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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*

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SUMMARY

Like the neuroblasts of the central nervous system, sensory organ precursors of the peripheral nervous system of the *Drosophila* thorax arise as single spaced cells. However, groups of cells initially have neural potential as visualized by the expression of the proneural genes *achaete* and *scute*. A class of genes, known as the 'neurogenic genes', function to restrict the proportion of cells that differentiate as sensory organ precursors. They mediate cell communication between the competent cells by means of an inhibitory signal, Delta, that is transduced through the Notch receptor and results in a cessation of *achaete-scute* activity. Here we show that mutation of either the bHLH-encoding genes of the *Enhancer of split* complex (E(spl)-C) or *groucho*, like *Notch* or *Delta* mutants, cause an overproduction of sensory organ precursors at the expense of epidermis. The mutant cells behave autonomously suggesting that the corresponding gene products are required for reception of the inhibitory signal. Epistasis experiments place both E(spl)-C bHLH-encoding genes and *groucho* downstream of *Notch* and upstream of *achaete* and *scute*, consistent with the idea that they are part of the *Notch* signalling cascade.

Since all competent cells produce both the receptor and its ligand, it was postulated that Notch and Delta are linked within each cell by a feedback loop. We show, that, like mutant *Notch* cells, cells mutant for E(spl)-C bHLH-encoding genes or *groucho* inhibit neighbouring wild-type cells causing them to adopt the epidermal fate. This inhibition requires the genes of the *achaete-scute* complex (AS-C) which must therefore regulate the signal Delta. Thus there is a regulatory loop between Notch and Delta that is under the transcriptional control of the E(spl)-C and AS-C genes.

Key words: *Notch, Delta, Enhancer of split*, neurogenic genes, cell fate

INTRODUCTION

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). This is particularly obvious in the imaginal peripheral nervous system (PNS) where the rows of spaced bristles are the direct result of the pattern of segregation of the sensory organ precursors. 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). Neural potential is given by the expression of proneural genes of which two members of the *achaete-scute* complex (AS-C), *achaete (ac)* and *scute (sc)* are required for bristle development (Ghysen and Dambly-Chaudière, 1988). These genes encode proteins of the bHLH type that are thought to regulate downstream genes necessary to execute the neural fate (Villares and Cabrera, 1987). Expression of *ac* and *sc* is first detected in clusters of cells at the sites of each large bristle or macrochaete, but is subsequently restricted to a single macrochaete precursor and ceases in the other cells that then become epidermal (Cubas et al., 1991; Skeath and Carroll, 1991).

It is thought that competitive cell interactions between competent cells lead to singling out of the neural precursor that produces an inhibitory signal preventing the other cells of the group from following the neural pathway (Wigglesworth, 1940; Doe and Goodman, 1985; Simpson, 1990). This signal would result in the repression of *ac* and *sc* and as a result the cells surrounding the precursor adopt the epidermal fate. The inhibitory signal involves the genes *Notch (N)* and *Delta (Dl)*. Animals mutant for *N* or *Dl* display neural hyperplasia and an excess of cells differentiate neural precursors at the expense of epidermis (Lehmann et al., 1983; Hartenstein and Posakony, 1990). This causes most cells expressing high levels of *ac* and *sc* on the thorax to become neural.

Both *N* and *Dl* encode large transmembrane proteins (Wharton et al., 1985; Kidd et al., 1986; Vässin et al., 1987; Kopczynski et al., 1988) that are members of large families of conserved proteins found throughout the animal kingdom (see reviews by Artavanis-Tsakonas et al., 1995, and Simpson, 1994). Genetic and molecular data indicate that the *Dl* protein

is a ligand for the *N* protein and that the latter functions as a cellular receptor (Fehon et al., 1990; Heitzler and Simpson, 1991; Rebay et al., 1993; Lieber et al., 1993; Struhl et al., 1993). *Notch* and *Dl* are initially expressed by all cells of the *ac-sc*-expressing cluster but this lateral signalling resolves, with time, into a situation where the future sensory organ precursor produces the signal and the surrounding presumptive epidermal cells, the receptor. It has been postulated that this process relies on a feedback loop between Notch and Delta within each cell: cells expressing little or no receptor send a strong signal and constitutively inhibit their neighbours (Heitzler and Simpson, 1991). Consequently a small bias in protein levels between cells could be amplified via the regulatory loop resulting in a single signalling cell.

A number of other genes display mutant phenotypes similar to those of *N* and *Dl*. These include a cluster of genes spanning about 60 kb of which at least seven are structurally related: *m*δ, *m*γ, *m*β, *m3, m5, m7* and *m8*. These genes make up the *Enhancer of split* complex (E(spl)-C) (*m8* is the original *E(spl)* but we refer to it as *m8* for simplicity; Delidakis et al., 1991; Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992; Schrons et al., 1992). Deletions of this region result in extensive neural hyperplasia in the central nervous system and the extent of the phenotype depends upon the number of genes deleted. All of these genes encode basic-helix-loop-helix (bHLH) proteins that are structurally similar and appear to provide partly redundant functions (Klämbt et al., 1989; Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992). These proteins are members of the *hairy (h)*-related bHLH group and act as negative transcriptional regulators that bind to variants of the E boxes (Ohsako et al., 1994; Tietze et al., 1992; Oellers et al., 1994).

A gene of a different class, *groucho (gro)*, also resides at this location and is the only one that mutates to give a zygotic lethal phenotype (Preiss et al., 1988; Delidakis et al., 1991). Removal of both maternal and zygotic components of *gro* results in a strong neurogenic phenotype like that of null *N* or *Dl* mutants (Schrons et al., 1992; Paroush et al., 1994). *groucho* encodes a ubiquitous nuclear protein displaying repeated WD40 motifs which may mediate protein-protein interactions (Tata and Hartley, 1993). The recent demonstration that the *gro* protein can physically associate with *h*-related proteins has led to the proposal that Gro acts as a transcriptional co-repressor (Paroush et al., 1994).

The pattern and timing of the expression of E(spl)-C proteins suggests that they accumulate in response to N signalling activity (Jennings et al., 1994). A strong argument for a role of these genes downstream of N comes from the study of double mutants for E(spl)-C genes and a constitutive form of N. Such genotypes result in a phenotype of neural hyperplasia, suggesting that absence of E(spl)-C genes can interrupt transmission of the inhibitory signal (Leiber et al., 1993).

In this report we present observations on the effects of E(spl)-C and *gro* mutants on the development of the sensory bristles of the imaginal PNS. Here, a cell by cell analysis of the behaviour of mutant cells when adjacent to wild-type ones is possible. The generation of mutant clones revealed that removal of E(spl)-C genes or *gro* leads to a strong phenotype of bristle hyperplasia. Loss of either the bHLH-encoding genes or *gro* has a comparable effect on bristle spacing but loss of *gro* by itself is associated with other, additional phenotypes. Cells mutant for either the bHLH-encoding genes or *gro* behave autonomously and differentiate bristles even when adjacent to wild-type cells. This is consistent with a role for these genes in the reception of the inhibitory signal. Since the cuticular derivatives of all cells of the mutant clones can be detected, epistasis experiments could be performed and these revealed that the E(spl)-C genes act downstream of *N* and upstream of *ac-sc*. Finally, we found, that, like N^- cells, mutant E(spl)-C cells affect the fate of adjacent wild-type cells causing them to differentiate as epidermis. This inhibition requires the AS-C genes, suggesting that the regulatory loop between *N* and *Dl* is under a transcriptional control involving genes of both the E(spl)-C and the AS-C.

