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Role of *Notch* and *achaete-scute* complex in the expression of *Enhancer of split* bHLH proteins

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SUMMARY

The proteins encoded by *Notch* and the *Enhancer of split* complex are components of a cell-cell interaction mechanism which is important in many cell fate decisions throughout development. One such decision is the formation of the sensory organ precursor cell during the development of the peripheral nervous system in *Drosophila*. Cells acquire the potential to be neural through the expression of the proneural genes, and the Notch pathway is required to limit neural fate to a single cell from a proneural cluster. However, despite extensive analysis, the precise pathways linking the proneural with *Notch* and *Enhancer of split* gene functions remain obscure. For example, it has been suggested that *achaete-scute* complex proteins directly activate *Enhancer of split* genes leaving the action of Notch in the pathway unclear. Using monoclonal antibodies that recognise products of the *Enhancer of split* complex, we show that these proteins accumulate in the cells surrounding the developing sensory organ precursor cell and that their expression is dependent on the activity of Notch and does not directly correlate with expression of Achaete. We further clarify the pathway by showing that ubiquitous expression of an activated Notch receptor leads to widespread accumulation of *Enhancer of split* proteins even in the absence of *achaete-scute* complex proteins. Thus *Enhancer of split* protein expression in response to Notch activity does not require *achaete-scute* complex proteins.

Key words: *Drosophila*, *E(spl)*, *Notch*, *achaete-scute* complex, PNS

INTRODUCTION

Cell-cell signalling mediated by the transmembrane protein Notch is essential for a wide variety of cell-fate decisions during development in both invertebrates and vertebrates. One of the best studied processes involving this pathway in *Drosophila* is neurogenesis, both in the formation of the central nervous system and in the embryonic and postembryonic development of the peripheral nervous system (PNS). Neurogenesis in *Drosophila* involves two antagonistic activities: one which promotes neural development and the other which prevents the majority of cells from adopting that fate (for recent reviews, see Campos-Ortega, 1993; Muskavitch, 1994; Artavanis-Tsakonas et al., 1995). The former is provided by proneural gene-products such as the basic helix-loop-helix transcription factors [Achaete, Scute and Lethal of scute (Villares and Cabrera, 1987)] encoded by the *achaete-scute* complex (*AS-C*). Deletions that remove this complex lead to a reduction in the central nervous system and a loss of peripheral sense organs (Jimenez and Campos-Ortega, 1990). The antagonising activity is mediated by the gene products that make up the Notch signalling pathway. These include the transmembrane protein Notch and the proteins encoded by the genes of the *Enhancer of split* complex [*E(spl)-C*], seven of which (m3, m5, m7, m8, mβ, mδ, mγ) are basic helix-loop-helix [*E(spl)bHLH*] proteins (Klambt et al., 1989; Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992). Deletions removing *Notch* or *E(spl)-C* result in neural hypertrophy; all of the cells in the neural ectoderm adopt the neural pathway whereas normally only about a quarter of the cells from this region become neural (Lehmann et al., 1983). Similarly, absence of Notch during postembryonic development leads to supernumerary sensory organ precursors (Hartenstein and Posakony, 1990; de Celis et al., 1991; Heitzler and Simpson, 1991).

The proneural activity of *AS-C* genes has been demonstrated most elegantly through the ectopic expression of one of these gene products, Lethal of scute (L'sc), in the imaginal discs of *Drosophila* (Hinz et al., 1994). This leads to the formation of ectopic sensory structures. It also results in the ectopic transcription of some genes in the Notch signalling pathway, including the *E(spl)bHLH m7* gene. In contrast, loss-of-function mutations or deletions of *AS-C* lead to reduction in *E(spl)bHLH* transcription (Kramatschek and Campos-Ortega, 1994; Singson et al., 1994). Clearly the transcription of *E(spl)bHLH* genes is regulated in response to the presence of

AS-C proteins and these observations, along with the finding that Achaete and Scute can interact with regulatory sequences in the *E(spl)m8, m7* and *m5* genes (Singson et al., 1994), led to the proposal that the proneural proteins directly activate *E(spl)bHLH* transcription. However, recently we have shown that accumulation of *E(spl)bHLH* proteins in the embryonic neurogenic region is dependent on Notch activity and that *E(spl)bHLH* proteins are not expressed in neuroblasts where *AS-C* proteins accumulate to their highest levels (Jennings et al., 1994 and Fig. 3A). These results agree well with the deduced function of *E(spl)bHLH* proteins during neurogenesis, which is to repress the neural fate (Lehmann et al., 1983; Knust et al., 1987; Tata and Hartley, 1995), and have prompted us to further investigate the relationship between the expression of *AS-C* and *E(spl)bHLH* genes.

