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A Goldfish *Notch-3* Homologue Is Expressed in Neurogenic Regions of Embryonic, Adult, and Regenerating Brain and Retina

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ABSTRACT Members of the *Notch* gene family are thought to be involved in the regulation of cell fate decisions in a variety of embryonic tissues, particularly in the developing central nervous system (CNS) in *Drosophila* and vertebrates. In goldfish the CNS continues to develop and add neurons well into adulthood and has the capacity to regenerate new neurons. Using probes derived from *Xenopus Notch* to screen an adult goldfish retinal cDNA library, followed by 5′ RACE, we isolated a partial cDNA for a goldfish *Notch* homologue, *G-Notch*. Sequence alignment supported assignment of *G-Notch* to the *Notch-3* class. Northern blot analysis revealed a single transcript of >8 kb, and RNase protection assays indicated that *G-Notch* is expressed in eye and brain but not muscle of adult goldfish. The spatiotemporal pattern of expression of *G-Notch* was defined from early embryonic stages to adulthood by *in situ* hybridization. Expression in the embryonic CNS was localized to neurogenic regions and was downregulated in differentiated cell populations. In adult goldfish, expression persisted in and adjacent to the germinal zones in the retina and the brain. Weak expression was seen in scattered cells in the inner nuclear layer of the retina, which might include neurogenic stem cells. Following retinal lesions (puncture wounds or laser lesions restricted to photoreceptors in the outer nuclear layer), *G-Notch* was upregulated in proliferating cell populations throughout the retina, in association with a generalized mitogenic response. In the region of the laser lesion, where earlier studies have demonstrated that photoreceptors are regenerating at 1–3 weeks following the lesion, *G-Notch* expressing cells were abundant in the outer nuclear layer. These observations suggest that retinal regeneration involves the re-expression of an important developmental signaling molecule in neuroepithelial cells resident in the differentiated retina. Dev. Genet. 20:208–223, 1997. © 1997 Wiley-Liss, Inc.

Key words: cell fate; neurogenesis; regeneration; retina; photoreceptors; optic tectum

INTRODUCTION

Vertebrates that grow continuously and have the capacity to regenerate could provide convenient models for defining factors involved in determining cell fates in the central nervous system (CNS). Goldfish and other teleosts displaying indeterminate growth maintain neuronal progenitor cells throughout their lives [Müller, 1952; Richter and Kranz, 1970a,b; Johns, 1977; Meyer, 1978; Raymond and Easter, 1983], and can regenerate CNS tissues [Kirsche, 1960; Kirsche and Kirsche, 1961; Richter, 1968; Richter and Kranz, 1977; Maier and Wolburg, 1979; Raymond *et al.*, 1988; Hitchcock and Raymond, 1992].

The goldfish retina contains several distinct, proliferative cell populations in the neuroepithelial lineage. Most neurogenesis occurs in the germinal zone (GZ), an annulus of proliferative neuroepithelial cells at the ciliary (iris) margin [Müller, 1952; Johns, 1977; Meyer, 1978]. Another site of neurogenesis is the outer nuclear layer, where scattered proliferative "rod precursors" maintain the density of rod photoreceptors (and hence visual sensitivity) by interstitial addition of new rods [Raymond and Rivlin, 1987; Powers *et al.*, 1988]. Although the cellular fate of rod precursors is normally restricted, they are thought to be pluripotent and have been implicated in the regeneration of all neuronal cell

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types in damaged retinas [Raymond *et al.*, 1988; Hitchcock *et al.*, 1992]. Müller glia derive from the same multipotent neuroepithelial progenitors that generate retinal neurons [Turner and Cepko, 1987]. Although normally quiescent, they become mitotic after retinal damage [Wagner and Raymond, 1991; Lewis *et al.*, 1992; Braisted *et al.*, 1994], but there is no direct evidence that they transdifferentiate into neurons. Finally, undifferentiated, very slowly dividing cells scattered throughout the inner nuclear layer have recently been identified in the retina of adult fish, and may constitute a retinal stem cell population [Braisted *et al.*, 1995; Julian and Korenbrot, 1996; Raymond and Hitchcock, 1997].

Proliferating neuronal progenitor populations and the ability to regenerate neural tissue are also characteristics of the adult goldfish brain. The optic tectum, for example, contains a multipotent germinal zone [Kirsche and Kirsche, 1961; Meyer, 1978; Raymond and Easter, 1983] and constitutively dividing populations of ependymal and radial glia [Stevenson and Yoon, 1980, 1981, 1982; Raymond and Easter, 1983].

Although studies using goldfish have yielded abundant information about the cellular aspects of neural development and regeneration, molecular characterization of these processes is rudimentary. Our strategy has been to pursue conserved genes that may play similar roles in analogous developmental events across evolution. For example, an early developmental event common to insects and vertebrates is the segregation of neural progenitors from within a field of equipotential "proneural" cells by a process of lateral specification [Artavanis-Tsakonas *et al.*, 1995; Simpson, 1995; Chitnis *et al.*, 1995]. This process is thought to be driven initially by small, possibly random intercellular differences in levels of expression of the transmembrane receptor Notch and its transmembrane ligand Delta, products of genes first characterized in *Drosophila*. Delta, expressed by a putative neural progenitor, binds to the extracellular domain of Notch on a neighboring cell, activating a signaling pathway that represses the activity of proneural transcription factors, as well as *Delta* expression itself, in the Notch$^+$ cell [Jennings *et al.*, 1995; Chitnis and Kintner, 1996]. Small differences in levels of *Notch* and *Delta* expression are thereby amplified so that ultimately a single cell within a proneural cluster commits to a neural fate, while its neighbors remain uncommitted and competent to participate in subsequent episodes of differentiation [Chitnis *et al.*, 1995].

The first characterized vertebrate *Notch* transcript, cloned from a *Xenopus* cDNA library, was shown to be expressed strongly in the embryonic neuroepithelia, including the germinal zone of the developing *Xenopus* retina [Coffman *et al.*, 1990]. Rat *Notch* cDNAs cloned subsequently also showed embryonic retinal expression [Weinmaster *et al.*, 1991, 1992]. *Notch* therefore seemed a good candidate for a gene that would be involved in the neurogenetic processes occurring in the embryonic and adult teleost CNS during both development and regeneration. We report here the cloning of a partial cDNA for a goldfish *Notch* homologue (*G-Notch*) which is expressed in neural progenitor populations through adulthood, and whose expression is enhanced and its pattern altered in the retina after injury and during photoreceptor regeneration.

MATERIALS AND METHODS

All standard molecular biology techniques are according to Sambrook *et al.* [1989] unless otherwise indicated. Stages of goldfish embryonic development are according to Kajishima [1960].

Isolation of *G-Notch* cDNA

An oligo d(T)-primed λ gt10 cDNA library (gift of Dr. Daniel Goldman), made from goldfish retinal poly(A)$^+$ collected 10 days after optic nerve crush, was screened at low stringency with random-primed probes derived from the coding region of a *X-Notch-1* cDNA clone (gift of Dr. William Harris). Final stringency washes were in 2× SSC/1% sodium dodecyl sulfate (SDS) at 55°C. Purified positive plaques were assayed by polymerase chain reaction (PCR) for the presence of a highly conserved ~600 bp cdc10/ankyrin (ANK) repeat region of *Notch*, using degenerate primers based on the *Notch/X-Notch-1* ANK repeat region flanking peptides TPLMIA and ITDHMD: ([*Eco*RI site underlined] 5′-CAGAATTCACXCCXYTXATGATHGC-3′; [*Hin*DIII site underlined] 5′-ACAAGCTTRTCCATRTGRTCXGTDAT-3′; D = G/A/T, H = A/C/T, R = A/G, X = G/A/T/C). Degenerate PCR cycle conditions were 94°C 3 min, followed by 30 cycles of (94°C, 30 sec)/(44°C, 30 sec)/(72°C, 60 sec); [$MgCl_2$] = 2.5 mM. This screen identified a 2056 bp clone (*GotchCDC*) encoding most of the intracellular region of a goldfish *Notch* homologue. Weakly conserved sequences at the 5′ end of *GotchCDC* (5′-CCACTGTCTGCTGTCAACTG-3′, = AVDSRQW; 5′-CATCCTCACCATCAGACAGA-3′ = LSDGED) were used as nested primers in a commercial 5′ RACE protocol (Promega, Madison, WI) to amplify and sequence 2372 bp of upstream sequence (*GotchTM*) from stage 19 embryonic goldfish RNA. RACE PCR conditions were 94°C, 3 min, followed by 30 cycles of (94°C, 30 sec)/(57°C, 30 sec)/(68°C, 8 min), [$MgCl_2$] = 2 mM. Sequencing was done both manually and with the use of an ABI automated sequencer (Applied Biosystems, Foster City, CA) by using primer walking and nested deletion methods. Fragments were completely sequenced in both directions and were assembled by MacVector (IBI, New Haven, CT) and Sequencher (Gene Codes, Ann Arbor, MI) software. Conceptual assembly of *GotchCDC* and *GotchTM* yielded a single open reading frame, referred to as *G-Notch* in this paper. *GotchCDC* and *GotchTM* are deposited in GenBank under the accession number U09191.

