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Evolution of *cis*-regulation of the proneural genes

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ABSTRACT The current state of knowledge concerning cis-regulatory sequences of the proneural genes of vertebrates and *Drosophila* is discussed. Many proneural genes have a complex modular arrangement of discrete enhancer elements. One unusual feature of these genes is that many distant enhancer elements, regulating expression in specific spatial locations, require input from previously synthesized protein from the proneural gene itself, in addition to other transcriptional activators. This is distinct from the auto-regulation, via E boxes in the promoter, that takes place in neural precursors. The selection of neural precursors from a field of cells expressing a proneural gene, is mediated by Notch signalling and requires up-regulation of proneural gene expression in the precursor concurrently with down-regulation in the surrounding cells. Although the way in which a single cell is selected remains unclear, a number of feedback loops have been uncovered that reinforce the choice. These are briefly surveyed. A specific regulatory element, the Sensory Organ Precursor element, that mediates selection of the precursors of the large sensory bristles, has been described in *Drosophila*. We report the conservation of this sequence in *Calliphora vicina*, a higher fly. In contrast, no such sequence is seen in the *achaete-scute* complex of *Anopheles gambiae*, a basal Dipteran species. We suggest that this enhancer may have arisen during the evolution of the cyclorraphous flies and present a hypothesis for its possible function.

KEY WORDS: *bHLH protein, lateral inhibition, auto-regulation, vertebrate, Drosophila, Calliphora*

Introduction

Proteins belonging to the basic Helix-Loop-Helix (bHLH) class of transcription factors have an important role in promoting differentiation of various cell types during embryonic development. They function by forming heterodimers with ubiquitously expressed E proteins and binding via the basic domain to to a DNA motif, the E box, to activate transcription of target genes (Murre *et al.*, 1989). The proneural bHLH proteins are expressed in the neuro-ectoderm and, together with E2A/Da, promote neural development. Loss of function of proneural gene activity causes a loss of neurons or neuronal stem cells, whereas ectopic expression can lead to development of ectopic neurons (Bertrand *et al.*, 2002). There are two subfamilies of proneural proteins, the *achaete-scute* class and the *atona*/class (Bertrand *et al.*, 2002). These are named after the *Drosophila* representatives. The *Drosophila achaete-scute* complex *(AS-C)* comprises four genes, *achaete (ac), scute (sc), lethal of scute (l'sc)* and *asense (ase)*, all of which are required for development of the central and peripheral nervous systems (Ghysen and Dambly-Chaudiere, 1988). The *ac-sc* family has only three representatives in vertebrates (*ASH* for Ac-Sc-Homologue), two of which, *MASH-1* and *XASH-3/CASH-4*, are involved in development of the nervous system (Bertrand *et al.*, 2002). In *Drosophila* the genes of the *atonal (ato)* family, *ato, cato, amos* and *tap,* specify subtypes of sense organs (Chan and Jan, 1999). Perhaps because the vertebrate genome has retained few *ac-sc* genes, the *ato* family in vertebrates is greatly expanded. Broadly speaking its members can be placed into three groups defined by differences in the basic domain; these are the neurogenins (NGN), the *ato* homologues (ATH), and *neuroD* (Hassan and Bellen, 2000).

Many proneural genes are expressed in broad domains from which neural precursors are selected in a spaced array. This is achieved through the ability of these genes to activate a process that restricts the number of precursors through cell-cell interactions. This is referred to as lateral inhibition and is mediated by the Notch signalling pathway (Chitnis ang Kintner, 1996; Kimble and Simpson, 1997; Lewis, 1998). Proneural proteins regulate expression of the ligand Delta; activation of Notch through ligand binding results in down-regulation of proneural gene activity. From a field of initially equivalent cells, this feedback loop allows a single cell to dominate and inhibit its neighbours. Subsequently auto-regulation allows this cell to accumulate high levels of proneural gene

Abbreviations used in this paper: AS-C, Achaete-Scute; SOP, Sensory organ precursor.

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expression. Since the proneural genes both activate lateral inhibition and are inhibited by it, it is not clear how a single cell comes to be chosen.

Here we briefly review current knowledge of the regulatory sequences governing proneural gene expression. We first discuss what is known about the *cis*-regulatory sequences of proneural genes of *Drosophila* and vertebrates and then examine in greater detail sequences controlling auto-regulation, both direct and indirect, and the importance of auto-regulation for neural precursor selection. A specific enhancer element, the SOP (sensory organ precursor) enhancer, that is involved in the singling out of bristle precursors, has been described in *Drosophila*. We review work relevant to the deployment of this enhancer, present evidence for its conservation in higher Diptera and speculate on its function.

Spatial regulation of proneural genes

The proneural genes display complex spatio-temporal expression during development of the many neural cells of the central (CNS) and peripheral nervous systems (PNS). The regulatory sequences of the AS-C of *Drosophila* have been the most intensively investigated. Coding sequences of the four AS-C genes, *ac, sc, l'sc* and *ase*, are embedded in about 100 kb of DNA. A number of independently-acting regulatory enhancer elements have been described governing expression of *ac* and *sc* in the proneural clusters of the imaginal wing and thorax (Gomez-Skarmeta *et al.*, 1995; Ruiz-Gomez and Modolell, 1987), see Fig. 1. These enhancers are scattered throughout the complex; many are shared by both *ac* and *sc*. A roughly delimited common *cis*-regulatory region has been shown to contain elements required for expression of both *ac* and *sc* in the embryonic CNS (Skeath *et al.*, 1992). These enhancers only account for a fraction of the locations at which these genes are expressed, so other elements probably remain to be discovered. The mouse *achaete-scute homologue (ASH), Mash1*, also appears to contain multiple elements spread over more than 36 kb regulating expression in different tissues (Verma-Kurvari *et al.*, 1996) (Fig. 1). Regions both upstream and downstream have been delineated for expression in the CNS, PNS, olfactory epithelium and retina (Verma-Kurvari, 1998).

