The *Notch* Gene Product Is a Glycoprotein Expressed on the Cell Surface of Both Epidermal and Neuronal Precursor Cells during *Drosophila* Development

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Abstract. The *Notch* locus of *Drosophila melanogaster* is one of a small number of zygotically acting "neurogenic" genes involved in the correct segregation of neural from epidermal lineages during embryogenesis as well as in other postembryonic developmental events. We have generated antibody probes against three regions of the *Notch* protein to study the expression of *Notch* and begin a biochemical characterization of the protein. Consistent with predictions based on DNA sequence data, here we gather evidence showing that *Notch* encodes a large, glycosylated surface protein with an apparent molecular mass of 300 kD: (*a*) all three antibodies detect *Notch* on Western blots as a high molecular mass, primarily full-length product; (*b*) immunoelectron microscopy localizes the *Notch* protein to the cell membrane; and (*c*) lentil lectin column binding demonstrates that the protein is glycosylated, indicative of its surface protein nature. In general, the distribution of the *Notch* protein coincides with that of the *Notch* transcript determined previously by in situ hybridizations. *Notch* is expressed in a much wider range of tissue types than those disrupted in the neurogenic mutant, as determined by antibody localization. Early labeling in the blastoderm appears ubiquitous except for the pole cells, but as development proceeds some distinctive features emerge: stronger staining is seen within the germ band layer where neuroblast delamination occurs, and the developing embryonic nervous system shows pronounced axonal staining. In third instar larvae, *Notch* is expressed in imaginal disks and in the central nervous system. Based on these results, certain models for how *Notch* controls the neuroblast cell fate choice are eliminated. We discuss how *Notch* may function in this choice as well as in other lineage fate determinations.

THE *Notch* locus in *Drosophila* appears to be essential for the correct differentiation of neuroblasts from dermatoblasts in the ectoderm. It has been shown that the lack of *Notch* activity causes cells that normally follow an epidermal pathway to be misrouted towards a neural fate (Poulson, 1937; Lehman et al., 1983). Animals homozygous for a deletion of the *Notch* locus die as embryos, showing a hypertrophy of the nervous system at the expense of epidermal structures (Poulson, 1937). Neuroblasts and some dermatoblasts derive from a region in the ventral part of the embryo, which is termed the neurogenic region. The embryological evidence gathered so far suggests that cells in the neurogenic region are equipotent and that the choice between neural and epidermal fates relies on cell interactions during the critical early differentiation phases (Hartenstein and Campos-Ortega, 1984; Technau and Campos-Ortega, 1987; Doe and Goodman, 1985*a*,*b*). The choice of a cell in the neurogenic region between a neural or an epidermal pathway does not depend exclusively on the *Notch* locus. *Notch* is one of six zygotically acting genes (the others are *Delta, Enhancer of split, mastermind, big-brain,* and *neuralized*) known to affect this process; the absence of any one of these gene products results in all the cells of the neurogenic region adopting their primary fate, that of a neuroblast. Furthermore, maternally active genes have been demonstrated to influence the normal differentiation of the neurogenic region (Perrimon et al., 1985; Schüpbach and Wieschaus, 1989).

Notch is genetically and molecularly the best characterized of these loci: it is defined by a series of noncomplementing embryonic lethal *Notch* alleles (Welshons, 1965; Welshons and Keppy, 1981) that show the neurogenic phenotype in the homozygous or hemizygous condition and show dominant wing notching, thickened wing veins, and bristle abnormalities in heterozygotes. Also, mapping within the limits of the *Notch* locus is a recessive class of mutations that affect either eye or wing morphology and the dominant *Abruptex* alleles which confer wing vein and bristle abnormalities (Welshons and Von Halle, 1962; Welshons, 1971; Foster, 1975; Portin, 1975). These alleles show complex comple-

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mentation patterns, although they have been shown to function as a single genetic unit (Welshons, 1965). Temperature-sensitive mutants demonstrate critical periods of *Notch* function for viability of the animal or correct differentiation of various structures (Shellenbarger and Mohler, 1978).

The *Notch* locus has been cloned and sequenced (Artavanis-Tsakonas et al., 1983; Kidd et al., 1983; Wharton et al., 1985*a*). The implied protein product if unprocessed would be a 2,703-amino acid transmembrane protein with several striking features. The putative extracellular domain of 1,700 amino acids contains a typical hydrophobic signal sequence followed by a 36-fold tandemly repeated array of an ~38-amino acid-long cysteine-rich sequence that is homologous to the mammalian EGF. Another cysteine-rich motif is also found in the extracellular portion of the protein. This motif is repeated three times and is also found in *lin 12*, a nematode gene involved in the control of certain cell fates (Yochem et al., 1988). Beyond the transmembrane domain, the ~1,000-amino acid intracellular domain contains sequence elements similar to those found in proteins known to bind nucleotide phosphates (McCormick et al., 1985) and possesses sequences homologous to portions of the yeast genes that are involved in the control of the cell cycle (Breeden and Nasmyth, 1987). These features are consistent with *Notch* being involved in cell surface events and prompted the hypothesis that *Notch*, along with some or all the other zygotically acting neurogenic genes, may be involved in a cell interaction mechanism that mediates and interprets cell-cell interactions necessary for the determination of neural and epidermal lineages (Wharton et al., 1985*a*). This idea is further strengthened by recent data showing *E(spl)*, another one of the neurogenic loci that genetically interacts with *Notch*, encodes a gene product with homology to a G-protein (Preiss et al., 1988; Hartley et al., 1988). DNA sequencing and transcript analysis have suggested certain predictions regarding the *Notch* gene product. To test and extend these predictions, we have raised antisera against *Notch* fusion proteins and used them to begin a biochemical characterization and localization of the *Notch* protein to gain further insight into the possible mechanisms of *Notch* function. We detect a large, glycosylated membrane protein with an apparent molecular mass of 300 kD which is distributed in all somatic cells in early embryogenesis, including both epidermal and neuronal precursor cells during gastrulation. We show its expression throughout embryonic development and its localization in third instar larvae and discuss possible roles for *Notch* function based on these results.

Materials and Methods

Drosophila Stocks

Embryos were from either wild-type Canton-S flies or from a cross of N^{5419} (Mohler, 1956) females with Canton-S males. Larvae and pupae were Canton-S or Sevelen. Flies were raised at 25°C under standard laboratory conditions.

Fusion Protein Preparation

Three regions from the 7-kb *Notch* exon were subcloned using standard techniques (Maniatis et al., 1982) into one of the pUR β-galactosidase (β-gal)[1] fusion protein expression vectors (Rüther and Müller-Hill, 1983) as follows: (*a*) the 0.8-kb Bgl II-Bam HI fragment in the EGF repeat region into the Bam HI site of pUR 278; (*b*) the 0.57-kb Sau 3A fragment in the N repeat region into the Bam HI site of pUR 278; and (*c*) the 0.5-kb Sal I-Xho I fragment in the intracellular region into the Sal I site of pUR 289. *Escherichia coli* host strains 71-18 and DH20 gave the highest level of fusion protein induction. Fusion proteins were induced and harvested as described in Carroll and Laughon (1987) with the following modifications: 0.1 mM isopropyl β-D-thiogalactopyranoside was used to induce the β-gal fusion proteins and the lysed cell pellet was sonicated to reduce viscosity before electrophoresis on 7.5% preparative gels. Excised bands were electroeluted, and the protein was concentrated by dehydration against polyethylene glycol and dialyzed against PBS.

Antiserum Production

New Zealand white rabbits were injected subcutaneously with 200 μg fusion protein emulsified in an equal volume of Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY) and boosted every 3 wk with 200 μg fusion protein emulsified in Freund's incomplete adjuvant. After the second boost, rabbits were bled 1 wk after each boost. Sera were collected and affinity purified. Positive and negative affinity columns were made using 1 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) coupled to 5 mg of the appropriate protein according to the manufacturer's instructions. Negative selection columns consisted of β-gal (Type VI; Sigma Chemical Co., St. Louis, MO) and induced bacterial total proteins (minus the fusion protein). The flow through sera from these columns were positively selected on a *Notch*-β-gal fusion protein column, extensively washed with PBS, and eluted from the column with 4 M guanidine-HCl. The eluted fraction was dialyzed against PBS and passed once more through the β-gal column to remove residual traces of anti-β-gal activity.

Titer and specificity of the affinity-purified antibody was tested by spot test analysis. 100 ng of fusion protein or pure β-gal was spotted on small squares (2 × 2 mm) of nitrocellulose, dried, and blocked in Blotto (5% Carnation nonfat dry milk in PBS). Varying dilutions of affinity-purified antiserum were applied to the squares for 45 min and further processed as for Western blot analysis (see below). Good response against the fusion protein with minimal or no anti-β-gal activity was detected. For most experiments a dilution of 1:100 was selected as optimal.

Western Blot Analysis

Protein extracts were prepared either by homogenization in NP-40 lysate buffer (2% NP- 40, 75 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole, pH 7) and clarified from cell debris or by isolating cells first by homogenization (Dounce; Kontes Glass Co., Vineland, NJ) and resuspension in lentil lectin column binding buffer (Wilcox, 1986) or SDS-PAGE sample buffer. Protease inhibitors PMSF (3.4 μg/ml) (Sigma Chemical Co.), leupeptin (1:1,000) (Boehringer Mannheim Biochemicals, Indianapolis, IN), Aprotinin (1:1,000) (Sigma Chemical Co.), and diisopropyl fluorophosphate (1:4,000) (Sigma Chemical Co.) were routinely added to the homogenization buffer. Proteins were separated on gradient SDS-polyacrylamide gels and transferred to nitrocellulose (Towbin et al., 1979). The nitrocellulose was stained with Ponceau S (Sigma Chemical Co.) (0.2% wt/vol in 3% vol/vol TCA) to visualize total proteins. The blot was blocked in Blotto, incubated vs. primary antibody overnight, washed in TBS (100 mM Tris, pH 7.5, 0.9% NaCl), incubated vs. horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:3,000) (Bio-Rad Laboratories, Richmond, CA) for 2 h, washed in TBS, and primary antibody signal was visualized using 0.2 mg/ml diaminobenzidine, 0.03% H_2O_2, 0.0008% $NiCl_2$ in TBS. For developmental Western blots, protein levels were normalized between different stages by loading equal amounts of total protein, as determined by a protein assay kit (Bio-Rad Laboratories). For the lentil lectin column Western blot, protein samples were obtained as described (Wilcox, 1986).

