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# The regulation and function of the helix-loop-helix gene, *asense*, in *Drosophila* neural precursors

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## SUMMARY

***asense* is a member of the *achaete-scute* complex (AS-C) of helix-loop-helix genes involved in *Drosophila* neurogenesis. Unlike the other AS-C members, which are expressed in subsets of the ectodermal areas (proneural clusters) that give rise to neural precursors, *asense* is one of a number of genes that are specifically expressed in the neural precursors themselves (the neural precursor genes). We have identified a mutant *asense* phenotype that may reflect this later expression pattern. As a step in understanding the determination of neural precursors from the proneural clusters, we have investigated the potential role of the AS-C products as direct transcriptional activators of neural precursor genes by analysing the regulation of *asense*. Using genomic rescues and *asense–lacZ* fusion genes, the neural precursor regulatory element has been identified. We show that this element contains binding sites for AS-C/daughterless heterodimers. Deletion of these sites reduces the expression from the fusion gene, but significant expression is still achieved, pointing to the existence of other regulators of *asense* in addition to the AS-C. *asense* differs from the other AS-C members in its expression pattern, regulation, mutant phenotype and some DNA-binding properties.**

Key words: *Drosophila*, neurogenesis, helix-loop-helix, proneural, neural precursor

## INTRODUCTION

In *Drosophila* development, neurogenesis begins with the delamination of selected cells from a morphologically homogeneous sheet of ectoderm to become neural precursors (Ghysen and Dambly-Chaudière, 1989). Regardless of their future identity, all neural precursors are characterized by expression of a set of genes known as the 'neural precursor genes' (Vaessin et al., 1991; Bier et al., 1992; Brand et al., 1993). To understand the processes involved in neural precursor determination, it may be instructive to determine how this characteristic neural precursor-specific pattern of expression is achieved. We have investigated the regulation of one such neural precursor gene, *asense* (*ase*).

Obvious candidates for regulators of *ase* and other neural precursor genes are *daughterless* (*da*) and the proneural genes of the *achaete-scute* gene complex (AS-C: *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*); Ghysen and Dambly-Chaudière, 1989; Campuzano and Modolell, 1992). The expression of *da* is ubiquitous (H. Vaessin et al., unpublished data), while *ac*, *sc* and *l'sc* are expressed in subsets of the patches of ectodermal cells from which neural precursors will arise (proneural clusters), and then briefly in the neural precursors themselves after they become singled out by the process of lateral inhibition (Romani et al., 1987; Cabrera et al., 1987; Romani et al., 1989; Cubas et al., 1991; Skeath and Carroll, 1991; Martín-Bermudo et al., 1991). In the PNS, loss of function of *ac* or *sc* removes specific subsets of the stereotypic arrangement of external sense organs (García-Bellido and Santamaría, 1978; García-Bellido, 1979; Dambly-Chaudière and Ghysen, 1987). The absence of *da* function results in the loss of all sense organs (Caudy et al., 1988a).

The three AS-C genes encode transcription factors of the basic-helix-loop-helix (bHLH) family (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; Caudy et al., 1988b; Murre et al., 1989a) and are known to dimerize in vitro with the product of *da*, also a bHLH protein. The resulting heterodimers bind to a 6 bp consensus sequence (the E-box; Murre et al., 1989a,b; Van Doren et al., 1991; Cabrera and Alonso, 1991), and AS-C/da-binding sites are present in the upstream regions of *hunchback* (*hb*) (Cabrera and Alonso, 1991) and *Enhancer of split* [*E(spl)*] genes *m7* and *m8* (H. Vaessin, unpublished data). The upstream region of *ac* itself also has such binding sites, which are required for auto- and cross-regulation by *ac* and *sc*, respectively (Van Doren et al., 1991, 1992).

*ase* is the fourth member of the AS-C. It too encodes a bHLH protein (Alonso and Cabrera, 1988; González et al., 1989) and appears to be important for a subset of larval sense organs (Dambly-Chaudière and Ghysen, 1987). But unlike *ac*, *sc* or *l'sc*, the expression of *ase* defines it as a

neural precursor gene (it is expressed in all neural precursors *after* their formation, Brand et al., 1993). We report that *ase* is also distinguished from the other AS-C genes by some aspects of its mutant phenotype and DNA-binding properties.

It can be postulated that the proneural genes achieve their function in early neurogenesis by activating some or all of the neural precursor group of genes. We have addressed this by identifying a regulatory element that directs the *ase* neural precursor pattern of expression and showing that this element contains binding sites for AS-C/da heterodimers. Removal of these sites, however, reduces but does not eliminate expression from an *ase–lacZ* fusion gene, suggesting that regulators other than the AS-C must also play a major role in *ase* activation and its neural precursor pattern.

## MATERIALS AND METHODS

### DNA constructs

For the genomic *ase* construct, a 4.8 kb *Bam*HI fragment from phage λsc53 (Campuzano et al., 1985) was subcloned into pBluescript (pBS-T8). This was transferred to the P-element transformation vector pW8 (Klemenz et al., 1987) to give G:4.8. For the shorter rescue fragment, G:4.8 was digested with *Xba*I and *Avr*II and religated to give G:3.5. For the *lacZ* fusion gene, a *lacZ* coding region with SV40 polyadenylation signal was excised from pC4βgal (Thummel et al., 1988) as an *Xma*I fragment. This was filled in with Klenow enzyme and inserted into pBS-T8 from which the coding region of *ase* had been removed by *Hin*dIII digestion and Klenow filling-in. The resulting construct (pBaseβgal) contained the 2 kb *ase* promoter region and its first six amino-acids fused in frame to the *lacZ* coding region. The fusion gene was transferred to pW8 as an *Asp*718I–*Not*I fragment to give F:2.0. Truncation of this was performed by excising *Acc*I–*Bam*HI and *Sna*BI–*Bam*HI fragments from pBaseβgal (the first site being in the *ase* promoter, the second in the *lacZ* coding region), adding *Bam*HI linkers to the 5′ ends, and reinserting them into pBaseβgal in place of the *Bam*HI promoter fragment (to give F:1.2 and F:560, respectively). The upstream fragment was isolated as a *Bam*HI–*Sna*BI fragment from pBS-T8, *Asp718*I linkers were added, and the fragment inserted into pWZ to give H:1.4. pWZ is a P-element vector that contains a mini*white* gene and a *lacZ* gene bounded by an *hsp70* minimal promoter and polyadenylation signal (unpublished data).

### P-element transformation

P-element transformation of flies was performed as described (Spradling, 1986). The helper P-element plasmid used was pUCπΔ2-3 (Laski et al., 1986). DNA concentrations were 500 ng/μl plasmid and 200 ng/μl helper. At least three independent lines were used for each construct.

### Fly stocks

*Drosophila* stocks were raised on standard cornmeal-yeast-agar medium at 25°C. All stocks are described in Lindsley and Zimm (1992).