Expression of *ato* in *Drosophila* is also regulated by a modular arrangement of enhancers, but in contrast to *ac-sc*, the regulatory region appears to be much smaller, about 15 kb (Sun *et al.*, 1998). Two regions located 9.3 kb upstream, and 5.8 kb downstream of the coding sequence, account for all known domains of expression (Fig. 1). Their spatial activity appears to be redundant; expression of the 3' element occurs earlier. The upstream region was further shown to contain independent modules for expression in different tissues. In a similar fashion, *Math1* (a mouse *atonal*/homologue) appears to be entirely regulated by two redundantly-acting enhancers, both located downstream of the coding sequence (Helms *et al.*, 2000) (Fig. 1). Two blocks of sequences, A and B, composed of 561 bp and 544 bp respectively, are remarkably strongly conserved between the chick, mouse and human (Ebert *et al.*, 2003). This sequence homology has been shown to extend to functional homology (Ebert *et al.*, 2003).

From the studies performed so far, it thus appears that *cis*-regulatory elements regulating the *ac-sc* genes of both *Drosophila* and vertebrates, are spread over a larger area than those regulating the *ato* genes. These two gene families diverged phylogenetically before the expansion of the *twist* superfamily and the *ato* superfamily (Bertrand *et al.*, 2002; Vervoort, 2001). The neurogenin genes *ngn1* and *Ngn2,* have also been found to harbour multiple regulatory elements governing expression in different tissues. The sequences of some of these are highly conserved between human, mouse and zebrafish (Blader *et al.*, 2003; Scardigli *et al.*, 2001).

The activity of some enhancers requires the presence of proneural protein

A recurring theme that has emerged from recent studies is that the activity of some of the *cis*-acting enhancer modules is dependent upon previously synthesized protein from the *ac-sc* or *ato* genes themselves. This was first demonstrated for the *ac-sc* genes of *Drosophila*. The *ac, sc* and *ase* genes have E box sequences at locations close to the transcriptional start sites, that have been shown to play a role in direct auto-regulation, notably in selected precursors following lateral inhibition (Cabrera and Alonso, 1991; Culi and Modolell, 1998; Martinez *et al.*, 1993; Van Doren *et al.*, 1992). Enhancer sequences are often very far from the transcription start site, e.g. the enhancer regulating expression leading to the two dorsocentral bristles on the thorax, is 6kb

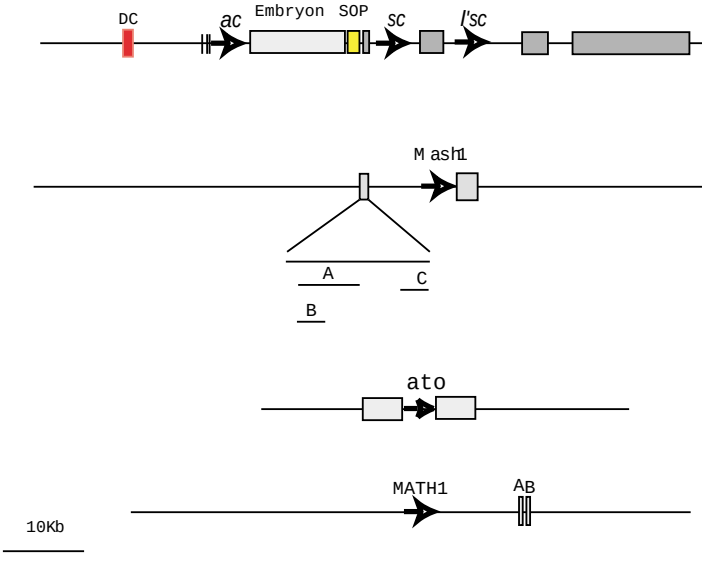

Fig. 1. Organisation of the regulatory regions of the *achaete-scute* and *atonal* genes of *Drosophila* and the *Mash1* and *Math1* genes of the mouse. *The structure of the AS-C of* Drosophila *is shown together with a few of the enhancer sequences that have been defined (Gomez-Skarmeta et al., 1995). The SOP enhancers of* scute *and* asense *are indicated in yellow and that of the enhancer for the dorso-central bristles in red. Regulatory sequences defined for* Mash1 *and* Math1 *of mouse (Helms* et al.*, 2000; Verma-Kurvari* et al.*, 1998), and* atonal *of* Drosophila *(Sun* et al.*, 1998), are indicated in grey.*

upstream of *ac* and 31kb upstream of *sc* (Garcia-Garcia *et al.*, 1999; Gomez-Skarmeta *et al.*, 1995). The trans-activator Pannier binds directly to the DNA sequences of the dorsocentral enhancer, but, through its intermediary Chip, also associates with Ac-Sc which in turn is bound to E boxes in the *ac* promoter to initiate transcription (Garcia-Garcia *et al.*, 1999; Haenlin *et al.*, 1997; Ramain *et al.*, 2000). Thus the activity of this enhancer requires the use of previously synthesized *ac-sc* protein, in order for the enhancer sequences to be brought into proximity of the promoter. In the embryo, a requirement for Ac-Sc for the function of their common regulatory region has not been tested. However it was noted that expression of *l'sc* enhances the level of *ac* expression without altering its spatial regulation (Skeath, 1992).

