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Transcriptional Heterochrony of *scute* and Changes in Bristle Pattern between Two Closely Related Species of Blowfly

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Temporal shifts in the expression of regulatory genes, relative to other events taking place during development, can result in changes in morphology. Such transcriptional heterochrony can introduce dramatic morphological changes that involve rather few genetic events and so has the potential to cause rapid changes during evolution. We have shown previously that stereotyped species-specific bristle patterns on the notum of higher Diptera correlate with changes in the spatial regulation of *scute* expression. *scute* encodes a proneural gene required for the development of sensory bristle precursors and is expressed before pupation in discrete domains on the presumptive notum at sites where the macrochaete precursors arise. Thus, for *Ceratitis capitata* and *Calliphora vicina*, species separated from *Drosophila melanogaster* by about 80 and 100 million years respectively, the domains of *sc* expression differ. In all three species, a second phase of ubiquitous *sc* expression, after pupation, precedes formation of the microchaete precursors. Here, we describe *sc* expression in *Phormia terranovae*, a species belonging to the family Calliphoridae that is closely related to *C. vicina*. We find that spatial regulation is almost identical between *P. terranovae* and *C. vicina*, in spite of their different bristle patterns. The timing of *sc* expression differs, however, between the two. The first spatially restricted phase of expression is slightly delayed and the second ubiquitous phase remarkably accelerated, such that there is a period of overlap. As a result, the last precursors from the first phase of expression arise at the same time as the first precursors from the second phase of expression and are morphologically indistinguishable from the late-arising microchaetes. These observations illustrate the power of developmental heterochrony in bringing about rapid morphological change.

***Key Words: achaete-scute*; bristle pattern; diptera; blowfly; heterochrony.**

INTRODUCTION

Many key genes that regulate embryonic development are known to have been conserved over long periods of evolutionary time. Differences in morphology between species are therefore likely to have arisen through changes in the deployment of these genes, which may be coopted to serve new functions in different genetic networks and their products acquire different molecular specificities (Duboule and Wilkins, 1998; Eizinger *et al.*, 1999). Another mechanism that can cause morphological change is heterochrony: phyletic changes in the development of some characters relative to others (Evans *et al.*, 1994; Gould, 1977, 1992; MacDonald and Hall, 2001; Patel, 1994; Richardson, 1995; Schlosser, 2001). These may be linked to temporal changes in the activity of specific gene(s) (Evans *et al.*, 1994; Wiltshire *et al.*, 1994). An experimentally contrived delay in transcription of *Hoxd-11* in the mouse, for example, leads to caudal displacement of the sacrum (Zakany *et al.*, 1997). Macroevolutionary change probably results from many small, incremental alterations in gene expression, but heterochonic shifts can conceivably lead to more rapid morphological change (Gould, 1977, 1992).

Comparison of closely related species may reveal discrete changes in gene activity that will help in the understanding of the evolution of developmental mechanisms. One good model in which to explore such changes is afforded by the regulation of sensory bristle patterns in Diptera. In higher flies, the large bristles are often organised into stereotyped spatial arrays in which each bristle occupies a defined position. Bristle precursors are born in a spatial array within