### Immunohistochemistry

Rabbit anti-β-galactosidase (Cappel) was used preadsorbed at 1:10,000. Anti-ase is a rabbit polyclonal antiserum against a peptide as described (Brand et al., 1993). Avidin-biotin amplification was used (Elite ABC kit, Vectalabs). Where possible, relevant stainings were performed in parallel for the same durations to allow an estimation of quantitative differences in expression.

### Mutagenesis

Site-directed mutagenesis was a PCR-mediated procedure described by Hemsley et al. (1989). The substrate for mutagenesis was a 580 bp *Bam*HI promoter fragment from fusion gene F:560 subcloned in pBluescript (pBS-Sna). Essentially, 25 bp oligonucleotides were synthesized to the sequences flanking each E-box. PCR was performed with each pair of primers using supercoiled pBS-Sna as the template, yielding a linear product lacking the 6 bp E-box sequence. The product was treated with Klenow enzyme, gel purified, 5′ phosphorylated with polynucleotide kinase and then circularized with DNA ligase. After bacterial transformation, plasmid DNA was isolated from a number of colonies and sequenced (Sequenase, USBiochemicals) using primers to the flanking polylinker. Further rounds of mutagenesis were performed as required for double and triple mutants.

### Protein synthesis

Bacterially expressed l'sc and da proteins were gifts from H. Vaessin and were fusion proteins produced by the pET3 system. sc and ase proteins were produced using the ProBond system (Invitrogen). Their reading frames were synthesized by PCR from plasmid templates and inserted into pRSET in frame with the $His_6$ leader peptide. Growth in BL21(DE3)pLysS bacteria, induction of protein, isolation of protein by binding to a $Ni^{2+}$ resin under denaturing conditions and renaturation are described in Jarman et al. (1993).

### DNA binding

30 bp oligonucleotides were synthesized for the four E-boxes and complementary pairs were annealed. 5′-Terminal G residues were added to the sequence as necessary; labelling was achieved with Klenow enzyme and α[$^{32}$P]dCTP. The sequences are as follows:

| | | |
|---|---|---|
| E1 (+307 bp) | ACAGAGCGCCAG | CAGCTG TCCTGATGCAAG |
| E2 (+183 bp) | TACGCAGGACCT | CAAATG CCTTCTGTTTTG |
| E3 (+45 bp) | GCTCGAGTTGAT | CAGATG TTAGTTTTCCCA |
| E4 (+18 bp) | CCACCCTTGAAC | CAGGTG GACTTTTTGGCT |

Electrophoretic mobility shift assays were performed as described (Jarman et al., 1993), and generally included 0–300 ng protein and ~100 pg oligonucleotide.

## RESULTS

### The phenotype of *ase*$^{1}$ and partial rescue by genomic fragments

To examine the function and regulation of *ase*, we first characterized its loss-of-function phenotype and the ability of genomic fragments to rescue this. Despite its expression in all neural precursors, flies lacking *ase* gene function show only reduced viability. Adults homozygous for the mutation *ase*$^{1}$ (formerly known as *sc*$^{2}$), a 17 kb deletion that is null for *ase*, have a reduced number of scutellar and abdominal bristles (Lindsley and Zimm, 1992; González et al., 1989). We have evidence, however, that most of the adult phenotype is due to a perturbation of the nearby *sc* gene rather than the loss of *ase*, presumably through the loss in *ase*$^{1}$ of part of the enhancer array that directs the expression of *sc* in the scutellar region and other areas (Ruiz-Gómez and Modolell, 1987). This is shown by the inability of these *ase*$^{1}$ defects to be complemented by *In(1)sc*$^{10\text{-}1}$ (which eliminates *ac*, *sc* but not *ase* function) — i.e. the affected bristles are also reduced in *In(1)sc*$^{10\text{-}1}$/*ase*$^{1}$ transheterozygotes (indeed, in such flies loss of scutellar and abdominal bristles

is exacerbated) (unpublished). Nevertheless, we have identified a previously unnoticed character, a defect in the stout row of mechanosensory bristles of the wing margin, that results from lack of *ase* rather than perturbation of *sc* (Fig. 2C–E). This *ase*$^{1}$ defect is seen neither in transheterozygotes with *In(1)sc*$^{10\text{-}1}$ nor in those with *Df(1)sc*$^{19}$ (a deletion of *ac*, *sc*, and *l'sc*, but not *ase*). Examination of the stout row in *ase*$^{1}$ flies shows a rather variable phenotype of deformed bristles. Examples of twinned and socketless bristles, and bristleless sockets can be observed. The defect can be quite severe, with buckling of the wing blade in areas of extensive fusion of sensillum material (Fig. 2D). There may also be missing bristles, but the other defects make this difficult to determine.

To look for rescue, a 4.8 kb genomic fragment that includes the *ase* transcription unit and 1.6 kb from the promoter region was introduced into flies by P-element transformation (Fig. 1, construct G:4.8). We observe that the thoracic and abdominal bristle phenotype is not rescued in *ase*$^{1}$ flies containing up to four copies of G:4.8 (five lines tested), which is consistent with this phenotype's resulting from a perturbation of *sc* function. Conversely, a single copy of the G:4.8 is sufficient to rescue the wing margin defect completely (Fig. 2E, four lines tested), thus confirming that this is a function of *ase*. From mosaic analysis, it had been reported that the stout row bristles were among the few adult external sense organs not affected in AS-C mutants (García-Bellido and Santamaría, 1978), but our results, and more recent mosaic results (M. Guo, M. B, A. P. J, and Y. N. J., unpublished), show that this is not so.

Embryos that lack *ase* are viable, but a specific subset of sense organs is missing (Dambly-Chaudière and Ghysen, 1987). Most consistently, an abdominal papilla, p5, is completely absent. Therefore, we scored this organ to assess the ability of the G:4.8 fragment to rescue the embryonic

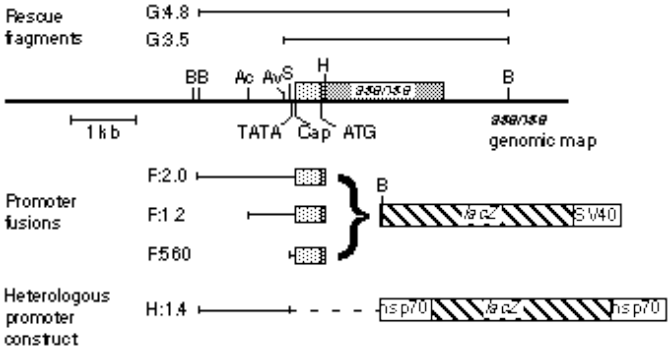


**Fig. 1.** A genomic map of *asense* showing the rescue and fusion gene constructs. The gene is depicted with the 5′ untranslated region unhatched. The transcription and translation start sites are indicated. The four E-boxes mentioned in the text are downstream of the indicated transcription start site. Restriction sites referred to in the making of the constructs are Ac, *Acc*I; Av, *Avr*II; B, *Bam*HI; H, *Hin*dIII; S, *Sna*BI. The fragments used for genomic rescue are depicted above the map. The fusion gene fragments shown below were each fused in frame to a *lacZ* coding sequence that had an SV40 poly(A) signal. For the heterologous promoter construct, H:1.4, the upstream promoter fragment was attached to a *lacZ* gene including a basal *hsp70* promoter.