In the present analysis, we have focused on the development of the adult PNS in the wing disc. Much of the adult body structure of *Drosophila* develops from groups of cells, the imaginal discs, which are set aside during embryogenesis and subsequently proliferate and differentiate during larval and pupal stages. During late larval stages the development of the adult sensory organs commences with the formation of a sensory organ precursor cell (SOP) (Ghysen et al., 1993). These arise at specific sites in the developing disc, which have been well documented for the wing and notal regions of the wing disc (Huang et al., 1991). The SOP subsequently divides in a stereotyped manner to give rise to the cells that make up the sensory organ (Hartenstein and Posakony, 1989). The genes of the Notch signalling pathway are involved both in the selection of the sensory organ precursor cell and in the subsequent fate decisions of its progeny (Hartenstein and Posakony, 1990; de Celis et al., 1991). Proneural gene products, such as Achaete, are first expressed in clusters of cells and then accumulate to highest levels in one cell of the cluster, which corresponds to the developing SOP (Cubas et al., 1991; Skeath and Carroll, 1991). Using antibodies that recognise some of the *E(spl)bHLH* proteins, we have investigated how their expression relates to the development of SOPs and whether their expression depends on Notch activity as it does in the embryo. In addition, we have addressed the role of proneural genes in the accumulation of *E(spl)bHLH* proteins; specifically, we have investigated whether the presence of an activated form of Notch can bypass the requirement for *AS-C* in *E(spl)bHLH* protein expression.

MATERIALS AND METHODS

Drosophila strains

"Wild-type" strains were Oregon R, *y w* or *cn*; *ry* depending on the genotype of other strains used in the experiments. The *Notch^{intra}* transgenic line was obtained from Gary Struhl (Struhl et al., 1993), the *neuralized-lacZ* enhancer trap line (*neu-lacZ^{B28}*) from Robert Whittle (Phillips and Whittle, 1993) and the *achaete-lacZ* reporter gene line from James Posakony (Van Doren et al., 1992). *Df(1)silver, In(1)scute$^{10-1}$ and Notch^{ts1}* are described in Lindsley and Zimm, (1992) as are the balancer chromosomes used.

Immunohistochemistry and immunofluorescence

Wing imaginal discs were dissected from third instar larvae and fixed for 30-45 minutes in 4% paraformaldehyde fixative. The fixation buffer and subsequent steps of the staining procedure were as described previously (Jennings et al., 1994) as were the procedures used for embryos. The following primary antibodies were used: mAbs 323 and 174 to detect *E(spl)bHLH* proteins; mAb990E5F1 to detect Achaete (Skeath and Carroll, 1992); and a rabbit polyclonal to detect β-galactosidase (Cappell). In experiments in which only the anti-Achaete antibody was used, the conditions were slightly modified to be optimal for this antibody (Skeath and Carroll, 1992). Secondary antibodies were from Jackson laboratories and were used at 1/250 final dilution. For bright-field double-label experiments in which discs were stained using mAb323 and anti-β-galactosidase, the secondary antibodies were both horseradish peroxidase conjugated and were added sequentially. For the first secondary antibody (i.e. anti-rabbit), the staining solution contained 5% nickel sulphate to produce a black precipitate and for the second (i.e. anti-mouse) the nickel sulphate was omitted so that a brown precipitate was produced. Following staining, the discs were transferred to 50% glycerol, where they were dissected free of other debris and finally mounted in 70% glycerol. Embryos were mounted in DePeX (Gurr, BDH) as described previously (Jennings et al., 1994). When fluorescent secondary antibodies were used, the discs were dissected in 50% glycerol as above and finally mounted in AF1 mountant (Citifluor Ltd, City University, London) for analysis using a Leica confocal microscope.

Experiments with Notch^{intra}

Notch^{intra} expression was induced in larvae containing the *Notch^{intra}* transgene (Struhl et al., 1993) by placing larvae at 37°C for 30 minutes followed by 20 minutes recovery at 22°C. The *sc$^{10-1}$* (Lindsley and Zimm, 1992) discs were obtained from a *sc$^{10-1}$/FM6* stock, from which *sc$^{10-1}$*/Y larvae were selected (*yellow^{+}* male larvae). Similarly, to analyze effects of Notch^{intra} in sc$^{10-1}$ discs, *yellow^{+}* male larvae were selected from the progeny of *sc$^{10-1}$/FM6; +/+* x *+/Y; Notch^{intra}/Notch^{intra}*.

Notch^{intra} expression was induced in embryos by subjecting them to a 30 minute heat shock at 37°C before fixation. For the experiments using *Df(1)svr*, an *FM7c* balancer chromosome carrying an *eve-lacZ* transgene was used to distinguish the genotype of embryos. Embryos were double stained with mAb323 and rabbit anti-β-galactosidase antibodies (Cappell); those embryos that had no detectable β-galactosidase were of the genotype *Df(1)svr*/Y and thus lacked all the *AS-C* genes. To examine the effects of Notch^{intra}, embryos were collected from the cross *Df(1)svr/FM7c LacZ;+/+* x *FM7c LacZ/Y;Notch^{intra}/+*. 50% of the *Df(1)svr /Y* embryos exhibited the phenotype shown in Fig. 7F, in agreement with the expected frequency of Notch^{intra} in the progeny.

