Developmental Biology **240**, 361–376 (2001)
doi:10.1006/dbio.2001.0482, available online at http://www.idealibrary.com on IDEAL®

ew metadata, citation and similar papers at core.ac.uk
brought to you by
provided by Elsevier - Publisher Conn

Scabrous Modifies Epithelial Cell Adhesion and Extends the Range of Lateral Signalling during Development of the Spaced Bristle Pattern in *Drosophila*

Olivier Renaud and Pat Simpson[1]

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, 67404 Illkirch Cedex, C.U. de Strasbourg, France; and Department of Zoology, Downing Street, University of Cambridge, CB2 3EJ, Cambridge, United Kingdom

The role of *scabrous* (*sca*) in the evenly spaced bristle pattern of *Drosophila* is explored. Loss-of-function of *sca* results in development of an excess of bristles. Segregation of alternately spaced bristle precursors and epidermal cells from a group of equipotential cells relies on lateral inhibition mediated by *Notch* and *Delta* (*Dl*). In this process, presumptive bristle precursors inhibit the neural fate of neighbouring cells, causing them to adopt the epidermal fate. We show that Dl, a membrane-bound ligand for Notch, can inhibit adjacent cells, in direct contact with the precursor, in the absence of Sca. In contrast, inhibition of cells not adjacent to the precursor requires, in addition, Sca, a secreted molecule with a fibrinogen-related domain. Over-expression of Sca in a wild-type background, leads to increased spacing between bristles, suggesting that the range of signalling has been increased. *scabrous* acts nonautonomously, and we present evidence that, during bristle precursor segregation, Sca is required to maintain the normal adhesive properties of epithelial cells. The possible effects of such changes on the range of signalling are discussed. We also show that the sensory organ precursors extend numerous fine cytoplasmic extensions bearing *Dl* molecules, and speculate on a possible role for these structures during signalling. © 2001 Elsevier Science

***Key Words:* *Drosophila, scabrous,* lateral inhibition, bristle, *Notch, Delta,* filopods, cell adhesion.**

INTRODUCTION

Patterns consisting of regularly spaced elements are a frequent occurrence in nature. The bristles of arthropods and the feathers of birds are good examples. A conserved role for the Notch signalling pathway in the generation of spaced patterns has been demonstrated in many animals (Fehon *et al.*, 1990; Hartenstein and Posakony, 1990; Heitzler and Simpson, 1991; Kidd *et al.*, 1986; Lewis *et al.*, 1998; Lieber *et al.*, 1993; Parks and Muskavitch, 1993; Parody and Muskavitch, 1993; Rebay *et al.*, 1993; Struhl *et al.*, 1993; Viallet *et al.*, 1998; Wharton *et al.*, 1985). During development of the sensory bristles in *Drosophila,* activation of Notch inhibits the bristle fate through repression of the bHLH-encoding proneural genes *achaete* and *scute* (Jennings *et al.*, 1994, 1995). *achaete* and *scute* are first expressed in discrete proneural fields of cells each with neural potential (Cubas *et al.*, 1991; Skeath and Carroll, 1991). All cells in the proneural field express both the receptor Notch, and its ligand, Delta, and have the means to transduce the signal downstream of activated Notch (Kimble and Simpson, 1997; Kooh *et al.*, 1993). Thus, each cell can be either a signalling or receiving cell. Ultimately, spaced, nonadjacent cells will become strong signalling cells and activate Notch in the cells surrounding them, which in turn become receiving cells. Activation of the receptor is linked to production of the ligand, since *Delta* is regulated by Achaete-Scute (Heitzler *et al.*, 1996; Kunisch *et al.*, 1994). This feedback loop can amplify any small difference between the cells. Such differences may arise through random fluctuations in amounts of ligand or receptor, or through the introduction of an external bias (Jan and Jan, 1995; Simpson, 1997). The differences are then reinforced by positive regulatory loops (Culi and Modolell, 1998; de Celis *et al.*, 1997; Wilkinson *et al.*, 1994).

[1] To whom correspondence should be addressed. Fax: 44 1223 33 66 76. E-mail: pas49@cam.ac.uk.

0012-1606/01 $35.00
© 2001 Elsevier Science
All rights reserved.

The inhibitory signal must act over several cell diameters because bristles are separated by about five epidermal cells. At the time of selection of the bristle precursor cells, there are only two or three intervening epidermal cells, not all of which are in contact with a precursor, but these divide again (Usui, 1993). The inhibitory signal must be maintained over this longer range, since the higher density of bristles, each separated by one or two cells, seen in hypomorphic *N* and *Dl* mutants suggests that the range of signalling is reduced (Hartenstein and Posakony, 1990; Heitzler and Simpson, 1991; Parks *et al.,* 1997). Indeed, Notch signalling in many other animals must extend over a longer range. This is puzzling because the ligand, Delta, is thought to be a membrane-bound protein. Delta contains nine EGF-type repeats in the extracellular portion and a small intracellular domain without homology to other known proteins (Chitnis *et al.,* 1995; Henrique *et al.,* 1995; Kopczynski *et al.,* 1988; Vassin and Campos-Ortega, 1987). Recently, proteolytic cleavage of Delta into several different isoforms has been demonstrated (Klueg *et al.,* 1998; Qi *et al.,* 1999). The generation of a soluble form of the ligand by cleavage would be one means by which the range of signalling could be extended. However, so far there has been no demonstration of diffusion or transport of a cleaved form of Delta. Rather, recent, extensive studies on the cellular trafficking of Delta and Notch suggest that cleavage of Delta is part of Delta inactivation and down-regulation of Notch–Delta signalling following receptor activation (Klueg *et al.,* 1998). Furthermore, work by Parks *et al.* (2000) suggests that activation of the receptor may require a membrane-bound form of Delta. These results are consistent with earlier observations, that, in *Drosophila* and vertebrates, Delta proteins lacking the intracellular domain display dominant negative behaviour (Chitnis *et al.,* 1995; Jen *et al.,* 1997; Klueg *et al.,* 1998; Sun and Artavanis-Tsakonas, 1996).

In this paper, we explore the role of *scabrous* in bristle spacing in *Drosophila. scabrous* encodes a protein with a domain related to fibrinogen, similar to that of the tenascins, vertebrate neuronal extracellular matrix proteins (Baker *et al.,* 1990; Ellis *et al.,* 1994; Mlodzik *et al.,* 1990). It acts during eye development and is thought to play a role in spacing of the ommatidia (ibid). *scabrous* mutants display a phenotype similar to hypomorphic *Notch* and *Delta* mutants with an excess of bristles (Baker *et al.,* 1990). It has been shown that *scabrous* is regulated by Achaete-Scute (Mlodzik *et al.,* 1990) and that it encodes a secreted protein (Hu *et al.,* 1995; Lee *et al.,* 1996). Furthermore, an association of Notch and Scabrous, resulting in stabilisation of the *Notch* protein, has been demonstrated, suggesting that the function of Scabrous is linked to the Notch signalling pathway (Powell *et al.,* 2001). It is thus a good candidate to be involved in inhibitory signalling over a range of several cell diameters.

Our results demonstrate that *scabrous* is not required for inhibition of cells situated adjacent to the bristle precursor cells but is needed, nonautonomously, for inhibition of cells not adjacent to the precursor. Furthermore, signalling over this "longer" range requires both Scabrous and Delta. We also show that Scabrous modifies the localisation of junction and adhesion proteins in the epidermal cells surrounding the bristle precursors. Finally, we describe fine cytoplasmic extensions bearing Delta, radiating from the bristle precursor cells, and discuss the possibility that they may allow membrane-bound Delta to reach more distant cells.

MATERIALS AND METHODS

Fly Strains

The wild-type stock was Oregon-R. For a description of mutants and inserts employed see Lindsley and Zimm (1992) and FlyBase Consortium (1999). The Gal4 and UAS lines used were: *sca*$^{537\text{-}4}$-*GAL4* (Hinz *et al.,* 1994), *pannier*^{MD237}-*Gal4* (Calleja *et al.,* 1996), *UAS-sca* (Ellis *et al.,* 1994), *neuralized-GAL4* (Jhaveri *et al.,* 2000; Venugopala Reddy *et al.,* 1999), *UAS-CADH*^{intra,3} (Sanson *et al.,* 1996), *UAS-lacZ* (Brand and Perrimon, 1993), and *UAS-GFP* (Brand and Perrimon, 1993).

Flies were raised on standard medium and maintained at 25°C.

Quantitative and Statistical Analysis

The distribution of microchaetes was examined under a microscope and recorded for each fly. Data from males and females were found to be similar and were pooled. The number of bristles varies from fly to fly, but within a range characteristic for each genotype. Drawings were made of each clone in mosaic flies by using a camera lucida. Data from the independent cultures were compared by using a Student's *t* test. Tests of significance were performed by using a paired, two-tailed, *t* test. Results were considered significant when a *P* value of 0.05 or less was returned.

Production of Mosaic Animals

Mutant clones were produced by mitotic recombination induced either by X-rays or by the FLP/FRT method (Golic, 1991; Golic and Lindquist, 1989; Xu and Rubin, 1993). Twenty-four-hour egg collections were made and larvae were X-rayed (1000R) or heat-shocked (1 h, 37°C) between 24 and 48 h after egg laying. Clones were marked with *pawn* (*pwn*), which labels bristles and epidermal cells. For a description of these markers, see Lindsley and Zimm (1992). Clones were induced in flies of the following genotypes: X ray-induced: *pr pwn/ pr pwn FLP*^{38}; *FRT82B kar*^{2} *ry*^{506} *Dl*^{RevA3}/ *FRT82B kar*^{2} *ry*^{506} *bx*^{34e}; *Dp(2;3)P*^{32}*[pwn*$^{+}$*]*; *pr pwn/ pr pwn FLP*^{38}; *FRT82B kar*^{2} *ry*^{506} *Dl*^{9P39}/ *FRT82B kar*^{2} *ry*^{506} *bx*^{34e}; *Dp(2;3)P32[pwn*$^{+}$*]*. FLP/FRT-induced: *y FLP*^{1}*/Y; FRT42D pwn y*$^{+}$*/ FRT42D P[w*$^{+}$*; hs-πM]45F; y FLP*^{1}*/+; FRT42D pwn sca*^{BP2}*/FRT42D P[w*$^{+}$*; hs-πM]45F.*

Immunohistochemistry and Cuticle Preparation

White prepupae were collected and left at 25°C in a moist chamber. Age is given in hours after puparium formation (APF). Staged pupae were dissected and stained as described previously (Usui and Simpson, 2000). Primary antibodies used in this study were guinea-pig polyclonal antiserum against the Delta extracellu-

© 2001 Elsevier Science. All rights reserved.

lar domain (1:4000 dilution, GP581, DSHB; Huppert *et al.,* 1997), mouse monoclonal antibody against Cut (1:200 dilution, 2B10, DSHB; Blochlinger *et al.,* 1990), mouse monoclonal supernatant against Scabrous used at 1:20 dilution (mAbsca1, DSHB; Lee and Baker, 1996), rat monoclonal antibody to *D*E-cadherin (1:40 dilution, DCAD2; Uemura *et al.,* 1996), rabbit antibody against HRP (1:200, Jackson; Jan and Jan, 1982), and rabbit antibody against GFP directly coupled with Alexa 488 (Molecular Probes). Secondary antibodies coupled with biotin, streptavidin-Cy3 (Jackson), HRP (Jackson), and Alexa Fluor (Molecular Probes), were used at 1:400. DAPI was used at 1:400. Nota were mounted in Fluoromount-G (Southern Biotechnology Associates), were observed by using a confocal microscope (Leica), and were processed with TCSTK software (developed by J.-L. Vonesch).

