Complex Cellular and Subcellular Regulation of Notch Expression during Embryonic and Imaginal Development of *Drosophila*: Implications for Notch Function

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Abstract. The *Notch* gene in *Drosophila* encodes a transmembrane protein with homology to EGF that appears to mediate cell–cell interactions necessary for proper epidermal vs. neural fate decisions. In this study, we examine Notch expression in detail throughout embryonic and imaginal development using confocal laser-scanning microscopy and specific mAb probes. We find that Notch is expressed in a tissue-specific manner as early as the cellular blastoderm stage, when cells of the presumptive mesoderm clearly express less Notch than adjacent ectodermal precursors. Notch is abundantly expressed during the initial determination of neuronal lineages, such as the embryonic neuroblasts and the precursors of sensory neurons in the imaginal disc epithelia, but expression quickly decreases during subsequent differentiation. These changing patterns of Notch expression do not correlate well with cell movements, and thus do not appear to support the notion that the major function of Notch is to maintain epithelial integrity via adhesive mechanisms. Our data suggest instead that Notch may act as a cell-surface receptor, perhaps functioning in the lateral inhibition mechanism that is necessary for proper spacing of neuronal precursors.

THE process of embryonic neurogenesis in the fruit fly *Drosophila melanogaster* first becomes apparent when when certain cells in the ventral ectoderm enlarge, migrate inward, and form the neuroblasts. Classical studies of the *Notch* gene (Poulson, 1937; Wright, 1970) have shown that this process is under genetic control: loss of function mutations at *Notch* result in hypertrophy of the embryonic central nervous system (CNS)[1] due to misrouting of epithelial precursors into a neuroblast developmental pathway. Subsequent embryological studies (Doe and Goodman, 1985) have suggested that this neural hypertrophy may be due to the failure of a lateral inhibition mechanism that normally functions to limit the number of ventral ectodermal cells that differentiate as neuroblasts. Genetic screens have revealed at least five other zygotically acting loci (*Delta, Enhancer of split, mastermind, big brain,* and *neuralized*) with similar mutant phenotypes; their common phenotype, the genetic interactions between these loci, and the molecular structure of their gene products suggest that together they function in a cellular interaction mechanism necessary for proper development of ectodermal derivatives (Artavanis-Tsakonas, 1988).

Sequence analysis and biochemical studies of the *Notch* locus have shown that it encodes an ~300-kD transmembrane protein (we refer to this protein as "Notch") that spans the cell membrane a single time (Wharton et al., 1985; Kidd et al., 1986, 1989; Johansen et al., 1989). The extracellular domain of Notch contains 36 tandemly arrayed epidermal growth factor (EGF)-like repeats, as well as three additional cysteine-rich repeats, termed Notch/lin-12 repeats, adjacent to the transmembrane domain. EGF-like repeats have been found in a number of proteins that interact extracellularly with other proteins (Furie and Furie, 1988; Davis, 1990). These structural considerations, together with genetic evidence of interactions between *Notch* and other neurogenic loci (Xu et al., 1990), have led to the hypothesis that Notch interacts biochemically with other proteins involved in ectodermal differentiation (Artavanis-Tsakonas, 1988). Indeed, we have recently provided evidence for apparently direct interactions between Notch and the product of the neurogenic locus *Delta* (Fehon et al., 1990), which also encodes a transmembrane protein with EGF-like repeats in its extracellular domain (Vässin et al., 1987; Kopczynski et al., 1988).

Studies using a temperature-sensitive allele of *Notch*, N^{ts1}, have shown that in addition to embryonic neurogenesis, *Notch* functions during imaginal development (Shellenbarger and Mohler, 1978; Cagan and Ready, 1989*a*) and oogenesis (Xu, T., R. Fehon, and S. Artavanis-Tsakonas, in preparation). Consistent with this finding, in situ hybridization and antibody localization experiments have revealed that Notch is

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1. *Abbreviations used in this paper*: AP, after pupariation; BMC, bristle mother cell; CNS, central nervous system; EGF, epidermal growth factor.

widely expressed during embryonic and adult development (Hartley et al., 1987; Markopoulou and Artavanis-Tsakonas, 1989; Johansen et al., 1989; Kidd et al., 1989). Although the diverse cells that express Notch often do not share an obvious developmental potential, they do correlate well with those cells that are known to be mitotically active. This result has raised the possibility that Notch may play a role in cell division in addition to its role in neurogenesis (Hartley et al., 1987; Markopoulou and Artavanis-Tsakonas, 1989). On the other hand, Notch interacts via its extracellular domain with the protein product of the *Delta* gene, suggesting that Notch could act as a surface receptor for ligands that carry signals between cells and/or as an adhesion molecule necessary for maintaining cellular contacts (Fehon et al., 1990). An adhesive function for Notch also has been suggested recently based on interpretations from genetic data (Cagan and Ready, 1989*a*; Hoppe and Greenspan, 1990; Greenspan, 1990). Thus, while several possible functions have been proposed for Notch, from the available data it has not been possible to determine which, if any, actually carries in vivo significance.

Much of the uncertainty regarding the cellular function of Notch results from our still imprecise knowledge of the timing and location of its expression in the developing embryo and larva. Previous studies of Notch expression (Johansen et al., 1989; Kidd et al., 1989) have used immunohistochemically stained whole-mount preparations and polyclonal antibody probes that do not provide sufficient resolution for a cell-by-cell expression analysis. In this study, we examine Notch expression using a combination of a highly specific mAb and the optical sectioning ability of the confocal microscope. These tools have allowed us to document in detail Notch expression throughout development, at the cellular and to some degree the subcellular level. Our results indicate that neuronal precursors in the embryo and the imaginal disc express Notch at the time of commitment to the neuronal fate, but then cease to express shortly thereafter. Thus modulation of Notch expression may be important to maintenance of neuronal fate. We find that the correlation between Notch expression and mitotic activity is not absolute: there are cells that are mitotically active but do not express Notch and vice versa. In addition, we present evidence that modulation of Notch expression does not coincide with developmentally regulated cell movements, as might be expected if Notch solely serves to maintain adhesive contacts between cells. Finally, optical sections of embryonic and imaginal tissues show that Notch is highly polarized toward the apical surface in some but not all epithelial cells.

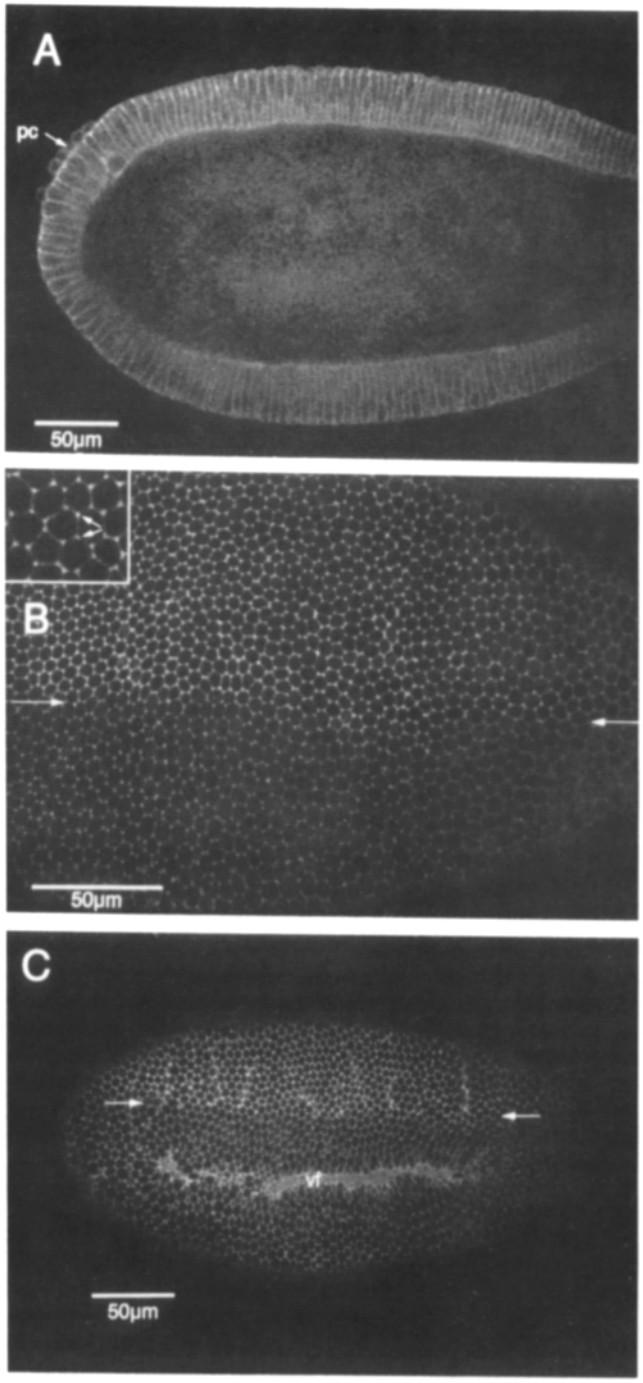

Figure 1. Notch is differentially expressed in the cellular blastoderm stage embryo. Confocal optical sections of three blastoderm stage embryos stained for Notch protein with mAb C17.9C6. (*A*) A mid-sagittal section through the blastoderm showing the single columnar layer of cells surrounding the blastoderm. All cells seem to express Notch, but note that there is more staining on the dorsal (*top*) side and just beneath the pole cells (*pc*). (*B*) Tangential section through the midlateral portion of the blastoderm. Note the discrete line of brighter dorsal and lesser ventral staining. Ventral-most cells will form the mesoderm during gastrulation. Also note the bright points of stain at the apices where three cells come into contact. (*C*) Tangential section through the ventral surface of the embryo at the beginning of gastrulation. Lengthwise fold (ventral furrow, *vf*) is the area in which cells will migrate inward to form the mesoderm. Note that these cells express less Notch than the more lateral and dorsal cells.

