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Functional relationships between *Notch*, *Su(H)* and the bHLH genes of the *E(spl)* complex: the *E(spl)* genes mediate only a subset of *Notch* activities during imaginal development

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SUMMARY

The basic helix-loop-helix proteins of the *Enhancer of split* complex constitute a link between activation of the transmembrane receptor Notch and the resulting effects on transcription of downstream genes. The Suppressor of Hairless protein is the intermediary between Notch activation and expression of all *Enhancer of split* genes even though individual genes have distinct patterns of expression in imaginal discs. A comparison between the phenotypes produced by *Notch, Suppressor of Hairless* and *Enhancer of split* mutations in the wing and thorax indicate that *Suppressor of Hairless* and *Notch* requirements are indistinguishable, but that *Enhancer of split* activity is only essential for a subset of developmental processes involving *Notch* function. Likewise, the ectopic expression of Enhancer of split proteins does not reproduce all the consequences typical of ectopic Notch activation. We suggest that the Notch pathway bifurcates after the activation of Suppressor of Hairless and that Enhancer of split activity is not required when the consequence of *Notch* function is the transcriptional activation of downstream genes. Transcriptional activation mediated by Suppressor of Hairless and transcriptional repression mediated by Enhancer of split could provide greater diversity in the response of individual genes to Notch activity.

Key words: *Drosophila*, *Notch*, *Suppressor of Hairless*, *Enhancer of split*, transcription, gene

INTRODUCTION

Notch function is essential for many cell fate decisions in *Drosophila* that involve the singling out of one cell from a group of competent cells (Muskavitch, 1994). In these processes, the Notch protein appears to function as a receptor in a cell-cell signalling pathway whose other components include the ligands Delta and Serrate, the intracellular transducer Suppressor of Hairless and the nuclear proteins encoded by the *Enhancer of split* complex (Artavanis-Tsakonas et al., 1995). Homologues of Notch, as well as other elements of the Notch pathway, have now been identified in other species and appear to act in an analogous manner, illustrating that this pathway is highly conserved (Artavanis-Tsakonas et al., 1995). The *Enhancer of split* genes [*E(spl)*] are required downstream of Notch (Vassin et al., 1985; de la Concha et al., 1988) since the accumulation of E(spl) proteins depends on Notch activation and they mediate the effects of constitutively active forms of Notch during neurogenesis (Lieber et al., 1993; Jennings et al., 1994; Heitzler et al., 1996). *E(spl)* is a complex locus that includes seven genes encoding related basic-helix-loop-helix (bHLH) proteins (m8, m7, m5, m3, mβ, mγ and mδ, see Fig. 1) which appear responsible for the actions of the locus during neural development (Knust et al., 1992; Delidakis and Artavanis-Tsakonas, 1992; Schrons et al., 1992). Detailed genetic analyses suggested that there is some overlap in the functions of these proteins (Delidakis et al., 1991; Schrons et al., 1992). Furthermore, no lethal mutations have been obtained in a single *E(spl)*bHLH gene preventing the assignation of specific function to individual genes. Consistent with the proposed functional redundancy, the patterns of expression of the different *E(spl)*bHLH genes are very similar, if not identical, during neurogenesis in the embryo (Knust et al., 1987, 1992).

The regulation of *E(spl)m8* gene expression appears to be mediated by Suppressor of Hairless [Su(H)], a DNA-binding protein (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995), which in tissue culture cells translocates from the cytoplasm to the nucleus when Notch interacts with its ligand Delta (Fortini and Artavanis-Tsakonas, 1994). The *E(spl)m8* upstream region contains several binding sites for Su(H), and its transcriptional activation by Notch is compromised by mutations that prevent Su(H) binding (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Thus,

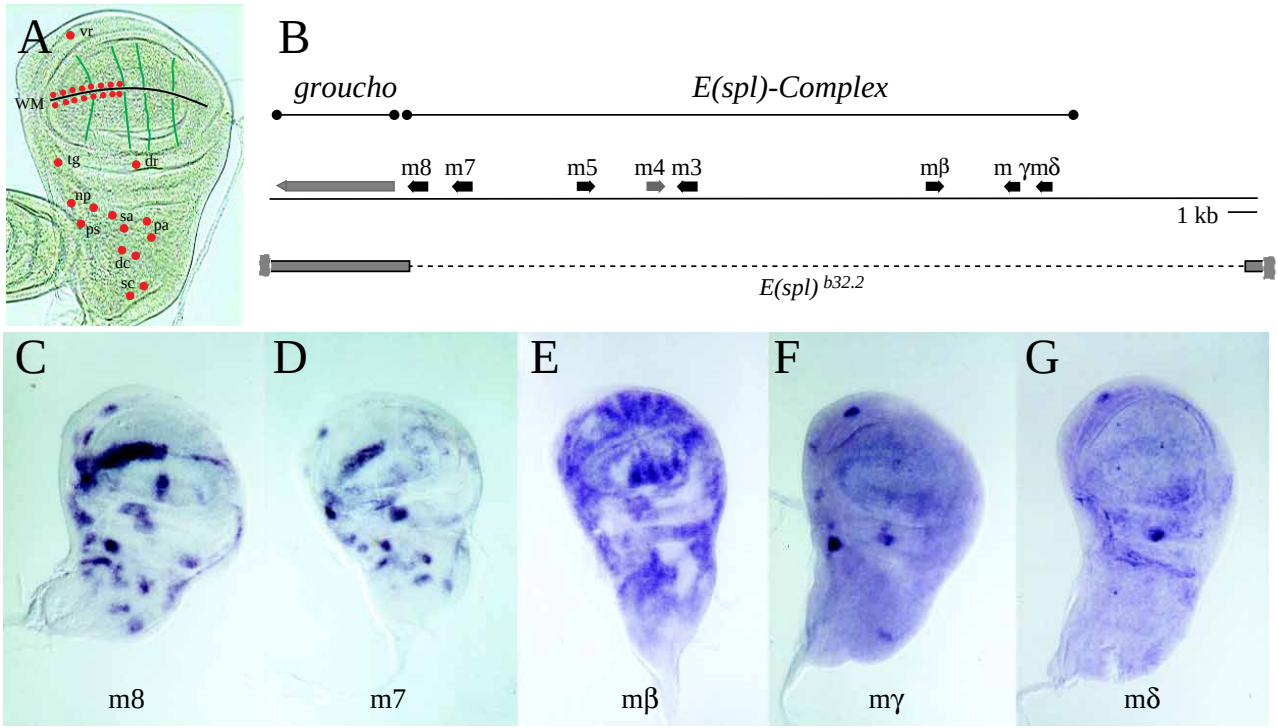

Fig. 1. Organisation of the *E(spl)-C* and expression of individual *E(spl)* genes in wing discs. (A) Fate map of a wing imaginal disc with the location of developing SOPs (red) veins (green) and wing margin, WM, (black) indicated. The positions of macrochaetae SOPs and some sensilla SOPs are indicated by red dots with the corresponding abbreviations: np, notopleural; ps, presutural; sa, supraalar; dc, dorsocentral; pa, postalar; sc, scutellar; dr, dorsal radius; vr, ventral radius; tg, tegula. (B) Map of the *E(spl)-C*. The line represents genomic DNA, distal is to the left, the black arrows indicate transcripts encoding the bHLH proteins and the grey arrow the unrelated m4 transcript. The neighbouring *groucho* transcript is indicated by a stippled arrow. The extent of the deletion in *E(spl)*$^{b32.2}$ is depicted, the shaded boxes indicate DNA remaining and dotted lines DNA deleted. This diagram is based on the following: (Delidakis and Artavanis-Tsakonas, 1992; Delidakis et al., 1991; Knust et al., 1992; Schrons et al., 1992). (C-G) Expression of *E(spl)* genes in wing imaginal discs from third instar larvae detected by in situ hybridisation, (C) *m8*, (D) *m7*, (E) *mβ*, (F) *mγ* and (G) *mδ*. All discs at the same magnification.

