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Notch-related genes in animal development†

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ABSTRACT The *Drosophila melanogaster* gene *Notch* is central to many cell differentiation events during development. It encodes a large transmembrane signal receptor protein that acts in a poorly understood mechanism of communication affecting the choice of alternative differentiation fates by cells in close proximity. Genes with homology to *Notch* have been isolated from the nematode *Caenorhabditis elegans* and a number laboratories, including our own, have isolated multiple vertebrate *Notch* homologs. In this article we briefly outline the current state of research on *Notch* and our contribution to it. First, we examine the structure of Notch-related proteins. We then examine the requirements for Notch activity in the development of different organisms and how genetic and transgenic studies are helping us to understand the mechanism(s) by which these proteins function. We present models for the action of Notch receptors during signal transduction and for the interaction of multiple vertebrate Notch receptors. Finally, we discuss current ideas about the role played by *Notch* in differentiation and cell-cell communication.

KEY WORDS: *development, neurobiology, neurogenic genes, embryo, differentiation, neural tube, ankyrin repeats*

The molecular analysis of animal development

In the past fifteen years the molecular analysis of mutations affecting embryo development, particularly in *Drosophila*, has resulted in a very rapid increase in our understanding of how genetic information is translated into three-dimensional form. It is increasingly clear that fundamental mechanisms of development are orchestrated by related genes in different animal phyla. Thus, many genes controlling development in *Drosophila* have homologs in vertebrates. Like *Drosophila*, vertebrates also possess Hox genes defining the characteristics of individual segments within segmented structures (Krumlauf, 1993), Pax genes specifying particular cell fates (Noll, 1993), and *wingless* (McMahon, 1992) and *hedgehog* (Smith, 1994) homologs acting in inductive interactions between neighboring groups of cells.

One group of functionally-related genes in *Drosophila*, the "neurogenic genes", controls a mechanism of communication enabling neighboring cells with similar developmental potential to choose radically different fates. The product of the neurogenic gene *Notch* plays a pivotal role in this communication mechanism by integrating extracellular signals and transducing them into the patterns of gene activity defining a cell's state of differentiation. Genes related to *Notch* and other neurogenic genes have been isolated from organisms as dissimilar as nematodes and humans. Thus, the communication mechanism defined by the neurogenic genes is probably fundamental to the development of most multicellular animals.

The characteristics that define the *Notch*-related genes

The *Notch*-related genes take their name from a dominant mutation of *Drosophila* first noted by Mohr in 1919 – flies heterozygous for this mutation develop notches in the outer edges (margins) of their wings (Mohr, 1919). In 1940 Poulson noted that, in embryos homozygous for a deletion of the *Notch* locus, almost the entire epidermis is transformed to a neural fate (Poulson, 1940). This mutant phenotype is now used to define members of the "neurogenic" gene group. In later years the *Notch* locus was intensively studied by genetic means (Welshons and von Halle, 1962; Welshons, 1965, 1971; Foster, 1975; Portin, 1975; Schellenbarger and Mohler, 1975; Lehmann *et al.*, 1983) and the *Notch* gene was cloned and sequenced (Wharton *et al.*, 1985b; Kidd *et al.*, 1986). The gene proved to encode a large protein of 2703 amino acid residues. Antibody studies and sequence analysis indicated that the protein is situated in the plasma membrane with one extracellular and one intracellular region (see Fig. 1). The extracellular region of the Notch protein is glycosylated and the intracellular region is variably phosphorylated on serine residues (Kidd *et al.*, 1989).

The archetypal Notch protein consists of a number of domains comprised of repeated elements (Fig. 1). At the extracellular, amino-terminal, end there are 36 repeats of a motif found in human epidermal growth factor (EGF repeats), some of

Abbreviations used in this paper: EGF, epidermal growth factor.

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†This work is dedicated to the memory of our beloved friend and colleague Jonas Dahlstrand.

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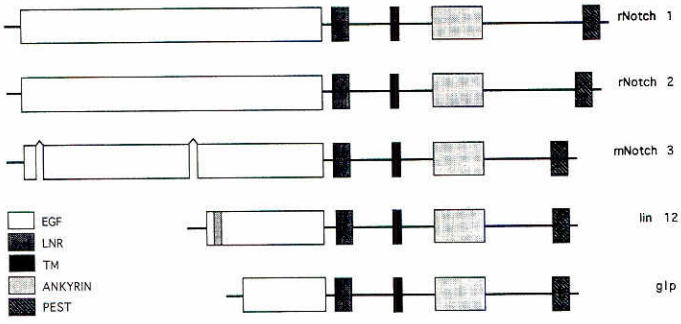

Fig. 1. Structural variation within the group of Notch and Notch-related proteins. *Schematic diagram of Notch and Notch-related proteins (N-terminus to the left) showing the series of conserved repeat regions (EGF, EGF repeat region; LNR, LN repeat region; TM, transmembrane domain; ankyrin, ankyrin repeat region; PEST, PEST region). The EGF repeat domain of mNotch 3 lacks one region equivalent in size to an EGF repeat composed of parts of EGF repeats 2 and 3 and another corresponding to EGF repeat 21. The shaded region in the lin-12 EGF repeat region indicates a non-EGF, cysteine-rich region. Abbreviations: rNotch 1; rat Notch 1 protein; rNotch 2, rat Notch 2 protein; mNotch 3, mouse Notch 3 protein.*

which are of the type that bind Ca^{2+} ions (Fehon *et al.*, 1990). Between the EGF repeats and the membrane lie three repeats of a motif yet only seen in Notch-related proteins – the "Notch/Lin-12" or LN repeats. Intracellularly lie 7 repeats of a motif seen previously in various proteins such as the yeast cdc10 and SWI 6 proteins controlling the cell cycle (Aves *et al.*, 1985; Breeden and Nasmyth, 1987a,b), the human cytoskeletal protein ankyrin (Lux *et al.*, 1990) and many others (Bork, 1993). These repeats are most commonly called *"cdc10/SWI 6"* or "ankyrin" repeats and are thought to mediate protein-protein interactions. We will refer to them as ankyrin repeats hereafter.