MATERIALS AND METHODS

Fly strains

For references to the origin of lesions associated with the following mutants, see Lindsley and Zimm, (1992) and Simpson, (1994). N^{55e11} is a null allele, associated with an insertion in the 5′ coding region and premature termination of transcripts. The N^{ts1} allele is associated with a glycine to aspartic acid change at position 1272 within the 32nd EGF-like repeat. It is a hypomorphic allele that fails to complement for all known effects of *N* locus mutations. Ax^{59b} is a gain of function *N* allele. It is associated with a changed cysteine residue at position 972 within the 24th EGF-like repeat. This is a highly conserved residue necessary for the secondary structure. The $ac^3\ sc^{10\text{-}1}$ chromosome is mutant for *ac* and *sc*. $Df(1)sc^{B57}$ is a deletion that removes all four genes of the *ac-sc* complex (AS-C) and causes lethality. $Df(1)sc^{H}$ removes *ac, sc* and *l(sc)* but *ase* remains functional.

$P[gro^+,ry^+]E8$ is a transposon carrying a wild-type allele of *gro* inserted at 89B. gro^{E48}, gro^{B88} and gro^{E73} are null alleles of *gro* and gro^{E107} is a hypomorphic allele (Preiss et al., 1988). The extent of different deletions of the E(spl)-C are shown in Fig. 1. $Df(3R)gro^{R8.1}$ and $Df(3R)\ gro^{32.2}$ were obtained by excision of a P element inserted between the gene *m8* and *gro* (Schrons et al., 1992). $Df(3R)E(spl)^{RA7.1}$ and $Df(3R)E(spl)^{RB251}$ were obtained as revertants of $E(spl)^D$ (Knust et al., 1987). $Df(3R)boss^{16}$ was characterized by Hart et al. (1990).

An AS-C^+ duplication on chromosome 3R was isolated. A $P[w^+$ *lacZ]* enhancer trap element at the *elav* locus, a hot spot for P element insertions, just proximal to the AS-C on the X chromosome was mobilized using the *P[ry+ Δ 2-3]* as the source of transposase. y $P[w^+$ *lacZ]*; *Ki* $kar^2\ ry^{506}$ *P[Δ2-3]/+* males were crossed en masse to $Df(1)sc^{B57}$ *w* sn^3*/FM7* females. Two *w* sn^3 sons were obtained from a total of 15000 flies. The suppressor mutations were genetically mapped to chromosomes 2 and 3 respectively. They correspond to autosomal duplications (named $Dp(1;2)sc^{+ip2}$ and $Dp(1;3)sc^{+ip3}$) of the tip of the X chromosome carrying the y^1 mutation and the AS-C genes. Meiotic and cytological mapping show that $Dp(1;3)sc^{+ip3}$ is inserted near the telomere of 3R.

Flies were maintained at 25°C.

Production of mosaic animals

Mutant clones were produced by mitotic recombination induced by either X rays or the FLP/FRT method (Golic and Lindquist, 1989; Golic, 1991; Xu and Rubin, 1993). Twenty-four-hour egg collections were made and larvae were X-rayed (1000 R) or heat-shocked (1 hour, 37°C) between 24 and 48 hour after egg laying. Clones were marked with *multiple wing hairs (mwh)* which labels epidermal cells, *forked* (f^{36a}) which labels bristles and *crinkled* (ck^{CH52}) or *pawn* (*pwn*), which label both sensory bristles and epidermal hairs. For a description of these markers see Lindsley and Zimm (1992). Thoraces were mounted between coverslips in Struhl's medium. Clones of mutants and deletions of the E(spl)-C on the right arm of chromosome III,

were made according to the following general scheme. The mutant was placed opposite *Dp(2;3)P32* that carries pwn^+ in flies that were homozygous for *pwn*. Recombination on 3R leads to a loss of the duplication and homozygosity of the mutant as well as the *pwn* marker; it was induced by either X-rays or by a heat shock to induce expression of the yeast FLP recombinase (placed on chromosome II) that in turn acts on the FRT target sequence present on 3R proximal to the mutant. Clones were induced in flies of the following genotypes.

X ray-induced: (1) f^{36a} / *Dp(3;Y;1)M2,* mwh^+ *y v;* emc^1 *mwh / mwh*

(2) ac^3 $sc^{10\text{-}1}$ f^{36a} / *Dp(3;Y;1)M2,* mwh^+ *y v;* emc^1 *mwh / mwh*

(3) N^{ts1} f^{36a}/ *Dp(3;Y;1)M2,* mwh^+ *y v; mwh*

(4) ac^3 $sc^{10\text{-}1}$ N^{55e11} f^{36a}/ *Dp(3;Y;1)M2,* mwh^+ *y Dp(1;1)Co,* N^+ *v;* emc^1 *mwh / mwh.* The use of *Dp(1;1)Co* in this cross ensures that the cells surrounding the clone are disomic for N^+ as they are in cross 1.

(5) *Df(1)*sc^{B57} N^{55e11} f^{36a} / *Dp(3;Y;1)M2,* mwh^+ *y v;* emc^1 *mhw/mwh*

(6) *Df(1)*sc^H N^{55e11} f^{36a} / *Dp(3;Y;1)M2,* mwh^+ *y v;* emc^1 *mwh/ mwh*

(7) ac^3 $sc^{10\text{-}1}$/+; *Dp(1;2)*sc^{19}, ac^+ sc^+ *cn bw sp /* ck^{CH52} *pr*

(8) *pr pwn; Df(3R)E(spl)*$^{RA7.1}$*/Dp(2;3)P32[*pwn^+*]*

(9) *pr pwn; Df(3R)*$gro^{r8.1}$*/Dp(2;3)P32[*pwn^+*]*

(10) *pr pwn;* gro^{E73}*/ Dp(2;3)P32[*pwn^+*].*

FLP/FRT-induced: (11) *pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(12) *pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} gro^{B88} */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(13) *pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} gro^{E48} */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(14) *pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} gro^{E107} */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(15) *pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} *P[*gro^+*,*ry^+*]E8(89B)* *Df(3R)*$gro^{b32.2}$ */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(16) *pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} *Df(3R)*$boss^{16}$ */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(17) *pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} *P[*gro^+*,*ry^+*]E8(89B)* *Df(3R)E(spl)*$^{RA7.1}$ */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(18) *pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} *P[*gro^+*,*ry^+*]E8(89B)* *Df(3R)E(spl)*^{RB251} */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(19) *pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} *P[*gro^+*,*ry^+*]E8(89B)* *Df(3R)*$gro^{r8.1}$ */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(20) Ax^{59b} */ Y ; pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} gro^{E48} */* FRT^{82B} *P[*N^+*]cos479* kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(21) Ax^{59b} */ Y ; pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} */* FRT^{82B} *P[*N^+*]cos479* kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(22) Ax^{59b} */ Y ; pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} *P[*gro^+*,*ry^+*]E8(89B) Df(3R)*$gro^{b32.2}$ */* FRT^{82B} *P[*N^+*]cos479* kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*]*

(23) ac^3 $sc^{10\text{-}1}$ */ Y ; pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} gro^{E48} */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*] Dp(1;3)*sc^{+ip3}*[y]*

(24) ac^3 $sc^{10\text{-}1}$ */ Y ; pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} *P[*gro^+*,*ry^+*]E8(89B) Df(3R)*$gro^{b32.2}$ */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*] Dp(1;3)*sc^{+ip3}*[y]*

(25) *Df(1)*sc^{B57} */ Y ; pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} gro^{E48} */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*] Dp(1;3)*sc^{+ip3}*[y]*

(26) *Df(1)*sc^{B57} */ Y ; pr pwn / pr pwn* FLP^{38}*;* FRT^{82B} kar^2 ry^{506} *P[*gro^+*,*ry^+*]E8(89B) Df(3R)*$gro^{b32.2}$ */* FRT^{82B} kar^2 ry^{506} *Dp(2;3)P32[*pwn^+*] Dp(1;3)*sc^{+ip3}*[y]*

Plasmid constructs and cotransfection analysis

A reporter plasmid pT5-0.8CAT was generated by blunt end ligation of an 894 bp *Eco*RI-*Hae*II fragment of upstream sequences of *achaete* including the transcription start, into the *Bgl*II cloning site of the CAT (chloramphenicol acetyltransferase) encoding vector pBLCAT3 (Luckow and Schütz, 1987). The sequences of the *ac* gene that were used had been previously reisolated from a wild-type (Canton-S) *Drosophila* genomic DNA library. The expression plasmids pP_{AC}-HLH-M5, pP_{AC}-HLH-M8, contain the full coding regions of the *m5* and *m8* genes of the E(spl)-C under the control of the *Drosophila* actin 5C promoter and are described by Oellers et al. (1994). The expression construct pP_{AC}-GRO was obtained from Michaela Dehio and Elisabeth Knust and was constructed similarly using a PCR amplified *Bam*HI fragment of 2016 bp that contained the *gro* coding region. pP_{AC}-DA was constructed by inserting a *Bgl*II fragment of 2.6 kb encompassing the *da* coding region prepared from the genomic clone pBS-da1Δ*Bam*HI (obtained from Harald Vässin).