it is proposed that the interaction of Delta with Notch results in activation of Su(H) and subsequently in expression of *E(spl)m8*. This and other E(spl) proteins are therefore nuclear effectors of the Notch signal and are likely to modulate the transcription of downstream targets. The effects of E(spl)bHLH proteins on transcription may involve interaction with the product of the neighbouring gene *groucho* (*gro*) (Hartley et al., 1988; Schrons et al., 1992; Paroush et al., 1994). Gro has sequence similarity to the yeast co-repressor TUP1 suggesting that the complexes formed between Gro and E(spl)bHLH repress transcription of target genes (Paroush et al., 1994). However, although the consequence of Notch activation in some processes is the transcriptional repression of specific downstream genes (e.g. *achaete, scute* expression during neurogenesis; Skeath et al., 1992; Ruiz-Gomez and Ghysen, 1993), in others it appears to be transcriptional activation (e.g. *wingless, cut and vestigial* expression during wing margin development; Rulifson and Blair, 1995; Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; de Celis et al., 1996; and *single-minded* in mesectodermal development; Martin-Bermudo et al., 1995; Menne and Klambt, 1994). The extent to which E(spl) proteins mediate these different transcriptional responses to Notch activity is not known.

During imaginal development *Notch* is required for the correct specification of many cell types, such as sensory organs, wing veins and wing margin in the wing disc, and most cell types during eye development (Shellenbarger and Mohler, 1978; Dietrich and Campos-Ortega, 1984; Cagan and Ready, 1989; Parody and Muskavitch, 1993; de Celis and Garcia-Bellido, 1994). Here we have investigated the regulation and function of individual *E(spl)*bHLH genes to determine whether they all respond to Notch activation and whether they participate in the different Notch-dependent processes occurring during wing development. Although normal expression of all *E(spl)* genes in the imaginal discs requires Notch-pathway activity, we find that individual *E(spl)* genes are expressed in distinct patterns, implying that other factors participate in regulating their expression in addition to Notch. This indicates a hidden complexity in the regulatory regions of these genes that could explain the conservation of the overall organisation of the complex between different species of *Drosophila* (Maier et al., 1993). In addition, we find that E(spl) proteins are only essential for a subset of the processes requiring Notch activity suggesting that Su(H) regulates other Notch downstream genes independent of the *E(spl)* genes. These findings provide a basis for the different effects of *Notch* on individual target genes and suggest that the specificity of the response to Notch resides in the organisation of the regulatory regions of *Notch* target genes.

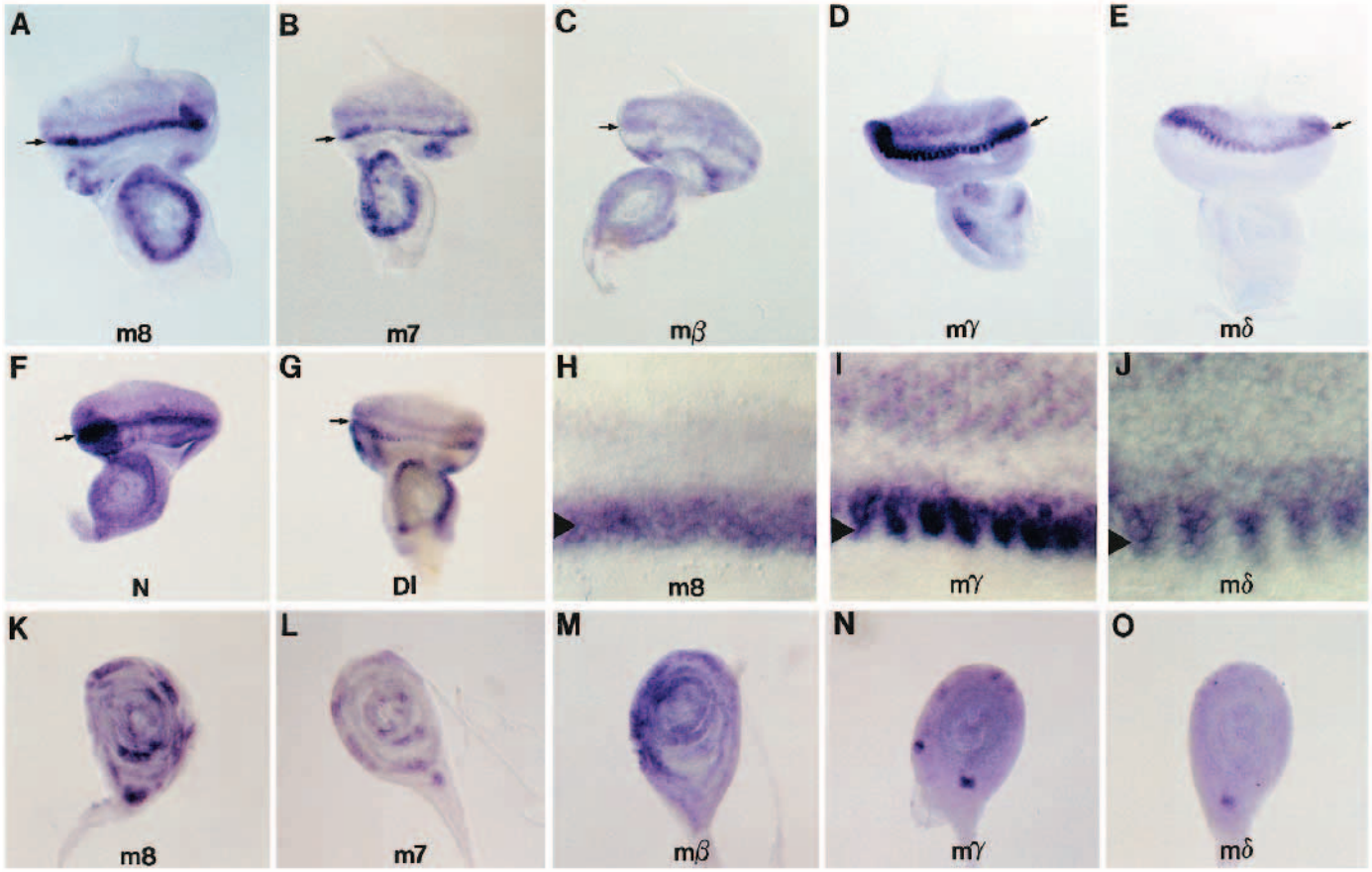

Fig. 2. The individual *E(spl)* genes have distinct patterns of expression in eye and leg imaginal discs. The expression of the mRNAs from the *E(spl)* genes *m8* (A,H,K,) *m7* (B,L) *mβ* (C,M) *mγ* (D,I,N) and *mδ* (E,J,O) was detected by in situ hybridisation in eye (A-J) and leg (K-O) imaginal discs. The expression of *Notch* (F) and *Delta* (G) mRNAs in the eye disc is also shown for comparison. (H-J) Higher magnification of a region of the discs shown in A, D and E, respectively. In the eye discs, each *E(spl)* gene has a unique pattern of expression, although in all cases high levels are detected close to the morphogenetic furrow (arrows, A-E, arrowheads H-J), as is also the case for *Notch* and *Delta* mRNAs. Expression at the furrow of some genes (*mδ*, *mγ* and *Delta*) appears in discrete clusters of cells and these can be seen at higher magnification in I (*mγ*) and J (*mδ*), and contrast with the more uniform and slightly more anterior expression of *m8* (H). The expression patterns observed for each mRNA were similar between leg discs and a representative example is shown (K-O).