RESULTS

We have previously described two antibodies (Jennings et al., 1994), one of which, mAb174, recognises specifically the *E(spl)* mδ protein. The other, mAb323, recognises at least 5 of the 7 bHLH proteins of the *E(spl)* complex and so provides an indicator of the cumulative expression of these proteins. We have used both monoclonal antibodies to examine the distribution of *E(spl)bHLH* proteins during PNS development in wing imaginal discs of third instar larvae (Fig. 1). *E(spl)bHLH* expression is dynamic and clearly relates to the development of the peripheral nervous system. For example, mAb323 reveals clusters of staining cells in three parallel lines in the anterior of the wing pouch of the disc, which correspond to the position of the developing sensory bristles of the wing margin (Fig. 1A,B,D). The wing margin staining is first detected in the centre of the disc and spreads to the periphery, consistent with the sequence of SOP formation (Huang et al., 1991). In addition, much of the staining in the notal region is in clusters

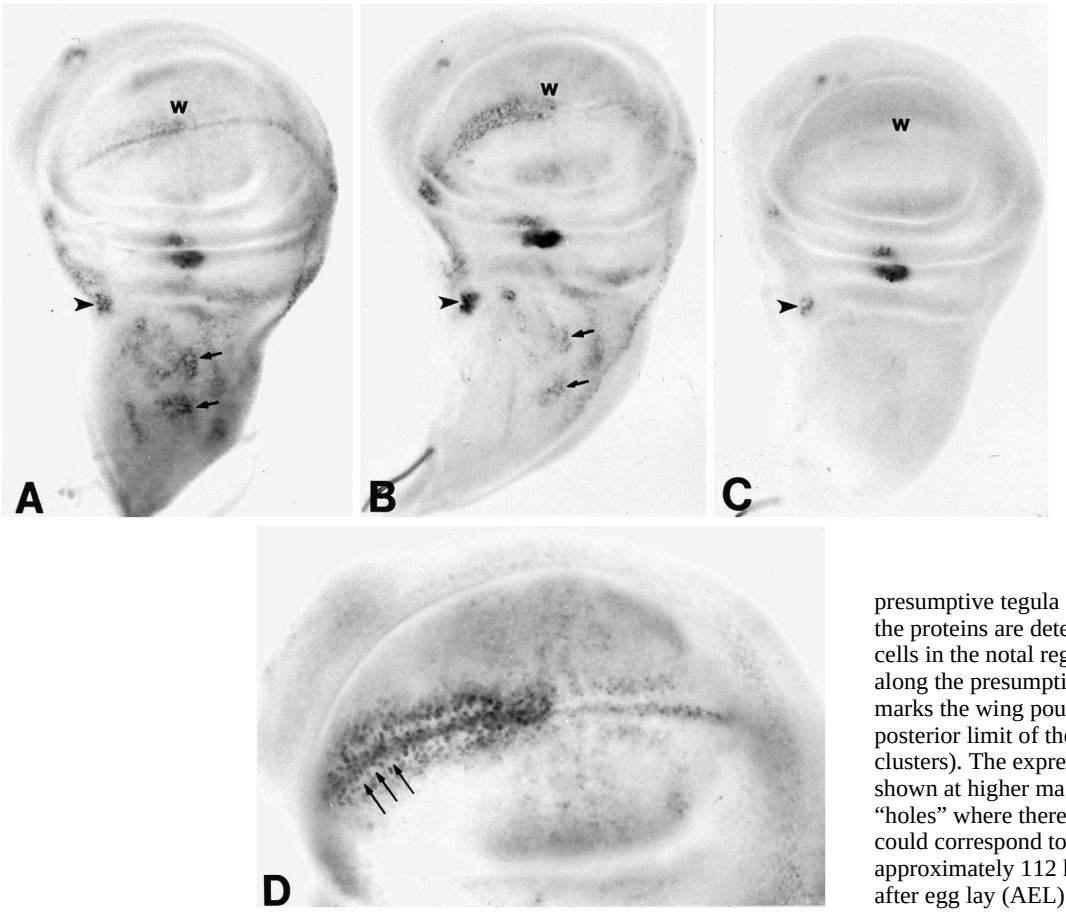

Fig. 1. Expression of *E(spl)bHLH* proteins in late third instar wing imaginal discs. The expression of *E(spl)bHLH* proteins in wing discs was detected using mAb323 (A,B,D) and mAb174 (C). The latter specifically recognises mδ only and detects protein at fewer sites (compare C with A,B; arrowhead marks the presumptive tegula cluster). Some of the sites where the proteins are detected include specific clusters of cells in the notal region (e.g. arrows) and two tracks along the presumptive anterior wing margin (w marks the wing pouch and is just above the posterior limit of the developing wing margin clusters). The expression along the wing margin is shown at higher magnification in (D), arrows mark "holes" where there is no detectable staining which could correspond to SOPs (see Fig. 3). Discs were approximately 112 hours (A) and 120 hours (B,C) after egg lay (AEL).

of cells that correlate with the positions where SOPs develop (Fig. 1A,B). The cells expressing mδ form a subset of those detected with mAb323: strong staining is detected at the positions where the dorsal radius, tegula and ventral radius campaniform sensilla develop (compare Fig. 1A,B with 1C) but not for example in the cells along the wing margin. The detection of mδ in a subset of positions in the wing disc suggests that the seven *E(spl)bHLH* genes are not expressed in an identical manner. In addition, some aspects of *E(spl)bHLH* protein expression detected by mAb323 appear distinct from SOP development, for example the proteins are detected all along the presumptive wing margin, not only at positions where the SOPs develop (Figs 1A, 2). This wing margin expression (which corresponds to the boundary between dorsal and ventral surfaces of the wing) is seen in discs from early third instar larvae, along with a more general expression throughout the notal region of the disc, suggesting that *E(spl)bHLH* proteins are involved in aspects of wing morphogenesis that are distinct from PNS development (S. J. Bray and J. F de Celis, unpublished data).