Two genes with structures similar to *Drosophila Notch* have been isolated and characterized in the nematode *Caenorhabditis elegans: glp-1* (Austin and Kimble, 1989; Yochem and Greenwald, 1989) and *lin-12* (Yochem *et al.*, 1988). Related genes have been cloned, partially or completely, from a range of vertebrates including humans (Ellisen *et al.*, 1991; Stifani *et al.*, 1992; Larsson *et al.*, 1994), rat (Weinmaster *et al.*, 1991, 1992), mice (Franco del Amo *et al.*, 1992; Reaume *et al.*, 1992; Kopan and Weintraub, 1993; Lardelli and Lendahl, 1993; Lardelli *et al.*, 1994), chick (Henrique and Ish-Horowicz, personal communication), the frog *Xenopus laevis* (Coffman *et al.*, 1990) and zebrafish (Bierkamp and Campos-Ortega, 1993) (Table 1). In Figure 1A we diagramatically display the structures of some of these homologs. A number of characteristics should be noted. First, Notch-related proteins are defined by their overall structure – extracellular EGF and LN repeats and intracellular ankyrin repeats. Second, the number of EGF repeats is variable, ranging from 10 in GLP-1 to 34 in mouse Notch 3 and 36 in *Drosophila* Notch and vertebrate Notch 1 and 2. This contrasts with the invariant number of LN and ankyrin repeats. Third, despite these differences, the overall structure of these proteins is highly conserved, in particular the intracellular ankyrin repeats which, for example, show 69% amino acid identity between rat Notch 1 and *Drosophila Notch* (Fig. 2).

The *Notch* gene family – a subset of the *Notch*-related genes

It is evident from inspection of Fig. 2 and from extensive mathematical analysis (Maine, Lissemore and Starmer, unpublished observations) that *Drosophila Notch* and the vertebrate *Notch 1*, *2*, and *3* genes form a subset – that we call the "*Notch* gene family" – within the larger set of "*Notch*-related genes". We define *Notch* gene family members as displaying the overall structure of *Notch*-related genes and showing gene product (i.e.

TABLE 1

CHARACTERIZATION OF VERTEBRATE *NOTCH* AND *NOTCH*-RELATED GENES

species	*Notch 1*	*Notch 2*	*Notch 3*	*int-3*
mouse	complete: Franco del Amo *et al.*, 1993 partial: Franco del Amo *et al.*, 1992 Reaume *et al.*, 1992 Kopan and Weintraub, 1993 Lardelli and Lendahl, 1993	complete: – partial: Lardelli and Lendahl, 1993	complete: Lardelli *et al.*, 1994	complete: – – partial: Robbins *et al.*, 1992
rat	complete: Weinmaster *et al.*, 1991	complete: Weinmaster *et al.*, 1992	–	–
human	complete: Ellisen *et al.*, 1991	complete: – partial: Stifani *et al.*, 1992 Larsson *et al.*, 1994	complete: – partial: Larsson *et al.*, 1994	–
Xenopus	complete: Coffman *et al.*, 1990	–	–	–
chick	complete: – partial: Henrique and Ish-Horowicz, pers. comm.	complete: – partial: Henrique and Ish-Horowicz, pers. comm.	–	–
zebrafish	complete: Bierkamp and Campos-Ortega, 1993	–	–	–

Summary of the data for the chracterization of vertebrate *Notch (Notch 1*, *2* and *3)* and *Notch*-related *(int-3)* genes at the nucleotide sequence level, as of August 1994. "Complete" indicates that the sequence of the entire coding region has been determined; "partial" that only a portion of the sequence is known. "—" indicates that published sequence information is not yet available.