Cotransfection of *Drosophila* Schneider cells (S2) by the calcium phosphate procedure was carried out using fairly standard protocols (Ashburner, 1989). Briefly, S2 cells were seeded at a density of 10^6 per ml into 90-mm culture dishes containing 10 ml of Schneider medium (GIBCO) adjusted with 10% FCS, penicillin and streptomycin. 10-15 μg of preformed DNA-$CaPO_4$ precipitates were added to the cells, consisting of 2 μg of the CAT reporter, 2 μg of an actin 5C-LacZ reporter used as an internal control for transfection efficiency and 0.1-5 μg of the different pP_{AC}-expression vectors. The samples were adjusted with Bluescript DNA. After 16 hours incubation at 22°C, the precipitates were washed out with culture medium and the cells further incubated for 24 hours. The washed cells were harvested and lysed by three cycles of freeze/thrawing into 100 μl of 250 mM Tris-HCl at pH 7.8, 5 mM DTT, 15% glycerol. The β-galactosidase activities were determined and served to standardize measurements of the CAT assays (Gorman, 1985) The results from individual experiments were expressed relative to the activity resulting from the reporter alone.

RESULTS

The phenotype of clones of cells mutant for the E(spl)-C or *gro* genes on the differentiated wing and thorax was assessed. The thorax bears two types of bristles, microchaetes and macrochaetes, whilst the wing is devoid of bristles over most of its surface and carries specialised bristles round the margin (Fig. 2). Bristles on the thorax are always spaced apart and they are separated by four or five epidermal cells, each of which secretes a fine non-sensory hair. Clones were labelled with markers for both bristles and hairs.

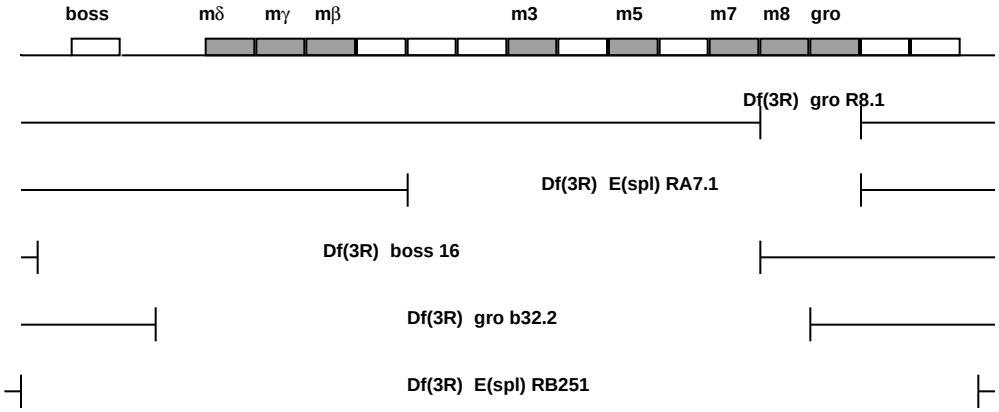

Fig. 1. The extent of E(spl)-C deletions used in this study (see Knust et al., 1992 and Schrons et al., 1992). The boxes represent known genes, bHLH genes are hatched.

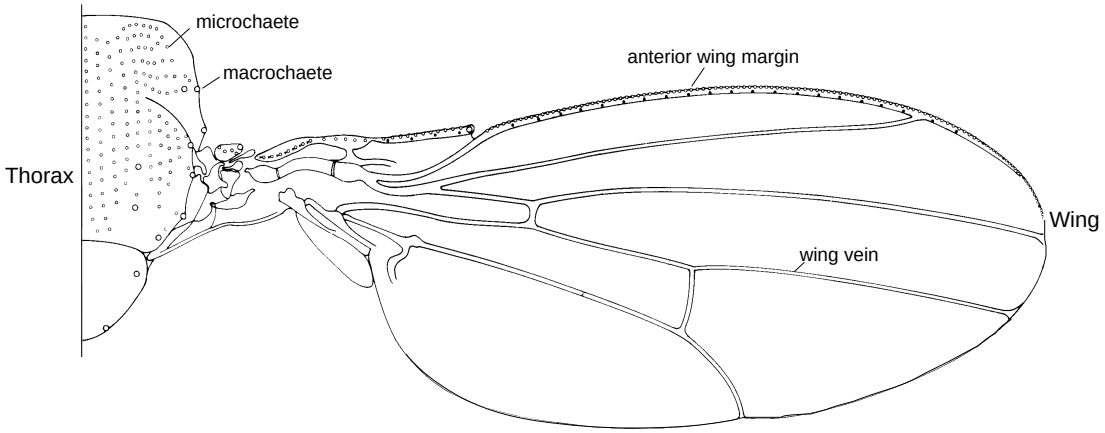

Fig. 2. Standard diagram of the wing and thorax. Bristles are indicated by circles.

Clones mutant for E(spl)-C bHLH-encoding genes or for *groucho* display bristle hyperplasia

E(spl)-C bHLH genes

Clones in which one to seven E(spl)-C bHLH-encoding genes were removed, were generated by means of FLP/FRT-mediated recombination of deficiency chromosomes (Fig. 1). Since most of these deficiencies also remove the gene *gro*, the transposon *P[gro$^+$, ry$^+$]E8* that carries a wild-type *gro* allele (hereafter referred to as *gro$^+$*) was added to the same chromosome arm as the deficiency. The mutant clones always caused bristle hyperplasia on the thorax, that is bristles developed at a greater density separated by fewer epidermal cells (Figs 3, 4).

Df(3R)gro$^{r8.1}$ (abbreviated to *r8.1*) removes the distal most bHLH gene, *m8*, and *gro*. Flies bearing this deletion, together with *gro$^+$*, are viable and have no apparent mutant phenotype. Furthermore, clones of this combination also have a wild-type appearance. *Df(3R)E(spl)$^{RA7.1}$* (abbreviated to *RA7.1*) removes the distal most four bHLH genes (*m3*, *m5*, *m7* and *m8*) and causes lethality in homozygotes even in the presence of the transposed copy of *gro$^+$*. Clones carrying *RA7.1* and *gro$^+$* display an increased microchaete density although the bristles are still separated by a number of intervening epidermal hairs (Table 1 and Figs 3A, 4A). *Df(3R)boss^{16}* (abbreviated to *boss^{16}*) removes six out of the seven bHLH genes (*m*δ, *m*γ, *m*β, *m3*, *m5* and *m7*). Clones of this deletion display a slightly stronger phenotype than the preceding combination: microchaetes are separated by fewer intervening hairs (Table 1). Finally the combination of *Df(3R)gro$^{b32.2}$* and *gro$^+$* (abbreviated to *b32.2 gro$^+$*) removes all seven of the bHLH-encoding genes. Clones of this genotype differentiate dense clumps of microchaetes that are frequently seen to be adjacent to one another (Figs 3B, 4D). Nevertheless a small amount of epidermis still differentiates and here and there bristles are seen to be intermingled with epidermal hairs. Clones mutant for *Df(3R)E(spl)^{RB251} gro$^+$* proved to be cell lethal, probably due to loss of the distal complementation groups included in this deficiency (Fig. 1; Schrons et al., 1992). The severity of the bristle hyperplasia thus increases as more and more bHLH-encoding genes are deleted.

