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Ectopic expression of individual *E(spl)* genes has differential effects on different cell fate decisions and underscores the biphasic requirement for Notch activity in wing margin establishment in *Drosophila*

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SUMMARY

A common consequence of Notch signalling in *Drosophila* is the transcriptional activation of seven *Enhancer of split* [*E(spl)*] genes, which encode a family of closely related basic-helix-loop-helix transcriptional repressors. Different E(spl) proteins can functionally substitute for each other, hampering loss-of-function genetic analysis and raising the question of whether any specialization exists within the family. We expressed each individual *E(spl)* gene using the GAL4-UAS system in order to analyse their effect in a number of cell fate decisions taking place in the wing imaginal disk. We focussed on sensory organ precursor determination, wing vein determination and wing pattern formation. All of the E(spl) proteins affect the first two processes in the same way, namely they antagonize neural precursor and vein fates. Yet, the efficacy of this antagonism is quite distinct: E(spl)mβ has the strongest vein suppression effect, whereas E(spl)m8 and E(spl)m7 are the most active bristle suppressors. During wing patterning, Notch activity orchestrates a complex sequence of events that define the dorsoventral boundary of the wing. We have discerned two phases within this process based on the sensitivity of *N* loss-of-function phenotypes to concomitant expression of *E(spl)* genes. E(spl) proteins are initially involved in repression of the *vg* quadrant enhancer, whereas later they appear to relay the Notch signal that triggers activation of *cut* expression. Of the seven proteins, E(spl)mγ is most active in both of these processes. In conclusion, E(spl) proteins have partially redundant functions, yet they have evolved distinct preferences in implementing different cell fate decisions, which closely match their individual normal expression patterns.

Key words: *Drosophila*, *E(spl)*, Wing development, Bristle development, Wing veins, Notch signalling

INTRODUCTION

Notch signalling plays an important role in a large number of cell fate decisions in all metazoans (Kimble and Simpson, 1997). Activation of the Notch receptor by an extracellular ligand sets off an intracellular response that culminates in the expression of a number of target genes. Transcriptional activation in response to Notch is usually dependent on the Suppressor of Hairless [Su(H)] transcription factor, which seems to be directly activated by Notch (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Schroeter et al., 1998; Struhl and Adachi, 1998). The most frequent target genes of Notch in *Drosophila* are seven *Enhancer of split* [*E(spl)*] genes, *E(spl)m8*, *E(spl)m7*, *E(spl)m5*, *E(spl)m3*, *E(spl)mβ*, *E(spl)mγ* and *E(spl)mδ*, which encode closely related proteins of the basic-helix-loop-helix family of transcription factors (Delidakis and Artavanis-Tsakonas, 1992; Jennings et al., 1994). E(spl)bHLH proteins act as transcriptional repressors in a complex with the co-repressor protein Groucho (Fisher and Caudy, 1998). Genetic and molecular analyses have so far detected little specificity amongst the seven E(spl)* proteins, proposing instead that they act in a redundant fashion (Delidakis et al., 1991; Schrons et al., 1992). Yet, considering the number of Notch-dependent processes and the context dependence of the outcome of Notch signalling, it is reasonable to hypothesize that a different subset of nuclear effectors, including perhaps a different subset of E(spl) proteins, might be acting in different instances. To date, suggestions of functionally distinct roles of the E(spl)bHLH proteins have come from their selective dimerization ability with other bHLH proteins and their distinct, albeit overlapping, patterns of expression in imaginal development (Alifragis et al., 1997; de Celis et al., 1996).

In an attempt to discern possible functional specialization among E(spl) proteins, we have chosen to focus on the wing disk, where *E(spl)* genes display distinct expression domains.

*Additional genes are included in the E(spl) locus, that do not encode bHLH products. As this work solely concerns the seven E(spl)bHLH proteins, we shall henceforth refer to them as E(spl) for brevity.

During the third larval instar (L3) and pupal periods, Notch activity is required for the specification and development of sensory organs in both notum and wing pouch domains of the wing disk, as well as in wing vein formation (de Celis et al., 1997; Huppert et al., 1997; Muskavitch, 1994). In both of these processes, Notch executes inhibitory signalling, diverting disk epithelial cells away from the neural and vein fates, respectively. Earlier in development, the basic coordinates of the wing disk are laid down by signalling from two organizing centres at the anteroposterior (AP) and dorsoventral (DV) boundaries, mediated by the secreted morphogens Decapentaplegic (Dpp) and Wingless (Wg), respectively (reviewed in Neumann and Cohen, 1997b). Establishment of the DV boundary takes place during L2 in a process that depends on Notch signalling. Activation of Notch at the future DV boundary directs the localized expression of *vestigial* (*vg*), *wg* and members of the *E(spl)* family (Diaz-Benjumea and Cohen, 1995; Jennings et al., 1995; Kim et al., 1996). *E(spl)* and *vg*, which encodes an unrelated nuclear protein, are direct targets of Notch activation via Su(H); the molecular mechanism of *wg* transcriptional activation is still unclear. Signalling by Notch and Wg subsequently interact in a complex process, which eventually leads to *cut* expression in DV boundary cells during late L3 (Micchelli et al., 1997; Neumann and Cohen, 1996). The combination of Wg and Dpp signalling is responsible for initiating a later round of *vg* expression from a 'quadrant' enhancer, which drives expression throughout the wing pouch except for the DV boundary (Kim et al., 1996, 1997; Neumann and Cohen, 1997a).

Among these Notch-dependent processes that take place in the developing wing disk, the role of E(spl) proteins is best understood in sensory organ precursor (SOP) selection. Neural precursor fate in the *Drosophila* wing depends on the expression of the proneural class of activator bHLH proteins encoded by the genes of the *achaete-scute Complex* (*ASC*) (Modolell, 1997). E(spl)bHLH proteins have been shown to directly repress the *achaete* gene, as well as to interact with proneural proteins making it likely that E(spl) downregulates proneural activity at many levels (Alifragis et al., 1997; de Celis et al., 1996; Giebel and Campos-Ortega, 1997; Jiménez and Ish-Horowicz, 1997; Nakao and Campos-Ortega, 1996). Of the seven members of the family, *E(spl)m8* and *E(spl)m7* are expressed in most proneural clusters of the wing disk, whereas *E(spl)mγ* and *E(spl)mδ* are only expressed in a subset (de Celis et al., 1996). A similar repressive action of E(spl) proteins probably accounts for Notch's vein suppression effect, where the candidate target gene is *veinlet/rhomboid* (de Celis et al., 1997). *E(spl)mβ* is strongly expressed in intervein regions during L3 and flanks vein domains, where *ve* is expressed. The role of *E(spl)* genes at the DV boundary is less clear. Four genes are expressed there, namely *E(spl)m8, E(spl)m7, E(spl)mβ* and *E(spl)mγ*, yet they appear to have little or no function, as clones mutant for the whole *E(spl)* locus do not display the dramatic wing scalloping caused by mutant clones for *N* or *Su(H)* (de Celis et al., 1996). Expression of the remaining two *E(spl)* genes, *E(spl)m5* and *E(spl)m3*, is not detectable in wing disks.