MATERIALS AND METHODS

Genetic strains

We have used the loss-of-function alleles N^{55e11}, N^{ts1} (Lindsley and Zimm, 1992), $Su(H)^{AR9}$ and $Su(H)^{1}$ (Schweisguth and Posakony, 1992). The *E(spl)* deficiency, $E(spl)^{b32.2}$, was originally named $gro^{b32.2}$ but has subsequently been renamed because it deletes all the *E(spl)* genomic region encoding the seven bHLH genes (Schrons et al., 1992) and retains some *gro* function since it complements the viable allele gro^{1}(data not shown). We confirmed that this strain was as originally reported using probes prepared from *m8*, *m5*, *m3* and *mδ*, and the proximal breakpoint of the deficiency on Southern blots of genomic DNA from flies heterozygous for this deficiency (data not shown). The cell markers used for clonal analyses were *forked* (f^{36a}) and *multiple wing hair* (*mwh*) (Lindsley and Zimm, 1992). We used two transgenes carrying the *f* wild-type allele inserted in 30B and 87F (designated P[f$^+$]30B and P[f$^+$]87F, respectively (a gift from P. Martin). The *Minute* alleles to generate M^+ clones were $M(1)o^{sp}$, *M(2)24F* and *M(3)95A* (Lindsley and Zimm, 1992). The GAL4 lines used were $GAL4^{c179}$ (a gift from A. Brand), $GAL4^{459.2}$ (Thomas et al., 1995) and $GAL4^{ptc}$ (Speicher et al., 1994). The UAS lines used were UAS-Ser (Speicher et al., 1994), UAS-m8 (Tata and Hartley, 1995) and those described below.

Generation of mitotic recombination clones

Mitotic recombination was induced by X-rays (dose 1000 R; 300 R/minute, 100 kV, 15 mA, 2 mm Aluminium filter). Irradiated larvae were timed in hours after egg laying (AEL). Clones were induced at the interval 48-72 hours AEL. Mutant clones in the X chromosome were generated in flies of genotype N^{55e11} $f^{36a}/M(1)o^{sp}$ and $N^{55e11}/Dp(1;Y;3)M2$, mwh^+f^{36a};*mwh*. Mutant clones in the 3R chromosomic arm were generated in males of genotypes f^{36a};*M(3)95A* P[f$^+$]*87F*/$E(spl)^{b32.2}$ and in the 2L arm in f^{36a};*M(2)24F* P[f$^+$]*37A*/$Su(H)^{AR9}$ males. Mitotic recombination proximal to the f^+ insertion produces homozygous mutant clones labeled with the cell marker *f*. Mutant clones in the thorax were recognized with the cell marker *f* ($E(spl)^{b32.2}$) or by the presence of naked cuticle (N^{55e11} and $Su(H)^{AR9}$).

Generation of UAS-mβ, UAS-m7 and UAS-mδ lines

A modified form of pUAST (Brand and Perrimon, 1993) was generated to include an optimal translation initiation site. In brief, pUAST was digested with *Eco*RI, the ends filled in and the plasmid religated to remove the *Eco*RI site. The resulting plasmid was then digested with *Bgl*II and *Xho*I and ligated with a [*Bgl*II-*Eco*RI]AUG cassette and an *Eco*RI-*Xho*I fragment containing the entire coding sequence of *mδ*, *mβ* or *m7* (the sequence of the AUG-cassette was AGATCTGTCGACATTACAAATGGAATC). The *Eco*RI/*Xho*I

E(spl) fragments were generated by polymerase chain reaction (5 cycles in the presence of *Pfu*I from Stratagene) from cDNA (*mβ*) or genomic (*mδ* and *m7*) clones (Delidakis and Artavanis-Tsakonas, 1992) using primers that spanned the initiation and termination sites and included an *Eco*RI or *Xho*I site, respectively (primer sequences available on request). The resulting UAS constructs were injected into *y w Drosophila* embryos to generate transformant lines using standard procedures (Rubin and Spradling, 1982). Once homozygous transformant lines with single inserts were established, we confirmed that they were capable of expressing the appropriate cDNA when crossed to GAL4 driver lines using in situ hybridisation (*m8*) or mAb323 (*mδ*, *mβ*, m7) to monitor the expression.

In situ hybridization and immunocytochemistry

In situ hybridizations were carried out using digoxigenin-labeled DNA fragments following the protocol of Cubas et al. (1991). To detect individual E(spl)bHLH, we used small fragments to minimise the possibility of cross-reactivity; none of the fragments contain the most conserved bHLH portion of the genes. Fragments used were from cDNA and genomic clones (Delidakis and Artavanis-Tsakonas, 1992) as follows with vector-derived restriction sites in brackets: 0.5 kb *Eco*RI/*Xho*I fragment from c-*m8-a*; 0.7 kb *Hin*dIII/(*Not*I) fragment from c-*mβ*-14a; 0.8 kb *Sph*I/(*Not*I) fragment from c-*m3*-15a; 0.7 kb *Nco*I/(*Not*I) fragment from c-*m5*-h; 0.4 kb genomic *Sac*I fragment for *m7*; 0.7 kb *Eco*RV/*Hin*dIII fragment from the 2.1 kb genomic *Hin*dIII fragment spanning *mγ* and a 0.4 kb *Nco*I/(*Nco*I) fragment from the 1.5 kb *Hin*dIII genomic fragment spanning *mδ*. Other fragments used were: 0.8 kb *Eco*RI fragment from the *wg* cDNA clone wgc14 (Baker, 1988); 2 kb *Eco*RI/*Sac*I fragment of T4 (*scute*; Cubas et al., 1991). Immunocytochemistry with the mAb323 antibody to detect *E(spl)*bHLH expression was performed as described in Jennings et al. (1995). Secondary antibody was from Jackson Immunological Laboratories (used at 1/250).

For analysis of *E(spl)* mRNA expression in *Su(H)*, mutant larvae were selected from the cross between $Su(H)^{1}$/*SM5-TM6B* and $Su(H)^{AR9}$/*SM5-TM6B* by the absence of the dominant *Tubby* marker carried on *TM6B*. The N^{55e11}/N^{ts1} larvae were generated by crossing N^{55e11}/Y; $Dp(1;2)w^{+51}$/*SM5-TM6B* with N^{ts1} females. *Tubby* female larvae (i.e. N^{55e11}/N^{ts1}; +/*SM5-TM6B*) were selected and maintained at 30ºC for 12 hours before processing for in situ hybridisation.

