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Altered Epidermal Growth Factor-like sequences provide evidence for a role of *Notch* as a receptor in cell fate decisions

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SUMMARY

In *Drosophila* each neural precursor is chosen from a group of cells through cell interactions mediated by *Notch* and *Delta* which may function as receptor and ligand (signal), respectively, in a lateral signalling pathway. The cells of a group are equipotential and express both *Notch* and *Delta*. Hyperactive mutant *Notch* molecules, (*Abruptex*), probably have an enhanced affinity for the ligand. When adjacent to wild-type cells, cells bearing the *Abruptex* proteins are unable to produce the signal. It is suggested that in addition to the binding of Notch molecules on one cell to the Delta molecules of opposing cells, the *Notch* and *Delta* proteins on the surface of the same cell may interact. Binding between a cell's own Notch and Delta molecules would alter the availability of these proteins to interact with their counterparts on adjacent cells.

Key words: epidermal growth factor, cell interaction, *Notch*, cell fate, *Drosophila*, *Delta*

INTRODUCTION

During embryonic development there are many instances where initially equivalent cells take up different fates as a result of interactions between themselves (Rubin, 1991; Horwitz and Sternberg, 1991; Greenwald and Rubin, 1992). In *Drosophila*, neural precursors segregate singly in a spaced pattern and are separated by intervening epidermal cells (Hartenstein and Campos-Ortega, 1984; Hartenstein and Posakony, 1989). There is evidence, however, that at each site more than one cell initially acquires the potential to become a neural precursor (Stern, 1954; Doe and Goodman, 1985; Simpson, 1990). The singling out of the neural cell is dependent upon cell interactions that involve the genes *Notch* and *Delta* (Lehmann et al., 1983; Heitzler and Simpson, 1991).

In the neuroepithelium of the adult thorax, the two genes *achaete* and *scute* confer the potential to develop as sensory organs (Garcia-Bellido, 1979; Ghysen and Dambly-Chaudière, 1988). If they are absent, the cells differentiate as epidermis (Garcia-Bellido and Santamaria, 1978). *achaete* and *scute* are expressed in clusters of cells at the sites of each of the large bristles, or macrochaetae, that occupy defined positions on the notum (Cubas et al., 1991; Skeath and Carroll, 1991). A single macrochaete precursor segregates from most of the clusters and the remaining cells cease expression of *achaete* and *scute* and differentiate as epidermis (Cubas et al., 1991; Skeath and Carroll, 1991). In the absence of *Notch* or *Delta*, a tuft of bristles develops at each site instead of the single one, suggesting that most *achaete/scute*-expressing cells adopt the neural fate (Heitzler and Simpson, 1991). *achaete* and *scute* are later expressed uniformly over the region where the equally spaced microchaetae, or small bristles, develop, and in the absence of *Notch* or *Delta*, a uniform field of densely packed adjacent microchaetae is formed.

It is thought that cells that are developing as neural precursors send an inhibitory signal preventing their neighbours from entering the neural fate (Wigglesworth, 1940; Doe and Goodman, 1985; Simpson, 1990). The cell autonomous behaviour of mutant *Notch* cells is in favour of a role for this protein as a receptor of the postulated inhibitory signal (Hoppe and Greenspan, 1990; Heitzler and Simpson, 1991). The signal itself could be mediated by *Delta* which behaves non-autonomously (Heitzler and Simpson, 1991). However, the *achaete/scute*-expressing cells of a cluster have equal developmental potential (Simpson and Carteret, 1989, 1990) and presumably all express both *Notch* and *Delta*, so the single presumptive neural cell has first to be singled out. The observation that wild-type cells will adopt the epidermal fate if adjacent cells express a lower level of *Notch* activity than themselves, but will produce neural precursors if adjacent cells express a higher level of *Notch* activity, suggests that *Notch* itself is involved in the initial choice of fate (Heitzler and Simpson, 1991). Cells mutant for *Notch* have an enhanced capacity to produce the inhibitory signal and one interpretation of this is that the signal and receptor might be connected via a feedback loop. Then, from a group of cells expressing both *Notch* and *Delta*, a cell that found itself with a smaller

amount of receptor (perhaps as a result of random physiological fluctuations) would have an increased ability to signal and perhaps induce the expression of more receptor in the adjacent cells. Such an initial bias could then be greatly amplified with time. The competition between the cells that is implied in this hypothesis is consistent with the observation that in *Notch* mutants the cluster of neural precursors forms earlier than the single precursor in the wild-type (Goriely et al., 1991).

Such a mechanism would imply considerable complexity in the functions attributed to the Notch molecule. The protein would have to interact with the Delta molecules of surrounding cells, modulate the activity of *Delta* within the same cell and furthermore after binding to Delta initiate a signal transduction event inside the inhibited cell that would eventually lead to the cessation of *achaete/scute* expression. *Notch* encodes a transmembrane protein of approx. 300×10^3 M_r and is composed of several distinct domains. The extracellular domain contains 36 copies of a cysteine-rich motif displaying EGF homology and also three copies of another cysteine-rich motif, *Notch/lin-12* (Wharton et al., 1985; Kidd et al., 1986). The intracellular domain of *Notch* contains five copies of a 33 amino acid repeat that is also found in *cdc10*, *SW16* and *SW14* (genes controlling the cell cycle in yeast; Andrews and Herskowitz, 1989), *lin12* and *glp1* (genes controlling cell fate decisions in the nematode that also show homology to the extracellular domain of *Notch*, Yochem et al., 1988; Yochem and Greenwald, 1989) and ankyrin (a cytoskeletal protein of the erythrocyte membrane; Lux et al., 1990). There is a remarkably high conservation of all of these domains across species pointing to an important functional role for each domain (Coffman et al., 1990; Ellisen et al., 1991; Weinmaster et al., 1991). *Delta* also encodes a transmembrane protein containing 9 EGF-type repeats in the extracellular portion but only a small intracellular domain that is without homology to other known proteins (Vässin et al., 1987; Kopczynski et al., 1988). It has been shown that these two proteins can mediate cell adhesion in a cell culture assay (Fehon et al., 1990). The *Notch* protein may therefore physically bind to Delta and perhaps act as a receptor, but no mechanism for generating intracellular signals is known.

Here we have studied the dominant *Abruptex* alleles of *Notch* (Welshons, 1971; Foster, 1975; Portin, 1975, 1981), which contain point mutations in the EGF-like repeats that are thought to modify the secondary structure of the protein (Kelley et al., 1987; Hartley et al., 1987). They represent a gain of function; mutant cells show the opposite phenotype to that of a loss of function, and differentiate fewer neural cells and more epidermis (see also Palka et al., 1990). In order to differentiate as epidermis, *Abruptex* cells require the *Delta* product. The results suggest that they may have an enhanced capacity to bind Delta. In contrast to cells mutant for loss of function alleles, cells mutant for *Abruptex* also fail to produce an inhibitory signal. From these studies we propose that in addition to the binding of Notch molecules on one cell to Delta molecules on an adjacent cell, the *Notch* and *Delta* proteins on the surface of the same cell may interact. Binding between a cell's own Notch and Delta molecules would alter the availability of the proteins to interact with their counterparts on adjacent cells. Such competitive protein-protein interactions could provide a molecular basis for the feedback mechanism between the signal and the receptor.