By ectopically expressing individual *E(spl)* genes in various domains of the wing disk, we have sought to identify differential effects in different processes: SOP selection, vein determination and wing patterning. We have indeed noted differential susceptibility of SOPs and veins to expression of *E(spl)* genes, which correlates with the wild-type expression pattern of the genes in question. Furthermore, we have documented repression of the *vg* quadrant enhancer by a subset of the E(spl) proteins, pointing towards a possible role of these proteins at the DV boundary. Finally, we have detected a positive role of some E(spl) proteins in the later phase of DV boundary formation, namely during expression of *cut*. Our results are consistent with two distinct episodes of Notch signalling occurring sequentially at the DV boundary.

MATERIALS AND METHODS

Drosophila strains

Notch alleles (N^{54l9}, nd^3 and N^{ts1}) and *wg-lacZ* (wg^{en11}) are described in FlyBase (http: //flybase. harvard. edu:7081/). *vg-lacZ* enhancers (boundary and quadrant) are described in Kim et al. (1996). *m8-lacZ* is described in Lecourtois and Schweisguth (1995). The *FRT* chromosomes bearing *P[πMyc], gro^{E48}, $Df(3R)gro^{b32.2}$* and N^{54l9} are described in Xu and Rubin (1993), Heitzler et al. (1996) and Baker and Yu (1997). The *GAL4* lines used were *32B, $ptc^{G559.1}$, ap^{md544}, omb-GAL4, h^{1J3}* and *455.2* (all described in FlyBase). We generated all *UAS* lines, except for *UAS-m8* (Nakao and Campos-Ortega, 1996).

Generation of *UAS-E(spl)* lines

Cloning into the pUAST vector (Brand and Perrimon, 1993) and generation of *UAS* transformant lines for the *E(spl)mβ* and *E(spl)mδ* genes is described in de Celis et al. (1996). The same procedure was followed to generate the *UAS-m5*, *UAS-m7*, *UAS-m3* and *UAS-mγ* transformant lines. Briefly, PCR fragments consisting of the coding regions only of the respective genes (sequence confirmed) were placed in a modified pUAST vector downstream of a synthetic oligonucleotide bearing an optimized translation start site. 5′ and 3′ UTRs are provided from the vector. Construction details are available on request. Transformants were obtained in a yw^{67c23} background.

N^{ts1} temperature shifts

Crosses for studying the effects of *UAS-E(spl)* in a N^{ts1} background were as follows:

For third chromosome lines, e.g. *UAS-mδ*:
N^{ts1} × 32B-GAL4 UAS-mδ / TM6B
For second chromosome lines, e.g. *UAS-mβ*:
N^{ts1} × UAS-mβ ; 32B-GAL4 / T(2;3) SM5, TM6B
For *m8-lacZ*:
N^{ts1}/ FM7c; 32B-GAL4 UAS-mδ / TM6B × m8-lacZ

Crosses were kept at 18°C and changed to new vials daily. Vials were placed at 29°C or 30°C at different developmental stages and for different durations as described in the Results. Developmental time is given as hours after egg laying (AEL); for consistency, we use the equivalent time at 25°C – development at 18°C takes approximately twice as long. For monitoring the expression of *cut*, *wg*, *Dll* and *m8-lacZ*, we compared side by side Tb^+ (*N^{ts1}/Y; 32B-GAL4 UAS-mδ/+*) male larvae and control *Tb* (*N^{ts1}/Y; TM6B/+*) males from the same vial.

Mosaic analysis

Clones were induced by heat shocking larvae (1 hour, 38°C) 48-96 hours AEL of the following genotypes:

y w^a N^{54l9} FRT18A / P[πMyc]5A10D FRT18A; hsFLP/ 32B-GAL4 UAS-mδ

hsFLP/+; FRT82B P[πMyc] 87E97E/ FRT82B kar^2 ry^{506} P[gro^+ry^+] Df(3R)$gro^{b32.2}$

hsFLP/+; FRT82B P[πMyc] 87E97E/ FRT82B kar^2 ry^{506} gro^{E48}

For the Myc-marked clones, larvae were picked as wandering third instar, heat shocked again for 90 minutes (38°C) to induce πMyc expression and then allowed to recover for 90 minutes before dissection.

In situ hybridization and immunocytochemistry

In situ hybridization with digoxigenin-labeled *wg* DNA and histochemical detection of β-galactosidase were done as described (Cubas et al., 1991). For immunocytochemistry, larvae were dissected in phosphate buffer and fixed as described in Xu and Rubin (1993). Antibody dilutions were as follows: Anti-Cut monoclonal antibody (kindly provided by Karen Blochlinger) 1/100; anti-Dll monoclonal antibody (kindly provided by Stephen Cohen) 1/1000; anti-N monoclonal antibody C17.9C6 (kindly provided by Spyros Artavanis-Tsakonas) 1/1000; rabbit anti-c-Myc (Santa Cruz Biotechnology) 1/1000. Horseradish-peroxidase-coupled secondary antibodies were from Jackson Immunological Laboratories (used at 1/250); diaminobenzidine was used for development. For mitotic clones, fixed tissues were reacted first with the anti-Cut antibody, developed with DAB+NiCl / $CoCl_2$ (black product) and subsequently with anti-cMyc or anti-Notch, developed with DAB alone (brown product).

Adult specimens

Wings were mounted in Aquamount mountant modified (BDH Laboratory Supplies). Nota were mounted in Hoyers (Wieschaus and Nüsslein-Volhard, 1986) and incubated overnight at 60°C.

RESULTS

Effects of ectopic expression of *E(spl)-C* genes

In order to obtain in vivo data on potential specific functions of E(spl) proteins, we ectopically expressed each one in mesothoracic imaginal disks using the *GAL4-UAS* system (Brand and Perrimon, 1993). The most prominent phenotypes observed were loss of sensory organs and loss of wing veins, in accordance with the gain-of-function phenotypes previously reported for *E(spl)m8*, *E(spl)m5* and *E(spl)mβ* (de Celis et al., 1996; Nakao and Campos-Ortega, 1996; Tata and Hartley, 1995). Qualitatively, all seven *E(spl)* genes produced these same overexpression phenotypes; however, the severity of these phenotypes depended on the individual E(spl) protein. E(spl)m5 stood out among the seven members as the most inactive protein, as it produced mild gain-of-function phenotypes only when two copies of the *UAS* transgene were used (six lines tested). We therefore concentrated our comparative analysis on the remaining six *UAS-E(spl)* bHLH transgenes.