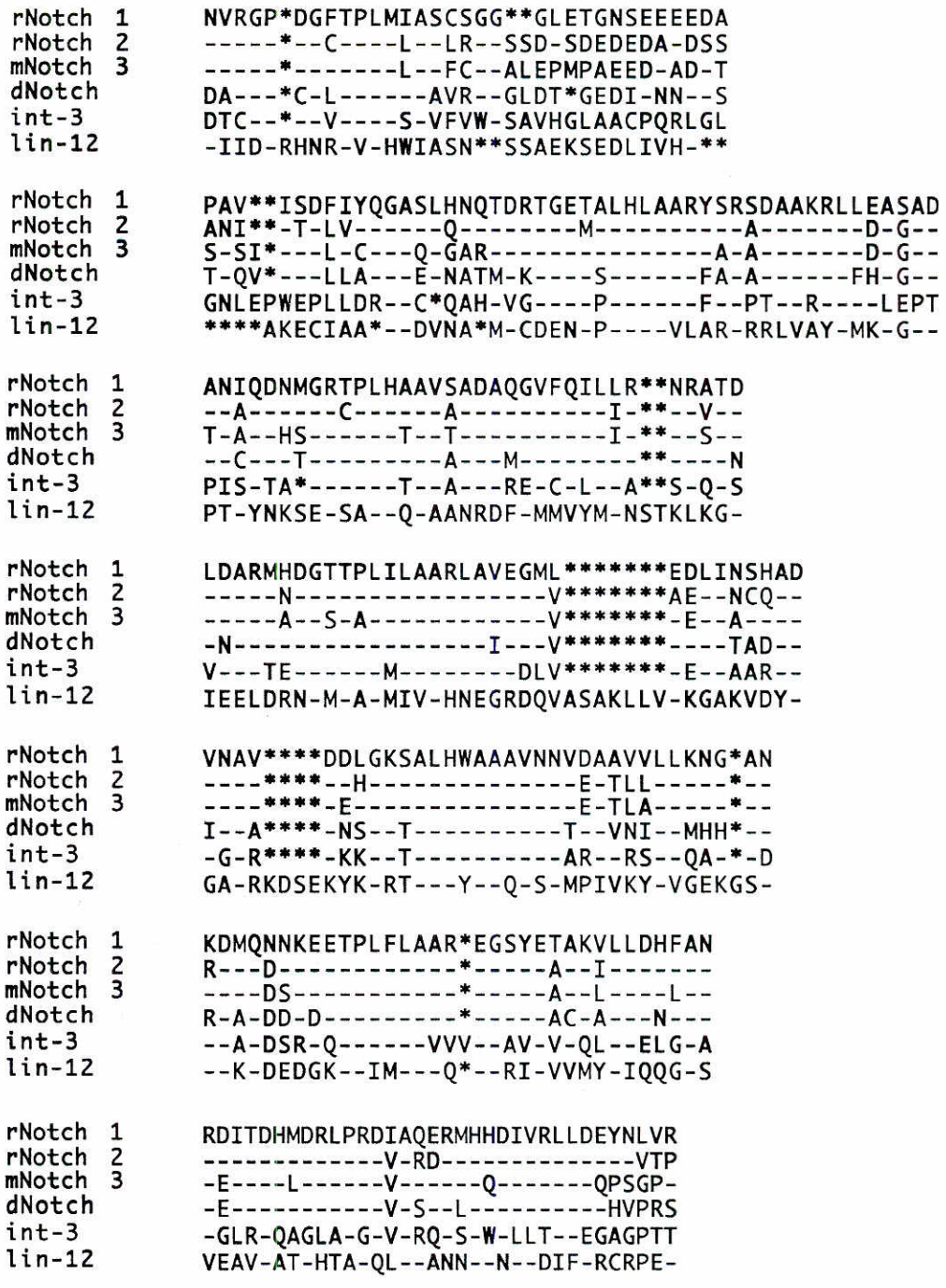

Fig. 2. Alignment of the ankyrin repeats from a variety of Notch and Notch-related proteins. *Comparison of amino acid sequences for the seven ankyrin repeats in the Notch (rNotch 1, rNotch 2, mNotch 3 and dNotch) and Notch-related (int-3 and lin-12) proteins, showing the degree of identity between residues. Alignments have been made against rNotch 1 amino acid sequence and so all dashes represent sequence homology to the top line. The levels of sequence identity in the ankyrin repeat region are: rNotch 1/rNotch 2= 78%; rNotch 1/mNotch 3= 75%; rNotch 2/mNotch 3= 77%; rNotch 1/dNotch= 70%, rNotch 1/int-3= 48%; dNotch/lin-12= 30%; rNotch 1/lin-12= 27%; int 3/lin-12= 25%. Abbreviations rNotch 1, rat Notch 1 (Weinmaster et al., 1991); rNotch 2, rat Notch 2 (Weinmaster et al., 1992); mNotch 3, mouse Notch 3 (Lardelli et al., 1994); dNotch: Drosophila melanogaster Notch (Wharton et al., 1985a); int-3, mouse int-3 (Robbins et al., 1992); lin-12, Caenorhabditis elegans lin-12 (Yochem et al., 1988).*

amino acid residue) identity with *Drosophila Notch*, equal to or greater than, the following levels:

EGF repeats	-45%
LN repeats	-40%
ankyrin repeats	-60%

(comparisons using the GAP program of Devereux *et al.* (1984) with gap weight = 3.0 and gap length weight = 1.0)

The *Notch* gene family is characterized by strong sequence conservation over great evolutionary distances (see Fig. 2) and, in contrast to many other genes, across almost the entire coding region. The sequence similarity is extensive, a fact illustrated by conservation within the EGF repeat domain. The individual EGF repeats of a *Notch* gene family member show higher homology to corresponding repeats in the other homologs than to their immediate neighbors (Coffman *et al.*, 1990; Weinmaster *et al.*, 1992; Lardelli and Lendahl, 1993) (see Fig. 3). This is also true for the mouse *Notch 3* gene that encodes only 34 instead of the usual 36 EGF repeats. The lower number of repeats apparently arose during evolution by deletion of exons encoding particular EGF repeats while the individual identity of the remaining

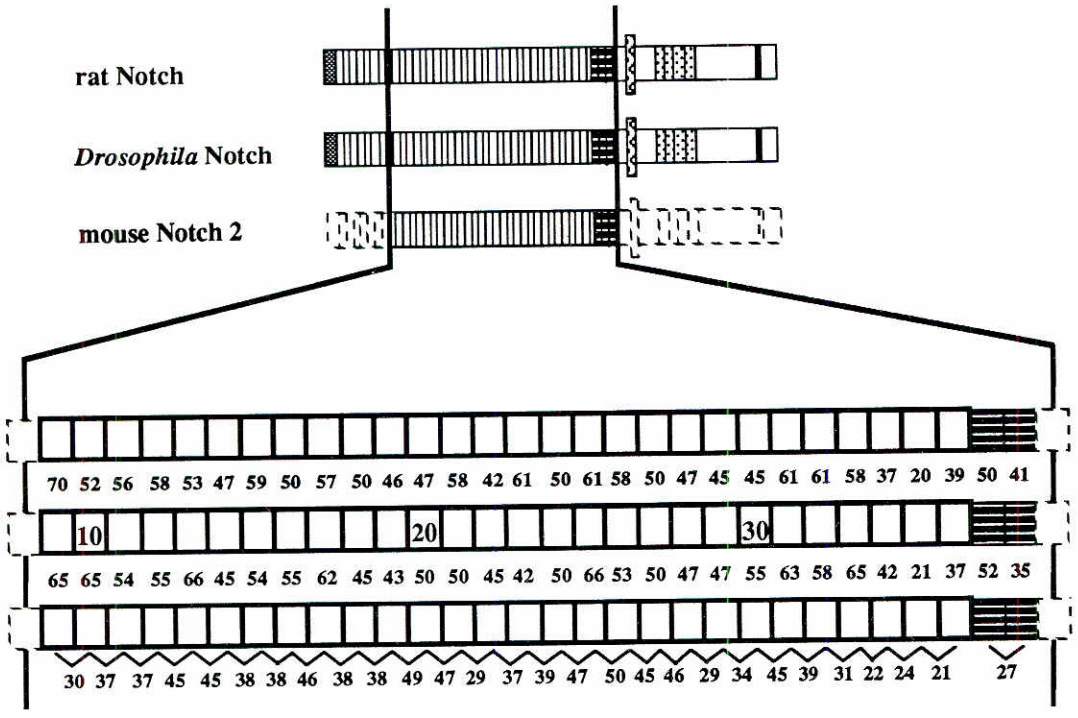

Fig. 3. Repeat homologies in the EGF and LN repeat regions of Notch proteins. *At the top, rat Notch 1, Drosophila Notch and mouse Notch 2 proteins are schematically depicted and a subset of the EGF repeats are enlarged below. Each plain box corresponds to an EGF repeat and every 10th box is numbered. Striped boxes denote LN repeats. The similarities between corresponding EGF or LN repeats are indicated as amino acid identity (%) in between the corresponding boxes. The amino acid identity between adjacent EGF or LN repeats in Notch 2 is shown at the base of the diagram. This Figure is modified from Lardelli and Lendahl (1993).*

repeats was maintained (Lardelli *et al.*, 1994). The maintenance of EGF repeat identity in invertebrate and vertebrate *Notch* genes implies that their function, and the function of the entire protein, is under strong selective pressure and is fundamental to embryo development in higher animals.

Between vertebrate species the *Notch 1*, *2*, and *3* homologs are highly conserved. For example, human and zebrafish Notch 1 proteins show 70% overall identity. Vertebrate *Notch 1*, *2*, and *3* genes all show approximately the same degree of identity to each other and so the duplication events that generated them probably occurred at approximately the same time during evolution. It has recently been shown that the multiple vertebrate *hox* gene clusters arose after the divergence of vertebrates from the primitive chordates (Garcia-Fernández and Holland, 1994) and it is reasonable to assume that the vertebrate *Notch* orthologues are also the result of the duplications of the genome that occurred at this time.

