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scute expression in *Calliphora vicina* reveals an ancestral pattern of longitudinal stripes on the thorax of higher Diptera

Daniela Pistillo*, Nick Skaer* and Pat Simpson†

Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK
Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, 67404 Illkirch Cedex, CU de Strasbourg, France
*These authors contributed equally to this work
†Author for correspondence at present address: Department of Zoology, Downing Street, Cambridge CB2 3EJ, UK (e-mail: pas49@cam.ac.uk)

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SUMMARY

In *Drosophila* the stereotyped arrangement of sensory bristles on the notum is determined by the tightly regulated control of transcription of the *achaete-scute* (*ac-sc*) genes which are expressed in small proneural clusters of cells at the sites of each future bristle. Expression relies on a series of discrete *cis*-regulatory elements present in the *ac-sc* gene complex that are the target of the transcriptional activators *pannier* (*pnr*) and the genes of the *iroquois* complex. Stereotyped bristle patterns are common among species of acalyptrate Schizophora such as *Drosophila*, and are thought to have derived from an ancestral pattern of four longitudinal rows extending the length of the scutum, through secondary loss of bristles. To investigate evolutionary changes in bristle patterns and *ac-sc* regulation by *pnr*, we have isolated homologues of these genes from *Calliphora vicina*, a species of calyptrate Schizophora separated from *Drosophila* by at least 100 million years. *Calliphora vicina* displays a pattern of four rows of bristles on the scutum resembling the postulated ancestral one. We find that *sc* in *Calliphora* is expressed in two longitudinal stripes on the medial scutum that prefigure the development of the rows of acrostichal and dorsocentral bristles. This result suggests that a stripe-like expression pattern of *sc* may be an ancestral feature and may have preceded the evolution of proneural clusters. The implications for the evolution of the *cis*-regulatory elements responsible for *sc* expression in the proneural clusters of *Drosophila*, and function of Pnr are discussed.

Key words: Diptera, Calliphora vicina, Sensory organ, achaete-scute, pannier

INTRODUCTION

Insects bear sensory organs, such as bristles, over the cuticle of the body. In some dipteran flies the bristles are organised into stereotyped spatial arrays in which each bristle occupies a defined position. A single species, *Drosophila melanogaster*, has been the focus of investigation into the genetic control of the arrangement of sensory bristles. In *Drosophila melanogaster* there are eleven large bristles, or macrochaetes, on each heminotum and these occupy stereotyped positions. Bristle precursor development depends upon expression of the *achaete-scute* (*ac-sc*) genes. The *ac-sc* complex of *Drosophila* contains four genes that encode related basic helix-loop-helix (bHLH) proteins, transcriptional regulators that work as heterodimers together with the product of the gene *daughterless* (Alonso and Cabrera, 1988; Ghysen and Dambly-Chaudiere, 1988; Gonzalez et al., 1989; Villares and Cabrera, 1987). Expression of these genes provides cells with neural potential, allowing them to develop into nerve cells. The large bristles on the notum arise from small clusters of cells expressing *ac-sc*, called proneural clusters, that prefigure the sites of each of the future bristles (Cubas et al., 1991; Skeath and Carroll, 1991). Within domains of *ac-sc* expression, single, spaced cells are chosen to become sensory organ precursors. *achaete* and *sc* share *cis*-regulatory enhancer sequences scattered over nearly 100 kb (Gomez-Skarmeta et al., 1995; Ruiz-Gomez and Modolell, 1987). These enhancer sequences respond to local positional cues, conveyed by transcriptional activators. One *trans*-acting factor is the product of the *pannier* gene (Ramain et al., 1993). Pannier is a transcription factor of the GATA family and acts in a selector gene-like fashion to regulate pattern in the medial, dorsal half of the notum (Calleja et al., 2000). It has been shown to directly activate transcription of *ac-sc* through binding to target sequences in the dorsocentral enhancer element, that drives expression in a cluster from which the dorsocentral bristles arise (Garcia-Garcia et al., 1999). The genes of the Iroquois complex are required for the bristles of the lateral half of the notum (Gomez-Skarmeta et al., 1996; Leyns et al., 1996).

Other species of Diptera, particularly those of the derived cyclorraphous Schizophora (that includes *Drosophila*), have different, but equally stereotyped bristle patterns. Many of these patterns are phylogenetically old, suggesting that they are stable over long periods of evolutionary time (McAlpine, 1981;

Grimaldi, 1987). Closely related species have similar arrangements of bristles, whereas evolutionarily more distant ones display more diverged patterns. *Ceratitis capitata* is separated from *Drosophila* by about 80 million years (McAlpine, 1981). It displays a stereotyped bristle pattern with some bristles occupying similar positions to those of *Drosophila* (Wülbeck and Simpson, 2000). The *scute* gene of *Ceratitis* is expressed in proneural clusters at the sites of each future bristle, suggesting a similar genetic organisation of the locus in this species (Wülbeck and Simpson, 2000).

Throughout the cyclorraphous Schizophora there is a basic arrangement of bristles on the dorsal notum (McAlpine, 1981). There are four rows of bristles on the scutum: the acrostichal (AC), dorsocentral (DC), intra-alar (IA) and supra-alar (SA) rows. The pattern of most species of Schizophora can be superimposed upon this basic 'ground plan', even though some species display all rows and others have only a subset (McAlpine, 1981). It has thus been postulated that the stereotyped bristle patterns of species such as *Drosophila* and *Ceratitis* are derived from an ancestral pattern similar to this 'ground plan' (McAlpine, 1981; Simpson et al., 1999). The cyclorraphous Schizophora are subdivided into two subordinate groups, the Calyptrata and the Acalyptrata. Both *Drosophila* and *Ceratitis* are acalyptrates. When compared with the Acalyptrata, Calyptrata generally bear more macrochaetes and many species display all four rows extending the full length of the scutum. *Calliphora vicina* is one such species with a pattern resembling the hypothetical ancestral one. It is separated from *Drosophila* by at least 100 million years.

To investigate evolutionary changes in *ac-sc* expression, we have isolated homologues of these genes, and also *pannier*, from *Calliphora vicina* and examined their expression patterns. We find that *sc* is expressed in two longitudinal stripes that prefigure the development of the AC and DC rows of bristles. This result suggests that a stripe-like expression pattern of *sc* may be an ancestral feature and may have preceded the evolution of the small discrete proneural clusters characteristic of *Ceratitis* and *Drosophila*. In contrast, bristles of the IA and SA rows of *Calliphora* arise from domains of *sc*-expressing cells some of which resemble proneural clusters. These observations reinforce the hypothesis that the stereotyped patterns are derived from an ancestral pattern of four rows of bristles on the scutum and suggest that this pattern may have been the result of a regulated expression of *sc* in four stripes. We have also examined the expression pattern of the *pannier* homologue in *Calliphora*. We find that it is expressed in a conserved domain in the medial dorsal notum, consistent with a possibly conserved selector gene function. The implication of these results for the evolution of the *cis*-regulatory elements and the function of Pannier in the regulation of *sc* expression in the proneural clusters of *Drosophila* is discussed.