We initially focussed our attention on the specification of wing veins and the specification of three types of external sensory organs: notum macrochaetae, notum microchaetae and anterior wing margin bristles. Between three and seven lines of each *UAS* transgene were tested for adult phenotypes and only quantitative variations were observed. More impressive were the phenotypic differences seen between different transgenes, representative examples of which are shown in Figs 1 and 2. *E(spl)mβ,* which is normally expressed in intervein regions (de Celis et al., 1997), produced the most dramatic loss of vein (Fig. 1D) when driven by *32B-GAL4*, which expresses uniformly in the wing pouch (Fig. 1O). *UAS-E(spl)mγ* had an equally severe effect on veins, while the remaining four had

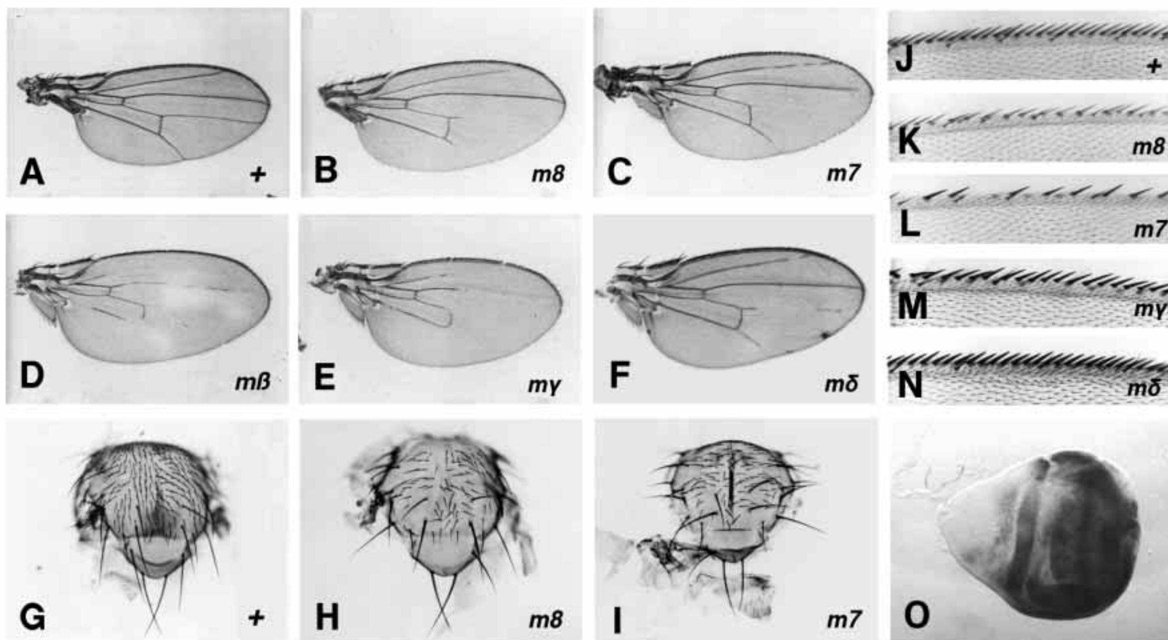

Fig. 1. Ectopic expression of *E(spl)* genes driven by *32B-GAL4.* (A-F) Wings of adult flies bearing one copy of the driver construct *32B-GAL4* alone (A) or along with one copy of a *UAS* construct expressing *E(spl)m8* (B), *E(spl)m7* (C), *E(spl)mβ* (D), *E(spl)mγ* (E) or *E(spl)mδ* (F). (J-N) Higher magnifications of the anterior wing margins of wild-type wings (J) or wings carrying *32B-GAL4* and *UAS-m8* (K), *UAS-m7* (L), *UAS-mγ* (M) and *UAS-mδ* (N). Note the severe loss of veins by *32B-mβ* in D and the severe loss of wing margin bristles by *32B-m7* in L. In contrast *32B-mδ* (N) and *32B-mβ* (not shown) give essentially wild-type wing margins. (G-I) Wild-type notum (G) for comparison with nota from *32B-m8* (H) and *32B-m7* (I) flies. Although macrochaetae are relatively unaffected in H and I (only one anterior scutellar missing), the density of microchaetae is significantly decreased. At this temperature no other *UAS-E(spl)* transgene resulted in microchaeta reduction. (O) Expression pattern of *32B-GAL4* in a late third instar wing disk, as reported by *UAS-lacZ* and X-gal staining. The absence of notum staining agrees with the fact that macrochaetae remain relatively unaffected, as their precursors are determined during this stage. *UAS-m5* caused no phenotypes in one copy, whereas *UAS-m3* resulted in late larval-pupal lethality making adult cuticle analysis with this driver line impossible. All flies shown were reared at 25°C. Anterior is up in all panels.

milder effects (Fig. 1A-F). Loss of notum microchaetae and wing margin bristles was also seen in some *32B-E(spl)* combinations, strongest with *E(spl)m8* and *E(spl)m7* (Fig. 1G-N). Interestingly, whereas *E(spl)m8* is more effective in abolishing the notum microchaeta fate, *E(spl)m7* is most active against wing margin bristles. We observed macrochaeta loss using the h^{1J3}-*GAL4* line (Fig. 2A-C). The phenotypes ranged from more than half of the macrochaetae deleted (by *UAS-m8*) to rare loss of one scutellar macrochaeta only (by *UAS-m*γ and *-m3*). A summary of the relative efficiency with which the different *E(spl)* genes affect each process studied is shown in Table 1. Some of these effects are evident from Figs 1 and 2, while others were obtained by comparing flies raised at higher temperatures, where the GAL4 system's expressivity increases, and/or flies bearing two copies of the responder transgene (not shown).

The differences in E(spl) activity described in Table 1 were observed with *GAL4* lines that exhibit moderate levels of expression. When stronger expressing *GAL4* lines were used, the differences were lessened. For example, with *ap-GAL4* (Fig. 2D-I) most *E(spl)* transgenes gave complete loss of macrochaetae and microchaetae, except for the very anterior edge of the notum; the sole exception (besides *E(spl)m5*) was *E(spl)m*δ, which allowed a few central microchaetae to form (Fig. 2F). With the scutellum-specific *455.2-GAL4*, all *UAS-E(spl)* (except *E(spl)m5*) resulted in severe loss of scutellar

