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Mutual Exclusion of Sensory Bristles and Tendons on the Notum of Dipteran Flies

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Summary

Background: Genes of the *achaete-scute* complex encode transcription factors whose activity regulates the development of neural cells. The spatially restricted expression of *achaete-scute* on the mesonotum of higher flies governs the development and positioning of the large sensory bristles. On the scutum the bristles are arranged into conserved patterns, based on an ancestral arrangement of four longitudinal rows. This pattern appears to date back to the origin of cyclorraphous flies about 100–140 million years ago. The origin of the four-row bauplan, which is independent of body size, and the reasons for its conservation, are not known.
Results: We report that tendons for attachment of the indirect flight muscles are invariably located between the bristle rows of the scutum throughout the Diptera. Tendon development depends on the activity of a transcription factor encoded by the gene *stripe*. In *Drosophila*, *stripe* and *achaete-scute* have separate expression domains, leading to spatial segregation of tendon precursors and bristle precursors. Furthermore the products of these genes act antagonistically: ectopic *sr* expression prevents bristle development and ectopic *sc* expression prevents normal muscle attachment. The product of *stripe* acts downstream of Achaete-Scute and interferes with the development of bristle precursors.
Conclusions: The pattern of flight muscles has changed little throughout the Diptera and we argue that the sites of muscle attachment may have constrained the positioning of bristles during the course of evolution. This could account for the pattern of four bristle rows on the scutum.

Introduction

The large sensory bristles, or macrochaetes, on the notum of *Drosophila* are found in an invariant, stereotyped pattern. This is due to the precise, spatially regulated expression of the genes of *achaete-scute (ac-sc)* complex (AS-C) in small, proneural clusters of cells at the sites of each future bristle [1–3]. Genes of the *ac-sc* family encode related basic-helix-loop-helix (bHLH) transcriptional regulators whose expression provides cells with neural potential [4]. Studies of the distribution of macrochaetes in other species of Diptera have provided insight into the origin of stereotyped patterns. Many cyclorraphous (higher) flies display a pattern of four longitudinal rows of bristles on the scutum [5, 6]. The four rows appear to be in homologous positions in different species, suggesting that an ancestor, common to most of today's species, may have possessed a four-row bauplan. Observations in *Calliphora vicina* indicated that such an ancestral pattern could have been generated from four stripes of *sc* expression [7]. Stereotyped arrangements may be derived from the ancestral pattern through loss of bristles, since nearly all species have arrangements that can be superimposed over the four rows. It is not known why the number of rows should be restricted to just four, a pattern that is independent of body size. Cyclorraphous flies are thought to have arisen about 100–140 million years (myr) ago [5, 8], so this pattern has remained remarkably stable. Is the positioning of bristles constrained by some other feature of the development and organization of the Dipteran mesonotum [9]? Alternatively, is it the result of a preferred direction of selection [10]?

The enlarged tergal wall of the mesothorax of Diptera is given over entirely to flight and houses the powerful flight muscles [11]. These attach to tendons that in insects develop from epidermal cells. The development of tendon cells is dependent on *stripe (sr)*, a gene encoding an early growth response *(egr)*-like transcription factor [12–14]. *stripe* is expressed in spatially restricted domains on the notum of *Drosophila* [15]. Interestingly, we find that the domains are situated between the inferred positions of the four bristle rows of the ancestral pattern and that expression of *sr* does not overlap with that of *ac-sc*. The spatially distinct expression domains of *sr* and *ac-sc* lead to a spatial segregation of the precursors of tendons and of bristles. Furthermore the two genes act antagonistically: ectopic *sr* expression prevents bristle development and ectopic *ac-sc* expression prevents normal muscle attachment. Examination of more than 300 species of Diptera revealed that tendons are invariably positioned between the rows of macrochaetes. We postulate that the positions of macrochaetes have been constrained during evolution by the sites of attachment of the flight muscles, resulting in a four-row bauplan.

Results

The Precursors of Macrochaetes and Tendons Develop at Different Locations Due to Spatially Separate Expression Domains of *stripe* and *achaete-scute*

By using plasmid rescue, we have characterized a Gal4 insertion in the *stripe (sr)* gene, sr^{MD710}, recovered during a screen for adult patterning genes in *Drosophila* [16] that fails to complement mutant *sr* alleles (Figure 1D). When crossed to *UAS-GFP*, sr^{MD710} shows an expression pattern on the prospective scutum identical to that pre-