Notch activity is required in numerous differentiation events during development

Genetic and molecular studies have begun to reveal the functions of *Notch*-related genes in animal development. The activities of these genes and their products (which we shall henceforth refer to as 'Notch activity') are essential for many cell fate decisions at different times and in different tissues. Below we outline the knowledge gained from studies of Notch activity in *C. elegans*, vertebrates and, finally, the organism that has contributed most to our understanding: *Drosophila*.

C. elegans

Two *Notch*-related genes have been identified in *C. elegans*: *glp-1* and *lin-12* (Yochem *et al.*, 1988; Austin and Kimble, 1989; Yochem and Greenwald 1989). *glp-1* activity is required for induction of proliferation of germline cells, induction of development of the anterior pharynx and formation of the hypodermis (Austin and Kimble, 1987; Priess *et al.*, 1987), while *lin-12* is necessary for early vulval morphogenesis (Sundaram and Greenwald, 1993a) and a number of binary cell fate decisions during post-embryonic development (Greenwald *et al.*, 1983). Intragenic mapping of mutations in *glp-1* has shown that the ankyrin repeats are essential for Notch activity (Kodoyianni *et al.*, 1992; Lissemore *et al.*, 1993). Indeed, transgenic expression of only the 7 ankyrin repeats and 52 flanking amino acid residues is sufficient to induce development of multiple pseudovulvae and other aberrant structures (Roehl and Kimble, 1993)

Attempts to find genes that interact with *glp-1* and *lin-12* have involved screens for mutations that suppress or mimic the phenotypes of *glp-1* and *lin-12* hypomorphs (Maine and Kimble, 1989, 1993; Sundaram and Greenwald, 1993b). A number of loci have been detected, at least some of which interact with both *glp-1* and *lin-12* (Lambie and Kimble, 1991; Sundaram and Greenwald, 1993b). The locus *lag-2* has been characterized genetically and nematodes mutant for *lag-2* display a phenotype that is the sum of the *glp-1* and *lin-12* null phenotypes. This gene apparently encodes an extracellular ligand of the GLP-1 and LIN-12 proteins that has homology to Delta and Serrate, ligands of *Drosophila* Notch (Tax *et al.*, 1994) (see below). Thus the receptor-ligand system represented by Notch-related proteins is evolutionarily ancient.

Transgenic manipulation of *C. elegans* indicates that the activities of *glp-1* and *lin-12* are at least partially interchangeable (Fitzgerald *et al.*, 1993). This suggests that the selective advantage for this organism of possessing two *Notch* homologs may be provision of a more diverse pattern of Notch activity and that the GLP-1 and LIN-12 proteins control cell fate through the same signalling pathway. The same evolutionary argument has been

suggested to explain the existence of the vertebrate *Notch 1* and *2* genes (Weinmaster *et al.*, 1992; Lardelli and Lendahl, 1993). However, the discovery that the third *Notch* homolog, *Notch 3*, lacks specific EGF repeats implies that its protein product has a unique extracellular ligand-binding profile and that not all vertebrate *Notch* genes are interchangeable (Lardelli *et al.*, 1994).

Vertebrates

The first demonstration of the potential importance of *Notch* genes for vertebrate development came from the isolation of oncogenic forms of human *NOTCH 1* (originally named *TAN-1*) (Ellisen *et al.*, 1991) and the mouse *Notch*-related gene *int-3* (Robbins *et al.*, 1992). In both cases truncated forms of the protein products lacking most or all of their extracellular domains are expressed. Expression of a truncated human NOTCH 1 protein from the ß-T cell receptor gene promoter can promote development of T-cell lymphoblastic leukaemias whereas expression of truncated int-3 protein from the mouse mammary tumor virus (MMTV) long terminal repeat promoter promotes breast tumor formation in mice (Jhappan *et al.*, 1992). These observations suggest that vertebrate Notch activity is important for terminal differentiation and cell proliferation. This is supported by the recent observation that, like *NOTCH 1*, the human *NOTCH 2* and *3* genes are located in regions of neoplasia-associated chromosomal translocation (Larsson *et al.*, 1994).

Following these studies, Coffman *et al.* (1993) showed that expression of an extracellularly truncated form of Notch 1 throughout early *Xenopus* embryos leads to excessive recruitment of ectodermal cells into the neural tube and to changes in cell fate in other tissues. Parallel experiments expressing a complete Notch 1 protein showed little effect on embryo development. It therefore appears that Notch activity is limited by the embryonic distribution of its extracellular ligand(s), that ligand binding is essential for intracellular signalling by Notch and that removal of the extracellular domain results in constitutive Notch signalling (see also the later section on models of Notch function). This is probably a universal paradigm for all *Notch* genes since transgenic experiments in *C. elegans* (Roehl and Kimble, 1993; Struhl *et al.*, 1993) and *Drosophila* (Lieber *et al.*, 1993; Rebay *et al.*, 1993; Struhl *et al.*, 1993) lead to similar conclusions. In addition, our laboratory has preliminary results showing that expression of an extracellularly-truncated form of Notch 3 in the neural tube of developing mice disturbs neural development, probably by promoting excessive cell proliferation (Lardelli, Williams and Lendahl, unpublished observations).

While investigation of the activities of the vertebrate *Notch* genes is only beginning, the expression patterns of these genes imply that they will also function in diverse developmental events. All the known *Notch* family genes are expressed early in embryogenesis (Williams *et al.*, 1995). Expression of *Notch 1* and *Notch 2* has been observed in the presomitic mesoderm and early somites, respectively (Reaume *et al.*, 1992; Swiatek *et al.*, 1994) (see Fig. 4). In addition, we have observed *Notch 1* expression in the cardiac primordium and *Notch 2* expression in the node and neural fold of embryos at 7.5 dpc. In contrast, *Notch 3* is apparently expressed in most, if not all, cells of the embryonic ectoderm and mesoderm from at least as early as 7.0 dpc but not in the node or primitive streak (Williams *et al.*, 1995). Relatively little is yet known about regulation of Notch expression, but it has recently been shown that the three mammalian

Fig. 4. Early somitic expression pattern of the mouse *Notch 2* gene. *Lateral view of a whole-mount in situ hybridization (Wilkinson, 1992) of a digoxigenin-labeled antisense cRNA probe to mouse Notch 2 transcripts in a 13-somite mouse embryo (8.5 days post coitum). The probe was transcribed from a 1 kb mouse Notch 2 cDNA clone (Lardelli and Lendahl, 1993). High levels of Notch 2 mRNA are observed in the two most caudal (i.e. most recently formed) pairs of somites. In more anterior somites Notch 2 transcripts are less abundant and has become localized to dorsal somitic cells – probably representing the dermomyotome. Bar represents 70 μm.*