Similarly one of the two enhancers of *ato* and of *Math1* respectively, require *ato* or *Math1* protein. The 3' and 5' enhancers of the *Drosophila* gene *ato* drive expression at the same locations, but only the 5' enhancer requires the use of previously synthesized *ato* protein (Sun *et al.*, 1998). A detailed analysis in the eye, where morphological markers allow careful timing, showed that the 3' enhancer comes on first, the 5' enhancer only later. E boxes are present in the 5' enhancer but it was not determined whether they are functionally relevant. It is possible that position specificity of expression is governed by the 3' enhancer, and that the 5' enhancer allows an increase in levels of activity by means of auto-regulation. However the 5' enhancer was shown to be modular, with specific sequences governing expression in different cells, suggesting that its activity is not due to auto-regulation alone. The situation for the vertebrate *Math1* gene is remarkably similar. Each of the *Math1* enhancers, A and B, is sufficient alone to drive expression in all known expression domains, except the spinal cord which is exclusively dependent on enhancer B (Helms *et al.*, 2000). Expression of enhancer B, but not that of enhancer A, particularly in the spinal tube, requires *Math1* protein. Indeed enhancer B contains an E box that is bound by Math1: activity of this enhancer is lost in the absence of *Math1* protein or if the E box is deleted (Helms *et al.*, 2000). Enhancer A also contains an E box, but deletion of this sequence is without effect. Enhancer B is not merely used to increase protein levels through auto-regulation. It contains a much more extensive region of conserved sequence than the small region around the E box and it is expressed in the spinal tube in the absence of prior activity of enhancer A. This poses the question of the origin of the *Math1* protein in the spinal cord that allows expression of enhancer B alone.

Thus two orthologous *ato* genes from phylogenetically distant species display a remarkable similarity in the organization and functioning of their regulatory regions. They differ from the *ac-sc* genes in that the relevant E boxes are present in the enhancer region itself, rather than close to the transcription start site.

Cross-regulation of proneural genes

Another common feature of proneural genes in different species is their ability to regulate one another. In vertebrates there appear to be cascades of proneural gene activity, not unlike those thought to regulate muscle development. Studies in *Xenopus* and mouse have shown that *Mash1* and the neurogenins are expressed earlier than the *ato* homologues,

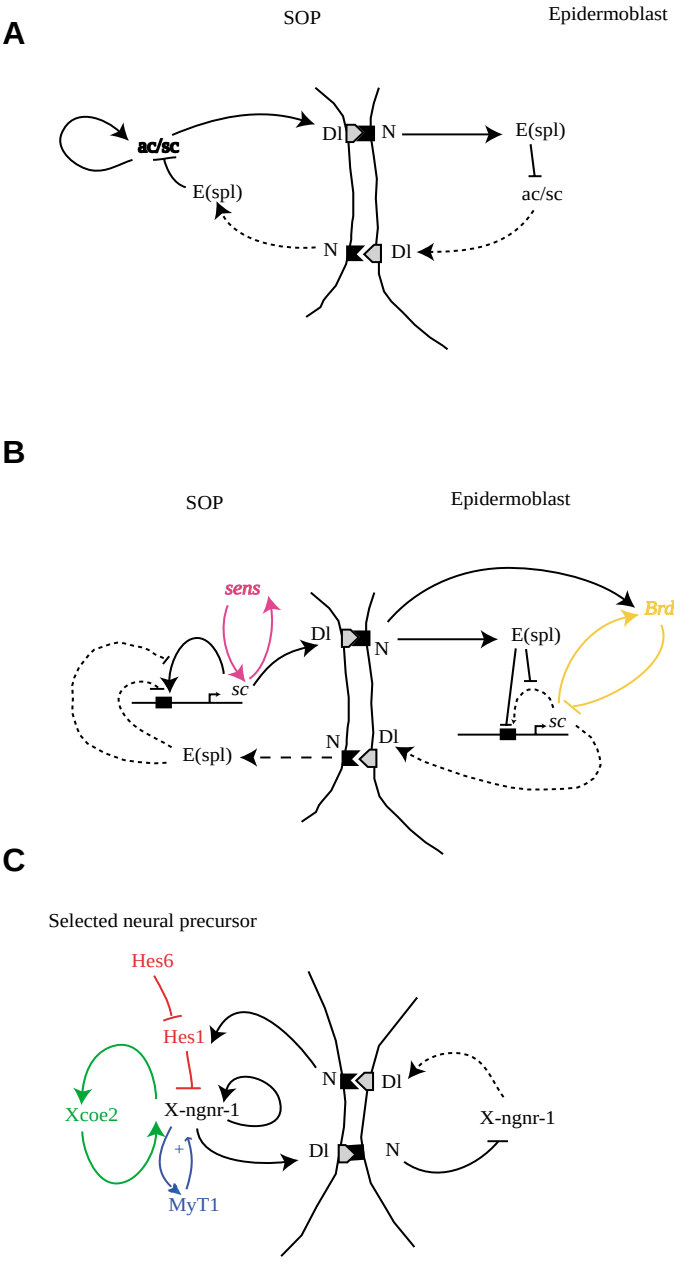

Fig. 2. Regulatory loops involved in the selection of neural precursors in *Drosophila* and vertebrates. (A) *The transcriptional loop involved in neural precursor selection. In some instances, such as the small bristles of* Drosophila, *this loop, together with stochastic fluctuations in protein turnover, may suffice to select single cells from a group of initially equivalent ones.* **(B)** *Additional loops and factors identified in* Drosophila, *that help to increase levels of proneural protein in the selected cell. The* senseless *gene (pink) is involved in indirect auto-regulation of* scute (*Nolo* et al., *2000), and* Bearded *genes (beige) may help to repress* scute *in non-selected cells (Lai* et al., *1997; Lai* et al., *2000).* **(C)** *Additional loops and factors identified in vertebrates that also help a selected cell to escape inhibition.* X-Coe2 *(green) helps maintain X-*ngnr-1 *expression in the selected precursors (Dubois* et al., *1997);* Hes6 *prevents activity of* Hes1 *(red) thus increasing levels of proneural activity (Bae* et al., *2000; Koyano-Nakagawa* et al., *2000) and MyT1 (blue) associates with X-ngnr-1 making the cell insensitive to inhibition (Bellefroid* et al., *1996*).