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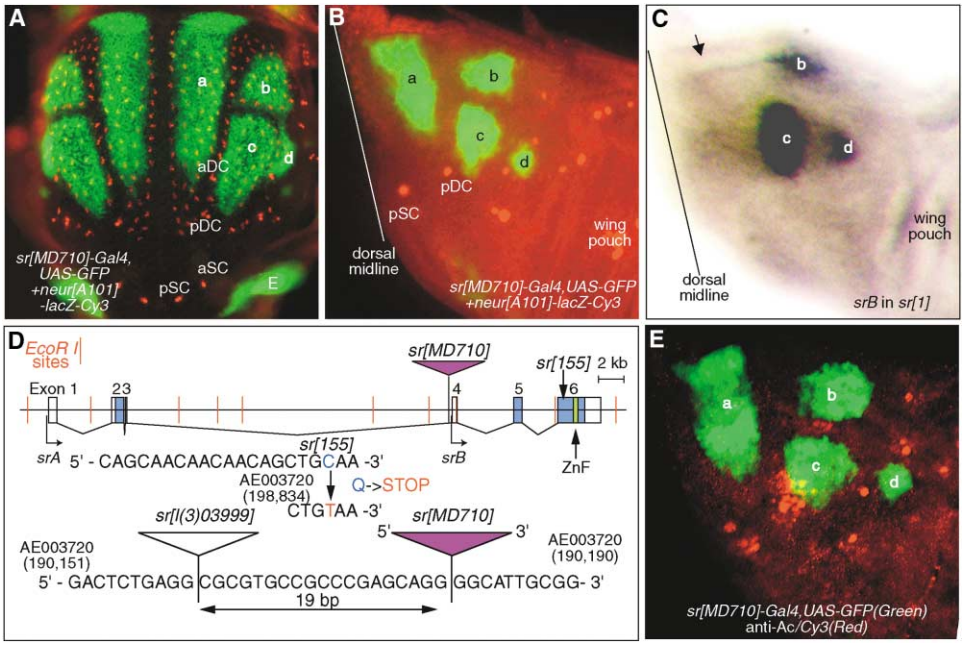

Figure 1. The Expression Patterns of *stripe* and *achaete* on the Notum

The expression of *stripe* on the pupal notum (A) and in the thoracic part of the third larval instar wing disc (B) are visualized with the use of *sr-Gal4* and *UAS-GFP*. Bristle precursors are labeled with $neur^{A101}$*-lacZ* and stained with anti-β-gal antibody. Macrochaete precursors are outside the domains of *sr* expression. (C) In situ hybidization with an *srB* probe [13] in a third instar larval wing/thorax disc of a sr^{1} mutant larva. The medial domain of expression (a) is missing. (D) *stripe* has been shown to contain a triple zinc finger domain in its C-terminal region [12, 13]. Two splice variants are produced at the *sr* locus; they differ in their 5′ region and share the 3′ region with the zinc finger motifs [13]. The Gal4 insertion line sr^{MD710} is located in the intron that separates the different 5′ regions from the common 3′ region, very close to *l(3)03999* (P1618), another P element insertion previously identified [13]. Sequencing of sr^{155} uncovered a single nucleotide substitution resulting in a stop codon (amino acid Q590 STOP of *srA* and Q316 STOP of *srB)*. This would result in a truncated protein lacking the zinc finger motifs required for transcriptional activity [12, 13]. (E) The spatial expression domains of *stripe* and of *achaete* (visualized with an anti-Ac antibody) show very little overlap in late third instar discs.

viously described (Figures 1A and 1B) [15]. Two splice variants are produced at the *sr* locus [13]. We have determined that the isoform *srB* is expressed in the imaginal disc and that *srA* comes on much later, after formation of macrochaete precursors is completed (not shown). *stripeB* is expressed at the time when the macrochaete precursors arise [1, 2], but notably, *sr* expression does not cover the sites where precursors of the macrochaetes develop, as shown by double labeling for *sr* and $neur^{A101}$*-lacZ* (Figures 1A and 1B). Additionally, double labeling for *sr* and *ac* expression in the imaginal disc reveals very little overlap between them (Figure 1E). Embryonic staining revealed that the expression domains of *sr* and *ac-sc* are also spatially separated during development of the larval peripheral nervous system (not shown).

We examined *sr* mutants to see whether a loss of *sr* function leads to ectopic bristles or ectopic *ac-sc* expression in the domains of *sr* expression. $stripe^{1}$ is a viable allele lacking the large expression domain in the medial (dorsalmost) region of the notum (Figure 1C). The loss of this domain is not accompanied by ectopic expression of *ac-sc* at this site in larval discs (not shown). We have determined that sr^{155} [17] is a null allele (Figure 1D). Flies homozygous for sr^{1} or transheterozygous for sr^{1}/sr^{155} display additional macrochaetes on the scutum at a very low frequency (an additional dorsocentral bristle was found in 1/100 sr^{1}/sr^{1} and 8/300 sr^{1}/sr^{155} hemithoraces). Thirty-four thoraces bearing one or more clones of cells mutant for sr^{155} were examined; collectively they covered the entire dorsal notum. No ectopic macrochaetes were seen; each of the eleven extant macrochaetes was identified at least twice within mutant territory (Figure 2A). We conclude that endogenous Sr is not normally required to repress bristle development.

In a reciprocal fashion, animals devoid of Ac and Sc $(In(1)ac^{3}\ sc^{10\text{-}1})$, which have no bristles on the notum, display a normal pattern of correctly attached flight muscles (not shown).

Mutually Antagonistic Activities of *stripe* and *achaete-scute*

In flies mutant for $ac^{Hairy\ wing1}$ (ac^{Hw1}), ectopic macrochaetes develop due to generalized overexpression of *ac* [18]. These bristles cluster at sites devoid of *sr* expression (Figure 2B). This suggests that when *ac-sc* proteins accumulate ectopically in the domains of *sr* expression, endogenous Sr is able to prevent bristle development. Consistent with this, there is a broader distribution of ectopic bristles over the notum of ac^{Hw1}; sr^{1} double mutant flies than in flies mutant for ac^{Hw1} alone (Figure 2C). In the medial notum, where *sr* expression is absent in sr^{1} (Figure 1C), many more bristles are found.