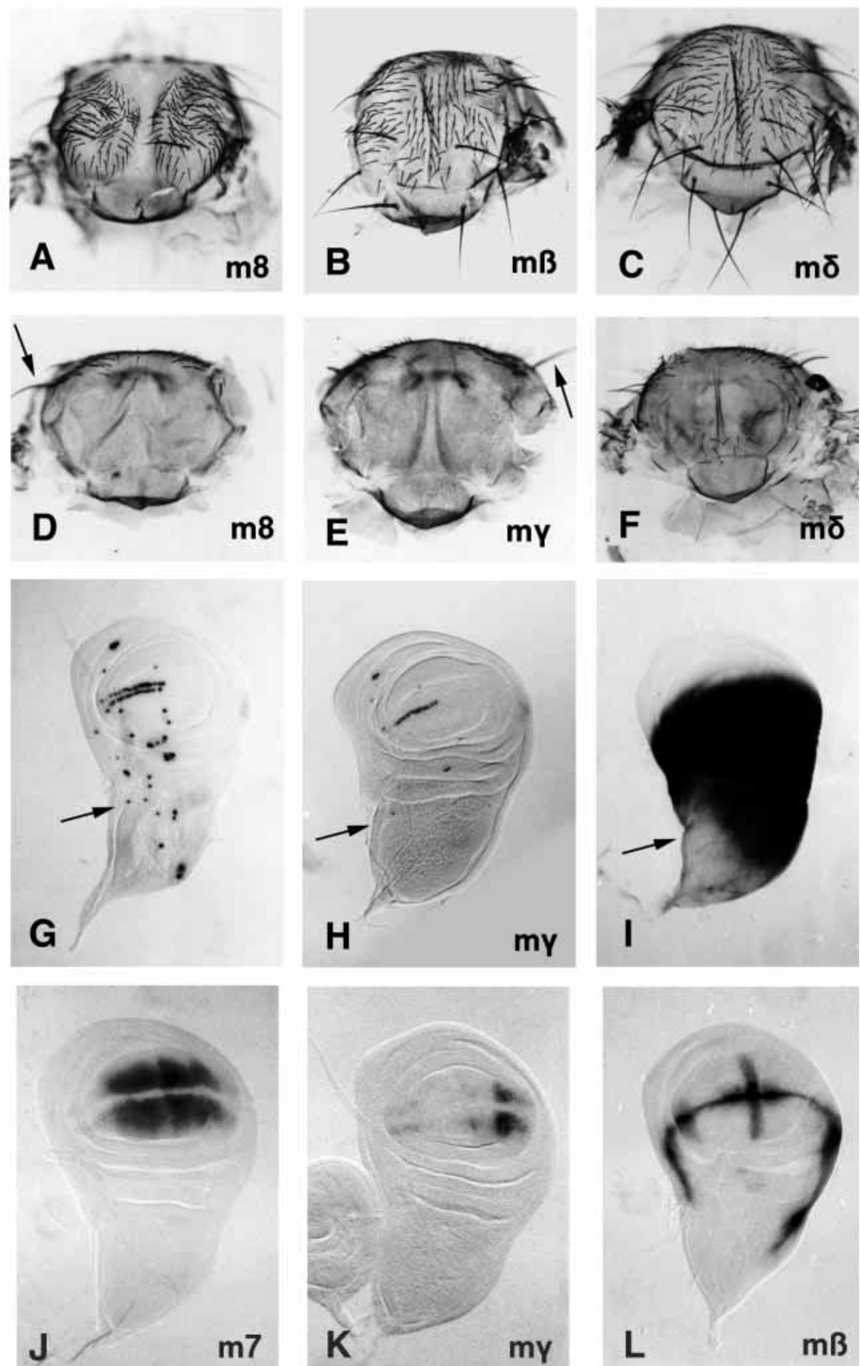

Fig. 2. Ectopic expression of *E(spl)* genes driven by h^{1J3}-*GAL4*, *ap-GAL4* and *omb-GAL4.* (A-C) Nota ectopically expressing *UAS-E(spl)* transgenes by h^{1J3}-*GAL4.* Note that although *UAS-m8* (A) and *UAS-m*β (B) cause extensive deletion of macrochaetae, *UAS-m*δ (C) is wild-type (cf. Fig. 1G). (D-F) Nota ectopically expressing *UAS-E(spl)* by *ap-GAL4.* Both *UAS-m8* (D) and *UAS-m*γ (E) completely remove macrochaetae and microchaetae, except for the anterior part (arrows) of the notum, where the driver line is apparently not expressed. *UAS-m*δ (F) is somewhat less severe, permitting the generation of several microchaetae at the centre. (G,H) Late third instar wing disks bearing the $neur^{A101}$-*lacZ* enhancer trap, which stains sensory organ precursors (SOPs). Whereas a wild-type disk (G) displays a characteristic pattern SOPs, most dorsal SOPs are abolished by expression of *UAS-m*γ by *ap-GAL4* (H). The latter is expressed only in the dorsal compartment as revealed by *UAS-lacZ* (I). In H, the ventral (uppermost) row of margin sensilla and the ventral radius are unaffected (compare with G). The only dorsal SMC not affected is the anterior notopleural (ANP, arrows in D, E, G and H). The corresponding region in I (arrow) shows that there is little or no expression of the driver transgene there. (J-L) Wing disks expressing *UAS-E(spl)* transgenes by the *omb-GAL4* driver; J and K also carry *vg(quad)-lacZ.* E(spl)m7 does not affect the expression of this enhancer – the disk in J shows an essentially wild-type pattern. E(spl)mγ strongly represses *vg(quad)-lacZ* in the middle of the wing pouch (K). *UAS-m*β does not affect the wild-type pattern of the *vg* boundary enhancer (L). (A-F) anterior is up; (G-L) anterior is left, ventral is up.

Table 1. Relative efficiency of different E(spl) proteins in various processes

Process	E(spl) proteins
wing vein deletion (*32B-GAL4*)	mβ=mγ> m8=m7>mδ
notum microchaeta loss (*32B-GAL4*, *ap-GAL4*)	m8>m7>>mγ=mβ = m3>mδ
wing margin bristle loss (*32B-GAL4*)	m7>m8>mγ>mδ>mβ
notum macrochaeta loss (*1J3-GAL4*)	m8>m7>mβ>mδ>mγ=m3
N/+ wing nicking suppression (*32B-GAL4*)	mγ=mδ>mβ=0>m7=m8
N^{ts}/Y (late upshift) wing nicking suppression (*32B-GAL4*)	mγ=mδ>mβ=m7=0>m8
N^{ts}/Y (early upshift) wing nicking enhancement (*32B-GAL4*)	m8=m7=mβ=mγ=mδ
vg(quad)-lacZ repression (*omb-GAL4*)	mγ=m3>>mδ=mβ=m7=m8=0

The *UAS-E(spl)* transgenes are ranked according to the severity of phenotype that they produce when assayed in the different processes shown. In some cases, *UAS-m3* could not be tested due to inviability of adults. '0' means that no effect was seen, e.g., ectopic expression of mδ, mβ, m7 and m8 did not affect the wild-type *vg(quad)-lacZ* expression pattern. In the rows describing 'wing nicking suppression', transgenes showing the opposite effect (nicking enhancement) are placed at the end of the rank (<0).

bristles (not shown). This observation argues in favour of functional interchangeability of the six proteins, but does not detract from the fact that at levels closer to physiological each protein has varying efficacy according to developmental context. We have excluded the possibility that *E(spl)* genes displaying low activity might be an artifact of not having recovered a strongly enough expressing transformed line: each transgene shows dramatic effects in some process, in which other transgenes are less active (see Table 1); in the case of E(spl)mδ, which appears less active than other proteins in Table 1, we have shown that it affects R8 cell specification in the developing eye much more strongly than any of the other transgenes (Ligoxygakis et al., 1998).

