**asense is a Drosophila neural precursor gene and is capable of initiating sense organ formation**

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**SUMMARY**

Neural precursor cells in *Drosophila* arise from the ectoderm in the embryo and from imaginal disc epithelia in the larva. In both cases, this process requires *daughtercless* and the proneural genes *achaete, scute and lethal-of-scute* of the *achaete-scute* complex. These genes encode basic helix-loop-helix proteins, which are nuclear transcription factors, as does the *asense* gene of the *achaete-scute* complex. Our studies suggest that *asense* is a neural precursor gene, rather than a proneural gene. Unlike the proneural *achaete-scute* gene products, the *asense* RNA and protein are found in the neural precursor during its formation, but not in the proneural cluster of cells that gives rise to the neural precursor cell. Also, *asense* expression persists longer during neural precursor development than the proneural gene products; it is still expressed after the first division of the neural precursor. Moreover, *asense* is likely to be downstream of the proneural genes, because (1) *asense* expression is affected in proneural and neurogenic mutant backgrounds, (2) ectopic expression of *asense* protein with an intact DNA-binding domain bypasses the requirement for *achaete* and *scute* in the formation of imaginal sense organs. We further note that *asense* ectopic expression is capable of initiating the sense organ fate in cells that do not normally require the action of *asense*. Our studies therefore serve as a cautionary note for the inference of normal gene function based on the gain-of-function phenotype after ectopic expression.

Key words: *achaete-scute* complex, helix-loop-helix, proneural genes, neural precursor genes, neurogenesis, *Drosophila*

**INTRODUCTION**

Neural precursor cells in *Drosophila* arise from undifferentiated ectodermal cells in the embryo or imaginal discs (reviewed by Campos-Ortega, 1988; Ghysen and Dambly-Chaudière, 1989; Campos-Ortega and Jan, 1991; Artavanis-Tsakonas, 1988; Simpson, 1990; Cabrera, 1992; Campuzano and Modolell, 1992). This process requires the action of the proneural genes such as those of the *achaete-scute* complex (AS-C), which encode members of the basic helix-loop-helix (bHLH) group of transcriptional factors. The proneural genes of the AS-C are expressed in small groups of cells in the epithelium, called proneural clusters, prior to the generation of a neural precursor; this expression is thought to make the cells competent to follow a neural fate. Through cell-cell interactions mediated by the neurogenic genes, expression of the proneural genes is then restricted to a single cell (the neural precursor) that will delaminate from the epithelium and contribute to the formation of the nervous system. Previous studies of genetic interactions, as well as interactions of the protein products in vitro or in yeast, suggest that AS-C products form heterodimers with the product of *daughtercless* (*da*) to activate genes that lead these precursor cells to execute a neural fate (Dambly-Chaudière et al., 1988; Murre et al., 1989; Cabrera and Alonso, 1991).

Another group of genes, the neural precursor genes, are activated in most or all newly born neural precursors (neuroblasts in the central nervous system (CNS), and sense organ precursors, SOPs, in the peripheral nervous system (PNS)), and may control different aspects of neuronal differentiation (Bier et al., 1989, 1992; Vaessen et al., 1991). We wanted to determine whether this group of genes is a part of the downstream genetic program activated by AS-C/da heterodimers. As a first step, we chose to study the expression and activity of one putative member of this group, the *asense* (*ase*) gene. *ase* is located in the AS-C and itself encodes a bHLH protein (Alonso and Cabrera, 1988; González et al., 1989). Previous studies (Alonso and Cabrera, 1988; González et al., 1989) have raised the possibility that *ase* is functionally distinct from the proneural members of the AS-C, *achaete* (*ac*), *scute* (*sc*) and *lethal-of-scute* (*l*′*sc*), because *ase* expression appears to be activated later than that of the proneural genes and it appears to persist for a longer period of time. However, the identity of the *ase*-expressing cells was not established in these studies.

Using antibodies against the *ase* protein, we have carried out a detailed examination of *ase* expression during embryonic and larval development. The *ase* expression closely follows proneural gene expression in space and time and is found in neural precursors shortly after their
formation, suggesting that ase is a member of the neural precursor group. We also show that normal ase expression requires the function of the proneural genes, whereas ectopic expression of ase induces sense organ precursor formation even in the absence of the proneural genes normally required for these precursors.

MATERIALS AND METHODS

Flies and culture conditions

Flies were crossed under standard conditions at 25°C, unless otherwise noted. Different aspects of the mutant stocks employed in this study are described by Lindsley and Grell, 1968; Lindsley and Zimm, 1992; García Bellido, 1979; García-Alonso and García Bellido, 1986; Caudy et al., 1988; Jiménez and Campos-Ortega, 1990. ase 
 was renamed from sc 
; it is a small deletion removing only the ase gene and a putative regulatory element of the sc gene (González et al., 1989; Jarman et al., 1993). As neurogenic mutations, the amorphic alleles Nse 
 and neu 
 were used (Lehmann et al., 1983).

sc 
 was marked with white 
 and maintained with In (1) dl 
. y Hw. Larvae for sc 
 wY / wY (y 
 / w 
) were identified in the third instar based on mouth hook and Malpighian tubule coloration. In (1) lac 
 larvae were obtained from a homozygous stock. Larvae carrying emc 
 Df(3)emc 
, a strong viable combination of emc alleles, were obtained as Tubby 
 larvae from a cross of emc 
 Df(3)emc 
 with Df(3)emc 
 TM6B, Tb.

Heat-shock protocol

Staging of embryos was done on the basis of timed collections and morphological criteria described in Campos-Ortega and Hartenstein (1985a). Larvae were staged for the time course of heat-shock induction of ase relative to the third instar molt, as described by Huang et al. (1991). This method of staging was chosen because it introduces the smallest scatter of age during third instar, puparium formation occurring at 48±3 hours (Huang et al., 1991). Time was converted to hours bpf (before puparium formation) by subtraction from 48. Pupae were collected at the white prepupae stage (= PF). Larvae and pupae were left to develop for the experiments described, we used two homozygous viable transformant lines, hs- 
 and hs- 
.

Histological techniques

Whole-mount hybridization with digoxigenin-labelled probes was carried out essentially as described (Tautz and Pfeiffle, 1989). A fragment containing only the coding region of ase (from pAse-Kpn, see below) was used as a probe; probe preparation by random primer labelling was according to the instruction of the manufacturer (Boehringer), except that a higher concentration of random primer (1 mg/ml) was used to increase labelling efficiency.

