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The expression and function of the *achaete-scute* genes in *Tribolium castaneum* reveals conservation and variation in neural pattern formation and cell fate specification

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SUMMARY

The study of *achaete-scute* (*ac/sc*) genes has recently become a paradigm to understand the evolution and development of the arthropod nervous system. We describe the identification and characterization of the *ac/sc* genes in the coleopteran insect species *Tribolium castaneum*. We have identified two *Tribolium ac/sc* genes – *achaete-scute homolog* (*Tc-ASH*) a proneural gene and *asense* (*Tc-ase*) a neural precursor gene that reside in a gene complex. Focusing on the embryonic central nervous system we find that *Tc-ASH* is expressed in all neural precursors and the proneural clusters from which they segregate. Through RNAi and misexpression studies we show that *Tc-ASH* is necessary for neural precursor formation in *Tribolium* and sufficient for neural precursor formation in *Drosophila*. Comparison of the function of the *Drosophila* and *Tribolium* proneural *ac/sc* genes suggests that in the *Drosophila* lineage these genes have maintained their ancestral function in neural precursor formation and have acquired a new role in the fate specification of individual neural precursors. Furthermore, we find that *Tc-ase* is expressed in all neural precursors suggesting an important and conserved role for *asense* genes in insect nervous system development. Our analysis of the *Tribolium ac/sc* genes indicates significant plasticity in gene number, expression and function, and implicates these modifications in the evolution of arthropod neural development.

Key words: *achaete-scute*, *Tribolium castaneum*, *Drosophila melanogaster*, Central nervous system

INTRODUCTION

The *achaete-scute* (*ac/sc*) genes are key components of the genetic regulatory network that governs the formation and patterning of the arthropod nervous system. *ac/sc* genes encode phylogenetically conserved basic helix-loop-helix (bHLH) containing transcription factors that are known to promote the initial commitment of cells to the neural fate in species as diverse as flies and spiders (Garcia-Bellido and Santamaria, 1978; Balcells et al., 1988; Stollewerk et al., 2001). The *ac/sc* genes are thought to initiate nervous system development in all arthropods. Therefore, many of the evolutionary modifications to nervous system pattern that exist within the Arthropoda are probably caused by modifications to *ac/sc* gene expression, regulation or function. As such, the *ac/sc* genes have become a model for deciphering the evolution and development of the arthropod nervous system (Skaer et al., 2002a).

During *Drosophila* nervous system development, the proneural *ac/sc* genes are first deployed in stereotyped patterns of ectodermal cell clusters (proneural clusters). Proneural *ac/sc* gene expression confers upon naive ectodermal cells the ability to acquire the neural precursor fate. Within each proneural cluster, one or more cells retain proneural *ac/sc* gene expression and commit to the neural precursor fate, while the remaining cells in the cluster take on an epidermal fate. Once formed, neural precursors activate expression of neural precursor genes and rapidly extinguish proneural *ac/sc* gene expression. Neural precursor genes are expressed in all neural precursors and appear to promote the division and differentiation of these cells (Dominguez and Campuzano, 1993; Jarman et al., 1993; Wallace et al., 2000).

Pioneering genetic and molecular studies led to the identification and characterization of the four *Drosophila ac/sc* genes. These genes exist in a complex spanning ~100 kb at the distal tip of the X-chromosome. Three of the genes, *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*), were found to promote neural precursor formation and were therefore termed 'proneural genes'; the fourth gene, *asense* (*ase*), is expressed only in neural precursors and is thus termed a neural precursor gene. Genetic studies established the essential role of *ac, sc* and *l'sc* in promoting the initial decision of ectodermal cells to acquire the neural precursor fate (Garcia-Bellido and Santamaria, 1978; Balcells et al., 1988). Expression studies

showed that *ac* and *sc* are expressed in identical patterns of proneural clusters during central (CNS) and peripheral nervous system (PNS) development, while *l'sc* is expressed in a broader and mostly complementary pattern of proneural clusters in the CNS and only minimally in the PNS (Cubas et al., 1991; Martin-Bermudo et al., 1991; Skeath and Carroll, 1991; Skeath and Carroll, 1992). In the CNS, the composite expression patterns of *ac*, *sc* and *l'sc* mark all proneural clusters and their associated neural precursors. In addition to their role in neural precursor formation, *ac* and *sc* play a separate role in specifying the individual fate of neural precursors. While *ac*, *sc*, and *l'sc* are functionally interchangeable with respect to neural precursor formation, only *ac* and *sc* can promote the proper gene expression profile and cell division pattern of the MP2 precursor (Parras et al., 1996; Skeath and Doe, 1996). Thus in the *Drosophila* CNS, *ac/sc* genes can regulate both the formation and individual fate specification of neural precursors.

Owing to their central role in the formation of the *Drosophila* CNS and PNS the *ac/sc* genes have become a focal point for understanding the evolution of nervous system pattern in arthropods (Skaer et al., 2002a). To date, *ac/sc* genes have been identified in the medfly *Ceratitis capitata* (Wulbeck and Simpson, 2000), the blowflies *Calliphora vicina* and *Phormia terranovae* (Pistillo et al., 2002; Skaer et al., 2002b), the malarial mosquito *Anopheles gambiae* (Wulbeck and Simpson, 2002), and the spider *Cupiennius salei* (Stollewerk et al., 2001). The number of insect proneural *ac/sc* genes differs, with three in *Drosophila*, two in *Ceratitis* and *Calliphora* and one in *Anopheles*, while each species has a single *asense* gene. Thus, the basic subdivision of *ac/sc* genes into proneural and *asense* genes and the functional roles these classes play in nervous system development appear well conserved. In support of the conservation of proneural *ac/sc* and *asense* function within arthropods, RNAi studies in *Cupiennius* suggest that one of the *ac/sc* genes carries out a proneural-like function (*Cs-ASH1*) while the other (*Cs-ASH2*) carries out an *asense*-like function (Stollewerk et al., 2001).

