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 daugh-terless 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 down-stream 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-Tsakonis, 1988; Simpson, 1990; Cabrera, 1992; Campbell 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 daugh-terless (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 (neur-oblasts in the central nervous system (CNS), and sense organ precursors, SOPs, in the peripheral nervous system (PNS)), and may control different aspects of neuronal differ-entiation (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 het-erodimers. 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*-2; 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 *neuK* were used (Lehmann et al., 1983).

*sc* was marked with white and maintained with In (1) *di* in *y Hw*. Larvae for *sc*/*1 w/Y (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 *eme*/*Df(sc2)127*, a strong viable combination of *emc* alleles, were obtained as *Tubby*- larvae from a cross of *eme*/*eme* with *Df(sc2)127*.*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 indicated times at 25°C before they were subjected to heat-shock treatment. For other staining, climbing third instar larvae were taken from uncrowded vials maintained at 18°C.

Heat-shock treatment of larvae and pupae was done for *hs-ase* with two 25 minute pulses at 39°C, separated by 30 minutes at room temperature. Larvae were collected into a basket inside an Eppendorf tube half-filled with water and incubated in a water bath. Pupae were placed on a floating agar dish. For hs-8*Gal4*-ase and hs-8*Gal4*-1*sc*, the incubation time was reduced to 15 minutes.

**Histochromic techniques**

Whole-mount hybridization with digoxigenin-labelled probes was carried out essentially as described (Tautz and Pfeifle, 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 CLSDESMIDAIADWWEAHPKSNSGACTLNSV, 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 antisera (Poly-sciences) 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-Biotipy (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)daX136 and Df(1)sc19 embryos. Homozygous embryos could be recognized because of the absence of anti-β galactosidase. For standard immunofluorescence staining, 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-biotipy. 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 Lumena 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 (Gonzalez 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 A1961-T1960 and G396S-C104 under standard PCR conditions, ligated to KpnI linkers (NEB) and cloned into bluescript SK+ to give pAse-Kpn, the sequence of which was determined by standard sequencing protocols (USB). Two conservative nucleotide exchanges were found: A901 to C, A745 to T. The fragment was then cloned into the KpnI site of pW1H (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 w Y flies. 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-4a and hs-ase10a alone (2 copies) or in combination (4 copies). To obtain hs-8*Gal4*-ase, an Asp718 fragment containing the coding region from pAse-Kpn was inserted into the Asp718 I site of pUAST (A. Brand and N. Perrimon) and injected into w* embryos. Of more than 30 independent transients, 15 were established as stocks and tested by crossing them to a stock producing Gal4 under control of a heat-shock promotor (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-8*Gal4*-ase 3 and hs-8*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-8*Gal4*-1sc* were obtained in an 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-l'sc 2-4a and hs-Gal4-l'sc 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-T1960; A2474-T1 TTG AAC CTG CCT CAG GCC ATT TCC TTC CCT AGC-2439 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 Hpal and directly inserted in place of the corresponding wild-type fragment in pAseKpn, from which the HindIII site of the polylinker had previously been removed, to yield pAse-PM. A deletion of the basic domain 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 (G2521-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 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 (Fig. 7): 'Point mutant': hs-Gal4-ase 1 from stage 1 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 se (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 germband retraction stages, when products of the other ASC genes are no longer detectable (Fig. 2C,D,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 ac. Shown are whole mounts of wild-type embryos hybridized with ac (A,D) or ase (B,E,G) digoxigenin-labelled probes to detect mRNA, or anti-ase antibodies (C,F,H,I) to detect ase protein. (A) Optical section through a stage 8 embryo hybridized with a probe detecting the expression of the proneural ac gene. (B) An embryo at a similar stage hybridized with a probe detecting ase mRNA. Whereas expression of ac 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 germband. (D–F) Ventral views of the abdominal part of the germband of stage 9 embryos. (D) ac 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 germband 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 *asense*

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

Previous studies failed to detect *ase* expression in the wing disc (González et al., 1989). We now find, however, that both *ase* RNA and protein are expressed in a pattern reminiscent of SOPs (Fig. 3A). Confocal double immunofluorescence visualization of *ase* and A101 confirms that the *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 *ase*. Onset of *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), *ase* is never seen in more than one cell within each proneural cluster. In double-label studies with antibodies against *ac* and *ase* (Fig. 3F,G), *ac* expression is detected in proneural
**Fig. 3.** *asense* 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; trl, 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).
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 divison, 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 legs (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 ase 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 ase**

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 l’sc (in Df(1)sc19 embryos) leads to elimination of some, but not all ase-expressing cells in the PNS (Fig. 5F). The subset of CNS 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} mutant, which do not express ac or sc and lack most of the external sense organs, and those of the mutant Im(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 (Lindsay 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 pupariation 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>l</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>l</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 AS-C (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 asense 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 (Rodríguez 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 (Rodríguez 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 pupariation 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 pupariation 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 transmamts 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 asense during and shortly after the period for normal neural precursor formation**