Polyclonal antibodies were raised by injecting two rabbits with the peptide CLESDESMDAIDWWAHEAHPKSNGACTNLSV, corresponding to a fragment from the C terminus of the putative ase protein. The terminal Cys residue was used to couple the peptide under oxidizing conditions to keyhole limpet haemocyanin carrier protein. Positive sera from one rabbit were further purified by affinity chromatography using bacterially produced ase fusion protein (see Jarman et al., 1993) on an Affigel column (Biorad). Before use, the antibodies were preabsorbed with homozygous ase 
 embryonic or larval tissue; final dilution of the antibody fractions was approximately 1:100. Western blot analysis of embryonic extracts was done as described in Blochlinger et al. (1991). 22C10 (1:50) and anti-ac (1:20) are mouse monoclonal antibodies kindly provided by Dros Seymour Benzer and Sean Carroll, respectively. Anti-β-galactosidase is a polyclonal rabbit antiserum (Polysciences) used at 1:10 000 to 1:50 000. Secondary antibodies coupled to HRP (1:500) or conjugated to Texas red or biotin (Multilabel grade, 1:200) were obtained from Jackson Immunoresearch, and Avidin-Bodipy (1:50) was purchased from Molecular Probes. Standard antibody staining protocols were used throughout as described in Hartenstein and Campos-Ortega (1986). Imaginal discs were dissected in 100 mM phosphate buffer pH 7.0, fixed for 30 minutes in 4% paraformaldehyde in PBS and processed for staining. ‘Blue balancers’ with lacZ insertions were used to allow identification of homozygous Df(2L)daKX136 and Df(1)sc 
 embryos. Homozygous embryos could be recognized because of the absence of anti-β galactosidase staining. For double immunofluorescence labelling, imaginal discs were simultaneously incubated with anti-ase and a mouse monoclonal anti-β galactosidase (Promega, 1:50) or anti-ac antibodies, followed by donkey anti-rabbit-biotin plus goat anti-mouse-texas red, and a third incubation with avidin-bodipy. Double-labelled discs were examined on a Biorad MRC 600 confocal microscope equipped with a Krypton/Argon laser. Data were recorded and processed using commercial software (CM, Biorad) on a Compaq PC and an IBM worm drive. Several optical sections from a z-series were combined to form the images of the entire discs, which were subsequently artificially colored using Luminera software. Standard light microscopic photography was done on a Nikon photomicroscope equipped with Nomarski optics.

Construction of hs-ase

A genomic BamHI fragment containing the ase gene was subcloned from the λsc53 phage (González et al., 1989); subsequent numbering refers to the coordinates used by these authors. The coding region was isolated from plasmid DNA using the primer pair A 
 and G 
 under standard PCR conditions, ligated to KpnI linkers (NEB) and cloned into blue script SK+ to give pAse-Kpn, the sequence of which was determined by standard sequencing protocols (USB). Two conservative nucleotide exchanges were found: A 
 to C, A 
 to T. The fragment was then cloned into the KpnI site of pWH1 (Blochlinger et al., 1991) in the sense and antisense orientation and transformed into flies by standard P-element-mediated transformation. G0 larvae were raised at 18°C and eclosing flies were individually tested for the presence of w+ transients by mating them to y w. 12 of 13 transients carrying hs-ase in the sense orientation showed upon heat shock the phenotypes that we describe here; 19 transients carrying the same KpnI fragment in the antisense orientation did not show a phenotype with or without heat shock. Stocks were established from these lines; in the experiments described, we used two homozygous viable transformant lines, hs- 
 and hs- 
 alone (2 copies) or in combination (4 copies). To obtain hs-Gal4-ase, an Aps718I fragment containing the coding region from pAse-Kpn was inserted into the Aps718 I site of pUAST (A. Brand and N. Perrimon) and injected into w+ embryos. Of more than 30 independent transformants, 15 were established as stocks and tested by crossing them to a stock producing Gal4 under control of a heat-shock promoter (hs-Gal4/CyO; obtained from Ed Giniger). All crosses showed extra sense organs after heat shock in CyO*, but not in CyO control flies. Lines hs-Gal4-ase 3 and hs-Gal4-ase 5 were examined in more detail by heat shocking larvae and pupae; they showed the phenotypes described in Fig. 8. Stocks carrying hs-Gal4-1sc were obtained in a analogous fashion by PCR isolation of the coding region of 1sc, sequencing,
cloning into pUAST (H. Vaessen) and transforming it into flies. Lines hs-Gal4-1sc 2-4a and hs-Gal4-1sc 13-3 were used.

**In vitro mutagenesis**

The point mutant was obtained by PCR mutagenesis. A fragment of 500 bp was isolated using the primer pair A1941-T1965; A2474 TT GTT AAC CTG CCT CAG GCC ATT TTC TCC CCT AGC-2359. Highlighted bases lead to exchange of Arg171 and Arg173 to Gly, and Val174 to Leu; the latter exchange is assumed to be insignificant since Leu occurs in this position in many bHLH proteins. The PCR product was digested with HindIII and HpaI and directly inserted into the reverse complementary typewild fragment in pAse-Kpn, from which the HindIII site of the polylinker had previously been removed, to yield pAse-PM. A deletion of the basic domain only or both the basic and HLH domain was obtained by ‘divergent PCR’ (Hemsley et al., 1989) with primers exactly flanking the region to be deleted and pAse-Kpn as a template. Primers ase10 (C2428-G2488) and ase11 (C2455-G2476) were used to delete the basic domain (pAse-bd−), and ase10 and ase12 (G2621-C2641) to delete both basic and bHLH domain (pAse-bHLH−); these three primers also contained a unique BglII site that introduces the amino acids Leu and Asp in place of the deletions. PCR products were cut with BglII, ligated and transformed. The HindIII-HpaI fragment was then inserted into the pAse-Kpn backbone, as described above for the point mutant, to yield pAse-bd− and pAse-bHLH−. All mutated plasmids were then sequenced to ensure the presence of the mutations. Mutated ase coding regions were then inserted into the Asp718 I site of pWH1 (pAse-PM, pAse-bd−) and pUAST (all three mutants) and P-element transformants were derived as above.

Stocks of homozygous transformants were tested as described by crossing to hs-Gal4-ase− females and comparing the size of the protein were confirmed by western blot analysis of embryonic extracts (Fig. 7): ‘Point mutant’: hs-Gal4-ase− PM 1; deletion of the basic domain: hs-Gal4-ase− PM 11.1 and hs-ase PM 1; deletion of the basic domain: hs-Gal4-ase− bd− 7.3 and 7.5, and hs-ase bd− 7.1, 7.2 and 7.4; deletion of the bHLH domain: hs-Gal4-ase− bHLH− 13.1a.