MATERIALS AND METHODS

Isolation of genes

Construction of cDNA libraries

Construction of embryonic cDNA libraries was performed using the cDNA Synthesis Kit, ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene) according to the instructions of the manufacturer. Total RNA was extracted with TRIZOL (Gibco BRL) according to instructions of the manufacturer from a 0- to 24-hour collection of *Calliphora* embryos. mRNA was purified using the Oligo(dT) Beads Kit (Dynall).

RT-PCR

Fragments of *Calliphora scute* (*sc*) (729 bp); *pannier* (*pnr*) (1194 bp), and *Delta* (*Dl*) (555 bp) were isolated by RT-PCR using the following degenerated primers (5′ to 3′, forward then reverse):

sc: AAYGCIMGIGARMGIAAYCG, CRTCRTCIGGIGTRCART-CYTC;

pnr: GAYTTYCARTTYGGIGARGG, GCIGYYTGIATIACRT-TRTGYTG;

Dl: CCIGGIACITTYWSIYTIATIRTIGARGC, RCAIGTICCICC-RTTIVCRCAIGG.

cDNA was generated from mRNA extracted from a 0- to 24-hour embryo collection using Superscript II reverse transcriptase (Gibco BRL). This was then used as a template. PCR was performed according the following general scheme: 94°C 1 minute; annealing temperature 1 minute 30 seconds; 72°C 2 minutes; 35 cycles; 10 minutes 72°C. PCR products were cloned into pGem T easy vector (Promega).

RACE

The 1194 bp fragment of *pnr* recovered by RT-PCR was extended by 5′ RACE PCR using the 5′/3′ RACE kit from Roche. A composite sequence of 1533 bp was generated.

Low stringency screening

Homologues for *lethal of scute* (*l'sc*; also known as *l(1)sc*) and *asense* (*ase*) were isolated by low stringency screening performed at 42°C in buffer containing 20% formamide, 5× SSPE, 0.5% SDS, 5× Denhart's solution. Washes were carried out at 50°C with 2× SSC, 0.5% SDS. A genomic *Calliphora* library (from M. Bownes) was plated and nylon replica filters (Amersham, Hybond-NX filters) were screened with a fragment containing the bHLH domain of *Drosophila virilis achaete* (*ac*) (from J. Modolell). Several phages containing either *l'sc* or *ase* were isolated. Complete coding sequences were subcloned into pBluescript vector (Stratagene).

High stringency screening

To recover the full sequence of *sc*, the *Calliphora* genomic library was screened at high stringency with the 680 bp fragment recovered by RT-PCR using Amersham Hybond-NX filters and conditions according to the manufacturer.

All sequences were submitted to GenBank. Accession numbers: *asense*, AY061875; *lethal of scute*, AY061876; *scute*, AY061877; *pannier*, AY061878; *Delta*, AY061879.

Sequence analysis

Sequences were compared using the ClustalX software. Alignments were performed using default ClustalX parameters, and percentage identities calculated from the resulting alignments (Thompson et al., 1997).

Rearing of *Calliphora*

Flies were kept at room temperature and fed with sucrose. Eggs were laid in fresh meat and kept at room temperature. Larvae were fed on fresh meat and kept at room temperature. White pupae were collected and staged at 25°C.

Labelling of RNA probes

Digoxigenin-labelled RNA probes (DIG-UTP, Roche) were generated using the standard protocol of Roche. The resulting RNA was resuspended in 100:l preHyb solution (50% formamide, 5× SSC, 0.1% Tween-20, pH 6.0). RNA was transcribed from linearised DNA templates.

Tissue preparation and staining

In situ hybridisation

Wing discs and pupal thoraces were dissected in phosphate-buffered saline (PBS) and fixed using a modified version of the protocol of Pattatucci and Kaufmann (Pattatucci and Kaufmann, 1992) in a solution of 4% formaldehyde, 5% DMSO in PBS. In situ hybridisations were performed using a protocol adapted from Wülbeck and Campos-Ortega (Wülbeck and Campos-Ortega, 1997).

Immunostaining

Wing discs and pupal thoraces were dissected in PBS, fixed in 4% formaldehyde/PBS for 20 minutes and stained. Mouse anti-22C10 and anti-HRP (horseradish peroxidase) primary antibodies were used at 1:200 dilution. Biotinylated anti-mouse secondary antibody was used and visualised using a standard ABC kit (Vector Chemicals). All preparations were mounted in 80% glycerol, 1× PBS.

Thoraces

Adult flies were collected 30-90 minutes after eclosion before the cuticle had tanned and darkened and stored in 70% ethanol, thereby allowing clearer visualisation of bristle patterns. Thoraces were dissected in 70% ethanol, transferred to 100% ethanol for 10 minutes and mounted under raised coverslips in Euparal (Fisher Chemicals).

RESULTS

Isolation and conservation of *achaete-scute* homologues

We have searched for *achaete-scute* homologues from *Calliphora vicina*. We were able to recover sequences specific to *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*) (Fig. 1). No sequences specific to *achaete* (*ac*) were recovered. Percentage identity with the *Drosophila* orthologues is as follows (overall/bHLH only): *sc*, 74.3/96.7; *l'sc*, 74.9/95.8; *ase* 71.1/90.2. Comparisons with *Drosophila ac* were also made (overall/bHLH only): *Calliphora sc* shared 68.7/87.5% identity, *l'sc* 66.2/87.2%, and *ase* 63/81.2%.

Examination of expression of these three proneural genes in *Calliphora* embryos by in situ hybridisation revealed expression of all three genes in the presumptive central nervous system similar to that seen in *Drosophila*. *l'sc* and *sc* are initially expressed in a dynamic pattern in clusters of cells, which are then progressively restricted to individual neural precursors; *ase* is expressed in single cells (not shown).