phenotype. Two independent transformant lines show a partial rescue of this organ (8.7% and 16% respectively in *ase*$^{1}$ larvae with two G:4.8 copies *vs.* 0% and 89% detected in *ase*$^{1}$ and wild-type larvae respectively). There may be a number of reasons for the lack of complete rescue. Expression of *ase* from G:4.8 may be inadequate, either because the pattern is incomplete (for example, if some regulatory elements are outside G:4.8) or the level is too low. Immunohistochemical staining of *ase*$^{1}$ embryos containing two copies of G:4.8 with an antibody specific for ase shows a pattern of expression that appears to be similar to that of the normal gene: essentially, expression is observed in neural precursors (Fig. 2A,B) (Brand et al., 1993). The level of expression, however, appears to be significantly lower than ase expression in wild-type embryos from parallel stainings (not shown). Given the genetic dependence of the adult phenotype of *ase*$^{1}$, it is also possible that the perturbation of *sc* may affect these larval organs.

A shorter genomic fragment that includes only 167 bp upstream of the transcription start site (Fig. 1, G:3.5) has also been tested for rescue of the adult phenotype. Expression of ase is even weaker from G:3.5 than from G:4.8 in both embryos and the imaginal discs (ectodermal primordia of adult epidermis) (not shown). Nevertheless, this low level of expression is sufficient to rescue the wing margin defect.

From the genomic rescues, we conclude that, while some of the regulatory sequences required for *ase* function may be absent from the two genomic fragments examined, an element directing the major pattern of expression in neural precursors has been located, and this is responsible for at least one of the functions of *ase*.

### *ase–lacZ* fusions

To study the action of the regulatory element present in G:4.8, we constructed a fusion gene consisting of the upstream region present in this rescue fragment, including its presumed TATA box and initiation codon (González et al., 1989), fused in-frame to a *lacZ* reporter gene (Fig. 1, F:2.0). This includes the 455 bp 5′ untranslated region (UTR) of the *ase* transcription unit as well as 1.6 kb upstream of the transcription start site.

In embryos containing this fusion gene, all identifiable neural precursors express β-galactosidase as they arise (Fig. 3A–C, three independent lines tested), although this is initially rather weak. In older embryos, owing to perdurance of the β-galactosidase protein, staining is seen in the progeny of the neural precursors (Fig. 3D). In the PNS, this is particularly strong in the neuron and its sister cells. β-galactosidase expression is also seen in other places that express *ase* — particularly in cells of the posterior midgut rudiment from stage 11 onwards [see Campos-Ortega and Hartenstein (1985) for staging], later in the anterior midgut and in some cells of the Malpighian tubules. Neural precursor expression is also seen in the imaginal discs (Fig. 3E). Thus, apart from the perdurance of the β-galactosidase, the pattern of expression is identical to that of ase (Brand et al., 1993).

Truncations were performed to give two fusion genes with 1.2 kb and 560 bp of sequence upstream of the translation start site, respectively (Fig. 1, F:1.2 and F:560) (four

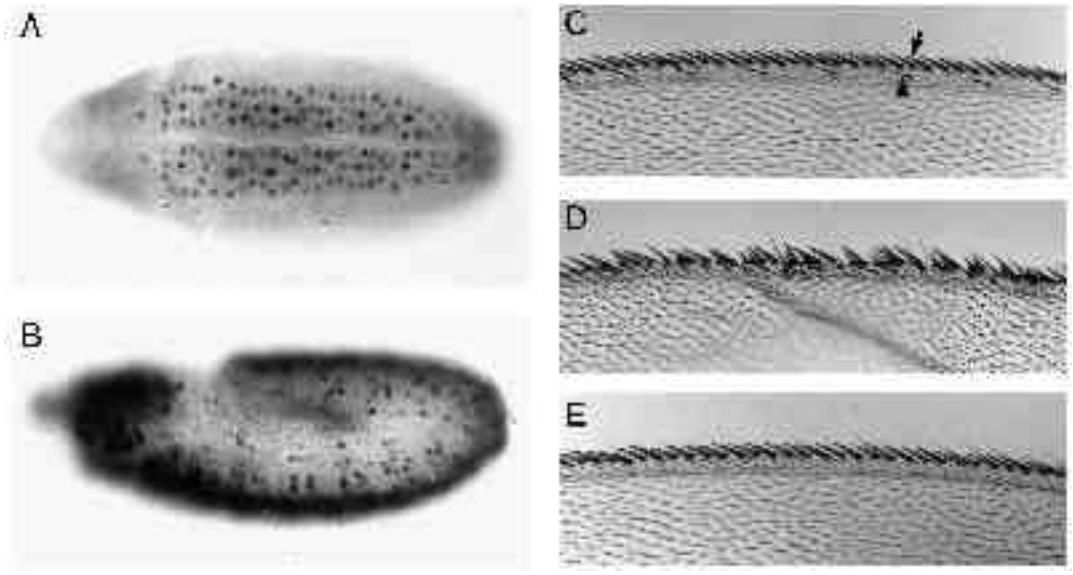


**Fig. 2.** Behaviour of the 4.8 kb *ase* genomic rescue fragment (G:4.8). The fragment supports a neural precursor pattern similar to wild-type *ase*. (A,B) Embryos containing the two copies of the rescue fragment in an *ase*-null background (*ase*$^{1}$) were stained with anti-ase serum. (A) Ventral view of a stage 10 embryo (Campos-Ortega and Hartenstein, 1985) showing the first wave of neuroblasts; some neuroblasts show cytoplasmic staining, presumably due to the breakdown of the nucleus during cell division (Brand et al., 1993). (B) Neural precursors of the PNS staining at stage 11. Neuroblast and ganglion mother cell staining is out of focus. (C-E) The fragment rescues a defect of *ase*$^{1}$ in the stout row of wing margin bristles. (C) Wild-type wing margin, with stout (arrow) and recurved (arrowhead) rows indicated. (D) Representative view of the defect in an *ase*$^{1}$ wing. (E) Wing margin of an *ase*$^{1}$ mutant fly bearing two copies of G:4.8. Note that the recurved row (an apparent defect of *sc*) is not rescued.

lines tested for each). The latter is equivalent to the shorter rescue fragment, G:3.5, and is close to the minimum that can be achieved given that the presumed TATA-box and transcription start site are 480 and 455 bp upstream of the initiation codon, respectively. Both constructs appear to support an identical pattern of β-galactosidase expression to the original fusion (F:560 shown in Fig. 4A–C). The 560 bp fusion is generally weaker, with a more pronounced background. It also shows an extra pattern element consisting of pair-rule-like stripes in the ectoderm from stage 8 onwards, which could indicate the loss of an inhibitory element.