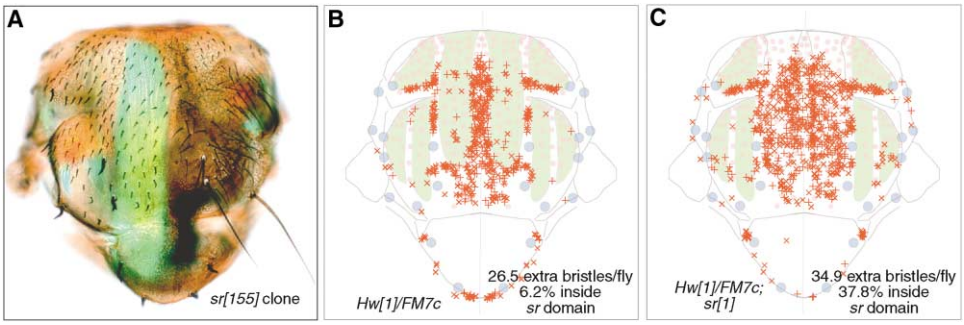

Figure 2. *stripe* Mutant Phenotypes

(A) Notum bearing a clone of cells mutant for sr^{155}, viewed under polarized light. The clone differentiates full-length bristles in a background of short *Ki Sb* bristles. The mutant cuticle is very uneven and displays black pigmentation. The black pigmentation extends into areas outside the known domains of *stripe* expression. Possibly low levels of Stripe are present elsewhere. Mutant, and even wild-type bristles at the mosaic border, may be displaced, possibly due to physical deformation. The underlying muscles are poorly differentiated and unattached; only the wild-type ones on the left are visible (green). Ectopic macrochaetes on 25 heminota of ac^{Hw1} (Hw^{1}) (B) and ac^{Hw1} (Hw^{1}); sr^{1} (C) mutant flies are shown as red crosses. Domains of expression of *srB* are indicated in green, and the positions of wild-type bristles are in pale blue. Ectopic bristles in ac^{Hw1} (Hw^{1}); sr^{1} flies occupy the domain of expression missing in sr^{1}.

In addition, there is a synergy between ac^{Hw1} and *sr* mutants: the double mutant displays more bristles than the sum of the two single mutants (Figures 2B and 2C). Thus the patterned distribution of bristles in ac^{Hw1} mutants is due to an antagonistic function of endogenous *sr* activity on bristle development.

To determine whether *sr* is able to repress *ac-sc* activity we looked at the consequences of ectopic and overexpression of Sr on bristle development. Note that *stripe* has been shown to induce its own expression when overexpressed in the embryonic ectoderm [19]. Overexpression in the endogenous *sr* pattern *(sr-Gal4/UAS-SrB)* resulted in a severe decrease in the number of small bristles or microchaetes (not shown). Overexpression in proneural *(ac-sc)* domains and then in bristle precursors using *sca-Gal4* resulted in a total lack of all bristles (Figures 3A and 3E). Staining with an antibody against Ac, nevertheless, revealed the presence of this protein in proneural clusters at levels indistinguishable from the wild-type (Figures 3B and 3F). However, whereas in wild-type flies *ac-sc* expression is then refined to single cells that become the bristle precursors, in *sca-gal4/UAS-SrB* flies *ac* expression is not refined. Furthermore, staining of these animals with an antibody to the *senseless* protein known to be required for precursor formation and maintenance [20] demonstrated that bristle precursors fail to form (Figures 3C and 3G). This suggests that Sr does not repress transcription of *ac-sc*.

To examine this further, we looked at the effects of Sr on Sc expressed from a heterologous promoter (UAS-SC). Overexpression of Sc induces additional macrochaetes (Figure 3D), but, when Sr is coexpressed with Sc, the number of bristles is strongly reduced (Figure 3H). These results suggest that Sr does not directly regulate transcription of *ac-sc* but can antagonize a stage in the formation and/or maintenance of the bristle precursors. Direct autoregulation of *ac-sc*, as well as positive feedback loops involving other factors, have been shown to be important in precursor formation [20–22]. Note therefore that factors acting downstream of the initial *ac-sc* transcription in proneural clusters can, in some cases, indirectly affect transcription of *ac-sc* in the precursors.

In a reciprocal fashion, when ectopically expressed, Ac-Sc impedes flight muscle attachment. *sr-Gal4/UAS-SC* flies have ectopic macrochaetes in the domains of *sr* expression (not shown). Endogenous *sr* protein appears insufficient to overcome the high levels of Sc in this genotype. We noted that instead of the usual six large bundles, the longitudinal flight muscle bundles are very much thinner and more numerous (Figures 3I and 3J) and that the animals are unable to fly, although they can walk.

Macrochaetes Are Excluded from the Sites of Flight Muscle Attachments in Diptera

By using a crossreacting RNA probe, we have looked at the expression of *sr* in two other species of Drosophilidae, *D. testacea*, and *D. ararama*. The latter is thought to be phylogenetically separated from *D. melanogaster* by about 60 myr. In both cases a conserved pattern of expression was observed (not shown). The indirect flight muscles have been described in a number of families of Diptera: they show only minor variations (our unpublished observations) [11, 23]. The conservation of muscle patterns and *sr* expression suggests that the location of tendons may be conserved in other Dipteran species. The pattern of attachment of the dorsal longitudinal and dorsoventral indirect flight muscles at the sites of *sr* expression in *Drosophila melanogaster* is schematized in Figure 4B [15, 24], and the muscle pattern is illustrated in *Anopheles gambiae* and *Calliphora vicina* (Figures 4C and 4D).