Materials and Methods

Immunofluorescent Staining

The fixation and staining procedures used in this study were essentially those described previously (Johansen et al., 1989). In brief, embryos were fixed in a 1:1 mix of heptane/4% paraformaldehyde (prepared in PBS by heating to 60°C for 1–2 h). After devitellinization in MeOH, the embryos were blocked with a 1-h incubation in PBS/1% normal goat serum (ngs/0.1% saponin, rinsed with PBS, and then incubated overnight at 4°C in PBS-ngs-saponin with a 1:10 dilution of culture supernatant from the anti-Notch monoclonal line C17.9C6 (see Fehon et al., 1990 for details of the production of this antibody). Embryos were then rinsed three times with PBS, incubated 1 h at room temperature in PBS-ngs-saponin, and then incubated either 2–4 h at room temperature or overnight at 4°C in fluorescein-conju-

gated goat-anti-mouse (#115-015-062; Jackson Immunoresearch Laboratories, West Grove, PA) at a dilution of 1:250 in PBS-ngs-saponin. After this incubation, embryos were again rinsed three times with PBS, incubated in PBS-ngs-saponin for 1 h at room temperature, and rinsed once with PBS. Embryos were mounted in 90% glycerol, 10% Tris base pH 8.0, 0.5% (wt/vol) *n*-propyl-gallate (Sigma Chemical Co., St. Louis, MO) under coverslips for viewing on the microscope. Larval and pupal tissues were dissected in M3 tissue culture medium (Fehon et al., 1990), rinsed in PBS, and fixed in 2% paraformaldehyde in PBS without heptane. Further incubations were performed as described for embryos. For Notch/β-galactosidase double-labeling experiments, preparations were simultaneously incubated with affinity-purified rabbit anti-Notch and anti-β-galactosidase (Promega Biotec, Madison, WI) mAbs and stained using specific fluorescein-conjugated goat anti-mouse and Texas Red-conjugated goat anti-rabbit 2° antibodies (Jackson Immunoresearch Laboratories). Notch/22C10 double staining was performed using the same rabbit anti-Notch antiserum and monoclonal 22C10 (kindly provided by Dr. S. Benzer, Cal Tech).

HRP-stained whole mounts were prepared using the same protocol with HRP-coupled secondary antibodies and the diaminobenzadine staining procedure described in Johansen et al. (1989). After staining, specimens were dehydrated in EtOH, cleared in methyl salicylate (Sigma Chemical Co.), and mounted in Permount (Fisher Scientific, Fair Lawn, NJ).

Microscopy

Confocal images were collected using the MRC 500 system (Bio-Rad Microsciences Division, Cambridge, MA) attached to a Zeiss Axiovert compound microscope. In all cases, the programs BASE and SCALE were run on images as they were collected (software provided by Bio-Rad Laboratories). Where noted in the text, optical sections were combined using the PROJECT program. For photo reproduction, image files were transferred by disk to a Macintosh IIfx computer using Apple File Exchange, converted to PICT file format using Image 1.29q (Wayne Rasband, National Institutes of Health), and arranged and annotated using Aldus Persuasion (Aldus Corp., Seattle, WA). Digital images were then printed to T-Max (Eastman Kodak Co., Rochester, NY) ASA 100 35-mm film using a Matrix Slidewriter film recorder (Agfa Matrix Division).

Nonconfocal images (Figs. 5 *B*, 6, *E* and *F*) were collected using a Leitz Orthoplan 2 microscope with DIC optics and epifluorescence. Video images were produced by a Dage Series 70 television camera and digitized using a Nu Vista capture board (Truevision, Inc., Indianapolis, IN) in an Apple Macintosh IIfx computer. Digitized images were then imported into Aldus Persuasion as described for confocal images.

Results

Embryonic Expression

Previous studies have shown that Notch expression in the *Drosophila* embryo is first detectable at the cellular blastoderm stage. Using an mAb that is specific for Notch, C17.9C6 (Fehon et al., 1990), together with the confocal laser-scanning microscope, we have examined in detail Notch expression in this and subsequent stages of *Drosophila* development. At the blastoderm stage we found antibody staining primarily at the surface of cells (Fig. 1), consistent with previous studies that have shown Notch to be a transmembrane protein (Wharton et al., 1985; Johansen et al., 1989; Kidd et al., 1989). Longitudinal sections through the blastoderm showed that Notch was expressed along the length of each cell from the apical to basal surface of the blastoderm (Fig. 1 *A*). In addition, though, there was increased staining just below the apical surface of each cell, especially beneath the pole cells. In tangential sections this staining was apparent as bright points at the apices where three cells come into contact (Fig. 1 *B*).

Also apparent in Fig. 1, *B* and *C* is a patch of cells in the ventral-most part of the embryo that expressed less Notch. This patch, centered on the ventral furrow, was 14–16 cells wide and therefore corresponded approximately to the cells that become internalized through the ventral furrow to form the embryonic mesoderm (Leptin and Grunewald, 1990). Once inside, the mesoderm spreads laterally and dorsally along the basal surface of the overlying ectodermal cells. At this stage, confocal sections showed that Notch expression in the mesoderm was clearly less than that of the surrounding ectoderm (Fig. 2, *A* and *B*). Thus mesodermal cells maintained reduced levels of Notch expression before and after migrating inside the embryo. In contrast, a previous report (Kidd et al., 1989) suggested that Notch is highly expressed in the mesoderm, especially in germ-band extended embryos. We believe that their reported mesodermal localization may be an artifact of whole-mount light microscopy, or due to differences in specificity of the previously used polyclonal antisera compared with the mAb antibody used here. To control for the possibility that these differences in staining were due to processing of particular domains of Notch, mAbs specific for the extracellular domain of the protein were also tested and found to give identical staining results (data not shown).

Simultaneous with gastrulation, germ band extension occurs, bringing the posterior end of the embryo dorsal and anterior toward the head. In optical sections taken at this stage, we found that all ectodermal cells, including the delaminating neuroblasts, expressed Notch in a nearly uniform manner and along all surfaces (Fig. 2 *A*). Thus, as was reported previously (Johansen et al., 1989; Kidd et al., 1989), Notch expression was not modulated in ectodermal derivatives during neuroblast segregation (Fig. 2 *A*). In contrast, neuroblast expression was not maintained following the inward migration of these cells. As a result, by 7 h of development Notch expression in developing neural tissues had dropped significantly while expression in the ectodermal epithelium remained high (Fig. 2 *B*). However, at 10–12 h, when the nerve cord had become distinct from the epithelium, Notch staining was apparent within the embryonic CNS (Fig. 2 *D*). Therefore, after a period from ~6–10 h during which Notch was expressed at low levels in the dividing embryonic neuroblasts, its expression appeared to increase in subsets of cells concomitant with the differentiation of the nerve cord. Expression was also apparent at the apical (luminal) surface of the hindgut epithelium (Fig. 2 *C*) and similarly in the foregut and salivary glands (not shown).

Notch expression in the CNS is shown in detail in Fig. 2 D and Fig. 3. Staining was conspicuous ventrally and laterally in clusters of cells, and dorsally within the neuropil, which contains the neuronal fibers (Fig. 2 *D*; Fig. 3, *A–C*). The cell clusters were segmentally arrayed in the periphery of the CNS, and therefore appeared to correspond to the most recent progeny of the embryonic neuroblasts, the ganglion mother cells and some differentiating neurons. The neuroblasts themselves could not be distinguished at this stage. Notch was also expressed on the axonal bundles coming from these clusters and going into the neuropil, as well as on neuronal fibers within the neuropil itself (Fig. 3 *C*). Expression was also apparent within the cephalic lobes of the CNS in the neuropil, peripheral cell clusters, and in the primordia of the optic lobes (Fig. 3 *D*).

