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, m6, 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 (Singh 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 Notchcretion transgenic line was obtained from Gary Struhl (Struhl et al., 1993), the neutralized-lacZ enhancer trap line (new-lacZ248) 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(scut)e1011 and Notch were 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; mAb990ESF1 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 PeRex (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**

Notch expression was induced in larvae containing the Notch; transgene (Struhl et al., 1993) by placing larvae at 37°C for 30 minutes followed by 20 minutes recovery at 22°C. The s10-1 (Lindsley and Zimm, 1992) discs were obtained from a s10-1/FM6 stock, from which s10-1/Y larvae were selected (yellow+ male larvae). Similarly, to analyze effects of Notch in s10-1 discs, yellow+ male larvae were selected from the progeny of s10-1/FM6; s10-1/Y; Notch. Notch 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)svrY and thus lacked all the AS-C genes. To examine the effects of Notch3, embryos were collected from the cross Df(1)svrFM7c LacZ; s10-1/Y; Notch/+. 50% of the Df(1)svr Y' embryos exhibited the phenotype shown in Fig. 7F, in agreement with the expected frequency of Notch3 in the progeny.

**RESULTS**

We have previously described two antibodies (Jennings et al., 1994), one of which, mAb174, recognises specifically the E(spl)m6 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
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...
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\textsuperscript{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\textsuperscript{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.

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\textsuperscript{10-1} mutation (sc10-1) 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\textsuperscript{intra}, we have used this to investigate whether AS-C proteins are essential for Notch activation of...
**Requirements for E(spl)bHLH expression**

The effects of Notch\(^{\text{intra}}\) on E(spl)bHLH protein accumulation were compared in discs from wild-type and \(sc^{10-1}\) 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\(^{\text{intra}}\) leads to widespread 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\(^{\text{intra}}\).

In both wild-type and \(sc^{10-1}\) 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\(^{\text{intra}}\) 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\(^{\text{intra}}\), 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\(^{\text{intra}}\) bypasses the requirement for AS-C in this process.

**DISCUSSION**

The development of the sensory organs of the adult wing and
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*\textsuperscript{intra}) results in high levels of *E(spl)bHLH* proteins throughout the developing wing disc. The effect of *Notch*\textsuperscript{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*\textsuperscript{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,
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 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 m6 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: m6 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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**REFERENCES**