We extended our analysis of the specificity of *UAS-E(spl)* transgenes using a number of molecular markers pertaining to wing patterning. Expression was driven in a domain centered around the AP boundary of the wing pouch by the *omb-GAL4* driver. Neither *wg-lacZ* (not shown) nor the boundary enhancer of *vg* (Fig. 2L) were affected by ectopic expression of *E(spl)* genes. In contrast, the *vestigial (vg)* quadrant enhancer was strongly repressed by E(spl)m3 and E(spl)mγ, but was unaffected by the other *E(spl)* transgenes (Fig. 2J,K). In addition to revealing the activity of our *UAS-m3* transgene, which was less active in other contexts, this finding suggests a role for the normal E(spl)mγ expression at the DV boundary, namely to repress *vg(quad)* expression. This is in agreement with the published observation that this enhancer contains a potential DNA binding site for Hairy/E(spl) proteins (Kim et al., 1996) and could account for the effects of *N* alleles on this enhancer reported by Go et al. (1998).

Different members of the *E(spl)-C* have opposing effects on wing notching

Although ectopic expression of *E(spl)* genes phenocopied activation of the Notch pathway in abolishing SOPs and veins, it did not generate ectopic wing margin in the same way as was observed with expression of Notch ligands or activated Notch itself (e.g. Diaz-Benjumea and Cohen, 1995). We were able to detect effects of *E(spl)* on the wing margin by studying their ectopic expression in genetic backgrounds with reduced *Notch* function. Flies heterozygous for a *N* null allele or hemizygous for the hypomorph nd^3 have distally nicked wings, as well as thickened wing veins, in accordance with the need for Notch in establishment of the DV organizer and restriction of vein fate. When *E(spl)* transgenes were expressed in these backgrounds under *32B-GAL4*, we observed an unexpected effect. *E(spl)mδ* and *E(spl)mγ* restored the missing distal margin and adjacent wing blade tissue, while *E(spl)m8* and *E(spl)m7* enhanced wing notching (Fig. 3). *E(spl)mβ* had a weaker effect, being able to rescue only nd^3 (not shown); finally, *E(spl)m5* had no effect and *E(spl)m3* was not tested due to pupal lethality with the *32B-GAL4* line. The transgenes that showed strong vein suppression effects in wild-type backgrounds still displayed this phenotype (Fig. 3B-E), presumably by overcoming the reduction in *Notch* activity.

We then tested the *32B-E(spl)* combinations in a more severe *N* loss-of-function background, by using the temperature-sensitive allele N^{ts1}. First, a series of temperature shifts was performed to define *Notch* requirements during different time periods. Flies were reared at 18°C and shifted to the non-permissive temperature (29°C) for 14 hours at various times AEL (see Materials and Methods). A broad temperature-sensitive period was observed for wing notching spanning from

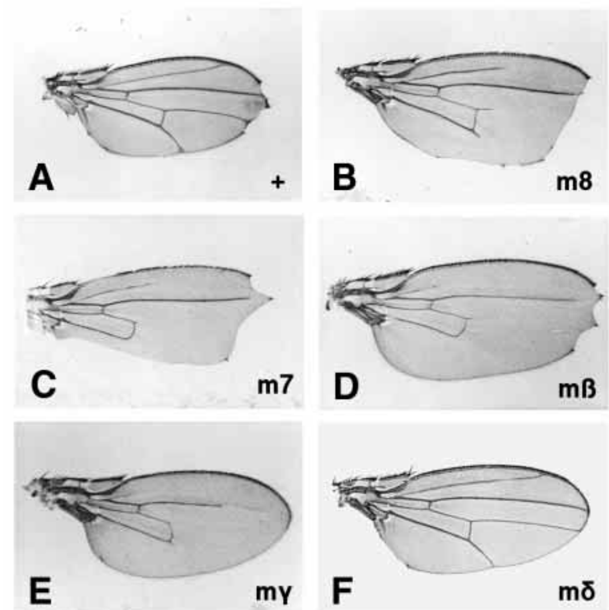

Fig. 3. *32B-E(spl)* expression in a *N*/+ background. (A) Wing heterozygous for the null allele N^{54l9}. (B-F) N^{54l9}/+ wings carrying one copy of *32B-GAL4* along with one copy of *UAS-m8* (B), *UAS-m7* (C), *UAS-mβ* (D), *UAS-mγ* (E) and *UAS-mδ* (F). Note the restoration of the wing blade in E and F, and the enhancement of wing notching in B and C.

60-108 hours AEL, i.e. from roughly mid-second instar to late third instar, in agreement with Shellenbarger and Mohler (1978). We compared the wing phenotypes of N^{ts1}/Y flies in the presence or absence of ectopically expressed *E(spl)* after shifting to the non-permissive temperature for 14 hours starting at early (72-84 hours AEL) or mid- (96-108 hours AEL) third instar larval stage. Early shifts of control N^{ts1} flies resulted in long paddle-shaped wings with extensive anterior and posterior scalloping (Fig. 4A). Late shifts produced wing nicking around the margin, with less extensive loss of adjacent tissue (Fig. 4B). In the presence of *32B-GAL4* and *UAS-m*δ or *UAS-m*γ*,* the late upshift regimen gave wings with no or slight margin nicking (Fig. 4D), similar to the rescue observed for *N/+* or nd^3/Y genotypes, while *UAS-m*β*, UAS-m7* or *UAS-m8* had no effect. Surprisingly, in the early shifts all five *E(spl)* transgenes gave enhancement of the N^{ts1} phenotype resulting in miniscule wings (Fig. 4C) that resemble the *vestigial* wing phenotype.

These data suggest that the broad temporal requirement observed for Notch during the third larval instar reflects at least two distinct phases of Notch activity in the process of wing margin specification with opposite response to overexpression of *E(spl)m*δ or *E(spl)m*γ. The effects of the various transgenes on wing notching are summarized in Table 1.

Ectopic *E(spl)m*δ and *E(spl)m*γ restore *cut* but not *wg* expression

Loss of function of the genes *wg* and *cut* results in wing scalloping phenotypes, such as those that we observed in our N^{ts1} experiment (Blochlinger et al., 1993; Couso et al., 1994). Because Notch signalling at the DV boundary is needed for the expression of *wg* and *cut*, we sought to determine whether the wing margin rescue produced by *E(spl)m*δ and *E(spl)m*γ might reflect restoration of expression of either of these genes.