The Larval Central Nervous System

As in the embryonic CNS, we found abundant Notch expres-

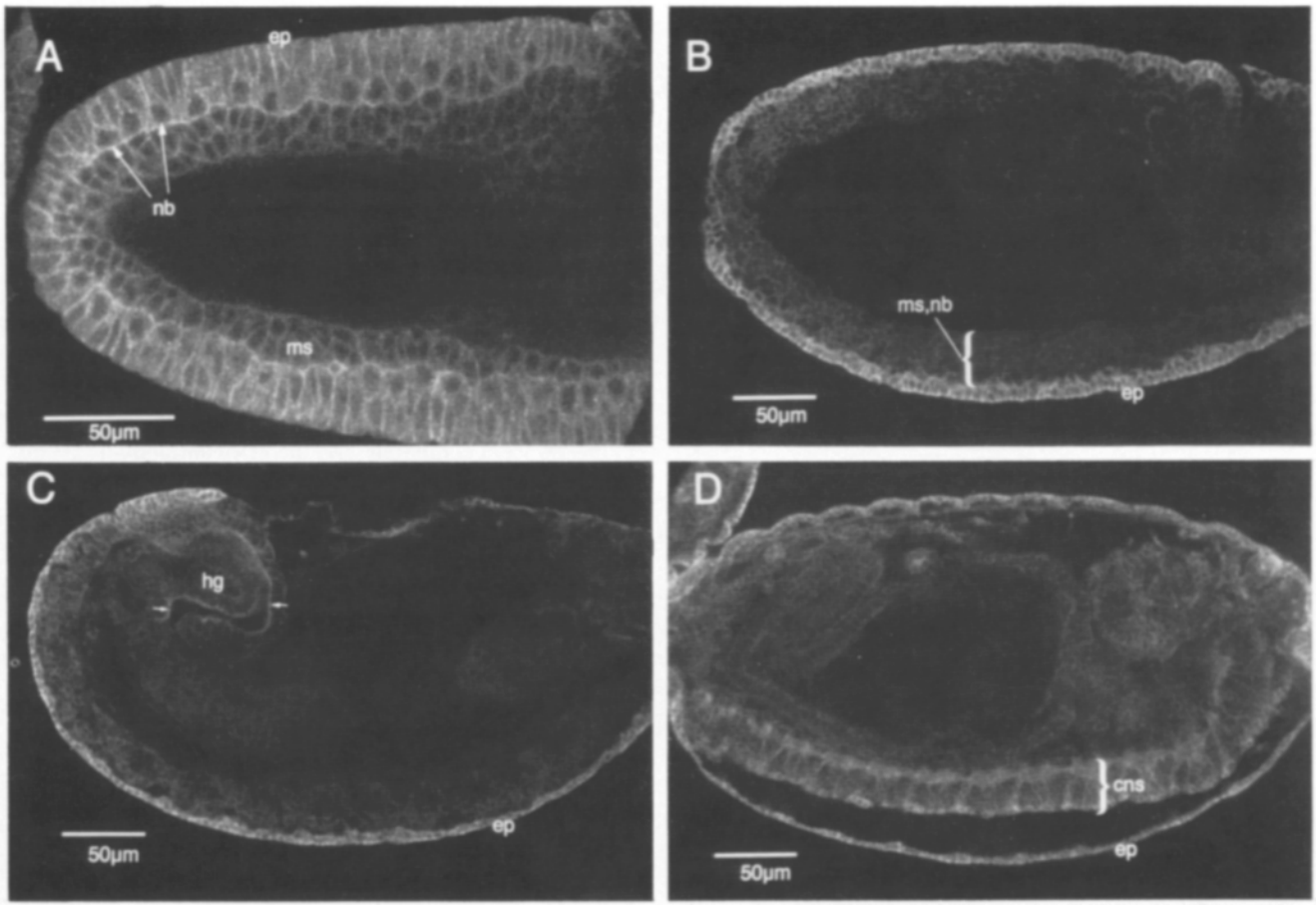

Figure 2. Notch expression in the neuroblasts changes during development of the embryonic central nervous system. Mid-sagittal confocal sections through *Drosophila* embryos. (*A*) Just after the start of gastrulation (~4 h old), the mesoderm has moved inward and spread along the inside surface of the blastoderm. Note that the mesoderm (*ms*) expresses Notch at a much lower level than the surrounding epidermal (*ep*) and neural (*nb*) precursors. Inwardly migrating neuroblasts (*nb*), which express Notch at a high level, are clearly visible. (*B*) A 7-h-old embryo, in which gastrulation is complete. While Notch expression is high within the epidermis at this stage, it is low both in the mesoderm and in the neuroblasts, which have essentially completed their inward migrations. (*C*) An 8-h embryo. At this stage, the hind gut (*hg*) is clearly visible in section. Note that while Notch is relatively uniformly expressed on the embryonic epithelial cells, it is primarily expressed on the luminal (apical) surface of the gut (*arrows*). (*D*) 13-h embryo in which the nerve cord has condensed and is therefore readily apparent. In contrast to *B* and *C*, at this stage there are strongly staining cells in the ventral part of the central nervous system (CNS). These cells appear to be the clustered progeny of the embryonic neuroblasts. In addition, there is staining in the epidermis.

sion in the optic formation centers and in the head, thoracic, and abdominal neuroblasts of the larval CNS, in agreement with previous studies (Johansen et al., 1989; Kidd et al., 1989). However, in contrast to embryonic expression, we detected no Notch staining in the larval neuropil. Notch expression in the formation centers was readily detectable from the first larval instar throughout larval development (Fig. 4 *A*). These epithelial structures produce the adult optic ganglia during metamorphosis (White and Kankel, 1978).

Staining of the larval neuroblasts, which generate adult specific neurons, was also apparent as early as the first larval instar. At this stage, small groups of cells expressed Notch in the head and in the thorax (Fig. 4 *A*). By the middle of the second instar, these expressing cells had grown to clusters consisting of a single large cell, the neuroblast, and a number of closely associated smaller cells, the ganglion mother cells and their progeny (Fig. 4 *B*), as has been previously reported (Johansen et al., 1989; Kidd et al., 1989). These clusters continued to grow throughout larval development, and by the middle of the third instar had formed large, stereotypically arrayed clusters (see Truman and Bate, 1988 for a description of the development of these cells; Fig. 4 *C*). Close examination of the neuroblast and its progeny at this stage showed intense Notch staining at the surfaces of these cells (Fig. 4 *D*). In these sections Notch appeared to be concentrated in regions of contact between the neuroblast and its progeny, with relatively little accumulation in regions of contact with surrounding cells. However we did not make quantitative measurements of staining in these regions and it is possible that this increased staining is simply due to the presence of two tightly opposed membranes that both express Notch. In addition, we detected a single stained bundle of axon fibers that emerged from each neuroblast-neuron cluster and headed toward the neuropil (Figs. 4 *B* and 5 *A*).

In addition to the formation centers and the neuroblasts, both of which constitute neuronal lineages, Notch was ex-

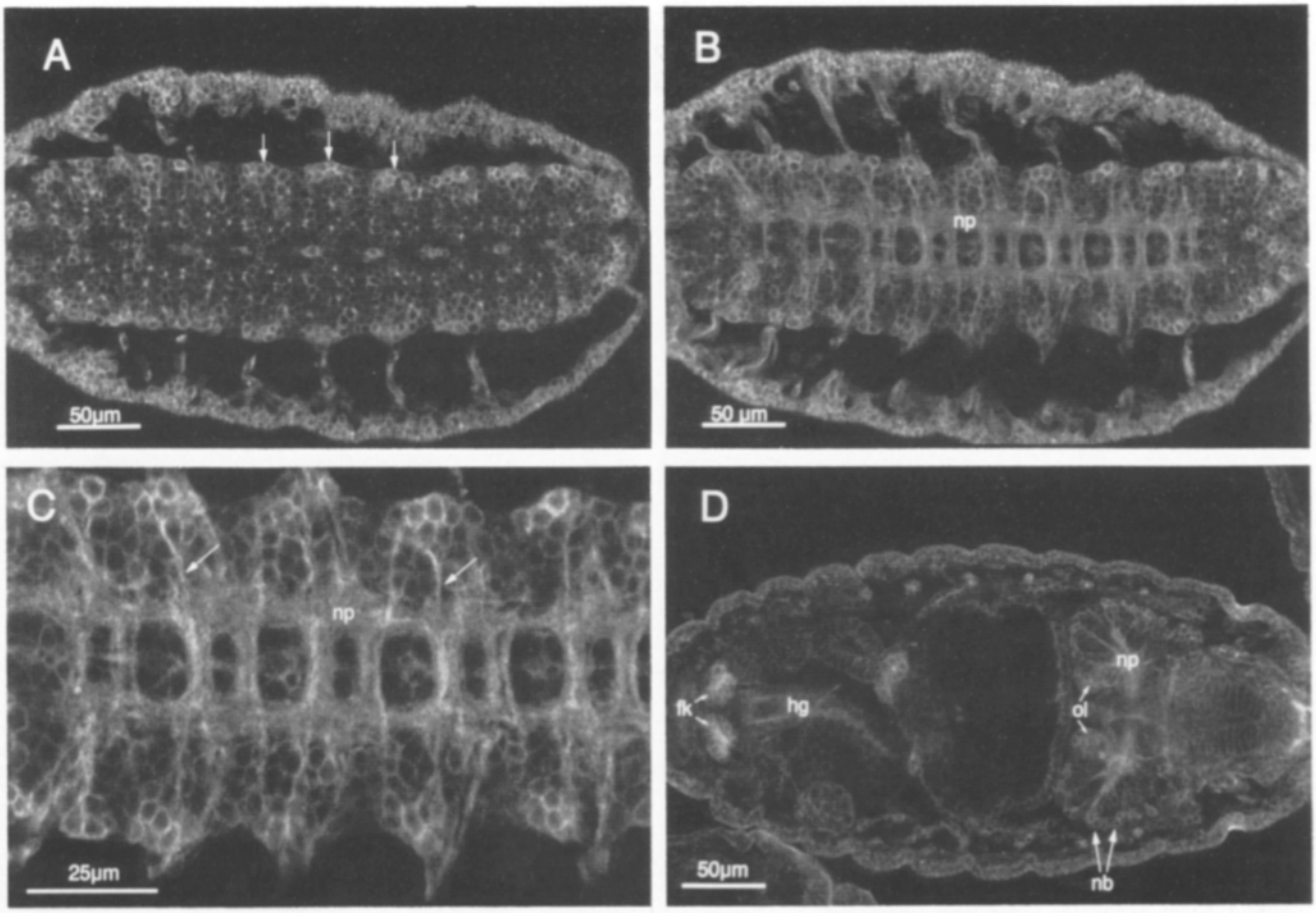

Figure 3. Expression in the embryonic CNS. *A* and *B* show frontal sections of the ventral part of the embryonic nervous system at various levels. (*A*) A superficial view of the nerve cord (most ventral part). Segmentally arrayed clusters of cells, the progeny of the neuroblasts, are marked with arrows. (*B*) A more dorsal view, at the level of the neuropil (*np*), a ladder-like structure that is composed of the developing axons from the embryonic neurons. At least some axons within the neuropil express Notch at readily detectable levels, although apparent staining differences within the neuropil could be due to differences in membrane density. (*C*) A higher magnification view of the section in *B*. Note the neuropil staining, the segmentally arrayed cell clusters, and the axonal bundles (*arrows*) going into the neuropil from these cells. (*D*) A dorsal section of an embryo at the same stage, showing Notch expression in the optic lobes (*ol*), the neuroblast progeny (*nb*), and the neuropil of the brain hemispheres. Also note polarized expression in the hindgut (*hg*) and the Filzkörper (*fk*).