**RESULTS**

**The embryonic expression pattern of asense RNA and protein**

The distribution of ase RNA has been studied previously by in-situ hybridization to sections (Alonso and Cabrera, 1988; González et al., 1989). It was noted that ase is expressed later than other genes of the ASc, though the identity of the ase-expressing cells was not established. We have raised antibodies against a C-terminal peptide of the ase protein for immunocytochemical localization of ase, and compared the protein distribution to the RNA pattern as determined by the more sensitive whole-mount in situ hybridization technique (Figs 1, 2). Staining for the antigen is eliminated in embryos deficient for the ase gene (ase−) (not shown), indicating that ase is the only member of the bHLH family recognized by this antibody. RNA was detected a few minutes before the protein; otherwise, we do not observe differences between the RNA and the protein pattern except in the eye disc. The following description is based on analyzing both.

Onset of asense expression in precursors of CNS and PNS

ase expression is first detected shortly after the onset of gastrulation in the nuclei of single cells that are in the process of segregating from the neuroectodermal epithelium, but not in the surrounding cells of the proneural cluster (Fig. 1B,C). By contrast, proneural genes such as sc, l ’sc (Cabrera et al., 1987; Romani et al., 1987) and ac (Fig. 1A) already show strong expression in proneural clusters. While ac is expressed in a subset of neuroblasts (Fig. 1D), the ase-expressing cells give rise to the entire first wave of segregating neuroblasts (SI) and most likely all of the second and third wave (SII and SIII) of neuroblasts as well (Fig. 1E,F). ase is also found in at least some of the ganglion mother cells, progeny of the neuroblasts (Fig. 1G,H). During germ band retraction (stage 16), ase is seen in a subset of ventroperipherally located cells of the ventral nerve chord and in conspicuously large and superficial cells of the brain hemispheres (Fig. 1I). These cells correspond in their position and appearance to larval neuroblasts and the optic lobe anlage (Truman and Bate, 1988). The restriction of ase expression to neural precursors and the prolonged presence before cell division set ase apart from the proneural genes.

In the PNS, expression of ase closely resembles the early expression of the PNS marker line A37, which marks all sense organ precursors (SOPs) and their progeny (Ghysen and O’Kane, 1989). A subset of these cells variably depends on ase (Dambly-Chaudière and Ghysen, 1987; Jarman et al., 1993). Of the first two SOPs to arise in the PNS anlage, the anterior one (the A cell; Ghysen and O’Kane, 1989) gives rise to a sense organ, lk or lh3, that partially depends on ase, while the posterior one (the P cell) produces the ASC-independent chordotonal organs (Dambly-Chaudière and Ghysen, 1987). We find ase to be expressed from early stage 10 onwards in both the A and P cells (Fig. 2A,B,E–G), as well as in most or all precursors that form subsequently (Fig. 2C,D,H,I). At early stages, a third cell of unknown fate may be seen in addition to the A and P cell. (Fig. 2F). We do not detect ase RNA or protein expression in proneural clusters in the ectoderm at any stage. Thus, even the proneural cluster for a partially ase-dependent SOP does not show detectable expression of ase.

Unlike ac or sc (Cubas et al., 1991; Skeath and Carroll, 1991), ase remains detectable during and after division of the SOP (Fig. 2G–I). Some time after the first division, the progeny of the A and P cells cease to be labelled; instead, additional labelled cells appear in other subepidermal positions of the PNS anlage (Fig. 2H). Due to the expression in daughter cells (secondary SOPs), newly segregating SOPs are not easily distinguishable after this stage. ase also remains detectable in a subset of PNS cells during and after germ band retraction stages, when products of the other ASC genes are no longer detectable (Fig. 2C,D,H,I).

**Expression in the midgut anlage**

Expression of ase is not confined to neural cells: from stage 10 onwards, expression is also observed in the midgut anlage (see below and Fig. 5), as are ac and l’sc (Romani et al., 1987; Cabrera et al., 1987). At late embryonic stages, these cells display a distinctive non-neuronal morphology (A. Jarman, unpublished), and at least the majority of them correspond to precursor cells for the imaginal midgut (Hartenstein et al., 1992).
Fig. 1. Embryonic CNS expression of ase mRNA and protein compared with mRNA of the proneural gene ase. Shown are whole mounts of wild-type embryos hybridized with ase (A,D) or ase (B,E,G) digoxigenin-labelled probes to detect mRNA, or anti-ase antibodies (C,F,H) to detect ase protein. (A) Optical section through a stage 8 embryo hybridized with a probe detecting the expression of the proneural ase gene. (B) An embryo at a similar stage hybridized with a probe detecting ase mRNA. Whereas expression of ase in A occurs in proneural clusters in the ectoderm (brackets in A), ase is detected in single cells (neuroblasts), indicated by arrowheads. (C) Detection of ase protein in an early stage 9 embryo (slightly later than B). Arrows in A–C indicate the position of the posterior tip of the germ band. (D–F) Ventral views of the abdominal part of the germ band of stage 9 embryos. (D) ase RNA is expressed in a subset of the neuroblasts (arrow) whereas all neuroblasts express ase RNA (E) and protein (F). At this stage, some variation in intensity, particularly at the RNA level, is observed in cells undergoing mitosis. (G,H) Higher magnification views of the ventral germ band of the embryos shown in B,C. Arrows point to neuroblasts that express ase RNA or protein; arrowheads indicate some ganglion mother cells, daughter cells of the neuroblasts. MS, mesoderm; N, neural primordium; Epi, epidermis. (I) Expression of ase protein after germ band retraction in the ventral nerve chord (arrow and bracket) and the brain lobes.
Postembryonic expression of \textit{asense}

SOPs in the imaginal discs arise in a well-ordered spatial and temporal manner and are readily detected by \textit{lacZ} expression due to an enhancer trap insertion, A101, in the \textit{neuralized} gene (Huang et al., 1991; Boulianne et al., 1991). We compared \textit{ase} expression with A101 expression in order to determine whether \textit{ase} is expressed in SOPs. We also compared the temporal pattern of \textit{ase} expression with that of the proneural \textit{ac} gene product (Fig. 3).