We assayed *wg* RNA expression in wing imaginal disks from N^{ts1} animals in the presence or absence of *32B-GAL4, UAS-E(spl)m*δ. Larvae were shifted to the restrictive temperature (30°C) at 96-108 hours (mid-late L3) for 16 or 22 hours and then immediately dissected. As reported by Diaz-Benjumea and Cohen (1995), these regimens resulted in specific *wg* loss from the DV boundary, but not from other regions of the wing disk. No difference in *wg* expression was observed between N^{ts1} control disks and N^{ts1} disks with ectopically expressed *E(spl)m*δ (not shown). To conclusively prove that no Wg activity is induced by ectopic *E(spl)*, we studied expression of

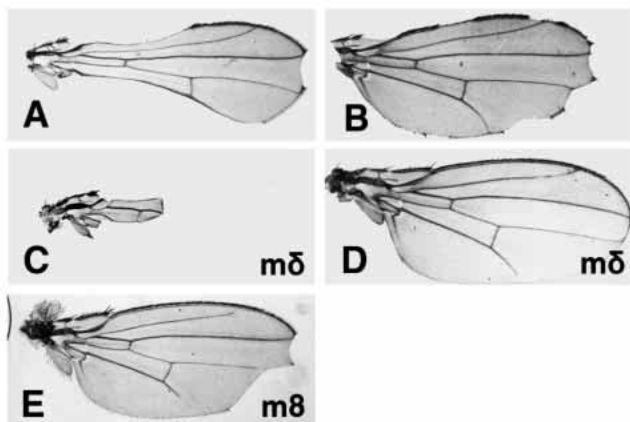

Fig. 4. Ectopic expression of *E(spl)* genes in a N^{ts} background. (A,B) Wings of N^{ts1}/Y individuals; (A) raised at 18°C and shifted to 29°C for 14 hours at 72-84 hours AEL; (B) similarly shifted to the non-permissive temperature at 96-108 hours AEL. (C,D) Wings of N^{ts1}/Y*; 32B-GAL4/UAS-m*δ flies; (C) shifted early, as in A; (D) shifted late, as in B. (E) Wing of a N^{ts1}/Y*; 32B-GAL4/UAS-m8* individual grown continuously at the permissive temperature (18°C). Note that nicking is never observed in N^{ts1} flies at the permissive temperature.

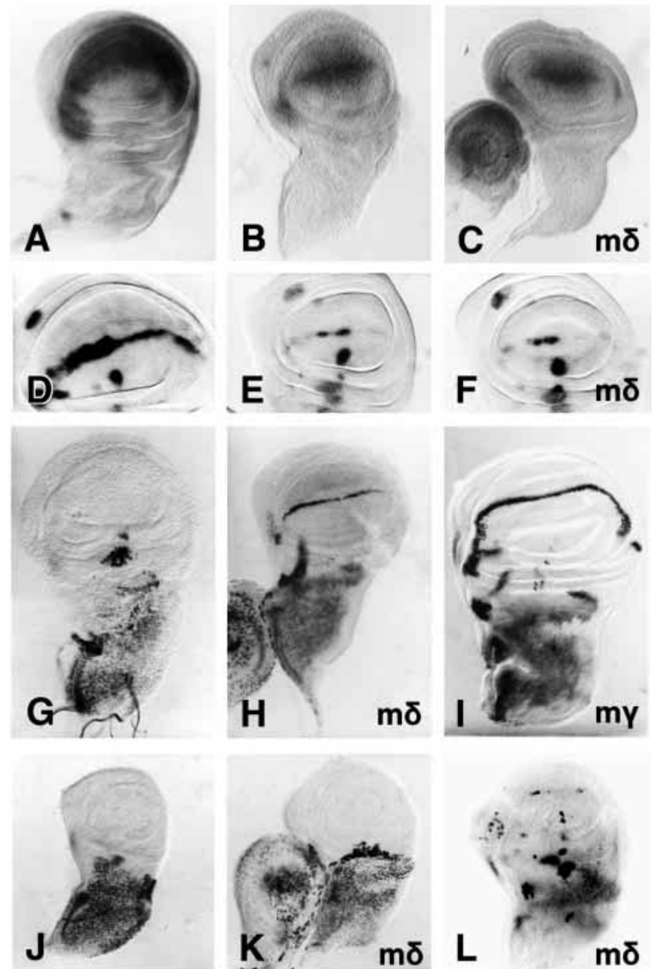

Fig. 5. Dll, *m8-lacZ* and Cut expression in N^{ts1} wing disks. N^{ts1}/Y flies were shifted to the restrictive temperature for 22 hours (B,C,E-I) or 48 hours (J,K) before dissecting. (A,D) Non-shifted controls (permissive temperature). (A-C) Disks were stained with an anti-Dll antibody. The wild-type disk (A) displays a broad band of Dll expression centered at the DV boundary. Loss of N function reduces the levels of Dll (B) and this is not modified by simultaneous expression of *32B-m*δ (C). Similarly the wild-type DV stripe of *m8-lacZ* (D) is severely reduced after loss of N function in the absence (E) or presence (F) of ectopic *m*δ. (G-L) Stained with an anti-Cut antibody. Whereas N inactivation in the absence of any transgene expression causes loss of Cut from the DV boundary (G), *32B-m*δ (H) or *32B-m*γ (I) restore Cut expression. This restoration is not possible if larvae are shifted to the restrictive temperature at an earlier stage; J carries no transgene, while K expresses *m*δ. The disk in L is from a N^{ts1}/Y; *32B-m*δ individual shifted to the restrictive temperature for 22 hours and then returned to the permissive temperature for 48 hours before dissecting. Note that blocking *Notch* activity does not affect Cut expression in the adepithelial cells of the notum. Also note that early-shifted disks (J-L) display significantly smaller wing pouches.

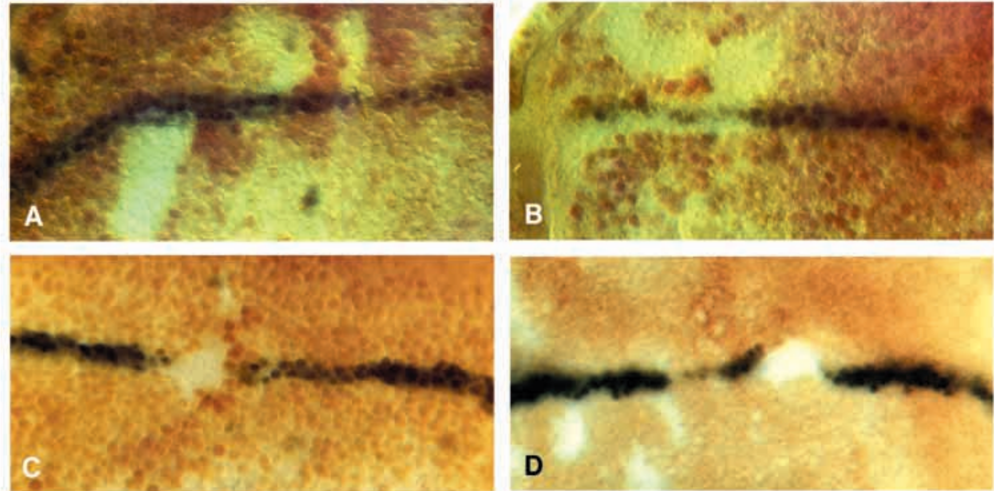

Fig. 6. Cut expression is affected by loss of *E(spl)* or *gro*. (A,B) Examples of *Df(3R)gro*$^{b32.2}$ homozygous mitotic clones revealed by the absence of πMyc staining (brown). Cut staining (black) is autonomously decreased within the clones. (C) Example of a *gro*^{E48} homozygous clone, revealed by the absence of brown πMyc staining. In this case, Cut staining (black) is completely abolished within the clones. Complete loss of Cut is also seen in *N*^{54l9} clones that are simultaneously expressing *UAS-m*δ via *32B-GAL4* (D). This specimen was counterstained with anti-Notch (brown).