pressed prominently in some subsets of apparently glial cells (Fig. 5, *A–D*). Most notably, Notch was expressed in segmentally arrayed plates of cells situated in the midline of the subesophageal, thoracic, and abdominal segments. Viewed dorsally, these cells appeared as a narrow line in each segment (Fig. 5 *C*), indicating that each plate was only a single cell diameter in width. However, when viewed laterally, these plates could be seen to extend many cell diameters in the dorsal-ventral dimension (Fig. 5, *A* and *B*). Most of the nuclei of these cells were in the dorsal- and ventral-most parts of the plate (data not shown), suggesting that these cells had cytoplasmic extensions running dorsoventrally within the plate. Truman and Bate (1988) identified these cells previously by their mitotic activity, but were not able to distinguish whether they were of neuronal or glial origin. Based on their morphology as revealed by Notch antibody staining, we suggest that these cells are of glial origin.

One remarkable feature of these presumptive midline glial cells was their apparent interaction with Notch-expressing axons produced by the progeny of the neuroblasts in the subesophageal, thoracic, and first abdominal segments. Lateral views showed that within these segments, axonal fibers entered the neuropil, turned horizontally toward the midline, formed bundles with fibers from other neuroblast clusters (Fig. 5, *A* and *B*), and then came into contact with the Notch-expressing midline glial cells of their respective segments. The end of each fiber bundle formed a "glomerular bulb" at the region of contact with the midline cell plate. These structures were hemisegmentally arranged and clearly visible in lateral views because of the antibody staining (Fig. 5 *B*). Thus, it appears that Notch-expressing axonal bundles traverse non-expressing cortex and neuropil layers to contact presumptive midline glial cells that also express Notch. Previous studies have shown that these neurons remain quiescent until metamorphosis, at which time they grow out and establish synapses characteristic of the adult CNS (Truman and Bate, 1988).

A second region of Notch expression was noted in glia-like cells situated at the neuropil-cortex interface of the thoracic segments that appeared to form "cups" surrounding the neu-

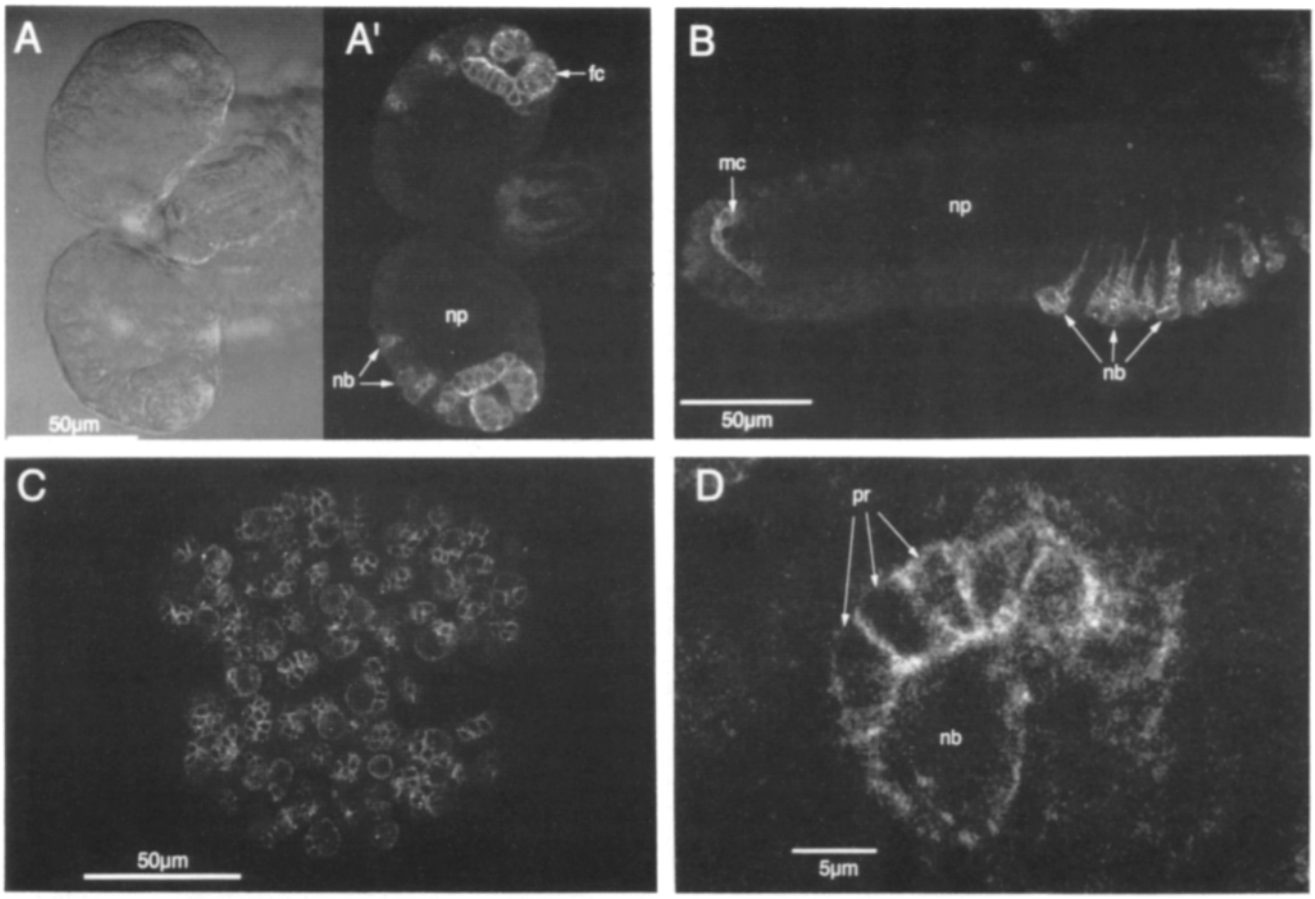

Figure 4. Notch expression in the formation centers and neuroblasts of the larval CNS. Notch-stained CNS preparations dissected from larvae of various stages. Two populations of cells are developing at this time; both the larval neuroblasts and the formation centers express Notch. (*A* and *A'*) The head region of a late first instar CNS shown with differential interference contrast optics on the left (*A*) and simultaneously in confocal fluorescence for Notch on the right (*A'*). Brightly stained epithelial structures are the optic formation centers (*fc*). A few stained neuroblasts (*nb*) are also visible. Unstained central region is the neuropil (*np*). (*B*) A mid-sagittal section of the thoracic and abdominal ganglia of a second instar larva. The stained clusters of cells in the thoracic and abdominal ganglia are the thoracic neuroblasts (*nb*) and their progeny. Just as in the embryo, there are bundles of axonal fibers coming from these cells and heading into the neuropil. Midline cells (*mc*) are also visible at this stage in the most posterior segment. (*C*) A frontal section of the thoracic ganglia from a mid-third instar larva, showing the thoracic neuroblasts. (*D*) A higher magnification view of one of these clusters, showing the neuroblast (*nb*) and its progeny (*pr*).

ropil and segmental nerves in the ventral portion of each thoracic segment (Fig. 5 *C*). These cells have not been identified previously in studies of mitotically active cells in the larval CNS (Truman and Bate, 1988), unlike the other Notch-expressing cells we describe here. Later, during pupariation, a similar pattern of expression was detected in cells that surround the neuropil within the cephalic and thoracic segments, thus forming a border of Notch-expressing cells between the cellular cortex and the neuropil (Fig. 5 *D*, *arrows*). A similar expression pattern of *Notch* mRNA was reported by Markopoulou and Artavanis-Tsakonas (1989).

The Wing Imaginal Disc

In the larval wing disc, as in all imaginal discs, Notch protein was expressed abundantly during larval development (Fig. 6; Johansen et al., 1989; Kidd et al., 1989). Using the ability of the confocal microscope to optically section whole wing discs, we were able to discern several novel features of Notch expression. First, Notch expression was highly polarized toward the apical surface in all disc epithelia (Figs. 6 *A*, 7 *D*, and 8 *A*), which consist of a single layer of tall columnar cells. In tangential sections through the surface of the disc epithelium (see Fig. 6 *B*), this apical staining was revealed to consist of an apicolateral ring around the top of each cell of the wing disc, forming a regular meshwork of stain. Although similar to the embryonic gut and the optic formation centers of the CNS (Fig. 7, *B* and *C*), this was in contrast to the embryonic ectodermal epithelium, which did not show strong subcellular polarity (Fig. 7 *A*). This apicolateral region is the zone in which intercellular junctions, such as adherens and septate junctions, occur (Poodry and Schneiderman, 1970; Fristrom and Fristrom, 1975), although we do not yet know the exact ultrastructural relationship between Notch and such junctions.