scute is expressed in stripes and clusters of cells in the developing notum

The bristle pattern of the dorsal notum of *Calliphora* is depicted in Fig. 2D. The four rows of large bristles on the scutum are labelled AC, DC, IA and SA for the acrostichal, dorsocentral, intra-alar and supra-alar rows, respectively. The transverse suture divides the scutum into pre-sutural and post-sutural domains. The scutellar suture also separates the scutum from the scutellum. The scutellum bears a single line of scutellar (SC) bristles round the lateral edge. The expression of *sc* in the developing notum was examined by in situ hybridisation. Expression starts at pupariation before the wing discs have started to evert and fuse along the midline. The general shape and morphology of the prospective notum is reflected in the shape of the discs, which bear strong similarity to the well-studied *Drosophila* discs (Usui and Simpson, 2000). Through examination of many different stages and comparison with *Drosophila*, we were able to determine the positions at which the various bristles arise. By 3 hours after puparium formation (h APF), two distinct longitudinal stripes of expression, aligned with the dorsal midline, are visible in the medial half of the future scutum at the positions of the future AC and DC bristles (Fig. 2A,D). Both stripes are interrupted by a band in which expression is absent; this corresponds to the future transverse suture (see Fig. 2D). A single stripe of expression along the posterior medial edge prefigures the row of SC bristles. In the lateral half of the notum expression appears in several broad domains and smaller clusters at the sites of the future IA and SA bristles (Fig. 2A,D). A stripe-like domain can be discerned but appears to include bristles of both IA and SA rows. In the lateral region, too, expression is lacking along the prospective transverse suture. The correspondence between the domains of expression and the future bristle rows is shown in Fig. 2D. Several clusters of expressing cells are also present in the region of the wing hinge and calypter, as well as on the wing blade, but we did not attempt to correlate these with specific sensory organs (Fig. 2A,B).

Expression is first observed in the DC stripe at, or just prior to, pupariation. It is rapidly followed by expression in the posterior domain of the prospective AC stripe, the posterior most IA and SA domains, and the more medial domain of the SC stripe (not shown). Expression is then observed more anteriorly, in the case of the AC, IA and SA domains, and laterally for the SC domain, and is finally visible at all sites of the prospective bristle organs by 3 hours APF (Fig. 2A).

By 8-10 hours APF, the stripes of *sc* expression are less coherent and later strong spots of high levels of expression are seen, which probably reflect the emergence of bristle precursors (Fig. 2B). At the same time *ase* starts to be expressed in single cells, the precursors of the bristles. By 10 hours, *ase* expression is widespread amongst the precursors (Fig. 2C,E). The rows of precursors arise from within each stripe or cluster of expression. However, *ase* expression is transient and the complete pattern of bristle precursors cannot be visualised at any one time. The order in which bristle precursors arise, as revealed by *ase* and high levels of *sc*, mirrors the progression of *sc* expression, and there is a general trend for precursors to arise in a posterior to anterior fashion. This holds true for the AC and DC domains, each of which have three presutural and three postsutural precursors. Exceptionally though, the final precursors to form in the post-sutural domain are the central ones of each triplet. These intercalate between the existing two, perhaps after growth of the epithelium by cell division generates more available space. This may also be the case for the three pre-sutural bristles in each row. By 16 hours APF *sc* expression has faded from the precursors.

22C10 is a marker of late precursors and the entire neural lineage, and is expressed later than *sc* and *ase* (Zipursky et al., 1984). The 22C10 antibody thus reveals neural precursors and staining for this marker reveals a similar pattern and time progression of precursor segregation (Fig. 2F). We also isolated a 555 bp fragment from the *Delta* gene of *Calliphora*. *Delta* has been shown to be downstream of Ac-Sc in *Drosophila* (Kunisch et al., 1994; Parks et al., 1997). In situ hybridisation with this probe, as well as staining with the cross-reacting

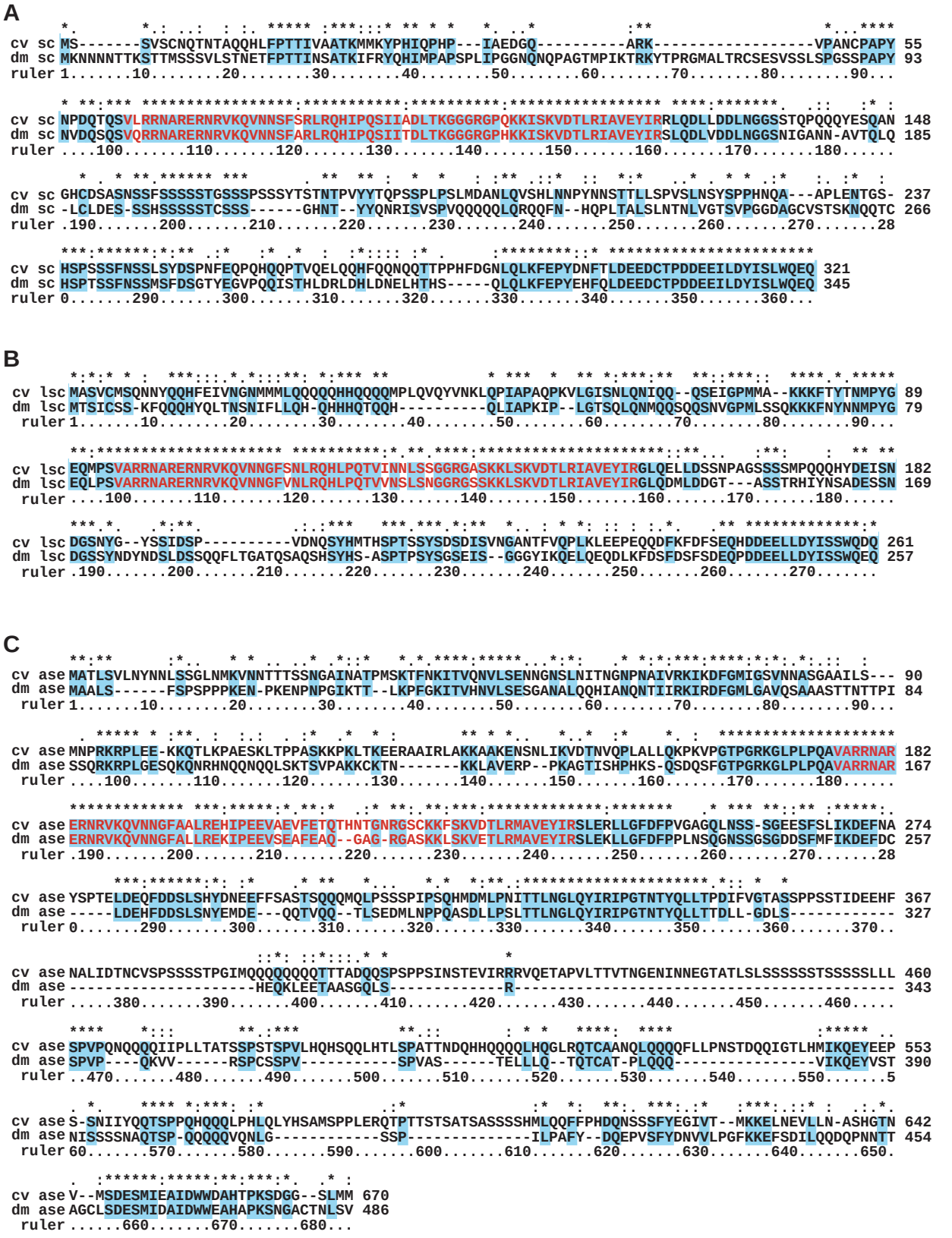

Fig. 1. Amino acid sequence comparison of the three *ac-sc* genes isolated from *Calliphora vicina* (cv) with their orthologues in *Drosophila* (dm). (A) *scute*, (B) *lethal of scute*, and (C) *asense.* The highly conserved basic helix-loop-helix (bHLH) motif is in red. The line above the sequences indicates conserved positions with one of three symbols: (*) indicates positions that have a single, fully conserved residue. These positions have also been shaded blue. (:) and (.) represent strong and weaker degrees of conservation respectively, according to the Gonnet Pam250 matrix (Benner et al., 1994).