which in their turn are expressed earlier than *NeuroD* (for review see (Bertrand *et al.*, 2002)). Similarly in *Drosophila*, *ase* is expressed in all neural precursors of the CNS and PNS, and *cato* is expressed in all sensory precursors, after the expression of *ac,sc*, *l'sc* or *ato* in proneural domains (Brand *et al.*, 1993; Dominguez and Campuzano, 1993; Goulding *et al.*, 2000; Jarman *et al.*, 1993). Hence, *neuroD, ase* and *cato* are thought to be neuronal precursor genes rather than proneural genes, since their activity is confined to neural precursors after they have been specified, whereas proneural genes are expressed in groups of cells before precursor selection, see below. In some of these cases it has been clearly demonstrated that the products of these genes directly regulate one another. Cross-activation has been observed for *Xath3* and *NeuroD*, and also for *ac* and *sc* where it has been shown to be direct (Martinez and Modolell, 1991; Perron *et al.*, 1999; Van Doren *et al.*, 1992). In the vertebrate neural tube, different populations of neurons along the dorso-ventral axis are generated by discrete non-overlapping domains of expression of *Ngn1, Math1* and *Mash1* (Gowan *et al.*, 2001). The borders between these domains are achieved by cross-inhibition: *Ngn1* and *Math1* mutually repress one anothers' activity and *Ngn1* represses *Mash1* (Gowan *et al.*, 2001). They do not inhibit themselves possibly due to the fact that each one encodes a factor from a different class of bHLH protein.

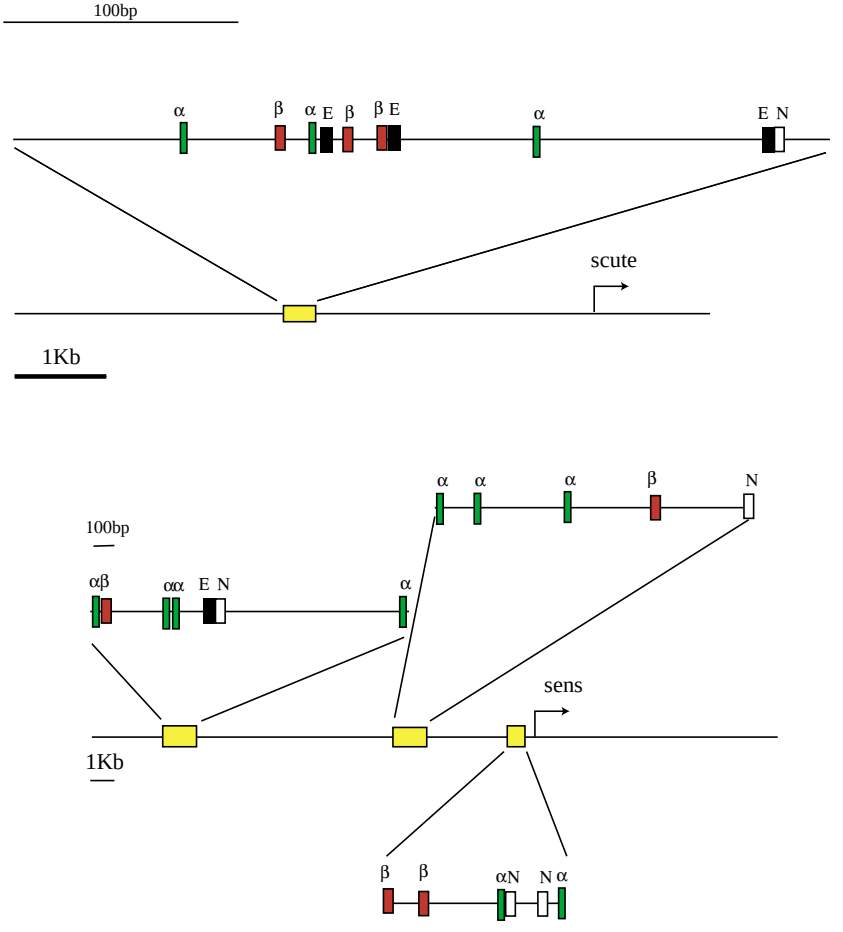

Fig. 3. Structure of the SOP enhancers of *scute* and *senseless* in *Drosophila*. *The position of the SOP enhancer of scute and the three apparent SOP enhancers of* senseless *are shown to scale. The number of binding motifs and their relative spacing is also indicated. A few isolated potential binding sites without significant clustering are found outside the yellow boxes. Accession number of the* senseless *gene region: AE003538.3.*

Selection of neural precursors

Lateral inhibition and the singling out of neural precursors

Proneural genes are expressed in broad territories in the neuroepithelium, from which neural precursors are selected in a spaced array. A hallmark feature of these genes is the ability to restrict their own activity to single neural precursor cells. In the *Drosophila* CNS the process is repeated several times, at the end of which remaining cells not recruited to become neural precursors adopt an epidermal fate. In vertebrates a similar process takes place but by the end all cells are recruited as neurons, for review see (Bertrand *et al.*, 2002). This process allows the maintenance of a pool of stem cells for a period of time sufficient to enable successive waves of neural progenitors to adopt different fates. Selection of neural precursors relies on interactions between cells mediated by the Notch signalling pathway. Notch is ubiquitously expressed and the *ac-sc* genes in *Drosophila*, and *Mash1* and NGNs in vertebrates, have been shown to activate transcription of the Notch ligands Delta and Serrate/Jagged, in a direct, dose-dependent manner (Bertrand *et al.*, 2002; Hinz *et al.*, 1994; Kunisch *et al.*, 1994). Thus all cells initially express both ligand and receptor, and, because activation of Notch leads to repression of the proneural genes, mutually inhibit one another. Ultimately, single, spaced cells escape inhibition, produce high levels of proneural protein and ligand, and repress their neighbours (Heitzler *et al.*, 1996; Heitzler and Simpson, 1991) (Fig. 2). These become neural precursors and, once selected, they maintain high levels of proneural protein through direct (and indirect) auto-regulation. Repression through the Notch signalling pathway occurs via the bHLH proteins of the *E(spl)* family in *Drosophila*, which may either directly bind target sequences, the N boxes, in the promoters of these genes, or associate with the *ac-sc* proteins turning them into repressors, see below. A number of *E(spl)* homologues (the *Hes* genes) have been found in vertebrates to act downstream of activated Notch. Putative binding sites for these proteins are conserved in the proneural genes, and Hes1 has been shown to form heterodimers with Mash1 but also to directly bind the *Mash1* promoter (see below).

