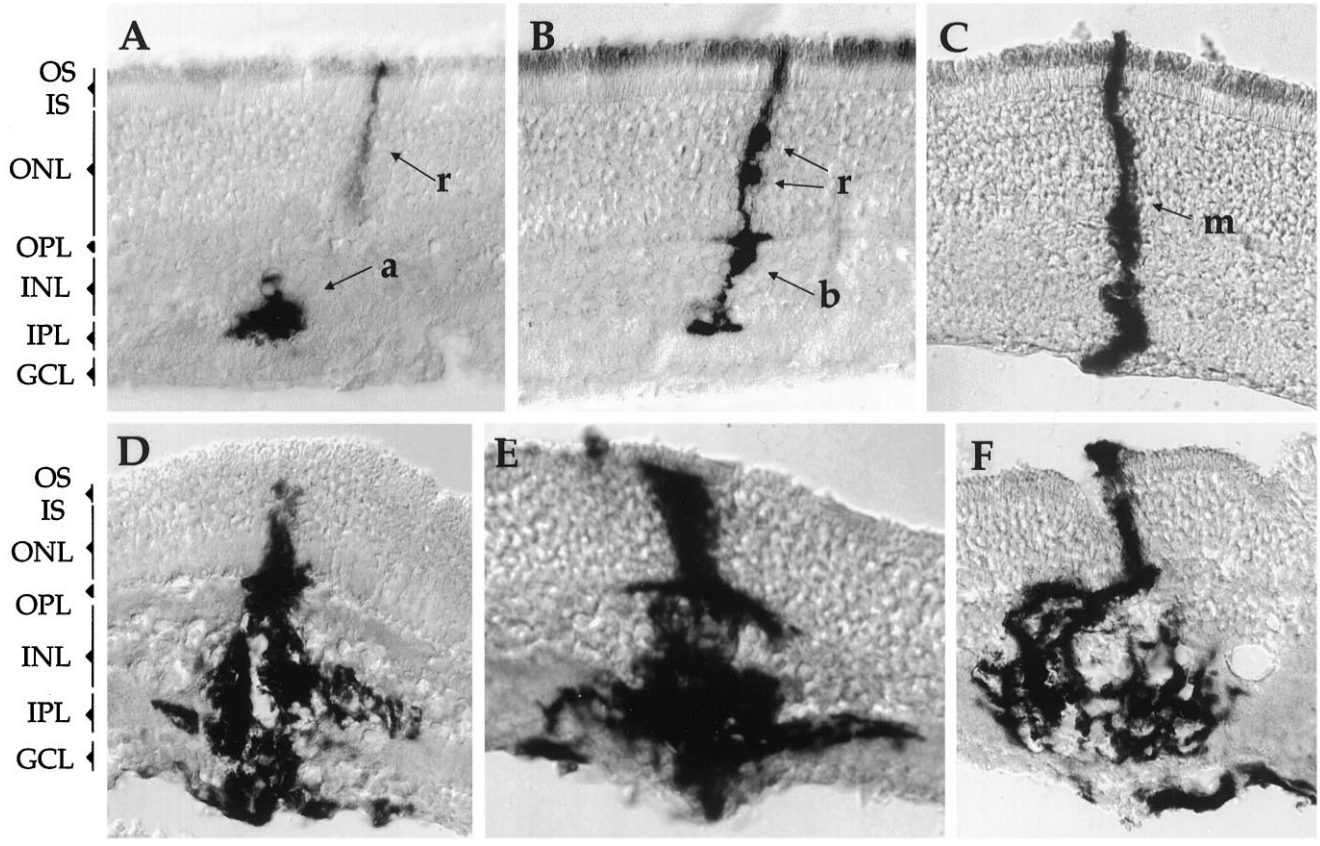

Figure 7. Clonal morphology of LIA- and mNIC-infected clones. P0 retinae were infected *in vivo* by intraocular injection. Retinae were harvested 3 weeks later, stained with X-Phos/NBT, and then sectioned to reveal clonal morphology. *A–C*, Control LIA-infected clones. Four cell types were observed: rod photoreceptor, amacrine cell, bipolar cell, and Müller glia. *D–F*, mNIC-infected clones. Note the grossly abnormal morphology within clones infected with the virus expressing an activated form of Notch 1. *OS*, Outer segment layer; *IS*, inner segment layer; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer; *a*, amacrine cell; *r*, rod photoreceptor; *b*, bipolar cell; *m*, Müller glia.