To explore further the roles *ac/sc* genes play during arthropod nervous system development and evolution we have focused on the red flour beetle *Tribolium castaneum* (Coleoptera), a species ~300 million years diverged from *Drosophila*. Here we present the isolation of the *Tribolium ac/sc* genes, and the characterization of their genomic organization, expression and function. We have identified two *Tribolium ac/sc* genes, *achaete-scute homolog* (*Tc-ASH*) and *asense* (*Tc-ase*), and determined that these genes reside 55 kb apart from each other and thus define the *Tribolium ac/sc* complex. Gene expression studies demonstrate that *Tc-ASH* is a proneural gene expressed in all proneural clusters and transiently in all neural precursors. Functional studies indicate that *Tc-ASH* is necessary for neural precursor formation in *Tribolium* and sufficient for neural precursor formation in *Drosophila*. These studies, however, do not support a role for *Tc-ASH* in specifying the individual fate of neural precursors, suggesting that the ability of *ac* and *sc* to regulate this process may represent a recent evolutionary specialization within the Diptera. We also show that *Tc-ase*, like other arthropod *asense* genes, is expressed in all neural precursors. Thus, these studies indicate significant plasticity in *ac/sc* gene number, expression and function since the divergence of *Tribolium* and *Drosophila*.

MATERIALS AND METHODS

Isolation of ac/sc genes from Tribolium castaneum

The bHLH regions of *Tc-ASH* and *Tc-ase* were amplified from *Tribolium* genomic DNA using degenerate oligonucleotide primers corresponding to the amino acid sequences RERNRVK/AVEYIR (forward/reverse). Full-length *Tc-ASH* was isolated by screening a *Tribolium* cDNA library, and full-length *Tc-ase* was isolated using several rounds of inverse PCR. Both *Tc-ASH* and *Tc-ase* were tested in high stringency Southern blots to verify their *Tribolium* origin.

BAC library screening and BAC sequencing

An arrayed *Tribolium* BAC library (Brown et al., 2002) was screened independently with *Tc-ASH* and *Tc-ase* resulting in 25 hybridization-positive BAC clones, 16 clones hybridized to *Tc-ase*, 7 clones hybridized to *Tc-ASH*, and 2 clones hybridized to both genes. A shotgun library was made from one of the BACs positive for both genes and 1820 paired sequencing reads were generated (SeqWright, Houston, TX). Reads were assembled into a single 128 kb contig using PHRED, PHRAP and Consed (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998). The accuracy of the assembly was verified using paired read analysis as well as comparison of virtual and actual restriction digests.

Rearing and preparation of *Tribolium castaneum*

Tribolium castaneum were purchased from Carolina Biological (Burlington, NC) and maintained at 30°C on white flour supplemented with 2% yeast. Embryos were collected by size exclusion on a standard testing sieve (Fisher Scientific, Chicago, IL) and fixed using standard protocols (Mitchison and Sedat, 1983).

Immunohistochemistry and RNA in situ hybridization of whole mount embryos

Immunohistochemistry and RNA in situ analyses were performed essentially as described previously (Skeath, 1998). Mouse 4D9 anti-Engrailed/Invected was used at a 1:5 dilution (Patel et al., 1989).

Germline transformation and RNAi

A full-length cDNA of *Tc-ASH* was subcloned into the pUAST vector (Brand and Perrimon, 1993) and five independent transgenic *Drosophila* lines were established by standard germline transformation protocols (Rubin and Spradling, 1982). *Tribolium* RNAi was performed as previously described (Brown et al., 1999) except that after injection embryos were incubated at 30°C for 18-28 hours without an oil overlay, in a humid box and then fixed.

Phylogenetic analysis

Phylogenetic analysis was performed using programs from the PHYLIP package (Felsentein, 1993). Protein sequence corresponding to the basic, first helix, and second helix of the bHLH region, aligned using CLUSTALW, was used in either distance (PROTDIST) or parsimony (PROTPARS) methods.

RESULTS

Identification of *ac/sc* homologs in *Tribolium castaneum*

The bHLH region of all known *ac/sc* homologs is well-conserved and can be distinguished from other bHLH regions by a number of highly conserved residues within the two