RESULTS

Distinct expression patterns of different E(spl)bHLH genes

If transcriptional activation of *E(spl)*bHLH genes depends exclusively on Notch activity, we might expect all *E(spl)*bHLH genes to have similar patterns of expression. This may be the case during neurogenesis in the embryo (Knust et al., 1987, 1992) but preliminary analysis, using antibodies in the imaginal discs, indicates that *E(spl)* genes could have distinct patterns of expression (Jennings et al., 1995). Using DNA probes that detect individual genes of the *E(spl)*-C, we have analysed the distribution of their mRNAs in the imaginal discs, with particular emphasis on the wing disc. Strikingly, we observe dramatic differences in the domains of expression (Figs 1, 2). As reported previously, the *m8* and *m7* mRNAs are detected in clusters of cells that correspond to the locations where sensory organ precursors (SOPs) develop (Fig. 1C,D; Hinz et al., 1994; Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). In addition, *m8* is also detected in cells at the dorsal/ventral boundary throughout the third instar. Expression of both *mγ* and *mδ* is also associated with the SOPs. However, these mRNAs are only detected in a subset of proneural clusters: particularly the dorsal and ventral radius for *mδ* and the dorsal and ventral radius, scutellum, tegula and at low levels along the anterior wing margin for *mγ* (Fig. 1F,G). Like *m8*, *mγ* is also present at high levels at the dorsal/ventral boundary in early stages (data not shown). The domain of *mβ* mRNA is the most distinctive, its expression is prominent in the wing blade and consists of several proximodistal stripes crossing the dorsal/ventral boundary that appear to be associated with the developing veins, and is also present at the dorsal/ventral boundary and wing margin. In addition, *mβ* shows a complex pattern elsewhere in the disc, with no simple association with developing sensory organs (Fig. 1E). We cannot detect expression of the *m5* gene in the wing imaginal disc and expression of *m3* occurs at low levels in association with some proneural clusters (data not shown).

The *E(spl)*bHLH genes also have distinct expression patterns in other discs. In the eye imaginal disc, expression of *m7*, *m8*, *mγ*, *mδ* and *mβ* genes is detected close to the morphogenetic furrow, (Fig. 2) with *m8* mRNA in a stripe of cells approx. 4-6 cells wide spanning the furrow (Fig. 2A,H), *m7* mRNA in a more modulated pattern in the same region (Fig. 2B) and *mγ* and *mδ* mRNAs in discrete clusters of cells that appear to lie just posterior to the furrow (Fig. 2D,E,I,J). The high levels of *E(spl)* expression close to the furrow correlate well with the presence of both *Notch* and *Delta* mRNAs in this region, with the latter present in clusters of cells somewhat resembling *mγ* and *mδ* (Fig. 2F,G). In more posterior regions of the disc, where the recruitment of undifferentiated cells into ommatidial units occurs, there is little expression of *m8* and *m7*, whereas *mγ*, *mδ* and *mβ* are all detected at quite high levels in subsets of cells (Fig. 2A-E,H-J). In the leg discs, *mβ* is particularly detected at the regions of the presumptive joints whereas *m8*, *m7*, *mγ* and *mδ* appear in clusters of cells in a similar pattern to that of *achaete/scute*, suggesting that they are again associated with developing SOPs (Fig. 2K-O). As in the wing disc, *mγ* and *mδ* are found at only a subset of these locations. The distinct patterns of expression that we observe raises the possibility that particular cell-fate decisions mediated by Notch during imaginal development involve specific *E(spl)*bHLH genes.

Notch signalling is essential for normal expression of all *E(spl)* genes

Previous analyses indicates that *m8* gene expression requires Notch activation and is mediated by Su(H) (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995); however, the dramatically different distribution of the mRNAs led us to investigate whether this is the case for other *E(spl)* genes. We examined expression of *mβ*, *m7*, *mδ*, and *m8* mRNAs in discs derived from larvae with loss-of-function mutations in the *Su(H)* gene ($Su(H)^{1}/Su(H)^{AR9}$). In all cases, their expression was severely reduced or absent in wing discs derived from mutant larvae (Fig. 3A,D,G,J) The results with *m8* contrast with those obtained using an *m8-lacZ* reporter gene, where β-galactosidase was found to accumulate to high levels in *Su(H)* discs in the places where proneural proteins are expressed (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). We do detect some residual *m8* in these domains, but the levels are much lower than those seen in

wild-type discs (Fig. 3A). Similar results were obtained when expression of *mβ* was analysed in discs with reduced *Notch* function (N^{ts}/N^{55e11}) even though, in these mutants, the wing pouch is less severely reduced compared to *Su(H)* mutants (Fig. 3K).

In general, the effects of *Su(H)* mutations on *E(spl)* expression were similar in leg (Fig. 3B,E,H) and eye (Fig. 3C,F,I) discs. However, we detected some residual expression in the eye disc, particularly for *m8* where the stripe of expression normally associated with the furrow remains quite strong (Fig. 3C). Similarly low levels of *m8* expression are detected in some patches in the leg disc (Fig. 3B).

E(spl)bHLH do not account for all the actions of Su(H)

Our data indicate that although Notch/Su(H) signalling is required for normal expression of each *E(spl)*bHLH, additional factors act on individual genes to confer distinct patterns of expression**.** To address the converse question, of whether E(spl)bHLH mediate all the effects of *Notch* and *Su(H)*, we have compared the phenotypes of cells lacking *E(spl)* function (using a deletion that removes all seven *E(spl)*bHLH genes, $E(spl)^{b32.2}$) with those of *Notch* and *Su(H)* mutant cells (Fig. 4). During development of sensory organs, Notch is required at two steps, first in the singling out of one sensory organ precursor (SOP) and second in the establishment of the correct fates in the four progeny cells. Thus, in the thorax, clones of *Notch* and *Su(H)* produce patches of naked cuticle (Fig. 4I-K; Dietrich and Campos-Ortega, 1984; Heitzler and Simpson, 1991; de Celis et al., 1991; Schweisguth, 1995), due to the differentiation of the resulting ectopic SOPs into neurons (Hartenstein and Posakony, 1990; Schweisguth, 1995). In contrast, cells lacking E(spl)bHLH proteins differentiate into dense clusters of bristles at the locations where the macrochaetae develop (Fig. 4L; Heitzler et al., 1996). Similarly, in mutant clones the microchaetae differentiate at much higher density forming large fields of bristles (Fig. 4L; Heitzler et al., 1996). This phenotype suggests that all cells in a proneural field become sensory organ precursors (SOP), each of which gives rise to a complete mechanosensory organ, and differs from the naked cuticle phenotype observed when *gro* function is removed (de Celis et al., 1991; Heitzler et al., 1996). This indicates that *E(spl)* function is required for the segregation of a single SOP but not for the correct differentiation of the progeny from each SOP unlike *Notch*, *Su(H)* and *gro*, which affect both processes. Occasionally we detect duplicated trichogens in clones of *E(spl)* mutant cells, but these are associated with fused tormogens suggesting that they arise from closely packed SOPs rather than from changes in the identity of SOP progeny cells.

A similar lack of consonance between the effects of removing *Notch* or *E(spl)* occurs in clones that reach the wing margin. Clones of *Su(H)* and *Notch* cause the loss of margin and blade tissue due to the requirement for *Notch* in the cells at the dorsal/ventral boundary (Fig. 4A-C; de Celis and Garcia-Bellido, 1994; Diaz-Benjumea and Cohen, 1995; de Celis et al., 1996). *E(spl)* mutant cells do not give this phenotype but only affect the differentiation of sensory structures along the wing margin without affecting its integrity (Fig. 4D). The mild nicking of the wing margin observed with deficiencies that delete *gro* in addition to three genes of the *E(spl)* complex are thus most likely attributed to the removal of *gro* (de Celis and Ruiz-Gomez, 1995; de Celis et al., 1996).