To assess the contribution of the remainder of the 2 kb promoter region excluding the 560 bp proximal fragment, we made use of a basal promoter (*hsp70* TATA box) fused to a *lacZ* coding region. The 1.4 kb upstream portion of the 2 kb fragment was inserted into this (Fig. 1, H:1.4). Of five independent lines analysed, none showed a neural precursor pattern of β-galactosidase expression in either embryos or imaginal discs. In two lines, H:1.4 supports β-galactosidase expression in a small set of late neural cells in the CNS, stomatogastric nervous system and part of the antenno-maxillary complex (Fig. 4). Although we have not excluded position effects, this pattern partly corresponds to a late component of expression observed for *ase* (Brand et al., 1993) and may indicate that a separate component of *ase* regulation is present in H:1.4.

## Behaviour of the fusion gene in mutant backgrounds

As a step in identifying genes that regulate *ase* through the identified promoter element, we have recorded the effect of mutation of the proneural genes on the neural precursor expression pattern of the 2 kb *ase–lacZ* fusion. In embryos carrying the deficiency *Df(2R)da*$^{KX136}$, PNS precursors appear to be absent although initially the neuroblasts of the CNS segregate normally (Ghysen and O'Kane, 1989; Bodmer et al., 1989; Brand and Campos-Ortega, unpublished data; Vaessin et al., unpublished data). We observe an overall reduction in the expression of the fusion gene in the segregating neuroblasts of *da* mutant embryos (Fig. 5A,C), although this is not as pronounced as the reduction seen for the endogenous gene (Brand et al., 1993). Patchy, weak staining is also seen in the epidermis, particularly in the areas that would normally give rise to PNS precursors (Fig. 5A,B).

The effect of removing the genes of the AS-C was analysed in the deficiency *Df(1)sc*$^{B57}$, which lacks all four genes of the complex. It results in the absence of much of the larval PNS, although the chordotonal organs and some multiple dendrite neurons are unaffected (Dambly-Chaudière and Ghysen, 1987). The CNS is also strongly affected later, but early on up to 75% of the neuroblasts segregate normally (Jiménez and Campos-Ortega, 1990). With the fusion gene in this background, the remaining neural precursors of the PNS express β-galactosidase as before. Those CNS neuroblasts that segregate also express β-galactosidase, but the onset of expression is significantly delayed, often until late in stage 10 (Fig. 5D). Normally, the neuroblasts express β-galactosidase before it is detectable in the PNS precursors. In mutant embryos, however, expression is seen in one of the two earliest arising PNS precursors (the P cell, which gives rise to the abdominal lateral