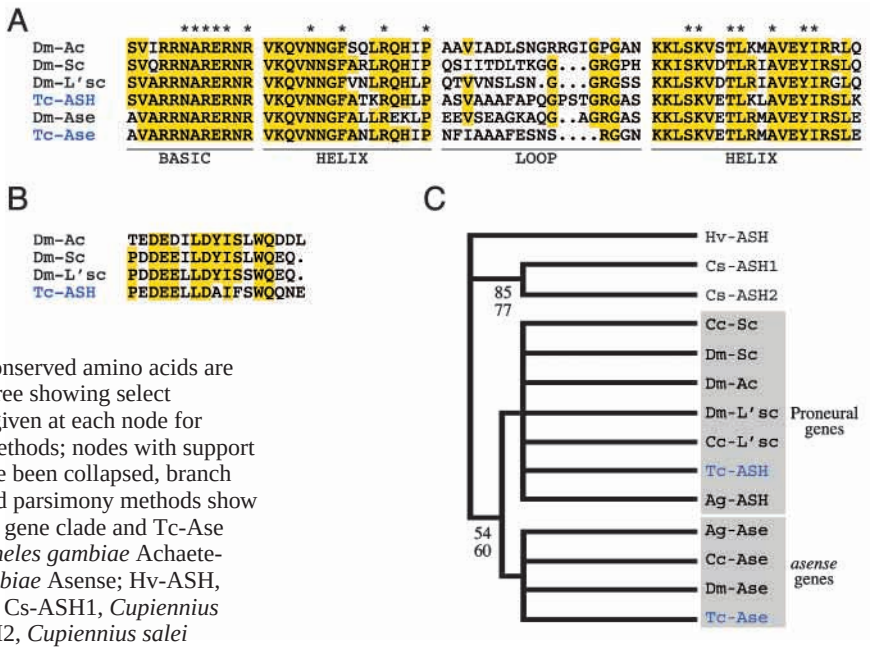

Fig. 1. *Tribolium castaneum* has two *ac/sc* homologs. (A) Comparison of the amino acid sequences from the bHLH domains of the *Tribolium ac/sc* genes (Tc-SH, Tc-Ase) with those from *Drosophila* (Dm-Ac, Dm-Sc, Dm-L'sc). Amino acids conserved in four or more proteins are highlighted in yellow and asterisks indicate residues conserved in all *ac/sc* bHLH domains. (B) Alignment of the C-terminal motif from *Drosophila* proneural *ac/sc* genes with the C-terminal motif of Tc-ASH. Conserved amino acids are highlighted in yellow. (C) Phylogenetic tree showing select invertebrate *ac/sc* genes; support values given at each node for distance (top) and parsimony (bottom) methods; nodes with support values less than 50 for either method have been collapsed, branch lengths are not to scale. Both distance and parsimony methods show that Tc-ASH groups within the proneural gene clade and Tc-Ase within the Asense clade. Ag-ASH, *Anopheles gambiae* Achaete-Scute Homolog; Ag-Ase, *Anopheles gambiae* Asense; Hv-ASH, *Hydra vulgaris* Achaete-Scute Homolog; Cs-ASH1, *Cupiennius salei* Achaete-Scute Homolog 1; Cs-ASH2, *Cupiennius salei* Achaete-Scute Homolog 2; Cc-Sc, *Ceratitis capitata* Scute; Cc-L'sc, *Ceratitis capitata* Lethal of scute; Cc-Ase, *Ceratitis capitata* Asense; Dm-Ac, *Drosophila melanogaster* Achaete; Dm-Sc, *Drosophila melanogaster Scute*; Dm-L'sc, *Drosophila melanogaster* Lethal of scute; Dm-Ase, *Drosophila melanogaster* Asense; Tc-ASH, *Tribolium castaneum* Achaete-Scute Homolog; Tc-Ase, *Tribolium castaneum* Asense.

helices (Fig. 1A, asterisks). We took advantage of these highly conserved residues and used degenerate PCR in combination with inverse PCR and RACE to isolate *ac/sc* genes from *Tribolium*. Extensive application of these methods identified two *Tribolium ac/sc* genes: *Tc-ASH* and *Tc-ase*. Conceptual translation shows that both genes encode the characteristic *ac/sc* basic and helical domains flanking the non-conserved loop domain (Fig. 1A).

Comparative sequence analysis of the *Tribolium* and *Drosophila* Ac/Sc proteins shows that within the bHLH region Tc-ASH shares 66% amino acid identity with Ac, 65% with L'sc, 61% with Sc, and 77% with Ase. In addition to the bHLH, Tc-ASH shares a C-terminal ~16 amino acid motif with Ac, Sc, L'sc and all other known proneural Ac/Sc proteins but not Asense proteins (Fig. 1B). Within the bHLH, Tc-Ase exhibits 77% amino acid identity with Ase, 71% with L'sc, 69% with Sc and 65% with Ac. Tc-Ase does not contain the C-terminal motif present in all proneural Ac/Sc proteins. These data suggest that *Tc-ASH* is a proneural gene and *Tc-Ase* an *asense* homolog.

In phylogenetic analysis, both distance and parsimony methods group all of the insect Ac/Sc proteins consistently into proneural and Asense clades (Fig. 1C). In these analyses, Tc-ASH always groups in the proneural clade and Tc-Ase in the Asense clade (Fig. 1C). However, the conserved bHLH region is short (just 48 amino acids) and contains a high degree of amino acid identity obscuring the phylogenetic relationships between individual Ac/Sc proteins within the proneural and Asense clades.

The *Tribolium ac/sc* genes exist in a complex

In *Drosophila*, the *ac/sc* genes exist in a complex, probably because they share regulatory elements (Gomez-Skarmeta et al., 1995). However, despite the identification of *ac/sc* genes in several species we know little about the genomic organization of *ac/sc* genes outside of the Diptera. To determine if *Tc-ASH* and *Tc-ase* exist in a complex we independently screened a *Tribolium* BAC library (Brown et al., 2002) with each *Tribolium ac/sc* gene. We identified 25 clones, two of which hybridized to both *Tc-ASH* and *Tc-ase* indicating that the two *Tribolium ac/sc* genes are linked.

To determine the precise molecular nature of the *Tribolium ac/sc* complex we sequenced one of the BAC clones that contained both *Tc-ASH* and *Tc-ase*. Sequence analysis of this 128 kb region revealed that *Tc-ASH* and *Tc-ase* reside 55.7 kb apart and are transcribed in the same orientation (Fig. 2). Within the sequenced region, there are no predicted genes upstream of *Tc-ASH* or between *Tc-ASH* and *Tc-ase*. In fact, the only other gene in the region is *cytochrome P450* (*cyt P450*), lying 8.5 kb downstream of *Tc-ase*. In *Drosophila*, *cyt P450* lies 5 kb downstream of *ase* suggesting a high degree of conservation in the genomic structure that surrounds *asense* in these species (Fig. 2). These data firmly establish the existence of the *Tribolium ac/sc* complex.

To investigate whether additional *ac/sc* genes reside near *Tc-ASH* and *Tc-ase*, we assembled the 25 BAC clones recovered in the screen into a 220 kb genomic contig. We then used degenerate PCR and low stringency hybridization to search this region for other *ac/sc* homologs. Despite exhaustive efforts, we failed to identify additional *ac/sc* genes. We also probed Southern blots of *Tribolium* genomic DNA at low stringency with *Tc-ASH*, *Tc-ase*, and *Drosophila ac* and *sc* to search for additional *ac/sc* genes in the *Tribolium* genome. Although these experiments consistently identified the bands that correspond to *Tc-ASH* and *Tc-ase*, they provided no evidence for the presence of other *ac/sc* genes in *Tribolium*. These data indicate that *Tc-ASH* and *Tc-ase* are probably the only *Tribolium ac/sc* genes and so define the *Tribolium ac/sc* complex.