To see whether tendons and macrochaetes are always spatially separate in other flies with different bristle patterns, we examined over 300 species spread over the phylogenetic tree of the Diptera (Figure 4A). Macrochaetes are not found in basal species (Nematocera) but are a feature of many higher flies, particularly cyclorraphous Brachycera [5]. Amongst more than 200 species of cyclorraphous flies examined, without exception the macrochaetes were found to be located outside

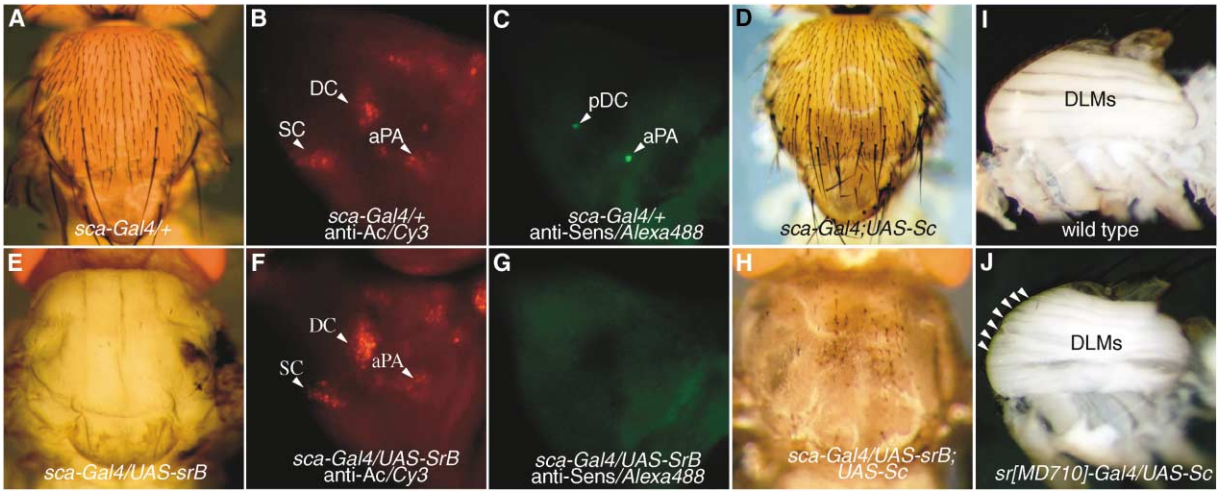

Figure 3. Phenotypes Resulting from Overexpression of *stripe* or *achaete*

sca-Gal4 drives expression in proneural domains and then in bristle precursors. Proneural clusters can be seen in (B), with an anti-Achaete antibody (red). Two emerging bristle precursors (pDC and aPA) can be seen in (C) with an anti-Senseless antibody (green). When *sca-Gal4* is used to drive *UAS-srB* [13], a loss of bristles occurs (E); compare with the control animal in (A), which displays a wild-type bristle pattern. *achaete* expression is normal in *sca-Gal4/UAS-SrB* flies (F), but the bristle precursors are absent (G). Overexpression of Scute using the *sca-Gal4* driver induces ectopic macrochaetes (D). However, overexpression of both of Stripe and Scute with this driver removes most of the ectopic bristles and also many of the extant ones (H). (I) Half section of the thorax of a wild-type fly showing the six large bundles of the dorsal longitudinal muscles. (J) Half section of the thorax of a fly resulting from overexpression of Sc in the *sr* expression domains. There appear to be more than six muscle bundles that are much thinner and less well organized than the wild-type ones. Abbreviations: DC, dorsocentral; SC, scutellar; aPA, anterior postalar; pDC, posterior DC; and aPA, anterior postalar.

the sites of muscle attachment. Examples are shown in Figure 5. The dorsal longitudinal muscles are attached between the acrostichal and dorsocentral bristle rows, the dorsoventral muscles between the dorsocentral and intra-alar bristles, and the tergal depressor of the trochanter (leg jump muscle) between the intra-alar and supra-alar rows (see Figure 6).

A number of fly species are ectoparasites, and, although some retain their wings, in others the wings are reduced or absent [25, 26]. The notal bristle patterns of these flies are quite diverged from those of most other Schizophora. The deer ked or louse fly *Lipoptena cervi* (Hippoboscidae; Figure 5H) emerges with wings and flies to a host. After the first blood meal the wings break