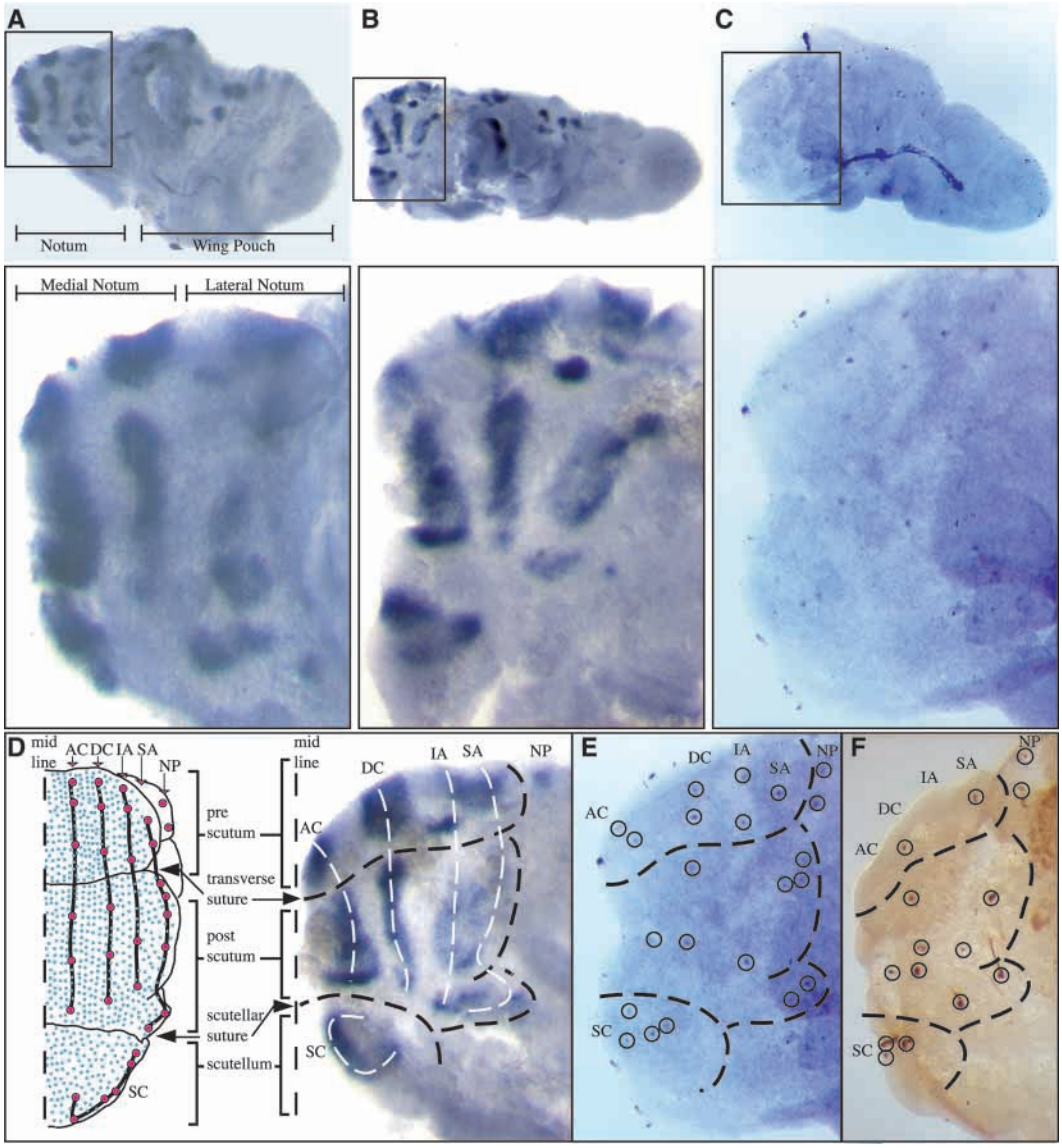

Fig. 2. Early expression of *scute* (*sc*) and *asense* (*ase*) in the wing disc of *Calliphora vicina*. (A,B,D) expression of *sc* transcripts, (C,E) expression of *ase* transcripts, (F) visualisation of 22C10 protein. Enlargements of the thoracic domain of the discs in A-C, indicated by the rectangles, are shown directly below. By 3 hours after pupariation, *sc* transcripts, visualised by in situ hybridisation (A) are present in two broad longitudinal stripes in the medial half of the prospective notum. These correlate with the positions of the future acrostichal (AC) and dorsocentral (DC) bristle rows, and are interrupted by a mediolateral band at the site of the prospective transverse suture in which expression is absent. In the lateral domain, at the sites of the future intra-alar (IA), notopleural (NP) and supra-alar (SA) bristles, *sc* is expressed in a series of clusters. By 8 hours (D) to 10 hours (B) after pupariation, *sc* expression is restricted to the sites of the future bristle organs, where small groups of cells have accumulated higher levels of the transcripts. A clear expression domain is seen in the scutellum; it is separated by an area in which *sc* is absent that corresponds to the prospective scutellar suture. In D a schematic drawing of the relationship between *sc* expression at 8 hours after pupariation and the morphology of the future adult thorax (macrochaetes are shown in pink, microchaetes in blue). At this stage the disc remains a highly folded epithelium which later expands longitudinally at the midline, and laterally and ventrally at the lateral edge of the notum. This explains the compact aspect of the acrostichal and scutellar domains, and close proximity of the intra- and supra-alar domains at this stage. *ase* expression in single cells (C,E), and visualisation of neurons with the 22C10 antibody 10 hours after pupariation (F), show that the early expression of *sc* prefigures the sites at which bristle precursors are born.

antibody against horseradish peroxidase, a neural marker, confirmed the above sequence of events (not shown).