Previous studies failed to detect \textit{ase} expression in the wing disc (González et al., 1989). We now find, however, that both \textit{ase} RNA and protein are expressed in a pattern reminiscent of SOPs (Fig. 3A). Confocal double immunofluorescence visualization of \textit{ase} and A101 confirms that the \textit{ase}-expressing cells are SOPs (Fig. 3B–E). Up to about 3 hours after puparium formation (apf), the oldest stage that we have examined, all SOPs that arise appear to express \textit{ase}. Onset of \textit{ase} expression at each position is detected shortly after A101 expression becomes detectable. Unlike A101, which occasionally labels 2–3 cells transiently before expression is restricted to the SOP (Huang et al., 1991), \textit{ase} is never seen in more than one cell within each proneural cluster. In double-label studies with antibodies against ac and \textit{ase} (Fig. 3F,G), ac expression is detected in proneural
**Fig. 3.** *ase* sense is expressed in neural precursors and their progeny in the wing imaginal disc. (A) A wing disc from a third instar larva approximately 12 hours bpf, hybridized with a probe detecting *ase* mRNA. Some prominent precursors are labelled. Notice that all staining occurs in isolated cells, although in the dorsal radius, the density of cells is high since many sense organs arise in this region. (B,C) Confocal microscope pictures of a disc at puparium formation that has been labelled in a double immunofluorescence experiment to detect expression of A101 (red) and *ase* (green). *ase* is detected in the neural precursor cells stained by A101 (Huang et al., 1991). (D,E) A dividing posterior scutellar (pSC) precursor is seen in the higher magnification view of the SC area showing that both daughter cells stain with anti-*ase*. (F–I) Double immunofluorescent detection of ac (red) and *ase* (green) in a late third instar wing disc about 6 hours bpf. Expression of ac is in proneural clusters (H) in the SC and DC areas, whereas in other areas, ac expression is already confined to the precursor. (I) *ase* colocalizes with ac only in the precursors. (F,G) An optical section from the same Z series through a more basal part of the epithelium. Here, precursor aPA will soon divide; it expresses *ase* (G), but ac is no longer detectable (F). Abbreviations for neural precursors: a, anterior; p, posterior; SC, scutellar; DC, dorsocentral; NP, notopleural; SA, supra-alar; tr1, 1st sensillum trichodeum, WM, wing margin; Teg, tegula; vR, ventral radius; dR, dorsal radius; L3–2, second sensillum campaniformia of the third vein (Huang et al., 1991).
Fig. 4. ase is expressed in neural precursors in imaginal discs and in the larval CNS. (A–C) Whole mounts of leg disc (A) and eye-antennal disc (B) or the larval CNS (C) from wild-type late third instar larvae stained with anti-ase antibodies. (A) A prominent group of cells at the base of the leg disc corresponds to precursors of the chordotonal organ (arrow). Also stained are the precursors of leg macrochaetae (arrowhead), others are not in focus. (B) Eye-antennal disc. Expression is detected in groups of cells that are probably precursors of the Johnston’s organ located in the anlage for the second antennal segment (arrowheads). (C) Larval CNS. Expression is seen in the neuroblasts and their progeny. Out of focus is the expression in brain lobe in a half circle of neurons.

clusters, whereas ase is expressed only in single cells that correspond to the SOPs. Close examination of the two ac-dependent precursors for the dorsocentral bristles (DC SOPs) revealed that onset of ase expression occurs in a late stage of cluster development, when ac expression increases in the single cell that will become the SOP. After ac expression disappears in the segregated cell prior to division, ase expression persists (Fig. 3H,I). Other SOPs of the notum do not require ac, though they appear to show the same relative onset of ac and ase (Fig. 3F–I). As in the embryo, ase remains detectable in both daughter cells during and after the first SOP division (Fig. 3D,E).

Expression of ase in the leg and antennal discs is also restricted to precursors that are labelled in A101 (Fig. 4A,B). As described previously (González et al., 1989), ase is detected in the prominent group of chordotonal organ precursors at the base of the leg discs (Fig. 4A), but it is also detected in the SOPs of the bristles. We also detect ase in large groups of cells in the antennal disc (Fig. 4B) that probably correspond to the precursors of the Johnston’s organs (Bryant, 1978), which are thought to be modified chordotonal organs (McIver, 1985). The expression pattern in the eye disc is unusual; ase RNA is readily detectable in the mature part of the eye disc, whereas no protein expression is detected. In the larval brain, ase protein and RNA are found in the CNS neuroblasts and in some of their progeny, and in a prominent horseshoe-shaped group of cells in the optic lobe anlage (Fig. 4C). These cells most likely correspond to the proliferation centers that give rise to the optic lobe (reviewed in Campos-Ortega and Hartenstein, 1985b). In ase-deficient flies, defects have been described in this part of the brain (González et al., 1989).

In summary, the pattern of asense RNA and protein distribution resembles that of other genes that are activated in all neural precursors (Vaessin et al., 1991; Bier et al., 1992).

Proneural mutants affect the expression of asense
The ase expression pattern shows a close temporal and spatial relation with the expression of the proneural genes of the AS-C. We examined ase expression in embryos and imaginal discs of proneural mutants.

Effect on embryonic expression of ase daughterless is required for the differentiation of all PNS sense organs and of most CNS cells. Although the neural precursors of these neurons form initially, they disappear shortly thereafter (Brand and Campos-Ortega, unpublished data; Vaessin, Brand, Jan and Jan, unpublished data). In da mutants (Df(2L)daKX136), expression of ase is strongly decreased even though the neuroblasts appear morphologically normal (Fig. 5B,D). This is also true in embryos lacking the maternal contribution of da (Brand and Campos-Ortega, unpublished data). Thus the expression of ase in neural precursors depends strongly on da.

The proneural genes of the AS-C are required for the formation of subsets of the PNS and CNS precursors (Jiménez and Campos-Ortega, 1990). Absence of ac, sc and lsc (in Df(1)sc19 embryos) leads to elimination of some, but not all ase-expressing cells in the PNS (Fig. 5F). The subset of PNS cells that expresses ase in Df(1)sc19 appears to correspond to the cells that do not require AS-C for their development, such as the precursors for chordotonal organs (CHOs), located in the posterior part of the segment (Fig. 5F). These observations indicate that ase is not expressed if the neural precursors are not formed; that is, expression requires the prior action of the proneural genes. The ase expression in the midgut anlage is also eliminated (Fig. 5H).