Immunohistochemistry

Whole Mounts. Embryos were dechorionated in 50% Clorox solution, washed with 0.7% NaCl, 0.002% Triton X-100 (TX-100), and fixed in a 1:1 heptane/fixative mixture for 20 min with vigorous shaking at room temperature. The fixative was either 4% paraformaldehyde in PBS or Bouin's fluid (0.66% picric acid, 9.5% formalin, 4.7% acetic acid). Vitelline membranes were then removed either by the heptane-methanol method (Mitchison and Sedat, 1983) or by hand using a tungsten needle. Larval disks and brains were dissected in insect Ringer's solution (110 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM Hepes, pH 7.2) and fixed for 20 min with 4% paraformaldehyde in PBS or Bouin's fluid. Tissues were then blocked

1. *Abbreviations used in this paper*: DAPI, 4,6-diamidino-2-phenylindole; β-gal, β-galactosidase; HRP, horseradish peroxidase; TX-100, Triton X-100.

in balanced salt solution, pH 6.95 (0.04 M NaCl, 0.05 M KCl, 0.01 M $MgSO_4$-$7H_2O$, 6 mM $CaCl_2$, 10 mM Tricine, 20 mM glucose, 50 mM sucrose, 0.2% BSA with 1% normal goat serum [Cappel Laboratories, Malvern, PA] and 0.1% TX-100). Incubations vs. affinity-purified antibody (1:100 in balanced salt solution, normal goat serum, TX-100) were at 4°C with shaking for 12–48 h. Samples were washed with PBS plus 0.1% TX-100, incubated for 2 h vs. HRP-conjugated goat anti-rabbit secondary antibody (1:200) (Bio-Rad Laboratories), washed with PBS plus 0.1% TX-100 followed by a rinse with PBS alone, and incubated for 10 min in an 0.2 mg/ml diaminobenzidine in PBS solution. HRP reactions were initiated by adding H_2O_2 to 0.015% and allowing the reaction to proceed for 5 min. Reactions were stopped by washing with PBS plus 0.005% soldium azide, and samples were mounted in 90% glycerol. Preparations double stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.) were incubated in a 10 μg/ml DAPI solution for 1 min and rinsed extensively just before mounting.

Frozen Sections. Larvae were washed in 70% EtOH, cooled on ice for 10 min, and frozen in OCT embedding medium (Lab-Tek; Miles Laboratories Inc., Elkhart, IN). Cryostat sections were melted onto poly-lysine-subbed slides and let stand for 30 min in a humid chamber. Slides were dehydrated for 20 min in −20°C acetone and then fixed in cold 2% paraformaldehyde in PBS for 5 min. Slides were washed in water, blocked in PBS, 3% normal goat serum, incubated vs. affinity-purified primary antibody (1:10 dilution) for 2 h at room temperature, washed, and incubated vs. fluorescein-conjugated goat anti-rabbit secondary antibody (1:250) (Cappel Laboratories). Slides were washed and mounted in 70% glycerol, 30% 10 mM Tris, pH 8, 1% *n*-propyl gallate.

Electron Microscopy. Embryos were fixed in 4% paraformaldehyde, 0.025% glutaraldehyde for 20 min and hand devittelinized. Antibody processing was as above, except 0.1% saponin replaced TX-100 as the detergent. Embryos were silver enhanced according to Liposits et al. (1984) with modifications by Steller (personal communication). Embryos were then postfixed in 2% Os_2O_4 for 2 h on ice, washed in PBS, and stained overnight in 2% uranyl acetate at 4°C. Embryos were then washed extensively in PBS, dehydrated through an alcohol series, and embedded in Spurr's medium. Gold sections were cut on an ultramicrotome (MT 2; Sorvall Instruments Div., Newton, CT) and viewed with a transmission electron microscope (10; Carl Zeiss, Inc., Thornwood, NY).

Results

Cloning of Fusion Proteins and Generation of Antibodies

Three domains of the putative *Notch* protein were selected as regions against which polyclonal antibodies would be raised (Fig. 1 *B*). These included the two classes of extracellular cysteine-rich repeats (EGF and N repeats) and an intracellular segment. Each of these was subcloned from exon E (Fig. 1 *A*) into one of the pUR β-gal fusion protein vectors (Rüther and Müller-Hill, 1983). Induction of β-gal fusion proteins by isopropyl β-D-thiogalactopyranoside gave rise to proteins of ~129, 122, and 122 kD for 0.8-kb Bgl II–Bam HI, 0.57-kb Sau 3A–Sau 3A, and 0.5-kb Sal I–Xho I fragments, respectively (Fig. 2). The fusion proteins were then purified by SDS-PAGE on preparative gels and injected into rabbits to generate antisera. Affinity-purified antiserum showed good response against the fusion protein with minimal or no response against pure β-gal, as judged by spot test analysis (see Materials and Methods).

All three antisera were tested on a Western blot of NP-40 supernatants of total homogenates extracted from 3–12-h-old embryos fractionated by SDS-PAGE. Each of the antibodies recognizes a high molecular mass band of ~300 kD, a size consistent with the expected full-length *Notch* product as predicted by DNA sequence analysis (Wharton et al., 1985*a*). This band can be resolved into two or three individual bands on a 5–20% gradient gel, possibly reflecting posttranslational modification events (Fig. 3). In addition, the intracellular antibody recognizes lower molecular mass bands that may be immunologically cross-reactive species or proteolytic fragments. The fact that all three antibodies recognize bands of the same apparent molecular mass suggests that they all recognize the *Notch* protein.

Specificity of the EGF Repeat Region Antibody

For the variety of analyses presented here, the EGF repeat region antibody has proven to be the one that gave the best immunocytochemical staining. The N repeat antibody cross reacts with protein in a mutant neurogenic embryo deleted for the *Notch* locus, and the intracellular antibody immunocytochemical staining is very weak. For these reasons, most of the histochemistry presented here was done with the EGF repeat region antibody. Immunoblot analysis suggested that our antibody is specific to *Notch*. Since several loci have been demonstrated on the sequence level to contain EGF-like repeats (Knust et al., 1987; Vässin et al., 1987; Rothberg et al., 1988), it was important to determine the specificity of the EGF repeat antibody for *Notch*. We tested the EGF repeat region antibody against 1-h embryo collections from a cross of N^{5419}/+ females with wild-type Canton-S males. N^{5419} is a deletion of polytene chromosome bands 3C6 to 10 (Lefevre and Green, 1972), resulting in elimination of the *Notch* locus. 25% of the offspring are neurogenic N^{5419}/Y male embryos. The embryos were aged 17 h, at which time the neurogenic embryos are easily distinguishable under a dissecting microscope. In wild-type embryos of this age, *Notch* protein is detectable on a Western blot (see below), and the neurogenic embryos show no evidence of necrosis. Equal numbers of neurogenic and phenotypically wild-type embryos were collected. NP-40 homogenates of these embryos were run on SDS-PAGE, electroblotted, and probed with EGF repeat region antibody. Fig. 4 *A* shows the presence of the 300-kD band in the phenotypically wild-type embryos (lane N^+) and the absence of this band in the neurogenic embryos (lane N^-). Ponceau S staining of the nitrocellulose after electroblotting suggested similar quantities of total proteins on both lanes as expected since equal numbers of embryos were run on each lane. This experiment suggests that at the level of immunoblot analysis the EGF repeat region antibody is specific to *Notch*.

Since polyclonal antibodies may recognize different epitopes on Western blots and in immunocytochemical stainings, we also analyzed this antibody's specificity at the level of protein localization within the tissues using the same cross of N^{5419}/+ females with Canton-S males. An overnight embryo collection was dechorionated, fixed in 4% paraformaldehyde or Bouin's fluid, devitellinized, and processed for whole-mount antibody staining. Fig. 4 *B* shows a wild-type embryo with the typical staining pattern of a stage 15 embryo with its prominently stained nervous system, while below it is a neurogenic embryo showing complete lack of staining. The same results were obtained when different fixation protocols were used. Thus, the EGF repeat region antibody appears to be specific for *Notch* also at the level of protein localization in the tissues.