If the proneural genes activate lateral inhibition and at the same time are inhibited by it, then one cell has to accumulate sufficient protein to escape inhibition. Exactly how this takes place remains unclear. In *Drosophila* the small thoracic bristles provide a good model system in which to investigate this process, since they arise from a single wave of *ac-sc* expression and remain at their site of origin. In this system it was found that by varying the relative levels of Notch, Delta or the Ac-Sc it was possible to bias the choice of neural fate to those cells producing more Ac-Sc/Delta, or less Notch (Heitzler and Simpson, 1991). This suggests that the transcriptional feedback loop

itself plays a role in the selection and that choice of the bristle precursors may be a stochastic process relying on random fluctuations in protein turnover (Heitzler *et al.*, 1996). This may represent the basal and simplest mechanism of singling out precursors (Simpson, 1997) (Fig. 2A). Selection of neuroblasts in the embryonic CNS of *Drosophila* is, however, largely independent of transcriptional regulation of *Delta* (Seugnet *et al.*, 1997). Furthermore post-transcriptional mechanisms may also operate for bristle precursor selection: ubiquitous expression of *sc* with a heterologous promoter, in the absence of the endogenous *ac-sc* genes, allows the formation of a spaced array of bristles (Rodriguez *et al.*, 1990).

Positive and negative feedback loops

A number of genes have been described whose activity reinforces lateral signalling by indirectly upregulating proneural gene expression in the neural precursors (Table 1, Fig. 2). These may participate in indirect autoregulatory loops that increase levels of proneural protein, potentiate the transcriptional activity of proneural proteins, down-regulate expression of *E(spl)/Hes* genes, or down-regulate expression of proneural genes in cells not selected as neural precursors. These factors would participate in the accumulation of sufficient proneural protein to allow the presumptive neural precursors to escape inhibition, but there is no evidence as yet, that any of these actually instigate the choice of fate.

Several other factors that have been shown to down-regulate proneural genes by blocking auto-regulation, such as *extramacrochaetae* and *Zic1*, do not appear to be involved in the selection of neural precursors (Cabrera *et al.*, 1994; Ebert *et al.*, 2003; Martinez *et al.*, 1993; Van Doren *et al.*, 1992).

Evolution of a specific enhancer element in *Drosophila* for the selection of bristle precursors

The SOP enhancer

A regulatory sequence, which we will call the SOP (Sensory Organ Precursor) enhancer, has been defined in the *sc* gene that is thought to mediate the process of neural precursor selection during development of the sensory bristles (Culi and Modolell, 1998). It drives auto-regulation of *sc* in the SOP itself and is probably also the target for repression in the inhibited cells. Sequences necessary for the activity of this enhancer were delimited to a 365-bp stretch nearly 3-kb upstream of the coding sequence of *sc* (Fig. 3). Comparison with *D. virilis* revealed a number of evolutionarily conserved sequences found in a 362-bp segment located 4.3-kb upstream of the structural *sc* gene of *D. virilis*. Three E boxes, binding sites for Ac-Sc/Da, are present, the most proximal of which is adjacent to an N box, a site that can be recognized by the *E(spl)* bHLH proteins (but see below). Three α boxes, motifs resembling the consensus binding sequence for transcription factors of the NFkB family and a T-rich motif of unknown function, β boxes, were also found.

During imaginal development expression of *ac* and *sc* is seen in proneural clusters preceding bristle development, and is then gradually restricted to the SOPs (Cubas *et al.*, 1991; Romani *et al.*, 1989; Skeath and Carroll, 1991). It is followed by expression of *ase* which is only seen in SOPs after their selection (Brand *et al.*, 1993; Dominguez and Campuzano, 1993; Jarman *et al.*, 1993). An SOP enhancer is present in the promoter of *sc* and *ase*, but not *ac*. Outside the AS-C, a number of other *Drosophila* genes known to