A closer look at the clusters of cells containing *E(spl)bHLH* proteins reveals that they often appear as rings, surrounding a cell that is not immunoreactive (Fig. 1A,B). To investigate whether the unstained cells within the clusters are SOPs, we used an enhancer trap fly-strain with a *lacZ* gene inserted in the *neuralised* gene, which gives β-galactosidase expression in all SOPs of the developing wing-notal disc (Huang et al., 1991; Phillips and Whittle, 1993). Double labelling wing discs with mAb323 and anti-β-galactosidase reveals that many of the cells expressing *E(spl)bHLH* proteins are surrounding SOPs (Fig. 2). It has been reported that *neu-lacZ* expression can sometimes be detected at low levels in >1 cell before the SOP enlarges (Huang et al., 1991). When this was observed occasionally at the site where the anterior-dorsocentral macrochaeta develops, it was accompanied by little or no *E(spl)bHLH* expression contrary to discs in which the SOP had resolved where the levels of *E(spl)bHLH* proteins were easily detectable in the surrounding cells (data not shown). We used confocal fluorescence to see whether the *E(spl)bHLH* proteins were indeed only present in the surrounding cells and not in the SOP, as suggested by the appearance of the clusters. Clearly, the β-galactosidase-expressing SOP does not express *E(spl)bHLH* proteins (Fig. 3). These are found in cells surrounding the SOP, although not all the expressing cells appear to be in direct contact with the SOP itself. Thus in general the highest levels of expression in the notal regions in late third instar discs are associated with cells surrounding SOPs.

Role of Notch activity in *E(spl)bHLH* protein accumulation

Expression of *E(spl)bHLH* proteins during SOP development is similar to that detected during neurogenesis in the embryo, where the proteins are found in cells surrounding the delaminating neuroblast. This expression in the embryo is dependent on Notch activity (Jennings et al., 1994). To investigate

Fig. 2. *E(spl)bHLH* proteins are detected in cells surrounding the SOP. Developing SOPs are detected in wing discs from *neuralised-lacZ* flies using anti-β-galactosidase antibodies (blue-black) and *E(spl)bHLH* protein expression is detected using mAb323 (brown). Throughout the wing disc much of the expression of *E(spl)bHLH* proteins is detected in cells surrounding SOPs.

whether a similar pathway operates in PNS development in wing discs, as seems likely based on the phenotype of *Notch* mutations (Hartenstein and Posakony, 1990; de Celis et al., 1991; Heitzler and Simpson, 1991), we used a temperature-sensitive allele of *Notch* (N^{ts1}) and transferred developing larvae to the non-permissive temperature for 12 hours prior to analysing their discs. The reduction in Notch function results in a dramatic loss of *E(spl)bHLH* expression throughout the wing disc (Fig. 4). However, we still detect a low level of protein in a number of cells consistent with the likelihood that some Notch protein is still active in these discs as has been reported in other studies using this allele (Xu et al., 1992). Conversely, when an activated form of Notch, $Notch^{intra}$ (Lieber et al., 1993; Struhl et al., 1993), is expressed throughout the disc under the control of a heat inducible promoter, *E(spl)bHLH* proteins are detected at high levels throughout the disc (Fig. 6B). Thus, as in the embryo, accumulation of *E(spl)bHLH* proteins in the wing disc occurs in response to Notch activity.

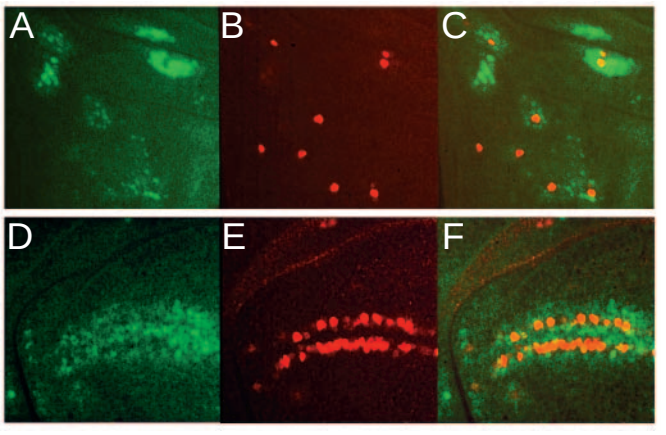

Fig. 3. *E(spl)bHLH* proteins are not present in the SOP. *E(spl)bHLH* proteins were detected with mAb323 (green) and SOPs using anti-β-galactosidase (red). Two regions of a wing disc are shown: (A-C) clusters in the wing/notal region including tegula and anterior notal wing process (anwp) cluster, (D-F) developing wing margin sensilla.