Anopheles is a basal dipteran (Simpson et al., 1999) that

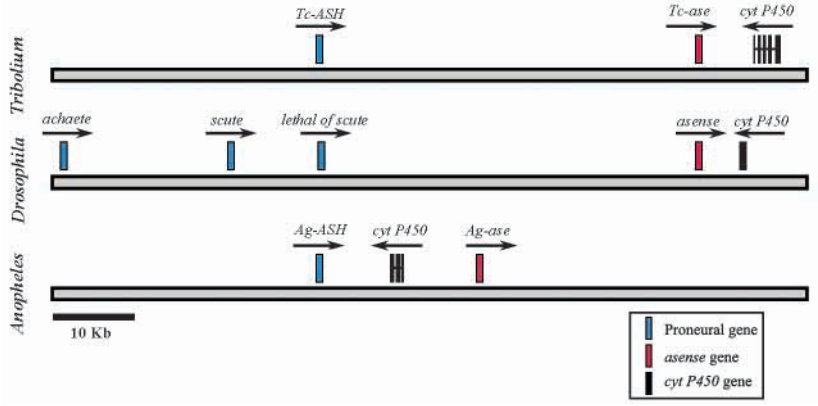

Fig. 2. Comparison of the *Tribolium*, *Drosophila*, and *Anopheles achaete-scute* complexes. (Top) The *Tribolium ac/sc* complex contains one proneural *ac/sc* gene, *Tc-AS* and one *asense* homolog, *Tc-ase*, that reside 55 kb apart. *Cytochrome P450* (*cyt P450*) lies downstream of *Tc-ase*. (Middle) The *Drosophila ac/sc* complex contains three proneural genes *ac*, *sc* and *l'sc* and one *asense* gene located 45 kb downstream of *l'sc*. As in *Tribolium*, *cyt P450* resides downstream of *ase*. (Bottom) The *Anopheles ac/sc* complex contains one proneural gene, *Ag-ASH*, and one *asense* gene, *Ag-ase*, that reside 22 kb apart. Unlike *Tribolium* and *Drosophila*, *cyt P450* lies between the *Anopheles ac/sc* genes. Proneural genes are in shown in blue, *asense* genes in red, and *cyt P450* in black. Arrows denote the direction of transcription. *Drosophila* map is adapted from Campuzano et al. (Campuzano et al., 1985) and the *Anopheles* map was constructed from available genome sequence data (Holt et al., 2002).

provides an important comparative link between the Diptera and other insect groups. Like *Tribolium*, *Anopheles* contains a single proneural *ac/sc* gene, *Ag-ASH*, and an *asense* homolog, *Ag-ase* (Holt et al., 2002; Wulbeck and Simpson, 2002). Analysis of the completed *Anopheles* genome sequence identified that *Ag-ASH* and *Ag-ase* lie 22 kb apart and are transcribed in the same orientation thus forming the *Anopheles ac/sc* complex [Fig. 2; see also Skaer et al. (Skaer et al., 2002a)]. As in *Drosophila* and *Tribolium*, *cyt P450* resides near the *Anopheles ac/sc* complex (Fig. 2). However, unlike *Drosophila* and *Tribolium* where *cyt P450* lies downstream of *ase*, *Anopheles cyt P450* resides between the *ac/sc* genes suggesting that a complicated rearrangement of this region occurred within the *Anopheles* lineage. The existence of *ac/sc* complexes in *Drosophila*, *Anopheles* and *Tribolium* suggests that the organization of *ac/sc* genes within gene complexes is a general feature of insects and perhaps all arthropods.

Tc-ASH has a proneural expression pattern and *Tc-ase* expression is restricted to neural precursors

We used RNA in situ hybridization to visualize the expression domains of *Tc-ASH* and *Tc-ase* during *Tribolium* CNS development. We find that *Tc-ASH* expression initiates prior to that of *Tc-ase* in ectodermal cell clusters throughout the CNS (compare arrows in Fig. 3A,C). Within each cluster *Tc-ASH* expression is progressively restricted to the presumptive neural precursor (Fig. 3A,B; arrowhead). The neural precursor then segregates into the interior of the embryo and shortly thereafter extinguishes *Tc-ASH* expression. These expression dynamics mirror those of all known insect proneural *ac/sc* genes confirming our initial identification of *Tc-ASH* as a proneural gene. Careful analysis of *Tc-ASH* expression throughout CNS development reveals that all neural precursors arise from *Tc-ASH*-expressing cell clusters. These results indicate that additional proneural genes are not required for neural precursor formation consistent with the idea that *Tc-ASH* is the only proneural *ac/sc* gene in *Tribolium*.

In addition to expression in proneural clusters, we also detect *Tc-ASH* expression in a transverse stripe extending outside of the neuroectoderm just posterior to the Engrailed stripe (Fig. 3A, asterisk). *Tc-ASH* expression in this non-neural domain does not give rise to *Tc-ase*-positive neural precursors (compare asterisks in Fig. 3A and C) raising the possibility