TABLE 1

GENES PARTICIPATING IN REGULATORY LOOPS WHICH INCREASE PRONEURAL FUNCTION IN NEURAL PRECURSORS

Collier	The *Xenopus* gene *Xcoe2* encodes a bHLH protein with a novel binding domain (Dubois *et al.*, 1997). It is expressed transiently after *X-ngnr-1*, but before *neuroD* and is absolutely required for neural precursor selection. Ectopic expression, even in naïve ectoderm leads to activation of *X-ngnr-1, Delta* and *neuroD*. This results in a spaced array of neural precursors. Thus, like proneural genes, *coe* is both sensitive to, and can activate lateral inhibition. It probably acts downstream of *X-ngnr-1* to maintain Notch/Delta signalling and high levels of *X-ngnr-1* in selected neural precursors.
senseless	In *Drosophila, senseless (sens)* is another gene that is absolutely required for neural precursor selection (Nolo *et al.*, 2000). It is expressed at low levels in proneural domains and at high levels in neural precursors. It is activated by *ac-sc* and can itself activate *ac-sc* and *ase*, thus initiating in an indirect autoregulatory loop like that resulting from *X-Coe-2* activity. Unlike *X-Coe-2,* it probably does not regulate *Delta*, but rather downregulates *E(spl)* in the neural precursor. It has also been shown that the product of one or more of the *E(spl)* genes can mediate repression of *sens* and that this repression is prevented by a Notch-independent function of Su(H) (Koelzer and Klein, 2003). *senseless* does not encode a bHLH protein, but a zinc finger protein, and by itself is unable to induce neurogenesis.
X-MyT1	This gene also encodes a zinc finger protein that is expressed downstream of *X-ngnr-1* (Bellefroid *et al.*, 1996). It is only able to promote neurogenesis in the presence of proneural bHLH proteins. Cells expressing both *X-ngnr-1* and *X-MyT1* are insensitive to lateral inhibition, so this factor may allow presumptive neural precursors to escape inhibition. *X-MyT1* does not activate *Delta* or *X-ngnr-1*.
Hes6	The product of this vertebrate gene is related to the *E(spl)* family of proteins, but differs in having a very short loop, as a result of which it binds neither E nor N boxes (Bae *et al.*, 2000; Koyano-Nakagawa *et al.*, 2000). It is activated by proneural genes and expressed before *neuroD* in neural precursors. It is a dominant negative regulator of Notch signalling that acts by associating with *Hes1* protein and abolishing its ability to repress transcription. It therefore indirectly promotes accumulation of proneural protein in the neural progenitors.
The Epidermal growth factor/ Ras-Raf pathway	This signalling pathway has been shown in *Drosophila* to promote positive interactions between cells in proneural domains that are essential for neural precursor selection (Culi *et al.*, 2001). Its activity is induced by regulation of expression of *rho/ve,* which activates EGFR signalling by releasing the ligand Spitz, by Ac and Sc. Activation of EGFR promotes direct auto-regulation of *ac* and *sc*.
Tramtrack and *phyllopod*	*phyllopod* encodes a novel nuclear protein that is required for the neural fate (Chang *et al.*, 1995; Dickson *et al.*, 1995). It is expressed in sensory organ precursors of *Drosophila* and is down-regulated in non-selected epidermal cells (Pi *et al.*, 2001). In contrast, *tramtrack*, which encodes aBTB/POZ-domain transcription factor, is expressed in non-neural cells where it is up-regulated by Notch signalling and represses the neural fate (Badenhorst *et al.*, 2002; Ramaekers *et al.*, 1997). *tramtrack* acts downstream of *phyllopod* which induces degradation of its protein product (Pi *et al.*, 2001).
Bearded	Ten genes of this family have been described in *Drosophila* (Lai *et al.*, 2000). They have been shown to repress gene activity by an unusual mechanism acting post-transcriptionally. This involves specific sequences in the 3' UTR region of their transcripts that can form RNA:RNA heteroduplexes with transcripts of other genes, including the proneural proteins, which contain complementary sequences (Lai and Posakony, 1997). This prevents accumulation of the heterologous transcripts by conferring transcript instability. They probably therefore down-regulate proneural gene activity. Indeed a dominant mutant form that is missing the relevant 3'UTR sequences, causes a phenotype similar to a loss of function of *Notch*. Thus absence of this regulatory activity confers insensitivity to lateral inhibition. The Brd genes are activated by Ac-Sc in proneural domains but they do not accumulate in neural precursors. They are also activated by Notch signalling which would occur in the inhibited cells. They thus probably help neural precursor selection by reducing Ac-Sc levels in the non-selected cells.

be involved in neurogenesis, carry stretches of sequence with a similar combination of binding sites: *pox*^{neuro}*, neuralized (neu)* and *Gliotactin* have 8, 13 and 17 E boxes respectively as well as a single α box and a single N box each. Remarkably, *senseless (sens),* which is indispensable for SOP selection, has three apparent SOP enhancers (Fig. 3).

Function of the SOP enhancer of scute *in* Drosophila

When coupled to a reporter gene, the 365-bp SOP sequence drives low level expression in small groups of cells preceding SOP selection, and then high levels in the SOPs (Culi and Modolell, 1998). It thus mimics *sc* expression which is gradually refined to single SOPs. This enhancer is thought to mediate singling out of the SOP. In the presumptive SOP itself, auto-regulation, by binding of Sc to the E boxes, contributes to maintaining the high levels of Sc required for precursor formation. Mutation of E1 and E2 abolishes expression in the SOP. Scute ensures high levels of expression of *Dl* in the SOP, thus activating Notch in neighbouring cells. Activation of Notch results in activation of the *E(spl)* genes, that in turn repress *sc* (Bailey and Posakony, 1995; Jennings *et al.*, 1995; Lecourtois and Schweisguth, 1995). In *Notch* mutants the number of cells with high activity of the SOP enhancer increases. Furthermore the enhancer is very sensitive to levels of E(spl). These observations suggest that the SOP enhancer sequence may also be the target for Notch signalling and repression of *sc*.

The mechanism of repression by E(spl) has been the subject of some debate. Members of this class of bHLH proteins contain a conserved proline in the basic domain and a WRPW tetrapeptide in the C terminus through which they recruit the co-repressor Groucho (Fisher and Caudy, 1998). The N box of the SOP enhancer is a binding site for the *E(spl)* proteins. An identical N box is found in the promoter of *ac*. Although *ac* does not have all the sequences characteristic of the SOP enhancer, it does have E boxes which function during auto-regulation, as well as the N box. The N box of *ac* has been shown to mediate transcriptional repression by the product of the gene *hairy*, which encodes a bHLH protein of the E(spl) family (Ohsako *et al.,* 1994; Van Doren *et al.,* 1994). However, *hairy* is not required for lateral inhibition, rather it is a regulator of the spatial expression of *ac*. Interestingly, loss of the N box in the SOP enhancer does not prevent the restriction of SOP enhancer expression to the SOPs (Culi and Modolell, 1998). Furthermore, although E(spl)m8 was shown to bind the enhancer, it is still able to mediate repression without binding. Recent studies have demonstrated that, in addition to direct binding, the *E(spl)* proteins can also bind indirectly by association with the *ac-sc* proteins that are in turn bound to DNA (Giagtzoglou *et al.*, 2003). In this manner they interfere with auto-regulation by preventing the transcriptional activity of Ac-Sc on the *sc* (and *ac*) promoter, but they would also prevent activation of other Ac-Sc target genes.

The vertebrate *Hes* genes show features of both the *hairy* and *E(spl)* genes of *Drosophila*. They are thought to be transcriptional repressors (Sasai *et al.*, 1992). In vertebrates, Hes1 has been shown, *in vitro*, to bind a single N box in the *hASH1* promoter to repress transcription (Chen *et al.*, 1997). This N box is identical (CACGCA) to the one in the SOP enhancer and to the N box of *Drosophila ac*. However, the *Hes* proteins are also thought compete for E2A and thus inhibit DNA-binding of proneural proteins (Sasai *et al.*, 1992).