The regular pattern of staining on the surface of the disc was interrupted at two places. First, mitotic cells, which are

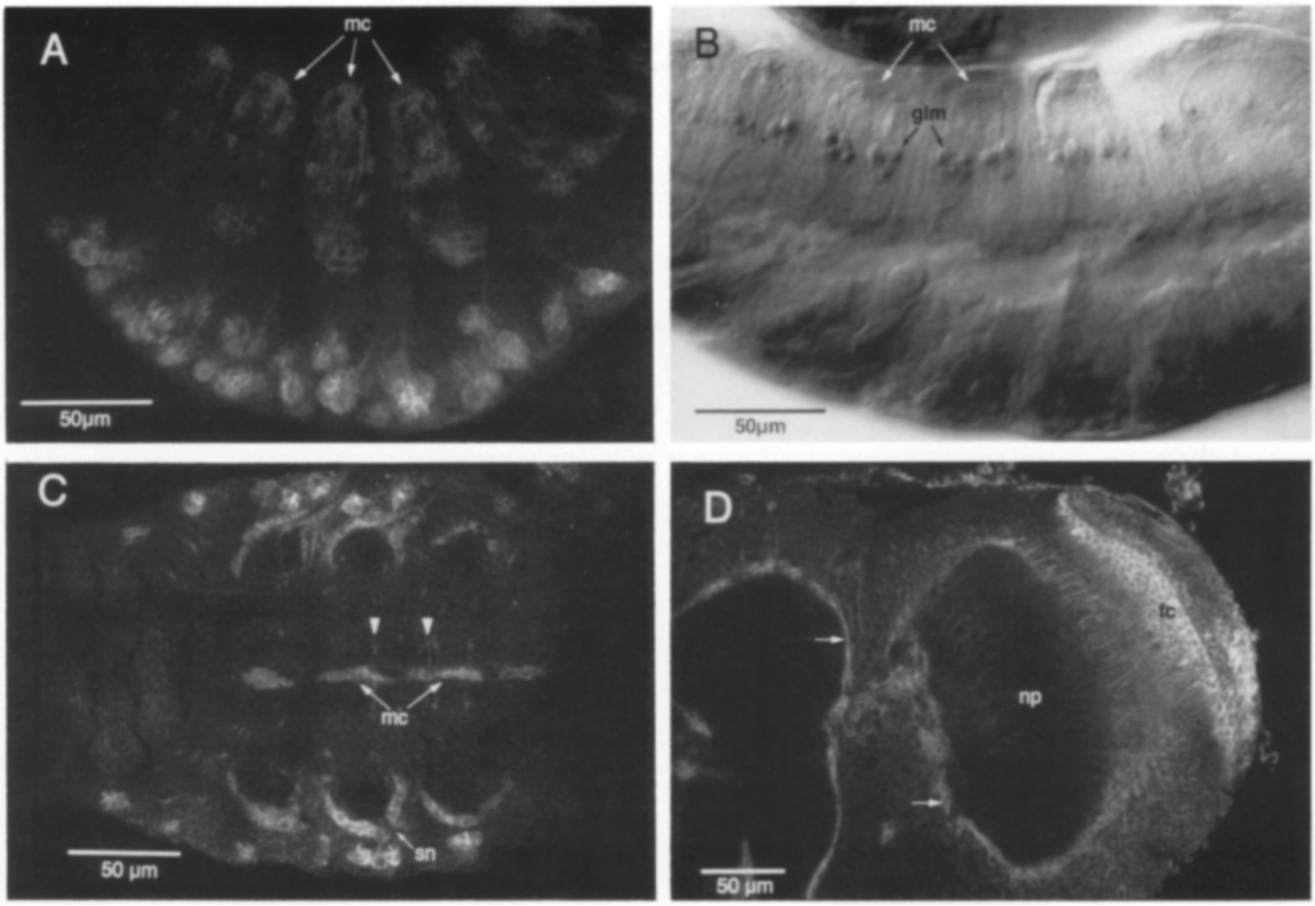

Figure 5. Notch is expressed by glia-like cells in the larval CNS. (*A*) A mid-sagittal view of the thoracic ganglia of a third-instar CNS, showing plates of midline cells (*mc*) that express Notch. This image is a projection of three adjacent optical sections created using the PROJECT program. (*B*) A view similar to that in A using DIC optics on a preparation stained for Notch using an HRP-coupled secondary antibody. The midline cells together with glomerular structures (*glm*) formed by axons from the progeny of the neuroblasts (see text for details) are clearly visible. (*C*) A dorsal view of the thoracic segments of a mid-third instar CNS preparation. The midline cell plates (*mc*) viewed from above appear as segmentally arrayed lines of cells only one cell diameter wide. Laterally, there are "cups" of cells that surround the segmental nerve (*sn*) that exits from each thoracic ganglion. Also visible are Notch-expressing neurons from the progeny of the larval neuroblasts (*arrowheads*). (*D*) Dorsal view of the brain hemisphere of a 24-h pupal CNS. The formation center (*fc*), which is in the process of forming one of the optic lobes, expresses Notch abundantly. Striated staining just below the formation center and in the adjacent neuropil region (*np*) is from the retinal axons. There is also staining at the interface between the neuropil and the surrounding cellular cortex (*arrows*). These cells presumably are glial in nature.

situated apically in the disc epithelium (Madhavan and Schneiderman, 1977), were clearly visible in stained preparations (Fig. 8 *C*). These cells did not differ in their expression of Notch, but were visible due to their large, rounded appearance (and the presence of mitotic figures; data not shown). Second, a double row of cells, situated on either side of the anterior wing margin ceased to express detectable levels of Notch at the end of the third larval instar (Fig. 6, *B* and *C*). These nonexpressing cells corresponded to the chemosensory bristle mother cells (BMCs; these cells are the precursors of the adult sensory neural structures; Hartenstein and Posakony, 1989), as was demonstrated by double labeling experiments using the A37 enhancer trap line (Ghysen and O'Kane, 1989) in which β-galactosidase (β-gal) is specifically expressed in the nuclei of BMCs: the cells that ceased to express Notch coincided with those that expressed β-gal (Fig. 6, *D* and *D'*). Thus, as the BMC's in late third instar larvae become committed to a neural fate, they cease to express Notch. Slightly earlier stages showed β-gal staining but no "holes" in the Notch expression pattern (data not shown), indicating that the A37 marker is expressed before alterations in Notch expression. It was not possible to follow Notch expression in the progeny of the BMC's after pupariation, because folding at the wing margin makes them difficult to observe. In addition, we do not know at this time if other BMCs cease to express Notch as they begin to differentiate because these cells do not form highly stereotyped arrays, making them more difficult to recognize.

We also confirmed and extended previous reports of increased Notch expression running in lengthwise bands down the wing blade and roughly correlating with the position of the presumptive wing veins (Johansen et al., 1989; Kidd et al., 1989). In the confocal microscope, these bands of expression first became obvious in the late third instar wing