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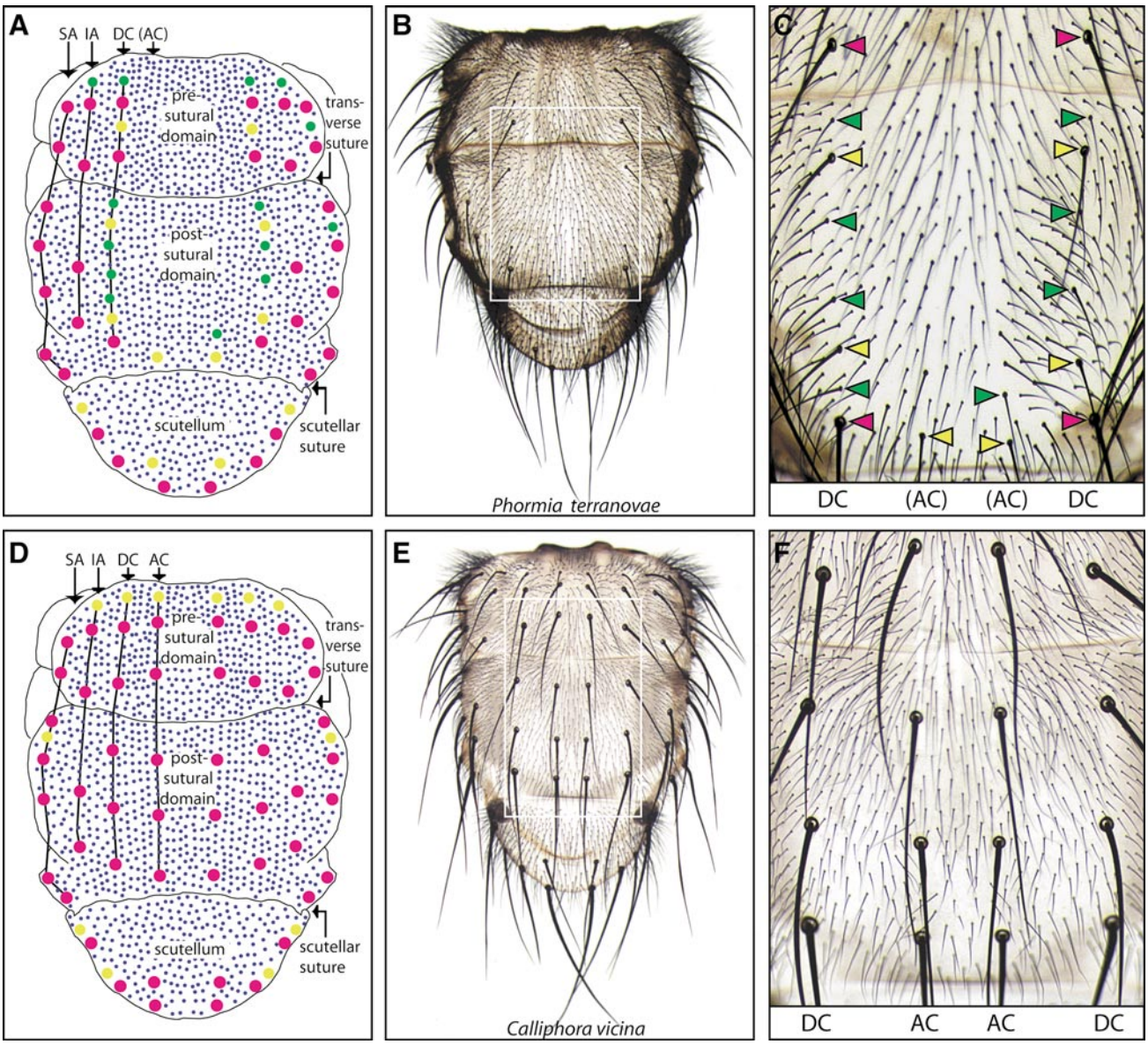

FIG. 1. Bristle patterns on the notum of *P. terranovae* (A–C) and *C. vicina* (D–F). (C) and (F) are enlargements of the boxed regions of (B) and (E), respectively. Pink dots/arrows indicate large macrochaetes, which are invariably present; yellow dots/arrows indicate intermediate macrochaetes which are often present; green dots/arrows indicate small macrochaetes which are frequently absent; blue dots indicate true microchaetes. *C. vicina* displays a pattern of four complete longitudinal bristle rows on the scutum. Only the intra-alar (IA) and supra-alar (SA) rows are complete in *P. terranovae*. The acrostichal row (AC) is represented by a single posterior bristle called the prescutellar, located just above the scutellar suture; the dorsocentral row (DC) generally bears large presutural macrochaetes, while all except the most posterior of the postsutural bristles are intermediate or small.

a two-dimensional epithelium and do not move from their site of origin. They are specified by the activity of the genes of the *achaete-scute* (*ac-sc*) family, and in several species it has been demonstrated that stereotyped bristle patterns result from precise spatial regulation of transcription of these genes (Cubas *et al.*, 1991; Gomez-Skarmeta *et al.*, 1995; Pistillo *et al.*, 2002; Romani *et al.*, 1989; Skeath and Carroll, 1991; Wülbeck and Simpson, 2000).