The mutant clones described above also caused an increase in the number of macrochaetes present at each site depending on the number of bHLH-encoding genes deleted. *RA7.1 gro$^+$* has relatively little effect, occasionally two instead of the single macrochaete could be seen (Table 1). The mutant phenotype for *boss^{16}* is only slightly stronger, whereas in the combination *b32.2 gro$^+$* a small tuft of macrochaetes is usually seen at each site (Table 1).

Table 1. Bristle spacing in clones mutant for E(spl)-C genes

Genotype	Genes deleted or mutant	Number of intervening epidermal cells*	Number of macrochaetes per tuft†
(19) r8.1 gro$^+$	*m8*	4.1±0.16	1.0±0.03
(17) RA7.1 gro$^+$	*m3 m5 m7 m8*	2.5±0.11	1.1±0.06
(16) boss^{16}	*m*δ *m*γ *m*β *m3 m5 m7*	2.1±0.13	1.6±0.07
(15) b32.2 gro$^+$	*m*δ *m*γ *m*β *m3 m5 m7 m8*	‡	3.1±0.15
(14) gro^{E107}	*gro*	4.1±0.15	1.0±0.08
(13) gro^{E48}	*gro*	0	§
(8) *RA7.1*	*m3 m5 m7 m8 gro*	¶	¶
(11) Wild type	none	4.3±0.12	1.0±0.01

Genotypes: number in brackets refers to the complete genotype, see Materials and Methods.
*More than 50 microchaete pairs were scored for each case.
†More than 15 tufts were scored for each case.
‡Bristles fairly densely packed with some epidermis present here and there.
§Very dense tufts of abnormally differentiated macrochaetae. We were unable to count the number of bristles.
¶No bristles formed, scar tissue.

The deletions employed also remove other genes not related to the E(spl)-C (see Fig. 1). However, while the phenotypes differ quantitatively in the severity of the bristle hyperplasia, they are qualitatively similar and thus the other genes deleted do not have any effect on the visible phenotype (see Discussion in Schrons et al. 1992).

Finally the bristles themselves often display abnormal morphology. This is only occasionally seen in the *boss^{16}* clones but is quite frequent in the combination *b32.2 gro$^+$*. Often two or more bristle shafts can be seen protruding from a single socket. This phenotype was not studied further.

In all cases of clones removing different combinations of bHLH-encoding genes, bristles were only found, albeit in greater numbers, at locations where bristles always develop, never at ectopic sites. No nicking of the wing margin was seen. On the wing the only phenotype observed is enlarged wing veins.

groucho

Several mutant alleles of *gro* were studied, which in all cases leave the bHLH-encoding genes intact. Clones mutant for

