

Vertebrate retinal ganglion cells are selected from competent progenitors by the action of *Notch*

Christopher P. Austin, Douglas E. Feldman, James A. Ida, Jr. and Constance L. Cepko

Howard Hughes Medical Institute and Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA

SUMMARY

The first cells generated during development of the vertebrate retina are the ganglion cells, the projection neurons of the retina. Although they are one of the most intensively studied cell types within the central nervous system, little is known of the mechanisms that determine ganglion cell fate. We demonstrate that ganglion cells are selected from a large group of competent progenitors that comprise the majority of the early embryonic retina and that differentiation within this group is regulated by *Notch*. *Notch* activity in vivo was diminished using antisense oligonucleotides or augmented using a retrovirally transduced constitutively active allele of *Notch*. The number of

ganglion cells produced was inversely related to the level of *Notch* activity. In addition, the *Notch* ligand Delta inhibited retinal progenitors from differentiating as ganglion cells to the same degree as did activated *Notch* in an in vitro assay. These results suggest a conserved strategy for neurogenesis in the retina and describe a versatile in vitro and in vivo system with which to examine the action of the *Notch* pathway in a specific cell fate decision in a vertebrate.

Key words: retina, ganglion cells, *Notch*, equivalence group, vertebrate

INTRODUCTION

At the earliest stages of development, the anlage of the vertebrate central nervous system (CNS) is composed entirely of mitotic progenitors. Little is known about the mechanisms by which these cells become determined to their fates and transform into the myriad differentiated cell types characteristic of the mature CNS. The retina is an attractive region of the CNS in which to study these issues because it is a relatively simple structure with well-characterized cellular architecture and is accessible to experimental manipulation (Dowling, 1987). Retinal lineage analysis has shown that progenitors are capable of producing overlapping combinations of cell types, suggesting that progenitors may be multipotential and that signals in a progenitor's environment contribute to the determination of cell fate (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990; Fekete et al., 1994). A number of diffusible factors have been shown to be capable of affecting differentiation of late-born cell types in the retina, including taurine (Altshuler et al., 1993) and 9-*cis* retinoic acid (Kelley et al., 1994), though their roles in normal development remain obscure.

The first cells produced during retinal development are the ganglion cells, the projection neurons of the retina. Extensive study of ganglion cell physiology (Kuffler, 1953; Dowling, 1987) and projections (Sperry, 1963; Bonhoeffer and Gierer, 1984) have demonstrated that ganglion cells transduce the retina's analysis of visual information and topographically map onto secondary visual centers in the brain. Despite this wealth

of functional data, little is known about how ganglion cells become committed to their fates during development. Ganglion cells have a number of features that make them attractive as a model for fate acquisition in the CNS. Because ganglion cells, like other CNS projection neurons, are born first during development, they arise from an environment that lacks other differentiated cells, thus simplifying cell type identification and possibly the complexity of environmental influences on cell fate. Ganglion cells also share antigenic, morphologic and functional characteristics with other CNS projection neurons and multiple markers exist with which to identify them in vivo and in vitro (Dowling, 1987).

In several invertebrate species, analogous early-born neurons are generated from a pool of progenitors with equivalent developmental potential, termed an 'equivalence group' (Kimble et al., 1979). Cell-cell interactions inhibit most of the cells in an equivalence group from differentiating into a primary cell fate and most take on secondary or tertiary fates. Only the cells that escape inhibition differentiate into the primary cell type. In *Drosophila*, proneural basic helix-loop-helix genes of the *achaete-scute* complex (*as-c*) (Skeath and Carroll, 1991, 1992) or *atonal* (Jarman et al., 1994) are required for establishment of neural competence of cells in an equivalence group. The inhibition of differentiation results from down-regulation of the *as-c* loci by the action of the neurogenic gene *Notch* (*N*) (Cabrera, 1990) and multiple other genes implicated in the transmission of the *N* signal, including *mastermind*, *deltex*, *Hairless*, *Suppressor of Hairless* and the *Enhancer of Split* complex (reviewed by Artavanis-Tsakonas et al., 1995). Definition of equivalence

groups has been accomplished by eliminating cell-cell interactions that normally mediate inhibition of differentiation by either generating neurogenic loss-of-function mutants (Heitzler and Simpson, 1991; Goriely et al., 1991) or ablating one or more cells in the equivalence group (Kimble, 1981). Elucidation of progenitor competence and the mechanisms of cellular specification in the vertebrate CNS have been more difficult, though evidence exists for a two-cell equivalence group in the zebrafish spinal cord (Eisen, 1992).

In the retina of *Drosophila*, genetic mosaic analyses demonstrated that, similar to the vertebrate retina, a progenitor's lineage did not predict the cell types that it produced (Ready et al., 1976; Lawrence and Green, 1979). Multiple cell fate decisions in the *Drosophila* retina have been shown to depend upon interactions among neighboring cells (Cagan, 1993). The R8 photoreceptor is the first cell type to terminally differentiate in the *Drosophila* retina, and the generation of the correct number and spacing of R8 cells requires *Notch* and other genes of the neurogenic group (Cagan and Ready, 1989; Baker et al., 1990; Baker and Rubin, 1992). Reduction of *Notch* activity in the region of the eye imaginal disc in which R8 differentiation is taking place promotes the immediate differentiation of most of the cells in the region as neurons with characteristics of R8, leading to the suggestion that these cells represent a large R8 equivalence group (Cagan, 1993).

In this paper, we show that the first-born neurons in the chick retina, the ganglion cells, are generated by a similar strategy to that operative in development of R8 in the *Drosophila* retina. The function of a critical component of the regulation of this developmental decision, Notch, is also conserved. The majority of progenitors in the early retina were found to be competent to differentiate as ganglion cells. Chick *Notch-1* was found to be expressed in the undifferentiated cells of the retina at this stage and alteration of *Notch* activity in either direction affected the number of ganglion cells produced in the retina. Antisense oligonucleotides were used to decrease Notch-1 level in vivo and in vitro, increasing recruitment into the ganglion cell pathway and doubling the thickness of the ganglion layer in vivo without affecting mitotic activity. Conversely, retroviral transduction of constitutively active Notch into progenitors decreased recruitment into the ganglion cell pathway in vivo and in vitro, and this effect could be eliminated by antisense treatment. Finally, *Drosophila* Delta was found to substitute for a putative Notch ligand in blocking differentiation of chick retinal progenitors into ganglion cells in a co-culture assay. The experimental system that we describe makes possible the manipulation of the Notch pathway activity in vivo and in vitro, with quantifiable effects on the differentiation of a specific vertebrate cell type.

MATERIALS AND METHODS

Experimental animals

White Leghorn chicken eggs were purchased from Spafas, Inc. (Norwich, CT) and incubated at 38°C in a rotating humidified incubator. Staging was done according to Hamburger and Hamilton (1951).

Retinal cell culture

The protocol for dissociation of retinal cells and culture in collagen gels was modified from Altshuler and Cepko (1992); cells were

cultured at a concentration of 25,000 cells/25 µl gel unless otherwise noted, with the addition of 100 µg/ml conalbumin (Sigma) and 10 µg/ml insulin. Gels were dissolved by addition of media containing 200 units/ml collagenase (Worthington); cells were transferred to polyornithine-coated slides and fixed in 4% paraformaldehyde in PBS for 15 minutes.

Cell pellets were made by centrifuging 10⁶ cells at 8000 g for 10 minutes. For explant cultures, retinæ were dissected and transferred directly to 200 µl medium. For marking of S-phase cells, 5 µCi/ml [³H]thymidine (Amersham) was included in the medium of explants for 1-2 hours before dissociation.

Immunohistochemistry and cell counting

After fixation, cells were blocked for 1 hour in 10% FCS, 5% donkey serum (Jackson Immunologicals, West Grove, PA) and 0.4% Triton X-100 in PBS. Primary antibody incubations were for 1 hour at room temperature in 10% donkey serum in PBS. Monoclonal antibodies and dilutions used to detect ganglion cells were as follows: 8A1, recognizing low molecular weight neurofilament (Barnstable, 1987), 1:1000; RA4 (McLoon and Barnes, 1989), 1:300; 4D5, recognizing the Islet-1 protein (Yamada et al., 1993), 1:50; 40.2D6 (directed against Islet-1) and 3A10 and E/C8 (both directed against neurofilament-associated proteins), obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA, 1:10; RMO 270.7, directed against low-molecular weight neurofilament (Carden et al., 1987), 1:10; 8D9, directed against NgCAM (Lemmon and McLoon, 1986), 1:10; anti-GAP-43 (Sigma Immunologicals), 1:1000. Polyclonal rabbit sera used were: anti-α₆-cytoA and anti-α₆-cytoB, directed against α₆ integrin (de Curtis and Reichardt, 1993), 1:500 and anti-Trk B (Santa Cruz Biotechnology, Santa Cruz, CA), 1:50. Each antibody was used to stain chick retinal sections from stage 14 (E1.5) to stage 35 (E8) and were found to be ganglion cell-specific. Rabbit anti-p27 gag antiserum (Spafas) was used at 1:100. Secondary antibodies used were Texas Red-conjugated donkey anti-mouse IgG, fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson Immunologicals), or anti-mouse Vectastain ABC Elite (Vector Labs), using DAB as chromogen. For autoradiography, slides were processed for immunocytochemistry, then dehydrated through graded alcohols, dipped in autoradiography emulsion (Kodak NTB-2) and exposed in light-tight boxes at 4°C for 2 days. After staining, slides were mounted in Gelvatol and coverslipped, and observed under UV illumination on a Zeiss axiophot microscope. At least 200 cells were counted in each well of a slide; each experimental condition was performed and counted in triplicate in each experiment, and each experiment was repeated at least three times.