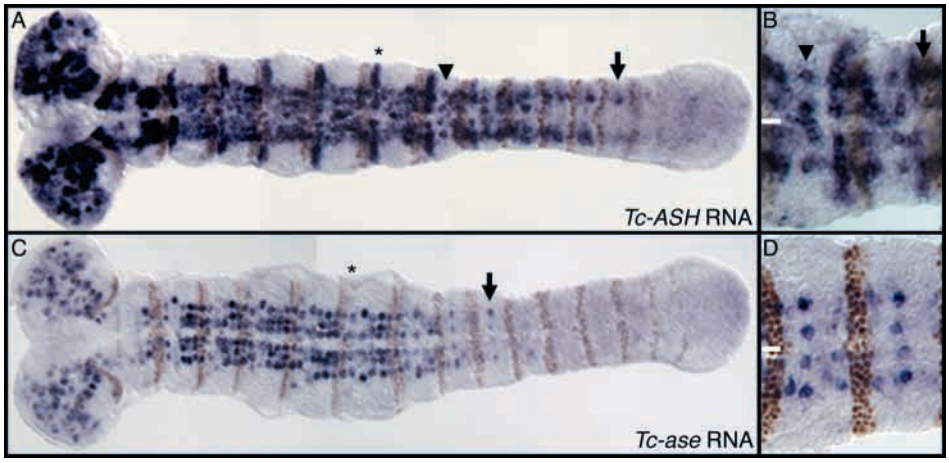

Fig. 3. *Tc-ASH* exhibits a proneural expression pattern while *Tc-ase* expression is restricted to neural precursors. (A-D) Ventral views of 20-hour *Tribolium* embryos. (A,B) *Tc-ASH* in situ hybridization (blue) and Engrailed protein (brown). (C,D) *Tc-ase* in situ hybridization (blue) and Engrailed protein (brown). (A) *Tc-ASH* is expressed in cell clusters in developmentally younger segments at the posterior (arrow); in developmentally older anterior segments *Tc-ASH* expression has resolved to single cells, the CNS neural precursors (arrowhead). In each segment *Tc-ASH* is expressed in a transverse stripe posterior to the engrailed stripe (asterisk). (B) High magnification view of two segments that shows *Tc-ASH* expression in proneural clusters (arrow) and single neural precursors (arrowhead). (C) *Tc-ase* is expressed in neural precursors (arrowhead) but not in ectodermal cell clusters. *Tc-ase* staining is not detected outside of the neuroectoderm (asterisk). (D) High magnification view of two segments showing *Tc-ase* expression. Note the position of *Tc-ase*-expressing neural precursors in D is identical to that of the large *Tc-ASH* expressing cells in B. Scale bars: 100 μm (A,C); 25 μm (B,D). Images in A and C are photomontages. Anterior, left.

that, as in *Drosophila*, proneural *ac/sc* genes may promote the formation of precursor cells outside of the nervous system (Carmena et al., 1995).

In contrast to *Tc-ASH*, in situ hybridization shows that *Tc-ase* expression is restricted to neural precursors and is not present in the ectodermal cell clusters from which these cells segregate. We find that all morphologically identifiable neural precursors activate *Tc-ase* after segregating from *Tc-ASH*-expressing cell clusters (Fig. 3C,D) and that these precursors maintain *Tc-ase* expression throughout embryogenesis. The restriction of *Tc-ase* expression to neural precursors mirrors the expression pattern of all known insect *asense* genes confirming our identification of *Tc-ase* as an *asense* homolog. The dynamics of *Tc-ase* expression also demonstrate that sequential waves of neural precursor formation form a grid-like pattern of seven anteroposterior rows and three dorsoventral columns of neural precursors, a pattern essentially identical to that observed in the *Drosophila* CNS.

Tc-ASH regulates neural precursor formation

Genetic studies indicate that the *Drosophila* proneural *ac/sc* genes promote neural precursor formation in the CNS and PNS (Garcia-Bellido and Santamaria, 1978; Garcia-Bellido, 1979; Dambly-Chaudiere and Ghysen, 1987; Jimenez and Campos-Ortega, 1990). To examine whether *Tc-ASH* promotes neural precursor formation in the *Tribolium* CNS we used double-stranded RNA interference (RNAi) to remove *Tc-ASH* function in early *Tribolium* embryos and then assayed neural precursor formation molecularly, by following *Tc-ase* expression, and by morphological examination. In *Tc-ASH* RNAi-treated embryos we observed complete loss of *Tc-ase* expression in 39.7% of embryos (n=23/58; Fig. 4B), partial loss in 43.1% of embryos (n=25/58; Fig. 4C) and wild-type *Tc-ase* expression in 17.2% of embryos (n=10/58). 96% of buffer-injected control embryos displayed wild-type *Tc-ase* expression (n=24/25, Fig. 4A). In these experiments, we observed near perfect correlation between loss of *Tc-ase* expression and the absence of morphologically identifiably neural precursors. These data indicate that *Tc-ASH* is necessary for neural precursor formation in the *Tribolium* CNS.

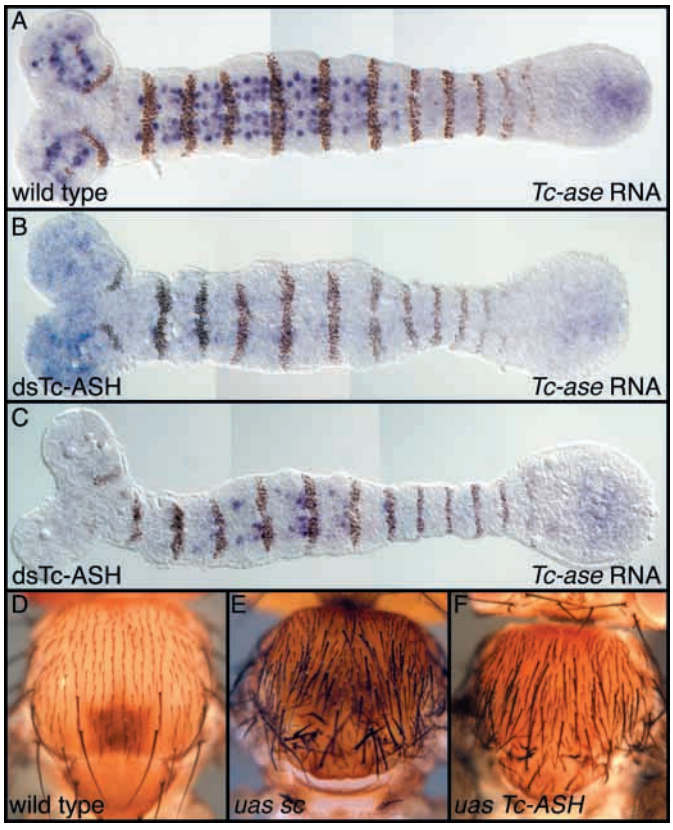

Fig. 4. *Tc-ASH* is necessary and sufficient for neural precursor formation. (A-C) *Tribolium* embryos injected with (A) buffer or (B, C) *Tc-ASH* double stranded RNA stained for *Tc-ase* RNA (blue) and Engrailed protein (brown). (A) Buffer-injected embryo displays the wild-type pattern of neural precursors. (B,C) *Tc-ASH* RNAi-treated embryos display either (B) a complete or (C) a partial loss of neural precursors as judged by *Tc-ase* expression. (D-F) Dorsal view of adult *Drosophila* nota. (D) Wild-type pattern of sensory bristles. (E,F) Ectopic sensory bristles result from misexpression of either (E) *UAS-Drosophila sc* or (F) *UAS-Tc-ASH* under the control of the *apterous-Gal4* driver line. A-C: anterior, left; D-F: anterior, up.