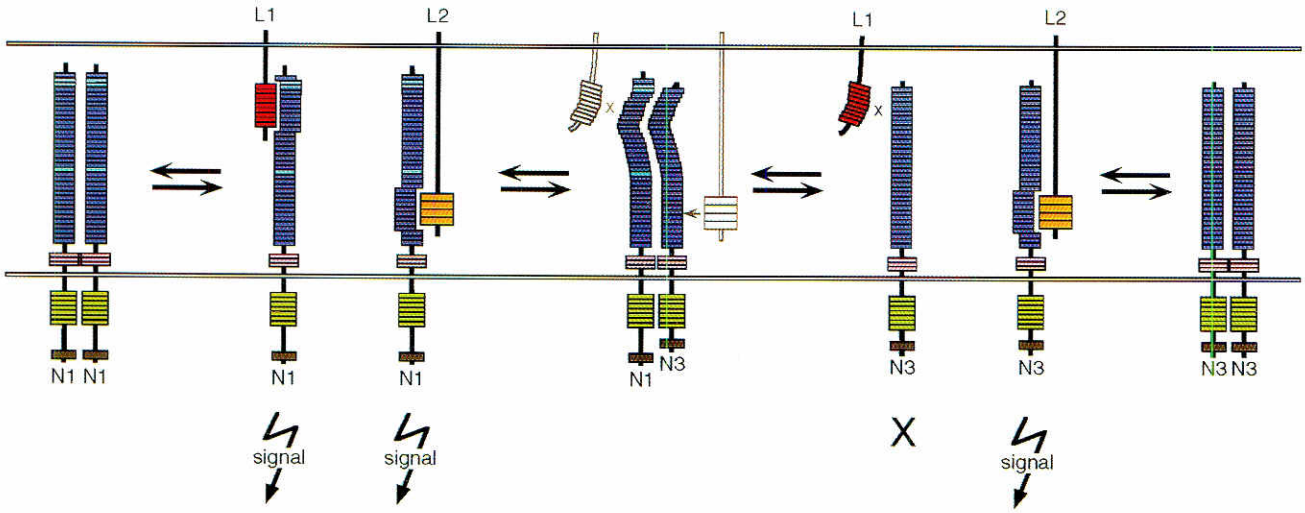

Fig. 5. Hypothetical model whereby the Notch 3 protein might modulate the signalling potential of Notch 1 (or Notch 2) – the 1 Ligand/1 Receptor (1L/1R) model. *Schematic representation of Notch receptors anchored in a plasma membrane of one cell contacting ligands residing in the plasma membrane of an adjacent cell. The model predicts that the Notch receptors are capable of forming homodimers and heterodimers which do not transmit a signal. Signal transmission (signal) occurs when a Notch monomer binds to a ligand and undergoes a change in conformation. Quiescent Notch dimers and active Notch/ligand complexes exist in equilibrium. Notch 1 (N1) is capable of binding to extracellular ligands, ligand 1 (L1) and ligand 2 (L2), causing transmission of a signal(s) (to the left in the Figure). Because of its shorter extracellular domain, Notch 3 (N3) can only bind to ligand 2 but not to ligand 1 (to the right in the Figure). In the presence of Notch 3, the formation of [ligand 1/Notch] complexes is decreased by the formation of Notch 1/3 heterodimers (in the center of the Figure). However, there is no change in the Notch dimer - [ligand 2/Notch] complex equilibrium since this ligand sees no difference in Notch 1 or Notch 3. The decrease in ligand 1 signalling caused by Notch 3 might be even more pronounced if ligand binding is necessary for Notch dimer disassociation. When heterodimerized with Notch 3, Notch 1 could be forced into a conformation incompatible with [ligand1/Notch 1] complex formation (in the center of the Figure).*

Notch homologs are regulated by tissue-tissue interaction and by exposure to retinoic acid (Mitsiadis *et al.*, 1995).

The overlapping and yet distinct expression patterns of the mouse *Notch* genes have previously led us to propose the possibility of heterodimerization between different Notch proteins as a way of expanding the regulative potential of Notch signalling (Lardelli and Lendahl, 1993; Lardelli *et al.*, 1994). Genetic data from *Drosophila* (see below) support the idea that Notch proteins may dimerize. The widespread and early expression of *Notch 3* that we have observed, and the lack of two specific EGF repeats in the Notch 3 extracellular domain, lead us to suggest that Notch 3 may function to modulate the activity of Notch 1 and 2 by heterodimerization and blocking of receptor-ligand interactions requiring these particular EGF repeats (Fig. 5). Given the access to cloned *Notch 1*, *2* and *3* genes this idea can now be tested experimentally.

To investigate the requirements for *Notch* activity during mammalian development, mutations of the endogenous genes are being introduced into the germline of mice by gene targeting. Mouse embryos lacking *Notch 1* die by 11.5 days of development (Swiatek *et al.*, 1994) apparently by defects in somitogenesis (Conlon *et al.*, 1995). Embryos lacking *Notch 2* die at birth and show reduction in lung and kidney size (T. Gridley, personal communication). Mouse embryos mutant for both *Notch 1* and *2* are being generated (T. Gridley, personal communication) and we are currently collaborating with the Gridley laboratory to mutate the endogenous *Notch 3* gene in mice. We have also constructed a dominant negative *Notch 3* transgene to test the function of *Notch 3* in the early neural tube (where *Notch 3* is highly expressed; Lardelli *et al.*, 1994).

Drosophila

Drosophila is the organism that has contributed most to our understanding of Notch activity and it is research with this organism that will probably give us the first detailed understanding of the intercellular communication mechanism in which Notch participates.

A variety of *Notch* mutations exist that affect particular aspects of *Drosophila* development. Deletion of one copy of the *Notch* gene results in the nicked wing phenotype, showing that *Drosophila* development is sensitive to *Notch* gene dosage (for review see Simpson *et al.*, 1992). Different single amino acid changes in the extracellular domain of Notch protein affect the development of different tissues. For example, the *split* mutation changes an isoleucine residue to threonine in EGF repeat 14 and results in eye and bristle defects (Hartley *et al.*, 1987; Kelley *et al.*, 1987) whereas the mutation *notchoid*^{3} changes a cysteine to phenylalanine in EGF repeat 2 and affects wing formation (Lyman and Young, 1993). These data suggest that the Notch extracellular domain interacts with different ligands in different tissues (see below).