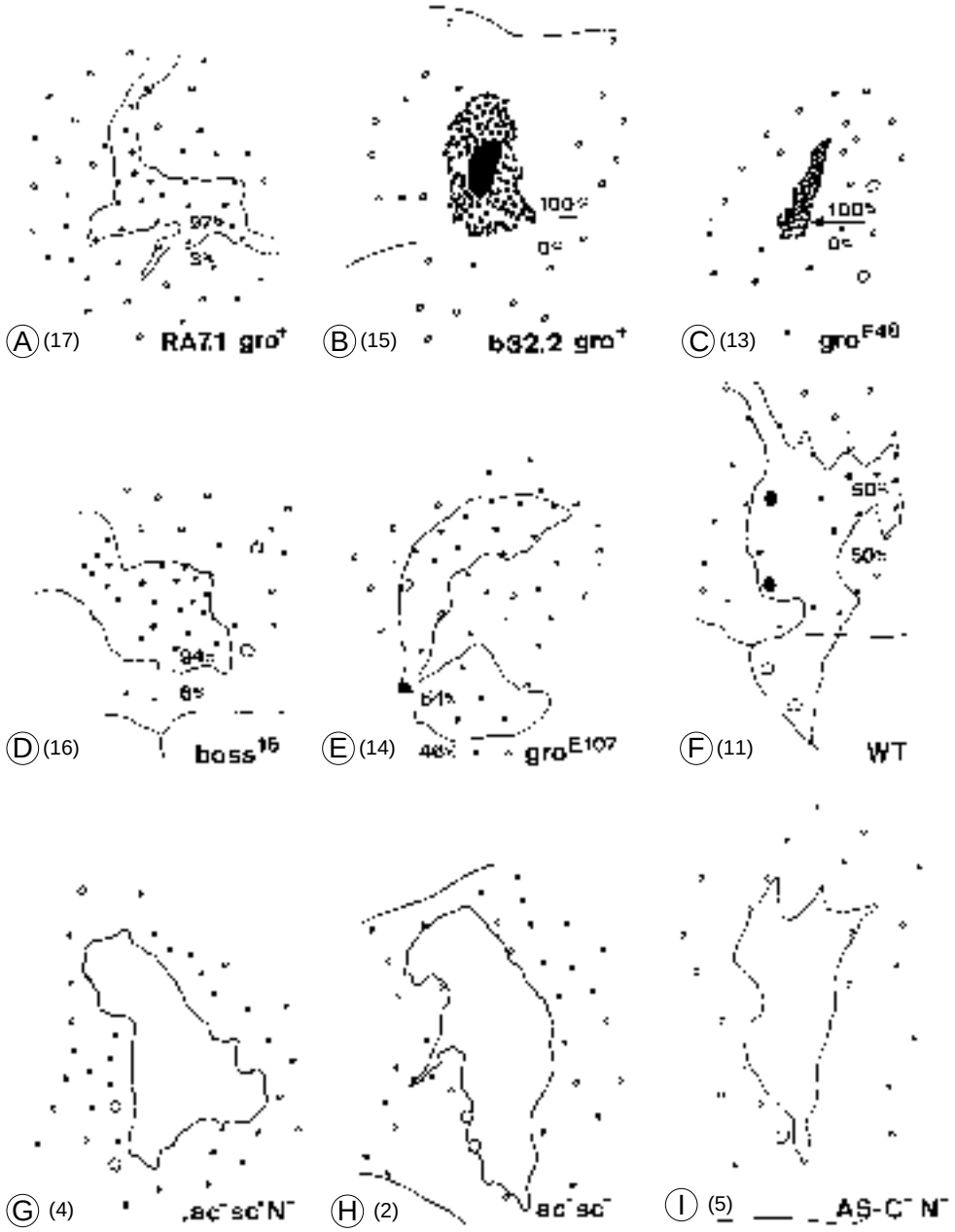

Fig. 3. Drawings of clones of different mutant combinations are presented. The numbers in parentheses refer to the complete genotype, see Materials and Methods. Open circles represent wild-type bristles and filled circles mark mutant bristles. It can be seen that some spacing between the bristles is retained for the weaker combinations *RA7.1 gro*$^{+}$ (A) and *gro*^{E107} (E). Little or no epidermal hairs are seen between the bristles of the strong mutants *b32.2 gro*$^{+}$ (B) and *gro*^{E48} (C). The percentage of mutant and wild-type bristles situated on the mosaic border are indicated inside or outside the clone border respectively. More than 100 bristles were scored for each genotype. Only in the case of the hypomorphic *gro*^{E107} (E) are a significant number of wild-type bristles seen along the mosaic border. Clones mutant for *ac* and *sc* (G,H and I) differentiate only epidermis and do not make any bristles. Thus the number of wild-type bristles could not be compared to the number of mutant ones. However, for the genotype *ac*^{3} *sc*$^{10\text{-}1}$ *N*^{55e11} (G) it was clear that wild-type bristles do not form along the clone borders: two bristles were seen for 36 clones. In contrast, wild-type bristles are seen at a high frequency on the borders of clones of the genotype *Df(1)sc*^{B57} *N*^{55e11} (I): several bristles were seen for every clone (*n*=17 clones). A positive or negative effect of these mutant clones upon their wild-type neighbours can also be seen by measuring the space beween the border of the clone and the nearest wild-type bristles. In control unmarked, non mutant clones (F) the border falls randomly between bristles, i.e. the spacing is not affected by the clone border. In this case the average distance between the nearest surrounding bristles, including those on the border, is 2.3±0.11 cell diameters (all bristles less than seven cell diameters away were scored; *n*=212 bristles scored). The figure for *Df(1)sc*^{B57} *N*^{55e11} (i) is 2.2±0.15 (*n*=113), which is no different from the controls. This reconfirms the conclusion that when all AS-C genes are removed the *N*$^{-}$ clones have no effect on the surrounding wild-type bristles. In contrast, the corresponding figures for clones mutant for E(spl)-C or *N* (genotype *N*^{ts1}, not shown) are 4.1±0.13 (*n*=158) and 4.0±0.13 (*n*=105) respectively. Such mutant cells therefore prevent wild-type cells for up to four cell diameters away from becoming bristles. This is about the same distance as the spacing between normal bristles (Table 1). A similar result was seen for cells of the genotype *ac*^{3} *sc*$^{10\text{-}1}$ *N*^{55e11} (G): 3.8±0.12 (*n*=131). The figure for *Df(1)sc*^{H} *N*^{55e11} (not shown) is intermediate: 2.8±0.16, demonstrating that activity of *ase* alone has some inhibitory activity but is less effective than *ac-sc* or *l'sc* plus *ase*.

gro^{E107}, a hypomorphic allele homozygotes of which die as white pupae, display very little effect on bristle density. However, clones mutant for *gro*^{B88}, *gro*^{E73} or *gro*^{E48}, three strong alleles that behave as nulls in genetic tests, show a complete transformation and consistently fail to differentiate epidermis (Table 1, Figs 3C, 4G). Thus microchaetes are densely packed and adjacent to one another, with no intervening epidermal cells. Only very occasionally one or two mutant epidermal hairs could be seen. This phenotype appears to be stronger than that observed when all seven bHLH-encoding genes are removed. Macrochaetes were also present as dense tufts of abnormal bristles. Several shafts per socket could often be seen and sometimes shafts were present without any socket. We have also analysed clones in which both E(spl)-C bHLH-encoding genes and *gro* are removed simultaneously. Clones mutant for *Df(3R)gro*$^{r8.1}$, which removes *gro* and only one bHLH-encoding gene, differentiate bristles. However, clones mutant for *RA7.1* which removes all bHLH-encoding genes (as

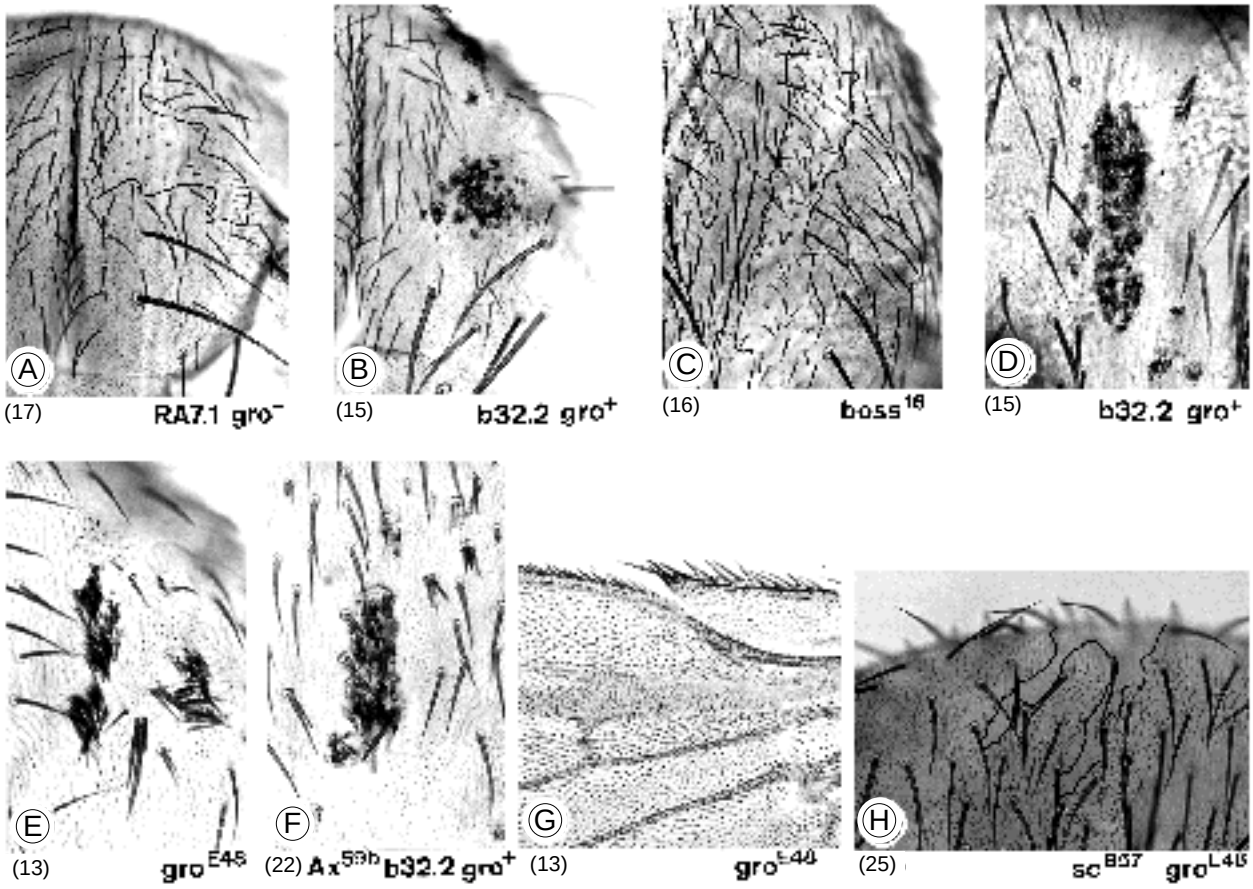

Fig. 4. Photographs of mutant clones. The clones, which are marked with *pawn*, are indicated with a black line. The numbers in parentheses refer to the complete genotypes, see Materials and Methods. Note that bristles in A and B are separated by intervening hairs. G shows a gro^{E48} clone on the wing blade that has differentiated ectopic bristles and an enlarged wing vein 2. H shows a double mutant sc^{B57} gro^{E48} clone that differentiated epidermis and should be contrasted with the gro^{E48} clone in E that differentiated bristles.

well as *gro*) fail to differentiate bristles and form scars on the cuticle, sometimes associated with a few mutant epidermal hairs (see also de Celis et al., 1991). Using $Df(3R)E(spl)^{BX22}$, which is genetically similar to *RA7.1*, Tata and Hartley (1995) showed that this phenotype is due to the formation of an excess of subepidermally located neurons at the expense of the cuticular parts of the bristle organs.