Retinal cell:S2 cell co-culture

Drosophila S2 cells and L49-3-6 Delta transformants were a gift of Dr Spyros Artavanis-Tsakonas, Yale University, New Haven, CT and were cultured according to Fehon et al. (1990). *Drosophila* and chick cells were mixed at a ratio of 50:1 (*Drosophila*:chick), spun at 8000 g for 10 minutes and the pellets cultured in 200 µl retinal media in a humidified 5% CO₂ atmosphere at 37°C for 24 hours. After incubation, pellets were dissociated, fixed, stained for NF and processed for autoradiography. Counts of the percentage of [³H]thymidine-labelled cells that also expressed NF were made. 3 pellets using untransfected S2 cells and 3 pellets using S2-Delta cells were made in each of 3 experiments; 100 cells were counted in triplicate for each experiment.

In situ hybridization

The in situ hybridization procedure was modified from Riddle et al. (1993), as follows: retinæ were sectioned at 15 µm and permeabilized with proteinase K (Boehringer-Mannheim, 1 µg/ml) for 10 minutes. The probe was a digoxigenin-labelled 1 kb chick *Notch-1* mRNA encoding a portion of the extracellular domain between the

EGF repeats and *lin12/Notch* repeats, transcribed from a plasmid supplied by Domingos Henrique and Julian Lewis at the Imperial Cancer Research Fund, Oxford, UK.

Western blotting

Retinal extracts were made by resuspending stage 24 chick retinae in RIPA buffer containing 3.4 µg/ml aprotinin, 25 µM leupeptin, 1 µg/ml pepstatin and 100 µM PMSF. 100 µg of total protein were run per lane, as determined by a bicinchoninic acid microassay kit (Pierce). Filters were incubated overnight at 4°C with an affinity-purified rabbit polyclonal antiserum directed against synthetic peptides from the *cdc10/ankyrin* repeat region of TAN-1 (a gift of J. Aster and J. Sklar) diluted 1:100. Specificity of the antiserum was assessed by preincubating the antibody with 1 mg/ml of the peptides against which the antibody was made, overnight at 4°C. Secondary antibody was 0.6 µCi/ml ¹²⁵I-labelled sheep anti-rabbit (Amersham) and signal was visualized using a PhosphorImager (Molecular Dynamics).

Antisense oligonucleotides

The sequences of *Drosophila Notch*, *Xotch*, rat *Notch 1* and 2, *Motch* and *TAN-1* were obtained from GenBank. Unpublished sequence data on *TAN-1* was obtained from Drs Jon Aster and Jeffrey Sklar at the Brigham and Women's Hospital, Boston. Unpublished partial sequence data on the epidermal growth factor-like repeat region and the *lin-12/Notch* repeat region (see Fig. 6A for explanation of terms) of a chicken *Notch-1* homologue (*CNotch-1*) was very kindly provided by Domingos Henrique and Julian Lewis, Imperial Cancer Research Fund, Oxford, UK. Antisense oligonucleotides were 23-mers with phosphothiorate linkages between all bases, were approximately 50% GC and had no base repeated any more than twice in succession. Oligonucleotides were designed against three distinct regions of the *Notch* sequence: (a) the EGF repeat region (oligo designated EGFR). *CNotch-1* sequence was used, with antisense sequence 5'-GTAGTCATTGACCCGCTGCACGC-3'. (b) the *lin12/Notch* repeat region (oligo designated LNR), with *CNotch-1* antisense sequence 5'-CCAGCACTGCAGTGACTGTGAGC-3'. (c) the 5' end of the *cdc10/ankyrin* repeat region (oligo designated CDCR). Chicken sequence was not available, so rat *Notch-1* sequence was used. Nucleotide sequence is highly conserved in this region and rat *Notch-1* sequence was used because it was most homologous to the chick *Notch-1* sequence in other regions. The CDCR oligonucleotide sequence differed at only 1-2/23 positions among the *Xenopus*, mouse, rat and human *Notch* homologues. The antisense sequence used was 5'-CCTCCGCTGCAGGAGGCAATCAT-3', representing bp 5871-5893 of rat *Notch-1*. For the oligonucleotide mismatch experiments, the chick LNR region sequence was used. Nucleotide substitutions were made only in position one of a codon, as the chicken flock used is outbred and position one is least likely to vary among strains of a species. Reading frame of the chick sequence was determined by comparison with other *Notch* homologue sequences in which the start site and reading frame have been defined. A substitution in the antisense nucleotide sequence was then made in position 1 of one, three or five codons, with maintenance of GC content.

Syntheses were done at the Howard Hughes Medical Institute Biopolymer Facility, after which the oligos were purified by running over a NAP-10 column, desiccated in a spin-vac overnight and resuspended in a minimum volume of distilled water. OD₂₆₀ readings were done to determine concentration. For each antisense oligonucleotide, a corresponding sense oligonucleotide was made and used in parallel in each experiment.

Injections of 5 µl of 250 µM oligonucleotide in DME were done into the vitreous and subretinal spaces of stage 16 embryos (E2; Hamburger and Hamilton, 1951), after Fekete and Cepko (1993), with harvest at stage 27 (E5). Neurogenesis is active throughout the retina at these early stages, with central retina developing before peripheral.

More ganglion cells would be expected in central regions and numbers of ganglion cells were therefore assessed independently in each region. In order to standardize the region of the eye analyzed, sections in which the diameter of the lens was the same were used and, on these sections, a central, intermediate and peripheral region, each with a specific retinal thickness, were counted. Because section thickness and magnification were also constant, this technique allowed the number of ganglion cells to be compared directly. Ganglion cells were identified on sections using antibodies to the Islet-1 protein, as this antigen was found to be completely specific to ganglion cells in the retina through E8 (C. P. A. and C. L. C., unpublished) and gives discrete nuclear staining which allows quantitation. Counting of individual cells in sections using NF or RA4 is not possible because both are process-associated and individual cells could not be distinguished. Counts were done of all Islet-1⁺ cells in a high power field using a hemocytometer mounted on the microscope; 3 fields of each type were counted for each retina and 5 sense- and 5 antisense-injected retinae of each oligonucleotide type were analyzed.

Explants were incubated for 24 hours in 200 µl medium containing oligonucleotide at 37°C. Concentrations of oligonucleotide in medium from 0.1-100 µM, with or without 0.1-25 µM DOTAP, were tested initially to determine optimal concentrations. DOTAP was found to have no effect in any condition, so was omitted from later experiments. 3 sense and 3 antisense explants were used for each experiment and each experiment was repeated 3 times for each oligonucleotide type.

RNase protection assay

After 24 hours of explant culture in oligonucleotide, retinal RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction, according to established protocols (Chomczynski and Sacchi, 1987). RNase protections were done using the RPA II kit (Ambion, Austin, TX), according to the supplied protocol. 1-20 µg RNA was used for each reaction initially, with 5 µg being used for most experiments. The *Notch* probe was a 257 bp transcript from a *PstI* digest of the chick *Notch-1* plasmid, with an expected protected region of 178 bp. The control probe was a 358 bp transcript from a *PvuII* digest of a chick *EF1α* cDNA with an expected protected region of 116 bp, provided by Randy Johnson and Cliff Tabin, Harvard Medical School. Probes were labelled with α³²P-CTP and gel-purified. The RPA II kit protocol was followed, with the following modifications. Hybridizations were done at 45°C overnight and RNase digestions were done with 5 U/ml RNase A and 200 U/ml RNase T₁ at 37°C for 30 minutes. Protected fragments were separated on a 6% acrylamide/8M urea gel and quantitated using a PhosphorImager (Molecular Dynamics, Mountain View, CA).

Retroviral construction and injections

The cDNA JK5T corresponding to BP 4045-7948 of *TAN-1* (Ellisen et al., 1991), was a gift from Drs Jon Aster and Jeffrey Sklar at the Brigham and Women's Hospital, Boston. To make the TANIC vector, JK5T was digested with *Bss*II and *Bsu*36I (Boehringer), and the resulting 2362 bp *TAN-1* fragment was blunted with T4 DNA polymerase and ligated into the *ClaI* site of RCAS(BP)A. pTANIC was transfected into line 0 chicken embryo fibroblasts by CaPO₄ precipitation. A control RCAS(BP)A-derived vector, APA, carrying the human placental alkaline phosphatase gene (Fekete and Cepko, 1993) was produced in parallel. Supernatants were harvested, concentrated by centrifugation at 20,000 g for 2 hours and frozen at -80°C until use. Viral titers were determined by infection of QT6 fibroblasts followed by staining for viral gag protein using monoclonal antibody 3C2 and HRP-conjugated donkey anti-mouse secondary antibody (Stoker and Bissell, 1987). Titer of TANIC was 10⁸ colony forming units (cfu)/ml and the titer of the APA control vector was 10⁷ cfu/ml.

Injections of TANIC and APA were done into stage 16 embryos (E2; Hamburger and Hamilton, 1951), as above for oligonucleotide

injections, except that 0.2-0.5 μ l of virus suspension was injected into the subretinal space only, of the right eye. Quantitation of ganglion cells in retinal sections was done in the same way as for oligonucleotide-injected eyes. Infected regions of retina were identified by staining with antibodies to the p27 gag protein (Spafas). Counts were done of all Islet-1⁺ cells in a high power field, in both infected and uninfected regions; 3 fields of each type were counted for each retina, and 5 TANIC- and 5 APA-infected retinæ were analyzed.