In addition to the large bristles (macrochaetes), the notum is also covered with numerous small bristles (microchaetes). At about 30 hours APF a second wave of *sc* expression takes place and is correlated with segregation of the precursors of the small bristles (Fig. 3A,B). Staining is fairly ubiquitous over the notum but is again excluded from the sutures. It is then refined to expression in single cells. *sc* expression at this stage reappears in the macrochaete precursors (Fig. 3A,B). By 33 hours APF *ase* staining is visible in single microchaete precursors (Fig. 3C), and by 50 hours 22C10 staining indicates that axonogenesis of the bristle neurons is taking place (Fig. 3D). We were unable to detect *l'sc* transcripts during development of the imaginal notum.

pannier is expressed in a conserved medial domain of the notum

We isolated a 1533 bp fragment of the *pannier* (*pnr*) gene from *Calliphora*. It shows 76.3% of overall conservation with *Drosophila pnr*, and 99% in the two zinc finger motifs. This is significantly greater than with any of the other four *Drosophila* GATA factors that display between 54% and 63% overall identity to *Calliphora pnr*. In situ hybridisation revealed expression in the wing discs at pupariation and throughout the period of *sc* expression and segregation of bristle precursors. It is expressed in a broad domain, similar to that of *Drosophila pnr*, which covers the medial half of the notum (Fig. 4A). The lateral boundary of *pnr* expression appears to be aligned with the DC row of precursors (Fig. 4B,C).

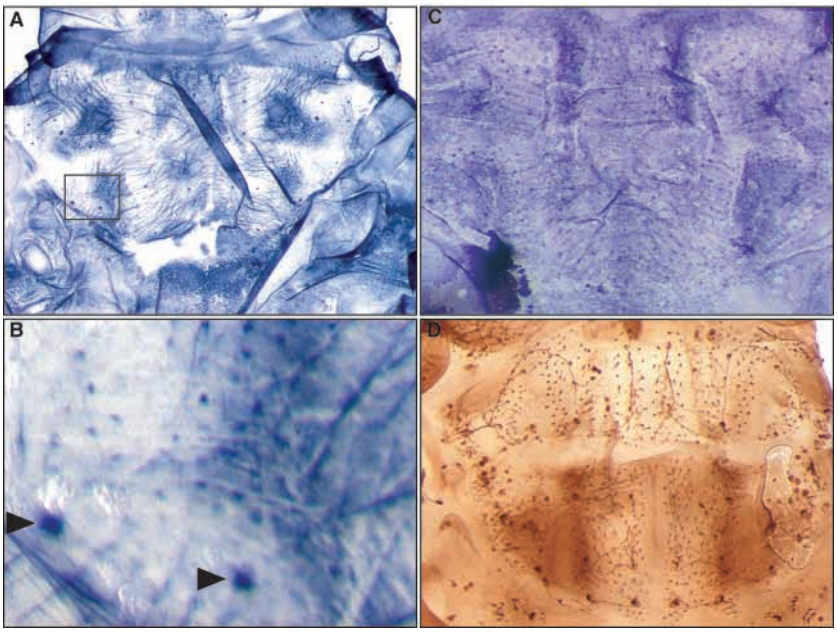

Fig. 3. Late expression of *scute* (*sc)* and *asense* (*ase*) in the pupal thorax of *Calliphora vicina*. After fusion of the two heminota along the midline, *sc* is re-expressed in the pupal thorax 29 hours after pupariation. Expression is initially widespread, and is excluded only from the sites of the prospective sutures, before becoming restricted to individual microchaete precursors by 31 hours after pupariation (A). *scute* is re-expressed in the macrochaete precursors (B: enlargement of the boxed area in A; macrochaete precursors are indicated by arrowheads) in the immediate vicinity of which down-regulation of *sc* in neighbouring cells is particularly clear. By 33 hours after pupariation, *ase* expression can be detected in microchaete precursors (C), and by 51 hours after pupariation, 22C10 staining reveals that axonogenesis has been initiated (D).

The number of bristles in the acrostichal row, but not the other three rows, varies with body size

We looked for variation in the bristle pattern between individuals of different size in our colony of *Calliphora*. Pupae of the same age were collected, weighed, and divided into four groups: 30 mg and smaller, 30-50 mg, 50-70 mg, and 70 mg and larger. They were left to hatch and the number of bristles per row on each individual was scored (Fig. 5F). The results indicate that there is some variation amongst the DC, IA and SA rows, and animals with fewer or additional bristles are found. Fig. 2D depicts the pattern seen in the majority of flies; we refer to this as the 'wild type'. In total, 10.5% of individuals showed differences from the 'wild type' in the DC row, 6.5% in the IA row and 19% in the SA row. However, this variation is small by comparison to that observed in the AC (58%) and scutellar (54%) rows. Moreover, the variation in bristle number in these two rows correlated strongly with size of the individuals. The AC row, for example, generally includes six bristles, three pre-sutural, and three post-sutural ones. Smaller flies, however, may have five bristles, and larger flies seven bristles, in this row (Fig. 5A,E). Bristles are most frequently lost from the anteriormost position (Fig. 5E) but may also be lost from other sites (Fig. 5D). Supernumerary AC bristles may appear throughout the row (Fig. 5A,B). Absence of bristles in the AC, SC, and DC rows is often associated with displacement of the other bristles (Fig. 5B,E). This indicates that the precise position of each bristle in the row is variable, although the lower level of variation in the DC row does suggest that a more robust patterning mechanism may operate in this domain. However, in other individuals, both supernumerary and missing bristles can be superimposed on top of the 'wild-type' pattern such that the 'wild-type' bristles are not displaced (Fig. 5A,D). Interestingly, bristle displacement was not observed in the IA and SA rows, where precursors arise from a more cluster-like expression of *sc*.

DISCUSSION

Conserved function of the three *achaete-scute* homologues of *Calliphora*

We isolated three orthologues of the *Drosophila* AS-C genes, which show clear homology to *sc*, *l'sc* and *ase*. Despite extensive screening by both PCR and of cDNA and genomic *Calliphora* libraries we were unable to find *ac*, so this gene may not be present in this species. It was not found in the acalyptrate, *Ceratitis capitata* (Tephritidae), and has so far only been described in the genus *Drosophila* (Alonso and Cabrera, 1988; Gonzalez et al., 1989; Villares and Cabrera, 1987) [see references in Takano (Takano, 1998)]. *achaete* displays closest similarity to *sc* of all three species. It shares a largely conserved expression pattern and has been shown to be functionally redundant with *sc* in *Drosophila* (Balcells et al., 1988; Gomez-Skarmeta et al., 1995; Martinez and Modolell, 1991). Thus, it may have arisen from duplication of the ancestral *sc* orthologue, some time after the separation of the Tephritidae and Drosophilidae.

Our results indicate strong conservation of the roles of the different *ac-sc* genes. In *Drosophila*, *l'sc* is essential for development of the central nervous system, and its loss results in lethality. We find that, as in *Drosophila*, *l'sc* is expressed in the central nervous system of *Calliphora* during embryogenesis, but is not expressed in the developing notum. Similarly, expression of *sc* in proneural domains in the presumptive notum is conserved and expression of *ase* is restricted to sensory precursors. This suggests that specialisation of the functions of these three genes predates the separation of acalyptrate and calyptrate Schizophora.