X-gal staining of pupal nota was performed as described in Ashburner (1989). For observation of cytoplasmic extensions, staged pupae were dissected, mounted, and directly observed by using both fluorescence and confocal microscopy. For examination of cuticles, nota of adult flies were dissected, dehydrated, and mounted in Euparal (Usui and Simpson, 2000).

Sections for Light and Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) overnight at 4°C and washed in the same buffer for 30 min, followed by postfixation for 1 h at 4°C in 1% osmium tetroxide with cacodylate buffer. They were then dehydrated in alcohol and embedded in Epon 812. For light microscopic analysis, semi-thin sections (2 μm) were stained with toluidine blue. Ultrathin sections (70 nm) were contrasted with uranyl acetate and lead citrate, and examined with a Philips 208 electron microscope.

RESULTS

Loss-of-Function of scabrous Results in More Closely Spaced Bristles; Gain-of-Function Causes Bristles to Be Spaced Farther Apart

The phenotype of *scabrous* was examined by counting the number of bristles on the scutum as well as the number of epidermal cells between bristles (Fig. 1). There is an excess of both macro- and micro-chaetes. Animals heterozygous for the null allele *sca*^{BP2} (Mlodzik *et al.,* 1990) display a bristle density that is slightly higher than the wild type; this is markedly increased in animals homozygous for *sca* mutants (Fig. 1). In addition to their higher density, the bristles of *sca* mutants are less evenly spaced than those of the wild type, and the acrostichal microchaetes fail to align into rows (Figs. 1A, 1D and 1E). Occasionally, in homozygotes of the null allele *sca*^{BP2}, double-bristle shafts and/or sockets are observed (Figs. 2A and 2B). Close examination revealed that these structures are due to abnormal fate assignments of the five cells of a single bristle organ. Staining with anti-Cut and anti-HRP indicates the presence of some bristle organs bearing two or more shafts or sockets at the expense of the neuronal and sheath cells (Figs. 2C and 2I). In addition to double shafts or sockets, *sca*^{BP2} homozygotes also display naked patches that are interspersed with areas of densely packed bristles (Fig. 1D). However, no naked patches are seen after staining with *neu*^{A101}*, lacZ* (Fig. 1B), or anti-Cut and anti-HRP (not shown), simply a very large excess of precursors. So, in some bristle organs, it is the cuticular parts that are missing as a result of transformation of the presumptive trichogen and tormogen cells. It is also noteworthy that the precise, consistent spatial arrangement of the different cells of the wild-type bristle organ is lost in the mutant (Fig. 2G), and, in addition, cells from a single lineage are occasionally situated at some distance from one another (Fig. 2E).

Thus, *sca* plays a role in both precursor segregation and the development of bristle organs; therefore, quantification of the external bristles alone as a means of comparison between different mutant alleles is misleading (Lee *et al.,* 1998). This explains why the density of bristles seen on the cuticle of *sca*^{BP2} null homozygotes is not as great as that seen in hypomorphic combinations such as *sca*^{BP2}/*sca*^{1} flies (Figs. 1D and 1E; Baker *et al.,* 1990).

We looked at the effects of over-expression of *sca* in the medial part of the notum using the GAL4-UAS system and the driver *pannier*^{MD237} (Brand and Perrimon, 1993; Calleja *et al.,* 1996). High levels of expression of *sca* are obtained with this driver (compare Fig. 3A with Fig. 5F). Over-expression in a wild-type background causes a slightly disorganised bristle pattern, but the bristles are much more evenly spaced than in loss-of-function mutants, and there are very few abnormally differentiated bristle organs and no naked patches (Fig. 1F). Notably, bristle density is decreased when compared with the wild type and there is a corresponding increase in the number of cells between bristles (Fig. 1F). So, with respect to the numbers of precursors segregating, over-expression on the notum results in a phenotype opposite to that observed in loss-of-function mutants. This phenotype requires the presence of the endogenous protein. No rescue of the mutant phenotype is seen within the *pannier* expression domain after over-expression of *sca* in *sca*^{BP2} flies (Fig. 1G). This suggests that uniform expression of *sca* is unable to mediate normal bristle spacing and that the small local differences in levels of Sca, due to regulation of the endogenous gene, are important.

Dynamics of scabrous Expression and Segregation of Bristle Precursors

Examination of *sca*^{BP2} homozygotes with *neu*^{A101}*, lacZ* that labels the bristle precursors, shows that an excess of more closely spaced microchaete precursors are generated during the early pupal stage. Precursor formation is complete in both wild-type and mutant nota by 15 h after pupariation (Fig. 1B). All of the precursors in the mutant, including the supernumerary ones, appear to form together over a short period of time. We were unable to detect an early stage with wild-type numbers of precursors, followed by a later stage with an excess of precursors. However, it is to be noted that precursor segregation is not synchronous over the entire notal epithelium, and so subtle timing

© 2001 Elsevier Science. All rights reserved.

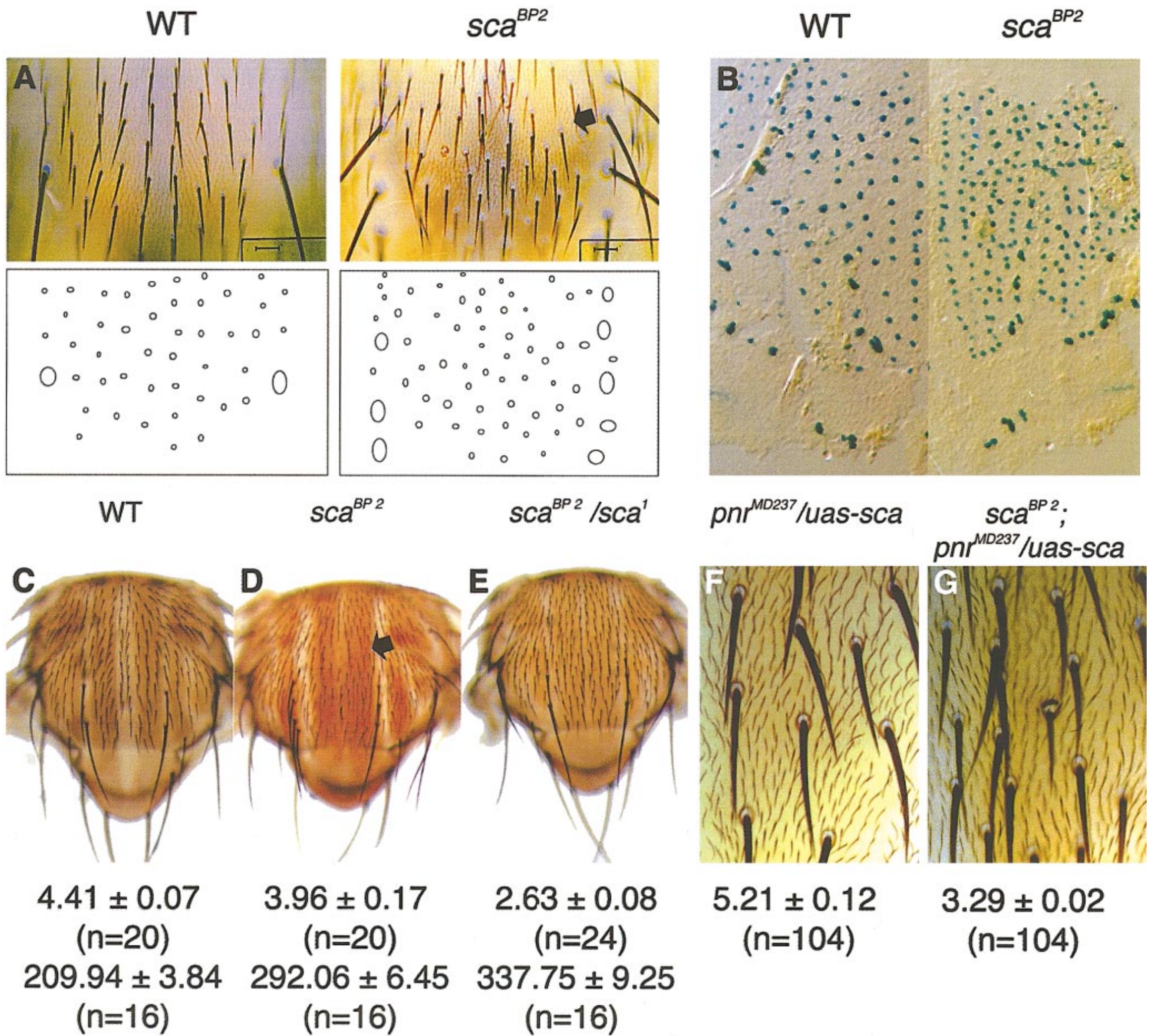
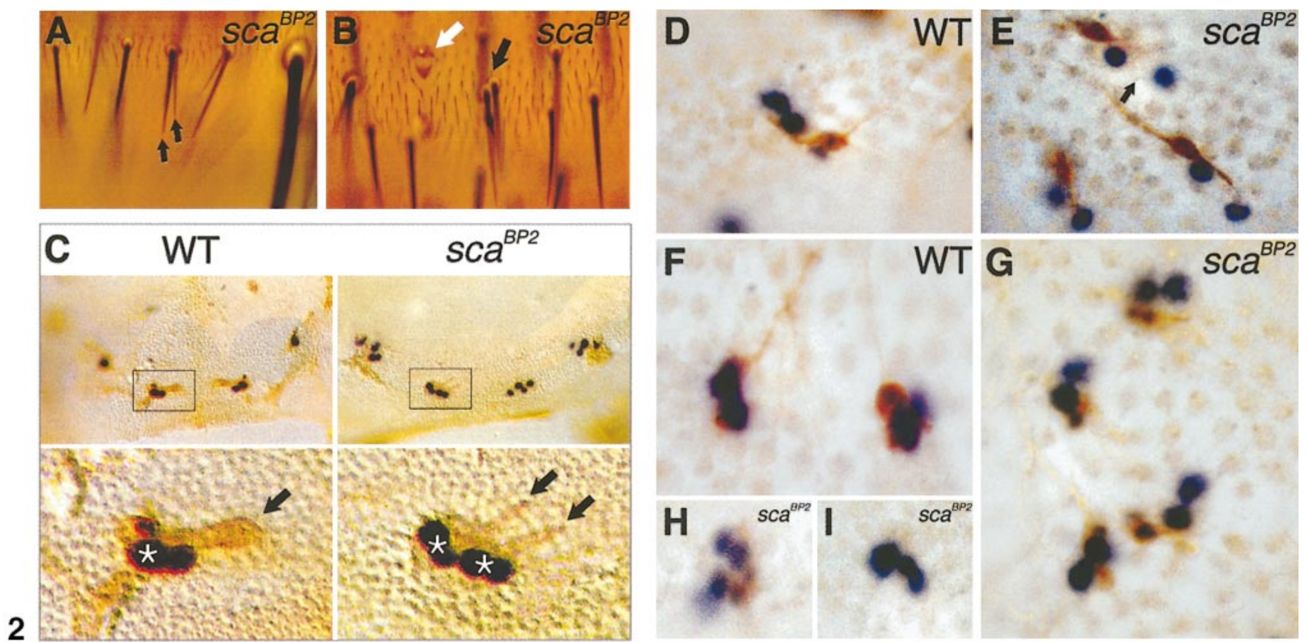