However, it is conceivable that the other EGF repeat–containing proteins or any other proteins that could potentially cross react with the *Notch* antibody are not present in neurogenic embryos. If, for example, a protein were epithelium specific, its absence in a *Notch* neurogenic embryo may be a consequence of the loss of a tissue type rather than directly

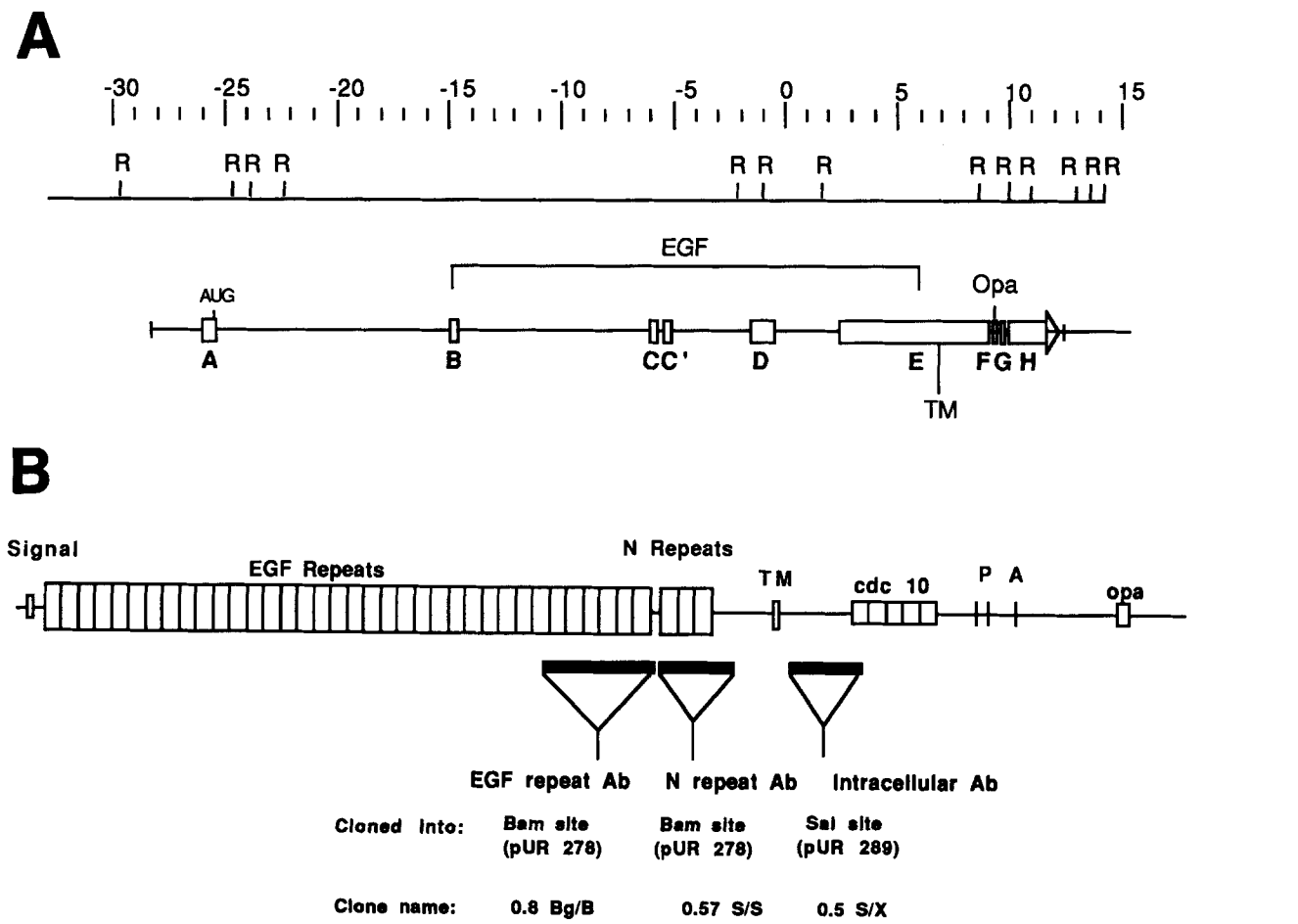

Figure 1. Schematic representation of the *Notch* gene and the *Notch* protein. (*A*) The *Notch* gene. A simplified restriction map showing only the Eco RI sites have been aligned with a schematic diagram of the locus where exons are indicated as boxes. The scale above the restriction map has been defined by Artavanis-Tsakonas et al. (1983), and each unit corresponds to 1 kb. The *Notch* gene consists of nine exons. The initiating AUG is located in exon A, the EGF-like repeats (see *B*) extend from exon B through part of exon E. Exon E also contains the transmembrane domain (*TM*). (*B*) Structure of the *Notch* protein and the regions against which antibodies were made. Indicated below the protein cartoon are the three regions against which antibodies were raised. Predictions based on DNA sequence analysis (Wharton et al., 1985*a*) suggest the protein is a transmembrane protein, indicated by signal sequence (*Signal*) and transmembrane domains (*TM*). The putative extracellular domain contains an array of 36 EGF-like repeats (*EGF Repeats*) followed by three other cysteine-rich repeats, termed Notch-repeats (*N Repeats*). Intracellularly, the protein shows five repeats homologous to the CDC10 gene in yeast followed by a region showing homology to nucleotide phosphate-binding domains (*P* and *A*). Towards the carboxy terminus there is a polyglutamine stretch known as an opa repeat (Wharton et al., 1985*b*). The three regions from exon E (see *A*) subcloned into the appropriate expression vector for antibody production included an 0.8-kb Bgl II–Bam HI fragment in the EGF repeat region, an 0.57-kb Sau 3A–Sau 3A fragment in the N repeat region, and an 0.5-kb Sal I–Xho I fragment from the primarily unique intracellular region. *Ab*, antibody.

due to deletion of the *Notch* locus. If the observed wild-type tissue staining were due to a protein other than *Notch*, which was "turned off" in a neurogenic embryo, presumably it would also be "off" in an *E(spl)* embryo, which has a similar neurogenic phenotype. Staining of embryos from an $E(spl)^{8D06}$ cross, where 25% of the embryos are homozygously deficient for the locus, revealed neurogenic embryos that now show a strong staining pattern (Fig. 4 *C*). The hypertrophied central nervous system is strongly stained, but gross disorganization of the neuropil is evident. This observation correlates with the in situ hybridization analysis that previously demonstrated the presence of *Notch* RNA in *E(spl)* neurogenic embryos (Hartley et al., 1987).

Developmental Expression of the Notch Protein

To determine the developmental profile of the *Notch* gene product in embryogenesis, homogenates of staged embryo collections were run on 5–20% gradient gels, electroblotted, and probed for the *Notch* antigen using the EGF repeat region antibody. In 0–2-h-old embryos, we fail to clearly detect *Notch* protein, but accumulation is evident in 2–4-h embryos and the protein increases in amount until it peaks at 8–10 h (Fig. 5, *arrow*). The *Notch* RNA developmental profile has previously been shown to peak in 6–7-h-old embryos (Yedvobnick et al., 1985). However, within the limits of resolution of these analyses the time difference observed between the protein and RNA peaks cannot be considered significant. Unlike the RNA, which starts decreasing between 8 and 9 h, protein levels remain high and begin decreasing appreciably only after 12–14 h in embryogenesis. In addition to the *Notch* protein, minor bands are also apparent in the Western blot shown in Fig. 5. The significance of these minor protein species is currently not known.

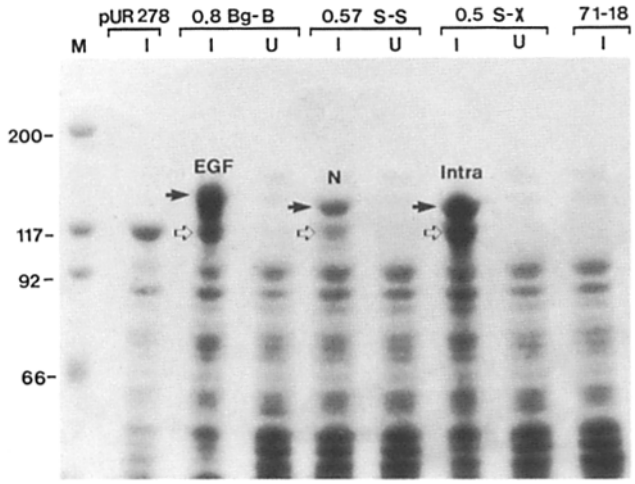

Figure 2. Expression of β-gal–*Notch* fusion proteins in *E. coli*. The figure shows a Coomassie blue staining of bacterial proteins that were harvested and separated by SDS-PAGE on a 7.5% gel. Host strain *E. coli* 71-18 was transformed with the appropriate expression vector clone. Cultures were either induced with isopropyl β-D-thiogalactopyranoside for fusion protein production (lanes *I*) or were uninduced (lanes *U*). Control host cells transformed with the expression vector only (pUR 278 I) showed a strongly induced band at 117 kD, the molecular mass of β-gal. Isopropyl β-D-thiogalactopyranoside induction gave rise to proteins of ~129 kD for 0.8-kb Bgl II–Bam HI (the EGF repeat fusion protein), 122 kD for 0.57-kb Sau 3A–Sau 3A (the N repeat fusion protein), and 122 kD for 0.5-kb Sal I–Xho I (the intracellular fusion protein) as expected (*solid arrows*). Some degradation of the fusion protein is apparent, as bands at the molecular mass of β-gal are also detected (*open arrows*). Only the larger of the two bands was cut out from preparative gels. Uninduced cultures (lanes *U*) showed no detectable levels of either band. Control host cells that had not been transformed with expression vector (71-18 I) showed no induced β-gal band. The molecular mass markers indicated in lane *M* are myosin (200 kD), β-gal (117 kD), phosphorylase B (92 kD), and BSA (66 kD).

Notch Is a Surface Protein

Although the molecular sequence analysis and the whole mount stainings (see below) suggested that *Notch* is a membrane protein, we wanted to confirm this on the ultrastructural level. Embryos were stained with the EGF repeat region antibody using an HRP-conjugated secondary antibody, and the staining was silver enhanced. Embryos were then processed for transmission electron microscopy. Fig. 6 shows two sections from an HRP-stained, silver-enhanced blastoderm embryo; the signal appears as an electron-dense stain that is clearly localized to the membrane of the somatic cells (for example see Fig. 6 *A*, *arrows*). Fig. 6 *B* shows the posterior end including pole cells from a mid-section of the same embryo shown in Fig. 6 *A*. As expected from the light microscopic examination of embryo whole mounts (see Fig. 8 *A*), somatic cells display a much stronger surface signal than the cell surface of the pole cells. We note that the basal side of the pole cell surface shows substantial signal. However, on the basis of the available data, we cannot exclude the possibility that this is due to a staining artifact, and thus the significance of this potentially interesting observation is not clear. Control experiments with secondary antibody alone showed no such staining, while an antibody that stains nucleoli showed silver grains in the nucleus when silver enhanced (data not shown), indicating that the method of whole embryo staining before sectioning and electron microscopy can, under the conditions we use, effectively visualize intracellular staining.