Mutation of the α and β boxes greatly reduce functioning of the SOP enhancer, suggesting that these motifs mediate protein binding and are required for activity. Factors binding to these have not yet been identified.

It is striking that *sens*, which is involved in the singling out of SOPs, contains three apparent SOP enhancers (Fig. 3). *senseless* is first expressed in small groups of cells prior to SOP selection, although expression is weak and the protein is not confined to the nucleus (Nolo *et al.*, 2000). It then accumulates to high levels in the SOP. This is consistent with its probable activation by Ac-Sc, indeed *sens* is not expressed in the absence of Ac-Sc. In turn, *sens* is required to maintain *sc* expression: clones of null alleles of *sens* lose *sc* expression by the time of pupariation even though Sc is present earlier on. Furthermore ectopic expression of *sens* (in the neuro-ectoderm) induces ectopic expression of *sc* and *ase*. However, in flies mutant for *ac* and *sc*, Sens, when expressed by means of a heterologous promoter, appears unable to activate *ase* (no bristles form). This suggests that *sens* may only be able to regulate *ase* in the presence of proneural gene activity. Other proneural genes, such as *amos* (an *ato* homologue) for example, can induce bristle formation when ectopically expressed, by activating both *sens* and *ase* without activating *ac* and *sc* (Lai, 2003; Villa-Cuesta *et al.*, 2003). Binding sites for Sens are present upstream of *sc* and *ac*, but are found at some considerable distance. Interestingly two well-conserved binding sites for Sens are found very close to the promoters of *E(spl)m4* and *E(spl)m5*, suggesting that Sens may act to repress Notch signalling in the SOP, which would indirectly promote expression of *sc* and *ase* in this cell.

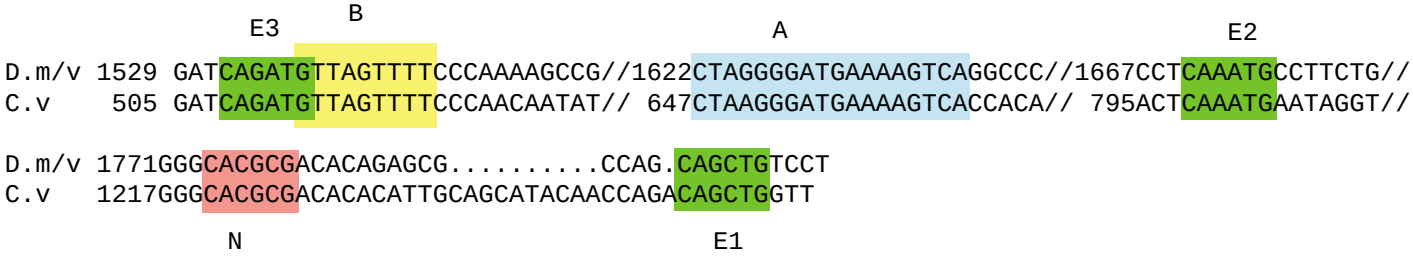

Fig. 4. Sequence of the SOP enhancer of the *asense* genes of *Drosophila melanogaster* and *Calliphora vicina*. *Conserved sequences have been aligned. Colours correspond to the binding motifs described in the text, namely the E boxes, N box and α and β boxes. Between the conserved motifs, (double slashes) there is no apparent conservation of sequence nor of length. Sequence submission number: AY362730. The conservation of the SOP enhancer of the asense gene in Drosophila concerns not only the binding sites but also the specific position of this enhancer in the 5' UTR of asense. Abbreviations: C.v., Calliphora vicina; D.m., Drosophila melanogaster; D.v., D. virilis.*

Conservation of the SOP enhancer in Diptera

The asense *gene of* Calliphora *contains a SOP enhancer*

The four genes in the AS-C of *Drosophila* are thought to have arisen as the result of three independent events of gene duplication, of which the last two probably occurred within the Diptera (Skaer *et al.*, 2002). *Anopheles gambiae*, a basal Dipteran species, phylogenetically separated from *D. melanogaster* by ~ 250 Myr, has only two AS-C genes, *AgASH* and *Ag-ase*. *AgASH* is thought to be representative of an ancestral gene that preceded the three genes *ac, sc* and *l(sc)*. Scrutiny of the AS-C in the recently published genome sequence of this species fails to uncover a combination of binding motifs comparable to the SOP enhancer of *Drosophila*. Three AS-C genes, *sc, l'sc* and *ase*, have so far been found in *Calliphora vicina*, a calyptrate cyclorraphous fly that is phylogenetically separated from *Drosophila* by ~ 100 Myr (Pistillo *et al.*, 2002). We have cloned and sequenced part of the AS-C from *Calliphora*. A sequence very similar to that of the SOP enhancer of *D. melanogaster*, much of which can be aligned, bearing 3 E boxes, an N box, an α box and a β box, was found upstream of the *ase* gene of this species (Fig. 4). Although 16kb upstream of the *sc* gene of *Calliphora* was sequenced, no sequence combination characteristic of the SOP enhancer was uncovered. The presence of the SOP enhancer in the AS-C of *Calliphora* and *Drosophila*, but not in *Anopheles*, suggests that this sequence may have evolved somewhere in the lineage leading to the cyclorraphous flies.

That an SOP enhancer should be present in the *ase*, but not the *sc* gene of *Calliphora* poses a paradox. How are the macrochaete SOPs singled out in this species? In accordance with the absence of the enhancer, *sc* is expressed in proneural domains but does not subsequently refine to single cells as it does in *Drosophila* (Pistillo *et al.*, 2002). On the other hand, right from the beginning of its expression, *ase* is expressed exclusively in single cells. So something other than these two genes must mediate lateral inhibition and SOP selection. *Calliphora Delta* is expressed in proneural domains and then refines to precursors, much as it does in *Drosophila*, so a gene other than *sc* appears to be regulating *Delta*. One possibility is that this could be an as yet undiscovered *ac* homologue. Although *ac* has not been found in the several cyclorraphous species examined, a phylogenetic tree built from a comparison of existing sequences suggests that such a gene may be present (Skaer *et al.*, 2002). Another possibility is that *sens* could mediate precursor selection by itself through the use of its SOP enhancers if these are conserved. We have not tried to recover a *sens* gene from *Calliphora*. However, it seems unlikely that *sens* alone can mediate singling out of SOPs in *Drosophila*. Senseless is not a proneural protein: it is unable to promote bristle development in the absence of bHLH proneural protein, and there is no evidence that it can regulate *Delta*. It can auto-regulate, but this has not been shown to be direct.