RESULTS

The majority of retinal progenitors are competent to differentiate as ganglion cells

Neurogenesis in the chick retina occurs between E2 and E10; ganglion cells are born first, between E2 and E6 (Prada et al., 1991). Though less than 5% of retinal cells are ganglion cells

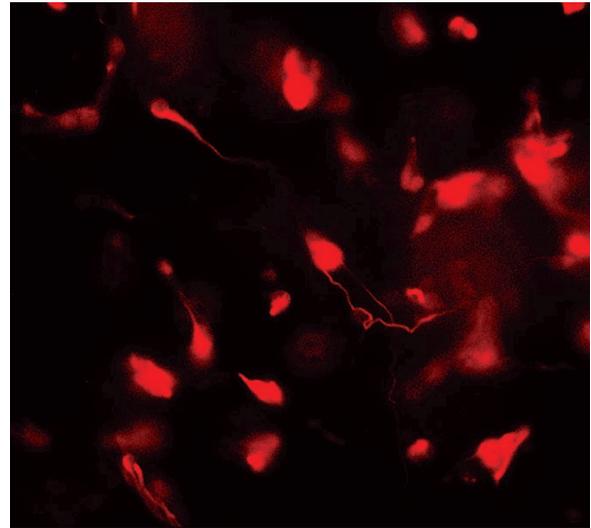
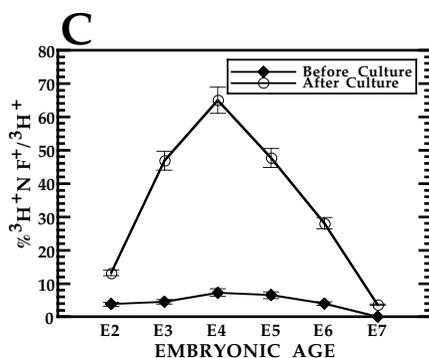
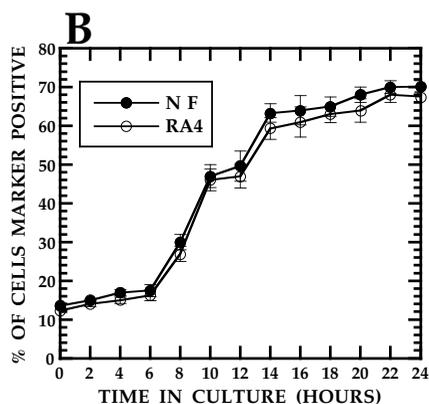
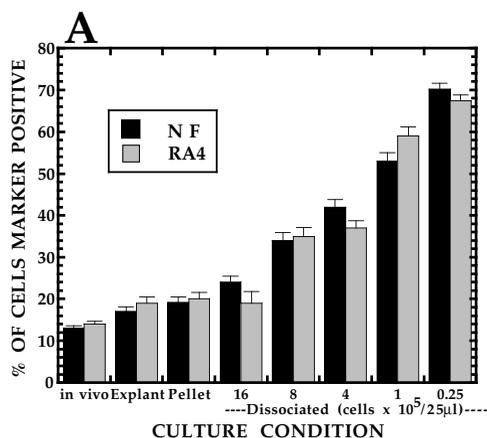


Fig. 2. Morphology of retinal cells in dissociated culture. Chick retinæ at E4 (stage 24) were dissociated and cultured at low density for 24 hours in collagen gels, then stained in situ with the ganglion cell-specific marker RA4 (McLoon and Barnes, 1989). Only some cells and processes are in focus because of the three-dimensional nature of the culture system.

in the adult chicken (Coulombre, 1955), the percentage of developing retinal cells that are ganglion cells was not known. This was determined by dissociating and immediately fixing chick retinal cells, followed by immunocytochemistry for the low molecular weight neurofilament (8A1; Barnstable, 1987), and the monoclonal antibody RA4 (McLoon and Barnes, 1989). Both antibodies recognize ganglion cells shortly after their terminal division in the retinal proliferative zone and remain specific for this cell type throughout retinal histogenesis (Barnstable, 1987; McLoon and Barnes, 1989; C. P. A. and C. L. C., unpublished observations). At E4 (stage 24), the

Fig. 1. (A) Density dependence of ganglion cell differentiation in culture. E4 (Stage 24) chick retinal cells were cultured for 24 hours under various conditions and stained for the ganglion cell-specific markers NF and RA4. The percentage of DAPI-stained cells that were NF⁺ or RA4⁺ were scored. Values plotted are means \pm s.e.m. of ≥ 3 experiments, with each condition done in triplicate in each experiment. 'in vivo', retinæ left in the animal for 24 hours; 'explant', retinæ cultured intact; 'pellet', retinæ dissociated and reaggregated into a pellet of 10^6 cells; 'dissociated', retinæ dissociated and placed in collagen gels at the indicated density. (B) Time course of ganglion cell marker expression in dissociated cultures. E4 (stage 24) chick retinæ were dissociated and cultured at a density of $0.25 \times 10^5 / 25 \mu$ l in collagen gels. Cells were harvested every 2 hours for the 24 hour culture period and stained for two ganglion cell-specific antigens, NF and RA4. (C) Developmental series of NF expression in dissociated cultures. Chick retinæ from E2 (stage 16), E3 (stage 20), E4 (stage 24), E5 (stage 27) E6 (stage 29) and E7 (stage 31) were incubated as explants for 1 hour in 5 μ Ci/ml [³H]thymidine, dissociated and cultured at a density of $0.25 \times 10^5 / 25 \mu$ l in collagen gels for 24 hours, stained for NF and developed for autoradiography. ³H⁺ cells that were also NF⁺ were scored. 'Before culture', cells fixed and stained immediately after dissociation. 'After culture', cells cultured for 24 hours, then fixed and stained. The percentage of ³H-labelled cells that were NF⁺ is shown (mean \pm s.e.m. for 3 experiments).

peak of ganglion cell genesis, $13.6 \pm 0.5\%$ (mean \pm s.e.m.) were neurofilament (NF)⁺ and $12.3 \pm 0.4\%$ were RA4⁺.

If cell-cell interactions were required for some aspect of ganglion cell differentiation, then the percentage of cells differentiating as ganglion cells might change with culture conditions that varied cell-cell contact. E4 retinæ were cultured for 24 hours in serum-free conditions in 3 ways: as intact retinæ (explants), as pellets (cells reaggregated through centrifugation), or as dissociated cells suspended in collagen gels at varied initial densities. The results of these experiments are in Fig. 1. From E4 (stage 24) to E5 (stage 27), the total number of cells in retinæ left in vivo increased by 10-fold, but the percentage of cells that express NF or RA4 did not change ($13.0 \pm 0.6\%$ NF⁺ and $14.0 \pm 0.7\%$ RA4⁺). Cells in explant culture, or cells dissociated and reaggregated into a pellet, showed a small increase, to 17–19% NF⁺ or RA4⁺. Cells cultured in collagen gels at the highest density showed a similar small increase in NF⁺ or RA4⁺ cells. As the cell density in collagen gels was progressively decreased over a 50-fold range, however, the percentage of cells that expressed NF⁺ or RA4⁺ after 24 hours increased (Fig. 1A). At the lowest density (0.25×10^5 cells/25 μ l), most cells were separated from their neighbors by several cell diameters; in this condition, the number of NF⁺ and RA4⁺ cells increased 5-fold during the 24 hours in culture, to $70.2 \pm 1.4\%$ NF⁺ and $67.4 \pm 1.4\%$ RA4⁺.

At E4, over 90% of the cells in the chick retina are mitotic progenitors (Dutting et al., 1983) and thus most of the NF⁺ and RA4⁺ cells present after 24 hours must have arisen from cells that were mitotic at the beginning of the culture period. This was confirmed by including [³H]thymidine in the culture medium to label mitotic cells. When only [³H]thymidine-labelled cells were scored after 24 hours in the lowest density culture, $75.4 \pm 1.0\%$ were found to be NF⁺, similar to the result from counting all cells. To exclude the possibility that differential cell division was responsible for the observed increase in the number of NF⁺ or RA4⁺ cells during the culture period, cells in various culture conditions were labelled with [³H]thymidine cumulatively throughout the 24 hour culture period. The percentage of cells that were labelled with [³H]thymidine after culture at lowest density, highest density, pellet and explant were the same, showing that differential mitosis in low density cultures does not explain the observed increase in cells expressing ganglion cell markers. Similarly, to exclude the possibility that differential death of cells not bearing the ganglion cell markers was responsible for the observed increase, cell recovery was quantified for each culture condition. The number of cells recovered after 24 hours was 80–90% of the number recovered if the cultures were harvested immediately after being made and this percentage did not vary according to cell density in culture. Furthermore, greater than 90% of the cells recovered after 24 hours of culture were viable as assayed by trypan blue exclusion.