The phenotypes of vein thickening observed in clones of *Su(H)* or *E(spl)* mutant cells suggest a requirement for these genes in establishing the normal vein thickness, a process that is also dependent on *Notch* activity (Fig. 4F-H; de Celis and Garcia-Bellido, 1994, and unpublished data). Thus at least some of *Notch* function in vein differentiation must be mediated by E(spl), a likely candidate being mβ.

Ectopic expression of E(spl) does not reproduce the effects of Serrate-mediated activation of Notch

The effects of Notch in sensory organ development and in the formation of the wing margin are reflected in the expression of the *achaete/scute* complex (*ac/sc*) and *wingless* (*wg*) genes, respectively. In proneural clusters, expression of *ac/sc* is repressed in cells that do not adopt the SOP fate presumably as a consequence of Notch activity (Skeath and Carroll, 1991; Cubas et al., 1991). Conversely *Notch* function at the dorsal/ventral boundary leads to the expression of several genes including *wg* (Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; de Celis et al., 1996). These differences might relate to the requirements for *E(spl)* in sensory organ development but not in wing margin formation. To investigate this possibility, we have compared the consequences of ectopic Notch and E(spl) activity on the transcription of *wg* and *sc*. To activate Notch ectopically, we have expressed Serrate in a central domain in the wing disc using two different GAL4 driver lines, $GAL4^{patched}$ and $GAL4^{459.2}$. It has previously been reported that these conditions result in Notch activation along the boundary of the ectopic Serrate domain in the ventral part of the disc as detected by the ectopic expression of genes such as *wg* (Fig. 5C,D; Kim et al., 1995; Thomas et al., 1995). Consistent with their expression at sites of Notch activation, we also find that E(spl)bHLH expression is induced by ectopic Serrate in a similar pattern to that seen for *wg* (Fig. 5C,D,G,H). In contrast, no ectopic activation of *wg* occurs when any of four different E(spl)bHLH proteins (m8, m7, mβ or mδ) are ectopically expressed using the same GAL4 driver lines (Fig. 5E and data not shown). Furthermore, the overgrowth and ectopic margin structures typical of ectopic Serrate expression in the $GAL4^{ptc}$ domain (Speicher et al., 1994) do not occur when E(spl) proteins are ectopically expressed using the same GAL4 line (Fig. 5A,B).

Notch-dependent repression of *sc* is, however, mimicked by ectopic expression of E(spl) m8 and mβ proteins. When GAL4 lines that drive expression in most of the thoracic region of the disc are used, the majority of the macrochaetae are absent from the thorax (not shown; see Tata and Hartley, 1995; Nakao and Campos-Ortega, 1996), and little residual *sc* expression is detected in the corresponding proneural clusters (Fig. 5I,J). The effects of ectopic Notch activation are expected to be the converse of those caused by mutations in *Su(H)*. This is the case for the effects on both *wg* and *ac*/*sc*. In *Su(H)* mutant discs, the stripe of *wg* mRNA associated with the dorsal/ventral boundary is absent (Fig. 5F) whereas *ac*/*sc* transcription persists at high levels in proneural clusters (Schweisguth and Posakony, 1992). Since Su(H) affects transcription of both *wg* and *ac*/*sc* whereas E(spl)bHLH only affect the latter, it is likely that Su(H) mediates transcriptional activation of Notch target genes other than *E(spl)* (Fig. 6).

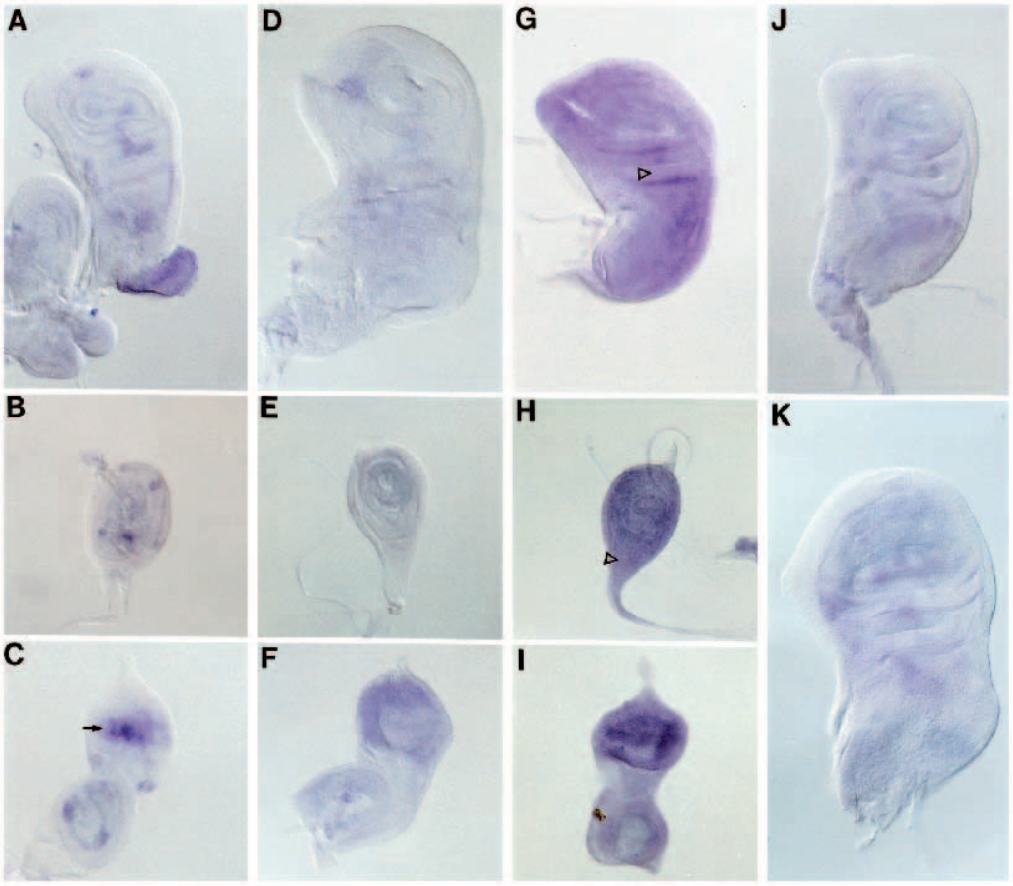

Fig. 3. Mutations in *Su(H)* or *Notch* abolish the majority of *E(spl)* expression. The expression of *E(spl)* genes in discs from $Su(H)^{1}/Su(H)^{AR9}$ (A-J) and N^{ts1}/N^{55e11} (K) mutant larvae was detected by in situ hybridisation under the same conditions as for the discs in Figs 1 and 2. (A-C) *m8*, (D-F) *m7*, (G-I) *mδ* (J and K) *mβ*. Little or no expression is detected in mutant wing (A,D,G,J and K) leg (B,E,H) or eye (C,F,I) imaginal discs, except for a stripe of *m8* expression in the eye disc (arrows in C) and a few weak patches of expression, most notably of *m8* in the wing and leg discs (A,B). The discs exposed to the *mδ* probe have been overdeveloped revealing weak residual expression in the eye, but no staining associated with sensory organ development (open arrowheads) in either the wing (G) or leg (H) imaginal discs.