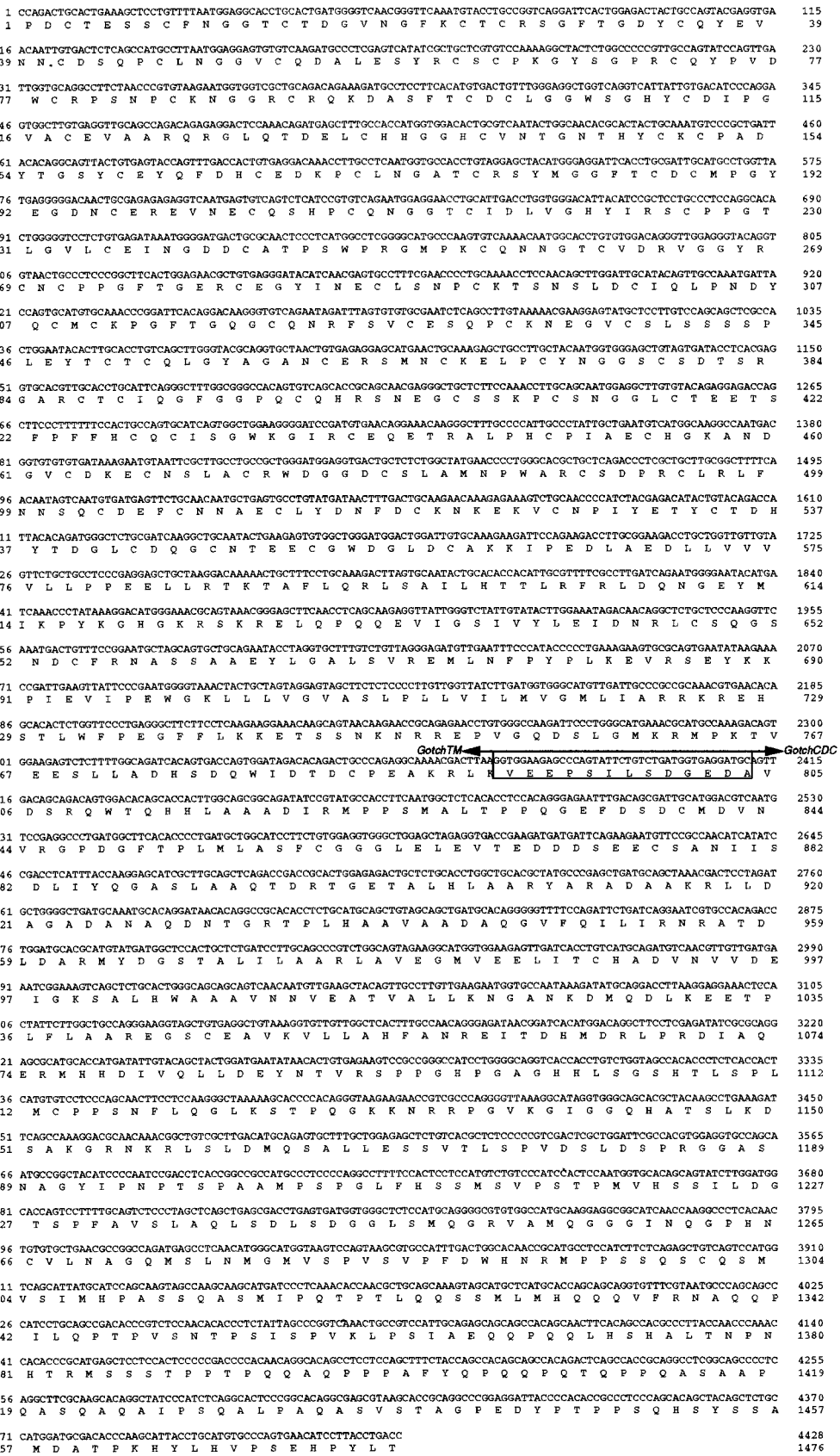

Fig. 1. DNA sequence and conceptual translation of *G-Notch*. The region of overlap between the component *GotchCDC* and *GotchTM* cDNAs is boxed.

RNase Protection

A 233-bp *Bst*EII restriction fragment of *G-Notch* corresponding to bp 2372–2605 (aaVEEP...LEVT) served as a template for synthesis of ^{32}P-labeled antisense RNA probes for RNase protection assays of total RNA from adult brain, retina, and flank muscle, performed according to manufacturer's protocols (Ambion, Austin, TX). Following electrophoresis, the gel containing protected fragments was dried onto Whatman paper and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) for 15 hr at −70°C with two intensifying screens.

Northern Blot

Total RNA was isolated from goldfish brain using Trizol Reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. Samples containing 10, 25, and 50 µg total RNA were mixed with sample buffer [Sambrook *et al.*, 1989] and denatured at 68°C, for 10 min. RNA was electrophoresed at 40 V for 4 hr at room temperature in a 1.0% agarose/1× MOPS/ 0.22 M formaldehyde gel [Tsang *et al.*, 1993], transferred to nylon membrane (Boehringer Mannheim, Indianapolis, IN) by downward neutral pH capillary transfer with 20× SSC [Chomczynski and Mackey, 1994], and ultraviolet (UV)-cross-linked (Stratalinker, Stratagene, LaJolla, CA). Digoxigenin-labeled antisense RNA probes were prepared from *GotchCDC* and *GotchTM* plasmids with the Genius System (Boehringer Mannheim, Indianapolis IN). The two probes were combined (each at 100 ng/ ml) and hybridized according to the manufacturer's instructions, except the high-stringency wash was at 68°C/ 0.1× SSC/0.1% SDS. Detection was with the CSPD chemiluminescent substrate (Boehringer Mannheim), and the blot was exposed for 30 min to Lumi-Film (Boehringer Mannheim).

Retinal Lesions

Goldfish (*Carassius auratus*), obtained from a local pet store or from Ozark Fisheries (Stoutland, MO) were anesthetized with 0.2% tricaine methanesulfonate, and the eyes were punctured multiple times through the sclera with an acupuncture needle [Sullivan and Raymond, 1991]. The fish were allowed to survive up to 15 days after the lesions.

An argon laser (System 920, Coherent, Palo Alto, CA) was used to produce retinal lesions as previously described [Braisted *et al.*, 1994]. Briefly, fish were anesthetized, the lens was removed through a slit in the ventral edge of the cornea, and the cornea was allowed to heal without suturing for 2–3 weeks. To improve the optics, a drop of methyl cellulose was applied to the cornea, and the retina was visualized with a slit lamp. Lesions (4 per retina) were produced with laser pulses using settings of 130 mW, 0.1 sec, and 500-µm spot diameter. The cell loss produced by the laser at these settings was confined primarily to the photoreceptor layer [Braisted *et al.*, 1994]. Fish survived for 1 day to 4 weeks following the lesions.