In contrast to observations with the bHLH-encoding genes, clones mutant for strong alleles of *gro* differentiate ectopic bristles. Thus microchaetes were found on the scutellum, in addition to the scutellar macrochaetes, and also on the wing blade (Fig. 4G). The development of ectopic bristles is reminiscent of the phenotype of *h* (Ingham et al., 1985). Indeed double heterozygotes for *h* and *gro* (h^{C1}/gro^{E48}) also differentiate ectopic microchaetes along wing vein 2: 31% of these flies bear between one and four bristles (n=42).

Clones were also associated with enlarged wing veins (Fig. 4G) and finally, for all three strong alleles, *gro* clones that touch the anterior wing margin are associated with wing outgrowths (not shown; see also de Celis et al., 1995).

The E(spl)-C genes participate in the *Notch* signalling pathway

Mutant cells display cell autonomy

Clones mutant for genes of the E(spl)-C thus differentiate an excess of bristles at the expense of epidermal hairs. Along the borders of the mutant clones, which can be precisely drawn, it was found that mutant cells in close contact with wild-type cells nevertheless formed bristles (Fig. 3). This was true for combinations removing only the bHLH-encoding genes and also for *gro* mutants. Thus, mutant cells cannot be induced to form epidermis by an inhibitory signal from their wild-type neighbours. This cell autonomy suggests that, like *N*, these genes are required for reception of the inhibitory signal.

E(spl)-C mutants are epistatic over a gain of function mutant of *Notch*

Loss of function mutants of *N* display a phenotype of bristle hyperplasia whereas gain of function mutants of *N* display the opposite phenotype: cells follow the epidermal pathway instead and differentiate epidermal hairs. Clones of cells doubly mutant for the *N* gain of function allele, Ax^{59b}, and either $b32.2\ gro^{+}$ or gro^{E48}, differentiate bristles and display a phenotype identical to that of $b32.2gro^{+}$ or gro^{E48} alone (Fig. 4H). Since they interrupt transduction of the signal, the E(spl)-C genes are therefore probably required downstream of *N* in the signalling cascade.

achaete-scute mutants are epistatic over E(spl)-C mutants

In the absence of the genes *ac* and *sc* no bristles develop and

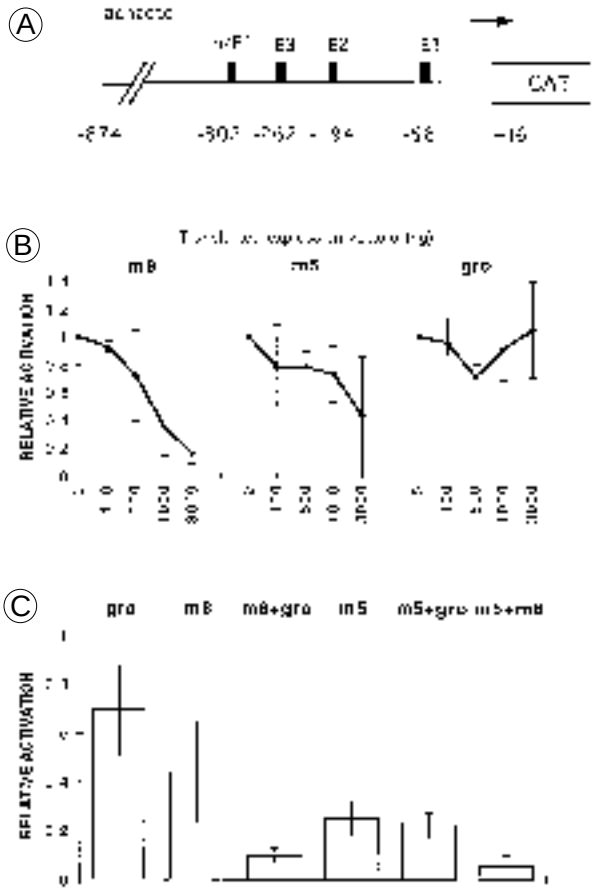

Fig. 5. The b-HLH *m5* and *m8* from the E(spl)-C can repress transactivation by *daughterless* of an *ac* reporter gene in *Drosophila* cultured cells and *gro* potentiates this effect. (A) Diagram of the known features of a minimal *ac* promoter present in the reporter construct used. These include three canonical E-boxes (E1-3) of class A recognised by b-HLH proteins (see Ohsako et al., 1994) which function as binding sites for the Da/Ac and Da/Sc b-HLH heterodimers, or Da homodimers in vitro (Van Doren et al., 1991; Cabrera and Alonso, 1991) and most probably in vivo (Van Doren et al., 1992; Martinez et al., 1993). The proximal most site, E1 shows higher affinity for these proteins (Van Doren et al., 1991) and displays extended similarities to target sites of proneural proteins which are present in other genes, and also appears to represent a site of major functional importance *in vivo* (Singson et al., 1994). A fourth site of binding for b-HLH proteins is present about 60 bp upstream of E3 and was indentified as a Hairy-specific binding site, h/E1 (Van Doren et al., 1994) or more generally as a class C site (Ohsako et al., 1994). The same site binds the b-HLH proteins *m7* (Van Doren et al., 1994), *m5* (Ohako et al., 1994), and appears to represent a variation of the sites bound by *m8* and *m5* (Tietze et al., 1992, Oellers et al., 1994) and the *h*-related mammalian HES proteins where it is referred to as the N-box. It was shown that the h/E1 site of *ac* by itself mediates repression in transfection assays or in transgenic flies (Van Doren et al., 1994; Ohsako et al., 1994). However, despite the fact that the b-HLH *m7* (Van Doren et al., 1994) and *m5* and *m8* (our results) display an effect of repression on the transcription of *ac*, they may not act exclusively through such a site. (B) Both *m5* and *m8* proteins repress the pT5-0.8 CAT reporter containing the natural *ac* upstream sequences in a dose-dependent fashion, whereas *gro* shows no effect at all. In these experiments the reporter has to be basally stimulated by the coexpression of *da* (500 ng of pP_{AC}-DA was used) resulting in a 30-fold increase in activity compared to the basal level. Under the conditions of our assays, higher amounts of expression vector for *m5* are needed to reach repression when compared to that by *m8*, whereas the combined expression of *m5* and *m8* showed strong synergistic effects. Also the expression of *h* in the same conditions leads to very similar repression of the reporter (not shown) in agreement with previously reported results (Ohsako et al., 1994). (C) Expression vectors resulting in mild repression effects (1 μg) were used in pairwise combinations of *m5*, *m8* and *gro*. The repression by *m8* was increased at least 4 fold by in the presence of transfected *gro* protein comparable to the effects of the combined *m8* and *m5* proteins. The potentiation by *gro* could not be reproducibly registered on *m5* under similar conditions. Figures are the mean and s.d. from three independent transfection experiments.

instead the cells all differentiate as epidermis. In contrast, cells mutant for E(spl)-C genes choose the neural fate over the epidermal one and the cells differentiate as bristles and do not make epidermis. We generated doubly mutant clones in which all seven bHLH-encoding genes *($gro^{b32.2}$ gro^{+})* and all of the *ac-sc* genes *(sc^{B57})* were simultaneously deleted, as well as clones mutant for both *gro* and all of the *ac-sc* genes *(sc^{B57} gro^{E48})*. A total of 21 clones of both genotypes were obtained: the phenotype of *ac-sc* prevails and cells clearly differentiate as epidermis, no bristles were seen (Fig. 4H). Thus the bristle hyperplasia seen in *E(spl)* mutant clones requires *ac-sc* activity indicating that the E(spl)-C genes act before *ac-sc*. Thus the E(spl)-C genes and *gro* act in the N signalling pathway to repress *ac-sc*.