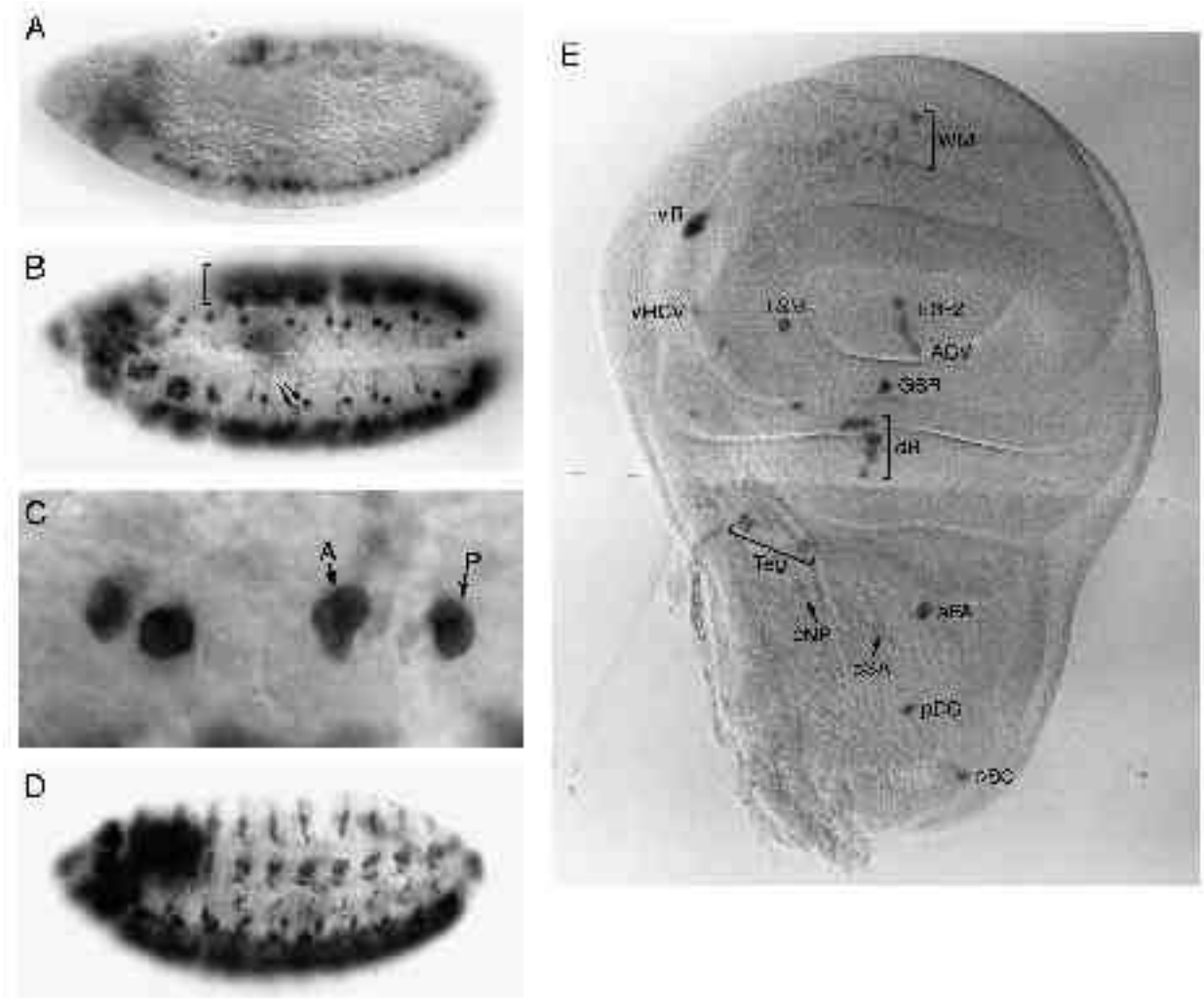


**Fig. 3.** 2 kb of the *ase* upstream sequence can drive expression of β-galactosidase in a neural precursor pattern. (A–D) Wild-type embryos containing the 2 kb *ase-lacZ* fusion gene (F:2.0) were stained with an antibody to β-galactosidase. (A) Lateral view of a stage 10 embryo. Neuroblasts are seen in cross section below the epidermis. Staining in the posterior midgut anlage occurs slightly later than this. (B) Stage 11 embryo, lateral view showing the earliest PNS precursors. These correspond to the A and P cells as seen in enhancer trap line A37 (Ghysen and O'Kane, 1998), which give rise to an external sense organ (des2) and a chordotonal organ (lch5) respectively (Dambly-Chaudière et al., 1992; Ghysen and O'Kane, 1989). The expression in the neuroblasts and ganglion mother cells of the developing CNS is bracketed. (C) The A and P precursors magnified. At this stage, each appears to consist of a pair of cells. (D) Lateral view of a stage 16 embryo showing perdurance in the PNS. Staining is most apparent in the neurons and inner support cells. (E) β-galactosidase staining of a wing disc of a wandering third instar larva of the same line, with identified precursors labelled [see Huang et al. (1991) for nomenclature]. The onset of staining appears to be somewhat delayed relative to *ase*, possibly reflecting a slower rate of synthesis of β-galactosidase and the cytoplasmic nature of the protein.

chordotonal organ, lch5, Ghysen and O'Kane, 1989) before it is seen in the neuroblasts. Therefore, although the remaining neuroblasts in *Df(1)sc*$^{B57}$ embryos segregate at the correct time, it appears that they do not activate the fusion gene normally.

In *Df(1)sc*$^{B57}$ embryos, the other early PNS precursor, the A cell (see Fig. 3), is reported to be absent, consistent with its being the precursor of an external sense organ that is dependent on AS-C gene function (Ghysen and O'Kane, 1989). Curiously, we observe in *Df(1)sc*$^{B57}$ embryos that the fusion gene is sometimes transiently activated in a large subepidermal cell in the position expected for this neural precursor (Fig. 5D) [11% of scored segments (*n*=147)]. The cell disappears soon after. We note that the A cell gives rise to a partially *ase*-dependent sense organ (Dambly-Chaudière and Ghysen, 1987; Dambly-Chaudière et al., 1992) (see Discussion).

In the deficiency *Df(1)sc*$^{19}$, all the members of the AS-C are deleted except *ase*. Its embryonic phenotype differs from *Df(1)sc*$^{B57}$ in a less severe reduction of the CNS and the additional presence of certain PNS elements that are partially *ase*-dependent. In the PNS, at late stages we now see high levels of fusion gene expression in the *ase*-dependent organs that form in addition to those elements seen in *Df(1)sc*$^{B57}$ embryos (Fig. 5G,H). In the neuroblasts, the delay in expression of the fusion gene is less pronounced

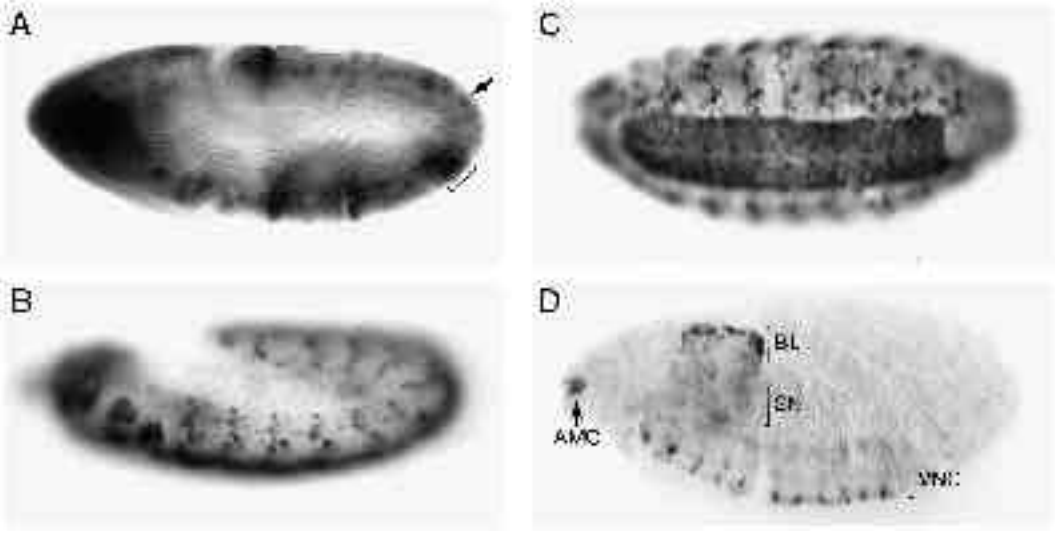


**Fig. 4.** A 560 bp *ase* fragment also supports a neural precursor pattern. (A–C) Wild-type embryos containing 560 bp *ase-lacZ* fusion gene (F:560). (A) Early CNS cross-section at stage 9, showing neuroblast staining (arrow) and extra epidermal stripes (bracket). (B) Early PNS, stage 11 but slightly later than Fig. 3B. (C) Ventro-lateral view of a stage 16 embryo showing perdurance of staining in the ventral nerve cord and ventral clusters of the PNS. (D) The remainder of the 2 kb promoter region (H:1.4) directs expression in a subset of the nervous system, as shown in this stage 16 embryo. A subset of ventral nerve cord (VNC) and brain lobe (BL) cells show expression, as do the antenno-maxillary complex (AMC) and stomatogastric nervous system (SN, mostly out of focus).

than for $Df(1)sc^{B57}$ embryos, possibly due to regulation by endogenous *ase* despite its delayed activation (Fig. 5F).

In summary, the reduced expression in $Df(2)da^{KX136}$ neuroblasts and the delayed expression in $Df(1)sc^{B57}$ neuroblasts suggests that activation of the F:2.0 fusion gene is at least partly due, directly or indirectly, to *da* and the genes of the AS-C, including *ase* itself. The remaining expression in these mutants, however, means that additional factors must also play a role.

## AS-C/da-binding sites in the 560 bp fragment

To determine whether the neural precursor expression pattern supported by the fusion gene can be attributed to direct regulation by the binding of proneural gene products, we examined the sequence of the 560 bp minimal *ase* fragment from fusion gene F:560 for potential binding sites (González et al., 1989). bHLH proteins bind to variants of a consensus sequence known as the E-box (CANNTG) (Murre et al., 1989b), and four such E-boxes are present in the *ase* sequence, each in the 5′UTR. To look for binding, oligonucleotides were synthesized (see Materials and Methods) and electrophoretic mobility shift assays were performed using bacterially synthesized proteins sc, l'sc, and da. Two of the sites, E1 (CAGCTG) and particularly E4 (CAGGTG), bind l'sc/da hetero-oligomers (presumably heterodimers) strongly (Fig. 6A). The E-box sequences at these sites match those previously identified as binding sites for AS-C/da heterodimers, all of which have the consensus $CAG^{C}/_{G}TG$, (Van Doren et al., 1991; Cabrera and Alonso, 1991; H. Vaessin, unpublished). The other two potential sites bind l'sc/da heterodimers only weakly (E3; CAGATG) or extremely weakly (E2; CAAATG), correlating with their decreasing similarity to the consensus. Heterodimers of sc/da bind in a similar fashion (Fig. 6B). In agreement with previous reports (Cabrera and Alonso, 1991; Van Doren et al., 1991), we find that AS-C homodimers bind very poorly, the strongest detected being a very weak binding of l'sc/l'sc to E1. The binding of da/da homodimers is also poor.