In mutants of neurogenic genes, neural precursors are formed at the expense of epidermal precursors (reviewed by Artavanis-Tsakonas, 1988; Campos-Ortega, 1988; Campos-Ortega and Jan, 1991; Simpson, 1990). Consistent with this, ase is expressed in most cells of the ectoderm in mutant
Fig. 5. Expression of ase is affected in neurogenic and proneural mutants. Shown are whole mounts of wild-type (A,C,E,G), daughterless deficient (B,D), ac, sc, and l’sc deficient (F,H) and strong neuralized (I) mutant embryos stained with anti-ase antibodies. (A,C) Expression of ase in a wild-type stage 9 embryo at low and higher magnification. Epidermal, neural and mesodermal primordia are indicated by brackets; arrows indicate neuroblasts, arrowheads indicate ganglion mother cells. (B,D) A stage 9 da embryo. Although neuroblasts are present, expression of ase is either severely reduced (arrow) or nearly undetectable (open arrows). (E) Stage 12 wild-type embryo, ase is expressed in the PNS primordium (bracket). Many neural precursors and their progeny stain with anti-ase antibodies. (F) A Df(1) sc19/Y embryo, which is deleted for ac, sc and l’sc, but not for ase. The number of cells in the PNS primordium is severely reduced; arrowheads point to the cluster of chordotonal organ precursors that continue to stain with anti-ase. (G,H) CNS and midgut staining in stage 12 wild-type (G) and Df(1) sc19 (H) embryos. Arrows point to midgut cells stained with anti-ase that are absent (asterisk) in the AS-C mutant embryo. CNS staining in this mutant embryo appears normal. (I) Embryo with the neurogenic mutation, neuKX9, ase is expressed in most cells of the neurogenic and PNS primordium.
embryos that lack neuralized function (Fig. 5I) or Notch function (not shown).

**Effect on ase expression in the imaginal discs**

We have examined ase expression in discs of the sc^{10-1}/Y mutant, which do not express ac or sc and lack most of the external sense organs, and those of the mutant In(1)ac^{3}, which express sc but not ac and have only a few of the sense organs missing. In both mutants, ase is expressed in the precursors of most or all remaining sense organs (Fig. 6), as was found in the embryo.

Wing discs derived from sc^{10-1} mutant larvae are devoid of ase-staining cells, with the exception of 1-2 cells located in the dorsal radius (Fig. 6B). No sense organ was detected externally in sc^{10-1} mutant wings in this region. It is possible that these ase-expressing cells give rise to an internal chordotonal organ that is known to reside in the radius (Miller, 1950). In the leg discs, the group of chordotonal organ precursors clearly expresses ase, as does a small subset of the precursors located on the more distal segments of the leg (Fig. 6D), consistent with the presence of a few bristles on sc^{10-1} adult legs (Lindsley and Grell, 1968; Held, 1991). In the antennal disc, the large group of putative Johnston’s organ precursors still express ase (Fig. 6A). The majority of cells that still express ase in sc^{10-1} discs may therefore be of the chordotonal type. For technical reasons, we have not
been able to examine ase expression in the precursors for the stout row of mechanosensory bristles at the wing margin, which is not affected in sc<sup>10-1</sup>. These precursors arise around 10 hours after puparium formation. In one case, we could detect expression of ase RNA in a row of large subepidermal cells, which we presume to be the stout row precursors (Fig. 6C). This indicates that ase expression in these cells is independent of ac and sc, and is consistent with the dependence of these sense organs on ase itself (Jarman et al., 1993).

Flies lacking a functional ac gene (In(1)ac<sup>3</sup>) retain all but the DC macrochaetae and express sc in their SOPs (Cubas et al., 1991; Skeath and Carrol, 1991). We find this to be reflected in the expression of ase in In(1)ac<sup>3</sup> wing discs: expression is lacking in the DC area, but is unaffected in the other SOPs (Fig. 5E). In contrast, flies carrying a combination of strong viable emc alleles, emc<sup>pel</sup>/Df(3)emc<sup>E12</sup> show a large number of additional bristles on the notum as a consequence of ectopic expression of AS-C genes (Cubas et al., 1991; Skeath and Carrol, 1991). Additional ase-expressing SOPs are observed in the notum portion of wing discs of this genotype (Fig. 5F).

In summary, ase activation only occurs upon SOP formation and both events may therefore be part of the same process. Also, ase-expressing imaginal SOPs can apparently be subdivided into four groups: those that require ase (stout row bristles), those that require ac (DCs), those that require sc (most other macrochaetae) and those that are independent of AC-S (such as the chordotonal organs). It appears that in most cases ase is temporally downstream both of the AC-S and of other proneural genes required for sense organ formation.

**Ectopic ase sense expression leads to appearance of duplicated and ectopic sense organs**

Ectopic expression of the proneural gene sc can result in placement of duplicated and ectopic bristles (Rodríguez et al., 1990). Since ase expression differs from that of proneural genes, we asked if this leads to a different phenotype under conditions of ectopic expression, when ase is under the control of a heat-shock promoter (hs-ase, Fig. 7).

**Construction and testing of hs-ase**

Stocks carrying 2 or 4 copies of hs-ase were constructed (Materials and Methods). Extracts from heat-treated embryos of these stocks contain an induced protein of the expected size (Fig. 7D) and expression is strong and ubiquitous in heat treated, but not untreated, imaginal discs (Fig. 7E). Although difficult to quantitate, the levels of protein achieved under these conditions appear to be much higher than wild type.

**hs-ase in wild-type background**

We restrict our analysis to the larval stage, because of the greater temporal and spatial resolution. Ectopic expression of ase after heat shock resulted in placement of many additional bristles in various parts of the fly’s body (Fig. 8) in a manner similar to that described after ectopic expression of sc (Rodriguez et al., 1990). The additional bristles are accompanied by corresponding internal parts of a sense organ, as determined by 22C10 staining of sensory neurons in adult nota (Fig. 8H). As reported for sc (Rodriguez et al., 1990), the type of sense organ formed, e.g. macrochaetae, microchaetae or campaniform sensilla, varies with the time of heat shock and the location in the fly (Table 1; Figs 8, 9).

For instance, a heat treatment before puparium formation (bpf) typically results in placement of macrochaetae on the notum but not on many other tissues, such as the ventral abdomen (Fig. 8B). After puparium formation (apf), however, numerous bristles are formed on the ventral abdomen, but no additional ones are found on the notum (Fig. 8D).