Higher Diptera of the Brachycera suborder often display two distinct categories of bristles of very different size: macrochaetes and microchaetes (taxonomists refer to bristles and hairs, respectively). Macrochaetes are generally

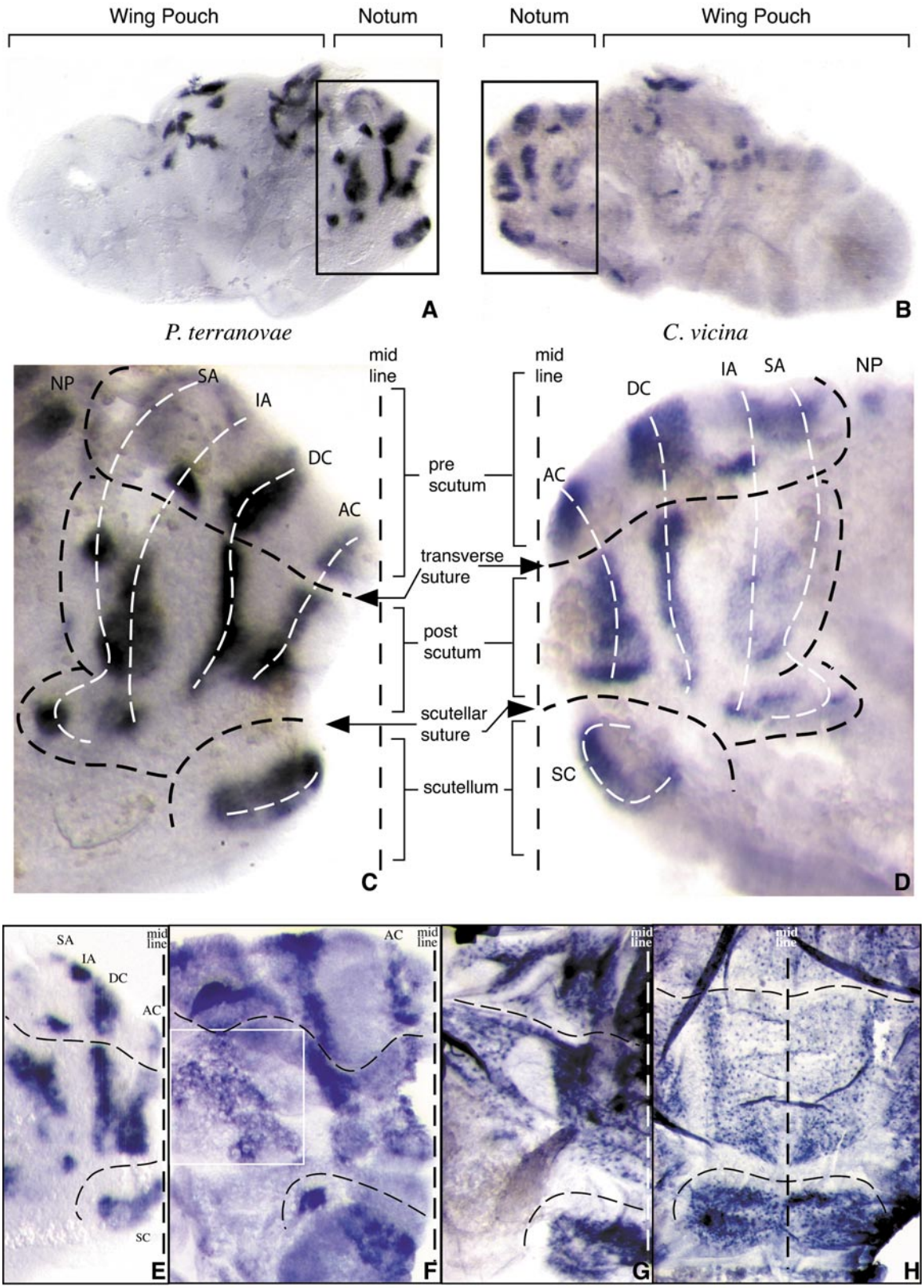

FIG. 2. Comparison of *scute* (*sc*) expression in the wing disc of *P. terranovae* at 5 h after puparium formation (APF) (A, C) and *C. vicina* at 8 h APF (B, D). Enlargements of the thoracic domain of the discs, indicated by the windows in (A) and (B), are shown directly below in (C) and (D). Unless labelled otherwise, dashed black lines indicate the morphology of the future adult thorax, and dashed white lines indicate the position of the future macrochaete rows. In both species, *sc* is expressed in two broad longitudinal stripes in the medial half

absent in the more basal suborder Nematocera (McAlpine, 1981; Simpson *et al.*, 1999). Many families of Brachycera bear macrochaetes but, interestingly, most families include species devoid of them. This means that either the macrochaetes have appeared independently several times in different lineages during the history of the Brachycera or they arose once and have been lost many times since. On the notum, macrochaetes are invariably arranged in longitudinal rows or in stereotyped patterns (McAlpine, 1981; Simpson *et al.*, 1999). Comparison of species within the derived Schizophora taxon suggests that changes in the positions of macrochaetes have taken place only gradually. Closely related species tend to have closely related patterns, whereas phylogenetically more distant species may display greater differences (Bernasconi *et al.*, 2000; Beverley and Wilson, 1984; Grimaldi, 1990; McAlpine, 1981; Simpson *et al.*, 1999). There has been a gradual tendency towards the evolution of stereotyped patterns. Neuronal specificity of macrochaetes is dependent on their position (Vandervorst and Ghysen, 1980), suggesting that bristle position is important for behaviour and that the genetic regulation underlying patterning is under strong selective pressure (Mackay, 1995, 1996; Skaer and Simpson, 2000). In contrast, the microchaetes do not display conserved patterns and are frequently randomly arranged, the number and position of each bristle varying between individuals of a species (Grimaldi, 1990; McAlpine, 1981).