MATERIALS AND METHODS

Fly strains

The molecular lesions associated with Ax^{59b}, Ax^{28}, Ax^{9B2} and *spl* are described in the text and given in Kelley et al. (1987) and Hartley et al. (1987). Ax^{SX1} was isolated in our laboratory and the lesions associated with this allele and also that of Dl^{9P39} have not been mapped. N^{ts1} is associated with an amino acid change in EGF repeat number 32 (Xu et al., 1992). The lesions associated with nd^1 and nd^2 are described in the text and given in Xu et al. (1990). Flies were raised on standard medium and maintained at 25°C.

Analysis of neural precursors

y Ax^{SX1} *v/FM6* and *y* Ax^{59b} *v/FM6* females were crossed to males of an enhancer trap transformant line, A101. A101 contains a *lacZ* gene insert at the *neuralised* locus (Boulianne et al., 1991) and exhibits staining in all sensory organ precursors (Huang et al., 1991). Of the larvae resulting from these crosses the *Ax* mutant males could be recognised by the *yellow* colour of the cuticle. Mutant and wild-type Oregon-R animals were selected at the wandering larval stage and the discs dissected and stained as in Huang et al. (1991).

Production of mosaic animals

Mutant clones were produced by X ray-induced mitotic recombination. 24-hour egg collections were made and larvae were irradiated with 1000 R of X-rays between 48 and 72 hours after egg laying. Clones were marked with *forked* (f^{36a}) and *yellow (y)* which label sensory bristles and *multiple wing hairs (mwh)* which labels epidermal hairs. For a description of mutations and rearrangements see Lindsley and Zimm (1992). $P(N^+)$ *Cos 479*, N^+ is described in Ramos et al. (1989). Thoraces were mounted between coverslips in Struhl's medium. Clones were induced in flies of the following genotypes:

(1) Ax^{59b} f^{36a}/*Dp(3;Y;1)M2,* emc^+ mwh^+ *y v;* emc^1 *mwh/mwh.*
(2) Ax^{SX1} f^{36a}/*Dp(3;Y;1)M2,* emc^+ mwh^+ *y v;* emc^1 *mwh/mwh.*
(3) Ax^{28} f^{36a}/*Dp(3;Y;1)M2,* emc^+ mwh^+ *y v;* emc^1 *mwh/mwh.*
(4) Ax^{9B2} f^{36a}/*Dp(3;Y;1)M2,* emc^+ mwh^+ *y v;* emc^1 *mwh/mwh.*
(5) *spl* f^{36a}/*Dp(3;Y;1)M2,* emc^+ mwh^+ *y v;* emc^1 *mwh/mwh.*
(6) nd^1 f^{36a}/*Dp(3;Y;1)M2,* emc^+ mwh^+ *y v;* emc^1 *mwh/mwh.*
(7) nd^2 f^{36a}/*Dp(3;Y;1)M2,* emc^+ mwh^+ *y v;* emc^1 *mwh/mwh.*
(8) f^{36a}/*Dp(3;Y;1)M2,* emc^+ mwh^+ *y* Ax^{59b}*;* emc^1 *mwh/mwh.*
(9) Ax^{SX1}*/Y; pr pwn; st* Dl^{9P39} *e/P(*N^+*) Cos 479,* N^+ bx^{34e} *Dp(2;3)P32,* pwn^+.
(10) Ax^{SX1}*/Y; pr pwn/+; st* Dl^{9P39} *e/P(*N^+*) Cos 479,* N^+ bx^{34e} *Dp(2;3)P32,* pwn^+.
(11) N^{ts1}*/Y; pr pwn; st* Dl^{9P39} *e/P(*N^+*) Cos 479,* N^+ bx^{34e} *Dp(2;3)P32,* pwn^+.

RESULTS

Cell autonomy and cell signalling

Our studies are restricted to the small sensory bristles, microchaetae, of the thoracic peripheral nervous system. The bristle precursors segregate in a spaced pattern (Harten-

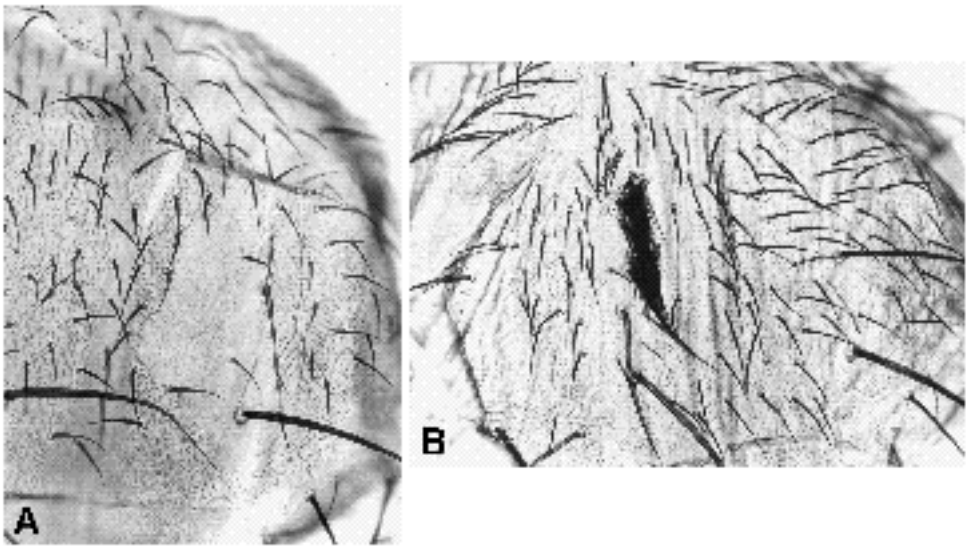

Fig. 1. Mutant phenotypes. (A) Photograph of a clone mutant for Ax^{59b} in an Ax^{59b} $f^{36a}/Dp(3;Y;1)M2$, emc^{+} mwh^{+} y v; emc^{1} mwh/mwh fly. In the case of this strong allele the mutant cells adopt the epidermal fate and so no bristles are formed. (B) Photograph of a clone mutant for both Ax^{SX1} and Dl^{9P39} in an Ax^{SX1}/Y; pr $pwn/+$; st Dl^{9P39} $e/P(N^{+})$ Cos 479, N^{+} bx^{34e} $Dp(2;3)P32$, pwn^{+} fly. The double mutant cells show the opposite phenotype to Ax Dl^{+} cells and adopt the neural fate. Bristles are thus formed and these clones are indistinguishable from Ax^{+} (N^{+}) Dl^{9P39} clones. 15 mutant clones of this genotype were obtained together with 6 clones in Ax^{SX1}/Y; pr pwn; st Dl^{9P39} $e/P(N^{+})$ Cos 479, N^{+} bx^{34e} $Dp(2;3)P32$,pwn^{+} flies that are therefore marked with pwn. Flies of the latter genotype are poorly viable. All clones showed a characteristic Dl^{9P39} phenotype. Note that Ax^{SX1}/Y males are non-viable but that either the addition of a N^{+} allele or a decrease in the number of copies of Dl^{+} from two to one allows viability, although the flies display a mutant phenotype of a decreased density of bristles. The flies used for this experiment have the double advantage of a single copy of Dl^{+} together with a N^{+} allele and their bristle pattern is wild type.