In analysing the effects of ectopic E(spl) expression, we have used four different proteins, m8, m7, mβ, and mδ. Although in normal conditions the expression of individual E(spl) proteins is associated with different processes in the wing disc, all these proteins produce similar effects when ectopically expressed, including bristle loss and truncation of veins (unpublished observations), which were originally noted for ectopic m8 expression (Tata and Hartley, 1995; Nakao and Campos-Ortega, 1996). Thus, despite their different expression patterns, it appears that the proteins themselves are capable of performing similar functions.

DISCUSSION

The bHLH proteins encoded by the *E(spl)* complex are components of the Notch signalling pathway. We find that different *E(spl)*bHLH genes have distinct, but Notch-dependent, patterns of expression in the imaginal discs, indicating that unidentified factors act in combination with Notch to regulate the expression of these genes. Furthermore, E(spl) function does not account for all the actions of Notch and Su(H). Thus, the mutant phenotypes produced by cells lacking *E(spl)* are a subset of those detected with cells mutant for *Notch* or *Su(H)*, and ectopic expression of E(spl) does not mimic all the effects produced by ectopic activation of Notch.

Regulation of E(spl)bHLH gene expression

Expression of *E(spl)* genes appears dependent on Notch activation: the presence of an activated form of the Notch receptor is sufficient to induce ectopic expression of *E(spl)* genes and disruptions to Notch signalling reduce *E(spl)* expression (Jennings et al., 1995, 1994). The most simple extrapolation of these results is that all *E(spl)* genes should be expressed in an identical manner. However, our analysis of the expression patterns of the different *E(spl)* genes in imaginal discs reveals distinct differences. In the wing disc, we detect expression from five of the genes: *m8* and *m7* are expressed in approximately all the domains where SOPs develop, *mγ* and *mδ* are detected at a subset of these locations and *mβ* is detected in a dramatically different pattern that relates in part to vein development. Despite these differences in their domains of expression, we find that transcription of all the *E(spl)* genes is affected by mutations that perturb the Notch pathway. Thus only very low levels of the mRNAs are detected in *Su(H)* mutant discs and similar results are obtained using mutations that reduce the function Notch itself. It is unlikely that the differences in the expression pattern are a consequence of individual genes responding to different thresholds of Notch activation since there is not a graded difference between the patterns. Therefore, we suggest that expression of *E(spl)* genes occurs in response to a combination of Notch activation, mediated by Su(H), and unidentified factors that provide additional positional information and must recognise particular *cis*-regulatory regions within the *E(spl)* complex. The residual *E(spl)* expression seen in *Su(H)* discs could indicate that the alleles used do not completely remove all Su(H) function. Alternatively, in some places, expression of *E(spl)* may be able

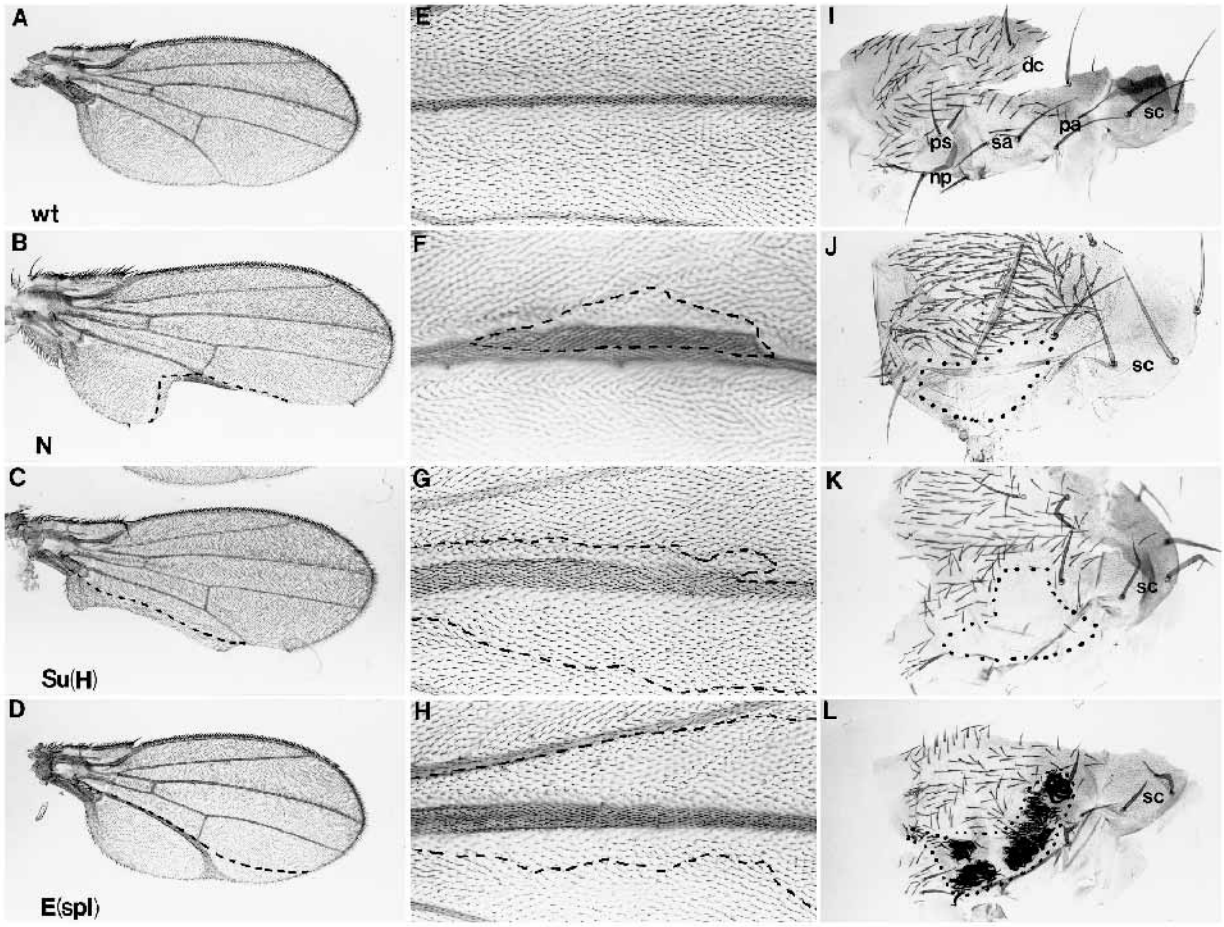

Fig. 4. Mutations in *Notch*, *Su(H)* and *E(spl)* do not produce identical phenotypes in the adult. Details of the wings (A-D), wing veins (E-H, L3 vein is shown) and thorax (I-L) of wild-type flies (A,E,I) and flies carrying clones of cells mutant for *Notch* [N^{55e11}, B,F,J] *Su(H)* [$Su(H)^{AR9}$, C,G,K] and *E(spl)* [$Df(3R)E(spl)^{b32.2}$, D,H,L]. The boundaries of the mutant cells are indicated by dashed lines in the wing. In the wing, clones of *Notch* and *Su(H)* mutant cells that abut the wing edge lead to scalloping of the wing (B,C) whereas only minor disruptions to the wing margin structures are produced by cells lacking *E(spl)* function (D). (E) Wild-type L3 vein. (F-H) Mutant L3 veins with Notch (F), Su(H) (G) and E(spl) (H) clones leading to thickening of the vein (compare F-H with E). (I) Wild-type hemithorax in which the positions of the macrochaetae are indicated by letters as in Fig. 1. In the thorax, *Notch* or *Su(H)* clones result in naked cuticle as shown within the dotted territory (compare J, K with I) whereas absence of *E(spl)* genes results in the differentiation of dense clusters of bristles (L). The scutelar macrochaetae (SC) in mosaic thorax are indicated in J-L.

to occur in the absence of Su(H)/Notch activation, suggesting that factors conferring positional information can be sufficient to promote *E(spl)* transcription.