Surface proteins are usually characterized by the presence of sugar groups, and so we tested for the presence of high mannose groups on the *Notch* protein by determining whether the protein binds to a lentil lectin affinity column. Embryo homogenates were passed through a lentil lectin affinity column, and the bound fraction competed off with methyl α-D-mannopyranoside. Fig. 7 *A* shows a Western blot of the affinity column eluates using the EGF repeat region antibody to visualize the *Notch* protein. Lane *Total* shows the total embryo homogenate before fractionation; lane *LL-bound* shows proteins that bound to the column and were competed off; lane *Flow-thru* shows the flow through that had not bound to the lectin column. The *Notch* triplet of bands is discernable in the lane containing total embryonic protein. All three bands are detected in the bound fraction, indicating that all three are characterized by the presence of high mannose sugar groups, indicative of their being surface proteins. No band was detected in the flow through lane. The same results were obtained when a Western blot of these protein samples was probed using the N repeat region antibody (Fig. 7 *B*).

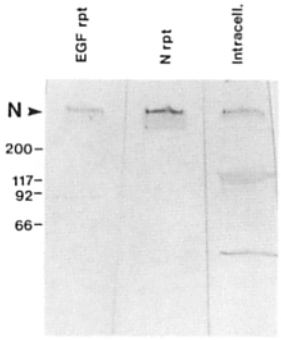

Figure 3. Detection of *Notch* protein on Western Blots. Three lanes of the same Western blot of 3-12-h wild-type embryonic homogenates were probed with antibodies to the EGF repeat (*EGF rpt*), N repeat (*N rpt*), and intracellular (*Intracell.*) regions, respectively. The *Notch* protein is detected by all three antibodies as a triplet of bands of molecular mass of ~300 kD (*arrowhead*). This may represent differentially glycosylated forms or reflect partial proteolysis of the protein. Primary antibody was detected using HRP-conjugated secondary antibody. The intracellular antibody recognizes, in addition, lower molecular mass bands that may be immunologically cross-reactive species or proteolytic fragments. In this particular Western blot, lower molecular mass bands are also detected with the EGF repeat region antibody, but in most immunoblots only the high molecular mass bands are recognized. Molecular mass markers are indicated in kilodaltons to the left.

Protein Localization in the Embryo

The *Notch* protein distribution in the developing embryo was examined using affinity-purified antibody raised against the EGF repeat region visualized by an HRP-conjugated secondary antibody (representative results are shown in Fig. 8). Very early, prenuclear migration embryos do not show any significant level of *Notch* protein, but by cellular blastoderm stage the protein is distributed in all cells throughout the embryo with the exception of the pole cells (Fig. 8 *A*). The signal is primarily localized to the cell surface (see also above) and is quite obvious even before complete cellularization, when the progress of the invaginating membrane can be followed by antibody signal. Such ubiquitous HRP labeling is seen throughout early embryogenesis, including cephalic and ventral furrow invagination, germ band extension, and early germ band retraction (Fig. 8, *A–C*). This expression correlates with the distribution of *Notch* RNA, which is widely dispersed throughout the blastoderm and early gas-

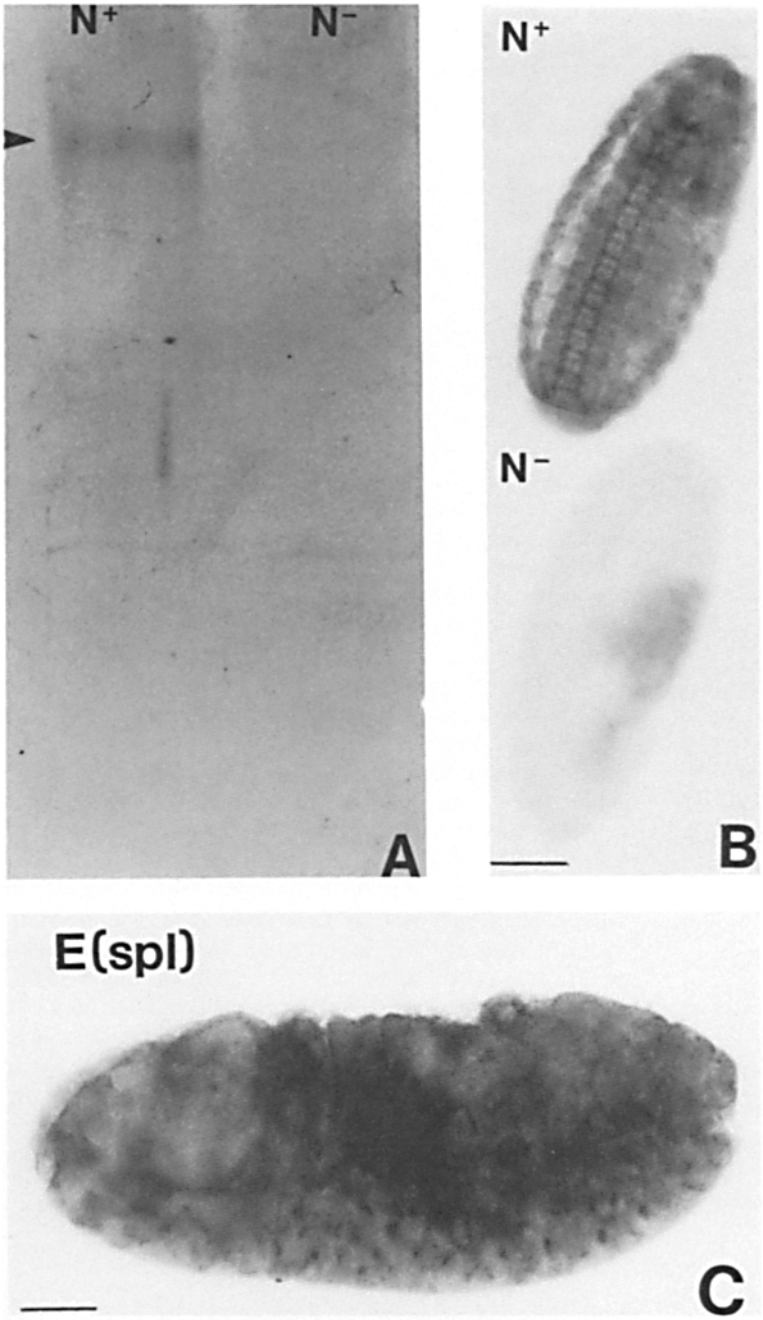

Figure 4. The EGF repeat region antibody is specific for *Notch*. (*A*) Western blot analysis of proteins from wild-type and *Notch* embryos. Embryos from a cross of $N^{5419}/+$ females were aged 17 h, at which time neurogenic embryos are easily separated from wild type. Homogenates of equal numbers of neurogenic and phenotypically wild-type embryos were run on 5–20% gradient SDS-PAGE and Western blotted. EGF repeat region antibody detects the 300-kD *Notch* band in the wild-type (N^+) lane, while no protein is detected in the neurogenic (N^-) lane. Antibody staining was visualized with an HRP-conjugated secondary antibody. (*B*) Whole mount embryo staining from a neurogenic N^{5419} cross. Embryos from the same cross as in *A* were fixed in 4% paraformaldehyde and probed with EGF repeat antibody visualized by HRP-conjugated secondary. The embryo at the top is a wild-type embryo and shows distinct staining, including prominent nervous system staining typical of later stages (see Fig. 8). The embryo at the bottom is a neurogenic embryo (higher magnification reveals lack of an epithelial layer) and is devoid of stain. (*C*) Whole mount staining of a neurogenic $E(spl)^{8D06}$ embryo. Embryos were fixed and stained as in *B*. EGF repeat region antibody stains neurogenic *E(spl)* embryos that are wild type for the *Notch* gene. Strong staining of the neuropil is detected in later stages although the central nervous system is grossly disorganized, as is typical for neurogenic embryos. Bars: (*B*) 200 μm; (*C*) 100 μm.

trula (Hartley et al., 1987). Thus, the *Notch* protein appears to be widely distributed, including the mesodermal and ectodermal layers of the germ band as well as all cells in the neurogenic region. However, as development proceeds, distinct and dynamic features of the antibody staining pattern become prominent. For example, although all cells still express *Notch*, a stronger region of staining in the whole mount is seen (Fig. 8 *C*, *arrows*) within the layer of the germ band where delamination of the neuroblasts occurs (Campos-Ortega and Hartenstein, 1985); as the germ band retracts, staining is eventually eliminated in the mesoderm and reduced in the epithelium. In addition to these structures, staining of the hindgut, foregut, and salivary gland invaginations is observed (Fig. 8 *D*). At later stages, protein is strongly localized within the developing nervous system (Fig. 8, *D–F*) with the pattern of expression developing into well-defined axonal staining of the ganglionic connectives and commissures. HRP signal showed a lower level of *Notch* expression throughout the cortex of the nerve cord where the cell bodies are located, although discrete clusters of cortex cells with axons extending toward the commissures can occasionally be distinguished (Fig. 8 *F*). Thus, the *Notch* protein may be preferentially localized to the axons or, since *Notch* is a membrane protein and the axonal tracts and neuropil contain a much higher density of membranes, this may account for the stronger *Notch* antibody signal in the axonal tracts. In addition, in some preparations, *Notch* product is also detected in peripheral nerves (Fig. 8 *F*).