FIG. 1. The bristle phenotype of *scabrous.* Nota of flies displaying *sca* phenotypes. Anterior is up on all panels. (A) The acrostichal region between the dorso-central bristles of the wild type and the null mutant sca^{BP2}. Note the presence of some naked patches (arrow) and the absence of bristle rows in the mutant. (B) Wild-type and sca^{BP2} nota at 15 hours APF, stained for neu^{A101}, a marker for the bristle precursors. An excess of more closely spaced precursors is seen in the mutant and no naked patches are present. (C–G) The bristle pattern of flies of different genotypes. The driver pnr^{MD237} was used to over-express Sca in wild-type (F) and mutant (G) flies; an enlargement of the acrostichal region is shown. The average distance, in number of epidermal cells, between microchaetes in the acrostichal region, as well as the total number of microchaetes on the scutum (C–E only), are shown below; each genotype displays a significant difference when compared to the wild type ($P < 0.05$, see Materials and Methods). The density of bristles is increased in all cases except (F) where it is decreased. Note, however, that naked patches are not seen in (F). A haplo-insufficient effect was also observed in flies heterozygous for sca^{BP2} (239 ± 6 bristles per scutum, $n = 36$).

differences in successive events may have escaped detection.

As previously described (Mlodzik *et al.,* 1990), we find that in the notum *sca* is expressed in a pattern similar to that of *ac-sc:* in proneural clusters before formation of the macrochaete precursors, in proneural stripes before forma-

© 2001 Elsevier Science. All rights reserved.



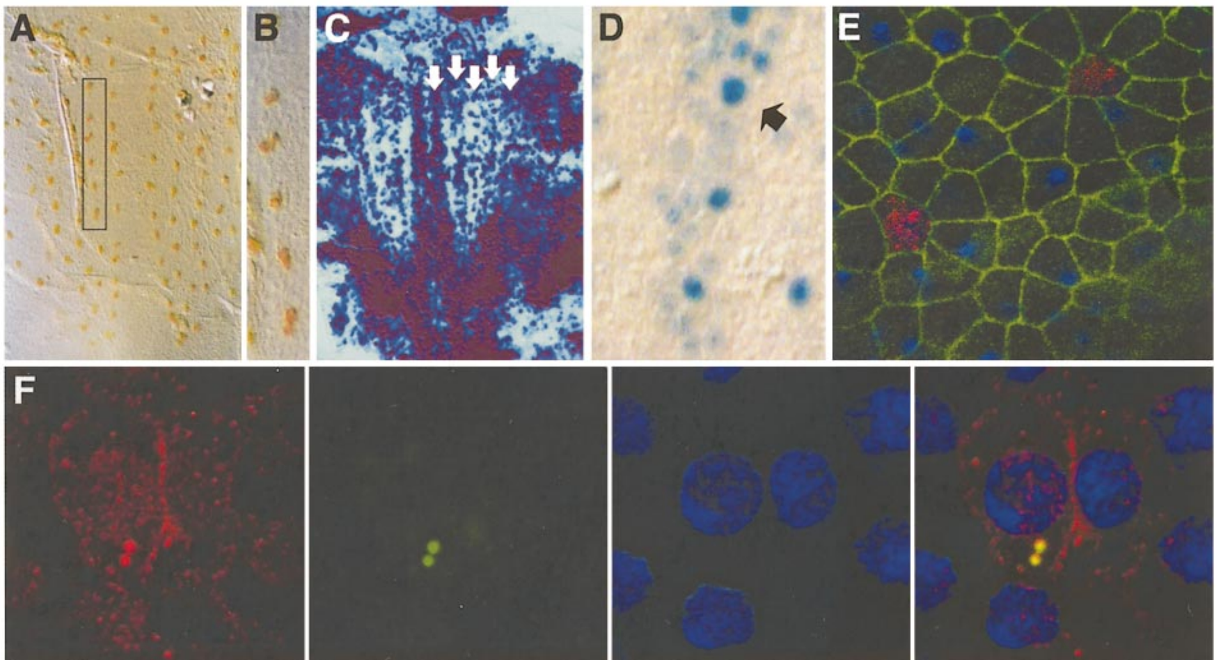


FIG. 2. The phenotype of bristle organs in *scabrous* mutants. (A, B) Cuticle of mutant flies showing abnormalities in the external structures of bristles. Black arrows point to duplicated shafts and sockets, the white arrow to an organ with duplicated, fused, sockets but no shaft. (C–I) Anti-Cut (black) and anti-HRP (brown) staining of wild-type and sca^{BP2} pupal nota 28 h APF. (C) The posterior scutellar macrochaete. Two socket and two shaft cells (asterisks) are visible in the mutant but the neuron is absent. (D–I) Acrostichal microchaetes. The precise spatial arrangement of cells of the bristle organ characteristic of the wild type is lost in the mutant where the four cells appear to be randomly arranged. (E) The trichogen and tormogen cells are no longer juxtaposed (arrow). (I) The neuron is missing.

FIG. 3. Expression pattern of *scabrous* in pupae. (A) Anti-Sca staining of a pupa at 16 h APF. (B) An enlargement of the microchaetes of row 2. Scabrous is localised in the bristle precursor. Double staining experiments show that Sca and Cut (antibody), that labels precursor cells, are coexpressed in the sensory precursors (data not shown). Note that some of the precursors have divided. (C) β-Galactosidase staining of a *sca-GAL4; UAS-lacZ* pupa at 12 h APF, showing the residual expression in the stripes corresponding to the rows of microchaetes (arrows). These are only visible after prolonged incubation. (D) β-Galactosidase staining of a *sca-lacZ* pupa at 15 h APF after prolonged incubation. Scabrous is strongly expressed in the microchaete precursor cells (arrow). Note that a ring of about nine epidermal

© 2001 Elsevier Science. All rights reserved.

tion of the microchaete precursors, and then in the precursors themselves (Fig. 3). Staining with an anti-Sca antibody, however, only reveals the strongest accumulation of protein in the bristle precursors; the protein is not detected in proneural domains (Figs. 3A and 3B). Double staining for Sca and *neu-lacZ* indicates that the two are coexpressed in the same cells (not shown). The use of *sca-GAL4; UAS lacZ* flies, on the other hand, allows detection of *sca* activity in the proneural domains, presumably due to the slow turnover and accumulation of Gal4 and galactosidase over time (Fig. 3C). Figure 3D shows staining in the proneural stripe corresponding to one of the microchaete rows where single precursors expressing high levels of *lacZ* can be seen. Note that each precursor is surrounded by a ring composed of eight or nine epidermal cells that appear to be arranged in a particular fashion. Staining with an antibody against *D*E-cadherin reveals an organisation of these cells into a rosette-like structure (Fig. 3E). However, when visualised with anti-*D*E-cadherin, these supracellular structures appear to be transient since they are only visible for some precursors in any one preparation. Rosettes were not seen in *sca* mutants, but this could be a consequence of the close spacing of precursors.

As in the eye (Baker and Zitron, 1995; Hu *et al.,* 1995), we observe that much of the Sca protein is in aggregates, possibly vesicles, in the cytoplasm. Rather little Sca could be visualized on cell membranes in our preparations. The expression of *Dl* on the notum is similar to that of *sca* (Parks *et al.,* 1997) and in fact we find that Sca often colocalises with Dl in aggregates inside the bristle precursor cells (Fig. 3F). Accumulation of Dl in vesicles has been previously described (Kooh *et al.,* 1993) and it is likely that our preparations reveal vesicles containing the two proteins.

We verified that *Dl* is expressed in *sca* mutants by means of an antibody to Dl and could see no apparent difference in *Dl* expression between wild-type or mutant imaginal discs and pupal nota (not shown).

Delta Signalling Can Extend Over Several Cell Diameters

Null mutants of *Notch* (*N*) or *Delta* (*Dl*) differentiate exclusively neurons on the notum (Hartenstein and Posakony, 1990; Heitzler and Simpson, 1991). So the ability of mutant cells to differentiate as epidermis when juxtaposed to wild-type cells was used as a test for autonomy. *Notch* was found to act autonomously since mutant cells could not be rescued to differentiate epidermis. In contrast, epidermal cells mutant for *Dl* were observed at the border with wild-type tissue. This indicates that cells mutant for *Dl* can be rescued by the presence of neighbouring wild-type cells, so demonstrating that *Dl* acts non-autonomously (Heitzler and Simpson, 1991). Strong hypomorphic mutants of *Delta* (*Dl*) display a phenotype of adjacent bristles with no intervening epidermal cells (Fig. 4A; Heitzler and Simpson, 1991). Rescue is observed round the edges of the clones where a band of mutant epidermal cells differentiate. Here, we have examined the extent of the rescue across the borders of clones mutant for Dl^{RevA3} and Dl^{9P39} cells (Heitzler and Simpson, 1991). We find that the band of mutant epidermis round the edges of the clones is on average 4.2 ± 0.29 ($n = 17$) cells wide. This is close to the distance separating bristles in the wild type. This almost complete rescue can also be measured by counting the number of cells between mutant bristles inside, and wild-type bristles outside, the clone, i.e., across the mosaic border. The number of intervening cells was found to be very similar because wild-type bristles are often located right on the clone border (Fig. 4A). These results indicate that Dl signalling from the wild-type cells can extend over several cell diameters.

scabrous Acts Nonautonomously

It was previously shown that *sca* acts nonautonomously in the eye (Baker *et al.,* 1990), so we checked for nonautonomy in bristle development by using clones of the null allele sca^{BP2}. *scabrous* loss-of-function mutants display an increased number of bristles but with epidermal cells in between. Therefore, unlike *N* and *Dl* mutants, the presence or absence of epidermis cannot be used as a test for autonomy. As a measure of rescue by wild-type cells, we looked at the number of epidermal cells between bristles, since in the mutant there are many fewer than in the wild type. The number of epidermal cells between mutant and wild-type bristles on either side of the mosaic border is shown in Fig. 4B. The result shows that, as in the case of *Dl,* there is a complete rescue: the distance is equivalent to that seen between wild-type bristles. Furthermore, we looked at the relative contribution of mutant and wild-type cells to the intervening epidermis. We found that epidermal cells of both genotypes contribute to the space between bristles (see legend to Fig. 4). We thus conclude that *sca* acts nonautonomously during bristle spacing.

---

cells are in contact with each precursor. (E) View of the epithelium of a 15-h pupa stained for Sca (red), *D*E-cadherin (green), and DAPI (blue). Epithelial cells around the precursor are arranged in a rosette like fashion. (F) Confocal optical section of nota dissected from staged pupae at 15 h APF showing a microchaete precursor located in the acrostichal region. Double labelling reveals that Dl (red) and Sca (green) colocalise. Nuclear staining (DAPI) is in blue. The image on the right shows a merge of all three. Sca and Dl are localised in the cytoplasm of the cell. Note that Sca and Dl colocalise in both planes (the z-axis is not shown).