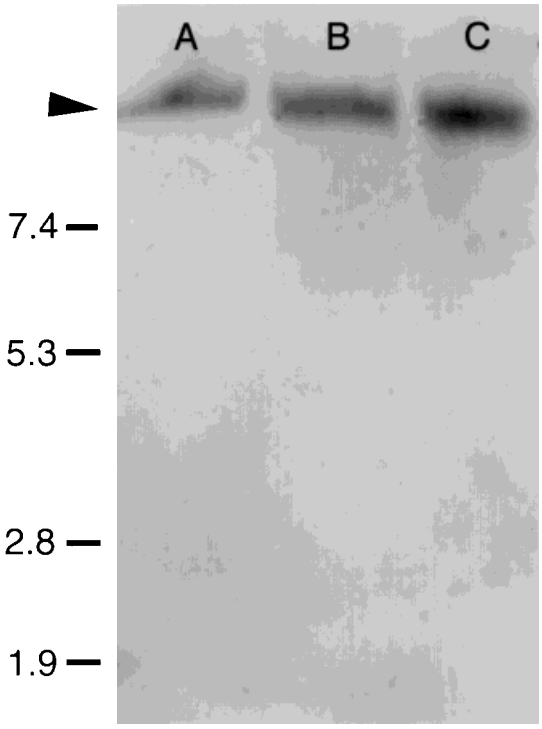

Fig. 2. Northern blot of goldfish brain RNA hybridized to an antisense RNA probe transcribed from *GotchCDC* and *GotchTM*. *Lanes A,B,C,* 10, 25, and 50 µg total RNA loaded, respectively. A single band of 8.5–9.0 kb hybridized to the probe *(arrowhead).*

In some cases, proliferating cells were labeled with bromodeoxyuridine (BrdU) injected intraocularly, to achieve an approximate intraocular concentration of 50 µM, at 24 hr prior to retinal fixation, as described previously [Barthel and Raymond, 1993; Braisted *et al.*, 1994].

Tissue Preparation, In Situ Hybridization, and Immunocytochemistry

GotchCDC and *GotchTM* plasmids were used to generate sense and antisense RNA probes labeled with digoxigenin (DIG)-UTP (Boehringer Mannheim) or ^{35}S-UTP. Results were identical using either template. Retinas were fixed for 1–3 hr in 4% paraformaldehyde/5% sucrose in phosphate-buffered saline (PBS) (pH 7.4). Tissue preparation, BrdU immunocytochemistry and nonradioactive in situ hybridization of whole embryos and sections was performed as described previously [Barthel and Raymond, 1993; Raymond *et al.*, 1995], except that 5-µm-thick cryosections were treated with proteinase K for 2 min, 45 sec. The DIG-labeled probes were visualized with a colorimetric alkaline phosphatase reaction using the substrate 5-bromo-4-chloro-3-indolyl phosphate with 4-nitroblue tetra-

Fig. 3. A: Canonical Notch protein, indicating conserved regions and region of homology to G-Notch. The latter was determined using BESTFIT (GCG Software Package, University of Wisconsin) to compare G-Notch to Notch proteins available in GenBank. **B:** Alignment of G-Notch to M-Notch-3 [Lardelli *et al.*, 1994] and Z-Notch [Bierkamp and Campos-Ortega, 1993], sequences that illustrate the overriding effect on Notch homology of phylogeny at the molecular level versus the organismic level. BESTFIT was used to determine the boundaries of the region of homology between G-Notch the other two Notch proteins as above. Multiple alignment of these regions was performed using the GCG PILEUP program. *Slashes*, amino acid identity to G-Notch. The conserved EGFL repeats [Davis, 1990], lin/Notch (LN) repeats, transmembrane (TM) domain, ANK repeats [Peters and Lux, 1993], and PEST (Pro/Glu/Ser/Thr-rich) domain [Rogers *et al.*, 1986] are indicated by name in both panels. Putative BNLS sequences [Dingwall and Laskey, 1991] are indicated in the alignment by asterisks. Numerous scattered glutamine (Q) residues near the C-terminal end of G-Notch probably represent a vestigial *opa* polyglutamine repeat region [Wharton *et al.*, 1985].

zolium chloride (Boehringer Mannheim). In some cases, whole embryos were embedded in methacrylate (Energy Beam Sciences, Agawam, MA) after in situ hybridization, and sectioned at 3 μm. Radioactive in situ hybridization to adult brain (10-μm-thick cryosections) was performed as described in Hieber *et al.* [1992].

Immunofluorescent detection of BrdU was performed as described [Barthel and Raymond, 1993], using a rat monoclonal anti-BrdU antibody (Accurate Chemical, Westbury, NY) and a secondary antibody conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA). A rabbit polyclonal antibody (FGP2, gift from Dr. Michal Schwartz) raised against goldfish glial fibrillary acidic protein (GFAP) visualized with a secondary antibody conjugated to FITC (Jackson ImmunoResearch, West Grove, PA) was used to label Müller glia.

RESULTS

Isolation and Sequence Analysis of *G-Notch*

An initial screen of a goldfish retinal cDNA library using *Xenopus Notch-1* (*X-Notch-1*) probes yielded a ~2-kb clone (*GotchCDC*) encoding a peptide with extensive homology to the intracellular region of Notch proteins. The 5′ end of *GotchCDC* encoded a region poorly conserved between mammalian Notch orthologues. This region was used to design primers for 5′ RACE to extend the sequence and confirm its identity. This strategy yielded a ~2.4-kb PCR fragment (*GotchTM*), from stage 19 embryonic cDNA, that was highly homologous to extracellular and transmembrane domains of Notch proteins. The region of overlapping DNA sequence between *GotchCDC* and *GotchTM* (20 bp exclusive of the RACE primer) is 100% identical between the two clones; conceptual assembly of the two clones yields a continuous open reading frame, indicating that they are part of the same transcript, hereafter referred to as *G-Notch* (Fig. 1). The truncated nature of the conceptual translation product, the absence of initiation and signal peptide sequences at the 5′ end of *G-Notch*, and of polyadenylation signal sequences at the 3′ end, all suggests that the ~4.4-kb construct represents only part of a full-length *G-Notch* cDNA. Hybridization of a mixture of *GotchCDC* and *GotchTM* probes to a single RNA band of 8.5–9 kb in adult goldfish brain (Fig. 2), lends further support to this inference.

The canonical *Notch* gene product is a large transmembrane protein whose extracellular domain contains a signal peptide, 36 EGF-like (EGFL) tandem repeats, and three tandem cysteine-rich lin12/Notch repeats (Fig. 3A). The intracellular domain contains six tandem cdc10/ankyrin (ANK) repeats separated from a C-terminal PEST region by a lengthy domain that is relatively unconserved apart from the presence of a putative bipartite nuclear localization signal (BNLS). G-Notch homology to other Notch proteins extends from the 26th EGFL repeat to the PEST region, encompassing well-conserved lin/Notch, transmembrane, ANK, and BNLS regions (Fig. 3A,B). The *opa* repeats found in the intracellular region of *Drosophila* Notch, on the other hand, are vestigial in G-Notch, as is true for other vertebrate Notch homologues.

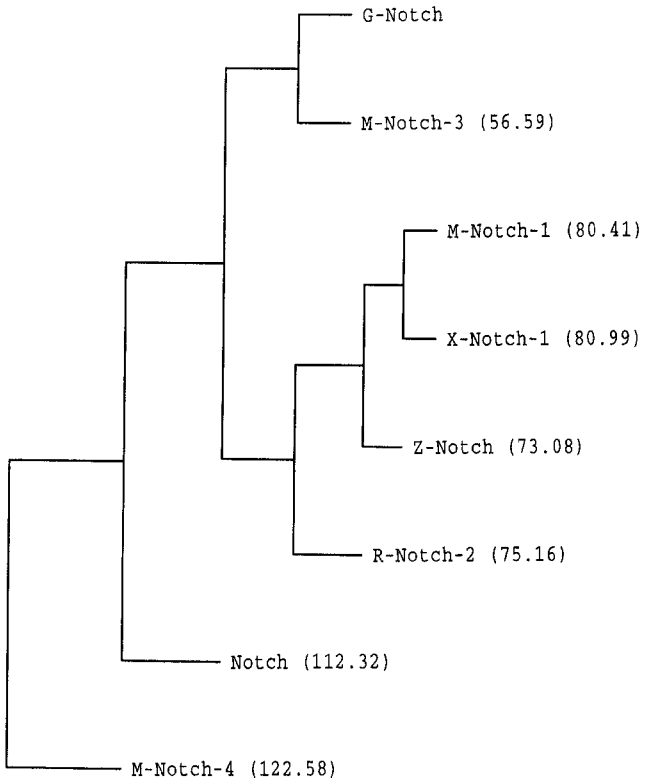

Fig. 4. Relatedness of representative Notch proteins to G-Notch and to each other. The region of most contiguous homology to *G-Notch* was identified for each homologue using BESTFIT and aligned using PILEUP. The DISTANCES program was used to generate a protein distance matrix from the aligned sequences, which was input to GROWTREE in order to generate a phylogeny tree. Numbers in parentheses next to sequence names indicate the relatedness of each Notch sequence to G-Notch, in terms of estimated number of amino acid substitutions per 100 amino acids, as calculated by DISTANCES. M-Notch-1, mouse Notch-1 [del Amo *et al.*, 1993]; R-Notch-2, rat Notch-2 [Weinmaster *et al.*, 1992]; M-Notch-4, mouse Notch-4 [Uyttendaele *et al.*, 1996]; Notch, *Drosophila* Notch [Artavanis-Tsakonas *et al.*, 1983]; X-Notch-1, *Xenopus* Notch-1 [Coffman *et al.*, 1990].