The classic phenotype seen from mutation of Notch is hypertrophy of the embryonic nervous system. Too many neuroblasts differentiate from the ectoderm at the expense of epidermal cell precursors. Notch activity is also required in the generation of the peripheral and somatogastric nervous systems, salivary

glands, gut, malpighian tubules, trachea, somatic musculature and the heart (Corbin *et al.*, 1991; Hartenstein *et al.*, 1992). A temperature-sensitive *Notch* allele (N^{ts1}) has allowed investigation of the developmental requirements for *Notch* at specific times in later embryo development and metamorphosis (Schellenbarger and Mohler, 1975). In the embryonic central nervous system (CNS), Notch is required for the development of the midline cells without which axonal commissures cannot form (Menne and Klämbt, 1994). During malpighian tubule development, Notch is required for selection of the "tip mother cell" from a group of precursors. In male flies, Notch is expressed in the region of gonadal proliferation at the tip of the testis and reduction of Notch activity reduces fertility (Xu *et al.*, 1992). Here, the action of Notch would appear to be directly analogous to GLP-1 function in the distal tip cell of the *C. elegans* gonad, in which GLP-1 is expressed in the mother tip cell. In female flies, complex patterns of Notch expression are seen in the ovary and reduction of Notch activity results in severe changes in ovary morphology and infertility. Reduction of Notch activity at specific times during development of ommatidia (the optical units of the compound eye) blocks differentiation of the cell types that would have occurred at that specific time. If Notch activity is later restored, differentiation continues in a fashion appropriate for the later time point (i.e. it does not resume where it was first interrupted) (Cagan and Ready, 1989). Further analysis of *Notch* mutations in *Drosophila* eye development has revealed a potential link between the Notch and Sevenless signalling pathways (Fortini *et al.*, 1993). The detailed study of embryonic requirements for Notch activity by Hartenstein *et al.* (1992) led them to suggest that a unifying principle of Notch activity might be a function in the generation or maintenance of an epithelial state.

It should be emphasized that not all processes requiring Notch function may be dependent on the signalling activity of Notch. It is conceivable that the large extracellular domain of Notch, and also its ankyrin repeats, play a role in cell-cell adhesion. Indeed, the experiments showing binding of Delta and Serrate to Notch (see below) were conducted using a cell adhesion assay (Fehon *et al.*, 1990) and mutations in collagen genes can suppress the *glp-1* mutant phenotype in *C. elegans* (Kramer *et al.*, 1988; Johnstone *et al.*, 1992). Notch and Delta have also been shown to affect axon pathfinding during *Drosophila* embryogenesis – a process that is thought to depend, at least in part, on different levels of adhesion between the axon growth cone and its substrate (Giniger *et al.*, 1993). This raises the possibility that the cell-adhesion properties of Notch may be a necessary part of Notch signalling and that local, cytoplasmic circuits of Notch signalling may contribute to axon guidance.

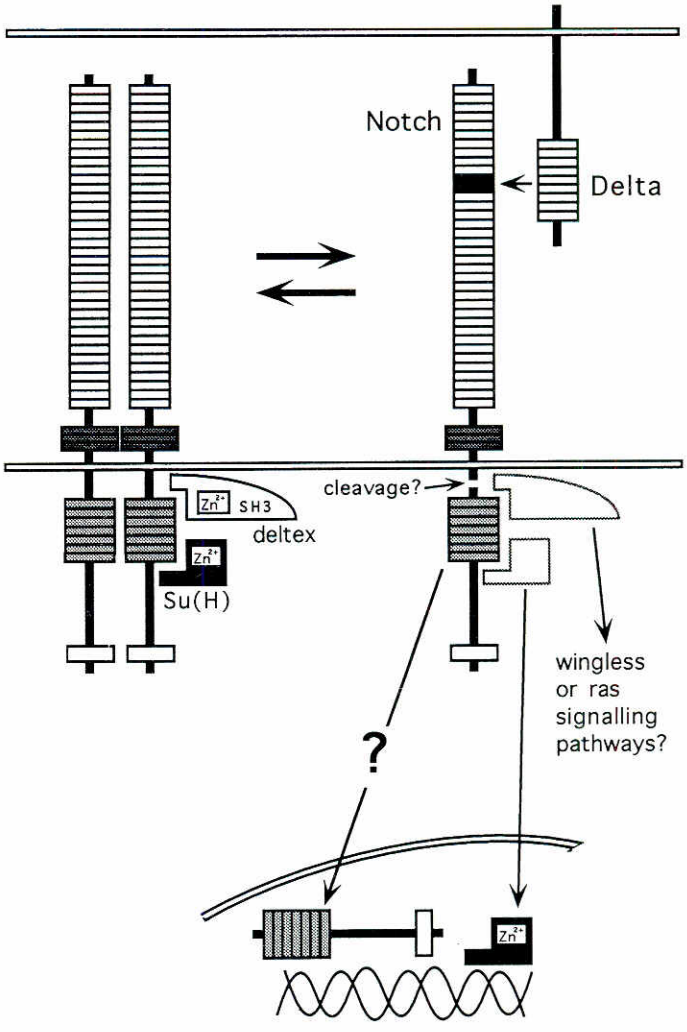

Fig. 6. An emerging understanding of Notch signal transduction. *The extracellular ligand Delta binds to Notch causing release of the zinc finger protein Su (H) from the intracellular ankyrin repeats. Su (H) is then transported to the nucleus, where it regulates gene expression, possibly by interaction with the basic helix-loop-helix protein E(spl). The possibility also exists that Notch activation results in cleavage and release of Notch's intracellular domain which is then actively transported into the nucleus to regulate gene expression. If both signalling pathways exist, then they may control separate aspects of Notch function. For example, transport of an intracellular Notch domain to the nucleus may control cell proliferation similarly to the yeast cell cycle protein cdc10 while the Su(H) signal may be required to block cell differentiation. The deltex protein has been shown to interact directly with the intracellular ankyrin repeats but has not been observed to be localized to the nucleus. It might possibly mediate the interaction of Notch with the ras and/or wingless signalling pathways.*

Proteins that interact with Notch receptors

Genetic screens for loci interacting with *Drosophila Notch* have revealed a number of genes, whose protein products presumably function in Notch signal transduction (Lehmann *et al.*, 1983). Most genes of the neurogenic group show genetic interactions with *Notch* (de la Concha *et al.*, 1988; Xu *et al.*, 1990; Lieber *et al.*, 1993) but only some of these have been demonstrated to encode proteins that interact directly with the Notch receptor. Nevertheless, the emerging picture is of a receptor interacting with multiple external and internal ligands and communicating with other signal transduction pathways.