The expression of *Jagged* in the developing eye also suggests a role in patterning. Its expression was seen as early as E12.5 in the dorsal regions of the optic vesicle and lens placode. Patterning along the dorsal/ventral (DV) and anterior/posterior axes is evident within several laminae of the retina. Retinal ganglion projections to the tectum depend on the DV position of ganglion cells in the retina. Some Eph family receptor tyrosine kinases are expressed in a gradient in the retina along the DV axis (Cheng et al., 1995; Holash and Pasquale, 1995; Kenny et al., 1995) and appear to control, in part, the targeting of ganglion axons within the tectum. Asymmetry is also observed in the distribution of photoreceptor cells. For example, in chick, rods are more abundant within the ventral side of the retina (Bruhn and Cepko, 1996), and in mice, blue cones are found almost exclusively in the ventral half of the retina (Szel et al., 1992). In addition, the optic fissure, through which blood vessels enter the retina, forms on the ventral side of the retina. Whether *Jagged* participates in the patterning events that result in the DV identity of the retina awaits further study. *Jagged* is also expressed in the presumptive ciliary body region before it is morphologically distinguishable from the adjacent neural retina or iris. It is later expressed in the folds of the ciliary body and in a subset of retinal neurons. In summary, *Jagged* expression is very dynamic during eye development and usually coincides with areas undergoing active morphogenesis.

In *Drosophila*, the role of the Notch pathway in tissue patterning and morphogenesis has been studied. It has been found that *Notch*, *Delta*, and *Serrate* are essential for wing margin formation and outgrowth (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; Rulifson and Blair, 1995; de Celis et al., 1996; Doherty et al., 1996). Serrate acts as a ligand required in dorsal cells to activate Notch at the DV boundary of the wing imaginal disk, whereas Delta acts as a ventral-to-dorsal signal. Downstream signaling components of the Notch pathway identified in studies of neurogenesis, such as *Su (H)* and the *E(spl)* complex, also participate in the formation of the wing margin. It remains unclear, however, how signals from Serrate in the dorsal compartment and Delta in the ventral compartment are integrated through Notch at the wing margin. Because *Jagged* and *Delta* are expressed similarly in two opposing regions in the developing eye, they may play a similar role in setting up the pattern in the periphery of the eye. Misexpression of *Jagged* or *Delta* in the developing eye may clarify their role in patterning.

Expression of *Notch 1* and *Delta* suggests a role in cell fate determination

The expression of *Notch 1* and *Delta* in the developing eye appears to be within undifferentiated progenitor cells of the neural retina. This is consistent with their expression in the other regions of developing CNS (Coffman et al., 1990; Weinmaster et al., 1991; Reaume et al., 1992; Myat et al., 1996). The scattered pattern of *Delta* expression is similar to that seen in the developing brain,

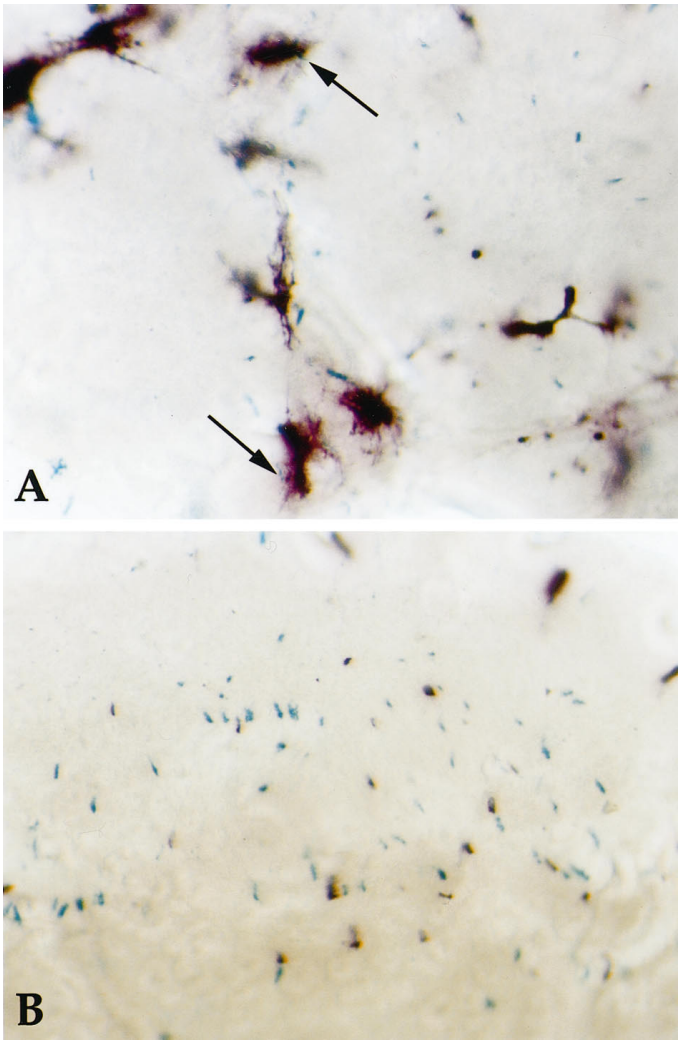

Figure 8. Morphology of mNIC- and mNEC-infected clones within retinal explants. E18 retinal explants were co-infected with BAG virus, encoding lacZ, and mNIC virus (*A*) or mNEC virus (*B*). The explants were harvested 2 weeks later and processed to visualize both BAG-infected clones (*blue*) and mNIC- or mNEC-infected clones (*purple*). Photographs were taken from the photoreceptor side of the retina. Note mNIC-infected clones were very large, with abnormal morphology, including extensive processes. In contrast, mNEC-infected clones appeared normal. The effect of the activated Notch appeared to be restricted to infected cells, because blue clones were normal even when located very close to mNIC-infected clones (*arrows*).