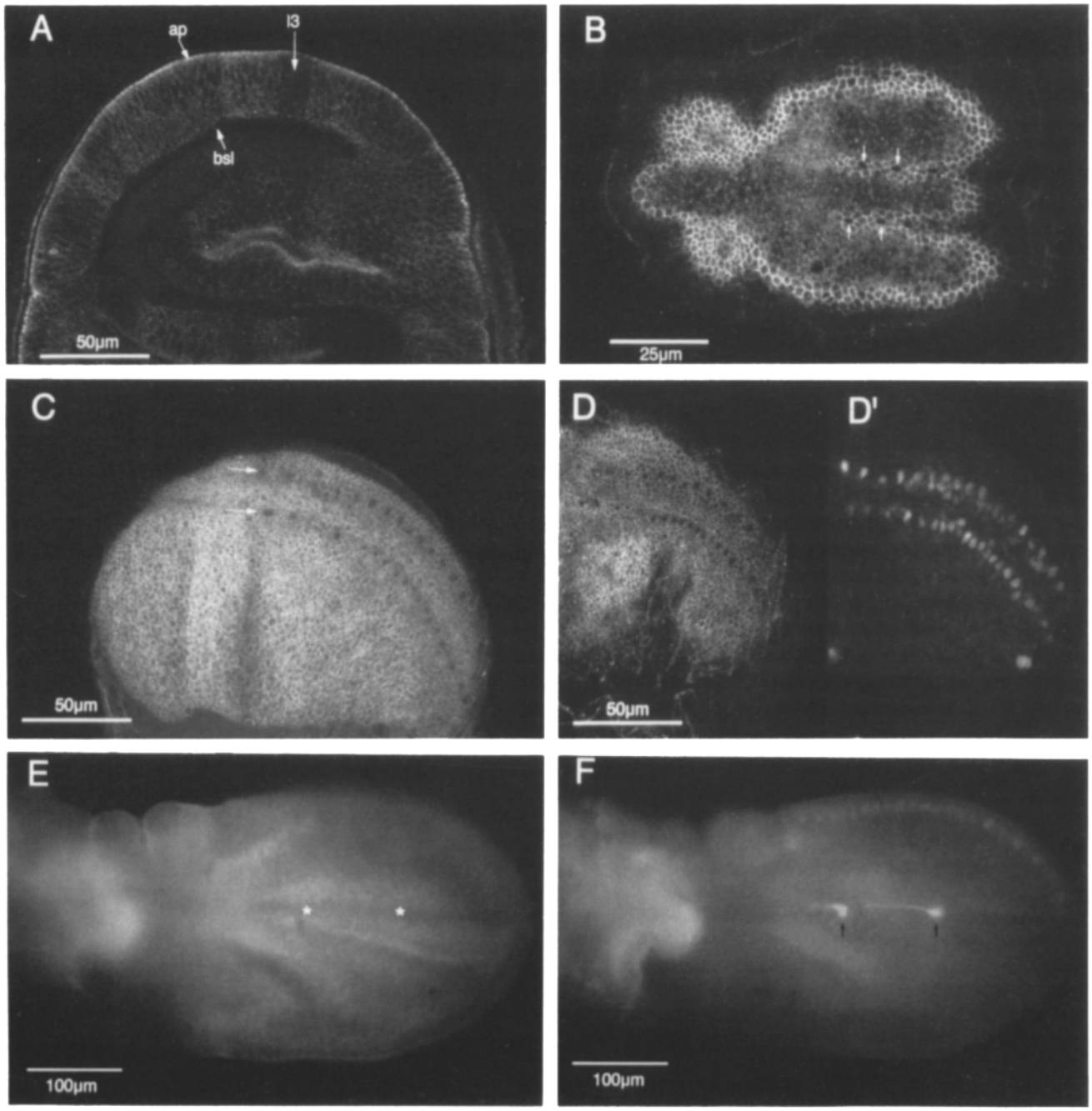

Figure 6. Notch expression in the wing imaginal disc. Images *A–D* are of the blade region of late third-instar imaginal wing discs and *E* and *F* are of 6 h AP wings. (*A*) Cross-section of the wing epithelium, which consists of a single-layer of columnar cells. As in the formation centers, Notch is expressed primarily at the apical (*ap*) rather than the basal (*bsl*) end of these cells. The future site of the third wing vein (*l3*), between heavier Notch staining on either side of this vein, is indicated. (*B*) Tangential section through the apical surface of the wing epithelium. Note that the protein is highly localized to the apicolateral point of contact between these cells, so that it forms a network of stain around the top of each cell. Arrows indicate some of the non-staining cells that are also visible in *C* and *D*. (*C*) A computer-generated projection of several such images that shows a double row (*arrows*) of cells that do not express Notch at this stage. (*D* and *D'*) Double labeling experiment using the A37 enhancer trap that specifically marks the bristle mother cells (BMCs). *D* shows a portion of the margin of a third instar wing stained with rabbit anti-Notch and Texas Red fluorescent secondary antibody. The characteristic two rows of unstained cells are readily apparent (*arrows*). *D'* shows a projection of several optical sections from the same disc stained for βgal expressed in the BMCs. The stained cells in D' correspond to the unstained cells in *D* (although in some cases they do not align precisely because Notch is expressed at the apical surface and βgal is expressed at a different focal plane in the nucleus). Thus the BMC's cease to express Notch as they begin to differentiate. (*E* and *F*) A 6-h AP wing disc stained for Notch (*E*) and with the 22C10 monoclonal antibody that labels developing sensory neurons (*F*). The region of the presumptive third vein in which the sensory neurons are developing (*asterisks* in E, *small arrows* in F) clearly corresponds to a band of less Notch expression.

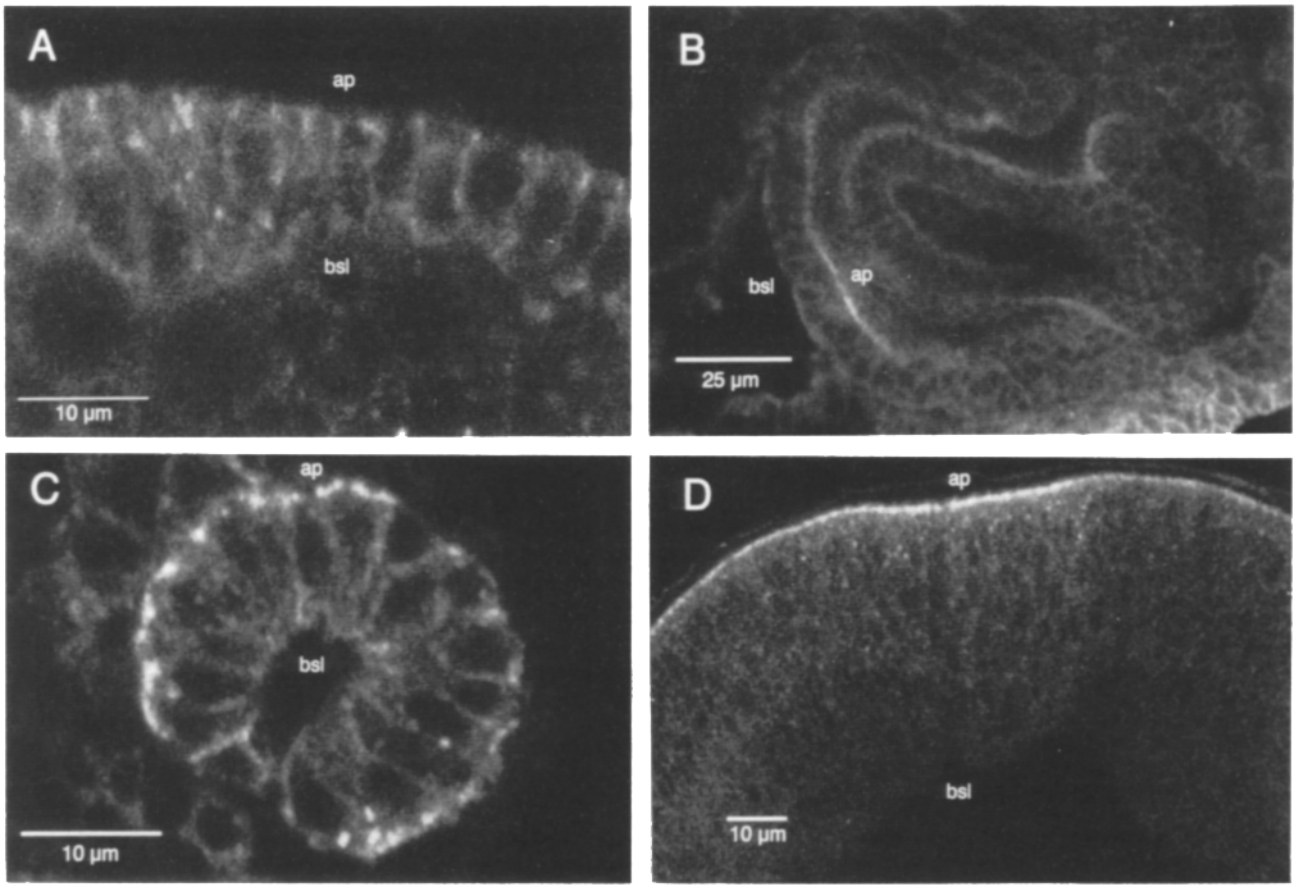

Figure 7. Notch is expressed in a polarized fashion in most epithelia. High magnification views of Notch expression in the embryonic ectodermal epithelium (*A*), the hindgut epithelium (*B*) the optic formation center of the first instar larva (*C*), and the wing imaginal disc epithelium (*D*). In *A*, the embryonic epithelium, Notch is expressed along the entire region of contact between cells, without marked polarity toward either the apical (*ap*) or basal (*bsl*) surface of the epithelium. In contrast, in the hindgut (*B*), Notch is localized primarily at the apical ends of these cells. In the optic formation centers (*C*), Notch is also polarized to the apical surface, although due to the high level of expression there is signal throughout the cell. This apical polarity is most striking in the imaginal discs, where almost all of the antibody staining is localized to the apical surface (*D*).

disc (Fig. 6 *A*). Optical sections showed that each band was due to an increased level of Notch expression throughout the cell, and not just at the apical end of the cell (Fig. 6 *A*). To ask if these bands correspond to the presumptive wing veins, we doubly stained pupal wings with anti-Notch antiserum and with the neural-specific antibody, 22C10 (Zipursky et al., 1984). By staining the neurons developing within the third wing vein, this monoclonal antibody served as a marker for this vein from 1 h after pupariation (AP) throughout pupal development (Schubiger and Palka, 1987). Between 1–18 h AP, observations of doubly stained wing discs (Fig. 6, *E* and *F*) clearly indicated that the third wing vein corresponds to a band of lower Notch expression between the two heavily expressing bands. Thus, neither of these bands correspond to the presumptive third wing vein, but instead to the region between this vein and adjacent veins.