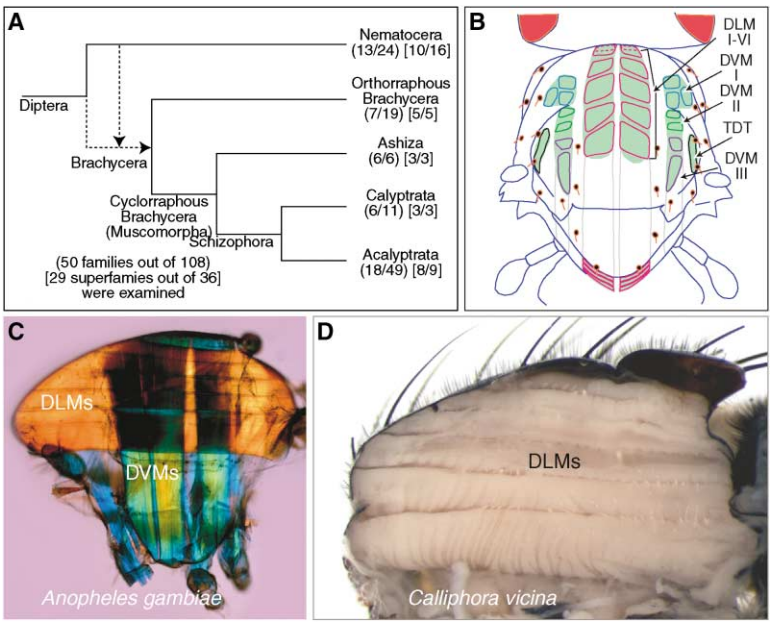

Figure 4. Macrochaetes Are Excluded from Muscle Attachment Sites in Diptera

(A) Simplified phylogenetic tree of the Diptera indicating the spread of families and superfamilies examined. The Nematocera are basal, and the acalyptrate Schizophora the most derived flies [8].

(B) Sites of attachment of the indirect flight muscles in *Drosophila* are shown by solid, colored lines. *stripe* expression domains are indicated in green. There are two main sets of muscle fibers. The six large fibers of the dorsal longitudinal fibers (DLM I to VI, pink), the wing depressors, extend longitudinally and their anterior ends attach at the site of the medial domain of *sr* expression. There are three groups of fibers belonging to the dorsoventral muscles, the wing levators (DVM I, II, and III, blue, green, and purple respectively), running perpendicular to the DLMs, that attach at the positions of the lateral domains of *sr* expression. The tergal depressor of the trochanter (TDT) is attached at the site of the most lateral *sr* domain (black). Macrochaetes are shown in orange.

(C and D) Photographs of a half section of the thorax of *Anopheles gambiae* and *Calliphora vicina*, respectively. When viewed with polarized light (C), the two tiers of six DLMs appear orange and the DVMs blue green.

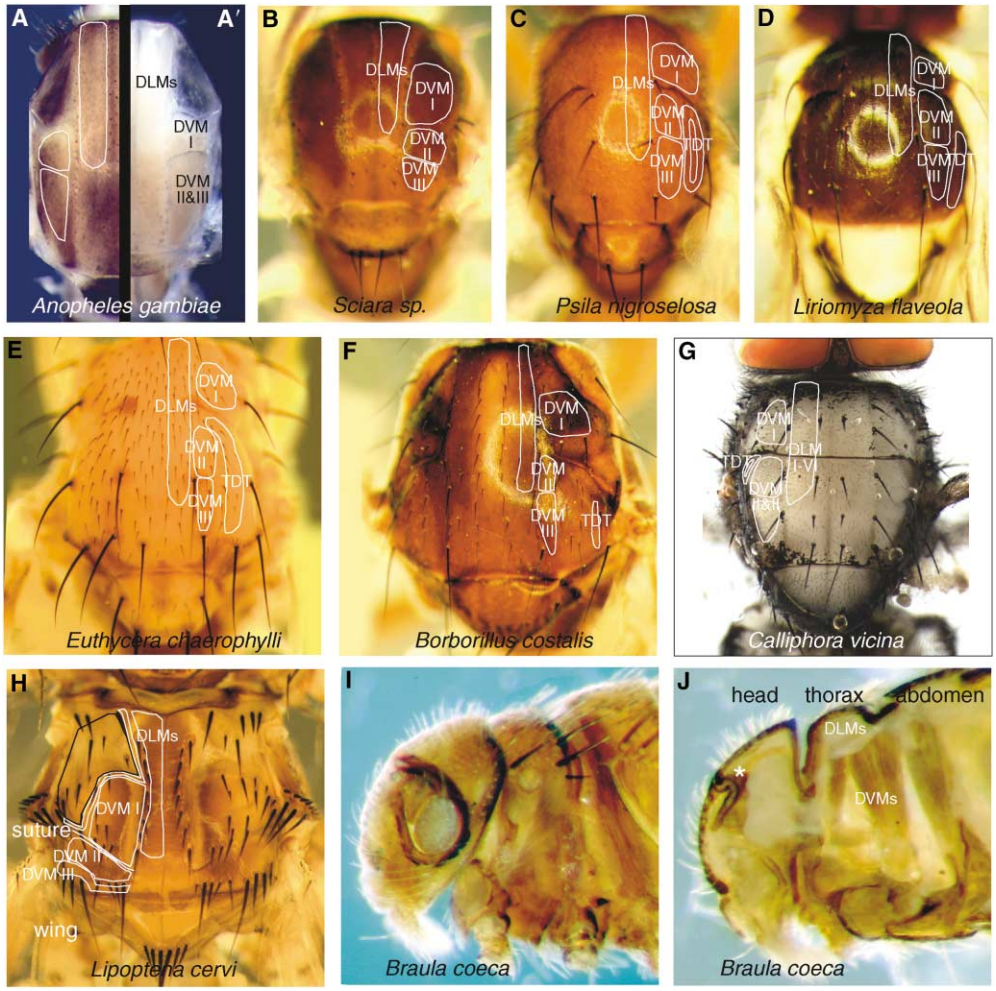

Figure 5. Representative Dipteran Species Showing Positions of Bristles and Sites of Muscle Insertion

Positions of bristles relative to the sites of flight muscle attachment (white lines) are shown for representative fly species (see Figure 4A for phylogeny).