where it is present only in newly postmitotic cells (Henrique et al., 1995). The timing of *Notch 1* and *Delta* expression correlates well with neurogenesis in the retina. Expression of both genes begins at the onset of neurogenesis and peaks around P0, when the largest number of neurons is generated (Alexiades and Cepko, 1996). Thereafter, their expression decreases and is gone completely in the differentiated retina by P27. Ahmed et al. (1995), however, reported that *Notch 1* is expressed in differentiated ganglion cells and inner nuclear layer cells, in addition to being expressed in undifferentiated cells of the retina. They also reported that *Notch 1* is expressed in PE and lens. These discrepancies may reflect the difference in the assays used, because Ahmed et al. (1995) used antibodies against Notch 1 for immunohistochemistry. Specificity of the antisera may be an issue because of the fact that at least three Notch proteins exist in mammals.

Notch activity and cell differentiation

Expression of an activated *Notch 1* allele in retrovirally infected cells led to aberrant differentiation of retinal cell types in an otherwise normal environment. Analogously, *Notch* has been shown to be involved in the differentiation pathway of multiple cell types in *Drosophila* and *Xenopus* retina. Expression of activated Notch appears to block cell differentiation (Cagan and Ready, 1989; Fortini et al., 1993; Dorsky et al., 1995). This block, however, does not appear to be irreversible in *Drosophila*. Once Notch activity subsides, cells may recover and differentiate according to the developmental cues present in a later environment (Fortini et al., 1993; Struhl et al., 1993). These studies suggest that Notch activity regulates the ability of cells to respond to differentiation signals. Other studies in mammalian cell cultures and *Xenopus* embryos also support the model that Notch blocks differentiation of neural and mesodermal cell lineages (Coffman et al., 1993; Kopan and Nye, 1994). Notch activity may not block differentiation of all cell types indiscriminately, however, because neuronal differentiation, but not glial differentiation, was blocked in cultures of differentiating P19 cells (Nye et al., 1994).

Among mNIC-infected clones, the most common "Christmas tree" morphology resembled aberrant Müller glial cells and/or undifferentiated neuroblast cells. Only Müller glia and undifferentiated neuroblasts have vertical processes extending across all retinal layers. Undifferentiated neuroblast cells do not have horizontal processes. The mNIC clones, however, had extensive horizontal processes. If these clones were indeed aberrant Müller glial cells, Notch may block neuronal differentiation but allow some aspects of glial differentiation to take place. Definitive identification of the mNIC-infected cells will require characterization by molecular markers. Unfortunately, we have been unable to detect the infected clones by indirect immunofluorescent staining, because of the low expression levels of the myc tag and AP in the infected clones. Thus, clones could only be located using the more sensitive AP histochemical staining, and the formazan precipitates that result obscure fluorescence and other detection methods that could be used to detect cell type-specific antigens within a clone. The low expression levels of the mNIC construct were unusual, because the control LIA and mNEC had much higher expression levels. One possible explanation is that a high level of Notch activity kills infected cells, and thus only those clones with low expression levels, e.g., attributable to a disadvantageous integration site, survive. In support of this idea is the finding that the titers of mNIC on NIH 3T3 cells or within the retina were 5- to 10-fold lower than the other two constructs used in this study and compared with other constructs that we have made.

Although the problem with detection has also prevented us from determining the number of cells per clone, it appears that the size of the mNIC clones was ~10 times larger than the control clones. Although the increased size may be attributable to the presence of very large cells, it seems that on sections of some mNIC clones there are more than the two cells that are observed in control, LIA-infected clones. It appears likely that the large clones are attributable to abnormal proliferation and abnormal morphology. Proliferation has been seen to result from other studies of truncated *Notch*. A truncated *Notch 1* homolog in human (*TAN-1*) and in mouse (*int-3*) have been shown to cause distinct forms of T cell lymphoma and mammary tumors, respectively (Ellisen et al., 1991; Jhappan et al., 1992). Not all cells, however, undergo proliferation in response to Notch activity. Early chick retinal cells do not appear to proliferate when activated Notch is introduced (Austin et al., 1995), nor do retinal cells

in *Xenopus* (Dorsky et al., 1995). In fact, in *Xenopus*, activated Notch arrested proliferation and blocked differentiation. Further study of the role of *Notch* in cell proliferation and of the mechanism by which CNS cells couple the cell cycle and differentiation will be required to understand these observations.

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