© 2001 Elsevier Science. All rights reserved.

Bristle Organs Are Never Adjacent in scabrous Mutants, Each Bristle Is Surrounded by Epidermal Cells

One important observation is that, although there is an excess of bristles, the bristle organs are never adjacent in *sca* mutants. They are always separated by at least one epidermal cell. Apparent cases of double bristles are attributable to abnormal differentiation of the bristle organ as described above. This means that *sca* is not needed for the generation of an alternating pattern of epidermal and neural cells. Such an alternating pattern is achieved by lateral inhibition. Lateral inhibition requires N and Dl and it has previously been shown that a regulatory loop involving these two molecules mediates the choice between epidermal and neural fates that prevents neural precursors from developing adjacent to one another (Heitzler and Simpson, 1991). The requirement for these two genes can be visualised along the borders of mutant clones, where mutant and wild-type cells are in contact. When next to *Dl* mutant cells, the wild-type cells adopt the neural fate; when next to *N* mutant cells, the wild-type cells adopt an epidermal fate (Fig. 4A; Heitzler and Simpson, 1991). Thus, at the time when they are choosing between neural and epidermal fates, these cells must have assessed the presence or absence of the *Dl* and *N* proteins in their neighbours, implying that these proteins play a role in the choice of cell fate.

If Sca were to act together with Dl as the signal involved in the segregation of single-spaced precursors, we would expect a bias in favour of wild-type bristles along the borders of *sca* mutant clones. In fact, both mutant and wild-type bristles can form along the borders of sca^{BP2} clones (Fig. 4B; Baker *et al.,* 1990). Unexpectedly, more mutant bristles are seen than wild-type ones. This may be simply attributable to the excess of mutant bristles. If the density of bristles in a mutant clone is greater than in the surrounding tissue, and if there is no bias for either genotype along the border, then one might expect to find a greater number of mutant bristles relative to wild-type ones along the border. We conclude that, in the case of *sca* mosaics, neural precursors can be chosen from cells of either genotype and that therefore cells choose a neural or epidermal fate regardless of whether or not they express *sca.* Thus, *sca* is not required to generate a pattern of spaced precursors, which would explain the fact that bristles are never adjacent in *sca* mutants.

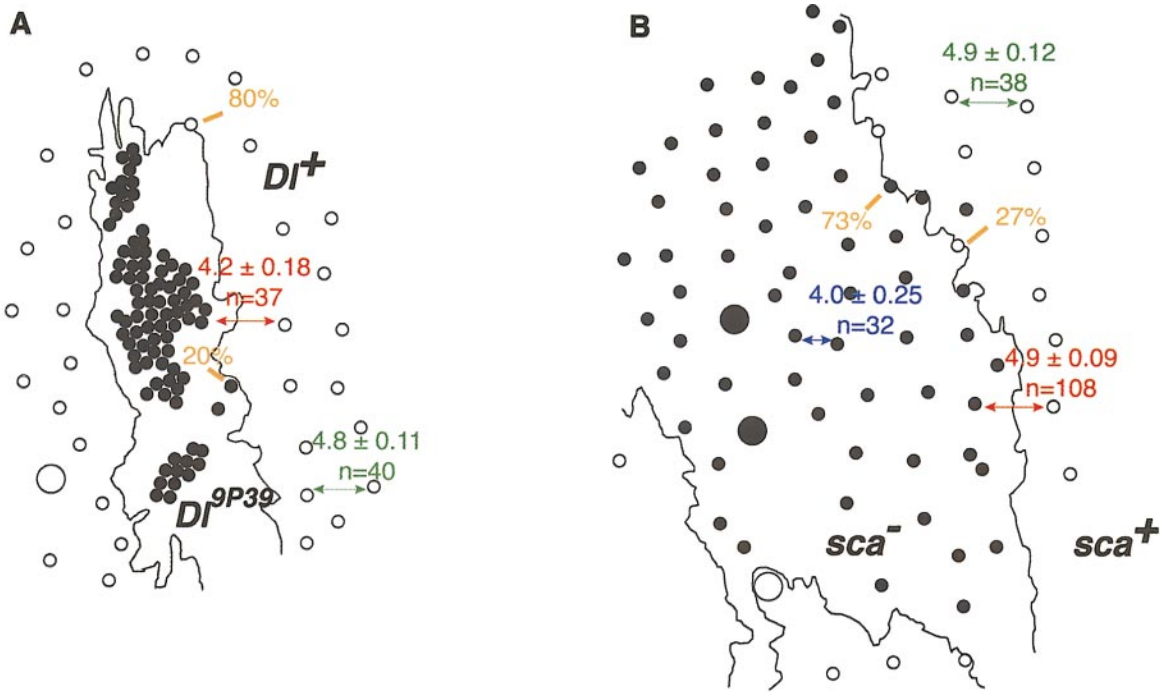
The Epithelium Is Disorganised in scabrous Mutants and the Distribution of DE-Cadherin Is Impaired

As cells mutant for *scabrous* behave nonautonomously and the *sca* protein is only detectable in the bristle precursors, Sca is likely to act on the epithelial cells surrounding the Sca-producing precursors. We examined the distribution of *D*E-cadherin in third instar imaginal wing discs and pupal nota (Kemler, 1993; Oda *et al.,* 1994; Takeichi, 1988; Woods *et al.,* 1997). In wild-type flies, confocal images of staining with an antibody to *Drosophila D*E-cadherin (Oda *et al.,* 1994) reveals an even hexagonal array of cell membranes (Fig. 5A). The protein is mainly located at the apical end of the cells at the level of the adherens junctions (Oda *et al.,* 1994; Woods *et al.,* 1997). Double staining with DAPI indicates that nuclei are located basally to these junctions. In *sca* mutants, *D*E-cadherin staining is much less intense, and does not appear to be localised at the apical ends of the

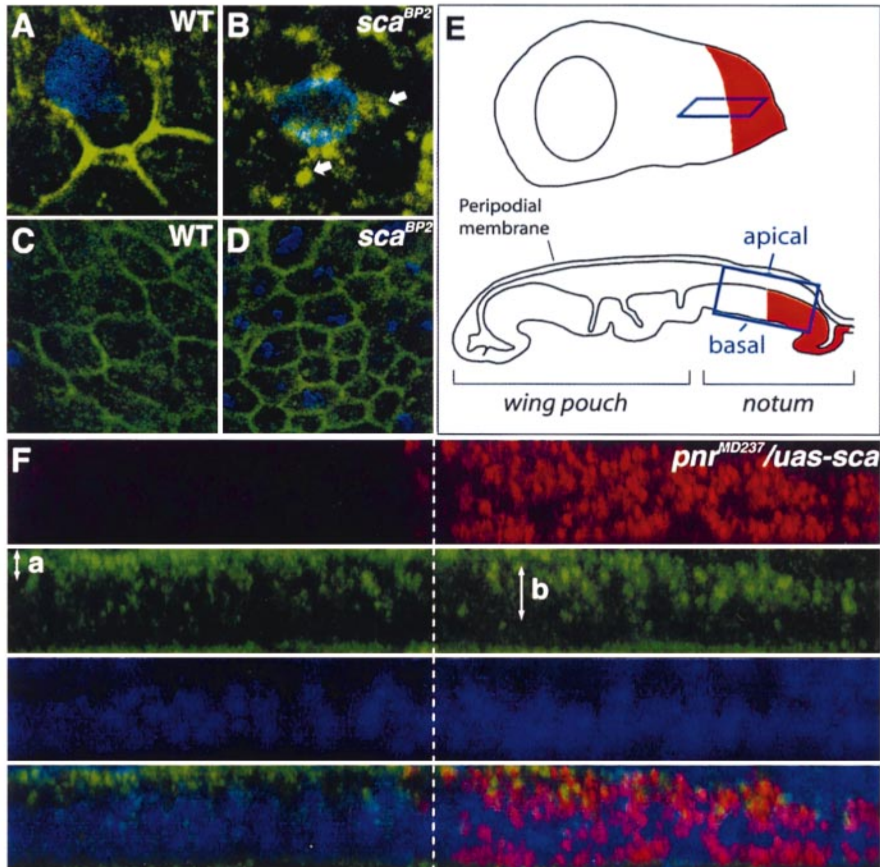
FIG. 4. Camera lucida drawings of mutant clones. (A) A Dl^{9P39} *pwn* clone. (B) A sca^{BP2} *pwn* clone. Mutant and wild-type bristles are represented by closed and open circles, respectively. Numbers and arrows indicate the average distance between bristles outside the clone (green), inside the clone (blue), and across the clone border (red). Note that, for both genotypes, the distance between microchaetes across the mosaic border is not significantly different from that measured for bristles outside. Both mutant and wild-type epidermal cells contribute to the space between mutant bristles inside, and wild-type bristle outside, the clone (on average 2.6 ± 0.21 ($n = 40$) wild-type and 2.12 ± 0.22 ($n = 40$) mutant cells were measured compared to 2.56 ± 0.27 ($n = 34$) wild-type and 2.29 ± 0.24 ($n = 34$) marked cells in a control clone). The frequency with which mutant bristles are found adjacent to wild-type hairs, along the clone borders, and vice versa, is given in orange, as a percentage of the total number of bristles scored [$n = 44$ for (A) and $n = 234$ for (B)]. Nonmutant control clones marked with *pawn* in the wild type, show values of 55.4 and 44.6%, respectively (not shown, $n = 171$). Along the mosaic border of clones mutant for *Dl* (A), the bristles are mainly wild type and mutant cells form epidermal hairs only along the edges of the clones.

FIG. 5. Mislocalisation of junctional proteins in *scabrous* mutants. (A–D) Confocal images of imaginal discs of third instar larvae (A, B) and pupal nota at 15 h APF (C, D). (A, B) Double labelled for *D*E-cadherin (green) and neu^{A101} which labels the precursor cells (blue). It can be seen that the distribution of *D*E-cadherin is irregular and punctate in the *sca* mutant when compared to the wild type. The white arrows indicate accumulation of the protein in clumps. The shape and size of cells are also quite variable in the mutant when compared to the wild type, as seen in (C) and (D), which are doubly labelled for *D*E-cadherin (green) and DAPI for nuclei (blue). Nuclei are expected to be located at a basal level of the cell but can be seen in a number of mutant cells in these images which were taken at an apical plane. (E) Drawing of the wing imaginal disc indicating the position of the cross sections (blue rectangle) made, giving the vertical optical reconstruction which is shown in (F). (F) Vertical optical reconstruction of the notum in a wing disc of a third instar larva of the genotype pnr^{MD237}*/UAS-Sca.* Sca, which is over-expressed in the domain of the *pannier* driver, is shown in red, *D*E-cadherin is in green, and DAPI in blue. The dashed white line indicates the boundary of the expression domain of *pannier,* so Sca is over-expressed to the right of this line as seen in the first panel. *D*E-cadherin can be seen to be localised apically in the control region (a), whereas in the region of over-expression (b), it is spread out over different apical–basal levels of the cells. The additional row of apical nuclei probably belong to cells of the peripodial membrane.

© 2001 Elsevier Science. All rights reserved.



4



5

© 2001 Elsevier Science. All rights reserved.