(A and B) Two species of Nematocera: *Anopheles gambiae*, family Culicidae and a species of the genus *Sciara*, family Sciaridae. The rows of thin, flimsy bristles in these species do not appear to be homologous to the macrochaetes of cyclorraphous flies [35], but are nevertheless situated on either side of the DLM tendons.

(C–G) Cyclorraphous flies in (C)–(G) belong to the families Psilidae, Agromyzidae, Sciomyzidae, and Sphaeroceridae (acalyptrates) and Calliphoridae (calyptrate), respectively. A negative correlation between the positions of bristles and tendons can be seen.

(H–J) Parasitic species of calyptrate (H) and acalyptrate (I and J) flies. The sites of attachment of the DLMs are reduced in *Lipoptena cervi* (Hippoboscidae), and a space where no muscles are attached is shown in black. Bristles are excluded from muscle attachment sites. The bee "louse" *Braula coeca* (Braulidae) is devoid of wings, and the mesothorax is reduced to a short mesonotal plate fused to the postpronotum, with no scutellum. The transverse row of stiff bristles is in a region where none of the reduced muscles attach. The asterisk in (J) indicates the ptilinum, a hallmark feature of the Schizophora.

off at the base and both direct and indirect flight muscles lose their attachments, break into fragments, and are completely lysed [27]. The individual we recovered was still in possession of its wings. There is only one clear longitudinal bristle row, the presumed dorsocentral row, which is situated between the dorsolongitudinal and dorsoventral muscles. In contrast bristles along the posterior margin of the scutum and the scutellum are arranged into transverse rows. Female *Puliciphora boringuenensis* (Phoridae, associated with ants) and male and female *Braula coeca* (the bee "louse," Braulidae), are minute, devoid of wings, and have a much-reduced mesothoracic segment with severely reduced flight musculature and no scutellum [26, 28]. There are no recognizable bristles belonging to the four rows of the bauplan; instead, there is a transverse row of bristles along the posterior border of the mesothorax, similar to that characteristic of abdominal segments (Figures 5I and 5J). In spite of the diverged patterns, bristles are not situated over the sites of muscle attachment in all of these species.

Discussion

In *Drosophila*, *sr* and *ac-sc* are expressed in spatially distinct domains on the notum. This is likely to be the

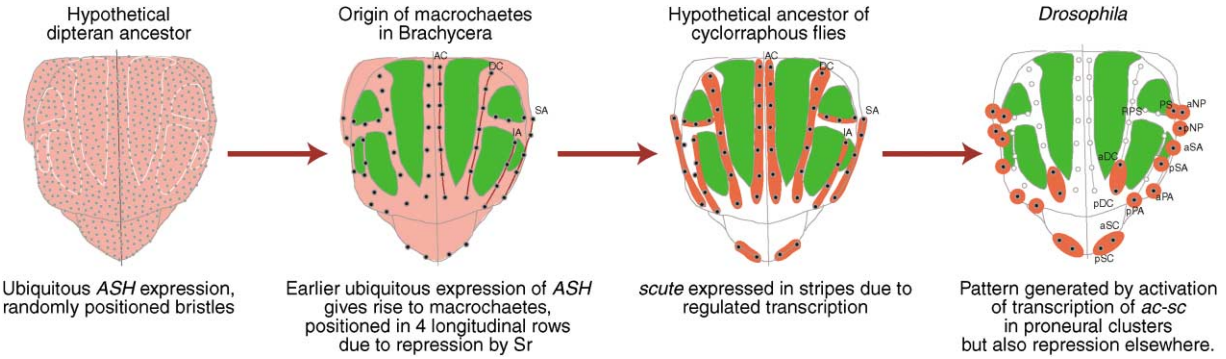

Figure 6. A Multistep Model for the Evolution of the *Drosophila* Bristle Pattern

The Dipteran ancestor is hypothesized to have had ubiquitous *ASH* expression (pink), and a random distribution of bristles [6, 37]. An earlier expression of *ASH* may have led to the appearance of macrochaetes in an ancestor of the Brachycera, and bristle development would have been prevented at the sites of tendons through the activity of *stripe* (green). During the evolution of cyclorraphous flies, *ASH* expression (red) may have been regulated to produce four stripes at the sites of the four bristle rows. The rows are labeled AC (acrostichal), DC (dorsocentral), IA (intra-alar), and SA (supra-alar). The stereotyped pattern in *Drosophila* is based on transcriptional activation at precisely defined sites, which may have involved the acquisition, perhaps in several stages, of discrete *cis*-regulatory modules [37]. Abbreviations: aDC and pDC, anterior and posterior dorsocentral bristles; aNP and pNP, anterior and posterior notopleural bristles; aSA and pSA, anterior and posterior supraalar bristles; aPA and pPA, anterior and posterior postalar bristles; and PS, presutural bristle.