Proneural clusters may have arisen from an ancestral pattern of longitudinal stripes of *scute* expression

An arrangement of four longitudinal rows of large bristles is characteristic of the scutum of a number of calyptrate flies and is thought to resemble an ancestral pattern or 'ground plan', from which the many different patterns seen in calyptrate and acalyptrate species are derived (McAlpine, 1981; Simpson et al., 1999). Thus an alignment of bristles into four rows may have been the first patterning event in a series of steps that culminated in the stereotyped bristle arrangements

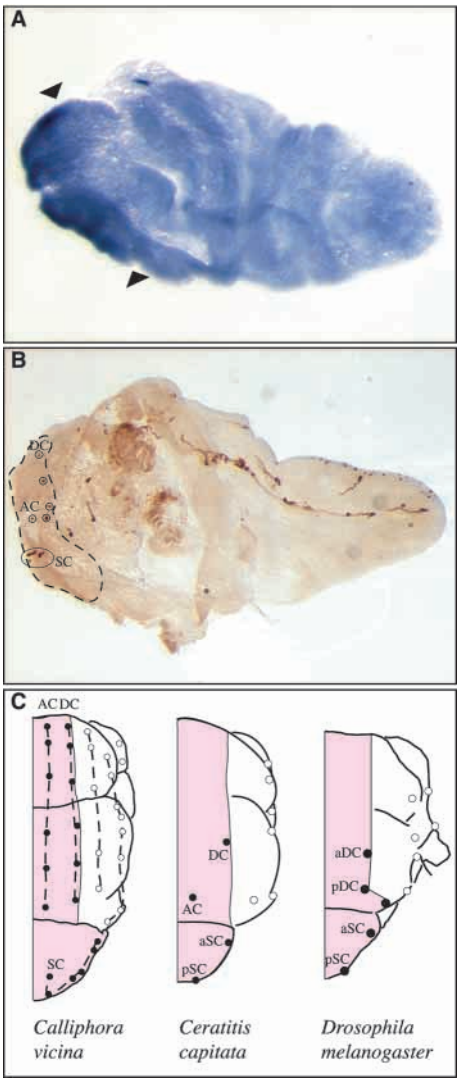

Fig. 4. Expression of *pannier* (*pnr*) in the wing disc of *Calliphora vicina*. (A) The boundary of *pnr* expression in the medial half of the developing notum at 8 hours after pupariation (arrowheads). The lateral limit of *pnr* expression appears to correspond to the position of the future precursors of the dorsocentral row, visualised in B by 22C10 staining. This seems to be a conserved feature with other species of cyclorraphous Schizophora: a similar correlation has been described in *Ceratitis capitata* and *Drosophila.* (C) A schematic representation of how the expression domains of *pnr* (shown in pink) in the wing disc correlate with bristle positions on the imaginal notum of the three species. Note that dorsocentral (DC) bristles are always positioned at the limit of *pnr* expression, but that the bristle patterns differ. aDC, anterior DC; aSC, anterior scutellar; pSC, posterior scutellar.

characteristic of *Drosophila*. The single AC and DC bristles of *Ceratitis capitata*, an acalyptrate species, come from two separate proneural clusters, suggesting a different developmental origin for AC and DC bristles, consistent with the hypothesis that each of them may be derived from an independent row (Wülbeck and Simpson, 2000). *Drosophila* does not bear any AC bristles, but does carry two DC bristles that interestingly arise from a single proneural cluster in a posterior to anterior sequence (Cubas et al., 1991; Skeath and Carroll, 1991). This cluster is controlled by a discrete *cis*-regulatory element, called the DC enhancer (Garcia-Garcia et al., 1999; Gomez-Skarmeta et al., 1995). The origin of this and the other positional enhancers is unknown. We have demonstrated that the row of DC bristles in *Calliphora* arises from a stripe of *sc*-expressing cells. It is thus tempting to speculate that a stripe-like domain may have preceded the cluster-shaped domain during the course of the evolutionary history of the lineage leading to *Drosophila*. If so, discrete regulatory elements may have been acquired to drive expression of *sc* in stripes on the scutum of a common ancestor of *Calliphora* and *Drosophila*. Identification of regulatory elements in *Calliphora* may help to resolve this hypothesis.

It is noteworthy that the two DC bristles of *Drosophila* are situated close to one another. If they are indeed derived from a complete longitudinal DC row present in an ancestor, through secondary loss of some of the bristles in the row, it seems likely that bristle loss would occur from the anterior downwards or from the posterior upwards, or both together. The DC enhancer may be derived from a single, discrete regulatory element that was responsible for a stripe of expression in the ancestor. If so, it is unlikely that bristles would be lost from the centre of the row, since this would entail a division of the stripe domain into two separate clusters of expression. We examined the distribution of DC bristles in 63 species of acalyptrate flies from 17 different families. 33% were found to have bristles missing from the anterior end of the row (see also Sturtevant, 1970), 8% from both anterior and posterior ends, and only one species (1.6%) had bristles missing from the middle of the row. The entire DC row was lacking in 1.6% of this sample. Similarly, examination of 52 species of calyptrate flies from 6 families, showed missing pre-sutural DC bristles in 9.6% of cases. The entire DC row was lacking in 7.7% of the animals.

The IA and SA bristles of *Calliphora* do not arise from stripes of *sc* expression but from apparent clusters. These resemble the proneural clusters of *Drosophila* and *Ceratitis* and are associated with a greater degree of determinacy of the positioning of these bristles (see below).

Stereotyped positioning of bristles along the anteroposterior coordinate of the scutum is a recent feature, whereas that along the mediolateral coordinate is of ancient origin

The pattern of four bristle rows appears to be an ancient, widespread one that has been retained regardless of considerable size differences between different species (McAlpine, 1981; Simpson et al., 1999). This suggests that bristle positioning along the mediolateral coordinate of the scutum was fixed a very long time ago. In contrast, anteroposterior patterning, that is the stereotyped positioning of bristles within rows, seems to have been acquired more recently in derived species. It is a characteristic of many acalyptrate flies such as *Drosophila* and *Ceratitis*, but is not a consistent feature of more basal species that frequently display a variable number of bristles within the rows. Such variability is thought to be an ancestral feature.