the Wg downstream gene *Dll* (Neumann and Cohen, 1997a) using immunocytochemistry as a more sensitive assay. Although Dll expression was not completely abolished after the 22 hours temperature shift, it was significantly reduced and no increase was seen with ectopic expression of *E(spl)m*δ (Fig. 5A-C). These results suggest that rescue of wing margin loss by *E(spl)m*δ ectopic expression is not due to restoration of *wg* expression at the DV boundary. We further confirmed that ectopic expression of *E(spl)m*δ did not restore expression of Notch-responsive genes, as the *m8-lacZ* boundary stripe was lost after heat inactivation of *N*^{ts1} and was not restored by concomitant expression of *E(spl)m*δ (Fig. 5D-F).

We used similar temperature-shift regimens to assay *cut* expression in *N*^{ts1} flies with or without *32B-GAL4, UAS-m*δ or *UAS-m*γ. When we shifted up at approximately 96 hours AEL, Cut protein accumulation in *N*^{ts1} disks was abolished after 22 hours (Fig. 5G). Reduction in Notch activity affected the DV stripe specifically and not the expression of Cut in adepithelial cells of the notum. In contrast, wing disks from *N*^{ts1} flies ectopically expressing *UAS-m*δ or *UAS-m*γ displayed an almost wild-type Cut stripe (Fig. 5H,I). In another experiment, the upshift was performed at approximately 72 hours AEL and the flies were maintained at the restrictive temperature until the late third instar (wandering stage), i.e. about 48 hours. In this case, both control and *E(spl)*-expressing animals lacked the boundary Cut stripe (Fig. 5J,K). To address whether the difference observed was due to the extended duration of the heat pulse, we upshifted larvae at ~72 hours AEL and kept them at the restrictive temperature only for 24 hours. After a further 2 days at the permissive temperature (i.e. ~one day equivalent at 25°C), they reached the wandering stage and were dissected. Again no margin Cut expression was observed regardless of whether they expressed ectopic *E(spl)* or not (Fig. 5L).

We therefore conclude that expression of *E(spl)m*γ or *E(spl)m*δ can promote or maintain *cut* expression in the absence of Notch activity during late L3. Yet if Notch activity is compromised during early L3, E(spl)mγ or E(spl)mδ cannot show this effect, suggesting two sequential Notch-dependent events in the activation of *cut*. In agreement with this hypothesis, *N* null mitotic clones induced during late L2 produced autonomous loss of Cut even in the background of *32B-GAL4, UAS-E(spl)m*δ (Fig. 6D). It is worth noting that, although in the 96 hour (late) *N*^{ts1} upshift the shape of the disks was normal, the disks shifted at 72 hours (early) had severely atrophic wing pouches regardless of the duration of the heat pulse (Fig. 5J-L). It thus appears that early requirements for *N* in the proliferation of the wing pouch, just as in DV boundary-specific gene expression, are not rescued by ectopic expression of *E(spl)m*δ or *E(spl)m*γ.

cut expression at the DV boundary needs *E(spl)* and *gro* activity

To test the hypothesis that E(spl) might be involved in *cut* expression, we analysed mitotic clones null for the *E(spl)-C*, using the deficiency of the locus *Df(3R)gro*$^{b32.2}$, which deletes all *E(spl)bHLH* genes but does not affect *gro*. Although such clones were not completely devoid of Cut protein, they frequently decreased the levels of Cut in an autonomous fashion (Fig. 6A,B). This result was unexpected given the inability of the same mutant clones to produce wing margin notching (de Celis et al., 1996); the lower levels of Cut must be sufficient for wing margin integrity. The interesting possibility therefore arises that some protein encoded by the *E(spl)-C*, most likely E(spl)mγ, is involved in *cut* expression, but acts redundantly with some other factor. If an anti-repression mechanism is active in *cut* expression (see Discussion), we would predict that clones null for the Gro co-repressor might also show defects in the activation of *cut*, since Gro appears to be a necessary co-factor for the activity of a number of repressors including E(spl) (Fisher and Caudy, 1998). We tested this by analysing clones homozygous for the null allele *gro*^{E48}. Interestingly, all clones impinging on the *cut* expression domain did not just decrease, but autonomously abolished *cut* expression (Fig. 6C). We, therefore, conclude that *gro*$^{+}$ is absolutely required for *cut* activation and that it is recruited not only by E(spl)mγ, but also via (an)other unidentified partner(s).

DISCUSSION

Functional specialization of E(spl) proteins

E(spl) genes are the most common transcriptional targets of Notch signalling known. One or more of these seven genes is

turned on in each instance of Notch signalling, yet, when viewed individually, they display distinct expression patterns, especially during postembryonic stages (Bailey and Posakony, 1995; de Celis et al., 1996; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). Even though functional analysis to date has suggested that the functions of the seven proteins are interchangeable, we have herein presented data showing that individual E(spl) proteins are most effective at influencing the cell fate determination events that occur within the domains of their highest respective expression. E(spl)m7 and E(spl)m8, which are most strongly expressed in proneural regions, are most effective at suppressing sensory organ fates. In contrast, E(spl)mβ is most effective at antagonizing vein fate, conforming with its expression in intervein regions. E(spl)mγ is active at the wing DV boundary to repress the *vg* quadrant enhancer and to activate *cut*. Finally, E(spl)mδ, which is strongly expressed in the morphogenetic furrow of the eye, is best at suppressing photoreceptor R8 fate (Ligoxygakis et al., 1998). What the functional specialization of E(spl)m5 and E(spl)m3 might be still remains unclear, as our analyses so far have focussed on the wing and eye disks, where these genes are normally not expressed.