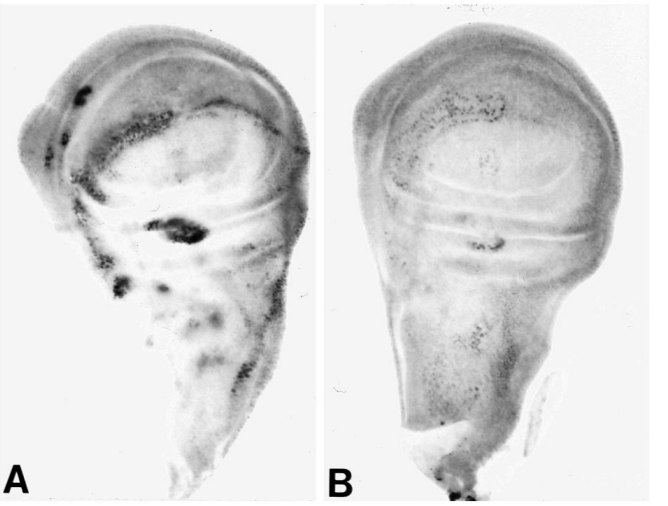

Fig. 4. Accumulation of *E(spl)bHLH* proteins is dependent on Notch activity. Wing imaginal discs from wild-type (A) and $Notch^{ts1}$ (B) larvae after 12 hours at 30°C were stained to detect *E(spl)bHLH* proteins with mAb323. Loss of Notch function (B) results in a reduction in *E(spl)bHLH* expression.

Relationship between *E(spl)bHLH* expression and proneural gene function

Expression of the genes of the *AS-C* is an important prerequisite for the development of the SOPs in the wing disc. The $In(1)scute^{10-1}$ mutation (sc^{10-1}) results in a failure of most adult external-sensory organ development due to aberrations in *achaete* and *scute* (Garcia-Bellido, 1979; Campuzano et al., 1985; Villares and Cabrera, 1987). Expression of *E(spl)bHLH* genes appears likewise affected, the *E(spl)bHLH m7* mRNA is barely detectable (Singson et al., 1994) and we find that *E(spl)bHLH* proteins are similarly reduced (Fig. 6C). One interpretation of these observations is that *AS-C* products directly regulate *E(spl)bHLH* expression. However, the expression patterns of the two gene families are not fully consistent with a simple relationship between *AS-C* products and *E(spl)bHLH* expression. The *AS-C* gene products, such as Achaete are first expressed in a cluster of cells and then accumulate to highest levels in the presumptive SOP (Cubas et al., 1991; Skeath and Carroll, 1992 and Fig. 5) whereas *E(spl)bHLH* proteins are detected at highest levels in the surrounding cells as the SOP develops (Figs 2, 3, 5). In addition, there are some clusters where the numbers of cells that express *E(spl)bHLH* proteins and Achaete are clearly different. This is most obvious in the case of the posterior-supraalar cluster which contains few (2-5) Achaete-expressing cells and >10 *E(spl)bHLH*-expressing cells (Fig. 5). Using an *achaete-lacZ* fusion gene (Van Doren et al., 1992) to mark the *achaete*-expressing cells, it is possible to see that the cells accumulating higher levels of *E(spl)bHLH* proteins are not those that express the highest levels of *achaete-lacZ* (Fig. 5C). These results indicate that proneural gene products are unlikely to be the primary determining force in *E(spl)bHLH* expression.

Since we have observed that *E(spl)bHLH* protein expression can be induced ubiquitously in wing discs and in embryos by the presence of $Notch^{intra}$, we have used this to investigate whether *AS-C* proteins are essential for Notch activation of

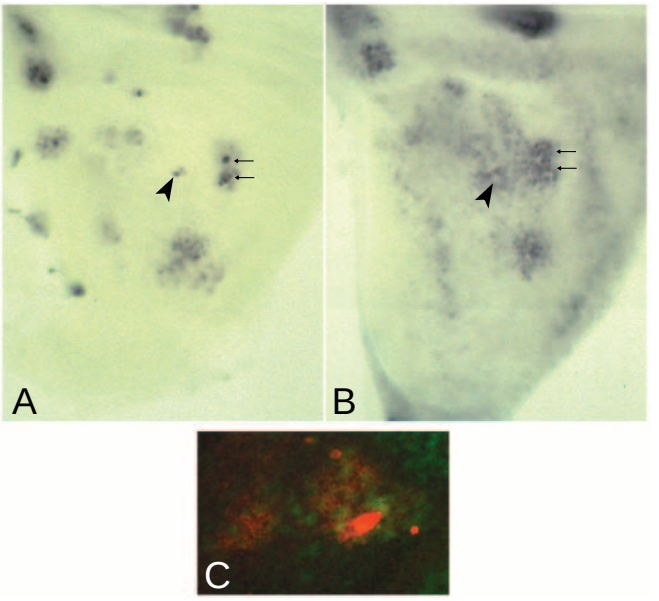

Fig. 5. Comparison of Achaete and *E(spl)bHLH* protein accumulation. Expression of Achaete (A) and *E(spl)bHLH* (B) proteins were detected in wing imaginal discs, only the notal region is shown. In both cases prominent clusters of cells are detected at similar locations (e.g. arrows). However the distribution of the proteins within the clusters is different. Arrowhead marks the posterior supraalar cluster. (C) Confocal immunofluorescence of a single proneural cluster, the anwp, in a disc from an *achaete-lacZ* transgenic strain. β-galactosidase (red) accumulates to high levels in one cell, the presumptive SOP, whilst *E(spl)bHLH* proteins (green) begin to be detected in surrounding cells.