Given the large increase in cells expressing ganglion cell

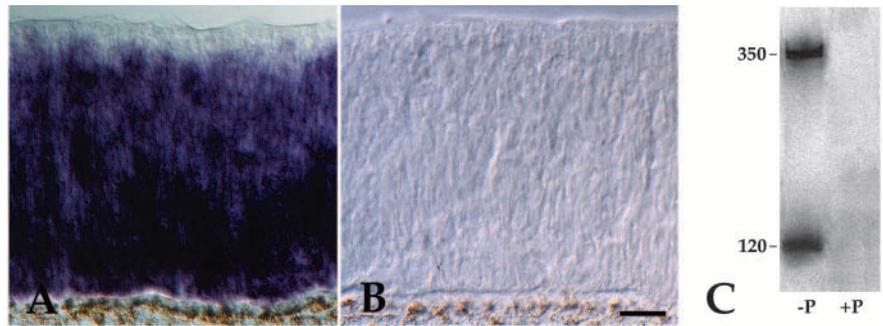


Fig. 3. Expression of *Notch* in the chick retina. (A) E4 chick retina after in situ hybridization with a digoxigenin-labelled antisense *CNotch-1* probe, with detection by alkaline-phosphatase-coupled secondary antibody. Pigmented epithelium is at the bottom, developing ganglion cell layer at the top. Bar, 100 μ m. (B) Adjacent section of E4 chick retina, after in situ hybridization with a digoxigenin-labelled sense *CNotch-1* probe. (C) Western blot of E4 chick retinal extract, stained with a polyclonal antiserum raised against peptides from the cdc10/ankyrin repeat region of TAN-1, the human Notch homologue. -P, antibody without blocking peptide; +P, antibody preabsorbed with TAN-1 peptides before staining.

markers over the short time in vitro, the time course of marker expression in the lowest density culture condition was examined by harvesting cells every 2 hours, and performing NF and RA4 immunocytochemistry. For the first 6 hours in culture, no significant change in the number of positive cells was seen. This was followed by a large increase in positive cells over the next 8 hours and a slow increase thereafter to 24

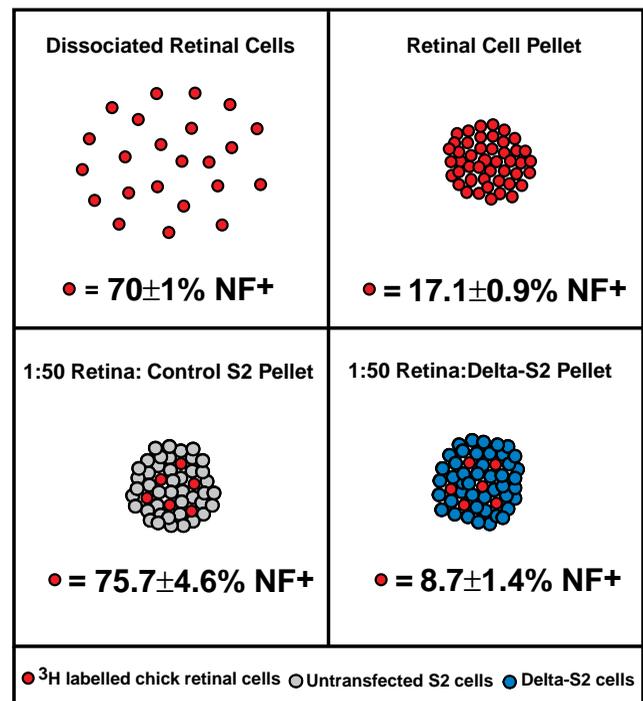


Fig. 4. Differentiation of chick retinal cells in pellets with S2 cells expressing Delta. E4 chick retinal cells were labelled as explants with [³H]thymidine, then dissociated and cultured in pellets with a 50-fold excess of *Drosophila* S2 cells that had been transfected with a full-length *Delta* cDNA (Fehon et al., 1990), or control untransfected S2 cells, for 24 hours. Pellets were dissociated and cells stained for NF and developed for autoradiography, and the percentage of ³H⁺ cells that were NF⁺ counted.

hours (Fig. 1B). Cells maintained in culture for another 48 hours showed no further change in the percentage of cells expressing NF or RA4 (data not shown).

To assess whether the expression of NF in dissociated culture was specific to progenitors from the period of ganglion cell genesis *in vivo*, progenitors from different age embryos were labelled with [³H]thymidine for 1 hour to mark cycling cells. Cells were then placed in dissociated cultures at the lowest density for 24 hours, followed by NF immunocytochemistry and autoradiography. The percentage of [³H]thymidine-labelled cells that were also NF⁺ after 24 hours in culture in this set of experiments increased from 13.2% at E2 to a maximum of 63.9% at E4 and decreased thereafter to 3.6% at E7 (Fig. 1C). To determine if the difference in differentiation reflected an intrinsic property of cells from different ages or a changing environment, dissociated cultures were made with [³H]thymidine-labelled cells mixed with a 20-fold excess of unlabelled cells from a different age; in each case the percentage of [³H]thymidine-labelled cells that expressed NF or RA4 was the same as the percentage when the labelled cells were cultured alone (data not shown). These results suggest that the competence of progenitors to differentiate into ganglion cells is correlated to the period of ganglion cell genesis *in vivo*.

Confirmation that the cells recognized by the NF and RA4 markers were ganglion cells was obtained in two ways. First, most NF⁺ or RA4⁺ cells were found to have morphological characteristics consistent with ganglion cell identity; they had a large cell soma and a single unipolar process many cell diameters long with a terminal enlargement resembling a growth cone (see Fig. 2). Second, eight other ganglion-cell-specific antibodies, directed against other neurofilament epitopes, neurofilament-associated proteins, α_6 -integrin, TrkB, NgCAM and Gap-43 (see Materials and Methods), were used to stain E4 (stage 24) retinal cells before and after 24 hours in low density culture and all showed a substantial increase over the 24-hour culture period (data not shown). These results suggested that the majority of early chick retinal progenitors were competent to differentiate as ganglion cells.

In order to begin to characterize the activity(s) responsible for the normal limitation of ganglion cell differentiation, co-culture experiments were done. Explants were co-cultured in a tissue culture well with dissociated cultures at the lowest density in collagen gels. No difference was seen in the number of NF⁺ or RA4⁺ cells in explants or dissociated cell cultures compared to each cultured alone, demonstrating that the inhibitory activity was not freely diffusible.

Notch-1 is expressed in the early chick retina

As the previous results indicated that an inhibitory activity that was not freely diffusible normally limited ganglion cell differentiation, we took a candidate molecule approach focusing on membrane-bound inhibitors of differentiation. As Notch had been shown to play such a role in *Drosophila*, we wondered if it might be the activity. To investigate this possibility, *in situ* hybridizations using a chicken *Notch-1* (*CNotch-1*) probe were performed. These revealed *CNotch-1* expression in a gradient across the proliferative zone of the E4 retina, highest at the ventricular surface, but no expression in the differentiated ganglion cell layer (Fig. 3A,B). As the only differentiated cells

at this age are ganglion cells (Prada et al., 1991), this suggested that *CNotch-1* is expressed in all undifferentiated cells of the early retina. Western blots on E4 chick retinal extracts, using an antibody to the human Notch homologue, TAN-1 (Fig. 3C), showed two bands of the predicted relative molecular mass (350 and 120 × 10³ M_r), with the same relative mobilities as the TAN-1 protein in its full-length and processed forms (Aster et al., 1994).

The inhibitor of ganglion cell differentiation is mimicked by Delta

If Notch activity was responsible for the inhibition of ganglion cell differentiation in pellet cultures, then replacement of retinal cells with cells bearing a Notch ligand should result in inhibition of ganglion cell differentiation. To test this possibility, we co-cultured *Drosophila* S2 cells transfected with a full-length *Delta* cDNA, or untransfected S2 control cells (Fehon et al., 1990), with chick retinal cells in a pellet. To label the chick cells, E4 (stage 24) retinæ were explanted into [³H]thymidine-containing medium for 2 hours. They were then dissociated and mixed in a pellet culture with either untransfected S2 cells or S2-Delta cells in a 1:50 ratio. Pellets were cultured for 24 hours, dissociated, stained for NF and processed for autoradiography. The results (Fig. 4) demonstrate several points. First, absence of cell-cell contact is not necessary to induce differentiation, because a high degree of ganglion cell differentiation was observed in retinal cells in contact with untransfected S2 cells. This suggests that one or more specific activities allowed by cell-cell contact inhibits ganglion cell differentiation. Second, *Drosophila* Delta protein, or another factor induced by Delta, is capable of inhibiting differentiation of chick retinal progenitors into ganglion cells, to an even greater degree than other retinal cells. Third, interspecies conservation of Delta between *Drosophila* and any putative chick homologue is sufficient to allow functional substitution, at least in this assay. This result is consistent with the finding of conservation of binding of Delta to *Xenopus* Notch EGF repeats 11-12 in an *in vitro* assay (Rebay et al., 1991). These data support a role for Notch in ganglion cell differentiation and together with recent data that a *Delta* homologue is expressed in the embryonic chick retina (D. Henrique and J. Lewis, personal communication) suggest that Delta may be the Notch ligand operative in the inhibition of ganglion cell differentiation.

Inhibition of Notch expression increases retinal ganglion cell number

These experiments suggested that the reason 70% of retinal cells differentiate as ganglion cells in low density culture is that they are relieved from inhibition by dissociation of Notch from its ligand(s). A decrease in Notch activity in the intact chick retina should then result in an increase in differentiation of ganglion cells. Antisense oligonucleotides were used to test this hypothesis. Phosphothiorate-modified 23-mer antisense oligonucleotides were designed to three regions of the *Notch* mRNA: the EGF repeat region (oligonucleotide designated EGFR), the lin-12/Notch repeat region (designated LNR) and the cdc10/ankyrin repeat region (designated CDCR) (see Fig. 6A). E2 (stage 16) chick eyes were injected with each of these oligonucleotides, with harvest at E5 (stage

27). Retinae were fixed, sectioned and stained with a monoclonal antibody to the Islet-1 protein (Yamada et al., 1993) as a marker of ganglion cells. This antibody is ganglion cell-specific throughout chick retinal development (C. P. A. and C. L. C., unpublished). Harvest was on E5, rather than later in development when other cell types are present, in order to examine the effect of a change in Notch level specifically on ganglion cell differentiation. By E5, the majority of ganglion cells have been born, but other cell types have not (Prada et al., 1991).