Four classes of Notch proteins have been described in mammals. Notch-1 and Notch-2 display minor differences in amino acid sequence [Weinmaster *et al.*, 1991, 1992]. Notch-3 and Notch-4 lack the full complement of EGFL repeats, with Notch-3 having 34 and Notch-4 having 29 [Lardelli *et al.*, 1994; Uyttendaele *et al.*, 1996]. Pairwise sequence comparisons and calculation of phylogenetic distances between representative Notch proteins (Fig. 4) suggest that G-Notch represents a member of the Notch-3 class. This assignment is necessarily provisional, since *G-Notch* sequence data do not

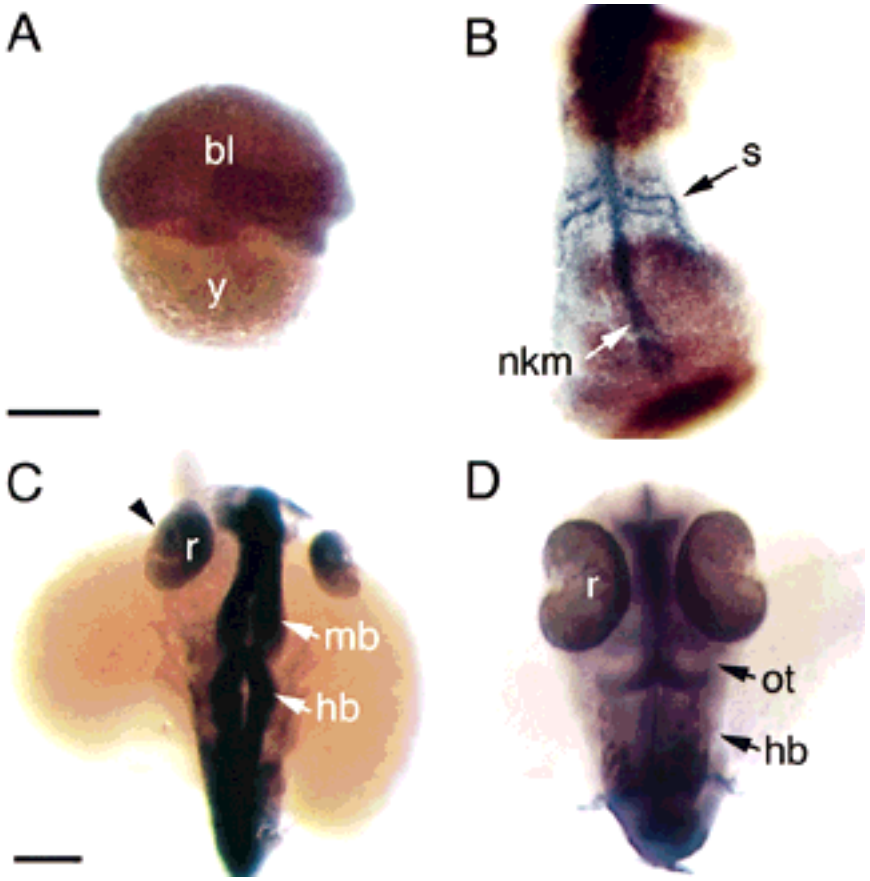

Fig. 5. Developmental expression pattern of *G-Notch* visualized in whole embryos by in situ hybridization; the reaction product is blue/violet. Anterior is up in all panels. **A:** Stage 12 (beginning of gastrulation); *G-Notch* is expressed throughout the blastoderm (bl), which has extended across approximately 50% of the yolk (y). **B:** Stage 15–16 (embryonic shield, 2–3 somites); the shield (neural plate equivalent) has been dissected away from the yolk. *G-Notch*-expressing somitic furrows (s) and the medial element of the presumptive neural keel (nkm) are evident; the lateral neural keel elements are too faint to see in this preparation. **C:** Stage 19 (optic cup and lens formation); swellings and constrictions of the neural tube mark future midbrain (mb) and hindbrain (hb) divisions. *G-Notch* expression is nearly uniform in intensity along the length of the neural tube, in the retina (r) and the lens placode (*arrowhead*). **D:** Stage 23 (beating heart); pigmentation obscures the relatively meager *G-Notch* expression remaining in the retina (r), although expression in midline (i.e., ventricular) regions of the brain remains distinct. Note expression also in the caudal margin of the optic tectum (ot), a region that contributes heavily to the adult tectal germinal zone. Bars = 100 µm (A) and 200 µm (C, also applies to B, D).

Fig. 6. Developmental expression of *G-Notch* visualized in sectioned embryos. **A–D:** Sections from embryos that were cryosectioned prior to in situ hybridization. **E–G:** From embryos raised in 0.003% 1-phenyl-2-thiourea to retard pigmentation, then processed whole for in situ hybridization, embedded in methacrylate and sectioned at 3 µm. **A:** Stage 22 (body pigmentation); a slightly oblique parasagittal section, showing *G-Notch* expression along the extent of the neural tube (nt; hindbrain, hb; midbrain, mb), in the otic capsule (oc), retina (r), and lens (l), and presumptive gill arches (ga). **B:** Enlargement of the area of the trunk bracketed in A, showing that *G-Notch* expression is restricted to neural tissue; epidermis (ep), notochord (no) and myomeres (my) are negative. **C:** Transverse section through a stage 22 hindbrain (hb) showing *G-Notch* expression restricted to ventricular regions. The roof of the fourth ventricle, (v4) has collapsed. Note also expression in the otic capsular (oc) epithelium. **D:** Stage 22 eye, showing absence of *G-Notch* expression in the earliest differentiating regions, i.e., around the choroid fissure (cf) and cellular layers nearest the vitreal cavity or lumen (lu). **E:** Stage 25 *(hatching)*; *G-Notch* is expressed in thin, presumptive ependymal layers *(arrowhead)* of the brain, e.g., the surfaces of the everted telencephalon (te). The retinal germinal zones (gz) express *G-Notch*, but expression has disappeared from the differentiating, laminated retina (r) and the lens (l). **F:** Stage 25 *(hatching)*; the ventricular lining of the hypothalamic ventricles *(arrowhead)* of the diencephalon (di) and the mesenchyme of the gill arches (ga) express *G-Notch*. *G:* Stage 25 *(hatching)*; Expression persists in the ventricular lining *(arrowheads)* of the midbrain (mb) and hindbrain (hb), in the otic capsule (oc) and mesenchyme of the head and pectoral fins (pf). Bars = 100 µm (A) and 100 µm (E, also applies to F and G).