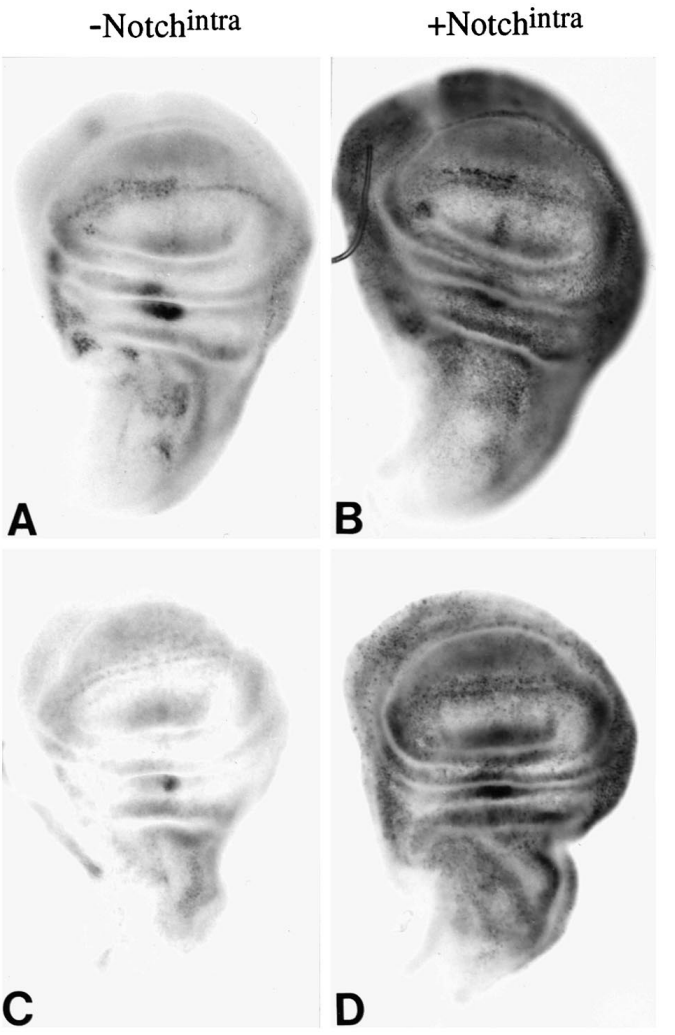

Fig. 6. Achaete and Scute are not required for ectopic accumulation of *E(spl)bHLH* proteins induced by Notch^{intra} in wing discs. *E(spl)bHLH* proteins were detected by mAb323 in wild-type (A,B) and $sc^{10\text{-}1}$ (C,D) wing imaginal discs. The absence of Achaete and Scute in $sc^{10\text{-}1}$ discs leads to loss of *E(spl)bHLH* expression (compare A and C). Ubiquitous expression of Notch^{intra} bypasses this requirement for *AS-C* and leads to ectopic expression of *E(spl)bHLH* proteins throughout the wing-disc in both wild-type (B) and $sc^{10\text{-}1}$ (D) discs.

E(spl)bHLH expression. The effects of Notch^{intra} on *E(spl)bHLH* protein accumulation were compared in discs from wild-type and $sc^{10\text{-}1}$ larvae. In both genotypes, *E(spl)bHLH* proteins are detected at high levels throughout the disc (Fig. 6B,D) and there is no distinguishable difference between them. Thus the presence of Notch^{intra} leads to wide-spread *E(spl)bHLH* expression whether or not *AS-C* proteins are present. However, not all cells have equal capacity to activate *E(spl)bHLH* expression in the presence of Notch^{intra}. In both wild-type and $sc^{10\text{-}1}$ discs, milder heat-shock conditions lead to high levels of *E(spl)bHLH* protein expression in the domains where *E(spl)bHLH* proteins are usually found (data not shown), suggesting that another component of the pathway is differentially active in certain regions, or that there is synergy with other patterning systems.

We extended our analysis to the developing embryo, where it is possible to study the effects of large *AS-C* deficiencies, which do not survive to larval stages. As in wild-type embryos, ubiquitous Notch^{intra} protein leads to wide-spread ectopic expression of *E(spl)bHLH* proteins in embryos that lack all the genes of the *AS-C* (e.g. *Df(1)svr* embryos which lack *AS-C* and neighbouring genes including *ventral nerve cord defective*). The level and extent of ectopic expression is indistinguishable from that seen in *AS-C*$^{+}$ embryos (Fig. 7D,F). For example, in both cases, mAb323 detects ectopic expression of *E(spl)bHLH* proteins in the amnioserosa. In the absence of Notch^{intra}, the *Df(1)svr* embryos have weaker and more patchy expression of *E(spl)bHLH* proteins than wild-type embryos (Fig. 7E). This correlates with the fact that fewer neuroblasts segregate in *Df(1)svr* embryos (Jimenez and Campos-Ortega, 1990), and agrees with previous observations that the expression of reporter genes containing *E(spl)bHLH gene* regulatory sequences is reduced (Kramatschek and Campos-Ortega, 1994). Thus, in the absence of *AS-C*, fewer cells are instructed to initiate neural differentiation, resulting in fewer cells initiating Notch signalling and *E(spl)bHLH* protein accumulation. Supplying cells with activated Notch via Notch^{intra} bypasses the requirement for *AS-C* in this process.