In SOP singularization as well as vein specification, the observed ectopic phenotypes of *E(spl)* are congruent with what would be expected by overactivation of Notch signalling, which suppresses both SOP and vein fates (Muskavitch, 1994). In the wing margin, we have detected two phases of Notch signalling: the early one is curiously antagonized by E(spl) activity, whereas the late one is assisted by E(spl)mγ and E(spl)mδ, but not by others. How can *E(spl)* have two opposing activities during sequential phases of wing margin specification? Our observation that some E(spl) proteins can repress *vg(quad)-lacZ* suggests a possible explanation. This enhancer ultimately depends on Notch signalling, as it requires prior expression of DV boundary-specific genes, like *wg*, that are turned on by Notch (Kim et al., 1996, 1997; Neumann and Cohen, 1997a). Perhaps inappropriate expression of *E(spl)* in the wing pouch enhances the tissue loss caused by heat-inactivated N^{ts1}, by decreasing the levels of Vg protein, which is needed for wing cell viability (Kim et al., 1996). This model is not fully consistent with the fact that we observed repression of *vg(quad)* only with E(spl)m3 and E(spl)mγ, whereas all E(spl) proteins tested were equally able to enhance the early N^{ts1}-induced tissue loss. The different genetic backgrounds of the two experiments may account for this. It is possible that in the sensitized N^{ts1} background mild repression of *vg(quad)* by any E(spl) protein could manifest itself as enhancement of wing blade loss. Ectopic expression of E(spl) proteins is not sufficient to have the same effect in a N^{+} background (Fig. 1), thus accounting for the fact that when the N^{ts1} upshift is done at a later time – after the establishment of *vg* expression – the same driver (*32B-GAL4*)-responder (*UAS-mγ* or *UAS-mδ*) combination gives wing blade rescue (Fig. 4). It should also be pointed out here that GAL4-driven expression is much more robust at 30°C; perhaps the effects of the early upshifts are due to concomitant N activity reduction and very high E(spl) accumulation at a time most sensitive for *vg(quad)* expression.

What the above model does not account for is the stronger effect of E(spl)m8 and E(spl)m7 on wing margin loss in the *N*/+ background (Fig. 3). This cannot be due to *vg* repression, as we have seen that these two proteins are not good repressors of either of the two known enhancers of this gene (Table 1). It appears that this effect stems from yet a different activity of E(spl) proteins. In fact, we have preliminary data (not shown) that high level *UAS-m8* expression in a wild-type background results in repression of *cut* during late larval/ pupal stages.

Mechanism of E(spl) protein involvement in *cut* activation

Notch signalling has been shown to be necessary and sufficient for *cut* [as well as for *vg, wg* and *E(spl)*] expression within the wing pouch (Diaz-Benjumea and Cohen, 1995; Jennings et al., 1995; Micchelli et al., 1997; Neumann and Cohen, 1996). Whereas *vg* and *E(spl)* are direct targets of the N-responsive transcription factor Su(H) (Bailey and Posakony, 1995; Kim et al., 1996; Lecourtois and Schweisguth, 1995; Neumann and Cohen, 1996), there is no evidence to date that *cut* is activated by a similar mechanism. Instead, the present work suggests that *cut* expression requires Gro and partly also depends on E(spl)bHLH factors. One possibility is that E(spl) (at least E(spl)mγ and E(spl)mδ), like a number of other transcription factors (Fisher and Caudy, 1998), might have a dual function as either a transcriptional repressor or an activator depending on context. Such an activation role has never been suggested before for either E(spl) or Gro. Alternatively, two models can be envisaged that reconcile a repressor activity of E(spl) proteins with their role in *cut* activation. In one, E(spl) could act by repressing a negative regulator of *cut* transcription. In the other, they could repress a negative regulator of Notch signalling. In the latter case, E(spl) expression would promote a positive feedback loop to enhance Notch signalling, thus increasing the signalling output from the severely compromised N^{ts1} receptor at the restrictive temperature. The fact that no restoration is observed in the expression of two other Notch targets, namely *wg* and *E(spl)m8-lacZ* (Fig. 5A-F), argues against this hypothesis. We therefore favour a direct role of E(spl)mγ and E(spl)mδ in *cut* expression, either as activators or as repressors of a repressor, but not as general positive regulators of Notch signalling. Detailed study of transcriptional regulators that bind the *cut* wing margin enhancer will provide leads to unravel this rather complex control mechanism.

Ectopic expression of E(spl)mγ/E(spl)mδ is not sufficient for *cut* expression in a wild-type background. Rather, it appears that the ability of ectopic E(spl)mγ/E(spl)mδ to induce *cut* is spatially restricted to the normal domain of *cut* expression (Fig. 5G-I). As activated Notch is sufficient to ectopically turn on *cut* (Neumann and Cohen, 1996), it follows that some other Notch-responsive event, besides *E(spl)* expression, must also contribute to *cut* expression. This is consistent with our findings that early reduction of Notch activity abolishes *cut* expression despite concomitant ectopic expression of E(spl)mδ (Figs 5J-L, 6D). Molecular analysis has shown that *cut* expression requires the transcription factor Scalloped (Sd) (Morcillo et al., 1996). *sd* is a candidate target gene of Vg (Williams et al., 1993), which in turn is initially activated by Notch independently of E(spl) (Kim et al., 1996). It is possible that expression of *vg* and *sd* at the wing margin during early L3 could make these cells competent for *cut* expression. This would only be initiated later, when a second pulse of Notch signalling during mid-L3 activates (or relieves repression of) *cut* via E(spl)mγ or another Gro-interacting protein.

Why are there seven *E(spl)* genes?

The existence of genes with overlapping functions is quite common in mammals, yet it is rare in *Drosophila*, where many single gene mutations produce phenotypic consequences. Even when structurally similar genes are encountered in *Drosophila*, many of the individual members have evolved discrete functions as evidenced by point mutant phenotypes – e.g., *engrailed* and *ase* (Dominguez and Campuzano, 1993; Simmonds et al., 1995). Unlike these, the *E(spl)* locus comprises a cluster of seven apparently redundant genes (no individual loss-of-function phenotypes characterized) interspersed with structurally unrelated genes. This organization is not a quirk of *D. melanogaster*, but is also found in the distantly related species *D. hydei* (Maier et al., 1993), raising the question of its importance through evolution.

In the case of another complex locus, the *achaete-scute Complex*, the presence of multiple genes with (partially) overlapping functions has been proposed to have been selected for, in order to allow the use of extensive lengths of transcriptional regulatory regions that would be difficult to act on a single gene (Modolell, 1997). It is clear that besides Notch activation, each of the seven *E(spl)* genes requires additional distinct transcriptional regulation, as their patterns of expression are different, yet their *cis*-regulatory regions do not appear to be very long (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Thus, it is unclear why these regulatory regions have been allocated to different coding regions instead of being used as different enhancers for the same gene. A possible hypothesis stems from our work that suggests that individual members of the E(spl) family do indeed possess some functional specialization. Perhaps the pleiotropy of Notch function, namely the number of target genes to be regulated in different instances of signalling, would be too much to be handled by one individual effector protein, and gene duplications were favoured to produce similar effectors with different specializations. It appears that having multiple E(spl) genes confers a selective advantage to the organism, but individual gene knock-outs might yield effects of such low expressivity and penetrance that they would be undetectable by the currently practiced gross phenotypic analysis. Study of the genomic organization of homologous genes in more phylogenetically distant arthropods should shed light on the evolution of this complex locus.

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