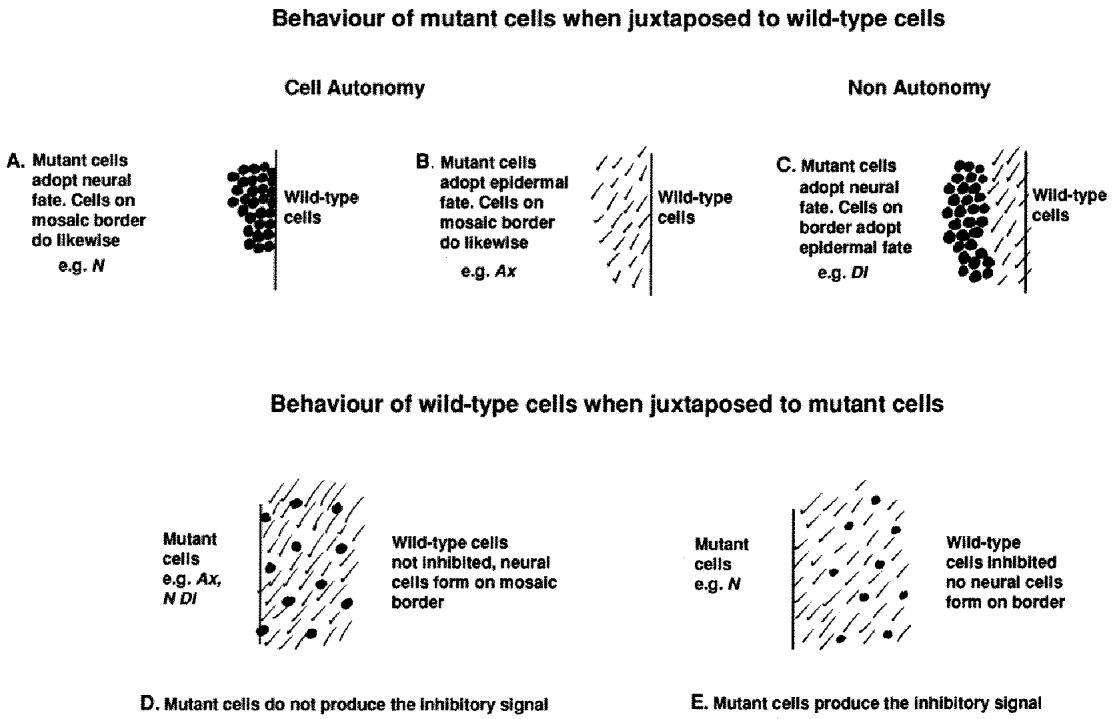

Fig. 2. Cell autonomy and cell signalling. In mosaic animals, mutant and wild-type cells are juxtaposed along the borders (represented by straight lines) of mutant clones. Mutant cells may differentiate as neural cells (bristles, indicated by black circles) as in A, or as epidermal hairs, as in B. If the mutant cells display the same phenotype along the mosaic border, as in A and B then they have not been influenced by their adjacent wild-type neighbours and are said to behave autonomously. In C the mutant cells differentiate as neural cells in the centre of the clone where they are not in contact with wild-type cells, but differentiate into epidermal cells along the clone border where they have been influenced by the neighbouring wild-type cells. These cells thus behave non-autonomously and we conclude that they have been prevented (inhibited) from differentiating as neural cells by a signal emanating from the wild-type cells. In a similar fashion wild-type cells can be influenced by their adjacent mutant neighbours. In D the wild-type cells have differentiated normally and produced neural cells along the mosaic border. The mutant cells were therefore unable to influence the fate of the wild-type cells. In E the wild-type cells along the mosaic border all differentiated as epidermis. In this case the mutant cells have produced a strong inhibitory signal preventing the wild-type cells from differentiating as neural cells. These experiments thus provide a measure of the signalling capacity of mutant cells.

stein and Posakony, 1989) and after differentiation are separated by 4-5 intervening epidermal cells. Each epidermal cell secretes a fine, non-sensory hair (Mitchell et al., 1990). Convenient markers are available for both hairs and bristles. The phenotype of mutant cells has been studied in clones. Mutant clones differentiate either an excess of bristles at the expense of epidermal cells, e.g. *Delta* (*Dl*), or an excess of epidermal cells and fewer bristles (e.g. *Abruptex* (*Ax*) Fig. 1A).

Along the borders of the clones, mutant and wild-type cells are juxtaposed. If the mutant cells on the border adopt the same fate as those in the centre of the clone that are not adjacent to wild-type cells, then they have not been influenced by their wild-type neighbours and are said to behave cell autonomously (Fig. 2A,B). If they adopt a different fate from those in the centre then they have been influenced through contact with their wild-type neighbours and display non-autonomy, e.g. clones mutant for *Dl* differentiate bristles in the centre but epidermis on the border (Fig. 2C). This is interpreted to mean that signals from the wild-type cells have prevented the mutant *Dl* cells from becoming neural.

It is thought that, in the neurogenic region of the disc, all cells have neural potential but that emerging neural precursors, once they have been singled out, produce an inhibitory signal preventing neighbouring cells from becoming neural and causing them to differentiate as epidermis. Wild-type cells initially have a choice between neural and epidermal fates. When juxtaposed to mutant cells, however, wild-type cells are sometimes inhibited by their mutant neighbours. In such cases they always adopt the epidermal fate (Fig. 2E). The behaviour of wild-type cells adjacent to mutant ones therefore provides a measure of the signalling capacity of the mutant cells.