DISCUSSION

The development of the sensory organs of the adult wing and

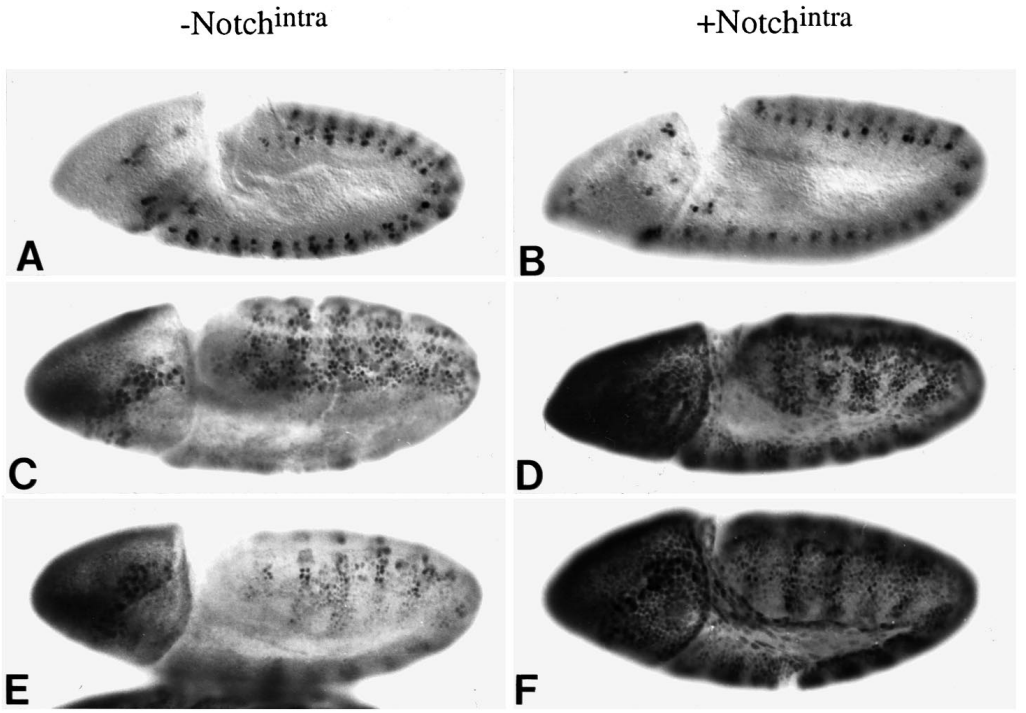

Fig. 7. Notch^{intra} causes widespread expression of *E(spl)bHLH* proteins in the absence of *AS-C* genes. The effect of Notch^{intra} on the expression of Achaete (A,B) and *E(spl)bHLH* (C-F) proteins in wild-type (A-D) and *Df(1)svr* (E-F) embryos was examined using monoclonal antibodies. In stage 9 wild-type embryos Achaete (A and Skeath and Carroll, 1992) and *E(spl)bHLH* proteins (C) are detected in the ventral neurogenic region. Ubiquitous expression of Notch^{intra} leads to accumulation of *E(spl)bHLH* proteins throughout the ectoderm and the amnioserosa (D) but does not alter the domain of Achaete expression (B). In *Df(1)svr* embryos, fewer cells in the neurogenic region contain detectable amounts of *E(spl)bHLH* proteins (E). When Notch^{intra} expression is induced in *Df(1)svr* embryos *E(spl)bHLH* proteins are detected throughout the ectoderm and amnioserosa (F). The levels and extent of ectopic *E(spl)bHLH* expression are similar to those detected in D. Each panel shows a dorso-lateral view of a stage 9 embryo.

notum in *Drosophila* is one of the many developmental processes that require Notch activity. Experiments in the embryo indicate that *E(spl)bHLH* gene-products are part of the same signalling pathway and are expressed in cells where Notch is activated (Lieber et al., 1993; Jennings et al., 1994). Here we have shown that the same relationship is seen in later stages of development: *E(spl)bHLH* proteins accumulate in the cells where Notch is required and their expression reflects Notch activity. Thus loss of Notch function leads to a reduction in *E(spl)bHLH* protein expression and the presence of ubiquitous activated Notch (Notch^{intra}) results in high levels of *E(spl)bHLH* proteins throughout the developing wing disc. The effect of Notch^{intra} on *E(spl)bHLH* expression in both the wing disc and the embryo is independent of the genes of the *AS-C*, arguing that *AS-C* proteins are not essential for the signalling pathway downstream of Notch.

Role of *AS-C* genes in regulating *E(spl)bHLH* expression

Expression of proneural genes in the wing disc and the ventral ectoderm of the embryo appears to confer on cells the potential to be neural, since their absence results in a failure of neural development (reviewed by Campuzano and Modolell, 1992; Campos-Ortega, 1993). The *E(spl)bHLH* proteins are thought to act antagonistically to prevent cells from adopting a neural fate, being components of the inhibitory pathway that is mediated by Notch signalling (Campos-Ortega, 1993; Muskavitch, 1994; Artavanis-Tsakonas et al., 1995). Thus, it is not surprising that the absence of *AS-C* genes should result in a reduction in *E(spl)bHLH* expression (Fig. 5 and Kramatschek and Campos-Ortega, 1994; Singson et al., 1994), since the *AS-C* genes are required to initiate the process that leads to Notch activation. However, the antagonistic action of the two and the observation that they accumulate to highest levels in different cells, argue against a simple relationship in which the *AS-C* genes directly regulate *E(spl)bHLH* expression. By supplying cells with an activated form of Notch, we have bypassed the requirement for *AS-C* and so have been able to show that these proteins are not essential for the activation of *E(spl)bHLH* expression. However, our data do imply that the *E(spl)bHLH* genes contain target sites for a transcription factor mediating the effects of activated Notch. High levels of *E(spl)bHLH* proteins are seen in regions where the mRNAs have not been detected under wild-type conditions, (e.g. amnioserosa in the embryo and wing disc pleural region) and in the tissues of *AS-C* mutants, which would otherwise have little or no *E(spl)bHLH* mRNA (Singson et al., 1994) indicating that transcriptional regulation must be involved. Two candidates for factors through which Notch could influence transcription have been proposed. One is the intracellular domain fragment of Notch itself; since Notch^{intra} is found in the nucleus, it has been suggested that activation of Notch could lead to proteolytic cleavage generating an active nuclear fragment (Weintraub et al., 1994). The other is the protein encoded by *Suppressor of Hairless*, which binds to DNA in vitro and translocates to the nucleus in tissue culture cells under conditions thought to mimic Notch activation (Brou et al., 1994; Fortini and Artavanis-Tsakonas, 1994).