We have also examined the ability of ase itself to bind to the E-box sequences in its 5′ UTR (Fig. 6C). Bacterially expressed ase protein binds as a heterodimer with da in a manner similar to l'sc and sc: ase/da binds well to sites E1 and E4, poorly to E3, and minimally to E2. Unlike sc, l'sc or da, we also observe the binding of ase/ase homodimers to site E1, and occasionally to E4. At equivalent or higher protein inputs, l'sc and sc barely show binding as homodimers. Thus, the binding properties of ase appear to differ from those of the other AS-C genes.

## In vitro mutagenesis of the AS-C/da-binding sites

On the evidence of the in vitro binding, we looked for an effect of deleting the E-boxes on the regulation of the fusion gene. Deletions of 6 bp were made to the 560 bp *ase–lacZ* fusion gene and flies were transformed. Deletion of single sites E1, E3 or E4 was found to have no detectable effect on expression in either embryos or discs (E2 was not done). Concluding that this may be due to redundancy between the binding sites, we then performed a double deletion of both strong sites (E1 and E4). With this construct (5 lines examined), neural precursor expression was still apparent, but the level of expression was discernably lower than the unmutated construct (Fig. 7). Also, we now observe much greater patchy expression in the ectoderm as though there is some deregulation of the fusion gene. Embryos containing a triple deletion of all three sites that showed appreciable binding (E1, E3 and E4) were indistinguishable from those with the doubly deleted construct (not shown).