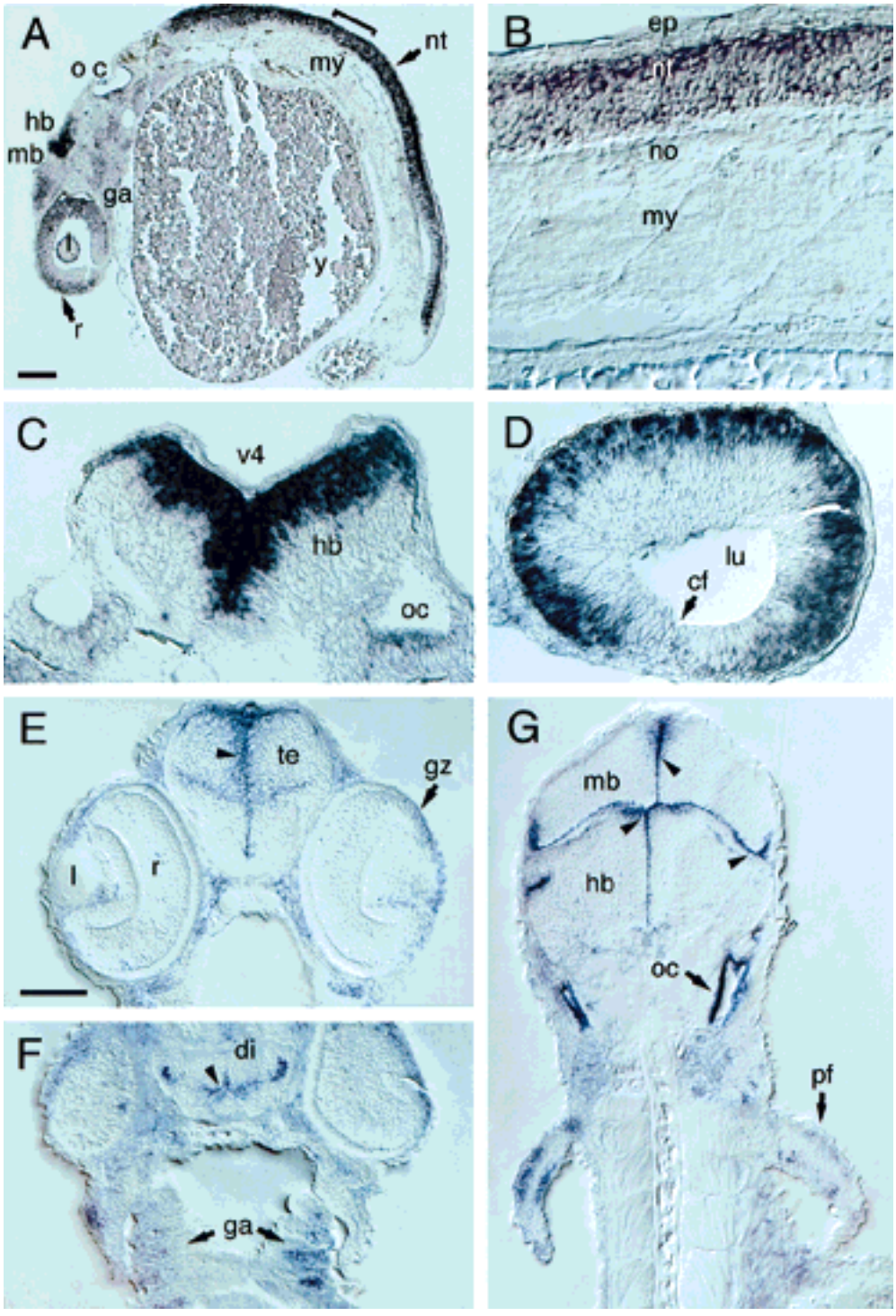
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Fig. 6.

yet extend into the extracellular region (EGFL repeats 2-21) where the two EGFL repeats that are absent in Notch-3 lie [Lardelli *et al.*, 1994].

Embryonic Expression of *G-Notch*: In Situ Hybridization

At the beginning of gastrulation (50% epiboly, stage 12) *G-Notch* is expressed throughout the goldfish blastoderm (Fig. 5A). Expression is seen in both the epiblast (presumptive ectoderm) and hypoblast (presumptive endo–mesoderm) germ layers (data not shown). By early neurulation (stages 15–16), *G-Notch* expression is localized to a midline stripe (Fig. 5B) and more faintly in two lateral stripes. Based on descriptions of zebrafish embryology [Schmitz *et al.*, 1993], these probably represent the tripartite elements of the presumptive neural keel. Mesodermal expression is concentrated in the posterior boundaries of the first somites (Fig. 5B], which as in zebrafish develop concurrently with the neural keel [Schmitz *et al.*, 1993].

As subdivisions of the brain become morphologically distinct, *G-Notch* is expressed robustly in neuroepithelia of all CNS structures including the optic cup (Fig. 5C, stage 19). Lens and auditory (otic) placodes also express *G-Notch*, although the epidermis does not (Fig. 5C). Although somitic expression is still visible at stage 19 (not shown), when body pigmentation begins (stage 22), differentiated myomeres have ceased expressing G-Notch (Fig. 6A,B). By this stage, the neuroepithelium has become a multilayered structure, and within it, *G-Notch* expression persists in the periventricular mitotic zone but has ceased in more lateral zones containing migrating and differentiating cells (Fig. 6C) and is not expressed in floor plate (data not shown).

At stage 22, retinal cell differentiation has begun at the choroid fissure and in the future inner cell layers throughout the retina [Sharma and Ungar, 1980] (P.A. Raymond, unpublished observations), and retinal *G-Notch* is downregulated in these regions (Fig. 6D). As early as the beating-heart stage (stage 23), *G-Notch* expression in the optic tectum of the midbrain is restricted to its midline and caudal margins (Fig. 5D), which persist as germinal zones in the adult tectum [Kirsche, 1960; Kirsche and Kirsche, 1961; Raymond and Easter, 1983]. In older embryos, expression in the CNS is primarily seen in the presumptive germinal zone (GZ) in the retina, and the presumptive ventricular lining (ependyma) of the brain (Fig. 6E,G). Mesenchymal and neural crest cell-derived *G-Notch* expression is also seen in various regions, including the head, gill arches, and fin buds of late embryos (Fig. 6E–G). By 10 days post-hatching, *G-Notch* expression persists only in a thin periventricular zone in the brain and in the spinal cord (data not shown).

G-Notch Expression in the Adult Goldfish CNS

RNase protection assays of total RNA from adult goldfish tissues indicated that *G-Notch* is expressed in adult brain and eyes, but not in adult skeletal muscle (Fig. 7). We subsequently performed in situ hybridization studies to localize this expression.

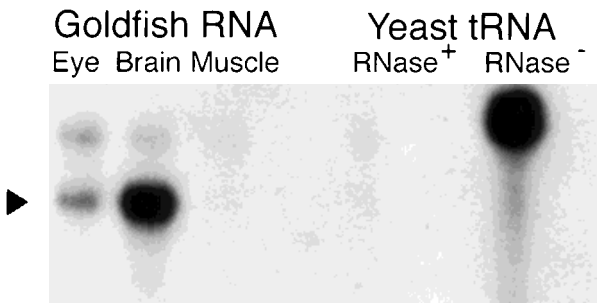

Fig. 7. RNase protection assay showing the presence of *G-Notch* transcripts *(arrowhead)* in the brain and eye but not flank muscle of adult goldfish. The larger fragment present in all lanes is a contaminating template cDNA. Yeast tRNA was included as a control.

Several sites of *G-Notch* expression were revealed by hybridization to parasagittal sections of adult brain. The ependymal lining of the forebrain in teleosts is in an atypical location: it underlies the pial, or outer, surface [Richter and Kranz, 1970b]. This unusual arrangement is due to an eversion of the telencephalic ventricle that is characteristic of teleost brain morphogenesis [Nieuwenhuys and Meek, 1990]. The everted ependyma of the forebrain expresses *G-Notch*, whereas expression ceases abruptly at the junction with the pial surface of the non-everted midbrain (Fig. 8B). However, *G-Notch* expression continues in the ependymal lining of the tectal (Fig. 8A,B) and hypothalamic ventricles (not shown). In the tectum, *G-Notch* is also expressed deeper in the ventricular wall, in the stratum periventricular (Fig. 8C,D). This layer corresponds to the "periependymal" zone wherein a persistent population of radial glia resides [Stevenson and Yoon, 1981, 1982].

Just as the retinal GZ forms the rim of the hemispheric retina, so the tectal GZ is located in the rim of each hemispheric tectal lobe. In the GZ of the tectum, as in the retinal GZ, the laminar cytoarchitecture collapses into a pseudostratified, primitive neuroepithelium of proliferating cells [Raymond and Easter, 1983]. In parasagittal sections the tectal GZ appears as a cluster of cells at the caudal junction of the tectum and the cerebellum; these cells strongly express *G-Notch* (Fig. 8E,F). The tegmental GZ, which may be a continuation of the tectal GZ [Kirsche, 1960; Raymond and Easter, 1983], also expresses *G-Notch* (Fig. 8E,F).