The thickness of the ganglion cell layer in the antisense-injected retinae was approximately twice that of the sense-injected control retinae (Fig. 5). The shape, size and total number of cells in the retina did not change with any oligonucleotide injection, however, and there were no ectopically located Islet-1⁺ cells. Staining with NF and RA4 confirmed the maintenance of approximately normal retinal architecture, although Nomarski optics revealed that the retina appeared less tightly organized and the individual cells had a slightly irregular shape (Fig. 5). Quantitation of Islet-1⁺ cells on sections showed a graded increase in ganglion cell layer thickness from center to periphery, reflecting the spatial gradient of development of the retina and the fact that the peripheral retina at this stage normally has very few Islet-1⁺ cells (Table 1). No change was seen in the contralateral eye of injected embryos, or in sense-injected eyes, relative to uninjected controls.

Similar results were obtained by explanting whole E4 retinae into medium containing 25 μ M oligonucleotide and culturing for 24 hours. Following the culture period, the explants were dissociated, fixed and stained for NF or RA4. Explants cultured without oligonucleotide showed a small increase in the number of positive cells during the culture period (Fig. 1A). In the presence of 25 μ M sense oligonucleotide, there was a similar small increase in the number of positive cells, which was not significantly different from the no oligonucleotide condition. In the presence of 25 μ M antisense oligonucleotide, however, there was a 66-80% increase in the percentage of cells staining for NF or RA4, relative to the no oligonucleotide condition (Fig. 6B). All three oligonucleotides gave qualitatively similar results and addition of the three oligonucleotides together at a concentration of 25 μ M gave no further increase in positive cells. A dose-response was seen in these effects, such that at 0.1 or 1 μ M, no change was seen; at 5 μ M, a 25-30% increase in NF⁺ or RA4⁺ cells was seen and at 100 μ M, there was evidence of toxicity.

That three antisense sequences directed against different regions of the *Notch* mRNA had the same effect suggested that the oligonucleotide effect was specific to the *CNotch-1* target mRNA. This specificity was tested further by introducing nucleotide mismatches into the lin-12/Notch repeat (LNR) oligonucleotide sequence and asking what effect these mismatches had on the observed antisense effect. LNR oligonucleotides were synthesized with one, three or five nucleotide mismatches introduced at regular intervals along the sequence and E4 retinal explants were incubated in a 25 μ M concentration of each for 24 hours. The efficacy of the antisense oligonucleotide in raising the percentage of NF⁺ cells decreased in a stepwise fashion with increasing sequence mismatches, so that the oligonucleotide with 5 mismatches (M5) gave almost the same percentage of NF⁺ cells

as the sense oligonucleotide (Fig. 6C). The effect on ganglion cell number declined both with decreased length of maximum complementary sequence and with decreased cumulative identity of the sequence, arguing that both appear to be important in determining the effectiveness of antisense oligonucleotides in bringing about specific degradation of their target RNAs, consistent with the conclusion reached by Woolf et al. (1992).

CNotch-1 mRNA in the oligonucleotide-treated explants was quantified by RNase protection (Fig. 7). Similar decreases in *CNotch-1* mRNA were seen with each antisense oligonucleotide, whereas no change was seen with any of the sense oligonucleotides (Table 2). Several attempts were made to quantify a decrease in Notch protein levels using Western blots and ELISA assays. These were unsuccessful due to problems in reproducibly quantifying Notch protein levels even in untreated, freshly excised intact retinae.

The effect of antisense oligonucleotides on the proliferative activity of retinal cells was assessed by the inclusion of [³H]thymidine for the duration of the explant culture period. After 24 hours, the explants were dissociated and the percentage of all cells that were ³H-positive was counted in antisense- and sense-treated explants. In three separate experiments, the percentage of cells that were ³H-positive was identical between sense-treated (51.2 \pm 2.3%) and antisense-treated (50.1 \pm 1.9%) retinae.

Expression of constitutively active *Notch* decreases ganglion cell number

If the hypothesis that *Notch* limits ganglion cell differentiation is correct, expression of a constitutively active form of Notch in the early retina should decrease ganglion cell production, if Notch activation is normally limiting. The intracellular domain of Notch, with or without the transmembrane domain, has been shown to be constitutively active (Rebay et al., 1993; Struhl et al., 1993, Lieber et al., 1993). We therefore constructed a retroviral vector encoding the intracellular domain of the human *Notch* homologue, *TAN-1*, and tested its effect on ganglion cell production.

The replication-competent retroviral vector TANIC carried the intracellular 2362 bp of *TAN-1*, encoding a protein starting 13 amino acids intracellular to the transmembrane domain and continuing to the C terminus. E2 (stage 16) chick retinae were infected in vivo with TANIC or a control vector carrying the human placental alkaline phosphatase gene (vector APA; Fekete and Cepko, 1993). To assess the effect of TANIC infection on ganglion cell production in vivo, infected retinae were harvested at E5 (stage 27) and sectioned. To identify infected cells, sections were processed for viral capsid protein (p27 gag) immunohistochemistry and only heavily infected (p27⁺) areas of the retinae were examined. Ganglion cells were visualized on sections by Islet-1 immunohistochemistry.

Infection of the retina was widespread at E5, as assessed by p27 staining. In p27⁺ areas of TANIC-infected retinae, the ganglion cell layer was thinned to half its normal width (Fig. 8). The ganglion cell layer was several cells thick in p27⁻ areas of TANIC-infected retinae and in APA-infected control retinae. In contrast, in p27⁺ areas of TANIC-infected retinae, the ganglion cell layer was generally one cell thick, with some

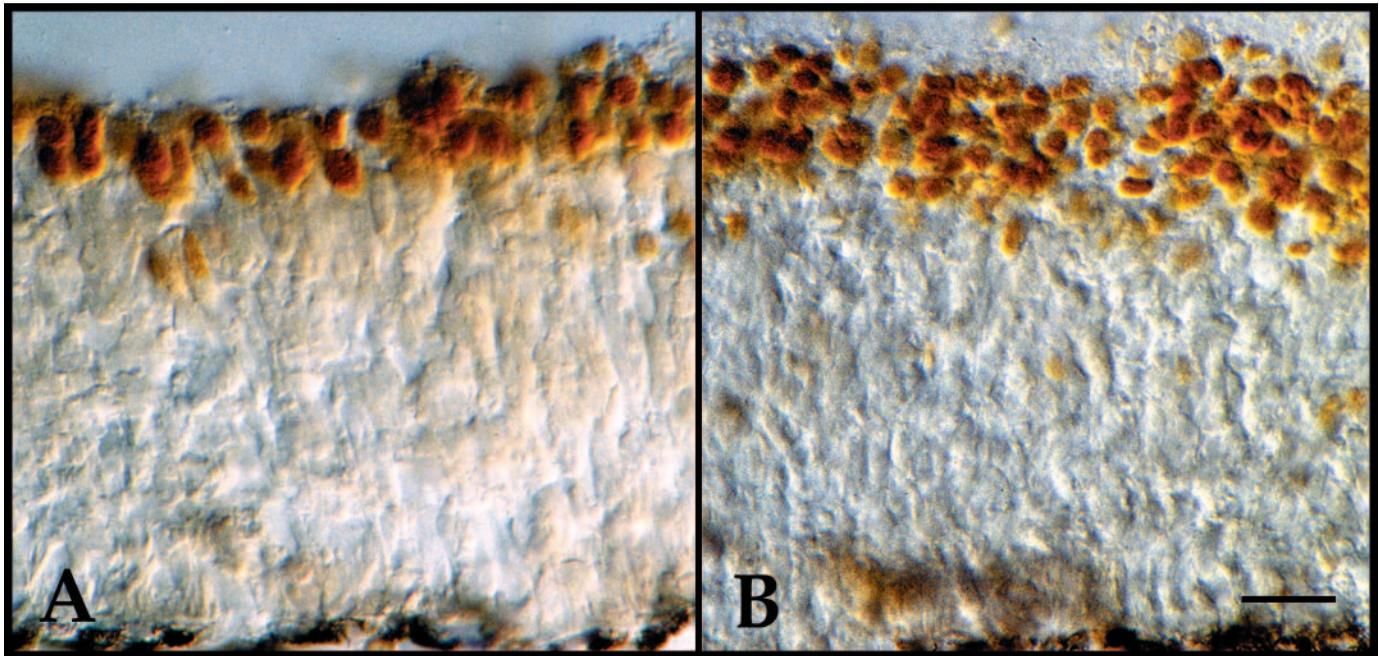


Fig. 5. Effect of *Notch* antisense oligonucleotide in vivo. Eyes of chick embryos were injected with either sense or antisense oligonucleotides at E2 and harvested at E5. Sections were stained with a monoclonal antibody to the Islet-1 protein as a marker for ganglion cell identity, using horseradish peroxidase coupled secondary antibodies for detection. Pigmented epithelium is at the bottom and developing ganglion cell layer at the top. (A) Eye injected with CDCR-sense oligonucleotide. (B) Eye injected with CDCR-antisense oligonucleotide (see text for definition of terms). Bar, 100 μ m.