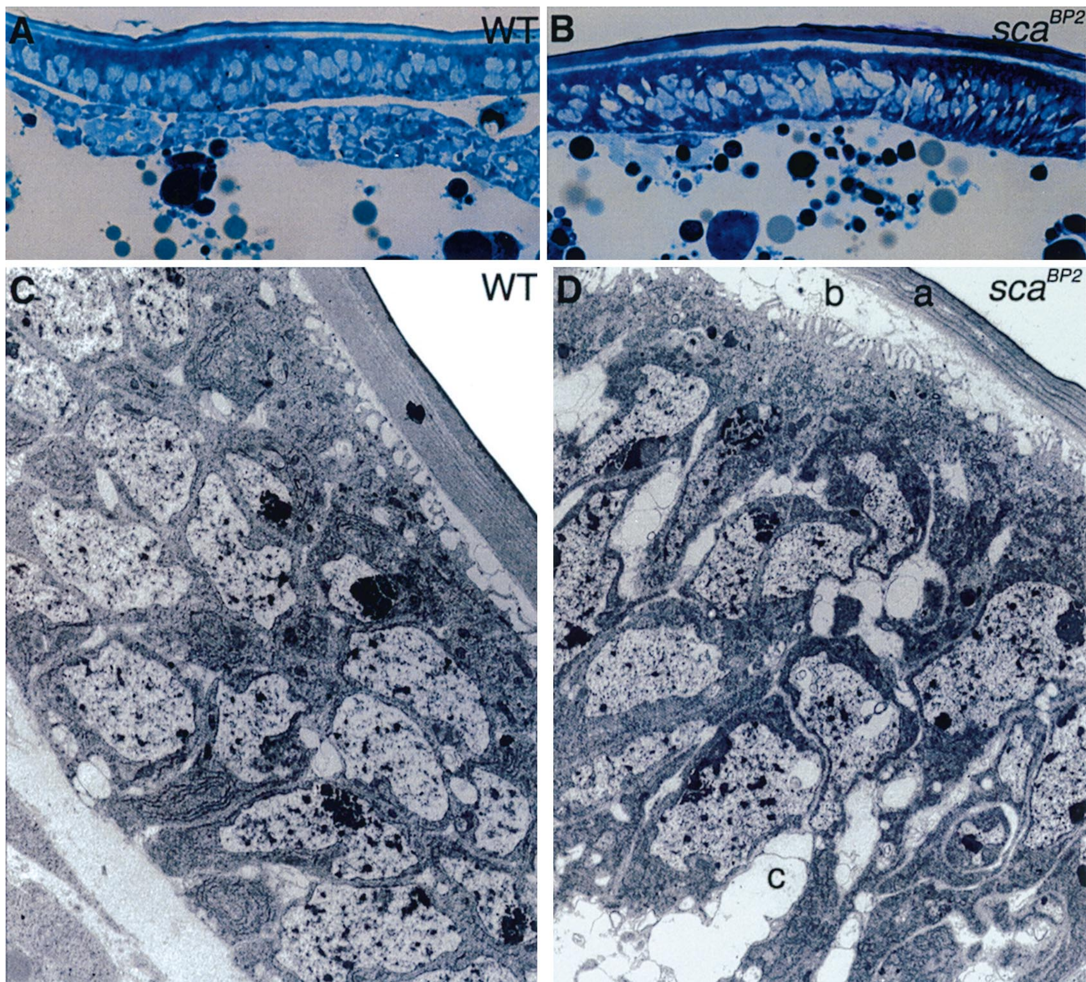


FIG. 6. The epithelium of *scabrous* mutants is disorganized. Semi-thin and transmitted electronic microscopy sections of the notum of wild-type (A, C) and sca^{BP2} (B, D) pupae (12 h APF). Transverse semi-thin sections of the notum are stained with toluidine blue. Note the uneven appearance of the mutant epithelium, and the holes that are visible between cells. Transverse TEM sections reveal that the mutant cuticle (a) is thinner, deposited in irregular layers, and separated from the epithelium (b). Holes (c) frequently separate cells. Mutant cells are of irregular shapes. Adherens and septate junctions are present in the mutant but are reduced in number and appear to be modified. Adherens junctions are often located less apically and septate junctions appear larger than normal (not shown). Scanning microscopy of the external cuticle does not show any difference between wild-type and mutant pupae (not shown).

cells. Instead, it reveals a pattern of small intense dots on the cell membranes at many different focal planes (Fig. 5B). In sca^{BP2}, sca^{WB1}, and sca^{BP2}/sca^{WB1} discs, the cell outlines are much less visible and in pupal nota, at any particular focal plane, the size and shape of cells appear to be variable (Fig. 5D). DAPI staining suggests that the nuclei may be found at different levels within the cells (Fig. 5D) and, unlike the situation in wild-type epithelia, *D*E-cadherin is sometimes found at the same apical–basal level in the cell as the nucleus. The distribution of Discs large, a protein localised in septate junctions (Woods and Bryant, 1991), as well as Armadillo, is similarly altered in mutant cells: the proteins seem to aggregate at different apical–basal levels (not shown).

A clearer picture of the organisation of the epithelium of *sca* mutants was seen in semi-thin and electron microscope sections of pupal nota (Fig. 6). While the epithelium remains a monolayer, the cells are arranged in much more jumbled fashion. They are of different shapes and sizes, and gaps, sometimes of quite substantial proportions, can be seen between cells. Adherens junctions are found at regular intervals at the apical end of the cells in the wild type, but they are only rarely seen at this level in the mutant suggesting mislocalisation. In addition, the cuticle secreted by the mutant cells is thinner, less evenly laid down, and frequently separated from the epithelium.

The phenotype of cells mutant for *sca* was rather

© 2001 Elsevier Science. All rights reserved.

unexpected. So we also looked at flies in which Sca had been over-expressed, and here too the distribution of *D*E-cadherin is slightly perturbed, although the phenotype is not as severe as that of *sca* mutants and is subtly different (Figs. 5E and 5F). Examination of mutant *sca* discs from early third instar larvae, before the onset of *ac-sc* and *sca* expression, showed a normal distribution of *D*E-cadherin (not shown). Discs mutant for ac^{3} $sc^{10\text{-}1}$ are devoid of Ac and functional Sc protein and fail to form bristle precursors (Campuzano *et al.,* 1985; Villares and Cabrera, 1987). *scabrous,* which is regulated by Ac-Sc is not expressed in the absence of Ac and Sc (Mlodzik *et al.,* 1990). The distribution of *D*E-cadherin in the epithelium of late third instar ac^{3} $sc^{10\text{-}1}$ discs appeared to be normal (not shown). Thus, in the absence of *ac-sc* expression and precursor segregation, *sca* is not required to maintain the organisation of the epithelium.

Expression of a Dominant Negative Form of DE-Cadherin Modifies Bristle Density in Wild-Type and Mutant sca Flies

To test whether an exogenous supply of *D*E-cadherin is able to modify bristle density, we looked at the effects of over-expression. Over-expression of wild-type *D*E-cadherin caused lethality. Over-expression of a dominant negative form of *D*E-cadherin, $\text{CADH}^{\text{intra,3}}$ (Sanson *et al.,* 1996) in all cells of the medial half of the notum, resulted in a slight increase in bristle density in a sca^{BP2} mutant background (number of intervening epidermal cells 3.37 ± 0.12, $n = 104$ cf. 3.96 ± 0.17, $n = 20$ for the mutant, $P < 0.01$). Interestingly, it caused a decrease in bristle density in a wild-type background (5.62 ± 0.13, $n = 104$ cf. 4.41 ± 0.07, $n = 20$ for the wild type, $P < 0.001$), similar to that seen after over-expression of Sca. Over-expression of $\text{CADH}^{\text{intra,3}}$, using the *sca-Gal4* driver, which would lead to high levels in bristle precursors, had no effect on bristle density (not shown), suggesting that changes in adhesion between presumptive epidermal cells is able to modify precursor numbers.

Bristle Precursors Extend Long Filopodia

In unfixed preparations using UAS-GFP and a driver expressed exclusively in the precursors, the bristle precursors were seen to display unusual morphology. Thin cytoplasmic extensions, or filopodia, project from the precursor cell between the surrounding epithelial cells (Figs. 7A–7E). These are visible under the light microscope and can extend for distances up to several cell diameters (Figs. 7A and 7C). The filopodia do not project away from the epithelium, rather they extend in a planar fashion. *scabrous* is not required for these extensions since they are present in *sca* mutants (Fig. 7D). We were unable to detect any significant differences between wild-type and mutant precursors, although occasionally in *sca* mutants the filopodia appeared to bend back on themselves (Fig. 7). The average length of filopods was 15 ± 0.7 ($n = 110$) in the wild type compared to 13 ± 0.7 ($n = 73$) for the mutant, (an arbitrary measure of distance was used). The average number of filopods per cell was 3.14 ± 0.27 ($n = 35$) for the wild type compared to 2.92 ± 0.34 ($n = 25$) for the mutant. Extension of filopods by the precursor cell is a dynamic process. The extensions are visible as soon as the precursors segregate at 8 h APF and are no longer present after division of the precursor at 16 h APF. After fixation of the tissue for antibody staining, we were able to detect the presence of some *Dl* molecules along the filopods (Fig. 7E).

DISCUSSION

scabrous Is Required for Inhibition of Cells That Are Not Adjacent to the Bristle Precursors

Our results indicate a function for *sca* in the inhibition of the neural fate, since in its absence an excess of neural precursors form. The *sca* mutant phenotype is very similar to that of hypomorphic *N* and *Dl* mutants and indeed Notch and Dl are known to be the main components of the signalling pathway regulating the spaced pattern of bristles. Thus, Sca is likely to positively modulate N signalling. During bristle precursor selection, like *Dl, sca* acts nonautonomously and is not required for reception of the signal which places its activity upstream of *N.* What are the respective roles of *Dl* and *sca* in the segregation of bristle precursors? Both genes are expressed in proneural domains and then at high levels in the bristle precursors, and their products act nonautonomously on neighbouring presumptive epidermal cells. Both proteins associate with N, but so far only Dl has been shown to be an activating ligand. Scabrous is a secreted molecule, whereas data accumulated to date suggest that active Dl is membrane-bound (Parks *et al.,* 2000). In the complete absence of Dl, all cells adopt the neural fate and thus bristle precursors arise adjacent to one another (Heitzler and Simpson, 1991). In the complete absence of Sca, there is an excess of bristle precursors but they are never adjacent and are always separated by at least one epidermal cell. This indicates that *sca* is not needed for the bristles to be spaced apart by a short distance, and that Dl, which is expressed in *sca* mutants, is able to inhibit cells immediately adjacent to the precursors without any help from Sca. In contrast, in the absence of Sca, Dl is unable to inhibit those cells not in direct contact with the precursor. This suggests a role for Sca in the inhibition of cells not adjacent to the precursor. We refer to these two, possibly separable events, as “short” and “long” range signalling.

Formally, there are three possible mechanisms for “long” range signalling. The first would be that *sca* is part of a relay mechanism whereby cells immediately adjacent to the precursor are inhibited by Dl and then relay the signal farther out by means of Sca. This hypothesis is very unlikely, however, because one would expect *sca* to be expressed in cells adjacent to the precursor following activation of N by Dl. In fact, *sca* protein is only detectable in

© 2001 Elsevier Science. All rights reserved.

the precursor itself, although *sca* is earlier expressed at low levels in the proneural domain. Furthermore, *sca* is expressed in the absence of Dl (Baker and Zitron, 1995) and therefore does not require a prior signalling event mediated by Dl.