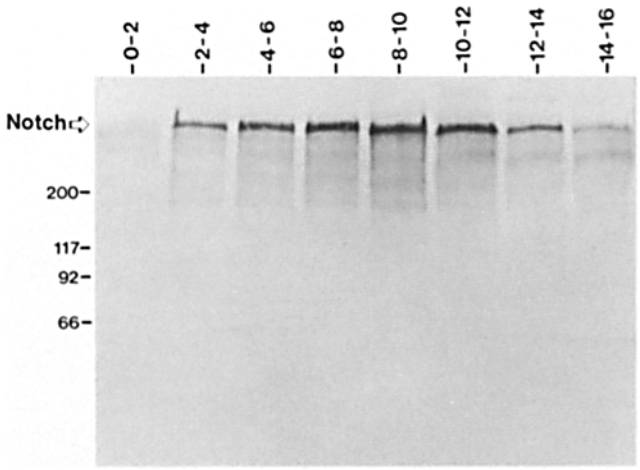

Figure 5. Developmental Western blot analysis. NP-40 supernatants from staged wild-type *Drosophila* embryo homogenates were fractionated by SDS-PAGE on 5–20% gradient gels, electroblotted, and probed for the *Notch* protein using affinity-purified EGF repeat region antibody visualized using HRP-conjugated secondary antibody. During embryogenesis, no significant levels of *Notch* protein are detected at 0–2 h. By 2–4 h, *Notch* protein is evident (*open arrow*) and continues to accumulate until it peaks at 8–10 h, after which by 12–14 h levels begin decreasing. Protein samples were normalized in each lane by loading equivalent amounts of total protein, as determined by a protein assay quantification test (Bio-Rad Laboratories). Molecular mass markers are indicated to the left in kilodaltons.

Although the overall correlation between RNA and protein distribution is good, the nervous system is one significant exception. RNA was detected at the base of the ventral cord, presumably due to dividing neuroblasts (Hartley et al., 1987), whereas the protein is strongly localized to the neuropil where the axons of many cells are packed closely together. *Notch* RNA in the central nervous system would not be localized to axons and may have escaped detection in the neuronal cell bodies due to low levels, while protein in the axonal tracts may be detectable due to the high cell membrane density there. Alternatively, the stability of the *Notch* protein may account for its detection by antibody even though those cells that are descendents of the neuroblasts are not actively transcribing *Notch* themselves.

Protein Localization in Third Instar Larvae

The distribution of the *Notch* protein in third instar larvae was examined in frozen sections as well as imaginal disk and central nervous system whole mounts using affinity-purified antibody raised against the EGF repeat region. Fig. 9 depicts cross sections through the anterior part of a third instar larva as visualized by immunofluorescence, while Fig. 10 shows whole mount stainings visualized by HRP reaction. Fig. 9 *A* shows an 8-μm frozen section through the central nervous system and proventriculus. Anti-*Notch* antibody was visualized using fluorescein-conjugated secondary antibody. The central nervous system shows a distinct pattern of staining: in this section signal is clearly detected in the region where the neuropil interfaces the cortex and is less evident, but present, in the cell body layer including the neuroblasts. More lateral sections reveal staining in the inner and outer proliferation centers of the optic lobe as expected from the in situ hybridization analysis (Markopoulou and Artavanis-Tsakonas, 1989). Fig. 10 *A* shows the neuroblast and progeny ganglion mother cell (Truman and Bate, 1987) staining in a whole mount of a dissected central nervous system where the primary antibody was visualized using an HRP-conjugated

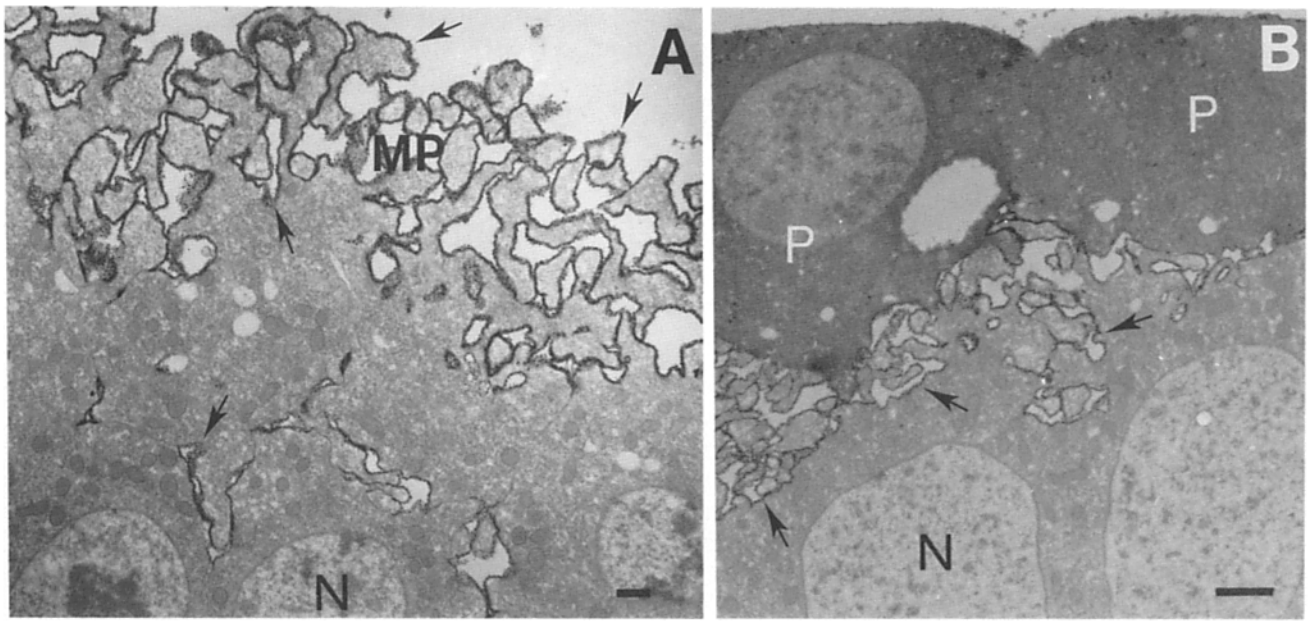

Figure 6. Notch protein localizes to the membrane. Electron micrographs show two sections from an early blastoderm stage embryo that has been stained by the EGF repeat region antibody. The antibody labeling by the HRP-conjugated secondary antibody has been silver intensified. (*A*) The antibody staining as revealed by the silver grains is confined to the cell membranes (*arrows*). (*B*) A section including pole cells of the same embryo shown in *A*. The outer surface of the pole cell membranes shows virtually no signal, while the somatic layer below reveals membrane localization of antibody signal. Signal on the pole cell surface facing the inner side of the embryo is also seen (see text for comments). *MP*, microvillar projections; *N*, nucleus; *P*, pole cell. Bars, 1 μm.

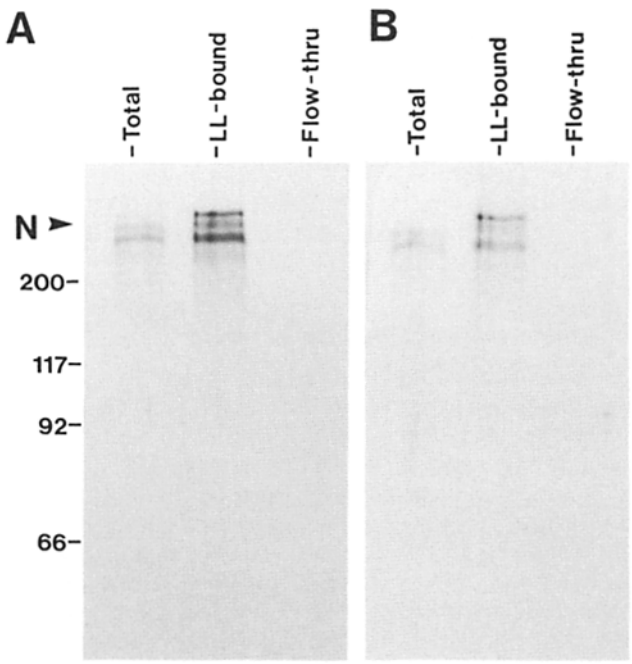

Figure 7. Notch is a glycosylated surface protein. Embryo homogenates were passed through a lentil lectin affinity column and the bound fraction was competed off with methyl α-D-mannopyranoside. An aliquot of total homogenate was removed before fractionation, and equal volumes of total proteins and flow through were loaded on lanes *Total* and *Flow-thru*, respectively; thus, these two lanes are normalized with respect to each other. Lane *LL-bound* contains protein eluted from the lentil lectin affinity column. Proteins were fractionated by 5–20% gradient SDS-PAGE, electroblotted, and probed with two different *Notch* antibodies. Markers are indicated to the left in kilodaltons. (*A*) Affinity-purified EGF repeat region antibody detected by HRP-conjugated secondary antibody was used to probe the Western blot. The *Notch* triplet of bands is detected in lane *Total*. All three bands are detected in the lentil lectin–bound fraction, indicating the presence of high mannose groups, while none were detected in lane *Flow-thru*. (*B*) Western blot probed with *Notch* repeat region antibody. The same results as in *A* were obtained: the *Notch* triplet was detected in the total protein and lentil lectin–bound fractions but not in the flow-through fraction, indicating that all three *Notch* bands are characterized by the presence of high mannose groups.

secondary antibody. Fig. 10 *C* is a higher magnification bright-field image of the EGF repeat region antibody staining focusing on the ventral ganglia neuroblasts, while Fig. 10 *D* shows a DAPI staining from the same preparation. DAPI stains all of the nuclei but, in cells that have been labeled by the HRP reaction product, the epifluorescence is partially blocked. Thus, the elucidation of antibody-positive cells is more sensitive using this technique (Karr and Kornberg, 1989). Small clusters of ganglion mother cells adjacent to the dividing neuroblast are stained with antibody. The stained region at the interface between cortex and neuropil is also visible in whole mount preparations as well as the optic lobe staining of the proliferation centers (Fig. 10, *A* and *B*). The protein pattern we observe in the larva corresponds closely with the in situ hybridization pattern (Markopoulou and Artavanis-Tsakonas, 1989), which has revealed a much more localized *Notch* expression than that seen in the embryo. These results indicate that the *Notch* protein and RNA in the central nervous system are closely correlated with regions known to be mitotically active (White and Kankel, 1978; Truman and Bate, 1987; Markopoulou and Artavanis-Tsakonas, 1989).