case too for other cyclorraphous flies. The expression pattern of *sr* is conserved in at least three *Drosophila* species, and the expression of *sc* during macrochaete formation in *Ceratitis capitata, Calliphora vicina*, and *Phormia terranovae* avoids the sites of muscle attachment as it does in *Drosophila* [7, 29, 30]. We demonstrate that when misexpressed in *D. melanogaster*, Sr and Sc antagonize one another's activities. Sr does not appear to repress transcription of *ac-sc* but may act downstream on one or more factors required to maintain high levels of proneural protein in the bristle precursors. However, in otherwise wild-type animals, loss of the endogenous *sr* or *ac-sc* gene products does not result in ectopic bristles or tendons. Nevertheless, two observations lead us to think that *sr* does have a role in repressing bristle development. Firstly, it is expressed early in the imaginal discs long before the tendon precursors form [31], and secondly, it appears to act redundantly with other repressors (our unpublished data). Macrochaetes are situated outside the sites of muscle attachment in all Diptera examined, suggesting that the spatial segregation of bristles and tendons has some significance for the flies. The mutually antagonistic properties of Ac-Sc and Sr would maintain this segregation, should the normal regulation of these genes, which involves a complex genetic network and many players [3, 4, 22, 32, 33], be impaired.

It is interesting to speculate that *sr* may have been part of an ancestral mechanism of bristle patterning. The macrochaetes on the scutum of most flies are derived from a bauplan of four rows that may have been present in a common ancestor [6]. Remarkably, in *Drosophila*, *sr* is expressed between the inferred rows of the postulated ancestral pattern. The pattern of indirect flight muscles and their attachments appears little changed throughout the Diptera, so the function of *sr* is likely to be phylogenetically ancient. The ancestor of the Diptera may have had randomly distributed bristles like those of extant basal flies (Nematocera) [5]. This could have resulted from ubiquitous expression of an *ac-sc* homolog *(ASH)*, as is the case for the scales of butterflies and a mosquito (Nematocera) as well as the microchaetes of higher flies [7, 29, 30, 34, 35]. If during the evolution of macrochaetes *sr* acquired a new function to repress bristle development, then repression by *sr*, in an animal with ubiquitous expression of an *ASH*, would have generated a pattern of rows (Figure 6).

The very precise positioning of macrochaetes in *Drosophila* is achieved by spatially restricted transcriptional activation of *ac-sc* in small proneural clusters that prefigure the sites of each bristle. Studies in *Calliphora vicina*, however, suggest that the four rows in a common ancestor of higher, cyclorraphous flies may have been generated from four stripes of *sc* expression [7]. If expression of an *ASH* was ubiquitous in the Dipteran ancestor, then this would imply a change in the transcriptional regulation of *ASHs* (Figure 6). The proneural clusters of *Drosophila* result from the activity of shared *cis*-regulatory enhancer sequences that respond to local transcriptional activators [3, 33, 36]. Nevertheless, a number of different repressors, such as the products of *extramacrochaetae, hairy*, and *u-shaped*, are also required to prevent levels of Ac-Sc accumulating outside the proneural clusters [4, 6, 32]. So bristle patterning in this species relies on both activation and repression of the activity of the *ac-sc* genes. We postulate that transcriptional activation may be a more recently derived patterning mechanism. If so, the *cis*-regulatory modules for activation of the AS-C may be of recent origin. The addition of these modules would have enhanced both the precision and robustness of the pattern. At least one of the AS-C enhancers present in cyclorraphous flies appears to be absent in *Anopheles gambiae*, a basal species [22]. The number of genes at the AS-C has increased throughout the Diptera by duplication, and it is conceivable that this may have provided material for the evolution of these modules [37].

Spatial Segregation of Bristles and Tendons: A Developmental Constraint?

Flies have remarkable powers of flight and the conservation of the pattern of flight muscles probably results from strong selective pressures. We have found that sites of muscle attachment to the epidermis are also conserved, indicating a similar location of tendons. In contrast, the positions of macrochaetes vary considerably throughout the higher flies [5]. Our survey of more than 300 species indicates, however, that this variation occurs only within the limits imposed by muscle patterning. Macrochaete patterns may therefore have been constrained during evolution by the sites of flight muscle attachment, thus accounting for the bauplan of four longitudinal rows at the origin of most patterns [6]. The concept of developmental constraint has been discussed extensively [9]. It proposes that certain phenotypic traits are not seen because the genetic mechanisms underlying development do not allow their formation. The alternative is that such traits are simply not favored by selection [10]. Here, we argue that bristle patterns may be constrained by the sites of muscle attachment.

Apart from the fact that they are mechanosensory organs, the function of macrochaetes is unknown. If the segregation of tendons and bristles is important for the function of either one, then one might expect their separation to be maintained by selection. We cannot know what selective pressures have operated in the past, and in an ancestor of the cyclorraphous flies, bristles and tendons may have been kept separate by the forces of selection. Subsequently, however, the genetic circuitry required for development could have evolved to an extent that in extant species they do not allow the development of bristles over the muscle attachment sites. One argument in favor of this comes from the study of *Drosophila* lines artificially selected in the laboratory. Selection for an increased number of macrochaetes on the scutum gives rise to flies with rather specific bristle patterns. Additional DC bristles form and some bristles situated on the lateral scutum [38–42]. The DC bristles may number as many as 40 and are either arranged in a cluster around the position of the wild-type ones [41] or are aligned into a longitudinal row extending anteriorly [38]. We have examined several of these lines (generously provided by Bruce Sheldon and Jesus Albornoz) and have ascertained that the bristles are not located over the sites of muscle attachment that are situated on either side of the ectopic DC bristles (our unpublished data). This suggests that artificial selection for ectopic bristles does not readily overcome the mechanism that prevents formation of bristles over muscle attachment sites. Therefore, Sr may limit the variation to generate different bristle patterns. A further observation consistent with an Sr-induced constraint is that in flightless ectoparasitic flies with diverged bristle patterns not arranged into longitudinal rows, the macrochaetes are nevertheless consistently excluded from the muscle attachment sites.