Calliphora appears to be intermediate with respect to this morphological feature. The number of bristles in the AC row of *Calliphora* varies between individuals: large flies may have more and small flies fewer AC bristles. It is clear that the precise position of each bristle is, to some extent, variable,

since the bristles are often displaced when compared with those on the contra-lateral side (Fig. 5B,E). The AC bristles arise from a stripe of *sc* expression, so spacing of the bristle precursors could be simply achieved through Notch-mediated lateral inhibition (Wigglesworth, 1940; Kimble and Simpson, 1997; Simpson, 1990). The distance between bristles is a function of the range of Notch signalling, so in larger animals there would be room for more precursors. This view is supported by the order in which the precursors arise within the post-sutural AC and DC rows. Each row has three post-sutural bristles, with precursors for the posterior and anterior-most forming first, followed by the central precursor. Formation of the central precursor may only be possible after growth of the epithelium has provided sufficient space between the other two precursors. It is also noticeable that 'missing' post-sutural bristles are invariably those located between the two early forming bristles, which are never lost, and that 'supernumerary' bristles are also added in the middle.

However, in other individuals the positions of the 'wild-type' bristles do not change, and 'additional' ones are superimposed on top of the 'wild-type' pattern. This observation leads us to postulate that there may be another mechanism(s), in addition to the regulation of *sc* transcription, which helps to position the precursors. In *Drosophila*, one or two precursors are selected from each proneural cluster by means of Notch-mediated lateral inhibition (Wigglesworth, 1940; Hartenstien and Posakony, 1990; Heitzler et al., 1996a; Heitzler and Simpson, 1991). However the choice is often biased to a cell at a specific position within the cluster (Cubas et al., 1991; Simpson, 1997; Skeath and Carroll, 1991); it is not known how this is achieved.

The function of *pannier* and the origin of *cis*-regulatory elements in the *achaete-scute* complex

The *pnr* gene of *Calliphora* was found to be expressed in a conserved domain, similar to that of *Drosophila* and *Ceratitis*, that covers the medial half of the notum. This suggests that *pnr* has retained its selector gene function (Calleja et al., 2000) in all three species. The bristle patterns and the domains of *sc* expression within the *pnr* expression domain differ, however, between the three species (Fig. 4). So, if the function of *pnr* has been conserved, other factors must have changed in order to account for these differences. It is not entirely understood how the broad domain of Pnr in *Drosophila* is translated into three small clusters of *ac-sc* expression, but this requires the activity of three discrete *cis*-regulatory elements, as well as modulation of Pnr function by at least one cofactor, the product of the *u-shaped* (*ush*) gene (Heitzler et al., 1996b; Ramain et al., 1993; Garcia-Garcia et al., 1999; Gomez-Skarmeta et al., 1995; Haenlin et al., 1997; Cubadda et al., 1997). Homologues of *ush* have not been isolated in *Ceratitis* and *Calliphora*, but the AC bristles of these species are situated within the domain where *ush* is expressed in *Drosophila*. Changes in the regulation of genes encoding cofactors for Pnr, such as *ush*, is thus a possible mechanism for evolutionary changes in bristle patterns.

Conclusions

Our observations suggest a model for the changes in gene regulation that may have occurred during evolution of the stereotyped bristle patterns of higher flies (Fig. 6). An ancestor of the Schizophora would have had a pattern of four longitudinal rows of large bristles on the scutum (McAlpine, 1981; Simpson et al., 1999). The bristle precursors would be spaced apart

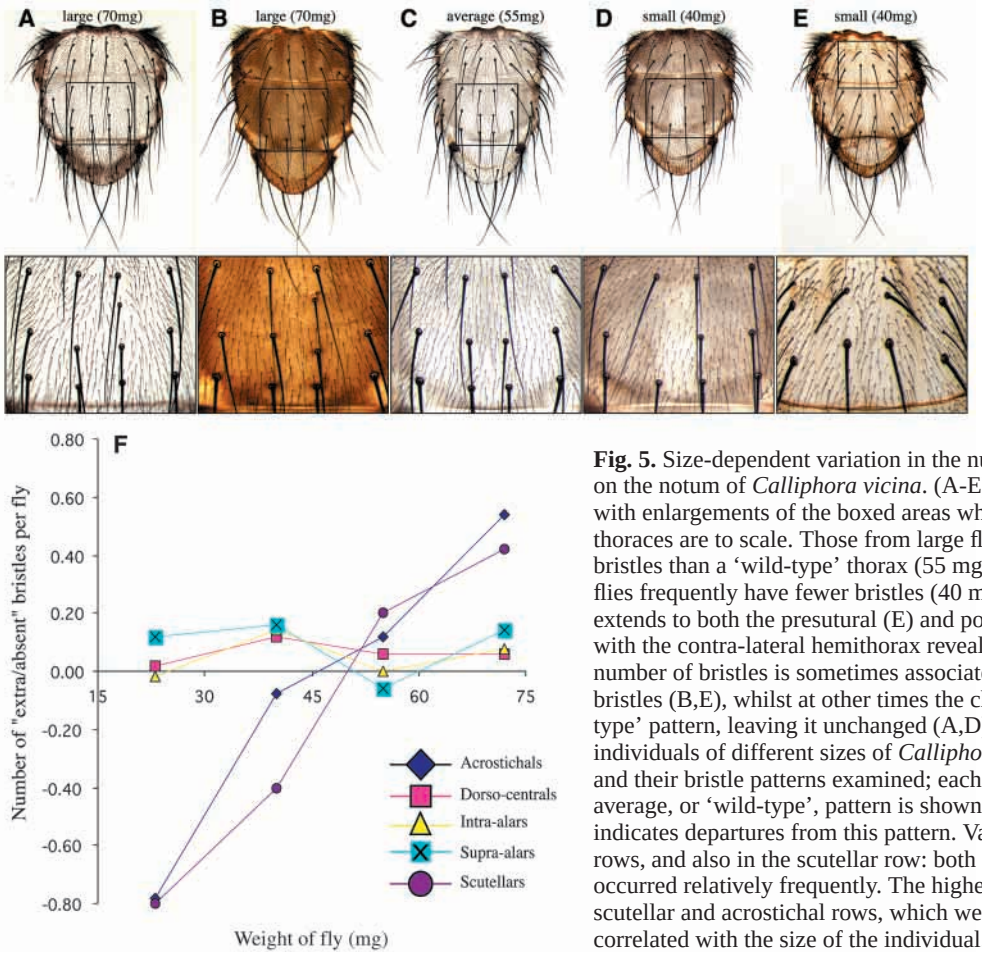

Fig. 5. Size-dependent variation in the number of bristles in the acrostichal row on the notum of *Calliphora vicina*. (A-E) Thoraces of adult *Calliphora* together with enlargements of the boxed areas which are shown directly beneath. All thoraces are to scale. Those from large flies (70 mg; A,B) regularly display more bristles than a 'wild-type' thorax (55 mg; C). Conversely, thoraces from smaller flies frequently have fewer bristles (40 mg; D,E). Variability in bristle number extends to both the presutural (E) and postsutural (A,B,D) domains. Comparison with the contra-lateral hemithorax reveals that an increase or decrease in the number of bristles is sometimes associated with a displacement of the 'wild-type' bristles (B,E), whilst at other times the changes are superimposed on the 'wild-type' pattern, leaving it unchanged (A,D). (F) Variation in bristle number between individuals of different sizes of *Calliphora vicina*. Individual flies were weighed and their bristle patterns examined; each bristle row was treated separately. The average, or 'wild-type', pattern is shown in Fig. 2D and Fig. 5C. The graph indicates departures from this pattern. Variation was discovered in all four scutal rows, and also in the scutellar row: both 'additional' and 'missing' bristles occurred relatively frequently. The highest variation was seen in the bristles of the scutellar and acrostichal rows, which were also the only rows in which variation correlated with the size of the individual.