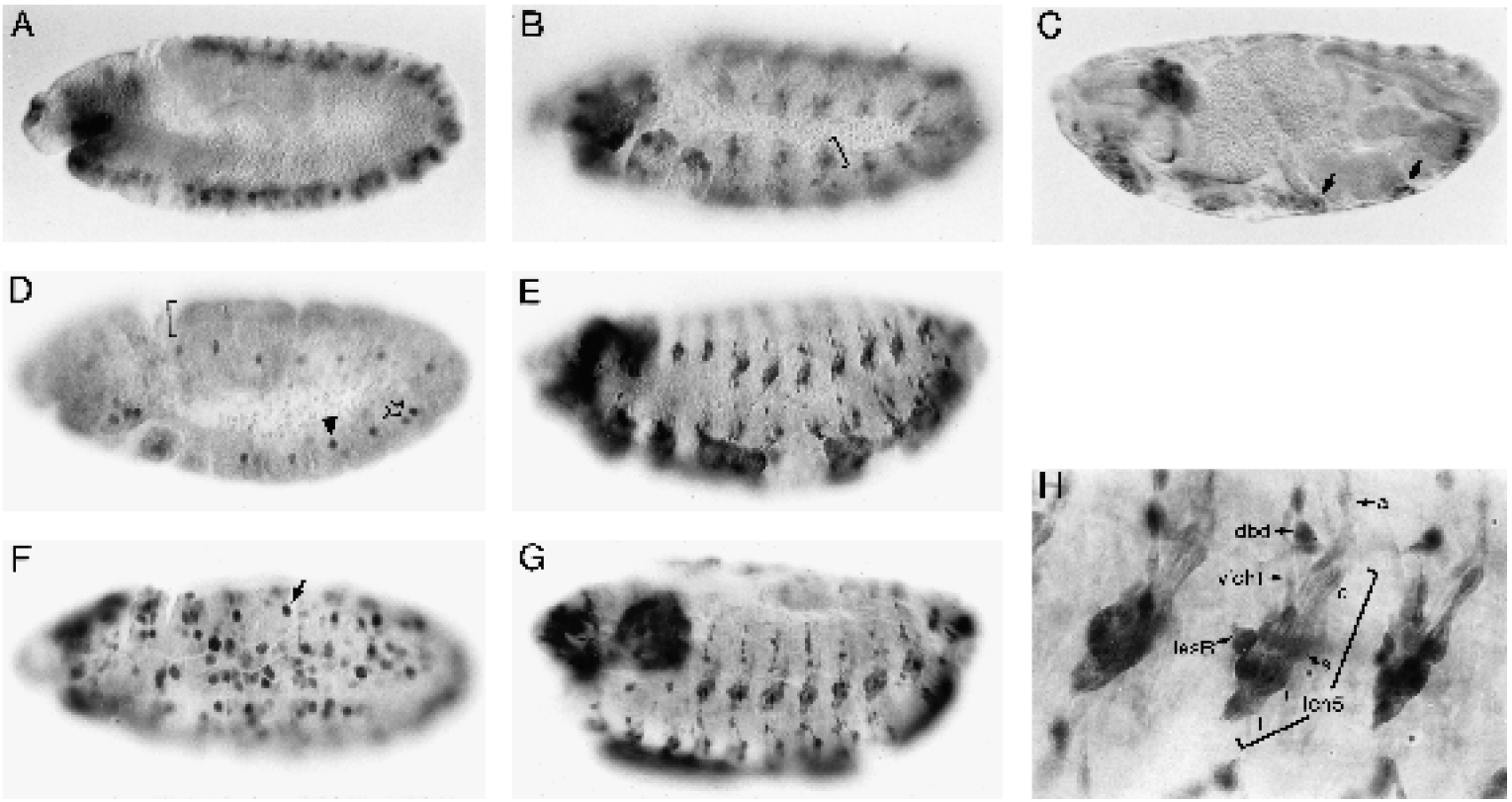


**Fig. 5.** Expression of the 2 kb *ase–lacZ* fusion gene in proneural mutants. (A–C) Expression in *Df(2R)da*$^{KX136}$ embryos. (A) Stage 10, showing expression in ectodermal cells as well as in neuroblasts. (B) Stage 11 PNS, showing residual patchy staining in the ectoderm (bracket). (C) At stage 16, some staining is seen in the remnant CNS (arrows), mostly corresponding to the late phase of *ase* expression, which is *da*-independent. (D,E) Expression in *Df(1)sc*$^{B57}$ embryos. (D) Staining in the neuroblasts (bracket) is delayed until after staining is seen in the first PNS precursor (P cell, arrowhead) in a stage 11 embryo (cf. Fig. 3B). Note that staining is seen in an additional cell (open arrow), which may correspond to the aborted formation of the A precursor. (E) At stage 16, β-galactosidase is seen in the remaining PNS (chordotonal organs and multiple dendritic neurons) and in the remnant of the CNS. (F–H) Expression in *Df(1)sc*$^{19}$. (F) The delay in neuroblast expression is less apparent, as seen in ventro-lateral view of a stage 10 embryo. The P precursor of lch5 is indicated by an arrow. Staining of the neuroblasts begins rather unevenly. (G). At stage 16, expression is additionally seen in those PNS elements that now form due to the *ase* function (external sense organs). (H) Magnified view of the lateral PNS cluster of three abdominal segments of a *Df(1)sc*$^{19}$ late embryo, showing perdurance of fusion gene expression in PNS elements with a variety of identities and origins. Indicated are the four cells of each chordotonal organ in lch5 (cap, c; sheath, s; neuron, n; and ligament, l), the extra attachment cells of lch5 (a), part of v′ch1, the dorsal bipolar dendritic neuron (dbd), and the *ase*-dependent external sense organ (lesB). The expression in the midgut primordium is abolished in *Df(1)sc*$^{B57}$, *Df(1)sc*$^{19}$ and *Df(2R)da*$^{KX136}$ backgrounds (out of focus).

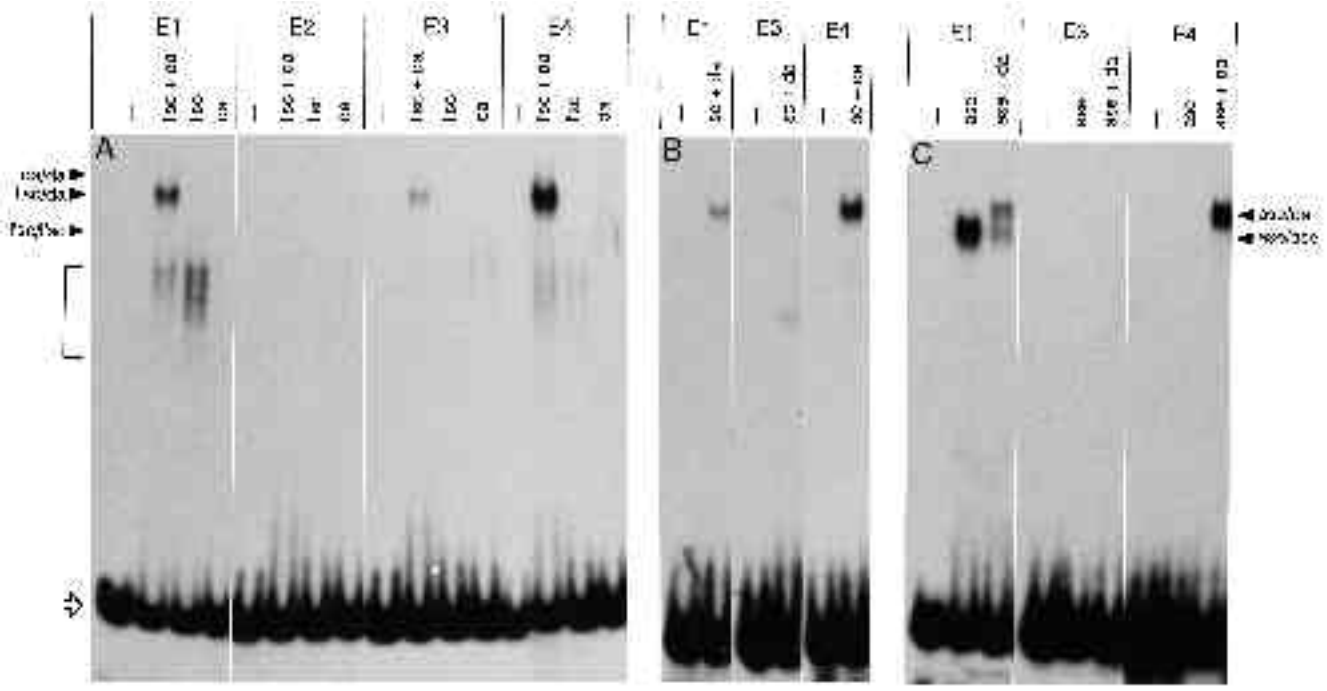


**Fig. 6.** Binding of AS-C and da proteins to E-boxes within the 560 bp *ase* regulatory fragment. Oligonucleotides to the four sites were used in electrophoretic mobility shift assays (Materials and Methods). (A) Binding of l'sc and da proteins. The heterodimer shifted species is indicated. The mobility of the homodimers is also indicated, but the very weak bindings of l'sc/l'sc homodimers to E1 and da/da to E4 are not apparent on this exposure. Bracketed shifts are due to bacterial components of the protein extracts. (B) Binding of sc and da proteins. The shifted band corresponds to the heterodimer. (C) Binding of ase and da proteins. In addition to ase/da heterodimer binding, strong binding of ase/ase homodimers to E1 is observed. The amounts of protein in each binding reaction is approximately equivalent (~200 ng), but comparisons of relative affinity between the different proteins is not possible as the proportion of active protein (correctly folded) in each extract is not known. Open arrow, free probe.

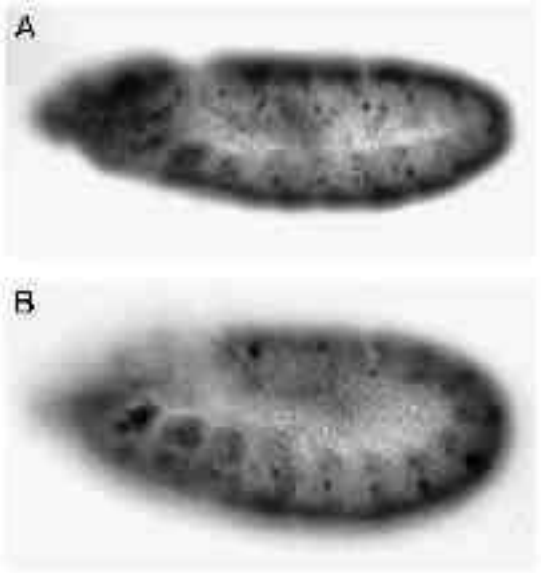


**Fig. 7.** Deletion of the strong AS-C/da-binding sites affects the expression of the 560 bp *ase–lacZ* fusion gene (F:560). (A) Unmutated F:560 showing staining of a stage 11 embryo. (B) A stage 11 embryo from a transformed line in which the two strong AS-C/da-binding sites, E1 and E4, were removed by 6 bp deletions (F:560Δ1,4). A reduction in expression level is seen, with increased patchiness of staining in the epidermis from which PNS precursors are arising. Note that the staining in the P precursor remains.