The variability we observe in the *Tc-ASH* RNAi phenotype appears to be a consequence of the technique as similar variability in RNAi phenotypes is seen for other genes in *Tribolium* (Brown et al., 1999), *Cupiennius* (Stollewerk et al., 2001) and *Drosophila* (unpublished observations). The loss of *Tc-ase* expression in these experiments is unlikely to arise from non-specific targeting of *Tc-ase* by double-stranded *Tc-ASH* because these genes share ≤65% nucleotide sequence similarity in the conserved bHLH region, and 100% of double stranded *Tc-ase*-injected embryos exhibit wild-type *Tc-ASH* expression (n=19). Although *Tc-ase* RNAi blocks *Tc-ase* expression, we have yet to identify a discernible phenotype in the embryonic CNS and injected animals are viable to adulthood (data not shown). This result is similar to the *Drosophila ase* phenotype in which no observable CNS defect has been identified and *ase* mutant flies are viable (Gonzalez et al., 1989; Jarman et al., 1993).

We next tested whether *Tc-ASH* is sufficient to promote neural precursor formation. In *Drosophila*, misexpression of proneural *ac/sc* genes in the developing notum leads to the formation of ectopic neural precursors that produce ectopic sensory bristles (Rodriguez et al., 1990). This assay is commonly used to test the proneural capabilities of *ac/sc* genes (Brand et al., 1993; Dominguez and Campuzano, 1993; Hinz et al., 1994; Grens et al., 1995; Wulbeck and Simpson, 2002). To assay the proneural potential of *Tc-ASH* in *Drosophila* we used the Gal4-UAS system and the *apterous-Gal4* driver line to misexpress *Tc-ASH* throughout the *Drosophila* notum (Brand and Perrimon, 1993; Calleja et al., 1996). In such flies, we observe the formation of many ectopic sensory bristles, a phenotype essentially identical to that observed when we misexpress *Drosophila sc* under identical conditions (compare Fig. 4E and F). These results demonstrate that *Tc-ASH* is sufficient to promote neural precursor formation in *Drosophila* and, together with our RNAi experiments, indicate that *Tc-ASH* is both necessary and sufficient to promote neural precursor formation.

Divergence of proneural *ac/sc* gene function between *Tribolium* and *Drosophila*

In the *Drosophila* CNS, *ac*, *sc* and *l'sc* exhibit essentially identical abilities to promote neural precursor formation,

however, *ac* and *sc* carry out functions distinct from *l'sc* in the fate specification of the MP2 neural precursor (Parras et al., 1996; Skeath and Doe, 1996). MP2 develops from a proneural cluster that expresses *ac* and *sc* but not *l'sc*. In *In(1) y^{3PL} sc^{8R}* embryos, *ac* and *sc* are not expressed in the MP2 cluster and a neural precursor forms in this position 17% of the time – roughly half of these precursors exhibit MP2-specific traits while the other half exhibit traits characteristic of other neural precursors (Table 1) (Parras et al., 1996; Skeath and Doe, 1996). In this background, expression of either *ac* or *sc* in the MP2 proneural cluster rescues both MP2 formation and fate specification to essentially wild-type levels (Table 1) (Parras et al., 1996; Skeath et al., 1996). In contrast, while *l'sc* expression rescues neural precursor formation almost completely, only 53% of these precursors exhibit MP2-specific traits (Table 1) (Parras et al., 1996; Skeath et al., 1996).

Tribolium has a single proneural *ac/sc* gene and a neural precursor identical to MP2 in position, morphology, and marker gene expression (data not shown). These data suggest that the roles of proneural *ac/sc* genes in specifying MP2 fate have changed since the divergence of *Drosophila* and *Tribolium*. The ability of *ac* and *sc* to specify MP2 fate may have arisen recently in the *Drosophila* lineage facilitated by the presence of multiple proneural *ac/sc* genes. Alternatively, the regulation of MP2 fate may be an ancestral function of proneural *ac/sc* genes that in *Drosophila* has been maintained by *ac* and *sc* but not *l'sc*. To test these models, we used *scabrous-Gal4* (Guo et al., 1996) to misexpress *Tc-ASH* in the MP2 proneural cluster in *In(1) $y^{3PL}$$sc^{8R}$* mutant embryos and assayed MP2 formation by the presence of Hunchback (Hb), a marker of all CNS neural precursors, and MP2 fate specification by the presence of Fushi-tarazu (Ftz) that specifically marks the MP2 precursor. We reasoned that if *Tc-ASH* specifies the MP2 fate in *Tribolium*, then we would observe a greater ability of *Tc-ASH* to rescue MP2 fate specification than is observed for *Drosophila l'sc* in this assay. Conversely, if *Tc-ASH* does not specify the MP2 fate in *Tribolium*, then we would observe little difference between the ability of *Tc-ASH* and *Drosophila l'sc* to rescue MP2 fate specification. We find that *Tc-ASH* expression rescues neural precursor formation in the MP2 position to 94%, however, only 46% of these precursors exhibit MP2-specific traits (Table 1). This phenotype is essentially identical to that of *l'sc* (Table 1) showing that *Tc-ASH* cannot specify the fate of the MP2 precursor in *Drosophila*. It is formally possible that MP2 fate specification in *Tribolium* requires the interaction of *Tc-ASH* with specific co-factors, but that, when misexpressed in *Drosophila*, does not specify MP2 fate due to a failure to interact with the *Drosophila* orthologs of these co-factors. However, because *Tc-ASH* can promote neural precursor formation in *Drosophila*, the most likely explanation for this result is that the ancestor of *Tribolium* and *Drosophila* proneural *ac/sc* genes did not play a role in the fate specification of MP2. These results support the model that the presence of multiple proneural *ac/sc* genes in *Drosophila* facilitated the ability of *ac* and *sc* to evolve new genetic functions, such as specifying the individual fate of neural precursors.