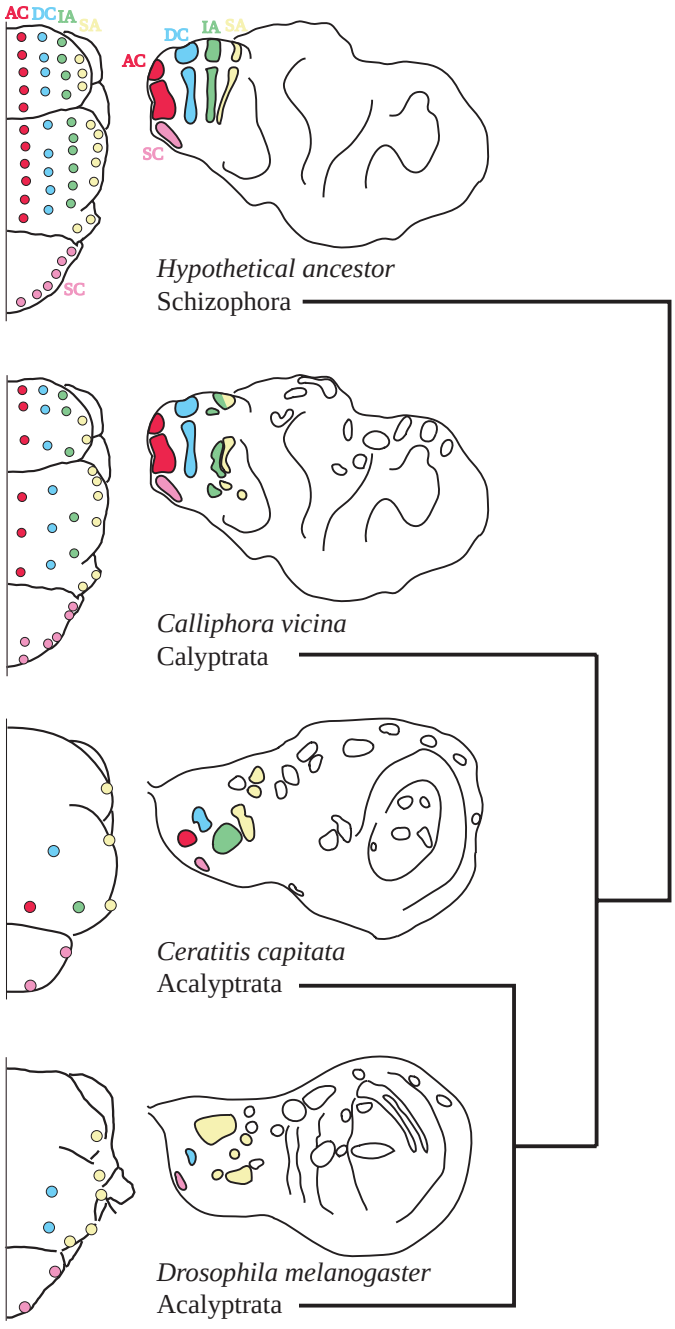

Fig. 6. A model for the evolution of proneural gene expression and bristle patterns within the higher Diptera. Representations of proneural gene expression in late larval/early pupal wing imaginal discs of different species are depicted on the right. The corresponding bristle pattern on the adult heminotum of each species is shown on the left. The acrostichal (AC) bristles and corresponding expression domains are shaded red, the dorsocentral (DC) blue, the intra-alar (IA) green, the supra-alar (SA) yellow, and the scutellar (SC) pink. Unshaded domains represent proneural expression not associated with bristles of the scutum and scutellum. The top diagram depicts a hypothetical ancestor of the cyclorraphous Schizophora. Proneural expression on the scutum is hypothesised to have been in four stripes, with a further stripe on the scutellum, giving rise to five rows of bristles each containing a variable number of spaced bristles. In *Calliphora vicina*, proneural expression corresponding to the AC, DC and SC bristle rows occurs in stripes, but that corresponding to the IA and SA rows is in proneural clusters. The number of bristles in the DC, IA and SA row is only very slightly variable. Bristles have a tendency to occupy more or less stereotyped positions. However, variability is quite common in the AC and SC rows, and a displacement of bristles from the stereotyped positions is also observed in the DC row. In *Ceratitis capitata* and *Drosophila melanogaster* expression of proneural genes occurs in clusters of cells that correspond to the positions of the bristles that occupy highly stereotyped positions (Cubas et al., 1991; Skeath and Carroll, 1991; Wülbeck and Simpson, 2000). The notal bristle pattern of *Drosophila* is extremely robust as changes are seen in less than 0.1% of individuals. Bristle rows are not present in many acalyptrates like *Ceratitis* and *Drosophila*, but the stereotyped arrangements may be derived from the pattern of rows in a common ancestor similar to that shown at the top, through secondary loss of bristles. Bristles are thus named AC, DC, IA or SA according to their presumed origin. The expression of proneural genes in clusters of cells in *Drosophila* is known to depend upon discrete *cis*-regulatory enhancer elements in the *achaete-scute* gene complex (Gomez-Skarmeta et al., 1995; Ruiz-Gomez and Modolell, 1987). One possibility is that these elements are derived from regulatory elements that allowed an expression of proneural genes in longitudinal stripes in an ancestor.

by lateral inhibition and the number of bristles in each row, and hence their position, would be variable. Four stripes of *sc* expression may have allowed the development of these rows, and they may have been a result of the activity of four discrete *cis*-regulatory elements. The two medial stripes would have been in the domain of *pnr* expression, and Pnr would have regulated activity of the two corresponding enhancers. During the course of evolution of the lineage leading to acalyptrate flies there has been a tendency to reduce the number of bristles through secondary loss (Grimaldi, 1987; Simpson et al., 1999; Sturtevant, 1970). At the same time the anterior-posterior positioning of individual bristles has become stereotyped. The *cis*-regulatory elements responsible for the stripes of *sc* expression may have been retained during this process and perhaps have been modified to drive expression in small proneural clusters. This development may have been accompanied by modulation of the activity of Pnr brought about by changes in the expression of regulatory cofactors such as Ush.

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