In order to force their wild-type neighbours into the epidermal fate, mutant *Notch* cells require *Delta*

Earlier studies showed that clones mutant for $Notch^{ts1}$ (N^{ts1}) differentiate as densely packed bristles and that along the mosaic borders the only bristles that form are of the mutant genotype. They therefore behave autonomously and at the same time they always inhibit neighbouring wild-type cells, causing them to take up the epidermal fate (Heitzler and Simpson, 1991, Fig. 2A). This was interpreted to mean that the *N* protein is required for reception of the inhibitory signal and that mutant *N* cells themselves produce a strong inhibitory signal. In contrast, cells mutant for Dl^{9P39} differentiate bristles unless they are in contact with wild-type cells, on the edges of mutant clones, where they differentiate as epidermis. Thus the only bristles formed on the clone borders are of the wild-type genotype (Heitzler and Simpson, 1991; Fig. 2C). This was interpreted to mean that the *Dl* protein is part of the inhibitory signal itself. In order to see whether mutant *N* cells require *Dl* in order to inhibit wild-type neighbours, we have examined the behaviour of cells doubly mutant for N^{ts1} and Dl^{9P39}. In the double mutant clones, both wild-type and mutant bristles are formed along the mosaic borders (Fig. 3B). Thus, in the absence of *Dl*, mutant *N* cells are no longer able to prevent neighbouring wild-type cells from adopting the neural fate. Furthermore occasionally a mutant and a wild-type bristle are found adjacent to one another (Fig. 3B), a situation that is never seen in clones mutant for either *N* or *Dl* alone. This is consistent with the idea that the mutant *N Dl* cell was unable to receive an inhibitory signal from its wild-type neighbour or to prevent the latter from differentiating as a neural precursor. These cells therefore behave as expected for cells deficient in both the signal and the receptor. This observation reinforces the case for *Dl* as the signalling molecule.

Neural precursors never form in the *Abruptex* mutants

The *Abruptex (Ax)* alleles of *N* are dominant and cause the development of fewer bristles. We have studied the four alleles Ax^{59b}, Ax^{SX1}, Ax^{9B2} and Ax^{28}. They can be ordered into an allelic series based on the density of bristles that is observed on the notum. Ax^{SX1} is hemizygous lethal and its phenotype was studied in homozygous mutant clones. Ax^{59b} is also lethal but animals develop as pharate adults with a differentiated cuticle. The density of bristles was estimated by counting the number of epidermal cells between bristles. An average of 8.9±0.13, 5.8±0.11, 6.7±0.11 was obtained for Ax^{SX1}, Ax^{9B2} and Ax^{28} respectively, compared to 5.0±0.1 for wild-type animals (Table 1). Hemizygous Ax^{59b} males are almost naked, occasionally one or two microchaetae are found and sometimes a single scutellar or postalar bristle differentiates.

In order to determine whether the lack of bristles reflects a failure of formation of the sensory organ precursors, we have studied precursor formation in the thoracic imaginal discs of hemizygous Ax^{SX1} and Ax^{59b} male wandering, late third instar larvae, using the marker A101. Of the 21 Ax^{59b} discs observed, no macrochaete precursors were visible whereas many of the notal and most of the triple row macrochaetae are present in wild-type larvae of this age (Fig. 4). Ax^{SX1} discs display an average of four macrochaete precursors (n=19) compared to 7 or 8 in wild-type discs of the same age. These results indicate that the neural precursors never form in the mutants as was also shown for some alleles in the wing by Palka et al. (1990) and suggests that the cells instead adopt the epidermal fate. This is consistent with the observation that homozygous mutant Ax^{59b} clones differentiate as epidermis. In contrast, in *N* null clones no cells differentiate as epidermis (Heitzler and Simpson, 1991).

Cells mutant for the *Abruptex* alleles of *Notch* are inhibited by their wild-type neighbours

Clones of cells homozygous for *Ax/Ax* are surrounded by heterozygous *Ax/+* cells. Along the clone borders, the homozygous *Ax/Ax* cells produced epidermis almost exclusively (Fig. 3C). This was also the case for the weaker alleles (such as Ax^{28}) which do allow the development of a fair number of bristles (see legend to Fig. 3C). Cells homozygous for these altered molecules of *Notch*, therefore, always adopt the epidermal fate when adjacent to cells that are heterozygous. In a separate experiment we also made marked clones of wild-type (+/+) cells that were surrounded by heterozygous Ax^{59b}/+ cells, which enabled us to examine the behaviour of heterozygous cells when adjacent to wild-type

