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-ASH*) 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 andConsed (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 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 (Felsenstein, 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 selecting 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 gambiense Achaete-Scute Homolog; Ag-Ase, Anopheles gambiense Asense; Hv-ASH, Hydra vulgaris Achaete-Scute Homolog; Cs-Ash1, Cupiennius salei Achaete-Scute Homolog 1; Cs-Ash2, Cupiennius salei Achaete-Scute Homolog 2; Co-Sc, Ceratitis capitata Scute; Cs-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.

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 I/sc and one asense gene located 45 kb downstream of I/sc. As in Tribolium, cyt P450 resides downstream of asense. (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).

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.

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 detected 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
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 identifiable neural precursors. These data indicate that *Tc-ASH* is necessary for neural precursor formation in the *Tribolium* CNS.

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 \( In(1) \ y^{3PL \ sc8R} \) 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).

*\textit{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\ sc8R}* 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-asense*, 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-asense* 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 asense* 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*,

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**Table 1. Tc-ASH cannot rescue MP2 specification in In(1)y^{3PL sc8R} embryos**

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>In(1)y^{3PL sc8R}</th>
<th>In(1)y^{3PL sc8R acc}</th>
<th>In(1)y^{3PL sc8R ac}</th>
<th>In(1)y^{3PL sc8R +Tc-ASH}</th>
</tr>
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<tbody>
<tr>
<td><strong>MP2 formation</strong></td>
<td>100% (309/309)</td>
<td>17% (34/198)</td>
<td>93% (134/144)</td>
<td>94% (117/125)</td>
<td>94% (271/289)</td>
</tr>
<tr>
<td><strong>MP2 identity</strong></td>
<td>100% (331/331)</td>
<td>9% (14/158)</td>
<td>98% (130/133)</td>
<td>53% (69/131)</td>
<td>46% (96/206)</td>
</tr>
</tbody>
</table>

*MP2 formation was assayed by Hunchback (Hb) expression that marks all neural precursors after they have formed.

1*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 1).

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 diptera, 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).

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 Te-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 ac; 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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REFERENCES