A second possible mechanism is that there may be two independent signals, one acting at a "short" range, Dl, and another at a "long" range, Sca. Two observations suggest that this, too, is unlikely. First, in the absence of *Dl,* all cells adopt the neural fate and so the process of bristle spacing is completely abolished. *scabrous* is expressed in cells mutant for *Dl* (Baker and Zitron, 1995) so this result indicates that Sca alone is unable to repress the neural fate. Second, our results indicate that *sca* is not involved in the process of lateral inhibition whereby a pattern of alternating neural and epidermal cells is generated. Along a border between wild-type cells and cells mutant for *Dl,* the precursors are nearly always selected from the pool of wild-type cells, rather than from the mutant cells. This is thought to be because the mutant cells produce little or no signal and are inhibited by the Dl-producing wild-type cells (Heitzler and Simpson, 1991). Along *sca* mosaic borders, the mutant cells can adopt the neural fate, indicating that they are not defective in the production of the activating ligand Dl. Furthermore, since wild-type precursors also form along the mosaic border, neural precursors can be chosen from cells of either genotype. Thus, cells choose the neural or epidermal fate regardless of whether or not they express *sca.* This would explain the fact that precursors are never adjacent in *sca* mutants and is not inconsistent with the observed *sca* phenotype of excess bristles. During segregation of both the normal component of precursors, as well as the additional precursors, adjacent cells have to choose between epidermal and neural fates. Any failure of this process would result in the presence of adjacent bristle precursors, a phenotype characteristic of *N* and *Dl* mutants, but not *sca* mutants. Segregation of single neural precursors surrounded by epidermal neighbours is thus probably mediated by *Dl* alone, which would explain why bristle spacing is completely abolished in the absence of Dl, even though Sca is present.

The third possibility is that "long" range signalling requires both Dl and Sca. Under this hypothesis, Dl would be the activating ligand in "long" range signalling, but would require Sca in order to inhibit cells not directly in contact with the precursor. This could be the case regardless of whether the signal originates exclusively in the precursor or additionally in proneural cells (see below). One observation in favour of this hypothesis is afforded by examination of flies mosaic for *Dl.* Along the edges of *Dl* mutant clones, mutant cells are able to differentiate as epidermis under the influence of an inhibitory signal from neighbouring wild-type cells. This "rescue," due to expression of *Dl* in the wild-type neighbours, can extend up to four cell diameters. If there is no relay mechanism and no other signal, then Dl must somehow be able to activate N several cell diameters away from the cell in which it is produced. Scabrous is of course present in both the Dl^+ and the Dl^- cells, but is unable to effect any rescue of cells mutant for *Dl* that are situated more than about four cells away from the wild-type *Dl*-expressing cells. Examination of *Dl* mutant clones in a mutant *sca* background would indicate the range of Dl signalling in the absence of Sca. Clones doubly mutant for *sca* and *Dl,* however, failed to differentiate the cuticular components of the bristles (for all allelic combinations tested) and so were uninformative (unpublished results).

We suggest that bristle spacing may be the result of two signalling events. The first step of lateral signalling involves *Dl* alone and allows a group of cells to choose a single neural precursor that will inhibit adjacent cells. This step proceeds normally in the absence of Sca. The second step would act to inhibit cells that are not adjacent to the precursor and would require the activity of both *Dl* and *sca.* This step is impaired in both *sca* and *Dl* mutants. In *Dl* mutants, in the absence of the inhibitory signal, lateral inhibition fails leading to adjacent precursors and a loss of epidermal cells. In *sca* mutants, an excess of precursors form, but they segregate singly and are spaced by at least one epidermal cell through the activity of Dl.

Examination of the nascent precursors with *neu-lacZ,* failed to reveal two temporally separate waves of precursor formation in *sca* mutants. Staining of wild-type pupal nota with *D*E-cadherin, however, may provide a visual correlate of the two groups of target cells. A rosette-like ring of wedge-shaped cells surrounds the bristle precursor; it is reminiscent of the cell preclusters that precede segregation of the R8 photoreceptor during development of ommatidia (Ready *et al.,* 1976; Wolff and Ready, 1991). These supra-cellular structures may allow more cells to enter into direct contact with the precursor during the first step of inhibitory signalling.

Local Discontinuities in the Concentration of Scabrous Are Essential for Bristle Spacing and a Gradient of this Molecule May Determine the Range of Inhibitory Signalling

Our results demonstrate a requirement for local discontinuities in the levels of Sca between cells during inhibitory signalling. This was also reported to be the case for eye development (Ellis *et al.,* 1994). Uniform expression of Sca under experimental conditions is unable to rescue the *sca* mutant phenotype. So the relative quantitative differences in the level of Sca between neighbouring cells is an essential feature of the spacing mechanism. We found that over-expression of Sca in a wild-type fly causes a phenotype opposite to that of the loss-of-function. The distance between bristles actually increases and there are correspondingly fewer bristles in the domain of over-expression. In these flies, although exogenous Sca is uniformly distributed, regulation of the endogenous gene will provide local differences in levels of the protein. The greater distance between bristles suggests an extension in the range of the inhibitory signal. Scabrous is likely to be present in a

© 2001 Elsevier Science. All rights reserved.

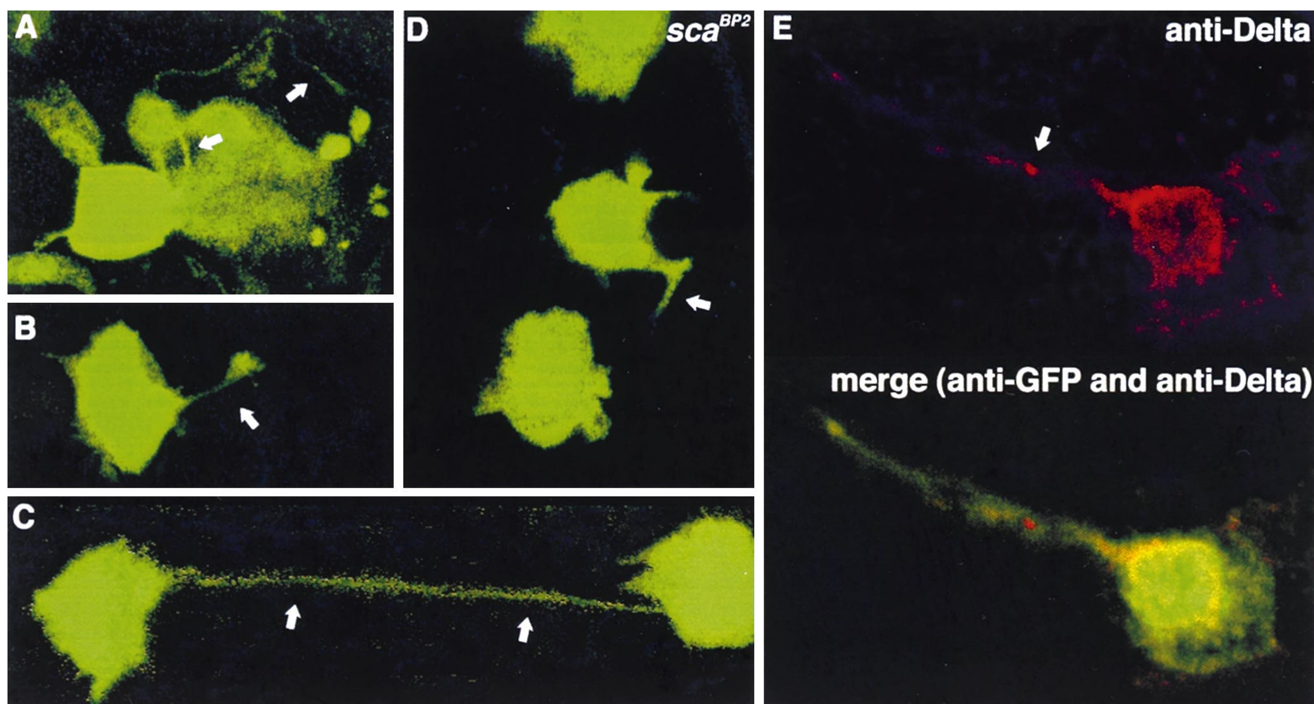


FIG. 7. Bristle precursors extend long filopodia. Confocal observations of the notum of wild-type (A–C) and mutant sca^{BP2} (D) pupae (13 h 30 APF) carrying *neu-GAL4/UAS-GFP.* Green precursor cells express GFP. Cytoplasmic extensions radiating from the precursor cells are seen that may extend for several cell diameters (arrows). In *sca* mutants, we occasionally observed U-shaped filopods (arrow in D) which may represent a defect in orientation. (E) Double labelling with an antibody against Dl (Red) and against GFP (green) in a pupae (14 h APF) carrying *neu-GAL4/UAS-GFP.* Cytoplasmic extensions of the precursor cells, although less numerous, can still be seen in these fixed preparations, that label with the Dl antibody (arrow). Note that a high concentration of *Dl* protein is observed at the base of the filopods. The top panel shows Dl staining; the bottom panel the merge of Dl and GFP.

gradient of decreasing concentration around each precursor. The molecule has been shown to have a very short half-life (Ellis *et al.,* 1994). Thus, it would decay rapidly after secretion and this would help maintain a graded distribution from the source. We postulate that such a gradient could define the range of the inhibitory signal.

We have observed a rescue of up to four cell diameters at the edges of clones of cells mutant for *Dl.* This suggests a signalling range that exceeds that required in wild-type flies, where bristles are usually only four to five cells apart. It is possible, however, that the signal range is longer than usual in this experimental situation because very large amounts of Sca are likely to be present in these clones due to the hyperplasia of precursors. It is, however, noteworthy that in other drosophilid species, such as *D. ararama,* the bristles are spaced by eight or nine cells, suggesting a possibly greater signalling range (unpublished observations).