Table 1. Change in islet 1⁺ cell number in retinae after antisense injection

Region of retina	CDCR*	LNR†	EGFR‡
Central	+108 \pm 14%§	+76 \pm 7%	+74 \pm 6%
Intermediate	+167 \pm 36%	+108 \pm 10%	+94 \pm 12%
Peripheral	+1019 \pm 58%	+481 \pm 29%	+341 \pm 26%

*Oligonucleotide from rat *Notch-1* in *cdc10*/ankyrin repeat region

†Oligonucleotide from chick *Notch-1* in *lin-12*/Notch repeat region

‡Oligonucleotide from chick *Notch-1* in EGF repeat region

§Mean \pm s.e.m. of percent increase in Islet 1⁺ cells in antisense- versus sense-injected retinae ($n=5$ for each, $P<0.01$ for each comparison).

areas being devoid of ganglion cells altogether. Quantitation of Islet-1⁺ cells was done by the same method as was used with the oligonucleotide-treated retinae. The number of Islet-1⁺ cells in TANIC-infected retinae was decreased in a center-to-periphery gradient consistent with the pattern of neurogenesis in the retina (Table 3).

Infection with TANIC had no effect on the proliferative activity of retinal cells. This was assessed by infecting with TANIC on E2 and either injecting the eye with [³H]thymidine or harvesting retinae as explants into medium containing [³H]thymidine at E4, with harvest 1 day later. Both sets of retinae were then dissociated and the cells stained for p27 and then processed for autoradiography. Results with each technique were similar. The percentage of cells that were labelled with [³H]thymidine was no different among uninfected (41.4 \pm 2.1%), APA-infected (44.3 \pm 3.3%) and TANIC-infected (42.5 \pm 3.1%) cells.

The fact that 70% of cells in the E4 retina differentiated as

ganglion cells in low-density dissociated cell culture or in a co-pellet with S2 cells, but not in a co-pellet with Delta-S2 cells, suggested that inactivation of Notch signalling by separation of Notch from its ligand was the critical change that caused differentiation in dissociated culture. This model predicts that cells expressing constitutively active Notch should be relatively insensitive to the effects of dissociation. To test this, retinae were infected with TANIC or APA at E2 and dissociated at E4. Some cells were processed immediately for p27 and NF immunocytochemistry, while other cells were cultured at the lowest density for 16 hours, then stained for p27 and NF. The effect of viral infection on ganglion cell differentiation was assessed by comparing NF immunoreactivity in virally infected (p27⁺) and uninfected (p27⁻) cells from the same retina. Prior to culture, the percentage of uninfected (p27⁻) cells that were NF⁺ in TANIC retinae was 14.2 \pm 2.1%, consistent with previous results (Fig. 1B). However, only 7.8 \pm 0.8% of the p27⁺ cells were NF⁺ in these retinae (a 45% decrease, $P=0.02$) (Fig. 9A). This decrease was similar to the decrease in Islet-1⁺ cells seen on sections (Table 3). In APA control-injected retinae, the percentage of uninfected and infected cells that were NF⁺ at the start of the cultures was the same (11.7 \pm 2.3% of the uninfected cells, versus 12.3 \pm 2.2% of infected cells, $P>0.10$). Similar results were seen after staining with RA4 (data not shown). The cells were then cultured at low density in collagen gels for 16 hours. At the end of this period, the percentage of uninfected (p27⁻) cells that were NF⁺ in TANIC-infected retinae was 58.2 \pm 2.5%, consistent with previous data (Fig. 1B), while the percentage of infected (p27⁺) cells that were NF⁺ was decreased 40%, to 34.9 \pm 3.4% ($P=0.01$) (Fig. 9A). Infection with APA had no effect on the percentage of cells expressing NF (Fig. 9A).

Fig. 6. Effect of Notch antisense oligonucleotides on ganglion cell differentiation in explants.

(A) Schematic representation of the proteins encoded by *Notch*, with locations of oligonucleotides directed against the corresponding mRNA (oligonucleotide sizes not to scale). All Notch homologues display a conserved structure. They are transmembrane proteins with an extracellular domain containing 36 epidermal growth factor-like repeats (EGFR), followed by three *lin-12/Notch* repeats (LNR). The cytoplasmic domain contains six *cdc10/ankyrin* repeats (CDCR), a polyglutamine (*opa*) sequence and a putative PEST sequence (Wharton et al., 1985; Kidd et al., 1986). Antisense oligonucleotides were directed against a sequence in each of the three conserved repeat domains. (B). E4 (stage 24) retinal explants were cultured for 24 hours

with no oligonucleotide, or in the presence of 25 μ M oligonucleotide, then dissociated and stained for neurofilament (NF). Oligonucleotides are named as in A. Results are expressed as the percent change in NF⁺ cells in oligonucleotide-treated explants compared to explants cultured without oligonucleotide. (C) Nucleotide mismatches decrease the ability of the LNR antisense oligonucleotide to increase numbers of NF⁺ cells in explant cultures. Methods as in B. M1=1 mismatch, sequence 5'-CCAGCACTGCAATGACTGTGAGC-3'; M3=3 mismatches, sequence 5'-CCAGCGCTGCAATGACTGTGAGC-3'; M5=5 mismatches, sequence 5'-CCAGCGCTGCAATGACTGTGAGC-3' (mismatches underlined).

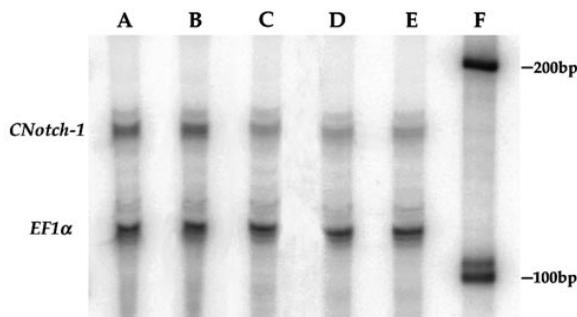
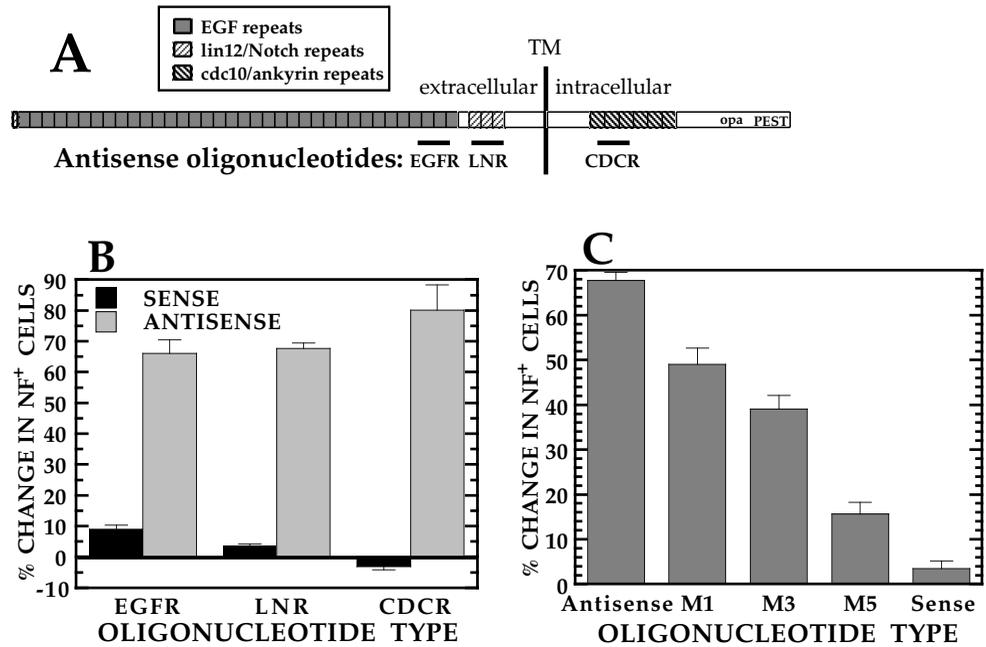


Fig. 7. RNase protection of *Notch* mRNA in oligonucleotide-treated explants. To measure a specific decrease in *CNotch-1* mRNA, the total RNA from treated explants was analyzed for the amount of *CNotch-1* and *EF1 α* RNA using RNase protection. *CNotch-1* probe and *EF1 α* control probe were included in the same tube and 5 μ g retinal RNA were used for each reaction. (A) No oligonucleotide treatment; (B) CDCR-sense oligonucleotide; (C) CDCR-antisense oligonucleotide; (D) LNR-antisense oligonucleotide; (E) EGFR-antisense oligonucleotide; (F) RNA molecular weight markers.

As a control for the specificity of the TANIC sequence in bringing about inhibition of ganglion cell differentiation, parallel dissociated cultures were made with TANIC-infected retinal cells, but with the addition of 25 μ M antisense CDCR oligonucleotide in the culture media. This treatment would be expected to bring about degradation of both TANIC and endogenous *CNotch-1* mRNA. Addition of CDCR antisense oligonucleotides to dissociated cultures eliminated the effect of TANIC infection on NF expression, restoring the percentage of p27⁺ cells that were NF⁺ to control levels (Fig. 9B).