Interestingly, there is a good correlation between the location of the genes within the *E(spl)* complex and their patterns of expression. Thus *mγ* and *mδ* have similar spatial expression and are located at the proximal end of the complex and likewise, *m8* and *m7* are located at the distal end and have related imaginal disc patterns. The relationship between the physical positions of the genes on the DNA and their expression is compatible with the possibility that there are shared regulatory elements interspersed through the complex. These putative *cis*-regulatory sequences would confer the response of individual genes to positional clues that must act in combination with Su(H)/Notch to generate the normal levels and pattern of expression of each gene. The expression of the *achaete-scute* complex genes is mediated by shared regulatory elements (Ruiz-Gómez and Modolell, 1987; Gomez-Skarmeta et al., 1995) and likewise, the distribution of regulatory sequences may be significant in the conservation of the organsation of Hom/Hox genes (McGinnis and Krumlauf, 1992). Shared regulatory sequences may provide a basis for the similarity of the *E(spl)* complex between Drosophilids (Maier et al., 1993) although whether or not this extends to more distantly related species awaits elucidation of the genetic organisation of *E(spl)* homologues in other organisms.

The expression of the individual *E(spl)* genes in distinct domains could indicate that the proteins have the ability to interact with specific target genes depending on the process. However, the ectopic expression of *m8*, *m7*, *mβ* or *mδ* produces very similar phenotypes of vein and bristle loss demonstrating that all the proteins have the potential to act on the same sets of target genes. It is thus not immediately evident why the *E(spl)* genes should be regulated differently. It is possible that they function with higher efficiency in the places and processes where they are normally expressed (Nakao and Campos-Ortega, 1996). It may also be important

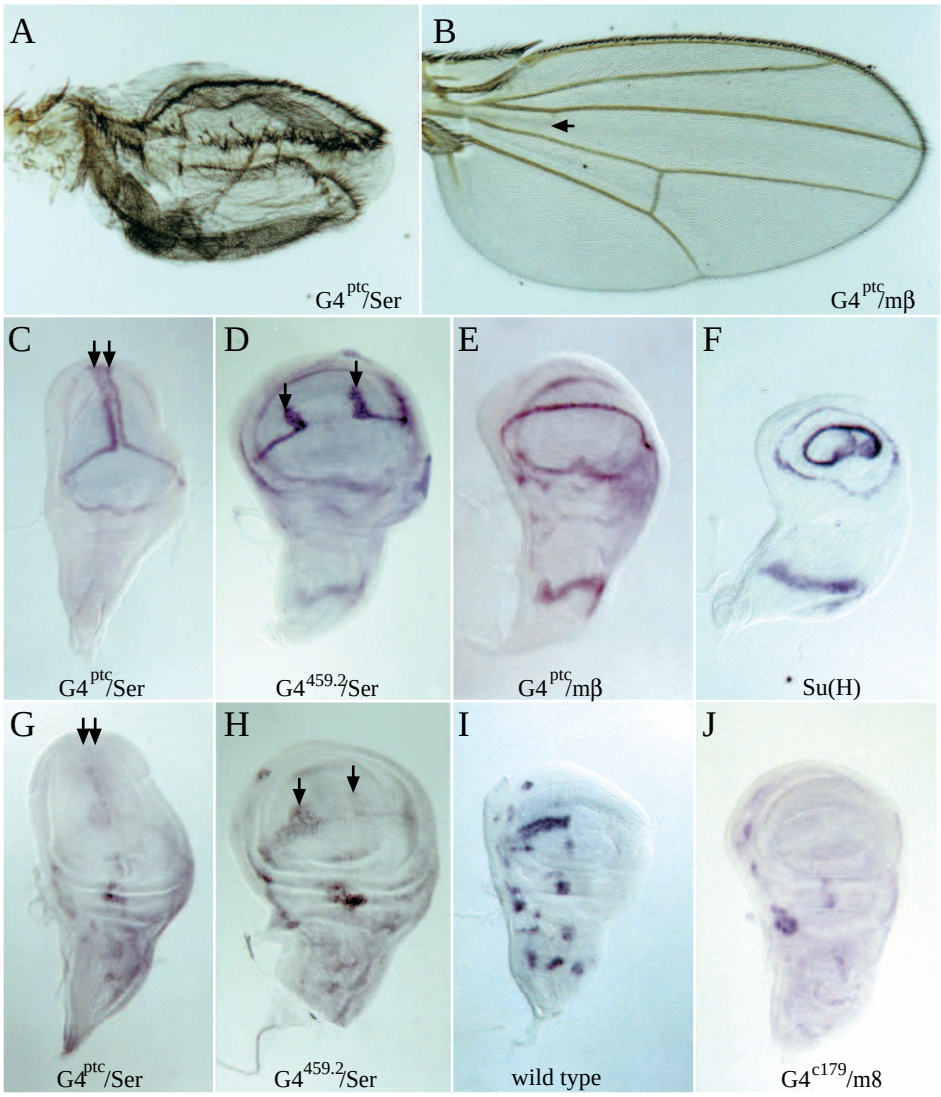

Fig. 5. Ectopic E(spl) does not mimic all the effects of ectopic activation of Notch. Effects of ectopic Ser (A,C,D,G,H) and E(spl)bHLH (B,E,J) were analysed in wings and wing imaginal discs and the Gal4 driver lines (G4) and UAS-cDNAs used are indicated in each panel. Wings from flies where ectopic Ser (A) was expressed under the control of the GAL4^{ptc} driver are mis-shapen with ectopic wing-margins on the ventral surface whereas ectopic expression of E(spl)mβ (B) in the same domain only results in loss of the anterior cross-vein (arrow). In the wing disc, ectopic Ser leads to ectopic stripes of *wg* mRNA in the ventral compartment (arrows C,D) and to ectopic expression of E(spl)bHLH in similar positions (arrows G,H). In C and G the ectopic Serrate expression has led to overgrowth in the ventral compartment of the disc. No such overgrowth is seen in discs where E(spl) proteins are expressed ectopically and the expression of *wg* mRNA is the same as in wild-type discs with an uninterrupted stripe along the dorsal-ventral boundary (E; mβ, identical results are obtained using m8 and mδ with this and with other GAL4 driver lines). In discs from *Su(H)* mutant larvae, the stripe of *wg* mRNA associated with the dorsal/ventral boundary is absent whereas other aspects of *wg* expression remains normal (F). A comparison of *scute* mRNA expression in wild-type (I) and in discs where E(spl)m8 protein is expressed at high levels throughout the wing pouch and most of the thorax (GAL4^{c179}, data not shown) demonstrates that ectopic m8 leads to a drastic reduction in the levels of *scute* mRNA (J).