In the retina, *G-Notch* expression was strongest in the GZ, where the densest population of retinal neuronal progenitors resides (Fig. 9A). Some recently-generated cells in the inner retinal layers near the GZ also express *G-Notch*. Weakly expressing cells were also occasionally found in the inner nuclear (INL) and ganglion cell (GCL) layers, but not in the outer nuclear layer (ONL), where rod precursors and photoreceptor somata reside (Fig. 9A, B). The precise identities of cells expressing

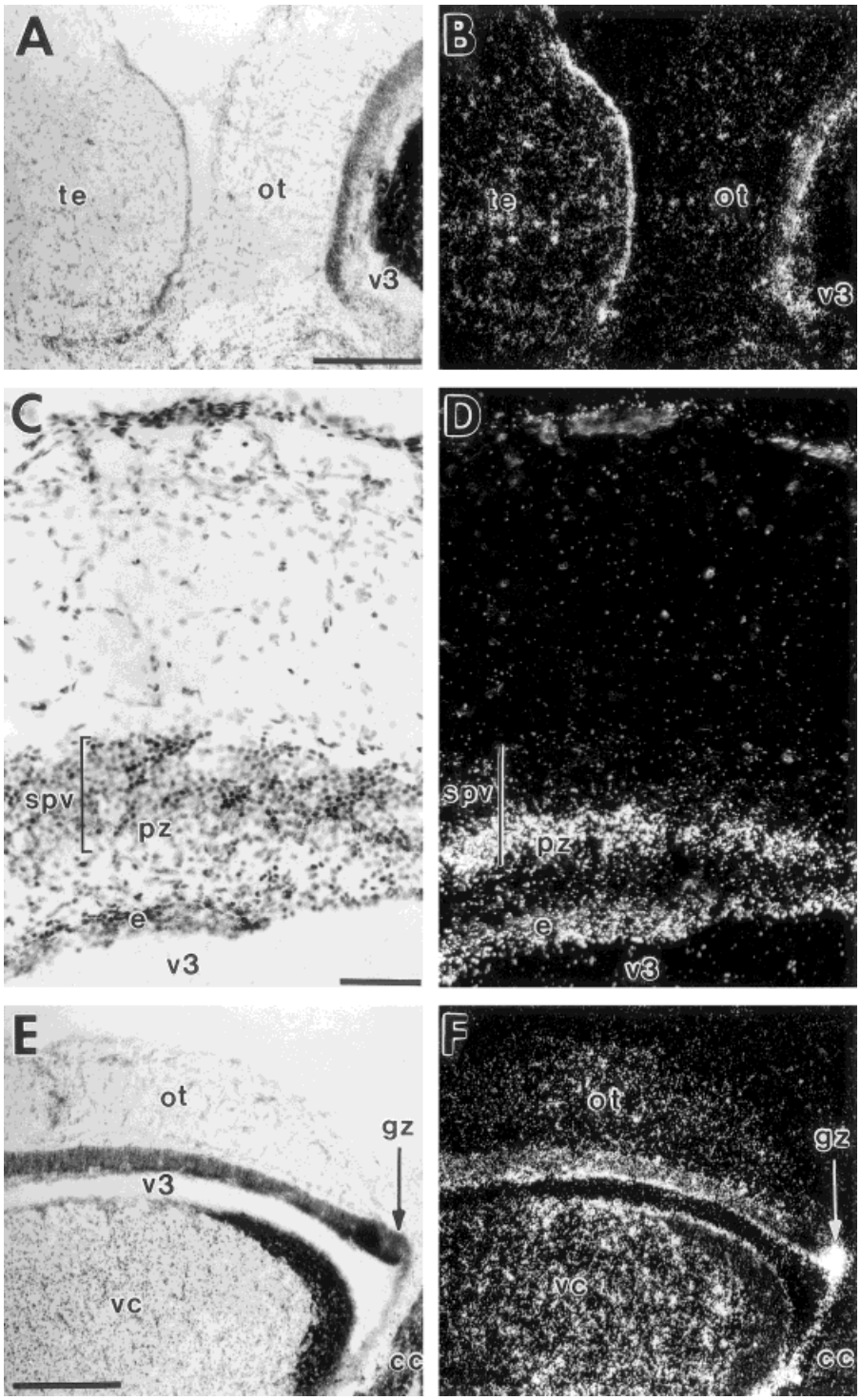

Fig. 8. Expression of *G-Notch* in adult goldfish brain correlates with proliferative regions. Anterior is left, dorsal is up. Brightfield (A,C,E) and darkfield (B,D,F) views of the same sections after radioactive in situ hybridization to *G-Notch* probes; the signal appears as white grains in darkfield illumination. **A,B:** Parasagittal section containing the junction of the forebrain (telencephalon, te) and the optic tectum of the midbrain (*ot*). The ependymal layer of the evaginated forebrain expresses G-Notch, as does the corresponding ventricular layer of the optic tectum (ot), which encloses the third ventricle (v3). **C,D:** Parasagittal section of the tectum, showing *G-Notch* expression mainly confined to the ependymal lining (e) of v3 and to the ventricular side (periependymal zone, pz) of the adjacent cellular layer, the stratum periventriculare (sp). **E,F:** Parasagittal view of the caudal end of the tectum, where the tectal germinal zone (gz) strongly expresses *G-Notch*. The tegmental germinal zone, which also expresses *G-Notch*, is just visible in the lower right part of the panel, where the valvular cerebellum (vc) and caudal cerebellum (cc) join. Bars = 100 μm (A and E) and 50 μm (C).

G-Notch in the adult retina were not determined in this study.

G-Notch Expression in Injured and Regenerating Retinas

G-Notch expression was investigated in retinas subjected to two types of focal injury, retinal puncture and laser lesion, both of which stimulate proliferation of intrinsic neuronal progenitor populations [Sullivan and Raymond, 1991; Braisted *et al.*, 1994]. With the parameters used, the laser lesions specifically ablate photoreceptors in the target region, while retinal puncture causes non-specific damage. In previous studies we have characterized the regeneration of both rods and cones in response to laser lesions [Braisted *et al.*, 1994]. In the present study we found that in both paradigms *G-Notch* expression increased locally within a few days in the retinal regions surrounding the lesioned area (Fig. 9B,D). As in uninjured retinas, BrdU labeling showed that only a subset of *G-Notch* expressing cells are mitotically active (Fig. 9C,D). Labeling with GFAP antibody, which marks Müller glia [Wagner and Raymond, 1991; Erickson *et al.*, 1992], revealed that many *G-Notch*-expressing foci of cells are associated with glial processes, but it does not appear that all *G-Notch*-expressing cells also express GFAP (Fig. 9E).

By 5 days after the laser lesion, novel *G-Notch* expression appears in the outer nuclear layer (ONL) of the injured retina (Fig. 9C,E). These are proliferating cells which within the next few weeks will give rise to regenerated cone and rod photoreceptors [Braisted *et al.*, 1994]. The origin of these clusters of cells, whether from rod precursors already located in the ONL or from neurogenic stem cells in the inner nuclear layer (INL) has not been established. It has been shown that Müller cell nuclei, normally found only in the INL, migrate into the ONL following ablation of photoreceptors [Braisted *et al.*, 1994]. Whatever their origin, it is clear that these cells which are responsible for regenerating photoreceptors have abundant *G-Notch* expression, similar to the dividing and differentiating neuroepithelial cells in the GZ at the growing margin. Beyond these local regenerative responses, the level of *G-Notch* expression, especially in the INL and in the GZ, increases throughout the retina. Thus, injury to the retina alters both the level and the location of *G-Notch* expression.

DISCUSSION

The sequence data presented here strongly suggest that *G-Notch* represents a member of the *Notch* family. *G-Notch* encodes a peptide containing all of the motifs present in a canonical Notch protein, and also their spacing. This high degree of structural conservation implies important functions for the various motifs, and indeed the conserved EGFL, ANK, and C-terminal regions have been found necessary for proper functioning of the Notch signaling pathway in fruit flies, amphibians, and mammals [Rebay *et al.*, 1993; Coffman *et al.*, 1993; Jarriault *et al.*, 1995].