After heat-shock induction of ase, the extra macrochaetae on the notum appear around the normal macrochaetae. To test whether some of these limitations of bristle induction might be due to an insufficient level/stability of ectopically produced protein, we produced transformants carrying ase under the control of a promoter containing yeast Gal4-binding sites, and crossed a heat shock-Gal4 construct into these flies (Fig. 7). In flies carrying both of these constructs (hs-Gal4-ase), penetrance and expressivity of the phenotype appeared to be increased, i.e. more bristles appear to be placed at a given time and position, making it in some cases difficult to assign the ectopic bristle to a cognate position (Fig. 8C); this correlates with a much higher level of protein as determined in western blots (Fig. 7D). In contrast, the spatial and temporal sensitivity of the tissue does not appear to be altered, indicating that the amount of ase product cannot be the only factor that is limiting the ability of cells to assume a neural fate.

**Extra sense organs are induced by ectopic expression of ase**

In order to determine the sensitive period for ectopic ase expression, we staged groups of larvae carrying two copies of hs-ase and subjected them to heat treatment at different times. It appears from these studies that the extra sense organs for a given position form at specific time windows (Table 1; Fig. 9). The clearest case is that of the DC bristles: the maximum overproduction of these organs is reached during and after the period of time when their precursors normally form (Romani et al., 1989; Skeath and Carrol, 1991; Cubas et al., 1991).

An intact DNA-binding domain is necessary to produce ectopic sense organs

Previous studies indicate that bHLH proteins dimerize via their HLH domain and that the basic domain preceding the HLH domain is required for sequence specific DNA recognition (Murre et al., 1989; Davis et al., 1990). To distinguish between the possibility that the ase product acts directly by binding to DNA to activate the genetic program for bristle formation and the alternative that it titrates negative regulatory factors of ac and sc such as emc by dimerization (Garrell and Modolell, 1990; Ellis et al., 1990; van Doren et al., 1992), we tested in our heat-shock experiments mutations of ase that abolish the DNA-binding moeity but retain the dimerizing HLH domain. Three different mutations were generated: a double mutant that changes two arginine residues of the basic domain to glycine (these
Fig. 7. An intact basic domain is required for ectopic sense organ formation after heat-shock induction of ase. (A–C) A summary of the structure of different ase (A,B) and l’sc (C) heat-shock constructs and their ability to induce extra bristles after a heat shock. (A) Top: Structure of the hs-ase construct. Expression is controlled via a heat-shock promoter, hsp. The arrow symbolizes the transcription start site of the ase gene under the control of the heat-shock promoter. The basic domain (bd) required for DNA binding and the helix-loop-helix domain (HLH) required for protein interaction are indicated by boxes. Heat shock of animals carrying hs-ase leads to abundant protein expression (shown in D) that is homogeneous and nuclear, as shown in the wing disc in (E). Heat shock during larval and pupal stages results in bristle formation, indicated by a plus in the column ‘extra bristles’, as shown in more detail in Figs 8 and 9. (A) Bottom: Structure of the hs-ase bd− construct, where the basic domain has been deleted. This construct is no longer able to induce bristle formation, although a protein of the correct size is still produced. (B) Structure and performance of a series of hs-Gal4-ase constructs. Expression of the yeast regulatory gene Gal4 is controlled by the heat-shock promoter. Gal4 protein binds, after induction through heat shock, to binding sites (indicated as black boxes) that are upstream of the start site of transcription in the pUAST vector. Various residues are crucial for MyoD to bind to DNA, Davis et al., 1990); a deletion of the six amino acids of the basic domain and a larger deletion of both the basic and the HLH domains (Fig. 7). Bacterially produced mutant proteins were no longer able to bind DNA in a bandshift assay (A. Jarman, unpublished), although normal ase does so when produced under the same conditions (Jarman et al., 1993). All three mutant versions were tested for their function in the fly with the hs-Gal4 system; the double mutant and the basic domain deletion were also tested under direct control of the heat-shock promoter. Western blot analysis of embryonic extracts indicates that the mutant ase proteins are of the expected size and that they are stable under the conditions of the heat-shock experiment (Fig. 7D). Larvae carrying the mutant constructs were then tested under standard heat-shock conditions, but the resulting flies showed no additional bristles. Thus, the DNA-binding domain of ectopically expressed ase is necessary for the production of additional bristles.

Gene activation after ectopic expression of ase
Given its ability to direct ectopic sense organ formation, we asked if hs-ase causes activation of other genes expressed in neural precursors, such as the A101 marker (Huang et al., 1991). Larvae carrying two copies of hs-ase and one copy
Fig. 8. Ectopic sense organs are formed after ectopic *ase* expression. Shown are dissected body parts of flies that have been heat treated during larval or pupal stages. (A) Notum of a wild-type fly, showing the regular arrangement of macro- and microchaetae. (B) Notum of a fly carrying two copies of hs-*ase* that was heat shocked around 12 hours bpf. Additional macrochaetae appear in the dorsocentral area (DC) and the scutellar area as a consequence of ectopic *ase* expression. (C) Notum of a fly carrying one copy of hs-*Gal4*-ase. The phenotype in the notum is more severe than in B. Most other tissues of this fly are indistinguishable from the wild type. (D) Abdominal pleura of a fly carrying 1 copy of hs-*Gal4*-ase after a heat shock 12-24 hours apf. Except for the central sternites, this area is normally devoid of bristles. After heat shock, numerous microchaetae densely cover the area; macrochaetae are not observed. The notum of this fly showed no extra sense organs. (E–G) Induction of *ase* can replace proneural gene function. (E) The notum of a fly lacking functional *ac* and *sc* protein (*sc*10-1) is devoid of macrochaetae and microchaetae. (F,G) In the presence of one copy of hs-*ase*, sense organ formation can be restored in *sc*10-1 flies. Macrochaetae are formed if the heat shock occurred during late third instar (F), whereas microchaetae are formed if the heat shock occurred during pupal life (G). (H) 22C10 staining of an adult notum of a fly that showed additional hs-*ase*-induced scutellar macrochaetae externally. External portions of ectopic macrochaetae are accompanied by corresponding internal sensory neurons and their axons (arrowhead). (I) Distal portion of a wild-type wing. Roman numerals indicate the vein number. Vein II is devoid of sense organs, whereas vein III shows a few campaniform sensilla in reproducible locations. (J) After heat-shock induction of *ase* at 8 hours apf, numerous microchaetae-like sense organs are formed preferentially along vein II, but not necessarily associated with the vein (open arrow). Earlier during the competence period for this phenotype (see Table 1, Fig. 9), macrochaetae-like sense organs appear, though they also seem to have less tendency to form along the veins. Additional sense organs of a different type, campaniform sensilla, are formed mainly along vein III. (K) A similar result is seen with hs-*ase* in a *sc*10-1 mutant background. Ectopic sense organs form (arrowheads) and are preferentially, though not always, located along the wing veins.
of A101 were subjected to a 30 minute heat shock at 39°C and wing imaginal discs were dissected 12 hours after the treatment. In the DC area, additional A101-positive cells were formed in 7 of 19 discs as a consequence of the heat shock (Fig. 10D). Thus, although A101 expression normally precedes that of ace, its expression is activated in the ectopic neural precursors induced by hs-ace.