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 moiety 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
ase wild-type and mutated coding regions were cloned into this vector. The phenotype after heat shock of the wild-type hs-Gal4-ase construct is stronger than for the hs-ase construct shown in A, although not qualitatively different (see text and Fig. 8). Asterisks in the second construct indicate the exchange of two Arginine residues that are crucial for DNA binding in myogenic bHLH proteins; this abolishes the ability to induce extra sense organs, as in the other constructs shown with a deleted basic domain, or a deleted bHLH domain. (C) Structure of the hs-Gal4-l'sc construct. Symbols are as above; the strong bristle phenotype is indistinguishable from hs-Gal4-ase bearing flies after heat shock in the presence of a hs-Gal4 construct (see Fig. 8). n.d., protein level not determined. (D) Western blot of a 12% SDS PAGE gel to detect wild-type and mutant aseproteins in total protein extracts from heat-shocked embryos at different times. Each lane contains the protein equivalent of five embryos from a mix of embryos containing different copy numbers of hs-ase or hs-Gal4-ase. Lane 1: one fourth of the embryos carried hs-Gal4-ase; no heat shock (control). Lane 2: hs-Gal4-ase, 1 hour after heat shock, Lane 3: hs-Gal4-ase, 3 hours after heat shock. Lane 4: hs-ase, 4 copies per embryo, 3 hours after heat shock. In spite of the 16-fold higher average copy number in this line, the intensity of the ase band is lower than in the other lanes. Lane 5: one fourth of the embryos carried the construct with a deleted basic domain (hs-Gal4-ase-bd−), 3 hours after heat shock. Lane 6: One fourth of embryos carrying the point mutant of the basic domain (hs Gal4-ase PM). Lane 7: One fourth of the embryos carrying the deletion of both basic and bHLH domain (hs Gal4-ase bHLH−). Proteins are stable and of the size predicted by the ase open reading frame (54×10^3 M_r for the wild-type protein (González et al., 1989) and 47×10^3 M_r for the bHLH deletion; the other mutant proteins are of expected size).

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 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 constructs, which in this case are different copy number constructs, are tested on the basic 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 ase, its expression is activated in the ectopic neural precursors induced by hs-ase.

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

Ectopic expression of ase can substitute for missing proneural gene function

The observation that ac is activated as a consequence of hs-ase raises the possibility that hs-ase induces ectopic ase, a neural precursor gene
organs indirectly via the activation of proneural genes. To test for this possibility, we placed one copy of hs-ase into the sc^{10-1} mutant. The nota of sc^{10-1} 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^{10-1}; hs-ase/+ 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)yr^{3PL}sc^{S1R/Y}; 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...
of the proneural \textit{ac}, \textit{sc} or \textit{l'sc} genes are clearly detectable, we consistently detect \textit{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 \textit{ase} is recognized by the antibody, as may be the case for an antibody directed against the \textit{l'sc} protein (Cabrera, 1990). Third, the expression of an \textit{ase-lacZ} fusion gene is also only detected in single cells (Jarman et al., 1993). However, of those SOPs that depend on \textit{ase} for their proper formation (Dambly-Chaudière and Ghysen, 1987; Jarman et al., 1993), we can reliably identify only the \textit{A} cell that gives rise to \textit{lk/h3}, and \textit{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 \textit{ase} expression in any of the proneural clusters, we cannot rule out that we have overlooked very transient expression in this particular case.

If \textit{ase} expression is indeed absent in proneural clusters for \textit{ase}-dependent sense organ precursors, how might one reconcile this paradoxical observation? Formation of \textit{ase}-dependent sense organs is somewhat suppressed if \textit{ac}, \textit{sc} and \textit{l'sc} function are eliminated (in \textit{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 \textit{ase}-dependent SOPs and may be involved in the selection of neural precursors from these clusters of cells; \textit{ase} expression in the selected precursors may then be essential for their further development. For different precursors, the function of \textit{ase} could be supplemented to different extents by the expression of the proneural genes in the precursor. Loss of \textit{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.

\textbf{Over-expression of \textit{ase} gives rise to a gain-of-function phenotype characteristic of proneural genes}

Our results show that ectopic expression of \textit{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 \textit{hs-ase} in the same tissues that respond to a heat-shock induction of \textit{sc} (Rodríguez et al., 1990). Also, induction of \textit{ase} restores bristles at or around their normal positions on the notum in the absence of \textit{ac} and \textit{sc}, and can thus functionally replace the proneural \textit{ac} and \textit{sc} genes. Consistent with this notion, we find that \textit{hs-ase} leads to apparently inappropriate activation of \textit{ac} and \textit{A101} (\textit{neuralized}), two genes that are normally active in early stages of precursor formation, prior to the normal activation of \textit{ase}. Thus, under conditions of overexpression \textit{ase} acts like a proneural gene. Similarly, \textit{l'sc} is not normally required for development of adult sense organs on the notum (García-Bellido and Santamaria, 1978), and it is not normally expressed there (M. Brand, unpublished). Nevertheless, \textit{hs-l'sc} behaves like \textit{hs-ase} or \textit{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 \textit{ase}, \textit{l'sc} or \textit{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 \textit{extramacrochaetae} (\textit{emc}) product needs to be reduced in order to allow \textit{ase} protein to become effective. \textit{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; Marí-Beffa et al. 1991). This is consistent with the finding that the \textit{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 \textit{emc} can interfere with binding of \textit{ac} or \textit{sc} to \textit{da} protein (Van Doren et al., 1992). However, simply raising the level of \textit{ase} protein through the use of the more efficient \textit{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 \textit{da} becomes limiting under the conditions of AS-C over expression.

\textbf{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, \textit{ase} and \textit{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 \textit{hairy}, where misexpression leads to artificial interference with the sex determination function of \textit{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 \textit{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 \textit{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 \textit{hhl-1} in \textit{C. elegans} can differentiate in

\textit{ase}, a neural precursor gene 15
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 a ‘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, lsc 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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