In the proventriculus, the diploid cells of the imaginal ring are prominently stained (Fig. 9 *A*) as well as a polyploid region in the inner cortex. In situ hybridization analysis confirms that *Notch* RNA is expressed in the proventriculus (Markopoulou and Artavanis-Tsakonas, 1989). Polyploid cells in the outer structure, which are midgut derived, are devoid of both RNA and protein signal. Similarly, the diploid cells of the imaginal disks show staining, as indicated in Fig. 9 *B*, which shows a horizontal section through the anterior of a third instar larva. Within the disks, the antibody signal is preferentially localized to the apical surface of the epithelium; peripodial membrane and basal disk surface show significantly less signal. Whole mount preparations of eye and wing disks reveal additional features of this staining. Particularly striking is the strong localization of *Notch* to the morphogenetic furrow in the eye disk and expression in an apparently discrete pattern in each ommatidial unit posterior to the furrow (Fig. 10 *E*). It should be noted that this pattern could be due to either differential *Notch* expression or merely a reflection of greater membrane density in the center of the ommatidium. Additional analysis is necessary to distinguish between these possibilities. The antennal and wing disks show a more uniform level of staining (Fig. 10, *E* and *F*), although the wing disk shows a slightly increased level of staining in two broad bands extending across the wing pouch (Fig. 10 *F, arrows*). This darker staining does not appear to be a reflection of the characteristic folding of the tissue in the pouch; other folded regions do not show this effect, and preliminary results in everting early pupal disks which are unfolding also show this staining pattern (data not shown). The identity of the cells within the limits of the two broad bands of staining in the wing disk is not known, although it is possible that they correspond to presumptive wing veins, as identified by Schubiger and Palka (1987). As with the larval central nervous system *Notch* expression, the imaginal disk staining also correlates with *Notch* RNA distribution (Markopoulou and Artavanis-Tsakonas, 1989) and corresponds to tissues known to be actively dividing (Madhavan and Schneiderman, 1977). Finally, we also observe immunoreactive material in muscle (data not shown). However, the mRNA in situ hybridization analysis failed to clearly identify *Notch* RNA in this tissue, leaving it unresolved whether the immunoreactive material is *Notch* protein or reflects a cross reacting epitope on an unrelated protein.

Discussion

Notch Antibodies

We have generated antibodies against three different regions of the putative *Notch* protein to study the expression of *Notch* and begin a biochemical characterization of the protein. All three antibodies detected *Notch* on Western blots as a large, ~300-kD protein, a size compatible with a full-length *Notch* protein as predicted from the sequence analysis. The *Notch* protein is retained on a lentil lectin column and can be competed off with methyl α-D-mannopyranoside, indicating the

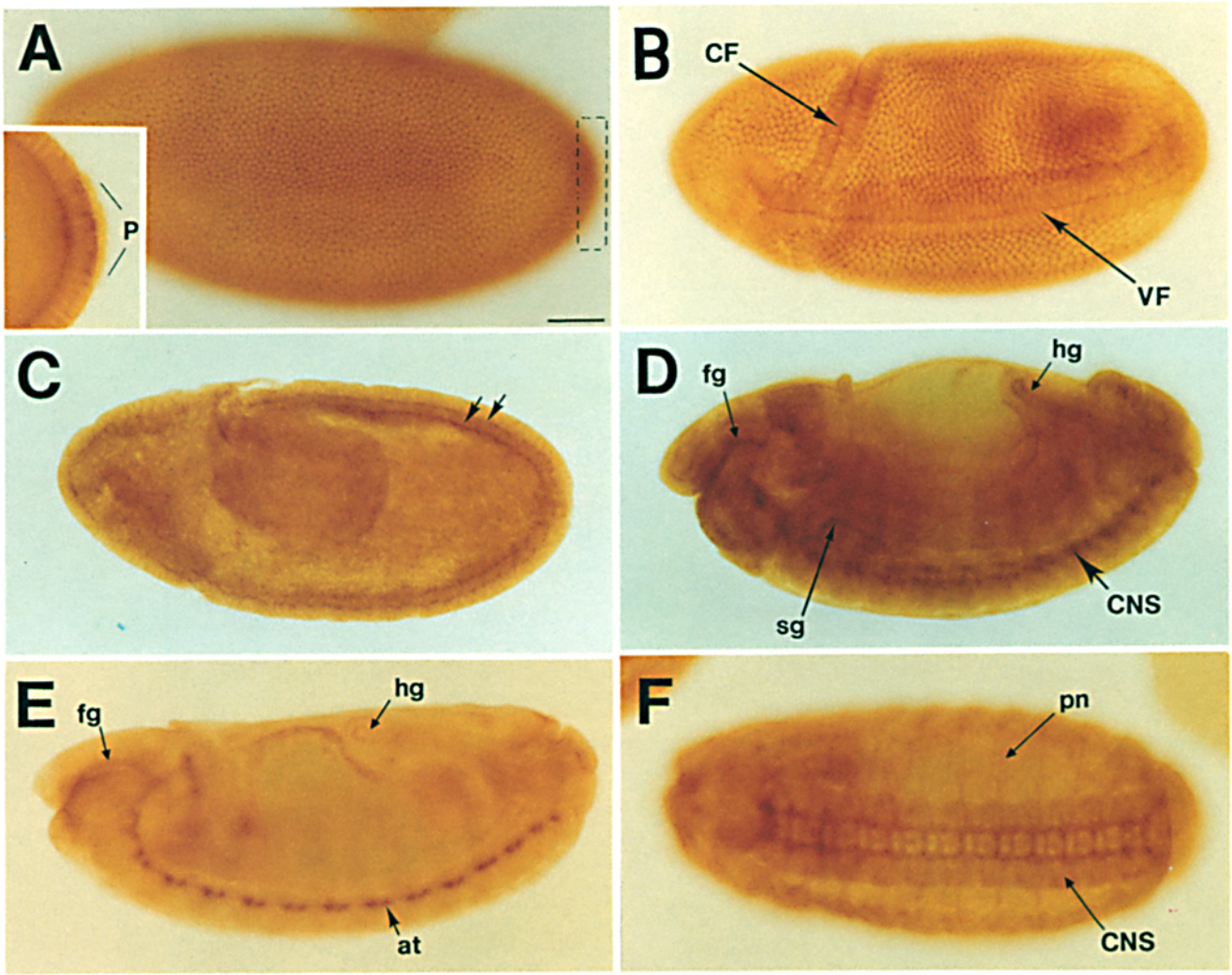

Figure 8. Development of *Notch* expression in whole mount embryos. Embryos at different stages were fixed in 4% paraformaldehyde or Bouin's fluid and probed with affinity-purified EGF repeat region antibody detected using HRP-conjugated secondary antibody. Embryos are oriented anterior left and dorsal up, except for *B*, which is slightly oblique to show more of the ventral aspect, and *F*, which is a ventral view. *A* and *B* are focused on the embryo surface; *C–F* are optical cross sections. (*A*) Cellular blastoderm stage. *Notch* protein is detected on the surface of all cells except the pole cells. Inset photograph shows an optical section of the pole cells region (approximately corresponding to the boxed area) of the same embryo. Pole cells (*P*) appear essentially devoid of stain. (*B*) Germ band extending. Ubiquitous *Notch* expression continues. Cephalic furrow (*CF*) and ventral furrow (*VF*) invaginations are apparent (*arrows*). *Notch* expression is detectable around all cells within the neurogenic region (see either side of ventral furrow). (*C*) Germ band extended. Widespread distribution of *Notch* protein is still detected in all somatic cells in the embryo, but a distinctive, stronger region of staining is detected in the layer of delaminating neuroblasts (*arrows*). (*D*) Germ band retracting. Levels of *Notch* expression in the epidermis and mesoderm decrease, while prominent staining of the developing neuropil in the central nervous system becomes evident. Strong staining in the foregut, hindgut, and salivary glands is observed. (*E*) Dorsal closure complete. *Notch* protein is localized primarily to the central nervous system axonal tracts as well as the foregut and hindgut. (*F*) Ventral view. Well-defined staining of the ganglionic connectives and commissures is observed; peripheral nerves also stain. The cortex of the nerve cord shows a lower level of staining. *at*, axonal tracts; *CF*, cephalic furrow; *CNS*, central nervous system; *fg*, foregut; *hg*, hindgut; *pn*, peripheral nerves; *sg*, salivary glands; *VF*, ventral furrow. Bar, 100 μm.

presence of high mannose groups. Additionally, unfixed live tissue culture cells show strong antibody staining at the surface (Fehon, R. G., K. M. Johansen, P. Cherbas, S. Artavanis-Tsakonas, manuscript in preparation). This, together with electron microscopic localization of *Notch* protein to the membrane, is strong evidence that *Notch* is a membrane-bound surface glycoprotein. These results are consistent with predictions based on DNA sequence data (Wharton et al., 1985*a*) that showed the potential translated product to consist of a 2,703–amino acid protein with signal sequence and transmembrane domains and multiple potential N-linked glycosylation sites, all characteristic features of membrane proteins. The detection of *Notch* protein on Western blots as a doublet or triplet suggests posttranslational modification, although limited proteolysis cannot be ruled out.