Spacing of the bristles in *Anopheles* does not appear to require the particular combination of binding motifs present in the SOP enhancer. Other basal flies and indeed most other insects have evenly spaced sensory organs, so if the enhancer has evolved only recently in higher flies it may, in addition, fulfill some other function. It appears to be important for development of the macrochaetes, which are the large sensory bristles present on some parts of the fly body. In this respect it is of interest to consider the derived mode of development of these organs. Macrochaetes are characteristic of higher flies which have two phases of *sc* expression: an early phase from which the macrochaete precursors arise and a later phase from which the microchaete (small bristle) precursors segregate (Cubas *et al.*, 1991; Huang *et al.*, 1991; Pistillo *et al.*, 2002; Simpson *et al.*, 1999; Skaer *et al.*, 2002; Skeath and Carroll, 1991). Indeed we have postulated that macrochaetes may have arisen as a result of the expression of *sc* at earlier stages of development. The basal species *Anopheles gambiae*, has only a single phase of expression from which all sensory organ precursors form (Wülbeck and Simpson, 2002). There is an important consequence of the early formation of macrochaete precursors, and that is the need to maintain high levels of proneural protein for a considerable time after the proneural domains have faded and before the initiation of metamorphosis and bristle differentiation. This requires maintaining high levels of expression in single isolated cells. Direct auto-regulation has been traditionally invoked for this maintenance, and also cross-activation between *ac* and *sc* in *Drosophila* appears to function almost exclusively in the SOP (Gomez-Skarmeta *et al.*, 1995; Martinez and Modolell, 1991). However it may be too dangerous for the cell to rely exclusively on this mechanism, since a loss of proneural protein through stochastic processes would result in a loss of the precursor. Thus, integration of other signals and factors, which may bind the α and β boxes, may have evolved to ensure maintenance. Such factors could act independently of the products of the proneural genes. In this context, it is of interest that the SOP enhancer is present in a number of *Drosophila* genes, such as *neu*, *sens* and *ase*, that are expressed mainly or exclusively in the SOP, after it has been singled out. In these instances the enhancer is not mediating lateral inhibition but is presumably contributing to maintenance of the neural fate of the precursor. In *Anopheles*, where there is a single phase of proneural gene expression, precursors of the bristles and scales form simultaneously only a short time prior to differentiation (Wülbeck and Simpson, 2002). Thus there is no requirement for a protracted period of precursor maintenance in this species.

A second specialised feature of macrochaetes is that the arrangement of these bristles in many cyclorraphous flies is invariant and very reproducible from one individual to another. In contrast in the lower flies, bristle patterns are not stereotyped: the number and position of bristles varies between individuals (Simpson *et al.,* 1999). The stereotyped pattern of macrochaetes appears to be due to the fact that selection of SOPs from the proneural clusters is not random but biased to specific cells (Cubas *et al.*, 1991; Simpson, 1997; Skeath and Carroll, 1991). It is thus interesting to speculate that the activity of the SOP enhancer could also be related to the more precise positioning of macrochaete SOPs.

Conclusions

The data summarised above underlines a notable peculiarity of the bHLH proneural genes, namely the fact that much of their transcriptional activity is regulated by mechanisms that require the presence of their own protein products. Many steps during the process of neural precursor selection are controlled via the proneural proteins. The proneural proteins (1) activate *Delta* thus mediating their own repression non-autonomously, (2) activate *Brd* genes whose products may down-regulate proneural genes in inhibited cells, (3) are required for direct auto-regulation which is necessary for neural precursor selection and maintenance of the neural fate

in the precursors, (4) are repressed by *E(spl)/Hes* genes in part, by a mechanism that relies on their association with the products of these genes, (5) are required for indirect autoregulatory loops with genes such as *Coe* and *sens*, which act to increase their levels in neural precursors and (6) are required for cooperative effects with proteins such as X-MyT1, which may allow also cells to escape lateral inhibition. In addition repression by post-transcriptional regulators, not involved in precursor selection, such as Extramacrochaetae/Id involves association with proneural proteins. The proneural genes also cross-regulate one another to allow cascades of proneural gene activity during neurogenesis, or to specify distinct domains of spatial expression.

Another notable feature is that some of the *cis*-regulatory modules governing spatial expression of the proneural genes, also require the presence of previously synthesized protein. In some of these cases, such as the dorso-central enhancer of the *Drosophila* notum and enhancer B of *Math1* in the spinal cord, it is not clear where the protein comes from, since it is not detectable by standard procedures beforehand. It is therefore likely to be present at low concentrations. One possibility is that proneural genes are activated in the neuro-ectoderm in early embryonic development and that they continue to be expressed at low levels thereafter in all cells derived from this tissue; low levels could easily be maintained by preventing auto-regulation for example. They would therefore be available at any point in later development without the need for activation *de novo*. These observations may provide support for the default model of vertebrate neural induction. This model postulates that neural development in the ectoderm is inhibited by BMP4/activin, and that induction is due to interference with BMP signalling which converts ectodermal cells from an epidermal to a neural fate (Chang and Hemmati-Brivanlou, 1998; Hemmati-Brivanlou and Melton, 1994). Perhaps the neural default state of the ectoderm represents the ancestral state present in a common ancestor of *Drosophila* and the vertebrates. If so, the direct regulation of transcription by some enhancers not requiring proneural protein, may be a novelty that has been introduced independently in some tissues of different animal lineages.

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