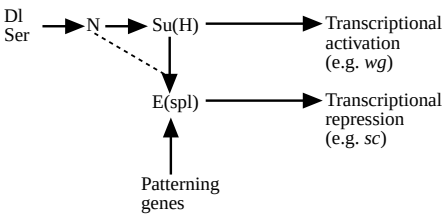

Fig. 6. Transcriptional activation and repression elicited by Notch activation. Based on the phenotypes of clones lacking *Notch* and *Su(H)*, it appears that Su(H) is involved in all the processes requiring Notch activation. Thus the binding of the Notch ligands Delta (Dl) or Serrate (Ser) to Notch (N) leads to activation of Su(H) which can then promote transcription of *E(spl)*bHLH genes and other target genes. E(spl)bHLH are only required for a subset of the processes mediated by Notch and these may be restricted to events involving transcriptional repression, such as the repression of *scute* during singling out of SOPs. Conversely, when the consequence of Notch activation is the transcriptional activation of downstream genes, such as *wg*, E(spl)bHLH proteins are not required, and Su(H) may act directly on target genes to promote their expression. It is not known whether the transcription of *wg* is a direct effect of Su(H) activity or whether other intermediate transcription factors are required. Expression of *vestigial* and *cut* is also activated at the dorsal-ventral boundary as a consequence of Notch activation. Similarly, there is as yet no evidence that E(spl)bHLH directly repress transcription of *scute*. The expression of *E(spl)* genes in distinct domains in the imaginal discs indicates that their transcription involves the combined effects of Su(H) and the products of unidentified patterning genes. The dotted line linking Notch with Su(H) indicates that Notch may participate directly with Su(H) in activating *E(spl)* expression.

to co-ordinate the Notch response with other signalling/patterning events occurring in the same cell, the separation of regulatory units to individual genes being an efficient way to accomplish this.

E(spl) proteins do not mediate all the effects of activated Notch

The processes that require *Notch* during the development of the wing and thorax include wing margin formation, vein differentiation and singling out of SOPs. All of these are affected to a similar extent by mutations in *Notch* or *Su(H)* consistent with Su(H) being a key transducer for the Notch signal. Expression of particular *E(spl)* genes is associated with all these processes and, in the developing veins, the absence of *E(spl)*, *Su(H)* or *Notch* results in the formation of thickened veins. However, in some of the other places where *Notch* and *Su(H)* are required, it appears that *E(spl)* function is dispensable. For example, Notch and Su(H) activity are essential at the dorsal/ventral boundary of the wing disc and we have shown that *m8*, *mγ* and *mβ* are transcribed there in a Notch-dependent manner, but two lines of evidence indicate that E(spl) do not mediate the effects of Notch in these cells. First, clones of cells lacking all *E(spl)* genes that abut the wing margin do not produce wing nicks, unlike similar clones of *Notch* or *Su(H)* mutant cells. Second, ectopic expression of four E(spl)bHLH proteins, two of which are normally detected in the cells at the dorsal/ventral boundary, fails to mimic the effects of ectopic Notch activation, mediated either by Serrate or Delta, in causing expression of *wg* or inappropriate growth of the disc (Thomas et al., 1995; Kim et al., 1995; Doherty et al., 1996). Thus although expression of E(spl)bHLH proteins at the dorsal/ventral boundary correlates well with *Notch* requirements, these proteins do not appear essential for *Notch* function, in so far as the maintenance of the integrity of the wing margin and *wg* expression is concerned. Since we have only tested the effects of E(spl) proteins singly, it remains possible that specific combinations of E(spl) proteins would elicit additional effects. However, both the functional redundancy implied by genetic analysis and the similarity between the effects produced by the individual proteins argue against combinatorial requirements. It is also possible that *E(spl)* function at the dorsal/ventral boundary overlaps with other repressor proteins expressed there, such as Extramacrochaetae or Hairy, in which case phenotypes might only be observed when all these proteins are absent too. Nevertheless the results from both loss and gain of function experiments argue that E(spl) proteins are not essential for *Notch* function at the dorsal/ventral boundary, even though they are present.

A further indication that E(spl)bHLH proteins are only required for a subset of *Notch* function comes from the effects of mutations on the development of bristles. *Notch* and *Su(H)* are essential for both the selection of a single SOP from the field of proneural cells and for the subsequent cell fate decisions in the SOP progeny. Thus, absence of Notch or Su(H) leads to naked cuticle, because all the progeny of the SOPs form neurons rather than bristle, shafts and sockets (Hartenstein and Posakony, 1990; Schweisguth, 1995). In contrast, clones of cells lacking *E(spl)* function give rise to dense clusters of macrochaetae, indicating that many/all the cells have become SOPs, but that the SOP progeny can make the appropriate cell fate decisions (Fig. 4; Heitzler et al., 1996). During the selection of the SOP, therefore, E(spl) appears to be a key mediator of *Notch* function and, indeed, E(spl) is necessary for activated Notch proteins to suppress SOP fate (Heitzler et al., 1996). This also correlates well with the effects of ectopic E(spl)m8 protein, which can efficiently suppress SOP formation (Tata and Hartley, 1995). The expression of *E(spl)* in the progeny of the SOP is not known, but the fact that sensory bristles are produced in their absence indicate that these genes are not essential for the assignation of cell fates in the progeny of the SOPs. Deficiencies completely removing *gro* and some *E(spl)*bHLH genes more completely mimic the phenotype of *Notch* mutations on sensory organ development, leading to patches of naked cuticle, while *gro* lethal alleles have similar but weaker phenotypes (de Celis et al., 1991; Heitzler et al., 1996). Thus the effects of Notch in the SOP progeny could be mediated by Gro interacting with other partners, whose expression may be dependent on Notch activation. The ability of ectopic E(spl)bHLH to misroute the fate of SOP progeny (Tata and Hartley, 1995; Nakao and Campos-Ortega, 1996) could be due to competition for interactions with Gro between E(spl) and other putative Gro partners, or to the ability of E(spl)bHLH proteins to interfere with *Notch* or *Delta* expression in the relevant cells. Other processes are also affected differentially by mutations removing *gro* or *E(spl)* genes. For example, at the dorsal/ventral boundary, cells lacking *gro* cause weak scalloping of the wing margin whereas cells lacking *E(spl)*bHLH genes do not. Furthermore, *gro* alleles also lead to phenotypes that are unrelated to those seen with *Notch* mutations, namely overgrowth of wing tissue in the anterior compartment, indicating that *gro* also functions in *Notch*-independent pathways (de Celis and Ruiz-Gomez, 1995).

The regulation of *E(spl)* gene expression appears to be a complex process that involves the integration of signals from the Notch pathway with additional unidentified factors that confer spatial specificity to individual genes. The E(spl) proteins only mediate a subset of Notch-dependent processes that may be those requiring transcriptional repression of downstream genes. The other processes that involve transcriptional activation appear to be independent of *E(spl)* even though in some cases E(spl) proteins are clearly present in the relevant cells. In contrast, Su(H) is required in both types of Notch-dependent gene regulation. The branching of the Notch pathway at the level of Su(H) implies that the response of downstream genes to Notch activation depends on whether the organization of their regulatory regions makes them targets of Su(H) or E(spl), and explains the diverse effects of Notch on transcriptional regulation (Fig. 6).

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