## DISCUSSION

Despite their different identities and origins, all neural precursors express a common set of products derived from the neural precursor genes (Vaessin et al., 1991; Bier et al., 1992). As a step in understanding how this is achieved, we have examined the regulation of *ase* as a representative of this group of genes. To this end, we have located the minimal *ase* regulatory region that is able to support its neural precursor pattern of expression. The transcription factors of the proneural genes (AS-C and da) are prime candidates for neural precursor gene activation and we have established that the *ase* regulatory element contains AS-C/da-binding sites. Since the proteins of the AS-C are expressed in proneural clusters, this raises the question of how *ase* expression is confined to the neural precursor. We discuss the possibilities for this regulation. Furthermore, in line with its differing expression pattern, we have also found other distinguishing features between *ase* and the other AS-C genes: we have characterized an *ase* function that suggests a role later in neurogenesis, after neural precursor formation. We also show a difference in DNA-binding properties of ase protein.

### The function of *ase* in the neural precursor

The AS-C genes are thought to be involved in the earliest stages of neural precursor formation. *ac*, *sc* and *l'sc* have an

expression pattern (in proneural clusters) and mutant phenotype (absence of sense organs and their precursors) consistent with this. Given the later expression of *ase*, one may ask whether this gene has a later function in neural precursors. However, the analysis of embryonic phenotypes (Ghysen and Dambly-Chaudière, 1987; Jiménez et al., 1990), the similar effects of ectopic expression of *sc*, *l'sc* or *ase* (Rodríguez et al., 1990; Brand et al., 1993) and the DNA-binding characteristics (Cabrera and Alonso, 1991; Van Doren et al., 1991; this report) all suggest a high degree of interchangeability between the products of the AS-C, including *ase*. Taken with its later expression pattern, one possible function of *ase* is to reinforce the effect of the other AS-C products in determining the neural precursor state.

We have obtained some observations, however, that suggest a later role for *ase* in some neural precursors that is more distinct than a simple boosting of proneural gene function. In flies lacking *ase*, we have identified that the stout row of bristles on the wing margin shows such morphological defects as stunting, fusing and twinning, as opposed to a simple absence of sense organs. Introduction of genomic *ase* fragments can efficiently rescue the wing-margin defect. This suggests a role for *ase* in the later development of the neural precursors of the stout row or their descendents after their formation. The reported misrouting of axons in the optic lobe of $ase^1$ flies may also point to a later function (González et al., 1989).

We have a similar observation concerning *ase* function in the embryo. Certain external sense organs depend on AS-C function, but can form even when *ase* is the only AS-C gene present (Dambly-Chaudière and Ghysen, 1987). Although this suggests an early proneural function for *ase*, we have found that, in the case of one such partially *ase*-dependent sense organ, its precursor (the A cell) appears occasionally to delaminate in the absence of any AS-C gene function, implying that here too the requirement for *ase* is in precursor survival/development rather than in formation. It also suggests that unidentified (proneural) factors are responsible for the initial formation of this precursor.

## Role of proneural genes in the neural precursor regulation of *ase*

Our analysis shows that 560 bp of the *ase* locus can direct a neural precursor pattern from a *lacZ* reporter gene — a portion that consists of the 5′ UTR of the transcription unit and the upstream TATA sequence. This segment contains multiple binding sites for AS-C/da heterodimers, suggesting that neural precursor genes are directly activated by the bHLH products of the proneural genes of the AS-C complex and *da*. Nevertheless, removal of these sites lowers but does not abolish *lacZ* expression in neural precursors. In contrast, Van Doren et al. (1992) have shown that mutation of similar AS-C/da-binding sites in an *ac* autoregulatory element leads to the complete loss of the proneural cluster expression that this *ac–lacZ* fusion gene normally exhibits (Martínez and Modolell, 1991). This difference may help to explain the different expression patterns of the two genes despite their shared possession of AS-C/da-binding sites; the proneural pattern of *ac* relies strongly on its AS-C/da-binding sites, while the neural precursor pattern of *ase* requires additional regulatory inputs.

As evident from the continued expression of the *ase–lacZ* fusion in mutants of AS-C and *da*, it is likely that the additional factors that regulate *ase* are also responsible for the remaining neural precursor formation seen in these mutants. Many other potential transcription factors are expressed in neural precursors and could be involved in neural precursor gene activation, including a number of zinc-finger-containing proteins, such as *hb* (Tautz et al., 1987; Jiménez and Campos-Ortega, 1990), *Krüppel* (*Kr*, Gaul et al., 1987) and *scratch* (E. Bier, unpublished) (see Cabrera, 1992). The stronger effect of the *da* mutant on fusion gene expression also suggests that da may have other unidentified bHLH partners, both in the CNS (responsible for the remaining neuroblast formation) and in the PNS (responsible, for example, for chordotonal organ formation). Indeed, we have recently isolated a candidate proneural gene for chordotonal organ formation (Jarman et al., 1993). The bHLH product of this gene, *atonal*, also binds to the E-boxes in the *ase* 5′-UTR as a heterodimer with da.

There may also be negative influences that prevent activation of *ase*, but not *ac*, in the proneural clusters by AS-C/da proteins. A number of observations indicate this, in particular the abnormal ectodermal expression that is seen when the AS-C/da-binding sites are deleted. It is possible that the AS-C/da-binding sites are adjacent to, or overlap with, binding sites for negative regulators. Close proximity of positive and negative sites is a common means of sharply defining expression patterns, as seen in the studies of *even-skipped* (Small et al., 1991), *Kr* (Hoch et al., 1992), *Ultra - bithorax* (Müller and Bienz, 1992), and *rhomboid* (Ip et al., 1992). A number of the neurogenic genes involved in lateral inhibition within the proneural clusters encode potential transcription factors [*E(spl)*, *neuralized*], and thesewould be candidate inhibitors of *ase* within the clusters.

## *ase* as a transcription factor

As would be predicted from their similar bHLH domains, the DNA-binding properties of ase protein are very similar to those of ac, sc and l'sc. For the four E-boxes in the *ase* promoter, the binding of ase/da heterodimers occurs in a similar fashion to that of sc/da and l'sc/da; that is, binding is strongest to a consensus of $CAG^{C}/_{G}TG$. Nevertheless, the upstream sequence is not simply an autoregulatory element — the pattern and level of expression from the 2 kb *ase–lacZ* fusion gene is essentially unchanged in $ase^1$ embryos (not shown). An apparent difference in binding properties, however, is that, unlike ac, sc or l'sc, ase can also bind well as a homodimer. It is known that a truncation allows the bHLH protein E12/47 to bind as a homodimer (Sun and Baltimore, 1991). Also, the removal of the C terminus from l'sc appears to increase homodimerization as measured by immunoprecipitation (Cabrera and Alonso, 1991). Interestingly, this C-terminal sequence is conserved in ac, sc and l'sc, but not in ase (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; González et al., 1989). It remains to be determined whether the ability of ase to bind to DNA as a homodimer reflects some aspect of its function, such as a potential later role in the developing neural precursors or their progeny.

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