DISCUSSION

To investigate the roles that the *ac/sc* genes play during arthropod nervous system development and evolution we have characterized the genomic organization, expression and function of the *Tribolium ac/sc* genes. The results in this paper indicate that the *Tribolium ac/sc* complex consists of two genes – *Tc-ASH*, a proneural gene, and *Tc-ase*, a neural precursor gene, that reside 55 kb apart from one another. We show that *Tc-ASH* is expressed in all CNS proneural clusters and their associated neural precursors. In addition, we find that *Tc-ASH* is required to promote neural precursor formation in *Tribolium*. These data support the model that *Tc-ASH* promotes the development of all CNS precursors in *Tribolium* and thus plays the role of multiple proneural *ac/sc* genes in more derived insects like *Drosophila*. The function of *Drosophila ase* is not well understood. However, the gene and its expression pattern are highly conserved in *Tribolium* suggesting a conserved ancestral function for *asense* genes.

Conservation and plasticity in *ac/sc* gene number in Arthropoda

Homologs of *ac/sc* genes have been described in a number of insect and non-insect species. These data, together with our own, support and augment the model proposed by Skaer et al., (Skaer et al., 2002a) in which the last common ancestor of arthropods contained a single prototypical *ac/sc* gene that carried out both proneural and *asense* functions. In support of this model, the sole *Hydra ac/sc* gene, *CnASH*, does not group with either the proneural or *asense* genes in phylogenetic analysis and contains motifs indicative of both the proneural and *asense* genes (Grens et al., 1995; Skaer et al., 2002a). In addition, phylogenetic analysis of the two *ac/sc* genes found in the chelicerate *Cupiennius salei* indicates these genes are more closely related to each other than any other *ac/sc* genes (Fig. 1C) (Stollewerk et al., 2001; Skaer et al., 2002a). These data raise the possibility that a single ancestral *ac/sc* gene underwent independent duplication events in chelicerates and insects (Fig. 5; duplications a and b). Given this possibility, it is interesting that one of the *Cupiennius ac/sc* genes, *Cs-ASH1*,

Table 1. *Tc-ASH* cannot rescue MP2 specification in *In(1) y^{3PL} sc^{8R}* embryos

			Proneural genes expressed		
	Wild type	*In(1) y^{3PL} sc^{8R}*	*In(1) y^{3PL} sc^{8R}+sc*‡	*In(1) y^{3PL} sc^{8R}+l'sc*‡	*In(1) y^{3PL} sc^{8R}+Tc-ASH*
MP2 formation*	100% (309/309)	17% (34/198)	93% (134/144)	94% (117/125)	94% (271/289)
MP2 identity†	100% (331/331)	9% (14/158)	98% (130/133)	53% (69/131)	46% (96/206)

*MP2 formation was assayed by Hunchback (Hb) expression that marks all neural precursors after they have formed.
†MP2 identity was assayed by Fushi-tarazu (Ftz) expression that specifically marks the MP2 precursor in each hemisegment.
‡These data are equivalent to those obtained by Skeath and Doe (Skeath and Doe 1996, table I).
Numbers in parentheses indicate the number of hemisegments in which a precursor in the MP2 position expressed the indicated gene.

exhibits a proneural-like expression pattern and appears to carry out a proneural-like function and the other, *Cs-ASH2*, exhibits an *asense*-like expression pattern and appears to carry out an *asense*-like function (Stollewerk et al., 2001). These data suggest that independent duplications of an ancestral *ac/sc* gene have independently given rise to proneural-like and *asense*-like functions in the chelicerate and insect groups. Alternatively, phylogenetic analysis may inappropriately partition chelicerate *ac/sc* genes from insect *ac/sc* genes because of evolutionary selection for species-specific amino acid changes in chelicerate as compared to insect proteins.

Within the insects, it has become clear that serial duplications of a single proneural *ac/sc* gene gave rise to multiple proneural *ac/sc* genes in the more derived groups. For example, *Tribolium* and the basal dipteran *Anopheles* each contain a single proneural *ac/sc* gene. However, *Ceratitis*, a more derived dipteran, contains two proneural *ac/sc* genes. Thus, a duplication of the ancestral proneural *ac/sc* gene occurred within the dipteran lineage after the divergence of *Ceratitis* and *Anopheles* (Fig. 5; duplication c). The presence of three proneural *ac/sc* genes in *Drosophila*, a highly derived genus of dipterans, identifies a second duplication event. The simplest explanation for these data is that the second duplication occurred after the divergence of *Drosophila* and *Ceratitis* (Fig. 5; duplication d). However, comparative sequence analysis suggests this duplication may have preceded the divergence of *Drosophila* and *Ceratitis* and that *Ceratitis* has either lost an *ac/sc* homolog or it has yet to be identified (Skaer et al., 2002a).

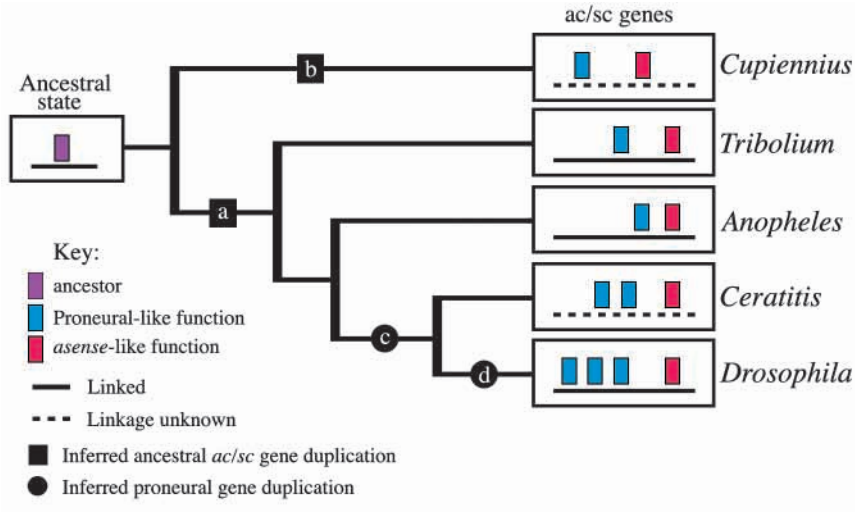

Fig. 5. A Model for *ac/sc* gene evolution. The *ac/sc* genes, their developmental functions (proneural-like, blue or *asense*-like, pink), and existence in complexes have been mapped on a standard species tree (Maddison et al., 2001). Branch lengths are not drawn to scale. See discussion for detailed description.