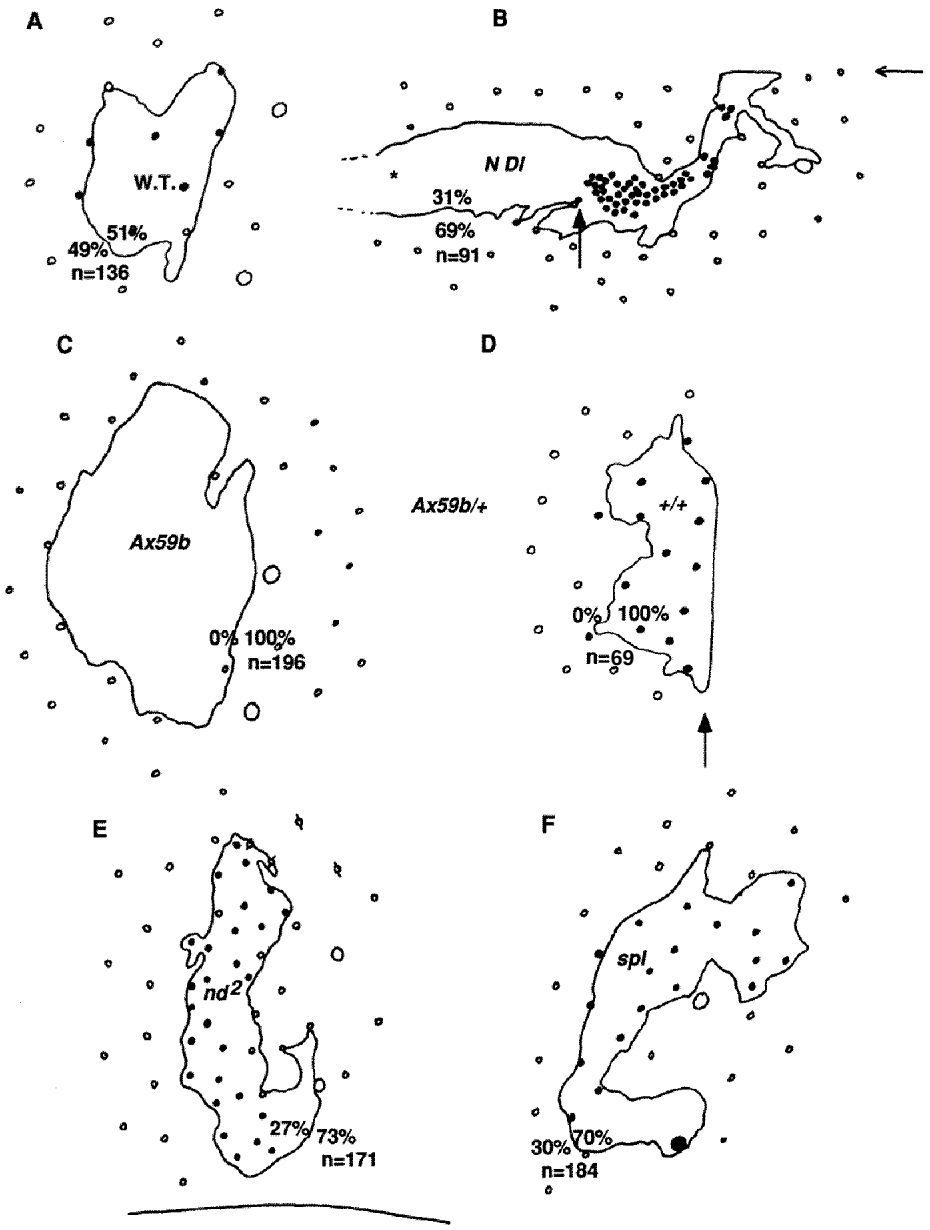

Fig. 3. Camera lucida drawings of mutant clones. Twin clones were made in which the bristles and hairs of the mutant clones are marked with f^{36a} and *mwh*, respectively, and the bristles of the wild-type +/+ twin clones with *y* (see Materials and Methods). Only the mutant f^{36a} clones are drawn. The percentages of marked mutant (f^{36a}) and non-marked (*y* or wild-type) bristles found on the clone margins are indicated (the figure for marked bristles just inside, and the figure for non-marked bristles just outside, the clone border). *n* = the number of bristles scored. On the borders of the control non-mutant clones in A f^{+} and f^{36a} bristles form with equal frequency. This indicates a random choice of fate, epidermal or neural, for wild-type cells. (B) A double mutant *N Dl* clone. Twin spots were not made in this experiment. Both mutant and wild-type bristles are found on the border. The mutant bristles are less frequent but this is probably not significant since both *N* and *Dl* affect the differentiation of the bristle organ itself (Hartenstein and Posakony, 1990) and in these clones the bristles sometimes do not form (Heitzler and Simpson, 1991) as in the area designated by an ∗. Adjacent mutant and wild-type bristles are sometimes found in these clones (arrow). (C) A clone homozygous for Ax^{59b}. Only an occasional bristle is differentiated. Clones homozygous for Ax^{SX1}, Ax^{28} and Ax^{9B2} were also made and a decreased density of bristles is seen for these alleles (see Table 1). *Ax/Ax* clones are surrounded by heterozygous *Ax*/+ cells. In the case shown, all of the bristles formed along the clone margin are of the Ax^{59b}/+ genotype. For Ax^{SX1}, Ax^{28} and Ax^{9B2} the frequency of homozygous mutant bristles found on the borders was 1%, 6% and 12% respectively (*n* = 99, 94 and 88). (D) A wild-type +/+ clone in an Ax^{59b}/+ background. In this instance bristles on the margin are all of the wild-type genotype. (The straight clone border on the right, (arrow), is along the thoracic midline, where the two heminota (formed separately) fuse together). Thus it can be seen that Ax^{59b}/+ cells can differentiate as neural cells when adjacent to Ax^{59b}/Ax^{59b} cells, but differentiate only epidermis when adjacent to +/+ cells. (E) A clone mutant for nd^{2}. The bristle density can be quite different from clone to clone although the average shows an increase (see Table 1). Fewer mutant bristles form on the border. Clones mutant for nd^{1} were also made and these displayed a higher bristle density. 95% of the bristles on the margin of nd^{1} clones were mutant. (F) A clone mutant for *spl*. Here the density of bristles is higher than wild-type and more mutant bristles form on the borders.

Table 1. Density of microchaetae on the notum in flies of different genotypes

	Number of doses Dl^+			
Allele	1	2	2	3
Oregon R	4.2±0.11	5.0±0.1	5.1±0.14	5.2±0.14
Ax^{59b}	20.5±0.55	lethal*	lethal*	lethal
Ax^{SX1}	7.9±0.12	lethal	lethal	lethal
Ax^{9B2}	3.4±0.13	5.8±0.12	5.8±0.12	6.2±0.13
Ax^{28}	3.8±0.14	6.7±0.11	6.3±0.10	6.2±0.11
spl	2.4±0.11	4.7±0.09	4.6±0.11	4.6±0.13
nd^1	4.4±0.13	4.5±0.13	4.9±0.13	4.8±0.14
nd^2	3.2±0.14	5.0±0.15	4.9±0.16	5.2±0.14

Each epidermal cell secretes a single hair. The density of microchaetae was estimated by counting the numbers of intervening hairs. >50 pairs of bristles were scored for each genotype.

*These animals develop to pharate adults that display only an occasional microchaete.

ones. Such heterozygous Ax^{59b}/+ cells, which are frequently able to develop as neural cells when not juxtaposed to wild-type cells, also develop as epidermal cells along these mosaic borders (Fig. 3D). *Abruptex* mutant cells that do have some neural potential are therefore prevented from becoming neural by an inhibitory signal from their wild-type neighbours.

Cells mutant for *Abruptex* do not influence the fate of neighbouring wild-type cells

Along the mosaic borders between wild-type cells and *Ax*/+ cells, the wild-type cells can adopt the neural fate and differentiate bristles (Fig. 3D). This means that the mutant cells have not influenced the fate of the wild-type cells. Since the *Ax*/+ cells adopt the epidermal fate along the border, **all** of the bristles found there are of the wild-type genotype. However, along mosaic borders with homozygous *Ax/Ax* cells, cells of the **same** *Ax*/+ heterozygous genotype produce bristles and simultaneously force the neighbouring *Ax/Ax* cells into the epidermal fate (Fig. 3C). Thus, *Ax*/+ cells adopt either the neural or the epidermal fate depending upon the genotype of their neighbours. They can inhibit *Ax/Ax* cells but are themselves inhibited by +/+ cells.

In order to adopt the epidermal fate *Abruptex* cells require *Delta*

If the *N* protein acts as a receptor for the inhibitory signal, then the gain of function *Ax* alleles, may correspond to constitutively active receptor molecules. The *Ax* phenotype can, however, be quite strongly modified by varying the number of doses of Dl^+, the postulated ligand (Table 1). The density of bristles produced by male flies mutant for different *Ax* alleles in the presence of one, two or three copies of Dl^+ was examined and in all cases the phenotype improves and the number of bristles increases when only one copy of Dl^+ is present. Furthermore male flies mutant for the alleles Ax^{59b} and Ax^{SX1} are lethal but they are rescued to viability when only a single dose of Dl^+ is present. (These two alleles display the lowest density of bristles on the thoraces of heterozygous flies; Table 1). These results suggest that the epidermal fate of *Ax* mutant cells is dependent upon the presence of *Dl*. To demonstrate this unambiguously we have constructed clones doubly mutant for *Ax* and *Dl* and examined their mutant phenotype in the centre of the clones. The phenotype of such a clone is shown in Fig. 1B. The mutant clones resemble Dl^{9P39} clones and produce densely packed bristles (see legend to Fig. 1B). Therefore *Ax* cells require *Dl* in order to take up the epidermal fate and the *Ax* molecules are not constitutive receptors. Rather, they seem to have a greater affinity for *Dl*.