The most useful of these three antibodies for tissue localization was the EGF repeat region antibody. Three lines of evidence suggested that the antibody is specific for *Notch*. First, on both the level of Western blot analysis and immunocytochemical staining, *Notch*-deficient embryos fail to reveal

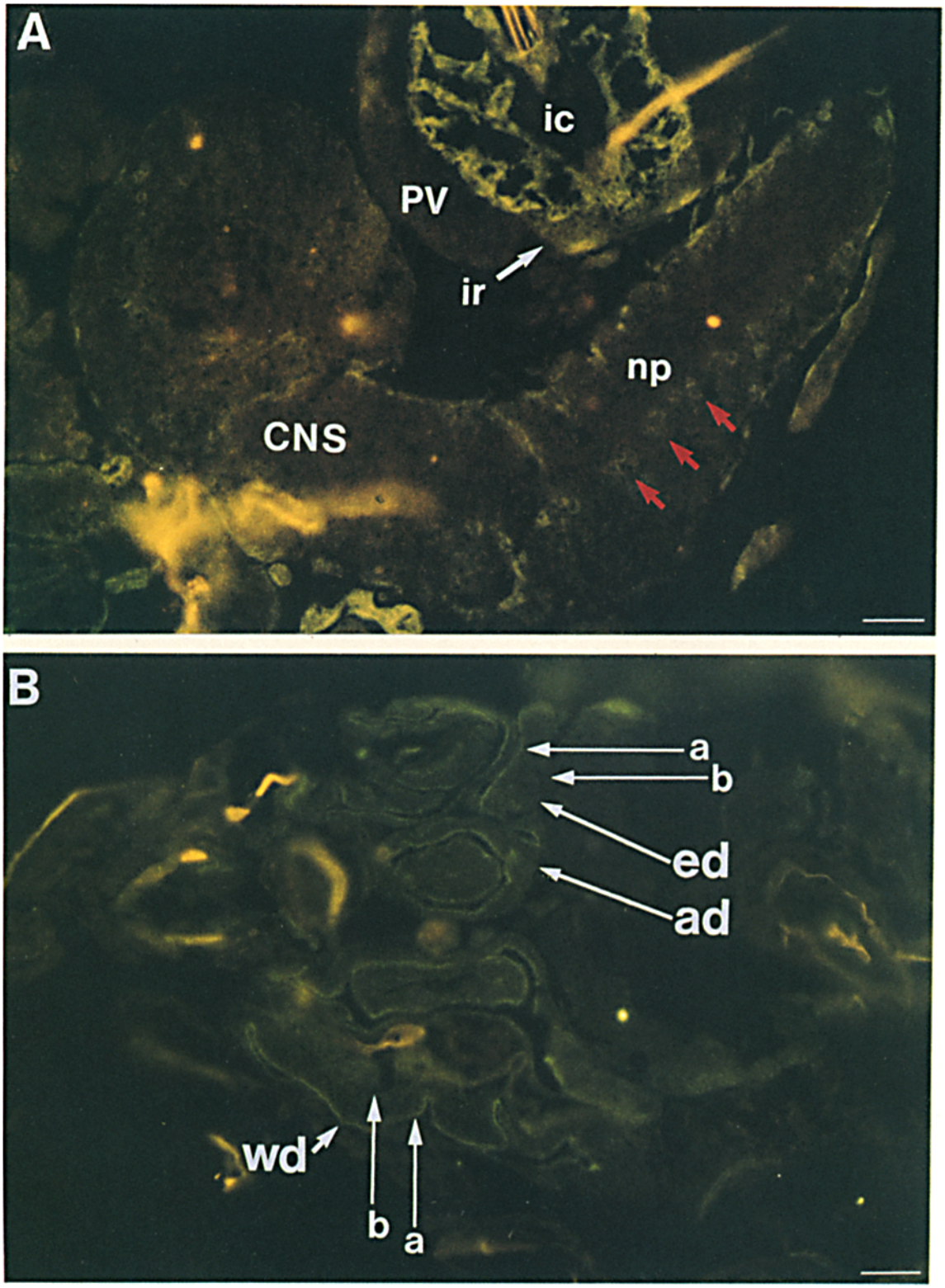

Figure 9. Notch expression in third instar larvae detected by immunofluorescence (frozen sections). Larvae were embedded in OCT, cryostat sectioned, dehydrated in acetone, and then fixed in 2% paraformaldehyde. *Notch* protein was detected with affinity-purified EGF repeat region antibody visualized with FITC-conjugated secondary antibody. (*A*) Cross section of central nervous system/proventriculus: well-defined staining of the cells at the interface between the cortex of the nerve cord and the neuropil is evident (*red arrows*); the neuropil itself appears devoid of signal. In the proventriculus, the diploid cells of the imaginal ring are prominently stained as well as a polyploid region in the inner cortex. *CNS*, central nervous system; *np*, neuropil; *PV*, proventriculus; *ir*, imaginal ring; *ic*, inner cortex. (*B*) Horizontal section showing anterior of larva: Imaginal disks show strong staining; clearly evident are eye, antennal, and wing disks. Antibody signal is preferentially localized to the apical surface of the epithelium, with much lower levels of staining seen in the basal disk surface and peripodial membrane. *wd*, wing disk; *ad*, antennal disk; *ed*, eye disk; *a*, apical surface; *b*, basal surface. Bars: (*A*) 50 μm; (*B*) 50 μm.

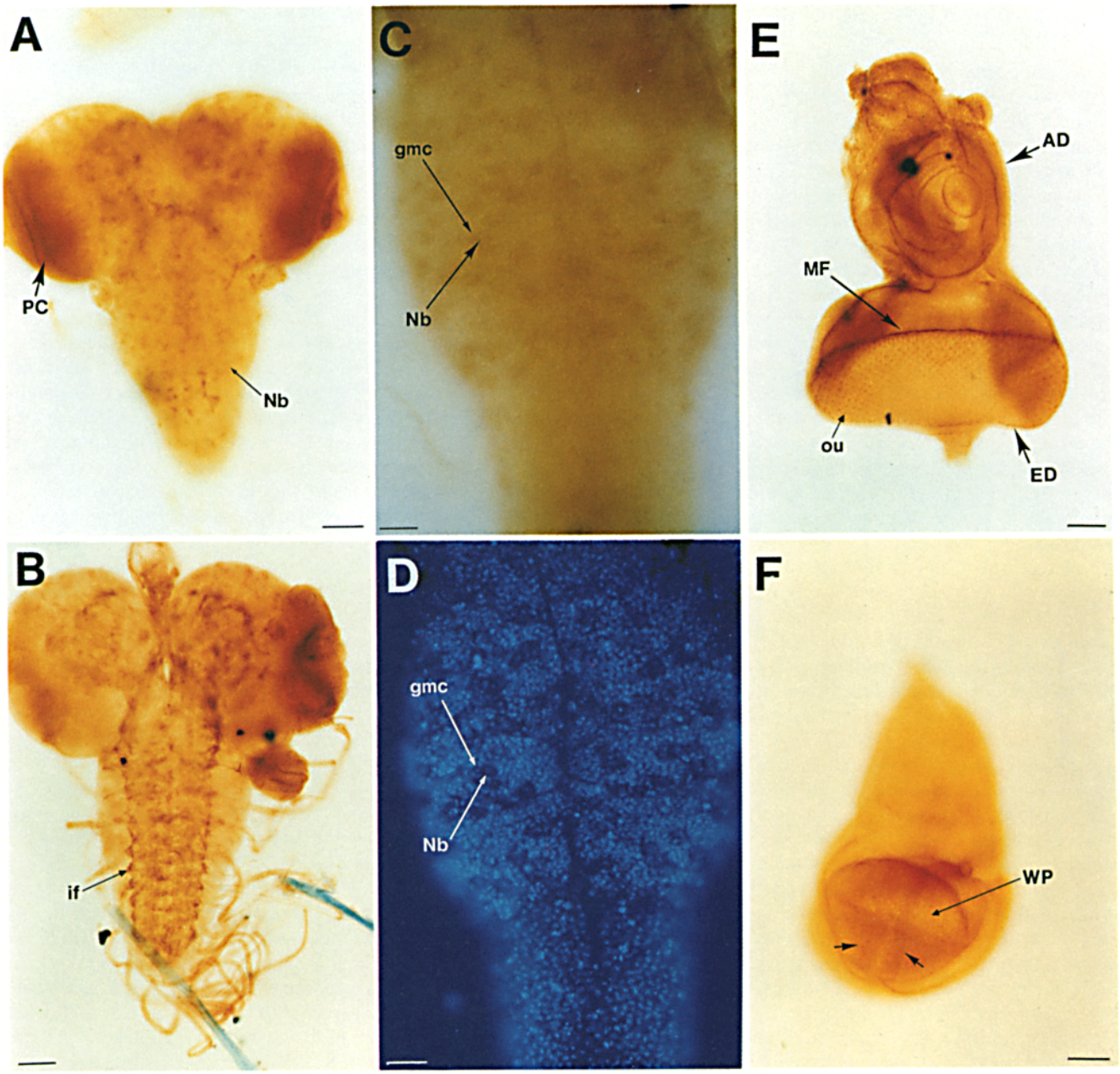

Figure 10. *Notch* expression in third instar larvae (whole mount preparations). Imaginal disks and brains were dissected in insect Ringer's, fixed in Bouin's fluid, incubated with affinity-purified EGF repeat region antibody, and visualized using HRP-conjugated secondary antibody. (*A*) Ventral surface of central nervous system: clusters of neuroblasts and ganglion mother cells show pronounced staining (*arrow*); neuroblasts (*Nb*) can be distinguished by their characteristic large size. The proliferation centers (*PC*) in the optic lobe of the brain hemispheres also show strong, localized staining. (*B*) Optical cross section of central nervous system: prominent staining of the region at the interface of the neuropil and the cortex of the nerve cord is seen (*arrow*). *if*, interface region. (*C*) Higher magnification of ventral ganglia: focus is on the ventral aspect of the ganglia, revealing antibody staining of large neuroblasts and adjacent, smaller ganglion mother cells (*gmc*). (*D*) Nuclear DAPI staining of ventral ganglia: DAPI epifluorescence of the same preparation in *C*. Cells that are antibody positive appear as dark "negative images" since the HRP reaction product partially blocks the epifluorescence. Large neuroblasts and adjacent ganglion mother cells can be more clearly identified in this manner. (*E*) Eye/antennal disk. General overall staining of both the eye and antennal disks (*ED* and *AD*, respectively) is evident. Additional features are detected in the eye disk: the morphogenetic furrow (*MF*) is strongly stained, as well as a discrete subset of cells in each ommatidial unit (*ou*) posterior to the furrow. (*F*) Wing disk. General overall staining of the wing disk is observed, although two broad bands extending across the wing pouch (*WP; arrows*) show a slightly higher level of staining. Bars: (*A*) 80 μm; (*B*) 80 μm; (*C*) 150 μm; (*D*) 150 μm; (*E*) 30 μm; (*F*) 50 μm.