The question of to which *Notch* class *G-Notch* belongs is probably not trivial, since it has been shown in mice that neither the expression patterns nor the functions of *Notch* orthologues are redundant [Swiatek *et al.*, 1994; Williams *et al.*, 1995]. *G-Notch* most likely encodes a member of the *Notch-3* class previously represented solely by a murine cDNA [Lardelli *et al.*, 1994]. Although specific functions of the *Notch* orthologues remain uncertain, mutations in the human *Notch-3* gene recently have been identified in a familial syndrome known as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) causing dementia and stroke with adult onset [Joutel *et al.*, 1996]. Sequence analysis of Notch-1 and Notch-2 proteins [Maine *et al.*, 1995] has shown that Notch paralogues (e.g., M-Notch-1 vs. X-Notch-1)

Fig. 9. Expression of *G-Notch* in the adult retina is upregulated after injury and during photoreceptor regeneration. In situ hybridization of retinal cryosections; the colorimetric reaction product has a purple/violet hue. **A:** *G-Notch* is expressed in the germinal zone (gz) at the margin of the retina and in the adjacent (immature) postmitotic cells. The differentiated retina is laminated, with photoreceptor nuclei in the outer nuclear layer (on), and various retinal neurons and glial cells in the differentiated inner nuclear (in) and ganglion cell (gc) layers. At the back of the neural retina is the retinal pigmented epithelium (rpe), which is filled with dark brown melanin. **B:** At 15 days following wounding (retinal punctures), *G-Notch* expression is upregulated in the germinal zone (gz) and inner nuclear (in) layer. The refractile profiles between the outer nuclear (on) layer and the retinal pigmented epithelium are photoreceptor processes (pr). A circumferential blood vessel (bv) surrounds the retina adjacent to the gz. **C:** In the region of a retinal puncture *(arrow)*, proliferating cells (light pink) have incorporated bromodeoxyuridine (BrdU), which was visualized by immunofluorescence *(arrowheads)*. The proliferating cells are found in all retinal layers. *G-Notch* expression is upregulated in the inner nuclear (in) and ganglion cell (gc) layers (although this double exposure of immunofluorescence and brightfield illumination makes the in situ signal difficult to appreciate at this low magnification). **D:** In the same section as in panel C, but at the retinal margin (which is located several hundred micrometers or more from the site of the nearest wound), proliferating neuroepithelial cells in the germinal zone (gz) and rod precursors in the outer nuclear (on) layer *(arrowhead)* are mitotically stimulated, as are glia (in the gc layer, *arrowhead*), a few cells in the inner nuclear (in) layer, and vascular cells (in the circumferential blood vessel, bv). **E:** Retina at 5 days following a laser lesion that destroys photoreceptors. The boundaries of the lesion are indicated by the large asterisks in the outer nuclear (on) layer; note the absence of refractile photoreceptor processes within the lesion. Note also the disruption in the rpe associated with the lesion, and the infiltration of inflammatory cells (if). The small asterisk in the ganglion cell (gc) layer indicates a displaced, pigmented cell. This section was processed for in situ hybridzation, followed by immunofluorescence using anti-GFAP, which is localized to radial Müller glial cells, whose processes span the retinal thickness *(arrows)*. Note the strong *G-Notch* expression in the outer nuclear layer (shown at higher magnification in the inset). Bars = 50 μm (A); 50 μm (B, also applies to C and D); 25 μm (E).

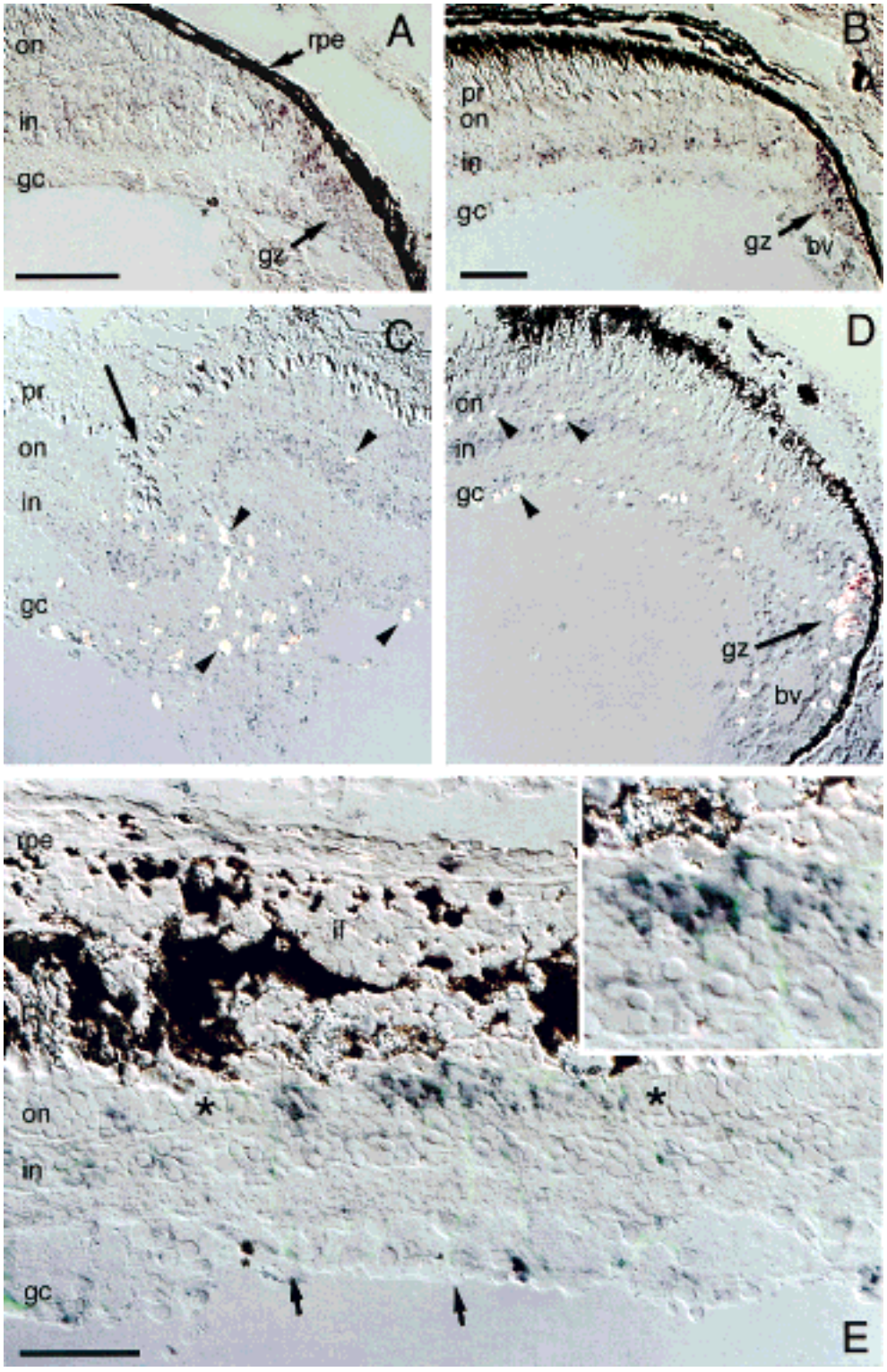
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Fig. 9.

are less divergent than Notch orthologues (e.g., M-Notch-1 vs. M-Notch-2). Thus we would predict that a goldfish Notch-3 would be more homologous to a mammalian Notch-3 than to a member of a different Notch class in goldfish or a closely related species. In agreement with this inference, G-Notch is more homologous to M-Notch-3 than to zebrafish Z-Notch, which our sequence analysis shows is in the Notch-1 class. As only a single *Notch* gene is present in *Drosophila*, it has been previously proposed that the evolution of *Notch* paralogues occurred between the divergence of arthropods and amphibians from the mammalian lineage [Lardelli and Lendahl, 1993; Lardelli *et al.*, 1994]; our analysis further localizes this event in time to before the evolution of teleost fishes.

Our expression data also support the identification of *G-Notch* as a *Notch* gene, particularly its expression in proliferative neuroepithelia. For example, expression in the proliferating ventricular zone of the embryonic murine neural tube is characteristic of *Notch-1, -2*, and *-3* [Lardelli *et al.*, 1994; Higuchi *et al.*, 1995]. However, assignment of *G-Notch* to a specific *Notch* class based on expression data is complicated by the paucity of such data for lower vertebrates, where only *Notch-1* expression patterns have been documented [Coffman *et al.*, 1990; Bierkamp and Campos-Ortega, 1993], and by inconsistencies in descriptions of rodent *Notch* orthologue expression patterns [Weinmaster *et al.*, 1992; Lardelli *et al.*, 1994; Higuchi *et al.*, 1995; Williams *et al.*, 1995]. *G-Notch* expression accords with that of *M-Notch-3* in that both (1) occur in the ectoderm and mesoderm of gastrulating embryos, (2) are absent in notochord and floor plate, (3) are strong in the embryonic periventricular neuroepithelium and the otic vesicles, and (4) occur in adult brain [Lardelli *et al.*, 1994; Williams *et al.*, 1995; Lindsell *et al.*, 1995]. However, expression patterns of *M-Notch-3* and *G-Notch* are not completely analogous. For example, *M-Notch-3* expression was detected in embryonic rodent epidermis and adult skeletal muscle, and not in the mostly undifferentiated E10.5 retina [Lardelli *et al.*, 1994; Williams *et al.*, 1995]. These discrepancies may reflect differences in probe sensitivities, species-specific adaptations of Notch-3 function, or alternatively, *G-Notch* might represent a novel class of *Notch*. The existence of a zebrafish *Notch-1* and of multiple *Notch* orthologues in mammalian species suggests that additional goldfish orthologues of *G-Notch* await discovery.