Table 2. Change in *CNotch-1* mRNA level after oligonucleotide treatment

Oligonucleotide type	Change in <i>CNotch-1</i> mRNA level*
CDCR antisense	-28.1 \pm 4.5%
LNR antisense	-32.0 \pm 9.4%
EGFR antisense	-24.7 \pm 5.3%
CDCR sense	+5.2 \pm 7.2%
LNR sense	+1.2 \pm 5.1%
EGFR sense	-2.9 \pm 3.5%

**CNotch-1* mRNA was measured by RNase protection with an *EF1 α* internal control and expressed as the difference in mRNA level between oligonucleotide treated and control retinae.

DISCUSSION

In the first differentiation event of retinal development, ganglion cells arise from a neuroepithelium devoid of other differentiated cell types. The work presented here shows that this occurs by selection from a larger group of progenitors with competence to produce ganglion cells, through the action of Notch. On the basis of these findings, we suggest that the majority of the early chick retina may constitute an equivalence group, the primary fate of which is the first born neuron in the retina, the ganglion cell. A similar suggestion has been made concerning the first born neuron in the *Drosophila* retina, R8 (Cagan, 1993). An equivalence group has been defined as a group of cells that have a common developmental potential, but which normally acquire different fates as a result of cell-cell interactions (Wigglesworth, 1940; Kimble et al., 1979). Initially described in nematodes, neural equivalence groups have been described in multiple invertebrate species

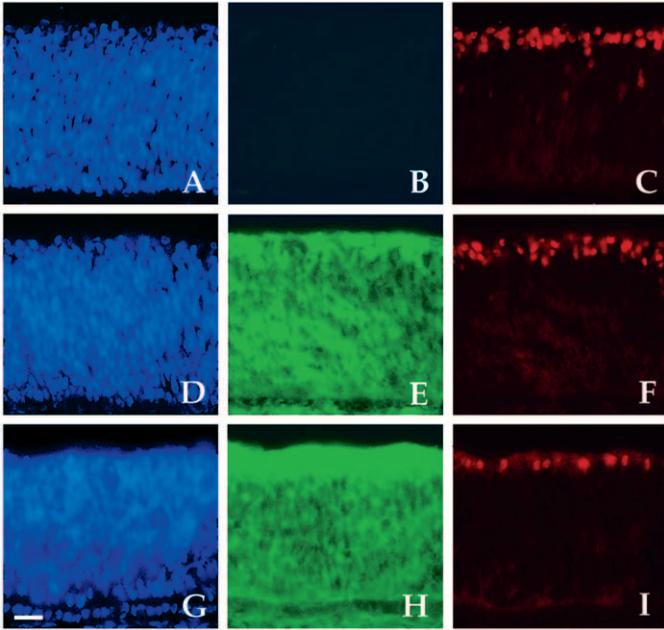


Fig. 8. Effect of infection with TANIC on ganglion cell differentiation in vivo. Retinae were infected with TANIC or APA control virus at E2 (stage 16) and harvested at E5 (stage 27). Sections were stained with DAPI to show cell nuclei (A,D,G), antibody to p27 gag protein as a marker of viral infection with FITC-conjugated secondary antibody (B,E,H), and antibody to the Islet-1 protein as a marker of ganglion cell identity with Texas-Red-conjugated secondary antibody (C,F,I). Top row (A-C) uninfected retina; middle row (D-F) retina infected with APA virus; bottom row (G-I) retina infected with TANIC virus. Quantitation of these data are in Table 3. Bar, 100 μ m.

Table 3. Change in Islet 1⁺ cell number in retinae after TANIC infection

Region of retina	% Decrease versus APA
Central	-41.3 \pm 2.6*
Intermediate	-45.9 \pm 3.1%
Peripheral	-93.8 \pm 9.4%

*Difference in mean \pm s.e.m. (fields counted/region/retina=5; retina n=5) of Islet-1⁺ cells in p27 gag⁺ regions of retinae.

(Muskavich, 1994; Taghert et al., 1984; Shankland and Weisblat, 1984) and in zebrafish (Eisen, 1992). In *Drosophila*, neuroblast specification in the embryonic ectoderm (Doe, 1992) and specification of the sensory mother cells of the sensory macrochaetae (Simpson and Carteret, 1990), both occur from well-defined proneural equivalence groups. Though we have shown that the majority of retinal progenitors have the competence to differentiate as a single cell type, we have not shown that the alternative fates that these cells may acquire are also equivalent. Support for the existence of a ganglion cell equivalence group will require these data and these experiments are now in progress.

The competence of the remaining 30% of cells remains to be defined. This is the percentage of cells that remain mitotic at the end of the culture period (C. P. A. and C. L. C., unpublished data), so ongoing mitosis may prevent differentiation of these cells. It is also possible that this 30% of progenitors do not have

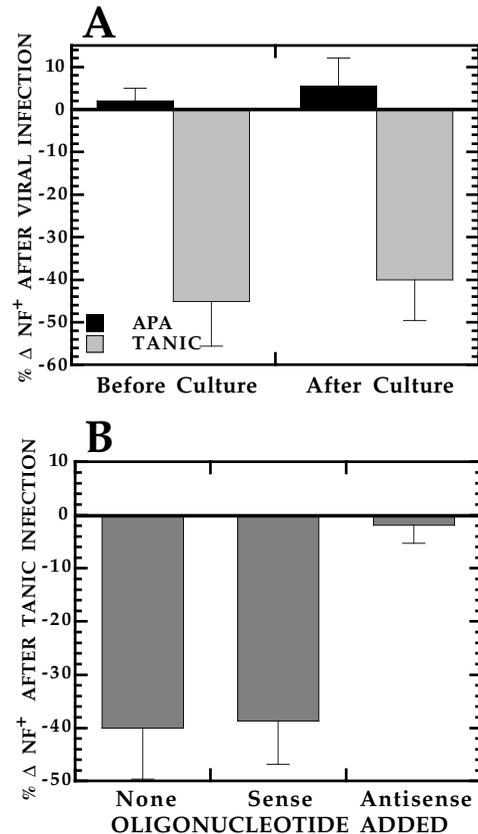


Fig. 9. Infection with TANIC decreases ganglion cell development in vitro. (A) The ability of cells to express NF in low density collagen gel cultures after infection with TANIC or the control APA virus was assessed. Retinae were infected in vivo with TANIC or APA at E2 (stage 16) and harvested at E4 (stage 24). Retinae were dissociated and either stained immediately for p27 gag and NF, or cultured for 16 hours at 0.25×10^5 cells/25 μ l in collagen gels prior to staining. Uninfected (gag⁻) and infected (gag⁺) cells from each retina were assayed for whether they expressed NF. Results are plotted as the percent difference in NF⁺ cells between uninfected cells and cells infected with APA or TANIC. (B) Antisense oligonucleotides directed against the intracellular domain of *Notch* eliminate the effect of TANIC in dissociated cultures. Techniques as in A, except that 25 μ M CDCR sense or antisense oligonucleotide was added to some of the cultures.

the competence to differentiate as ganglion cells, or that all progenitors may not be competent simultaneously. The center-to-periphery gradient of retinal development (Prada et al., 1991) might indicate that the population that was cultured was heterogeneous, though culturing central and peripheral retinal cells separately did not increase the percentage of differentiated cells (data not shown). Technical reasons may also explain why all cells did not differentiate in the lowest density dissociated cultures. Some cells migrated into clumps during the culture period, allowing residual cell-cell contact; if this was the case, Notch does not appear to have been operative, because addition of Notch antisense oligonucleotides to dissociated cell cultures did not change the percentage differentiating as ganglion cells (data not shown). In addition, it remains possible that a non-Notch inhibitory factor was still active in the lowest density dissociated cultures, or that an inducer of ganglion cell differentiation was limiting.

Notch activity regulates ganglion cell number

Members of the *Notch/lin-12* family encode transmembrane proteins that are thought to act as receptors for lateral inhibitory interactions influencing cell fate choice among cells in equivalence groups in invertebrates (see Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995 for recent reviews). *Notch* homologues have been found in *Xenopus*, human, rat, mouse and zebrafish (Coffman et al., 1990; Ellisen et al., 1991; Weinmaster et al., 1991, 1992; Franco del Amo et al., 1992; Bierkamp and Campos-Ortega, 1993; Lardelli et al., 1994). Chicken *Notch-1* (*CNotch-1*) was found to be expressed in all undifferentiated cells in the retina where cell fate decisions were being made, in a gradient with the highest expression in the ventricular zone where cytokinesis takes place (Sidman, 1961). This expression pattern is similar to that reported for mouse *Notch-1* (Guillemot and Joyner, 1993).

The experiments using antisense oligonucleotides are the first demonstration of the effect of a tissue-specific decrease in Notch activity during vertebrate development. Differentiation of progenitors into ganglion cells was increased by antisense treatment, doubling the thickness of the ganglion cell layer in the central retina and precociously extending the region of the peripheral retina in which ganglion cell differentiation occurs. Absence of normal differentiation in the presence of activated Notch has led to the suggestion that Notch signalling inhibits differentiation (Coffman et al., 1993). Our results show directly that ongoing Notch activity is required to prevent competent cells from undergoing differentiation in vivo. The only previous report of a decrease in vertebrate Notch function described mouse *Notch-1* mutants created by homologous recombination (Swiatek et al., 1994). In homozygotes, there was widespread cell death and embryonic lethality before neuronal differentiation in the CNS took place. The neural tube and optic vesicle, both of which normally express *Notch-1* and are entirely proliferative at this stage, appeared normal up to E9 in the *Notch-1* knockout, suggesting that *Notch-1* activity is not required for the partitioning of neural from non-neural ectoderm, nor for early proliferation events in the mouse CNS. Consistent with this finding, we observed no change in the proliferation of chick CNS retinal progenitors after treatment with Notch antisense oligonucleotides.