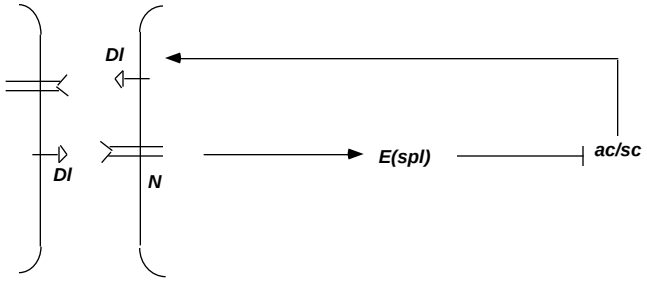

Fig. 6. Schema depicting the proposed regulatory loop between N and Dl during lateral inhibition. All cells express *ac, sc N* and *Dl*. Delta is visualized as a signal protein that acts as a ligand for the receptor N. After binding of Dl to N, a signal is transduced to the interior of the cell to E(spl)-C genes that then repress, perhaps directly, *ac-sc* expression. The transcription of *Dl* probably depends upon *ac-sc* activity.

The bHLH-encoding genes *m5* and *m8* mediate transcriptional repression of an achaete reporter; *groucho* potentiates this effect

The results described above show that both *gro* and the bHLH-encoding genes of the E(spl)-C mediate repression of *ac* and *sc* during lateral inhibition and bristle spacing. Using a cell transfection assay we found that the activation of a minimal *achaete*-CAT reporter construct (see legend to Fig. 5) is greatly attenuated by co-expression of either the *m8* or the *m5* products, but is not affected by co-expression of the *gro* protein. However, the combination of *m8* and *gro* consistently caused a greater repressing effect than *m8* alone suggesting that *gro* potentiates the repression mediated by the bHLH proteins on proneural gene activity. Therefore *m5* or *m8* can mediate repression by themselves, whereas a repressing effect of *gro* can only be achieved in combination with the bHLH proteins. A synergistic effect on repression was observed when *m5* and *m8* were added together.

Cells mutant for genes required in the *N* signalling pathway inhibit their wild-type neighbours; this inhibition requires the AS-C genes

Cells mutant for E(spl)-C genes influence the fate of adjacent wild-type cells

It was shown earlier that clones of cells mutant for *N* influence the fate of neighbouring wild-type cells and force the latter to adopt the epidermal fate (Heitzler and Simpson, 1991). Thus, along the borders of *N* clones no bristles are formed by wild-type cells. In the case of E(spl)-C mutant clones, too, a similar bias was found when compared to marked but non-mutant control clones (Fig. 3). Thus for a strong combination, such as *b32.2 gro*$^{+}$, where all seven bHLH-encoding genes are absent, all bristles along the mosaic border were found to be mutant (Fig. 3B). Clones of *gro*^{E48}, a strong allele, behaved similarly (Fig. 3C). Thus, like *N*$^{-}$ cells, cells mutant for E(spl)-C bHLH-encoding genes or *gro* prevent neighbouring wild-type cells from adopting the neural fate.

The effect of mutant cells on their neighbours requires the AS-C genes

The experiments described above and previously show that clones mutant for either *N* or E(spl)-C genes prevent nearby wild-type cells from adopting the neural fate and this can be seen by the absence of bristles of the wild-type genotype along the clone borders. In order to see whether genes of the AS-C are required for this inhibition we studied clones simultaneously mutant for *N* and different combinations of AS-C genes. When all four genes of the AS-C are removed (together with *N*, as in the genotype *Df(1)sc*^{B57} *N*^{55e11}) then wild-type bristles do form along the clone border (Fig. 3I). Therefore, in the absence of all the AS-C genes mutant *N* cells no longer affect the fate of their wild-type neighbours. However, when two of the AS-C genes, *l'sc* and *ase*, are provided (genotype *ac*^{3} *sc*$^{10\text{-}1}$ *N*^{55e11} Fig. 3G), or even only one gene, *ase* (genotype *Df(1)sc*^{H} *N*^{55e11}), then once again the *N*$^{-}$ cells prevent adjacent wild-type cells from entering the neural pathway. (Note, that, in the absence of *ac* and *sc*, mutant clones differentiate only epidermis, whether or not *N* is present (Heitzler and Simpson, 1991)). Therefore we conclude that activity of the AS-C genes is required in order for mutant *N* cells to influence the fate of their wild-type neighbours.

The relative amount of AS-C proteins can influence the choice between epidermal and neural fates

In order to see whether the quantity of Ac-Sc can influence the choice of fate, we analysed clones of cells heterozygous for *ac* and *sc* in a background of wild-type cells. We observed that 73% of the bristles forming adjacent to the clone border arise from cells that have two copies of the AS-C (n=113), a slight but significant departure from the expected 50% observed in the case of wild-type clones (n=212). These results confirm those of Cubas et al. (1991) who used a different technique, and show that cells with greater levels of Ac-Sc are more likely to become neural.

DISCUSSION

E(spl)-C bHLH-encoding genes are required to restrict the proportion of cells that differentiate as sensory organ precursors

Mutation of the bHLH-encoding genes of the E(spl)-C leads to a phenotype of bristle hyperplasia. Thus, as for the neuroblasts of the embryonic CNS, these genes are required to limit the number of neural precursors from a field of competent cells. When all seven of the bHLH-encoding genes are deleted, almost all cells choose the neural pathway and only a few epidermal cells differentiate. This results in dense tufts of adjacent macrochaetes and a uniform field of microchaetes. As observed in the embryo, weaker phenotypes result from the loss of fewer E(spl)-C bHLH-encoding genes. This is consistent with the suggestion that these genes contribute additively to the same genetic function (Schrons et al., 1992; Delidakis et al., 1991).

groucho mutants uncover the repressing function of both E(spl)-C bHLH-encoding genes and *hairy* in the imago

groucho mutants also cause bristle hyperplasia on the thorax, showing that this gene, too, functions during the neural-epidermal decision. For strong alleles the phenotype is almost completely penetrant and practically no mutant cells differentiate epidermis. Therefore, in spite of the physical proximity of *gro* and the bHLH-encoding genes, *gro* is separately required for the spacing of neural precursors. Using a cell transfection assay we demonstrate that *gro* potentiates the repressing activity of *m8* on an *ac* reporter, but that *gro* does not repress by itself. This suggest that Gro cooperates with *m8* in the repression of *ac*. Indeed it has recently been shown that the *gro* protein associates with a number of *h*-related bHLH proteins including the E(spl)-C proteins (Paroush et al., 1994). However, it is noteworthy that only the simultaneous removal of both *gro* and all of the bHLH-encoding genes mimics the *Notch* null phenotype and results in a complete failure to differentiate the bristle organ. Thus *gro* alone or the bHLH proteins alone allow(s) formation of the bristles, albeit with morphological abnormalities. Perhaps the bHLH proteins are slightly functional in the absence of Gro, and perhaps Gro can act in conjunction with other bHLH proteins.

Unlike the E(spl)-C bHLH-encoding genes, *gro* has an effect on the spatial expression of *ac-sc*. Clones mutant for *gro* display ectopic microchaetes on the wing blade and scutellum presumably caused by derepression of *ac-sc*. This phenotype is similar to that of *h*, another transcriptional repressor of *ac* (Van Doren et al., 1994; Ohsako et al., 1994). The synergism between *h* and *gro*, seen in double heterozygotes, favours the hypothesis that these two genes function together to repress *ac-sc* in the imago. This would fit with observations showing that the *gro* and *h* proteins function together during segmentation in early embryogenesis, where a similar synergism between the two mutants has been described (Paroush et al., 1994). In addition to genetic studies, the demonstration of direct protein-protein interactions between Gro and *h*-related bHLH proteins, led Paroush et al. (1994) to propose that Gro functions as a corepressor during transcriptional repression by these proteins.