Within five hours of heat-shock induction of ace, we also find very strong ectopic expression of ace in all cells of the notum (Fig. 10A,B). Thus, hs-ace is capable of activating at least one proneural gene. Moreover, although hs-ace induces relatively few bristles, it can activate ace expression in many more cells of the wing disc.

Ectopic expression of ace can substitute for missing proneural gene function

The observation that ace is activated as a consequence of hs-ace raises the possibility that hs-ace induces ectopic sense organs after heat-shock induction of ace

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The percentage of body halves showing formation of extra sense organs of the indicated type following a heat-shock treatment is given as a function of time relative to puparium formation. The number of body halves with extra sense organs in each case is given in parentheses. The total number of larvae/pupae scored at each time point is indicated on the bottom. We attempted to score only well-isolated areas where macrochaetae arise. Even so, additional chaetae tended to avoid the location of the extant bristles and were often located in between extant chaetae (see Fig. 8B), as was observed for heat-shock induction of sc (Rodríguez et al., 1990). For this reason, closely situated chaetae of the same area were scored together, e.g. aDC and pDC, were scored as DC. MsCh: the mesopleural chaetae are microchaeta-like. This area is normally free of chaetae. MicNo: increased microchaetae on the notum. EWSO: extra wing sensory organs. This group includes both chaetae and campaniform sensilla (see Fig. 8I–K). Early in the period, chaetae tended to be more macrochaetae like and were found in vein and intervein areas, and sometimes additional wing vein material was observed. Late in the period, chaetae tended to be microchaetae like and located along the veins, mostly vein II (Fig. 8J,K). WVD: small deltas on the tip of wing veins. AbPl: microchaetae-like sense organs on abdominal pleura (Fig. 8D), an area normally devoid of chaetae.
organoids indirectly via the activation of proneural genes. To test this possibility, we placed one copy of hs-ase into the sc/M mutant. The nota of sc flies are completely devoid of sensory organs (Fig. 8E), and no expression of ase (see above; Fig. 6B) or A101 (Cubas et al., 1991) is detected. After heat treatment, however, sc flies show formation of sense organs. Again, heat-shock treatment at different times results in placement of different bristles (Fig. 8F; macrochaetae; Fig. 8G, microchaetae). Sense organs were also formed after heat-shock induction of ase in flies carrying a deficiency for ac and sc, (Df(1)3PEscY; hs-ase+/+, not shown). Thus, ectopic expression of ase can replace the missing proneural function of ac and sc, the genes that are normally required for the formation of sense organs. It is still possible, however, that other proneural genes are activated in this case.

Ectopic expression of l’sc also leads to extra bristle formation

Having observed that removal of ac and sc did not abolish the ability of hs-ase to induce bristles, we asked whether ectopic expression of l’sc, the remaining proneural gene in the AS-C, also induces bristles. l’sc is not required for the formation of the bristles on the notum (García-Bellido and Santamaria, 1978), nor could we detect any l’sc RNA expression in wild-type wing discs (not shown). Nevertheless, supernumerary bristles are encountered in hs-Gal4-l’sc flies. Although not examined in detail, the temporal and spatial characteristics of sense organ induction due to hs-Gal4-l’sc closely resemble those due to hs-Gal4-ase. Thus, overexpression of ase or l’sc results in gain-of-function phenotypes that differ from what would be expected on the basis of their loss-of-function phenotypes.

DISCUSSION

ase is expressed in all neural precursors

Previous analyses of in situ hybridization to sections indicated that the pattern of expression of ase RNA differs from those of the proneural genes, but the identity of the cells involved remained largely uncharacterized (Alonso and Cabrera, 1988; González et al., 1989). We have found that ase RNA and protein are expressed in neural precursors of the CNS and the PNS in the embryo and in imaginal discs. In addition, ase is expressed in at least one nonneural cell type, the imaginal midgut precursors, where it also closely follows the expression of the proneural genes. The expression of ase in neural precursors requires the function of daughterless, and it is downstream of AS-C function in at least a temporal sense. This is consistent with analysis of the ase promoter, which indicates a potentially direct action of AS-C/da proteins on ase transcription (Jarman et al., 1993). Compared with the proneural genes, ase differs in its later onset of expression during precursor development, the exclusive expression in all neural precursors and the continued expression after the first precursor division. These properties of ase suggest that it belongs to the group of neural precursor genes (Vaessin et al., 1991; Bier et al., 1992). In the following, we discuss differences and commonalities between ase and the proneural genes of the AS-C.

Proneural clusters and ase expression

We were unable to detect ase expression at the proneural cluster stage, either in the embryonic CNS, PNS or imaginal disc tissue. It seems unlikely that ase expression in proneural clusters has been overlooked for the following reasons. First, at stages and under conditions where clustered expression

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**Fig. 10.** Activation of neural markers after heat-shock induction of ase. Whole-mount wing discs are stained with anti-ac and anti-ase (A,B) or anti-β-galactosidase antibodies for detection of A101 expression pattern (C,D). (A,B) ac expression is activated ectopically after heat-shock induction of ase. Larvae with two copies of hs-ase were heat shocked, dissected 5 hours after the heat shock, and stained in a double-label immunofluorescence experiment with anti-ac and anti-ase. (A) High expression of ac is induced in ectopic sites; compare to Fig. 3H for a wild-type pattern of ac. (B) Ectopic expression of ase is already weakening at this stage. (C,D) The SOP marker A101 is activated in ectopic SOPs after a hs-ase. (C) Wing disc from a late third instar larva of the A101 insertion line stained for β-galactosidase expression. The DC area, indicated by a bracket, contains the precursors for aDC and pDC. (D) β-galactosidase staining of a disc from a larva with two copies of hs-ase and one copy of the A101 insertion, 12 hours after ase induction. A pair of additional A101 staining cells is observed in the DC area (arrows).
of the proneural ac, sc or l’sc genes are clearly detectable, we consistently detect ase in only single cells. Second, the pattern of RNA and protein expression are identical, thus making it unlikely that only a specific form of ase is recognized by the antibody, as may be the case for an antibody directed against the l’sc protein (Cabrera, 1990). Third, the expression of an ase-lacZ fusion gene is also only detected in single cells (Jarman et al., 1993). However, of those SOPs that depend on ase for their proper formation (Dambly-Chaudière and Ghysen, 1987; Jarman et al., 1993), we can reliably identify only the A cell that gives rise to lk/h3, and ac expression in the proneural cluster associated with this sense organ is very faint and transient (Ruiz-Gómez and Ghysen, 1993). Thus, although we do not detect ase expression in any of the proneural clusters, we cannot rule out that we have overlooked very transient expression in this particular case.