Though the antisense oligonucleotides caused a decrease in *CNotch-1* mRNA level of only 25-30%, this relatively small decrease led to a doubling in ganglion cell number, suggesting that progenitors are sensitive to small changes in Notch activity. This conclusion is consistent with (1) the effects of small changes in gene dosage of either *Notch* or *Delta* in *Drosophila* (Artavanis-Tsakonas, 1988; Muskavitch, 1994), (2) the suggestion that differentiation of cells within a proneural equivalence group occurs in response to small random variations in Notch level (Seydoux and Greenwald, 1989; Heitzler and Simpson, 1991) and (3) the fact that such small initial variations may be amplified by subsequent effects on expression of both ligand and receptor (Wilkinson et al., 1994). All of these data are supportive of the idea that the Notch protein level is actively regulated, with a short half-life (Aster et al., 1994), as had been suggested by the presence of a PEST sequence, typical of rapidly degraded proteins (Rogers et al., 1986). It is also possible that the RNase pro-

tections underestimated the effect on Notch protein levels, as we were unable to reliably measure Notch protein; alternatively, *CNotch* RNA level may have been decreased to a greater extent in the subpopulation of cells that differentiated.

While the antisense oligonucleotide treatment led to a doubling in the number of ganglion cells, to an absolute level of 40%, dissociated culture led to 70% of the cells becoming ganglion cells. This difference could be explained by a failure of the oligonucleotides to reduce *CNotch-1* mRNA to a greater degree. Alternatively, it is possible that an additional inhibitory signal is responsible for the submaximal ganglion cell differentiation in explants, but is inactivated in the dissociated cultures along with Notch.

Infection of retinal progenitors early in the period of ganglion cell generation with a retrovirus (TANIC) encoding the intracellular domain of the human Notch homologue (TAN-1) produced the opposite effect to that seen after antisense oligonucleotide injection. In the central retina, the number of ganglion cells was decreased by 40% and in the peripheral retina by 90%. This effect on differentiation was cell autonomous, affecting only those cells expressing viral antigens, consistent with the prevailing model of Notch as a receptor of cell-fate-determining signals (Artavanis-Tsakonas et al., 1995). In addition, progenitors expressing activated Notch were resistant to the effects of dissociation on ganglion cell differentiation, as would be expected if the reason for differentiation in dissociated culture was inactivation of Notch by dissociation from its ligand. The fact that the control level of ganglion cell differentiation could be restored by incubating TANIC-infected dissociated cells with Notch intracellular domain antisense oligonucleotide was somewhat surprising, given the partial effect on differentiation seen in the other experiments. This finding suggests that the viral RNA was either low in amount and/or more sensitive to antisense degradation.

Some progenitors may be insensitive to Notch signalling, because constitutive activation of Notch with TANIC, or supplying excess ligand in the Delta-S2:retinal cell co-pellet assay, decreased the number of ganglion cells by half, but did not eliminate them. Determining the mechanism by which cells escape Notch inhibition in these assays should help answer the question of how cells escape Notch inhibition during normal development. Stochastic fluctuations in Notch level have been hypothesized to lead to differentiation (Seydoux and Greenwald, 1989; Heitzler and Simpson, 1991), but this cannot explain the results with TANIC. It is unlikely that sequence divergence of *CNotch-1* from *TAN-1* was responsible for TANIC resistance, as *Notch* homologues are highly conserved in this region. Some cells that expressed the viral p27 gag protein may not have expressed TAN-1, though expression of gag is highly correlated with expression of the nonviral gene (Fekete and Cepko, 1993); we were unable to address this directly because our antibodies did not detect Notch protein on sections. Alternatively, because Notch may be a multifunctional receptor, with activation by different ligands leading to distinct effects on cell fate (Couso and Martinez Arias, 1994; Artavanis-Tsakonas et al., 1995), ligand-independent perturbations of Notch activity such as were done here may have altered multiple Notch pathways simultaneously, resulting in an intermediate phenotype. Some ganglion cells would be expected after in vivo infection because, at the time of infection, 20% of ganglion cells were

postmitotic (Prada et al., 1991) and thus resistant to retroviral infection. More complete TANIC infection was expected in the retinal periphery, because fewer cells were postmitotic there at the time of infection and greater suppression of ganglion cell production was indeed observed in the periphery (see Table 3). Finally, a Notch-independent ganglion cell-inducing signal may explain the ganglion cell persistence.

In the dissociated cell culture experiments, a characteristic of viral transduction may have led to the observed TANIC resistance. Because the virus was replication competent, viral spread occurred after the initial infection, so infected cells varied in their time of exposure to activated Notch. Viral spread continued to occur in the dissociated cultures, as demonstrated by a two-fold increase in the percentage of cells that stained for the viral gag protein during the culture period (data not shown). Given the short latency to ganglion cell differentiation in dissociated culture (see Fig. 1B), many cells that were infected with TANIC shortly before or during culture would have differentiated before viral expression occurred, so would show both gag and neurofilament expression at the end of the culture. This heterogeneity would be minimized by the use of a replication-incompetent viral vector carrying the same construct and these experiments are now in progress.

We do not know what the cells expressing TANIC that are diverted from the ganglion cell pathway eventually become, nor do we know at what cells' expense the extra ganglion cells are produced in the antisense experiments. All harvests were done at or before the E5, in order to examine effects specific to ganglion cell genesis. No other cell types are produced before E6 (Prada et al., 1991) and none express specific markers until E7 (C. P. A., C. L. C. and S. Bruhn, unpublished data), so it was not possible to assay for other cell types. This has made assessment of the equivalence of these cells' alternative fates difficult. Recent experiments in the *Xenopus* retina (Dorsky et al., 1995) suggest that most cells transfected with an intracellular domain *Xotch* mRNA (*XotchΔE*) do not immediately switch into another cell fate but remain undifferentiated, at least temporarily. A related question is whether Notch plays a role in the genesis of later-born cell types in the chick retina. *Notch* is required for the correct differentiation of multiple cell types in the *Drosophila* retina (Cagan and Ready, 1989; Fortini et al., 1993) and *CNotch-1* was observed to be expressed in the chick retina by in situ hybridization throughout the period of generation and differentiation of every retinal cell type (data not shown). It is therefore likely that Notch plays a role in other cell fate decisions in the retina and preliminary results suggest that this is so in the postnatal rat retina (Z. Z. Bao and C. L. C., unpublished data).

Lastly, multiple aspects of this work suggest that perturbations of Notch activity do not affect mitotic behavior of progenitor cells. The gross morphology, size and cell number of retinae injected with antisense oligonucleotides or TANIC were the same as in control retinae and labelling with [³H]thymidine demonstrated directly that mitotic activity was not affected by these perturbations and suggested that Notch antisense effects were restricted to postmitotic cells. Consistent with this hypothesis, Coffman et al. (1993) found that the increase in neural and muscle tissue seen after injection of blastomeres with *XotchΔE* mRNA was unaffected by inhibition of cell division by aphidicolin and hydroxyurea and Dorsky et al.

(1995) reported no BrdU incorporation beyond what is normally observed in a small number of *XotchΔE*-transfected retinal cells.

Competence, Notch and acquisition of vertebrate neuronal cell fate

A working model for cell fate acquisition in vertebrate retinal development can be suggested based on these and related data. Cell lineage studies have shown that vertebrate retinal progenitors are capable of producing multiple cell types throughout development (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). Cell birthdating studies (e.g., Prada et al., 1991) have shown discrete periods in development during which each cell type is produced and differentiation of progenitors in culture reflects these birthdays (Reh and Kljavin, 1989; Adler and Hatlee, 1989). Intrinsic properties of progenitors have been suggested to at least partially control when different cell types are produced, as early progenitors appear to have limited competence to produce later-born cell types (Watanabe and Raff, 1990). The competence of progenitors to produce a given cell type in the retina may therefore be acquired and lost in a temporal pattern mirroring the order of birth of the different cell types, and more progenitors may be competent to produce each cell type than normally do so in vivo. Cell fate acquisition in this model would be regulated in part by changes in progenitor competence and in part by selection from among this group by the action of environmental factors. Competence may be mediated by the action of such genes as the *as-c* homologues proposed to be involved in acquisition of neuronal competence in vertebrates (e.g., *XASH-3* (Turner and Weintraub, 1994; Ferreira et al., 1994). Selection may occur through the action of positive or negative environmental factors in concert with regulation of the Notch pathway, many members of which have been identified in vertebrates in addition to the *Notch* homologues themselves (Sasai et al., 1992; Lindsell et al., 1995; Matsunami et al., 1989; D. Henrique and J. Lewis, personal communication). Though Notch clearly acts to inhibit differentiation as shown in a number of in vivo and in vitro systems, and we have shown here that inhibition of Notch is sufficient to induce differentiation, its effects on cell fate may be more complex (Artavanis-Tsakonas et al., 1995). These events appear to be regulated independently from mitotic activity of progenitors, at least in early neurogenesis, because neither differentiation (Harris and Hartenstein, 1991) nor Notch-induced developmental events (this paper, Dorsky et al., 1995; Coffman et al., 1993) appear to require cell division.

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