Cells mutant for *split* predominantly adopt a neural fate

The *split (spl)* mutation is also associated with a lesion in the extracellular domain of *N* (Kelley et al., 1987; Hartley et al., 1987). Clones mutant for *spl* on the thorax display a loss of function phenotype: more bristles are formed (Fig. 3F). Along the borders of such clones, bristles are predominantly mutant although wild-type bristles also form (Fig. 3F). The mutant phenotype of *spl* males can also be modified by altering the dosage of Dl^+, and more bristles are formed when the number of copies of Dl^+ is decreased

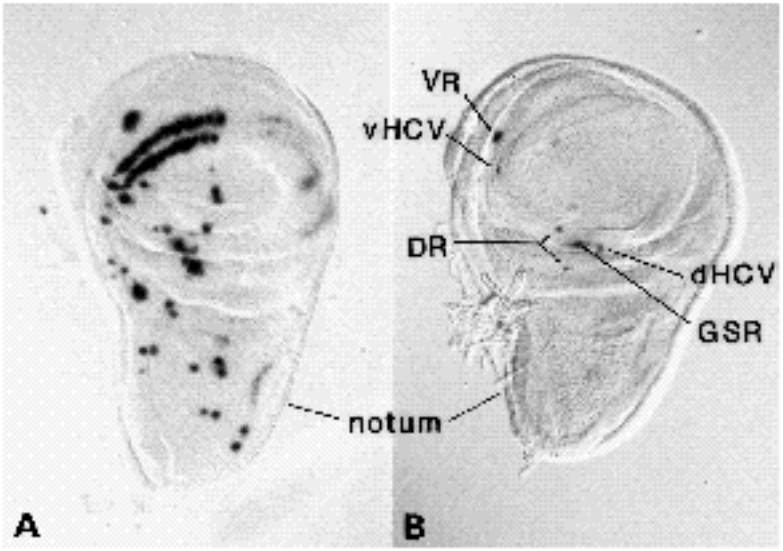

Fig. 4. (A) Photograph of a wild-type thoracic disc taken from a larva at the wandering stage and stained with the enhancer trap insert A101. Eight sensory organ precursors corresponding to the macrochaetae are present in the notum and two rows of adjacent bristle precursors along the wing margin can be seen. (B) Photograph of a thoracic disc from an Ax^{59b} larva at a stage similar to that in A. No bristle precursors are apparent. The stained spots correspond to several sensillae that are not lost in the mutant. For nomenclature of sensory organs see Huang et al. (1991).

(Table 1). Therefore, in contrast to *Ax*, *spl* mutant cells may have a lowered affinity for *Delta* and require more Delta molecules in order to approach the normal bristle density.

Mutations in the intracellular domain of *Notch*

Two alleles of *N* are known to have lesions in the intracellular domain (Xu et al., 1990). These are the recessive alleles *notchoid*^{1} (nd^1) and *notchoid*^{2} (nd^2). Animals mutant for nd^1 show a weak loss of function phenotype and produce a slight excess of neural cells (see legend to Fig. 3E). Animals mutant for nd^2 have a somewhat variable phenotype with a slightly increased or a slightly decreased density of bristles in different regions of the thorax (not shown), but in our collection of nd^2 clones the overall density of bristles is increased (Fig. 3E). Cells mutant for nd^1 can to some extent influence their wild-type neighbours since fewer than the expected number of wild-type bristles are found along the mosaic borders (see legend to Fig. 3E). Cells mutant for nd^2, on the other hand, like *Ax*, fail to prevent their wild-type neighbours from becoming neural and more bristles than expected are of the wild-type genotype (Fig. 3E). Male flies mutant for nd^2 differentiate a greater density of bristles when the dosage of Dl^+ is decreased, whereas nd^1 flies are unaffected by varying the dosage of Dl^+ (Table 1).

DISCUSSION

In the wild-type epithelium, spaced neural precursors are chosen from amongst equipotential cells. It was hypothesised that presumptive neural precursors produce an inhibitory signal that prevents neighbouring cells from realising their neural potential (Wigglesworth, 1940; Doe and Goodman, 1985). In neuroepithelia mutant for recessive loss of function alleles of *N* or *Dl* an excess of neural cells develops at the expense of epidermis (Lehmann et al., 1983). Cells mutant for *N*, but not *Dl*, autonomously adopt the neural fate when adjacent to wild-type cells in mosaic epithelia, it was therefore postulated that the *N* protein may function as a receptor for the inhibitory signal and that the *Dl* protein may constitute the signal itself and act as a ligand for *N* (Hoppe and Greenspan, 1986; Heitzler and Simpson, 1991).

We show here that, on the thorax, cells mutant for the dominant *Ax* alleles of *N* display the opposite phenotype to loss of function alleles and produce fewer or no neural cells and instead adopt the epidermal fate (see also Palka et al., 1990). This suggests that the *Ax* lesions alter the *N* protein in such a way that it becomes hyperactive. The molecular lesions associated with seven *Ax* alleles have been determined and each has a single amino acid change in one of the extracellular EGF-like repeats (Kelley et al., 1987; Hartley et al., 1987). Two lethal alleles, Ax^{59b} and Ax^{59d}, cause changes in a highly conserved cysteine residue required for the disulfide bonds between different repeats that are necessary for the secondary structure. It is likely, then, that the conformation of the extracellular domain is altered in the *Ax* proteins. *Notch* functions as a dimer but the *Ax* lesions do not affect dimerisation (Kelley et al., 1987).

Hyperactive *Ax* molecules require the *Dl* protein in order to send the cells into an epidermal fate. Also, lowering the gene dosage of Dl^+ suppresses the *Ax* phenotype. This suggests that mutant *Ax* cells have an enhanced capacity for ligand binding. Thus they do not behave as constitutive receptors. This is consistent with the observation that the phenotype of some *Ax* alleles is decreased in the presence of extra copies of N^+ (Portin, 1975; Foster, 1975). These experiments therefore provide further evidence for an interaction between the *N* and *Dl* proteins.