Extracellular interactions

Two proteins, Delta and Serrate, have been demonstrated to interact with Notch's extracellular domain. In a cell adhesion assay using truncated Notch proteins, Notch EGF repeats 11

and 12 were shown to be sufficient for binding to both Delta and Serrate (Fehon *et al.,* 1990). However, genetic screens have identified mutations outside these repeats that affect cell adhesion when the whole Notch protein is used (Lieber *et al.,* 1992) and deletion of EGF repeats other than 11 and 12 can produce wing phenotypes similar to those seen when *Delta* is mutated (Rebay *et al.,* 1993). Nevertheless, the huge size of Notch's extracellular domain and the strong conservation of the EGF repeats suggest that more ligands may exist. Mutations in the *Drosophila* EGF receptor gene *(faint little ball)* (Schejter and Shilo, 1989) have been shown to interact with *Notch* but it is not known whether the Notch and EGF receptor proteins interact directly (Baker and Rubin, 1992). There are also genetic data implicating wingless as a possible Notch ligand (Couso and Martinez Arias, 1994).

As mentioned earlier, the Delta and Serrate proteins share homology with the product of the *C. elegans* genes, *lag-2* and *apx-1* (Mello *et al.,* 1994; Tax *et al.,* 1994), thus providing strong evidence that the basic mechanism of cell-cell communication defined by the *Drosophila* neurogenic genes is conserved in nematodes. This is also the case in vertebrates where genes with homology to Delta (Bettenhausen *et al.,* 1995; Chitnis *et al.,* 1995; Henrique *et al.,* 1995) and Serrate (Lindsell *et al.,* 1995) have been isolated.

Intracellular interactions

Both mutant and transgenic analyses have demonstrated the importance of the ankyrin repeats for Notch function (Lieber *et al.,* 1993; Lyman and Young, 1993; Rebay *et al.,* 1993; Struhl *et al.,* 1993). The ankyrin motif is found in a diverse range of proteins (reviewed in Bork, 1993) and appears to be involved in protein-protein binding. Possibly the best example of this is the protein product of the gene *cactus* in *Drosophila*. This gene was cloned using a degenerate oligonucleotide based on the *Drosophila Notch* ankyrin repeat sequence (Kidd, 1992) and possesses a series of 6 ankyrin repeats. The cactus protein is cytoplasmic and forms part of the signal cascade controlling dorso-ventral patterning in the embryo. It binds to the transcription factor dorsal (Steward, 1984) and holds this protein in the cytoplasm until signalled to induce ventral-type development. It then releases dorsal, which is subsequently translocated to the nucleus to regulate transcription (for review on dorsal-ventral patterning see Steward and Govind, 1993).

Recent work indicates that Notch probably acts in an analogous fashion to cactus. Work in the laboratory of Artavanis-Tsakonas has shown that the product of the *Suppressor of Hairless* [*Su(H)*] gene binds to the Notch ankyrin repeats (Fortini and Artavanis-Tsakonas, 1994). The Su(H) protein possesses a Zn^{2+}-finger domain common to many transcription factors and, upon binding of Delta to Notch's extracellular domain, is translocated to the nucleus (Fortini and Artavanis-Tsakonas, 1994). Intriguingly, Lieber *et al.* (1993) have shown that the Notch ankyrin repeats are flanked by nuclear-localization signals and truncated Notch proteins lacking extracellular and transmembrane domains have been observed to be localized to the nucleus (Fortini *et al.,* 1993; Lieber *et al.,* 1993). It is thus possible that one aspect of Notch-signalling may be cleavage and release of the intracellular domain which is then transported to the nucleus to regulate transcription. However, there is not any published evidence yet for cleavage of Notch during signal transduction. It was recently shown that the intracellular domain can interact with the mouse homolog of Su(H) and activate transcription from a specific promoter (Jarriault *et al.,* 1995).

The *deltex* gene of *Drosophila* was identified in a genetic screen as interacting with *Notch* (Xu and Artavanis-Tsakonas, 1990). Its protein product possesses a Src homology 3-binding (SH3) motif in a protein domain distinct from the domain that binds to Notch's ankyrin repeats and a C3H2C3-type zinc finger motif implicated in interactions with membrane proteins (Busseau *et al.,* 1994; Matsuno *et al.,* 1995). These structures suggest that deltex may be involved in the interaction of Notch with other signalling pathways such as those defined by the ras and wingless proteins. Consistent with this, deltex remains cytoplasmic when expressed in the presence of truncated Notch proteins lacking ankyrin repeats (Diederich *et al.,* 1994).

Models of *Notch* function

Detailed transgenic studies using *Notch* genes mutated *in vitro* have begun to reveal how Notch receptors function at the molecular level. Ligand binding to the extracellular domain is required for Notch activation (Coffman *et al.,* 1993; Lieber *et al.,* 1993; Rebay *et al.,* 1993). Deletion of the ankyrin repeats results in a protein that can suppress Notch signalling (i.e. a dominant negative effect), whereas removal of all extracellular sequences leads to production of a constitutive signal (i.e. a "dominant active" effect) (Lieber *et al.,* 1993; Rebay *et al.,* 1993). Significantly, removal of all extracellular sequences *except* for the region between the LN repeats and the transmembrane domain (which contains two cysteine residues postulated to be necessary for dimerization of Notch receptors; Kidd *et al.,* 1989) does not generate a constitutively active protein. Conversely, mutation of either of these two cysteine residues to serine in an otherwise normal Notch receptor gives greater than normal signalling, but this is dependent upon the presence of Delta protein. Removal of the LN repeats apparently overcomes this ligand dependence and allows constitutive Notch signalling (Lieber *et al.,* 1993).

A possible mechanism of Notch function begins to emerge from these data which we refer to as the 1Ligand/1Receptor (1L/1R) model (Fig. 5). In the 1L/1R model, a quiescent Notch dimer disassociates to form a complex with one ligand molecule (such as Delta) and one Notch receptor. The ligand/Notch complex then undergoes a conformational change that activates intracellular signalling possibly involving Su(H), Deltex and/or cleavage of the intracellular domain as indicated above (Fig. 6).