Scabrous Modulates the Adhesive Properties of Epidermal Cells

We have shown that one property of Sca is to modulate adhesive parameters of the epithelial cells surrounding the precursor. Discrete changes in the distribution of *D*E-cadherin, Discs large, and other junctional proteins (Tepass, 1996; Uemura *et al.,* 1996; Woods and Bryant, 1991; Woods *et al.,* 1997) were seen in the epidermis of *sca* mutants, that are associated with mislocalisation of the adherens and septate junctions and some disruption of epithelial organisation. The monolayered nature of the epithelium is retained, consistent with the fact that the morphogenetic changes of metamorphosis are not impaired in *sca* mutants. This phenotype is only observed in late third instar discs and early pupae, when bristle precursors are forming. If *ac-sc* activity is removed, as in ac^{3} $sc^{10\text{-}1}$ mutant discs, the late third instar disc epithelium is wild type. In ac^{3} $sc^{10\text{-}1}$, the absence of Ac and Sc entails a loss of Sca, whose expression is dependent on Ac-Sc (Mlodzik *et al.,* 1990). The epithelial defects resulting from a lack of Sca protein thus coincide with *ac-sc* expression and the process of lateral inhibition. Scabrous may therefore be required to maintain normal epithelial integrity by counteracting the effects of a protein(s) activated during precursor segregation. It is therefore likely to act through association with a protein(s) whose expression is regulated by Ac and Sc. *Notch* is ubiquitously expressed in the epithelium, but is

© 2001 Elsevier Science. All rights reserved.

activated and probably up-regulated in future epidermal cells surrounding the precursors (de Celis *et al.*, 1997; Wilkinson *et al.*, 1994). A recent report from Powell *et al.* (2001) demonstrated that Sca binds N and as a result N is stabilised at the cell surface in S2 cells. Interestingly, in N^{ts1} mutants at 29°C, where the activity of N is strongly reduced, the distribution of *D*E-cadherin in the disc epithelium is also discretely altered and the epithelium appears similar, but not identical to that of *sca* (unpublished observations). This suggests that the epithelial defects seen in *sca* mutants may be the result of a failure to stabilise *N* protein in epithelial cells. These results are consistent with the idea that Sca acts through N, and with earlier observations linking N to epithelial cell adhesion (Hartenstein *et al.*, 1992; Hoppe and Greenspan, 1986; Kidd *et al.*, 1989).

scabrous is not required for inhibition of cells that are adjacent to the precursor. Furthermore, the requirement for *sca* in the fly is quite restricted: it is not expressed in many other tissues where Notch signalling takes place in its absence. While it is expressed in neural precursors in the embryo, loss of the protein there seems to be without consequence, perhaps because the interactions involve adjacent cells. This leads us to hypothesise that the effects of Sca on cell adhesion and the stabilisation of N may be specifically required when the levels of Dl are limiting. Delta is expressed in proneural domains and can still be detected in presumptive epidermal cells after precursor segregation (Parks *et al.*, 1997). Nevertheless the amount of Dl remaining in presumptive epidermal cells appears to be insufficient, by itself, to repress the neural fate. In the absence of Sca, or in flies carrying hypomorphic alleles of *Dl,* the space between bristles is decreased (Heitzler and Simpson, 1991; Lee *et al.*, 1998; Parks *et al.*, 1997). Furthermore, in epidermal cells, the transcription of *Dl* progressively declines due to repression of its regulators *ac* and *sc* following N activation, whereas in the bristle precursors the levels of Dl increase as the levels of Ac and Sc rise (Culi and Modolell, 1998; Heitzler *et al.*, 1996; Kunisch *et al.*, 1994). This leaves two possibilities. One is that all of the signal originates in the precursor cell, in which case Dl must be transported, by some as yet unknown means, from the precursor to cells not in direct contact with the latter. The other, is that the concentration of *Dl* molecules remaining on the presumptive epidermal cells is insufficient to inhibit by itself, but can do so if helped by Sca. Dl from both groups of cells may participate in the wild type. In either case, stabilisation of N may therefore be a means to increase the chances of receptor activation in the presence of limiting amounts of Dl.

Adhesion and the Cell Lineage of the Bristle Organ

Changes in cell adhesion could also be the cause of the abnormal bristle organs seen in *sca* mutants, where two or more cells of the bristle organ lineage adopt the same fate at the expense of the others. In the wild type, spatial arrangements of the cells of the bristle organs are stereotyped as a result of the nonrandom orientation of the mitotic spindles at each division (Gho *et al.*, 1999). In *sca* mutants, the cells are often randomly arranged and in some cases appear to drift apart from one another. This would be likely to prevent the precise cell–cell interactions, mediated by the *N* signalling pathway, necessary for the assignment of the correct cell fates (Jan and Jan, 2000).

The Bristle Precursor May Contact Distant Epidermal Cells by Means of Filopodia

We have observed that the precursor cells have a quite distinctive shape, reminiscent of neurons with a number of filopodial-like extensions that fan out in a planar orientation. We do not know whether, during bristle precursor segregation, the epidermal cells of the notum extend similar filopodia. Oriented epidermal outgrowths have been described in the epidermis of other insects and also in the wing pouch and peripodial membrane of *Drosophila* imaginal discs, where it has been suggested that they may function during signalling (Gibson and Schubiger, 2000; Locke and Huie, 1981; Ramirez-Weber and Kornberg, 1999). Some of these structures project basally and others extend long, straight and polarised structures. Their morphology differs from that of the extensions we observe.

It is not known whether Dl from the precursor cell is able to reach cells that are not adjacent to the *Dl*-expressing precursor, but one means by which this could occur is via cytoplasmic extensions. This has been suggested for Lag-2 signalling in the nematode germ line (Hall *et al.*, 1999). Indeed, we were able to detect the presence of *Dl* molecules on the filopodia. Although formation of filopodia will depend on properties of the neural precursor itself, subtle changes in junctional contacts and adhesion between surrounding epithelial cells may help to orient or stabilise these structures. The changes in the bristle density of both wild-type and *sca* flies that are seen after expression of a dominant negative form of *D*E-cadherin, suggest a role for adhesion molecules in bristle spacing. Preliminary observations indicate that Sca is not required for the extension of filopods. This is consistent with the nonautonomy of *sca* mutant cells. If filopodia are the means whereby nonadjacent cells are inhibited, and if Sca were to be required for extension of filopodia from the Sca-producing bristle precursors, then *sca* would be expected to behave autonomously. Further studies are necessary to determine the molecular basis of Sca function, but one possibility is that binding of Sca to N leads to discrete modifications in epithelial structure that allow Dl molecules on the cytoplasmic extensions to form stable ligand-receptor complexes. The colocalisation of Sca and Dl in cytoplasmic vesicles may indicate cellular trafficking of protein complexes that include Dl, N, and Sca.

© 2001 Elsevier Science. All rights reserved.

ACKNOWLEDGMENTS

We thank the following organizations for financial support: l'Institut National de la Santé et de la Recherche Médicale, le Centre National de la Recherche Scientifique, l'Hôpital Universitaire de Strasbourg, the Human Frontiers Scientific Programme Organization (RG0374/1997-M), the Human Capital and Mobility Programme of the European Community (FMRX CT 96 0065), l'Association pour la Recherche contre le Cancer, and the Wellcome Trust (29156). O.R. is the recipient of a predoctoral fellowship from the Ministère de l'Education Nationale, de la Recherche et de la Technologie (97440). We thank Annie Bauer, Claudine Ackerman, and Nadine Arbogast for technical help, Nick Baker, Marek Mlodzik, Matthew Freeman, Bénédicte Sanson, Manuel Calleja, and the *Drosophila* stock centers at Bloomington for generously sending us *Drosophila* stocks, Kazuya Usui for help with the technique for pupal dissection, Jean-Luc Vonesch and Didier Hentsch for help with confocal microscopy, Nadia Messadeq for help with sectioning and electron microscopy, and Mark Muskavitch, Hiroki Oda, Marc Bourouis, and the DSHB for antibodies. We especially thank Veronica Rodriguez for her gift of *neu-GAL4* before publication, and Francois Schweisguth and Elizabeth Georges for help with the interpretation of immunostained preparations. Discussion and comments on the manuscript from Michel Labouesse, Peter Lawrence, Helen Skaer, Ross Cagan, and the members of our group are gratefully acknowledged.

REFERENCES

Ashburner, M. (1989). "*Drosophila:* A Laboratory Handbook and Manual (Two volumes)" Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Baker, N. E., Mlodzik, M., and Rubin, G. M. (1990). Spacing differentiation in the developing *Drosophila* eye: A fibrinogen-related lateral inhibitor encoded by *scabrous. Science* **250,** 1370–1377.

Baker, N. E., and Zitron, A. E. (1995). *Drosophila* eye development: *Notch* and *Delta* amplify a neurogenic pattern conferred on the morphogenetic furrow by *scabrous. Mech. Dev.* **49,** 173–189.

Blochlinger, K., Bodmer, R., Jan, L. Y., and Jan, Y. N. (1990). Patterns of expression of cut, a protein required for external sensory organ development in wild-type and *cut* mutant *Drosophila* embryos. *Genes Dev.* **4,** 1322–1331.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118,** 401–415.

Calleja, M., Moreno, E., Pelaz, S., and Morata, G. (1996). Visualization of gene expression in living adult *Drosophila. Science* **274,** 252–255.

Campuzano, S., Carramolino, L., Cabrera, C. V., Ruiz-Gomez, M., Villares, R., Boronat, A., and Modolell, J. (1985). Molecular genetics of the *achaete-scute* gene complex of *D. melanogaster. Cell* **40,** 327–338.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta. Nature* **375,** 761–766.

Cubas, P., de Celis, J. F., Campuzano, S., and Modolell, J. (1991). Proneural clusters of *achaete-scute* expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Dev.* **5,** 996–1008.

Culi, J., and Modolell, J. (1998). Proneural gene self-stimulation in neural precursors: An essential mechanism for sense organ development that is regulated by Notch signaling. *Genes Dev.* **12,** 2036–2047.

de Celis, J. F., Bray, S., and Garcia-Bellido, A. (1997). Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the *Drosophila* wing. *Development* **124,** 1919–1928.

Ellis, M. C., Weber, U., Wiersdorff, V., and Mlodzik, M. (1994). Confrontation of *scabrous* expressing and non-expressing cells is essential for normal ommatidial spacing in the *Drosophila* eye. *Development* **120,** 1959–1969.

Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A., and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta,* two EGF-homologous genes in *Drosophila. Cell* **61,** 523–534.

Flybase Consortium. (1999). The FlyBase database of the Drosophila Genome Projects and community literature. *Nucleic Acids Res.* **27,** 85–88.

Gho, M., Bellaiche, Y., and Schweisguth, F. (1999). Revisiting the *drosophila* microchaete lineage: A novel intrinsically asymmetric cell division generates a glial cell. *Development* **126,** 3573–3584.

Gibson, M. C., and Schubiger, G. (2000). Peripodial cells regulate proliferation and patterning of *Drosophila* imaginal discs. *Cell* **103,** 343–350.

Golic, K. G. (1991). Site-specific recombination between homologous chromosomes in *Drosophila. Science* **252,** 958–961.

Golic, K. G., and Lindquist, S. (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell* **59,** 499–509.

Hall, D. H., Winfrey, V. P., Blaeuer, G., Hoffman, L. H., Furuta, T., Rose, K. L., Hobert, O., and Greenstein, D. (1999). Ultrastructural features of the adult hermaphrodite gonad of *Caenorhabditis elegans:* Relations between the germ line and soma. *Dev. Biol.* **212,** 101–123.

Hartenstein, A. Y., Rugendorff, A., Tepass, U., and Hartenstein, V. (1992). The function of the neurogenic genes during epithelial development in the *Drosophila* embryo. *Development* **116,** 1203–1220.

Hartenstein, V., and Posakony, J. W. (1990). A dual function of the *Notch* gene in *Drosophila* sensillum development. *Dev. Biol.* **142,** 13–30.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C., and Simpson, P. (1996). Genes of the *Enhancer of split* and *achaete-scute* complexes are required for a regulatory loop between *Notch* and *Delta* during lateral signalling in *Drosophila. Development* **122,** 161–171.

Heitzler, P., and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila. Cell* **64,** 1083–1092.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375,** 787–790.

Hinz, U., Giebel, B., and Campos-Ortega, J. A. (1994). The basic-helix–loop–helix domain of *Drosophila* lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell* **76,** 77–87.