The Eye Imaginal Disc

Extensive developmental genetic analyses have clearly defined a role for the *Notch* gene during the development of the eye imaginal disc (Dietrich and Campos-Ortega, 1984; Cagan and Ready, 1989*a*). In summary, these experiments have shown that *Notch* is required for the correct differentiation of the photoreceptors and the surrounding pigment and bristle cells. At the end of the third larval instar and during the first 12–18 h of pupal development, we found that Notch was expressed by all cells of the eye epithelium in a typically polarized fashion toward the apical end of the cell (Fig. 8 *A*). We also noted that there appeared to be more staining in the region of the morphogenetic furrow (Fig. 8 *A*) and (in some preparations) in clusters of cells posterior to the furrow, as has been previously noted (Johansen et al., 1989; Kidd et al., 1989). However, this antibody staining pattern was remarkably similar to that reported for cobalt sulfide (see Cagan and Ready, 1989*a* for example), a nonspecific membrane stain, implying that the apparent differences in Notch expression within and posterior to the morphogenetic furrow may simply be the result of increased membrane surface area in these regions. Thus at this stage both presumptive photoreceptors and pigment cells express Notch at apparently equal levels (Fig. 8 *B*). However we did note an apparent overall greater staining for Notch anterior to the furrow in comparison to cells posterior to the furrow (Fig. 8, *A* and *B*). We could also identify mitotic cells, apparently orga-

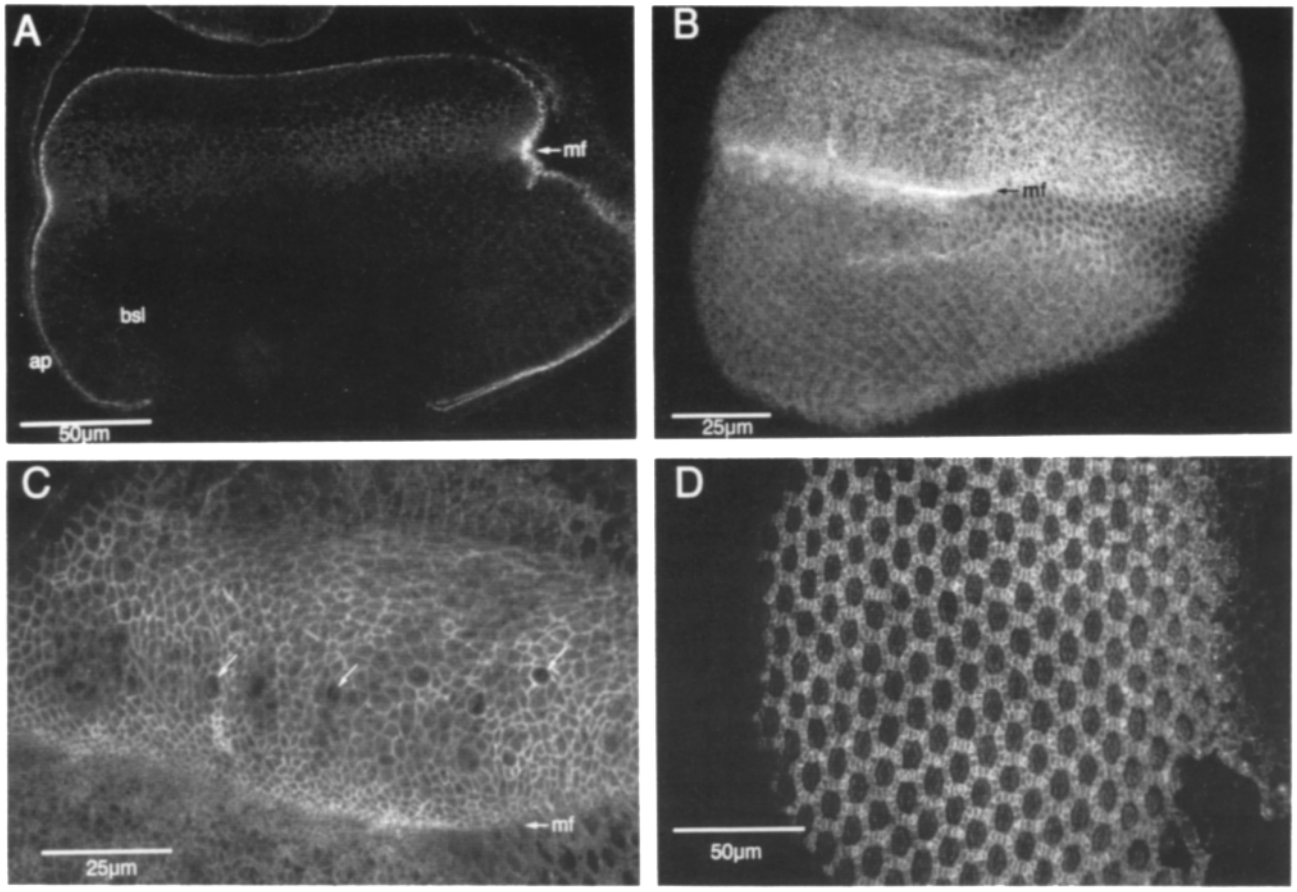

Figure 8. Notch expression in the eye imaginal disc. Confocal images of Notch expression in late third instar (*A–C*) and a 24-h pupal eye disc. Anterior is at top in *A–C* and to the right in *D*. (*A*) Shows an optical section through the eye disc. As with the other imaginal epithelia, Notch is expressed primarily at the apical (*ap*) rather than the basal (*bsl*) surface of the eye epithelium. There is also apparently greater antibody staining in the region of the morphogenetic furrow (*mf*) at the apical surface and more basally in the cells that compose this region. (*B*) Shows a surface view of the eye (constructed using the PROJECT program). Staining appears essentially uniform both anterior and posterior to the furrow. At higher magnification in *C*, the region anterior to the furrow shows conspicuous mitotic cells at the surface of the epithelium (*arrows*). In *D*, staining is apparent in cells outlining each ommatidium. These consist of 2° pigment cells, 3° pigment cells, and the cells of the bristle apparatus. Within each ommatidium, two 1° pigment cells and four cone cells are visible but are stained at a much lower level than the surrounding cells (refer to Cagan and Ready, 1989*b* for a complete description of eye development).

nized in rows parallel and anterior to the furrow, because of their large and rounded appearance at the surface of the disc epithelium (Fig. 8 *C*).

In contrast to late larval and early pupal development, by 24 h AP we noted distinct differences in expression of Notch at the apical surface of the eye disc (Fig. 8 *D*). By this stage of eye development, the photoreceptors have differentiated and are covered by four cone and two primary pigment cells (Ready et al., 1976; Cagan and Ready, 1989*b*). Surrounding the primary pigment cells, there are secondary and tertiary pigment cells, and the precursor cells that form the bristles of the eye. Antibody-stained eye discs showed that the cone and primary pigment cells expressed little or no Notch at this stage. In contrast, the secondary and tertiary pigment cells as well as the cells of the bristle apparatus expressed Notch abundantly (Fig. 8 *D*). This is the stage at which interactions occur to determine the exact fate of cells that will form the secondary and tertiary pigment cells (Cagan and Ready, 1989*a*). Furthermore, disruptions of *Notch* gene function at this stage result in abnormal differentiation of secondary and tertiary pigment cells but leaves the photoreceptor cells undisturbed. Thus, there is a good correlation between Notch expression as revealed by antibody staining and the requirement for *Notch* gene function in these cells.

Discussion

Using the increased resolution afforded by confocal microscopy together with a specific mAb, we have been able to examine Notch expression with unprecedented detail throughout embryonic and imaginal development, and have found that its expression is more restricted at the tissue and subcellular level than previous studies had indicated. In the cellular blastoderm we find that the presumptive mesodermal cells in the ventral-most part of the embryo clearly express less Notch than cells of the lateral and dorsal blastoderm (Fig. 1 *C*). Accordingly, the mesoderm also expresses less Notch once it has invaginated and spread against the inner surface of the blastoderm (Fig. 2 *A*). This result suggests that Notch expression may be regulated positively or negatively by

genes responsible for establishing the dorsal-ventral embryonic axis. In addition, Hartley et al. (1987) have shown that *Notch* mRNA is present in the mesoderm and the ectoderm in roughly equal abundances at the time of ventral furrow formation, while our antibody analysis indicates that there is much less Notch protein in the mesoderm at this and subsequent stages. Although neither study is quantitative, this apparent discrepancy between mRNA and protein could indicate that negative regulation of Notch expression in the mesoderm occurs at the translational level. We also note that the presumptive neuroblasts, which express Notch during their inward migration (Fig. 2 *A*), are in contact with mesodermal cells on their inner surface and highly expressing ectodermal cells on their outer surface. This provides a unique developmental context, and could serve as a signal to the neuroblasts and possibly to the mesoderm as well.

Tissue-specific Notch expression is also obvious in the larval CNS, where Notch is expressed by neuroblasts and their progeny, differentiating adult neurons, as they extend axons into the neuropil (Fig. 4 *B*). In addition, there is expression within two sets of apparently glial derivatives in the CNS: a segmentally arrayed set of cell plates that rest within the midline and express Notch from the middle of the third instar on into pupal development, and "cups" of cells that surround the segmental nerves of the thoracic ganglia (Fig. 5, *A–C*). Interestingly, we find that developing adult neurons (the progeny of the thoracic neuroblasts) appear to interact intimately with the midline plates by forming bulbous contacts with these cells that persist for much of the third larval instar and into pupal development. That both the developing neurons and the midline cells express Notch throughout this period suggests that Notch could serve some role in the processes of establishing and/or maintaining contacts between these cells.