What role(s) might *G-Notch* play in its various sites of expression? Although *Notch* expression often correlates with cellular proliferation, it has been shown that the two events can be uncoupled without altering *Notch* expression patterns [Coffman *et al.*, 1993]. Furthermore, in the *Xenopus* retina, *X-Notch-1* expression overlaps only partially with the mitotic germinal zone [Dorsky *et al.*, 1995], as we have reported here in goldfish for *G-Notch*, indicating that no simple relationship exists between mitosis and *Notch* expression. Notch has more compellingly been implicated in processes of commitment and early differentiation of cells, which are proximal events to terminal mitosis. The accumulated evidence suggests that Notch is involved in cell fate decisions not as a specifier of particular cell fates, but as an antagonist to differentiation in a variety of developmental contexts, among them neurogenesis [Fortini *et al.*, 1993; Chitnis *et al.*, 1995; Chitnis and Kintner, 1996; Nye *et al.*, 1994] and myogenesis [Conlon *et al.*, 1995; Nye *et al.*, 1994; Kopan *et al.*, 1994]. Alterations of Notch activity in the *Drosophila* CNS and ommatidia [Cagan and Ready, 1989; Rebay *et al.*, 1993], in the retina of chick [Austin *et al.*, 1995], and in the *Xenopus* neural plate [Coffman *et al.*, 1993; Chitnis *et al.*, 1995] produce shifts in numbers of the specific cell types normally produced during the period of disruption. These shifts are characteristic of a factor that inhibits differentiation, in that reduction in activity leads to overproduction of a cell type, while increased activity causes its underproduction. This function is particularly vital wherever sequential fate choices are made, as in the developing CNS. In the embryonic goldfish retina we observed a central-to-peripheral and vitreal-to-lumenal gradient of *G-Notch* expression that approximately parallels the differentiation of goldfish retinal cells; for example, the ganglion cell layer in the central retina differentiates first [Sharma and Ungar, 1980], concurrent with downregulation of *G-Notch* expression in the inner retina. Similarly the laminar organization of the neural tube develops in a peripheral-to-ventricular gradient, complementing the pattern of *G-Notch* expression. Thus, the pattern of *G-Notch* expression is consistent with a role for G-Notch in the maintenance of the undifferentiated state in these embryonic tissues.

G-Notch expression patterns in adult goldfish brain and retina argue for a similar role for this gene in postembryonic CNS growth and regeneration. In the brain *G-Notch* is expressed in the tectal germinal, ependymal, and periependymal zones, all of which contain proliferative cell populations. The goldfish tectal GZ consists of proliferative cells with neuroepithelial morphology, whose progeny, as determined by ^{3}H-thymidine uptake studies, are both neuronal and glial [Raymond and Easter, 1983]. The tectal GZ is also essential for tectal regeneration; cell proliferation in deep tectal layers (i.e., the ependyma and periependyma) alone is insufficient to restore tectal morphology following brain lesions [Kirsche, 1960; Kirsche and Kirsche, 1961; Richter, 1968; Richter and Kranz, 1977; Stevenson and Yoon, 1980]. Ependymal cells and radial glial cells of the periependyma also have mitotic potential [Stevenson and Yoon, 1981, 1982; Raymond and Easter, 1983]; a role for *G-Notch* in the maintenance of relatively undifferentiated pools of cells in the GZ and periventricular glial layers of the brain is therefore plausible. Expression of *G-Notch* in both ependymal and periependymal glial progenitor zones might even

reflect an underlying relationship between the two cell types, since explants of ependymal cells from proliferative regions of adult songbird forebrain produce radial glia [Goldman *et al.*, 1994].

In the adult retina, *G-Notch* expression in the GZ may be functionally analogous to that seen in the tectal GZ and in the embryonic retinal neuroepithelium, since these are all sites of neurogenesis. Similarly, if the *G-Notch*+ cells in the differentiated INL correspond to the normally quiescent stem cell-like populations reported in the INLs of both trout and goldfish [reviewed in Raymond and Hitchcock, 1997], G-Notch expression might act to keep them in an undifferentiated state. Since this function of Notch requires its interaction with Delta or Delta-like ligands produced by adjacent cells [Gu *et al.*, 1995; Chitnis *et al.*, 1995; Myat *et al.*, 1996], it would be illuminating to obtain teleost *Delta* genes and examine their expression patterns in adult retina and in brain regions that express *G-Notch*. Another vertebrate Notch ligand recently identified is Jagged, a transmembrane protein with structural similarities to the *Drosophila* Notch ligand Serrate [Lindsell *et al.*, 1995]. In rat embryos, *Jagged* is expressed in bilaterally symmetric, longitudinal stripes in the ventricular zone of the spinal cord, overlapping the expression domains of rat *Notch-1*, *-2*, and *-3*. Since *Jagged* is also expressed in many other tissues during embryonic development in the rat, including the dorsal root ganglia, eye, ear, kidney, pancreas, limb bud, and skin [Lindsell *et al.*, 1995], it would also be instructive to identify a teleost *Jagged* homologue and compare the expression patterns to those of *G-Notch*.

We also observed *G-Notch* expression in a subset of cells in the adult ganglion cell layer (GCL). Although the identities of these cells were not determined, *M-Notch-1* expression has been reported in the INL and GCL of adult rat retina, which apparently lacks neural progenitor cell populations [Ahmad *et al.*, 1995]. The function of Notch in this context remains to be defined, but since both of these retinal layers contain glial cells, which are capable of proliferation, it is plausible that *Notch* is expressed in these cells.

Proliferation of neuronal progenitor populations is a common reaction to retinal injury in goldfish in a variety of lesion paradigms, chemical as well as mechanical [reviewed in Raymond and Hitchcock, 1997]. In this study we observed widespread upregulation of *G-Notch* expression in injured retinas along with novel expression in the photoreceptor cell layer, associated with regeneration of photoreceptor cells. In surgically lesioned goldfish retinas, it has similarly been shown that regenerating cells upregulate expression of other developmentally regulated genes: *pax6* and *vsx1*, both members of the paired-class homeobox family of transcription factors [Hitchcock *et al.*, 1996; Levine *et al.*, 1994]. Disrupting the integrity of the retina and the pigmented epithelium is likely to release growth factors normally sequestered by the extracellular matrix [Sullivan and Raymond, 1991; Raymond *et al.*, 1992], which can stimulate proliferation [Gao and Hollyfield, 1992; Raymond *et al.*, 1992; Lewis *et al.*, 1992] and may alter expression of these regulatory genes. The upregulation of *G-Notch* expression, like the stimulation of mitotic activity, was not confined to the region at and immediately surrounding the retinal wound. Presumably most of the superfluous cells that proliferate outside the injured region either fail to survive or do not differentiate, although this has not been verified. A possible role of increased *G-Notch* expression in these undamaged regions would be to insure that differentiated cells are not overproduced in response to liberated growth factors.

We also noted that *G-Notch* expression in the INL and ONL was contiguous with GFAP immunoreactivity, which is also upregulated in injured retinas [Erickson *et al.*, 1992; Braisted *et al.*, 1994]. Both Müller glia, which express GFAP, and the putative stem cell in the INL proliferate in response to injury [Lewis *et al.*, 1994; Wagner and Raymond, 1991; Braisted *et al.*, 1994]. An alternative role of *G-Notch* expressed at the junction of glia and neural progenitors could be a mechanical function. There is evidence from *Drosophila* that the ligand-binding properties of Notch-like proteins could serve as guidance cues for migrating cells or growing axons [Giniger *et al.*, 1993; Menne and Klambt, 1994]. Outward migration of proliferating INL cells along Müller glial processes occurs in goldfish larvae before the appearance of rod precursors in the ONL [Raymond and Rivlin, 1987], and Sharma [1975] has described a similar process of neuronal migration along radial fibers in the developing optic tectum of the trout. The retinal Müller glia and the periependymal radial glia of goldfish may represent a scaffold-like component of a cell-migratory mechanism for constructing new tissue. If so, G-Notch could play roles that are both instructive (guidance on radial glia) and prohibitive (suppression of differentiation) in developing and regenerating CNS.

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