the existence of cross reacting material. Second, staining of *Notch* gynandromorphs show strong overall staining except in the male patch, which is totally devoid of stain and consists of cells homozygous for a *Notch* deletion (Hoppe, P., and R. Greenspan, personal communication). Finally, the protein pattern is consistent with the in situ RNA localization in the embryo and larvae as previously described by Hartley et al. (1987) and Markopoulou (1987). Thus, we conclude that the EGF repeat region antibody specifically recognizes the *Notch* protein.

Notch Expression in the Neurogenic Region

In the neurogenic region, the lack of *Notch* activity results in the misrouting of dermatoblasts into a neural pathway. The simplest hypothesis regarding *Notch* expression then would be that it acts in the neurogenic region to repress the developmental potential of ventral epidermal cells to become neuroblasts and thus its expression could be limited to those cells destined to give rise to epidermal structures (Wright, 1970). In situ hybridization analysis has shown that *Notch* is expressed throughout the neurogenic region as could be judged by the apparently ubiquitous labeling (Hartley et al., 1987). However, given that dermatoblasts outnumber and are intermixed with neuroblasts and that the resolution of the in situ analysis is limited, the possibility remained that *Notch* expression could be restricted to those cells destined to give rise to epidermal structures. Moreover, one could argue that the presence of the transcript does not necessarily imply the presence of the protein. Using antibody probes, we indeed detect the *Notch* product in all cells within the neurogenic region and thus conclude that the simplest model for *Notch* action, namely that the presence of *Notch* protein in a cell represses that cell's potential to develop as a neuroblast, is not correct.

The question of how *Notch* is involved in this cell fate choice when by localization it does not appear to differentiate cell types thus remains open. There are a number of conceivable ways to attain an asymmetry with respect to *Notch* expression in a subset of cells that we may not have detected in this study. For instance, *Notch* protein could be differentially limited to part of the cell surface. Alternatively, the protein could be somehow modified or processed in cells destined to develop as neuroblasts. Although in this study *Notch* was detected only as a primarily full-length product, we have evidence for processing of the *Notch* product in cell lines (Fehon, R. G., K. M. Johansen, P. Cherbas, and S. Artavanis-Tsakonas, manuscript in preparation) and the sensitivity of the present assays may not have been sufficient to detect a low level of protein processing in the embryo. Finally, molecular and genetic data, such as interactions between the neurogenic loci (Lehman et al., 1983; Portin, 1975; Hartley et al., 1987; Xu, T., and S. Artavanis-Tsakonas, personal communication), suggest that *Notch* is engaged in both homophilic and heterophilic interactions and these may serve to modulate its function in cell–cell communication: a mode of action compatible with the "receptor-like" structure of the *Notch* gene product and one that has been described for other EGF repeat–containing molecules. For example, it has been suggested that cytotactin, which contains EGF repeats most homologous to *Notch* (Jones et al., 1988), is functionally and developmentally modulated by protein–protein interactions with fibronectin (Tan et al., 1987). By analogy, the signal mediated by *Notch* may depend on its interactions with other proteins. The initial differentiation of a cell towards the neural lineage may be a stochastic event but, once that cell has delaminated into the interior of the embryo, its contact with new cells or extracellular matrix may trigger events that result in preventing the cells remaining behind from developing as neuroblasts. In this respect, it may be significant that the cell layer corresponding to the delaminated neuroblasts in the embryo does show distinctive, pronounced antibody staining.

Embryonic and Postembryonic Notch Expression

Previous studies using temperature-sensitive alleles of the *Notch* locus (Shellenbarger and Mohler, 1978) and mosaic analyses (Dietrich and Campos-Ortega, 1984; Markopoulou, 1987) have shown that its expression is required throughout embryonic and imaginal development. The *Notch* protein distribution as revealed by the antibody staining as well as the developmental profile of the transcripts (Artavanis-Tsakonas et al., 1983; Kidd et al., 1983) and in situ analysis (Hartley et al., 1987; Markopoulou and Artavanis-Tsakonas, 1989) support this conclusion since *Notch* is present at later stages in a variety of tissue types. The correlation between the protein distribution and the RNA localization in embryos (Hartley et al., 1987) and larvae (Markopoulou and Artavanis-Tsakonas, 1989) is overall very similar. *Notch* protein and RNA are widely dispersed in early embryos, but later they show a more restricted pattern in the nervous system, salivary glands, and gut structures. Postembryonically, the imaginal disks and central nervous system show strong antibody staining in a pattern consistent with RNA in situ localization. In general, detection of *Notch* protein and RNA correlates with mitotic activity; this is especially striking in third instar larvae, where analyses (White and Kankel, 1978; Madhavan and Schneiderman, 1977; Truman and Bate, 1987) have clearly defined dividing cells in the imaginal disks and central nervous system, all of which show strong *Notch* expression (Markopoulou and Artavanis-Tsakonas, 1989). One notable exception to this correlation with dividing cells in the embryo (Hartley et al., 1987) is observed in the axonal localization of *Notch* protein in the neuropil. In this case, *Notch* is present in postmitotic cells that have already been determined with respect to tissue type, and yet these cells still share one feature with undifferentiated cells that are undergoing incorporation into a given tissue: the axonal tracts are just being established in the embryo and cell–cell interaction is vital for the correct pioneering of nerve fibers and fasciculation of individual axons (Jessell, 1988). Another exception to this correlation could be in the muscle, but we have not yet rigorously tested the antibody's specificity to the *Notch* protein in the larva as was done for the embryo and so we cannot rule out potential cross-reactivity to other proteins at this stage.

The question arises as to how *Notch* is functioning in these various tissues. Thus far, all transcriptional evidence suggests that the *Notch* message is qualitatively the same and that the same gene product is functioning throughout development, albeit with different developmental consequences depending on the stage and the particular tissue. The failure of cells to properly incorporate into the developing tissue due to impaired cell–cell interaction, for instance, could result in tissue-specific defects. This implies a context-dependent *Notch* function (Hartley et al., 1987; Artavanis-Tsakonas, 1988; Markopoulou and Artavanis-Tsakonas, 1989) and could account for *Notch's* pleiotropic effects. Our working hypothesis is that the *Notch* protein plays an essential role in mediating the cell–cell interaction required by cells for their correct differentiation and the information that *Notch* relays depends on the location and contacts of the cell and its neighbors. Moreover, the widespread distribution of the protein in dividing or differentiating tissues suggests that *Notch* may play a fundamental role in providing the signals necessary for regulative differentiation.

The data that we have accumulated here support the notion that the *Notch* protein exists primarily as an unprocessed, full-length glycosylated surface protein. Nevertheless it should be emphasized that the resolution of the present analysis does not allow us to eliminate the possibility that some or even all of the functional aspects of *Notch* action may be mediated by processed forms of the protein. In fact, as alluded to earlier, preliminary work involving specific cultured cell lines has provided us with some evidence of processing. However, to address rigorously this important issue more analysis as well as additional reagents such as monoclonal antibodies are needed.

Any model developed to explain *Notch's* mechanism of action must take into account the results from mosaic analysis and cell transplantation studies. Experiments with large patches of mosaic *Notch* tissue suggested that *Notch* acts cell-autonomously, although it was not possible to score the borders of these patches (Stern and Tokunaga, 1968; Dietrich and Campos-Ortega, 1984; Hoppe and Greenspan, 1986; Markopoulou, 1987). In contrast, the result of transplantation data (Technau and Campos-Ortega, 1987), showing that cells from a *Notch* mutant that would normally have been misrouted into the neural pathway can in fact give rise to epidermal lineages when transplanted into a wild-type host, suggests that *Notch* acts nonautonomously while at the same time lends support for the hypothesis that cell interactions play a critical role in these determinative events. This apparent contradiction can be resolved by invoking local nonautonomy (Technau and Campos-Ortega, 1987). In other words, *Notch* is behaving nonautonomously only around the cell's immediate environment. The features of the *Notch* protein revealed by the present analysis, although not directly addressing the issue of cell autonomy, are certainly consistent with a molecule that functions to mediate the interaction between neighboring cells.

Studies by Stern and Tokunaga (1968) and Doe and Goodman (1985*a*,*b*) strongly suggest that during the development of both the embryonic and imaginal nervous systems each neuronal precursor cell, derived from the epithelium, produces an "inhibitory field;" that is, a signal that prevents adjacent epithelial cells from entering neural pathways as well. The question of the involvement of the neurogenic genes in the establishment of such an inhibitory field has been raised but remains open (Doe and Goodman, 1985*b*; Campos-Ortega, 1985). As far as *Notch* is concerned, the available evidence does not yet provide us with a clear biochemical function apart from strongly indicating an involvement in cell interactions. In that context, we can conceive scenarios where *Notch* variously plays the role of a signal and/or a receptor, is involved in adhesion, or has in fact more than just one biochemical role.

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