Because loss of Notch function causes all cells of the neurogenic region to enter a neural developmental pathway, early workers suggested that *Notch* function might be important for epidermal rather than neuronal cell lineages (Wright, 1970). However, *Notch* expression may be necessary for the correct development of both lineages, because a variety of expression data have recently shown that Notch is expressed in neuroblasts as they commit to a neuronal fate (Hartley et al., 1987; Johansen et al., 1989; Kidd et al., 1989). We show here that although Notch is expressed in the embryonic neuroblasts as they segregate, its expression subsequently decreases rapidly (Fig. 2 *B*). Notch expression in these cells remains low until differentiation of the CNS is well underway, at which time expression increases again (Fig. 2 *D*). An analogous situation occurs in the wing when the double row of BMCs differentiate from the surrounding wing epithelium (Fig. 6 *C*). Once these cells become committed to a neuronal fate (as assayed by the expression of β-gal in the A37 line), Notch expression rapidly decreases. These two observations suggest that while modulation of Notch expression does not seem necessary for initial specification of neuronal fate, it may be necessary for maintenance of this determined state. In other words, neuronal cells may need to decrease Notch expression shortly after they segregate from epidermal cells in order to differentiate correctly.

Given the complex genetic interactions between *Notch* and the other neurogenic loci, we would obviously like to know the relationship between the expression of *Notch* and these other genes. In particular, recent evidence that Notch and Delta (the protein product of the *Delta* gene) interact directly at the cell surface (Fehon et al., 1990) raises the question of the precise colocalization of these two proteins. Studies of Delta expression are currently in progress (Kooh, P. J., Muskavitch, M., personal communication). However, we already know from in situ analyses that *Delta* mRNA expression overlaps with, but is not identical to, expression of *Notch* mRNA in the embryo (Hartley et al., 1987; Vässin et al., 1987; Kopczynski and Muskavitch, 1989). More definitive statements about colocalization of these two proteins as well as the products of the other neurogenic loci, and therefore the possible in vivo significance of interactions between them, will require protein expression analyses.

The optical sections presented here further show that in the imaginal epithelia and the embryonic hindgut Notch expression is tightly localized to the apicolateral point of contact between cells, while in the embryonic epidermis and the neuroblasts it is much more generally localized along regions of contact between expressing cells (Fig. 7). Where Notch is found to be expressed in a polar fashion, it forms a ring near the top of each cell (see Fig. 6 *B*) that coincides approximately with the location of the apical junctional ring of epithelial cells (Poodry and Schneiderman, 1970; Fristrom and Fristrom, 1975). This apical ring represents a very small proportion of the total surface area of contact between expressing cells, and therefore implies that the subcellular distribution of Notch is regulated in these tissues. In vertebrate epithelial systems two mechanisms have been implicated in the generation of surface polarity: transmembrane proteins may be transported from the Golgi complex preferentially to one part of the cell; in addition, interactions with the underlying cytoskeleton may serve to restrict transmembrane proteins to a particular domain of the cell surface (for review see Nelson, 1989). We speculate that the observed polar Notch expression may be due to interactions with underlying components of the cytoskeleton. Consistent with this notion, we note that the intracellular domain of Notch includes a repeated sequence motif (the "*cdc10* motif") that is found in the cytoskeletal protein ankyrin (ankyrin appears to link transmembrane proteins to the actin-spectrin cytoskeleton; Lux et al., 1990), and that the subcellular localization of filamentous actin (Warn and Robert-Nicoud, 1990) appears to correlate well with the localization of Notch in cellular blastoderm embryos (Fig. 1 *B*) and in imaginal epithelia (Rebay, I., R. G. Fehon, and S. Artavanis-Tsakonas, unpublished observations). Possible interactions between Notch and the cytoskeleton are currently being examined.

Previous studies of *Notch* have led to the speculation that it may serve a role in mediating cellular interactions, perhaps by promoting adhesion between cells that is necessary for the transduction of specific signals mediated by other cell surface components (reviewed in Greenspan, 1990). In fact, we have shown that while Notch alone does not promote aggregation between expressing cultured cells, Notch can interact heterophilically with Delta at the cell surface to promote cell aggregation (Fehon et al., 1990). On the other hand, our observations of Notch expression in the embryo and the larva have failed to show a good correlation between morphogenetic movements and changes in Notch expression, as might be expected if Notch provides a major component of the adhesive force that holds cells together. For example, pre-

sumptive embryonic neuroblasts express Notch as they delaminate from the epidermis and modulate their expression of Notch only after this migration has been completed. In addition, BMC's rapidly cease to express Notch (Fig. 6, *C* and *D*), yet remain firmly integrated into the wing epithelium without losing either apical or lateral surface contacts with adjacent cells. Thus, rather than suggesting that Notch functions primarily as "glue" between cells, these data may be more consistent with the notion that Notch serves a direct function in transducing signals between cells, possibly by serving as a surface receptor for extracellular ligands.

Our observation that BMCs decrease Notch expression as they begin to differentiate may be consistent with the hypothesis that Notch serves as a cell-surface receptor. Within the imaginal discs, BMCs differentiate in highly patterned arrays whose proper spacing depends on lateral inhibition: each BMC suppresses its immediate neighbors from adopting a similar fate (reviewed by Simpson, 1990). Such a mechanism implies the existence of an inhibitory signal produced by the BMC and a receptor for this signal that is required in adjacent cells. In genetic terms, mutations in the receptor should act autonomously: that is, because a membrane-bound receptor cannot diffuse, when wild-type and mutant cells are adjacent in an epithelium there should be no rescue of mutant phenotype in the receptor-minus cells. Analyses of *Notch* clones in embryos and imaginal discs have shown that *Notch*$^-$ cells do behave autonomously (Dietrich and Campos-Ortega, 1984; Hoppe and Greenspan, 1990; Markopoulou and Artavanis-Tsakonas, 1991) as expected for such a receptor. In addition, based on genetic interactions Baker et al. (1990) propose that Notch interacts with the *scabrous* gene product, a secreted protein with similarity to fibrinogen, which may constitute a lateral inhibitory signal produced by R8 photoreceptors in the eye and BMC's in other imaginal discs. In the wing, *scabrous* is expressed specifically in regions where neurons are formed, such as in a double row along the anterior margin of the wing (Mlodzik et al., 1990). Thus the cells that express *scabrous* appear to correlate closely with those that show decreased Notch expression (Fig. 6, *C* and *D*), although double-labeling experiments will be required to confirm this correlation. Implicit in the lateral inhibition model is the notion that the BMC must not respond to the inhibitory signal it produces: if Notch functions as a receptor, then decreased Notch expression in the BMCs (as we have observed; Fig. 6, *B* and *C*) would provide a simple mechanism to prevent self-inhibition.

Lateral inhibition is also thought to occur between neuroblasts and presumptive epidermal cells during embryonic neurogenesis (Doe and Goodman, 1985; Artavanis-Tsakonas, 1988). By analogy with the BMCs in the wing, if Notch functions in epidermal cells to receive an inhibitory signal produced by the neuroblasts at this stage, then decreased Notch expression in the neuroblasts may be necessary to prevent self-inhibition by the neuroblasts. If so, our results are consistent with the hypothesis that Notch functions more in the stabilization of neuronal fate than in its specification: Notch expression is not regulated in the neuroblasts until after they segregate nor in the BMCs until after they express neural specific markers. Indeed, genetic studies indicate that *Notch* function is required to maintain epidermal fate well after the neuroblasts segregate from the epidermis (Hoppe and Greenspan, 1990; Greenspan, 1990). Experiments are currently underway to directly test these hypotheses by inducibly expressing Notch in cells, such as the neuroblasts, that do not normally express this protein, and to examine the subcellular distribution of Notch in the regions of cell contact (Fehon and Artavanis-Tsakonas, unpublished).

It is important to note that if such a Notch-mediated receptor system exists, the *scabrous* product may not be the only Notch ligand, nor must Notch be the only receptor for such signals. Mlodzik et al. (1990) propose that *scabrous* encodes a partially redundant function that may function primarily in imaginal development, and we have shown that Notch interacts with Delta in a manner consistent with receptor-ligand interactions (Fehon et al., 1990). In fact, based on interactions between wild-type and *Notch*$^-$ or *Delta*$^-$ cells in the wing, Heitzler and Simpson (1991) have proposed that Notch may be involved in the reception of inhibitory signals mediated by Delta. Given the structural complexity of the extracellular domain of Notch, with 36 repeated EGF-like units, and the phenotypic pleiotropy of *Notch* mutations, we envision that Notch may interact with multiple ligands during embryonic and imaginal development. Thus, the precise function of Notch in mediating cell–cell interactions may depend on a variety of context-dependent factors, such as the ligand with which Notch interacts and perhaps even the subcellular localization of these proteins.

In summary, tissue-specific Notch expression is readily evident throughout embryonic development, in both epithelial and neuronal cell lineages. Although little is currently known about the elements that control Notch expression during development, our results imply that in the blastoderm Notch expression may be regulated directly or indirectly by genes responsible for establishing the dorsal–ventral body axis. In subsequent stages, regulation of Notch expression appears to be complex, and may be necessary for the maintenance of neuronal and epithelial cell fate. Subcellularly, Notch expression is tightly restricted to the apical–lateral point of contact between most, but not all, epithelial cells. Previous studies (Johansen et al., 1989; Kidd et al., 1989) that have used whole mount preparations failed to detect much of this tissue and subcellular specific expression, and have therefore suggested that specificity of Notch function may come about because of interactions between Notch and other, more specifically expressed gene products. However, our observations of specific Notch expression at the tissue and subcellular levels imply that in addition to specific interactions with other proteins, the developmental complexity of Notch function may come about through precise regulation of the timing and location of Notch expression.

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