Another indication that the *Dl* and *N* proteins interact comes from studies of *Dl*-expressing and *N*-expressing cells that have been shown to bind together in culture (Fehon et al., 1990). These authors have further demonstrated that only the EGF-like repeats, numbers 11 and 12, are required for this adhesion (Rebay et al., 1991). The *Ax* lesions cluster to a 226 amino acid coding region containing six EGF repeats (Kelley et al., 1987). This region includes repeats 23-29 and is therefore some distance away from repeats 11 and 12. In fact, Notch molecules in which all of the extracellular parts of the protein, except for EGF repeats numbers 11 and 12, are missing, have been found to bind more effectively in the cell culture assay (Rebay et al., 1991). So one possible explanation for the behaviour of the *Ax* proteins would be that they display a shape change that renders repeats 11 and 12 more accessible. (The amino acid changes in the *Ax* molecules do not render the mutant repeats more similar to repeats 11 and 12). In contrast, our results suggest that cells mutant for *spl* have a lowered affinity to bind *Dl* and the molecular lesion in *spl* is much closer to repeats 11 and 12: a single amino acid change in repeat number 14 (Kelley et al., 1987; Hartley et al., 1987).

Unlike many cell interactions that involve ligand-expressing cells on the one hand and receptor-expressing cells on the other, the equivalent developmental potential of the neuroepithelial cells means that initially they must be expressing both *N* and *Dl* (Stern, 1954; Doe and Goodman, 1985; Simpson and Carteret, 1990; Kidd et al., 1989; Fehon et al., 1991). This means that prior to the signalling that occurs between the neural precursors and the future epidermal cells, it is first necessary to choose the neural precursor from amongst a group of equipotential cells. This initial choice also requires *N* and *Dl*. We have postulated that the level of receptor (*N*) that a cell is expressing can influence the amount of signal (*Dl*) produced such that if one cell finds itself with a marginally smaller amount of receptor it would consequently produce more signal and an, initially small, bias could thus be introduced which would be amplified with time (Heitzler and Simpson, 1991). Thus, the observation that cells mutant for *N* always inhibit neighbouring wild-type cells means that absence of the receptor results in more signal, as the consequence of a feedback loop between signal and receptor. We show here that in order to inhibit neighbouring wild-type cells, mutant *N* cells require *Dl*. This observation provides further evidence in favour of this model.

The *Ax* mutations cause the opposite phenotype to loss of function alleles, more epidermis and fewer neural cells, but in addition, and unlike N^- cells, they do not inhibit their wild-type neighbours. Along the borders between mutant and wild-type cells, the neural precursors are always pro-

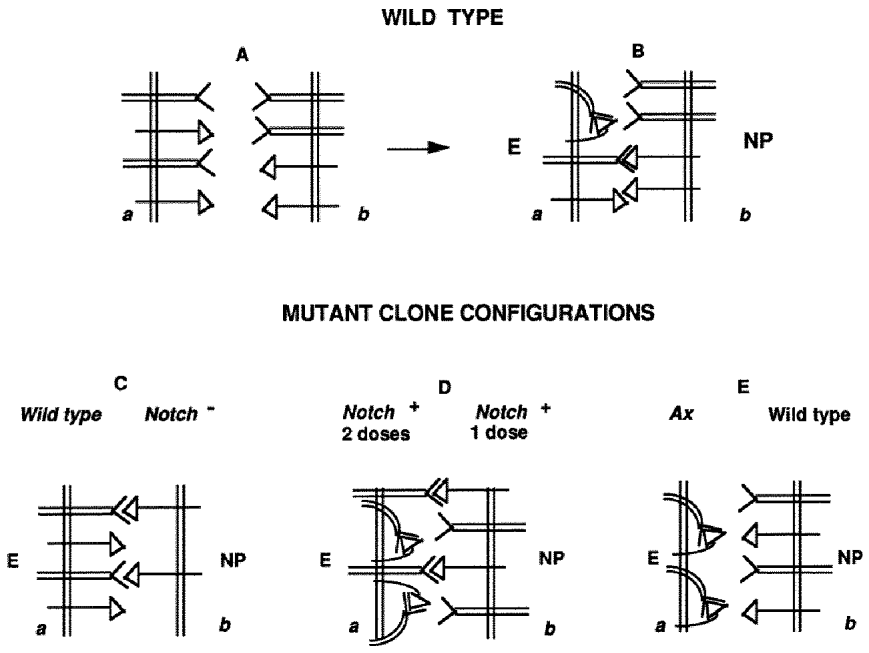

Fig. 5. A molecular model for how the mechanism of lateral inhibition may function. (A) In the wild-type epithelium two equipotential cells *a* and *b* express equal amounts of the receptor protein N (⟩═) and a signal molecule Dl (◁—) that acts as a ligand for N. The N and Dl molecules of opposing cells and also those on the same cell membrane can bind together. (B) In the membrane of cell *a* some of the N molecules are bound to the cell's own Dl. This impairs the cell's signalling ability as those Dl molecules are no longer available to interact with neighbouring cells. Cell *a* with a greater number of bound N molecules adopts the epidermal fate. The possibility of an autocrine signal is thus suggested. Cell *b* with fewer or no bound N molecules becomes a neural cell. (C) A wild-type cell *a* is juxtaposed to a mutant cell *b* devoid of *N* protein. In cell *b* the Dl molecules are always available to interact with the N molecules of neighbouring cells and so this cell sends a strong inhibitory signal. The N molecules of cell *a* are bound to the Dl of cell *b* and so cell *a* becomes an epidermal cell. (D) In a cell with a greater number of N molecules such as cell *a*, the receptor will bind to Dl molecules within the cell's own membrane as well as to its neighbours and so this cell will fail to signal and will become an epidermal cell. Cell *b* with fewer or no bound N molecules will become neural. (E) In the case of the *Ax* molecules of *N*, which seem to show an enhanced affinity for Dl, we postulate that the receptor is altered in such a way as to be able only to bind the Dl molecules within the same cell membrane. This implies the existence of an autocrine signal that would send the *Ax* cell, *a*, into the epidermal fate.

duced by wild-type cells. Furthermore the ability of mutant cells to signal depends on the capacity of neighbouring cells to do likewise. Thus *Ax/+* cells will produce neural precursors when adjacent to *Ax/Ax* cells but adopt the epidermal fate when next to +/+ neighbours. Formally then, when adjacent to wild-type cells, cells mutant for *Ax* behave similarly to cells carrying a larger amount of the normal N^+ product (Heitzler and Simpson, 1991). Therefore, whether due to a greater amount of wild-type protein or to a hyperactive mutant *N* protein, there seems to be a correlation between the level of activity of *N*, and a decreased ability of cells to signal. We have suggested that the *Ax* molecules have an increased affinity for Dl and therefore, enhanced binding is somehow associated with a lowered signalling capacity of the cells.