In addition to macrochaetes, higher flies have microchaetes, small mechanosensory bristles that are generally not patterned. Basal flies do not have macrochaetes (long, stout, thick bristles), and their thin, flimsy bristles may be located anywhere on the scutum. Puzzlingly, microchaetes are not excluded from the sites of muscle attachment. It is not known whether the two classes of bristles have different functions, but they differ in morphology and, at least in *Drosophila*, mode of development. Firstly, formation and maintenance of macrochaete precursors (the probable point of intervention by Sr) requires a specific regulatory sequence not used for microchaete development [21] (our unpublished data). Secondly, the microchaetes develop later, when a second Sr isoform, SrA, [13] is coexpressed with SrB (our unpublished data).

Macrochaetes seem to have arisen in the Brachycera [5], and their appearance may have been caused by the acquisition of an additional, earlier phase of *ASH* expression [30]. The macro- and microchaetes of cyclorraphous flies arise from two temporally distinct phases of *sc* expression, whereas all notal sensory organs of *Anopheles gambiae*, a basal species, arise from a single, late phase of *AgASH* expression [1, 2, 7, 29, 30, 35]. Amongst the derived taxa, however, there are species at scattered phylogenetic positions, devoid of macrochaetes [5]. So it is not clear whether these structures have arisen many times or whether they arose once and have been lost in a number of lineages [5, 6]. A common ancestor of the monophyletic cyclorraphous flies is likely to have existed more than 100–140 myr ago [5, 8], so if macrochaetes evolved only once, the four row bauplan must have been strongly selected for. On the other hand, if any early accumulation of ASH were to be antagonized by Sr, then the bristles would consistently be restricted to non-*sr*-expressing areas, and macrochaetes arranged in similar patterns could have arisen many times independently [43].

In addition to being restricted to areas outside the muscle attachment sites, in many species the number as well as the position of individual macrochaetes is highly stereotyped. Amongst acalyptrate flies there has been a tendency to reduce the number of macrochaetes to just a few [44]. This means that even at some locations devoid of *sr* expression, macrochaetes do not develop. Many stereotyped patterns are phylogenetically ancient; for example, the pattern in the Drosophilidae has been conserved for at least 40 myr [45]. This suggests that in addition to exclusion from muscle attachment sites by Sr, the precise positioning of bristles may be maintained by selection. Studies of *Drosophila* hybrids have provided evidence of stabilizing selection for the identical bristle pattern seen between these two species [46]. Given their scattered phylogenetic locations, the reduction/loss of wings and flight muscles in ectoparasitic species is almost certainly a result of convergence. The fact that these modifications are associated with bristle patterns that have diverged from those of winged species again suggests the patterns common to flying Diptera are subject to selective pressures. We propose that stereotyped macrochaete patterns may be the result of two independent forces. First, a constraint induced by flight muscle attachment may restrict the bristles to certain locations that form the basis of the four-row bauplan. Second, selective pressures may operate to maintain precise positions of individual bristles.

Experimental Procedures

Fly Strains

The molecular structure of the sr^{MD710} insertion was determined by sequencing of a 1.2 kb Pst1 genomic fragment obtained by plasmid rescue and that of sr^{155} by sequencing all exons by single embryo PCR (Figure 1D). The line *sca-Gal4*, together with *UAS-SrB* [19], *UAS-SC*, or *UAS-GFP*, and the enhancer trap line $neur^{A101}$ were used. Embryos were grown at 18°C and shifted to 25°C during larval development. For mutant strains, see FlyBase.

Mutant Clones

Loss-of-function clones of sr^{155} were generated either by X-rays in *Ki Sb*/sr^{155} flies (1000 rads: 100 kv, 10 mA for 5 min, 1.5 mm aluminium filter, Philips MG102 constant potential X-ray system, beryllium window) or by the FLP/FRT technique [47]: *HS-FLP; FRT82B* sr^{155}/*FRT82B Ki Sb* flies.

In Situ Hybridization and Antibody Staining

In situ hybridization was performed as in [19], with some modification: dissected wing discs were fixed in 4% formaldehyde/PBS, and then after washing in PBS-Tx, they were kept in hybridization buffer until use. No methanol or proteinase K treatment was employed. The anti-Achaete antibody was obtained from the Hybridoma Bank and the anti-Senseless antibody from H. Bellen. Standard antibody staining was applied. Fluorescence images were taken with a Leica microscope using FW4000 software. Images were processed with Photoshop (Adobe).

Fly Collection

Flies were collected from the Cambridge backs by using nets and a Malaise trap and were kept in 70% alcohol. Some species were obtained from the collection held in the Cambridge Museum of Zoology. A list of species examined and their classification is available on request. Thoraces were sectioned along the midline revealing the flight muscles and their points of insertion, which were examined by polarized light. Some darkly pigmented flies (e.g., Figure 5G) were bleached prior to examination.

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