The observation that truncated Notch proteins lacking intracellular domains block signalling may suggest, in analogy with tyrosine kinase receptor proteins (Kazlauskas, 1994), that Notch receptors are active as dimers (i.e. Notch receptors might act in a 1L/2R fashion). Cooperative interactions between Notch molecules have also been proposed to explain the "negative complementation" phenotypes of the *Abruptex* alleles of *Notch* (Welshons, 1971; Foster, 1975; Portin, 1975). These dominant alleles map to the EGF repeats (Hartley *et al.,* 1987; Kelley *et al.,* 1987) and encode activated Notch molecules (Palka *et al.,* 1990; de Celis and Garcia-Bellido, 1994). They can be divided into two negative complementation groups where possession of one

allele from each group is lethal, but flies with two alleles from the same complementation group survive. While these observations indicate that normal Notch function requires interactions between Notch molecules, one further experiment by Leiber *et al.* (1993) gives strong evidence in favor of an 1L/1R model. They find that the dominant negative action of a Notch receptor lacking ankyrin repeats is not affected by mutation of one of the membrane-proximal cysteine residues thought to be necessary for dimerization. Thus, blockage of Notch signalling apparently does not occur by the formation of inactive dimers but by competition for ligands (Lieber *et al.,* 1993). The validity of this model depends on whether the two membrane-proximal cysteine residues are actually essential for Notch dimerization. While the evidence for this is considerable, it has not been demonstrated biochemically yet.

The 1L/1R model is relevant to understan the oncogenic effect of fusion of the human *Notch 1* and *ß T cell receptor (TCRß)* genes in t(7;9) (q34;q34.3) translocations. While the protein product of the fusion has yet to be characterized, DNA sequence data suggest that part of the extracellular region of TCRß is fused to a truncated Notch 1 protein that includes the membrane-proximal cysteine residues, the LN repeats and two EGF repeats (Ellisen *et al.,* 1991). The data of Lieber *et al.* (1993) imply that a Notch protein truncated in this way should not be active. Therefore, activation of the TCRß/Notch 1 fusion is probably due to the presence of the TCRß sequences itself and/or binding of proteins to these sequences, in analogy with observations from other receptor chimeras (Rodrigues and Park, 1994). This model allows for an interesting clinical perspective and it may be possible to use Notch 1 receptor chimeras as a tool to block differentiation in response to a variety of extracellular ligands.

Deletion of amino acid residues between the ankyrin repeats and PEST motif (Fig. 1) of Notch's intracellular domain has no, or only mild, effect on Notch activity (Lieber *et al.,* 1993; Rebay *et al.,* 1993). A low selective pressure for the maintenance of this region during evolution may explain the size variation observed between the intracellular domains of the vertebrate *Notch* genes (Lardelli *et al.,* 1994).

Notch, lateral inhibition and cell differentiation

In *Drosophila*, neural cells arise in the embryonic neuroectoderm and in the imaginal epithelia from clusters of cells expressing proneural genes (Cabrera, 1990; Skeath and Carroll, 1992). All cells expressing proneural genes have the potential to become neural but only one cell in each cluster will do so (for review see Jiménez and Modolell, 1994). The process by which this cell is selected is called "lateral inhibition" and requires the activity of *Notch*, *Delta*, *Enhancer of split* and other "neurogenic genes". Without neurogenic gene function all the cells in a proneural cluster adopt a neural fate (see Simpson *et al.,* 1992).

Lateral inhibition is one example of a common theme in developmental biology – the generation of a coarse, broad primary pattern which is then refined by local secondary mechanisms (another example is the cascade of pattern refinement carried out by the segmentation gene hierarchy during the division of the *Drosophila* embryo into segments). The lateral inhibition mechanism involves subtle, competitive interactions among neurogenic gene products, especially Notch and Delta, at cell surfaces. Notch and Delta interactions not only occur between molecules on the surface of juxtaposed cells but within the same cell membrane (Simpson *et al.,* 1992; Heitzler and Simpson, 1993).

The conservation of Notch and Delta molecules during evolution implies that the lateral inhibition mechanism defined by the *Drosophila* neurogenic genes is fundamental to the development of all animals (Artavanis-Tsakonas *et al.*, 1995). The LIN-12- and GLP-1-controlled unequal cell divisions of *C. elegans* resemble the Notch-controlled epidermal/neural fate choice of *Drosophila* embryogenesis. However, in vertebrates there is not yet any clear data demonstrating the action of *Notch* genes in an event resembling lateral inhibition. Despite the expression of vertebrate *Notch* genes in the CNS primordium – the neural plate – (Coffman *et al.,* 1993; Williams *et al.,* 1995), the CNS forms by invagination *en masse* of all neural plate cells rather than delamination of individual neural precursors as in *Drosophila*.

In our analysis of mouse *Notch* gene expression during neural development, we found that all three genes are expressed in the ventricular zone of the CNS. *Notch 3*, in particular, is expressed at very high levels (Lardelli *et al.,* 1994). It is in the ventricular zone that unequal division of neural precursor cells takes place to produce new proliferative cells and migratory daughter cells that move outward along the radial glia finally to differentiate into one of the neural cell types (McKay, 1989).

In *Drosophila*, *Notch* expression is down-regulated once a neural precursor cell has been selected and begins to delaminate (Kooh *et al.,* 1993). A constitutively active *Notch* transgene inhibits *Drosophila* neural differentiation (Lieber *et al.,* 1993; Struhl *et al.,* 1993). In vertebrates, the higher levels of *Notch* expression in the ventricular zone of the neural tube may result from down-regulation of *Notch* in daughter cells as they migrate radially away from this zone to terminally differentiate. If this is true, then expression of a constitutively active *Notch* gene in the vertebrate neural tube should block cell differentiation and increase cell proliferation (since all daughter cells formed in the ventricular zone will become new proliferative cells). We are currently testing this hypothesis by expressing a constitutively active *Notch 3* gene in the neural tube of transgenic mice. Preliminary results indicate a rapid and dramatic increase in cell proliferation.

Conclusions

The available data suggest that the Notch receptors and their ligands represent an evolutionarily old and very well conserved system for signal transduction and can be found in species as diverse as flies, worms and vertebrates (Artavanis-Tsakonas *et al.,* 1995; Simpson, 1995). The existence of multiple *Notch* and *Delta* homologs in vertebrates raises the possibility that these genes may have diversified in function so that they now perform roles for which there are no analogues in lower animals, e.g. reflecting the increasing complexity of CNS structures. While research in *Drosophila* and *C. elegans* will reveal a basic mechanism of Notch function, considerable work remains to investigate the extracellular ligand specificity for the different vertebrate *Notch* homologs, the possibility and implications of heterodimerization between them, and whether the individual receptors

transmit signals by identical, overlapping or separate intracellular pathways.

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