One interpretation of our data is that the *AS-C* proteins are only required upstream of Notch to initiate the signalling process, which in turn leads to the accumulation of *E(spl)bHLH* proteins (Fig. 8). This implies that *AS-C* proteins must be able to influence signalling via the Notch receptor. One way they could achieve this is by increasing the levels of Delta,

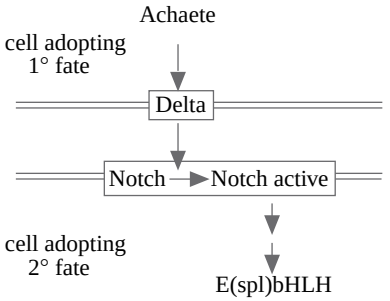

Fig. 8. Model of the pathway linking Achaete, Notch and *E(spl)bHLH* proteins. Arrows indicate positive responses. *E(spl)bHLH* proteins are expressed in response to activation of Notch, their expression prevents the cell adopting the neural fate. Two arrows between Notch and E(spl)bHLH indicate that this may involve intermediates. Notch is activated by its ligand, e.g. the Delta protein, present on the surface of adjacent cells. Expression of proneural proteins, e.g. Achaete, may result in increased synthesis of Delta in the cell adopting the neural fate.

the proposed ligand for Notch, as suggested by the finding that *AS-C* proteins can bind to regulatory sites in the *Delta* promoter (Kunisch et al., 1994) and can induce Delta expression (Hinz et al., 1994). Although this model accounts for the majority of previous results, it does not explain the observation that expression of reporter genes containing *E(spl)bHLH* regulatory sequences is severely impaired by mutations that disrupt Achaete/Scute-binding sites. However, the observation from in vitro binding experiments that many different bHLH proteins can recognise the same targets as Achaete and Scute (Ohsako et al., 1994) suggests an explanation for this apparent anomaly. The sites identified in *E(spl)bHLH* genes may not be targets for AS-C proteins in vivo, but rather for another of the bHLH transcription factors of the class A type.

A more complex explanation to account for our findings and the binding-site data is that *AS-C* proteins act co-operatively with the factor that transduces the Notch signal to activate *E(spl)bHLH* expression. This requirement might be overcome in the cells expressing $Notch^{intra}$ if it produces a higher level or longer lasting signal than wild type. However, the observation that, in clusters such as the posterior supraalar, many more cells express *E(spl)bHLH* proteins than express Achaete (or Scute which shows a similar distribution to Achaete; Cubas et al., 1991), makes it unlikely that *AS-C* proteins are required and we favour the explanation that *AS-C* genes are only required indirectly for expression of *E(spl)bHLH* genes.

Distinct expression of different *E(spl)bHLH* proteins

The *E(spl)* complex contains seven closely related bHLH proteins, but the significance of the different proteins remains unclear. Genetic evidence indicates some redundancy in their functions, since saturation mutageneses of the region have failed to uncover a lethal mutation in any of these genes (Ziemer et al., 1988). In addition, using combinations of transgenes and deficiencies, adult flies have been generated that lack at least one of the *E(spl)bHLH* genes (Delidakis et al., 1991; Schrons et al., 1992). Furthermore, in the embryo, the expressions of the mRNAs of the seven genes appear very similar at least during neurogenesis (Knust et al., 1992). However, our data indicate differences between the expression of *E(spl)bHLH* proteins in the wing disc. An antibody that detects only mδ stains strongly the positions at which the campaniform sensilla develop but not those where the wing margin sensilla develop, whereas an antibody that detects a broader spectrum of the *E(spl)bHLH* proteins stains both (and, in addition, regions that are not involved in SOP development). Similar results have been obtained in the eye disc: mδ is detected in a subset of the cells staining positive with mAb323 and the distribution of m7 and m8 mRNA is not identical (data not shown and A. Preiss unpublished observations). Thus, there are at least some differences between the expression domains of the different *E(spl)bHLH* proteins, suggestive of roles in different developmental processes. These different expression domains are incompatible with a simple hypothesis that expression of all *E(spl)bHLH* genes is solely dictated by Notch activity. We therefore postulate additional factors that cooperate with the Notch signal to activate each gene: these factors could interact selectively with the *cis*-regulatory regions of the *E(spl)bHLH* genes to activate their expression at different times/places. The identity of these postulated spatiotemporally restricted transcription factors remains to be elucidated.

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