In contrast to the plasticity in proneural *ac/sc* genes within insects, *asense* genes appear to be well conserved. A single *asense* gene exists in *Tribolium* and *Anopheles* as well as in the derived dipteran species *Ceratitis* and *Drosophila*. In addition, *Cupiennius* contains a single non-orthologous *ac/sc* gene with *asense*-like properties (*Cs-ASH2*). Thus, the potential that the *asense* function evolved independently in insects and chelicerates suggests an important role for the *asense* function in arthropod neural development.

The existence of *ac/sc* genes in complexes in *Drosophila*, *Anopheles* and *Tribolium* suggests that this genomic arrangement has been conserved in most if not all holometabolous insects. Shared cis-regulatory regions probably explain why proneural *ac/sc* genes remain linked in insects and perhaps other species. However, this does not explain why *asense* is retained in the *ac/sc* complex as the regulation of *asense* expression is distinct from that of the proneural *ac/sc* genes. This phenomenon may be explained by the presence of proneural *ac/sc* gene cis-regulatory regions surrounding the *asense* gene. In this model, chromosomal rearrangements that separate *asense* from the *ac/sc* complex would probably disrupt proneural *ac/sc* gene expression and neural precursor formation, thus leading to decreased viability. Consistent with this idea, cis-regulatory regions that drive proneural *ac/sc* gene expression in the *Drosophila* PNS appear to flank the *ase* gene (Dambly-Chaudiere and Ghysen, 1987; Gomez-Skarmeta et al., 1995). Thus, the modular cis-regulatory regions that control proneural *ac/sc* gene expression may also be responsible for the evolutionary conservation of the *ac/sc* complex. Alternatively, other as yet unidentified genomic forces may preserve the linkage between *asense* and proneural *ac/sc* genes.

These findings raise a number of interesting points. First, they highlight the potential for evolutionary plasticity of *ac/sc* genes. Significant changes in *ac/sc* gene number and expression have occurred over relatively short evolutionary distances and have been correlated with modifications to neural pattern and/or gene function. For example, alterations to *ac/sc* gene expression in Diptera appear to account for the different patterns of sensory organs found on dipteran species (Wulbeck and Simpson, 2000; Skaer et al., 2002b). In addition, our data on the role of proneural genes in MP2 fate specification suggest that the increase in *ac/sc* gene number in *Drosophila* appears to have facilitated the evolution of new developmental roles for *ac* and *sc* in this lineage. Second, the possibility that independent duplication events in chelicerates and insects each gave rise to proneural-like and *asense*-like genes, indicates that dividing these genetic functions between two genes may be developmentally advantageous. Third, the hypothesis that the last common ancestor of all arthropods contained a single ancestral *ac/sc* gene suggests it may be possible to identify direct descendants of the prototypical *ac/sc* gene in extant basal members of each arthropod group. The recent emphasis on the development of genomic resources in non-model organisms should greatly aid progress along this line of inquiry. Thus, continued analysis of *ac/sc* gene expression, organization and function in arthropods should provide additional insight into the genetic basis of the development and evolution of nervous system pattern.

Conservation and plasticity in *ac/sc* gene function and expression

The work presented in this paper together with studies on *ac/sc* gene function in *Drosophila* provide strong evidence that serial

duplications of proneural *ac/sc* genes in the dipteran lineage led to the diversification of proneural *ac/sc* gene function in *Drosophila*. Our work and that of others demonstrate that in *Drosophila*, *ac* and *sc* carry out functions distinct from *l'sc* in specifying the individual fate of the MP2 precursor (Parras et al., 1996; Skeath and Doe, 1996). We show that *Tc-ASH* can function in *Drosophila* as a proneural gene but like *Drosophila l'sc* fails to specify efficiently the MP2 fate in the CNS. Together these results suggest the ability of *ac* and *sc* to specify MP2 fate in *Drosophila* arose after the divergence of *Drosophila* and *Tribolium*. These data provide an example whereby a subset of duplicated genes has evolved a new genetic function while the entire set of duplicate genes has retained the ancestral function.

In addition to functional changes, the generation of multiple proneural *ac/sc* genes in the insects was paralleled by modifications to the expression profiles of these genes. In *Anopheles*, a basal dipteran, and *Tribolium* a single proneural *ac/sc* gene is expressed in all CNS proneural clusters. In more derived Diptera the presence of multiple *ac/sc* genes allows for more complex proneural *ac/sc* gene expression patterns. For example, *Ceratitis* contains two proneural *ac/sc* genes, *l'sc* and *sc*; *l'sc* is expressed in all CNS proneural clusters while *sc* is expressed in a subset of these clusters. In *Drosophila*, *ac* and *sc* are expressed in the identical pattern of proneural clusters and their expression is largely complementary to that of *l'sc*. The sum of proneural *ac/sc* expression in each species then marks all CNS proneural clusters despite differences in the expression pattern of individual proneural *ac/sc* genes. Thus, in *Drosophila*, the complete expression pattern of proneural *ac/sc* genes is divided between the largely complementary expression profiles of *ac* and *sc* relative to *l'sc*. The division of labor between proneural *ac/sc* genes in *Drosophila* has resulted in mutually exclusive expression patterns for *ac* and *sc* relative to *l'sc* in proneural clusters like MP2. This spatial separation of proneural gene expression probably facilitated the potential for *ac* and *sc* to acquire developmental functions distinct from *l'sc*.

Together our work and that of others on arthropod *ac/sc* genes highlights the utility of studying *ac/sc* genes in elucidating the genetic basis of the development and evolution of arthropod nervous system pattern. These studies illustrate the dynamic nature of *ac/sc* gene number, expression and function over a relatively short evolutionary time. Based on this, future work on *ac/sc* genes in additional arthropod species should continue to provide insight into the molecular basis of the evolution of arthropod nervous system development.

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