Hoppe, P. E., and Greenspan, R. J. (1986). Local function of the *Notch* gene for embryonic ectodermal pathway choice in *Drosophila. Cell* **46,** 773–783.

© 2001 Elsevier Science. All rights reserved.

Hu, X., Lee, E. C., and Baker, N. E. (1995). Molecular analysis of *scabrous* mutant alleles from *Drosophila melanogaster* indicates a secreted protein with two functional domains. *Genetics* **141,** 607–617.

Huppert, S. S., Jacobsen, T. L., and Muskavitch, M. A. (1997). Feedback regulation is central to Delta-Notch signalling required for *Drosophila* wing vein morphogenesis. *Development* **124,** 3283–3291.

Jan, L. Y., and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and grasshopper embryos. *Proc. Natl. Acad. Sci. USA* **79,** 2700–2704.

Jan, Y. N., and Jan, L. Y. (1995). Maggot's hair and bug's eye: Role of cell interactions and intrinsic factors in cell fate specification. *Neuron* **14,** 1–5.

Jan, Y. N., and Jan, L. Y. (2000). Polarity in cell division: What frames thy fearful asymmetry? *Cell* **100,** 599–602.

Jen, W. C., Wettstein, D., Turner, D., Chitnis, A., and Kintner, C. (1997). The Notch ligand, X-Delta-2, mediates segmentation of the paraxial mesoderm in *Xenopus* embryos. *Development* **124,** 1169–1178.

Jennings, B., de Celis, J., Delidakis, C., Preiss, A., and Bray, S. (1995). Role of Notch and achaete-scute complex in the expression of Enhancer of split bHLH proteins. *Development* **121,** 3745–3752.

Jennings, B., Preiss, A., Delidakis, C., and Bray, S. (1994). The Notch signalling pathway is required for Enhancer of split bHLH protein expression during neurogenesis in the *Drosophila* embryo. *Development* **120,** 3537–3548.

Jhaveri, D., Sen, A., Reddy, G. V., and Rodrigues, V. (2000). Sense organ identity in the *drosophila* antenna is specified by the expression of the proneural gene *atonal. Mech. Dev.* **99,** 101–111.

Kemler, R. (1993). From cadherins to catenins: Cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* **9,** 317–321.

Kidd, S., Baylies, M. K., Gasic, G. P., and Young, M. W. (1989). Structure and distribution of the Notch protein in developing *Drosophila. Genes Dev.* **3,** 1113–1129.

Kidd, S., Kelley, M. R., and Young, M. W. (1986). Sequence of the notch locus of *Drosophila melanogaster:* Relationship of the encoded protein to mammalian clotting and growth factors. *Mol. Cell. Biol.* **6,** 3094–3108.

Kimble, J., and Simpson, P. (1997). The LIN-12/Notch signaling pathway and its regulation. *Annu. Rev. Cell Dev. Biol.* **13,** 333–361.

Klueg, K. M., Parody, T. R., and Muskavitch, M. A. T. (1998). Complex proteolytic processing acts on delta, a transmembrane ligand for notch, during *Drosophila* development. *Mol. Biol. Cell* **9,** 1709–1723.

Kooh, P. J., Fehon, R. G., and Muskavitch, M. A. (1993). Implications of dynamic patterns of Delta and Notch expression for cellular interactions during *Drosophila* development. *Development* **117,** 493–507.

Kopczynski, C. C., Alton, A. K., Fechtel, K., Kooh, P. J., and Muskavitch, M. A. (1988). *Delta,* a *Drosophila* neurogenic gene, is transcriptionally complex and encodes a protein related to blood coagulation factors and epidermal growth factor of vertebrates. *Genes Dev.* **2,** 1723–1735.

Kunisch, M., Haenlin, M., and Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the *Drosophila* neurogenic gene *delta* is enhanced by proneural proteins. *Proc. Natl. Acad. Sci. USA* **91,** 10139–10143.

Lee, E. C., and Baker, N. E. (1996). gp300sca is not a high affinity Notch ligand. *Biochem. Biophys. Res. Commun.* **225,** 720–725.

Lee, E. C., Hu, X., Yu, S. Y., and Baker, N. E. (1996). The *scabrous* gene encodes a secreted glycoprotein dimer and regulates proneural development in *Drosophila* eyes. *Mol. Cell. Biol.* **16,** 1179–1188.

Lee, E. C., Yu, S. Y., Hu, X., Mlodzik, M., and Baker, N. E. (1998). Functional analysis of the fibrinogen-related *scabrous* gene from *Drosophila melanogaster* identifies potential effector and stimulatory protein domains. *Genetics* **150,** 663–673.

Lewis, A. K., Frantz, G. D., Carpenter, D. A., de Sauvage, F. J., and Gao, W. Q. (1998). Distinct expression patterns of notch family receptors and ligands during development of the mammalian inner ear. *Mech. Dev.* **78,** 159–163.

Lieber, T., Kidd, S., Alcamo, E., Corbin, V., and Young, M. W. (1993). Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes Dev.* **7,** 1949–1965.

Lindsley, D. L., and Zimm, G. G. (1992). "The Genome of *Drosophila melanogaster.*" Academic Press, San Diego, CA.

Locke, M., and Huie, P. (1981). Epidermal feet in pupal segment morphogenesis. *Tissue Cell* **13,** 787–803.

Mlodzik, M., Baker, N. E., and Rubin, G. M. (1990). Isolation and expression of *scabrous,* a gene regulating neurogenesis in *Drosophila. Genes Dev.* **4,** 1848–1861.

Oda, H., Uemura, T., Harada, Y., Iwai, Y., and Takeichi, M. (1994). A *Drosophila* homolog of cadherin associated with armadillo and essential for embryonic cell–cell adhesion. *Dev. Biol.* **165,** 716–726.

Parks, A. L., Huppert, S. S., and Muskavitch, M. A. (1997). The dynamics of neurogenic signalling underlying bristle development in *Drosophila melanogaster. Mech. Dev.* **63,** 61–74.

Parks, A. L., Klueg, K. M., Stout, J. R., and Muskavitch, M. A. (2000). Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development* **127,** 1373–1385.

Parks, A. L., and Muskavitch, M. A. (1993). Delta function is required for bristle organ determination and morphogenesis in *Drosophila. Dev. Biol.* **157,** 484–496.

Parody, T. R., and Muskavitch, M. A. (1993). The pleiotropic function of Delta during postembryonic development of *Drosophila melanogaster. Genetics* **135,** 527–539.

Powell, P. A., Wesley, C., Spencer, S., and Cagan, R. L. (2001). Scabrous complexes with Notch to mediate boundary formation. *Nature* **409,** 626–630.

Qi, H., Rand, M. D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T., and Artavanis-Tsakonas, S. (1999). Processing of the notch ligand delta by the metalloprotease Kuzbanian. *Science* **283,** 91–94.

Ramirez-Weber, F. A., and Kornberg, T. B. (1999). Cytonemes: Cellular processes that project to the principal signaling center in *Drosophila* imaginal discs. *Cell* **97,** 599–607.

Ready, D. F., Hanson, T. E., and Benzer, S. (1976). Development of the *Drosophila* retina, a neurocrystalline lattice. *Dev. Biol.* **53,** 217–240.

Rebay, I., Fehon, R. G., and Artavanis-Tsakonas, S. (1993). Specific truncations of *Drosophila* Notch define dominant activated and dominant negative forms of the receptor. *Cell* **74,** 319–329.

Sanson, B., White, P., and Vincent, J. P. (1996). Uncoupling cadherin-based adhesion from *wingless* signalling in *Drosophila. Nature* **383,** 627–630.

Simpson, P. (1997). *Notch* signalling in development: On equivalence groups and asymmetric developmental potential. *Curr. Opin. Genet. Dev.* **7,** 537–542.

© 2001 Elsevier Science. All rights reserved.

Skeath, J. B., and Carroll, S. B. (1991). Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes Dev.* **5,** 984–995.

Struhl, G., Fitzgerald, K., and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74,** 331–345.

Sun, X., and Artavanis-Tsakonas, S. (1996). The intracellular deletions of Delta and Serrate define dominant negative forms of the *Drosophila* Notch ligands. *Development* **122,** 2465–2474.

Takeichi, M. (1988). The cadherins: Cell–cell adhesion molecules controlling animal morphogenesis. *Development* **102,** 639–655.

Tepass, U. (1996). Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of *Drosophila. Dev. Biol.* **177,** 217–225.

Uemura, T., Oda, H., Kraut, R., Hayashi, S., Kotaoka, Y., and Takeichi, M. (1996). Zygotic *Drosophila* E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the *Drosophila* embryo. *Genes Dev.* **10,** 659–671.

Usui, K. (1993). The study of cell cycle and determination: Sensory mother cells of adult bristles are determined during G2 arrest in *Drosophila melanogaster.* In "Graduate School of Science." Hokkaido University, Hokkaido.

Usui, K., and Simpson, P. (2000). Cellular basis of the dynamic behavior of the imaginal thoracic discs during *Drosophila* metamorphosis. *Dev. Biol.* **225,** 13–25.

Vassin, H., and Campos-Ortega, J. A. (1987). Genetic analysis of *Delta,* a neurogenic gene of *Drosophila melanogaster. Genetics* **116,** 433–445.

Venugopala Reddy, G., Reiter, C., Shanbhag, S., Fischbach, K. F., and Rodrigues, V. (1999). Irregular chiasm-C-roughest, a member of the immunoglobulin superfamily, affects sense organ spacing on the *Drosophila* antenna by influencing the positioning of founder cells on the disc ectoderm. *Dev. Genes Evol.* **209,** 581–591.

Viallet, J. P., Prin, F., Olivera-Martinez, I., Hirsinger, E., Pourquie, O., and Dhouailly, D. (1998). Chick *Delta-1* gene expression and the formation of the feather primordia. *Mech. Dev.* **72,** 159–168.

Villares, R., and Cabrera, C. V. (1987). The *achaete-scute* gene complex of *D. melanogaster:* Conserved domains in a subset of genes required for neurogenesis and their homology to *myc. Cell* **50,** 415–424.

Wharton, K. A., Johansen, K. M., Xu, T., and Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43,** 567–581.

Wilkinson, H. A., Fitzgerald, K., and Greenwald, I. (1994). Reciprocal changes in expression of the receptor lin-12 and its ligand lag-2 prior to commitment in a *C. elegans* cell fate decision. *Cell* **79,** 1187–1198.

Wolff, T., and Ready, D. F. (1991). The beginning of pattern formation in the *Drosophila* compound eye: The morphogenetic furrow and the second mitotic wave. *Development* **113,** 841–850.

Woods, D. F., and Bryant, P. J. (1991). The discs-large tumor suppressor gene of *Drosophila* encodes a guanylate kinase homolog localized at septate junctions. *Cell* **66,** 451–464.

Woods, D. F., Wu, J. W., and Bryant, P. J. (1997). Localization of proteins to the apico-lateral junctions of *Drosophila* epithelia. *Dev. Genet.* **20,** 111–118.

Xu, T., and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117,** 1223–1237.

Received for publication April 9, 2001
Revised July 18, 2001
Accepted October 5, 2001
Published online November 14, 2001

© 2001 Elsevier Science. All rights reserved.