This has led us to the suggestion that, in addition to the binding of N molecules on one cell to Dl molecules on an adjacent cell, the *N* and *Dl* proteins on the surface of the same cell may interact. After co-transfection of cultured cells with both *N* and *Dl*, the two proteins were in fact found to co-localise (Fehon et al., 1990). Binding of a cell's Dl molecules to its own N molecules would reduce the availability of the *Dl* protein to interact with the N molecules of neighbouring cells and consequently lower the cell's signalling capacity. In a cell with a greater number of N molecules relative to Dl, N would bind to Dl molecules within the cell's own membrane, as well as to those of adjacent cells. In this fashion it would have fewer Dl molecules left to bind to adjacent cells and this would impair the cell's ability to signal (Fig. 5C). However, cells carrying the null allele N^{55e11} and thought to be devoid of protein, signal constitutively (unpublished results). In this case the absence of N would leave the *Dl* protein intact and free to bind to the *N* protein of adjacent cells (Fig. 5B).

There are several possible ways to explain the behaviour of the *Ax* proteins that appear to have an enhanced affinity for the ligand. It is possible that they are altered in such a way as to only be able to bind Dl in certain configurations. For example, they may only bind the Dl molecules of the same cell, which would simultaneously reduce the availability of the cell's Dl molecules to interact with the N receptor of neighbouring cells (Fig. 5D).

In the wild type, competitive protein-protein interactions of this kind could provide a molecular basis for the postulated feedback loop. If a cell finds itself with its N molecules bound to its own Dl molecules then it would signal less efficiently to neighbouring cells and adopt the epidermal fate (Fig. 5A). In our model, cells with the greatest number of bound N molecules, whether linked to Dl from the same cell or adjacent cells, differentiate as epidermis. This raises the possibility that binding of the N and Dl molecules of the same cell could lead to activation of the receptor in an autocrine fashion. At the present time we have no way of testing for an autocrine signal.

It is also possible that *Ax* molecules may no longer be able to bind Dl molecules from the same cell but only those of surrounding cells. If activation of the receptor is only achieved by this paracrine mechanism, then all the bound molecules of N will transduce the signal. In this case it would be necessary to invoke a different mechanism to account for the incapacity of these cells to signal.

Although, clearly, a number of other models could also account for the results, there are some advantages to the idea of competitive protein interactions. The signalling

mechanism that is mediated by *N* and *Dl* allows the cells to choose between alternative fates and eventually one cell of a group adopts the neural fate and the others become epidermal. The decision to be a neural or an epidermal precursor remains labile for a considerable time and following experimental intervention cells can change their fate (Doe and Goodman, 1985; Technau et al., 1988). We have suggested that small relative differences in the number of bound N molecules between cells could cause a bias that would eventually allow one cell to dominate. If this mechanism were to entail competitive protein-protein interactions of N and Dl, both between cells and within the same cell, then the process would be easily reversible and could occur without the need for new synthesis and transport to the membrane of these large proteins. Once established, however, subsequent maintenance of the two developmental programs would probably involve new synthesis of Dl in the neural precursor and of N in the epidermal cells. It might also lead to the repression of *Dl* expression in the future epidermal cells and of *N* expression in the neural precursors. It has in fact been observed that *N* expression disappears from the bristle precursors in the developing wing (Fehon et al., 1991).

If the N and Dl molecules within the same cell membrane interact, it is possible that this requires the participation of the internal domains of the proteins. Binding of the two molecules would probably involve a certain amount of movement laterally within the membrane. In this context it is interesting to note that the 33 amino acid SW16 repeats present in the intracellular domain of N are found in a number of other proteins including ankyrin. The ankyrins are integral membrane proteins that are thought to be involved in the movement of other proteins within the cell membrane (Lux et al., 1990). SW16 repeats are also found in several mammalian transcription factors where they are thought to be involved in protein-protein interactions in various cellular compartments (Thompson et al., 1991; Haskill et al., 1991). They could, therefore, control the association with other proteins and the functional activation of N as a signalling molecule but perhaps also be involved in an association with Dl. For the moment no mutant alleles with lesions that map to the SW16 repeats are known.

We have, however, studied the behaviour of cells mutant for nd^1 and nd^2 whose lesions are in the intracellular domain of the *N* protein. Two lesions are associated with nd^1: a three base pair insertion at the 3′end of the opa repeat that results in the addition of an extra glutamine, and a missense mutation in the codon of amino acid 2453 resulting in a threonine to isoleucine change (Xu et al., 1990). The nd^2 mutant is the result of the deletion of a single base pair in codon 2690 causing a frameshift changing the 14 carboxy-terminal amino acids into a new sequence of 23 amino acids (Xu et al., 1990). $notchoid^1$ has rather little effect on bristle density but is associated with an enhanced signalling capacity as is the case for loss of function protein null alleles. This suggests that in this case nd^1 proteins would not be able to interact with and sequester the *Dl* proteins of the same cell. Consistent with this hypothesis the phenotype of nd^1 is unaffected when the dosage of Dl^+ is varied. $notchoid^2$ animals show a slightly greater or slightly lower bristle density in different areas of the notum. Cells mutant for nd^2 show a very poor ability to inhibit adjacent wild-type cells, suggesting that, in contrast, in these cells the Dl molecules are not available to interact with neighbours. Altogether these results argue in favour of a role of the intracellular domain of *N* for the interactions between receptor and signal molecules within the same cell membrane. Further analysis with *N* proteins in which modifications in specific parts of the protein have been introduced may enable a partial assignment of the different domains to different functions.

There are many parallels between events leading to the segregation of neural precursors in *Drosophila* and the specification of cell fates in the vulva of the nematode. In the latter case a diffusible inductive signal from the anchor cell is capable of specifying different fates amongst the equivalent target VPC cells, depending on the distance of the target cells from the source of the signal. This signal provides the nearest VPC cells with the possibility of taking up the 1° fate, whereas cells further away from the source adopt the 2° fate (see Horvitz and Sternberg, 1991, for review). This is then reinforced by a lateral signal, that is mediated by *lin-12*, a *N*-like protein, and which ensures that only one cell adopts the 1° fate. It is thought that *lin-12* acts as a receptor for the lateral signal (Seydoux and Greenwald, 1989). During selection of the neural precursor in *Drosophila*, it would seem that *ac* and *sc* provide an intrinsic 'inductive signal' for the neural fate that can be considered as the 1° fate for these cells. This signal is also probably graded and may be stronger in the centre of the cluster of equivalent cells. Lateral signalling involving *N* and Dl would then ensure that only a single cell adopts the 1° fate. In gain of function mutants of *lin-12*, isolated VPCs can adopt the 2° fate: single VPCs, in the absence of the anchor cell, produce *lin-12* activity, so *lin-12* is probably active in the 2° cell in an autocrine fashion (Greenwald and Seydoux, 1990).

It is possible that N could function in a similar signalling pathway in many different cell types and the outcome of such cell interactions would depend upon the developmental history of the cells and the regulatory proteins that they are expressing. The existence of highly homologous *N* proteins in other organisms in which both extra- and intra-cellular domains are conserved points to a similar role for *N* in these organisms (Coffman et al.,1990; Weinmaster et al., 1991; Ellisen et al., 1991).

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