If ase expression is indeed absent in proneural clusters for ase-dependent sense organ precursors, how might one reconcile this paradoxical observation? Formation of ase-dependent sense organs is somewhat suppressed if ac, sc and l’sc function are eliminated (in Df(1)sc19 embryos, Dambly-Chaudière and Ghysen, 1987, or in the stout row of the adult wing margin, M. Guo, M. B., A. P. J., L. Y. J. and Y. N. J., unpublished). One or more proneural genes may be expressed in the proneural clusters of ase-dependent SOPs and may be involved in the selection of neural precursors from these clusters of cells; ase expression in the selected precursors may then be essential for their further development. For different precursors, the function of ase could be supplemented to different extents by the expression of the proneural genes in the precursor. Loss of ase, if not compensated for completely, may then result in the formation of aberrant bristles, as observed in the case of the malformed bristles of the wing margin (Jarman et al., 1993), or it may lead to not fully penetrant absence of the sense organ, as has been found in the few embryonic sense organs.

**Over-expression of asense gives rise to a gain-of-function phenotype characteristic of proneural genes**

Our results show that ectopic expression of ase can promote formation of sense organs and that this expression is effective only at the time when sense organs normally would form. In addition, ectopic sense organs are induced by hs-ase in the same tissues that respond to a heat-shock induction of sc (Rodríguez et al., 1990). Also, induction of ase restores bristles at or around their normal positions on the notum in the absence of ac and sc, and can thus functionally replace the proneural ac and sc genes. Consistent with this notion, we find that hs-ase leads to apparently inappropriate activation of ac and A101(neuralized), two genes that are normally active in early stages of precursor formation, prior to the normal activation of ase. Thus, under conditions of overexpression ase acts like a proneural gene. Similarly, l’sc is not normally required for development of adult sense organs on the notum (García-Bellido and Sантamaria, 1978), and it is not normally expressed there (M. Brand, unpublished). Nevertheless, hs-l’sc behaves like hs-ase or hs-sc with respect to temporal and spatial sensitivity of the responding tissue. This demonstrates the ability of different AS-C functions to replace one another under the gain-of-function condition. Some functional redundancy has been observed in the analysis of loss-of-function phenotypes of the AS-C (Jiménez and Campos-Ortega, 1987; Dambly-Chaudière and Ghysen, 1987), but our gain-of-function results suggest complete interchangeability of the AS-C members in initiating sense organ development.

Although ase, l’sc or sc overexpression can place sense organs on some parts of the body normally devoid of sense organs, such as the thoracic pleura, parts of the wing blade or the ventral abdomen, the placement of sense organs is far from random, pointing to constraints on neural competence. Some of these constraints are known. It is likely that the *examacrocystetae (emc)* product needs to be reduced in order to allow ase protein to become effective. emc is known to be a negative regulator of AS-C gene function (Moscoso del Prado and García-Bellido, 1984; García-Alonso and García-Bellido, 1986; Mari-Belgra et al. 1991). This is consistent with the finding that the *emc* RNA (Cubas and Modolell, 1992; Van Doren et al., 1992) and protein (Brand et al, unpublished) appear to be low in areas of active neurogenesis. Evidence from in vitro experiments suggests that emc can interfere with binding of ac or sc to da protein (Van Doren et al., 1992). However, simply raising the level of ase protein through the use of the more efficient hs-Gal4-ase constructs does not overcome such temporal or spatial constraints; it only appears to increase penetrance and expressivity of sense organ formation. We are currently testing the possibility that da becomes limiting under the conditions of AS-C over expression.

**Comparison of the neurogenic and the myogenic networks of bHLH proteins**

Our studies of members of bHLH proteins in fly neurogenesis indicate that a detailed knowledge of the pattern of expression and of the loss-of-function phenotype are important for interpreting the gain-of-function phenotypes. Clearly, under conditions of ectopic expression, ase and l’sc can activate the neural fate for cells that do not normally require their function. A similar situation was noticed in the case of the bHLH gene *hairy*, where misexpression leads to artifactual interference with the sex determination function of sc (Parkhurst and Ish-Horowicz, 1991). This behaviour could perhaps be a general property of bHLH proteins. In vertebrate myogenesis, all of the known myogenic factors with bHLH domains are able to switch cultured cells to a myoblast fate, and thus appear to be interchangeable like the genes of the AS-C. These genes have thus been called ‘master regulators’ of myogenesis (reviewed by Olson, 1990; Weintraub et al., 1991). Nevertheless, like the AS-C in *Drosophila* neurogenesis, the analysis of the distribution of the myogenic factors in vivo indicates temporally and spatially distinct patterns of expression in skeletal muscle primordia (Sassoon et al., 1989; Bober et al., 1991; Ott et al., 1991). Moreover, targeted gene disruption of some myogenic genes gives surprisingly mild effects; indeed, the results vary from abnormal rib development in the case of myf-5 disruption to normal muscle development in the case of MyoD disruption (Braun et al., 1992; Rudnicki et al., 1992). Similarly, body wall muscles that normally express the MyoD like gene *hlh-1* in *C. elegans* can differentiate in
the absence of that gene (Chen et al., 1992). Thus, as a group, the myogenic bHLH proteins are important for muscle development. However, assigning the role of the ‘master regulatory gene’ to an individual member solely based on gain-of-function phenotype can be misleading.

In fly neurogenesis, the loss-of-function phenotypes and the distribution of most of the gene products are known. As is the case for the myogenic regulators, the expression patterns and gain-of-function phenotypes of ac, sc, l’sc and ase are more widespread than the requirement for these genes, as evidenced by the loss-of-function phenotypes. Likewise, differences in temporal onset of normal expression such as observed between the proneural genes and ase seem not to affect the similar performance of these genes under the gain-of-function condition. Members of the AS-C have the same or very similar protein properties, but differ in their mode of regulation. This suggests that the task of the ‘master regulator’ of neurogenesis is shared by several genes rather democratically.

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