Our results suggest that, in the imago, *gro* is required at two stages in the regulation of *ac*: for its spatial expression together with *h*, and also during lateral inhibition together with the E(spl)-C bHLH proteins. Clones of null *gro* alleles show a stronger phenotype than that seen when all seven of the bHLH-encoding genes are removed. This suggests that, during lateral inhibition, *gro* may have other partners in addition to the seven E(spl)-C bHLH-encoding genes described so far. Furthermore,

a novel phenotype, that of wing outgrowths, is associated exclusively with *gro* clones and is not seen for either *h* clones (Ingham et al., 1985) or for E(spl)-C bHLH clones. This suggests the existence of another unknown partner for Gro.

E(spl)-C genes act in the Notch signalling pathway downstream of *Notch* and upstream of *achaete-scute*

Considerable evidence has now accumulated showing that Dl and N function together as ligand and receptor during lateral signalling leading to the spacing of neural and sensory organ precursors. We have found that in the imago E(spl)-C mutants show a phenotype indistinguishable from that of *N* and *Dl*, and that, like *N* mutants, they behave autonomously in genetic mosaics. This indicates that the mutant cells are unable to respond to an inhibitory signal emanating from the neighbouring wild-type cells, and that they are therefore defective in signal reception. It has indeed been shown that E(spl)-C mutants are epistatic over a constitutive *N* mutant in embryos, strongly suggesting that E(spl)-C genes act after *N* in the signalling pathway (Lieber et al., 1993). Our observation on *Ax* E(spl)-C double heterozygous clones is consistent with this hypothesis and with the fact that the E(spl)-C genes encode nuclear factors. It has been reported, that, in the embryo, some of the E(spl)-C bHLH proteins are only detectable as a consequence of N signalling: they are not seen in *N* mutant embryos and are ectopically expressed in response to ubiquitous expression of an activated form of N (Jennings et al., 1994). This suggests that these genes may be transcriptionally regulated in response to receptor activation.

Transduction of the signal would lead to repression of the AS-C genes; in their absence cells would differentiate as epidermis by default. The simplest hypothesis is that the E(spl)-C genes function before *ac-sc* and this is demonstrated by the epistasy of *ac-sc* mutants over E(spl)-C mutants: double mutant clones differentiate as epidermal cells. This suggests a requirement for *ac* and *sc* at the same time or after E(spl)-C genes, in accordance with a role of *ac-sc* as regulators of the neural fate. The E(spl)-C genes would thus be part of the N signalling pathway and could directly repress *ac-sc*. This would explain the repression of *ac* transcription on a reporter gene observed in our cell transfection assay. It has been proposed that the *Suppressor of Hairless* protein is a direct mediator of N signalling (Fortini and Artavanis-Tsakonas, 1994; Schweisguth, 1995). This protein behaves like a transcription factor that could transcriptionally regulate the E(spl)-C genes (Tun et al., 1994). Our results also show that the E(spl)-C genes are not required for the differentiation of epidermis, in their absence cells can develop into either sensory organs or epidermis, depending on the presence or absence of *ac-sc*.

It should be noted, however, that previous reports have shown that transcription of the E(spl)-C genes may depend on the *ac-sc* genes themselves (Singson et al., 1994; Kramatschek et al., 1994). It is possible that such a dependance is a non-autonomous, indirect consequence of the regulatory loop between N and Dl described below (see next section). This would not, however, account for the observations of direct binding of AS-C proteins in vitro on the *m8* and *m7* promoters and that activation of *m8* in vivo requires a bHLH binding site (Oellers et al., 1994; Singson et al., 1994; Kramatschek et al., 1994). Whether or not direct regulation of the E(spl)-C genes by the AS-C gene products takes place in the imaginal discs, the fact that *ac-sc* is epistatic over E(spl)-C genes argues strongly that *ac* and *sc* act after E(spl)-C genes to specify the neural fate.

The E(spl)-C and AS-C genes participate in a regulatory loop between Notch and Delta

Results from several laboratories are in favour of the idea that nascent neural or sensory organ precursors send an inhibitory signal via the ligand Dl, that is transduced within the presumptive epidermal cells from the receptor N to the AS-C genes by a linear signal cascade involving E(spl)-C. However, before this situation can prevail, it is first necessary to choose the presumptive neural precursor from amongst the cells of the proneural clusters, all of which express *ac-sc* and have the potential to go into the neural fate. These cells also each express both *N* and *Dl* (Kooh et al., 1993). We have previously shown that cells mutant for *N* produce a strong inhibitory signal that forces neighbouring wild-type cells into the epidermal fate (Heitzler and Simpson, 1991). Consistent with its role as the signalling molecule, Delta is required for this signal, mutant *N* cells that are simultaneously mutant for *Dl*, no longer inhibit their neighbours (Heitzler and Simpson, 1993). These results suggested the existence of a feedback loop between signal and receptor within each cell whereby activation of N would decrease the ability of a cell to inhibit its neighbours via Dl. Thus an initial small difference between the cells would be amplified by the postulated regulatory loop. The results presented here indicate that this mechanism requires both the E(spl)-C and the AS-C genes and is thus under transcriptional control.

Cells mutant for E(spl)-C genes, that act downstream of *N*, also inhibit adjacent wild-type cells. Furthermore inhibition of wild-type cells requires the AS-C genes themselves: cells mutant for *N* can no longer inhibit their neighbours if none of the AS-C genes are present. The production of signal therefore relies on the activity of at least one of the AS-C genes. Indeed inhibitory activity is restored to the mutant *N* cells by the addition of *l'sc* and *ase*, or *ase* alone, though less efficiently. (Note that, in the absence of *ac* and *sc*, the cells do not have neural potential and so clones do not make bristles, even in the presence of the *l'sc* and *ase* genes. Perhaps this is because the levels of proneural protein are insufficient for bristle development. The activity of *l'sc* and *ase* is sufficient, however, to provide signalling ability. It is probable, that, in the wild-type wing disc, Ac and Sc themselves are responsible for production of the signal).

If the signal depends upon *ac* and *sc*, then it follows that *Dl* must be controlled by these genes. Indeed it has been observed that, in thoracic discs, *Dl* expression is present first in a proneural cluster-like pattern and then at high levels in the sensory organ precursor, as visualized both by in situ hybridisation and by expression of a *Dl* enhancer trap line or by *Dl-lacZ* fusions (Huang, Ghysen and Dambly-Chaudière, personal communication; Haenlin et al., 1994). Furthermore, discs that are mutant for *ac-sc* fail to express *Dl* (ibid). The *ac-sc* genes may in fact act directly on the transcription of *Dl*: it has recently been shown that AS-C products activate transcription of *Dl* by binding to specific sites in its promoter (Kunisch et al., 1994). Thus cells with more doses of the AS-C genes are

more competitive for the neural fate because they express more Dl. We have previously shown that cells with a higher amount of Dl relative to their neighbours have a greater chance of becoming neural (Heitzler and Simpson, 1991). Thus the initial bias between the competent cells may depend on the levels of Ac-Sc, which are known to vary between cells of the proneural cluster (Cubas et al., 1991). We therefore suggest that the feedback loop between N and Dl is mediated through activation of E(spl)-C genes and repression of AS-C genes, as shown in Fig. 6. This lateral signalling ensures that only one cell will ever become a precursor.

Notch and *Dl* homologues have been described in a number of different species, including mammals (see Artavanis-Tsakonas et al., 1995, and Simpson, 1994 for review). The *Caenorhabditis elegans N* homologue *lin-12*, has been shown to play a role in lateral signalling that is very similar to that seen in *Drosophila* (Seydoux and Greenwald, 1989) and indeed it has been demonstrated that a feedback loop operating between Lin-12 and its ligand Lag-2, is also under transcriptional control (Wilkinson et al., 1994). Recently, a study of early nervous system development in *Xenopus*, provides evidence for a role of *X-Notch-1* and *X-Delta-1* in a lateral inhibition process limiting the number of cells that differentiate as neurons (Chitnis et al., 1995).

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