In those species examined to date, *Drosophila melanogaster*, *Ceratitis capitata*, and *Calliphora vicina*, precursors for the macrochaetes arise earlier in development than those for the microchaetes (Cubas *et al.*, 1991; Huang *et al.*, 1991; Pistillo *et al.*, 2002; Simpson *et al.*, 1999; Skeath and Carroll, 1991; Usui and Kimura, 1993; Wülbeck and Simpson, 2000). This is probably true of most Brachycera, since the macrochaetes are spaced farther apart from one another than are the microchaetes, suggesting that there has been a longer interval for division of the intervening epidermal cells (Lawrence and Hayward, 1971). Two temporally separate waves of precursor segregation have been described, one largely before, and one after the pupal moult. The macro- and microchaete precursors thus arise at different times during early pupal development.

The two categories of bristles arise from two temporally distinct periods of *sc* expression. The *ac-sc* genes encode related basic helix–loop–helix (bHLH) transcriptional regulators whose activity provides cells with neural potential (Alonso and Cabrera, 1988; Gonzalez *et al.*, 1989; Villares and Cabrera, 1987). In *D. melanogaster* and *C. capitata*, the stereotyped pattern of macrochaetes on the notum is the result of expression of *ac* and *sc* during the last larval instar in small proneural clusters of cells that prefigure the sites of each of the future bristles (Cubas *et al.*, 1991; Romani *et al.*, 1989; Skeath and Carroll, 1991; Wülbeck and Simpson, 2000). The *ac* and *sc* genes of *D. melanogaster* share *cis*-regulatory enhancer sequences scattered over nearly 100 kb, that respond to local positional cues, conveyed in part by transcriptional activators such as Pannier and the products of the *iroquois* genes (Garcia-Garcia *et al.*, 1999; Gomez-Skarmeta *et al.*, 1995, 1996; Haenlin *et al.*, 1997; Leyns *et al.*, 1996; Ramain *et al.*, 1993; Ruiz-Gomez and Modolell, 1987). The macrochaetes of *C. vicina*, a species separated from *D. melanogaster* by about 100 million years, are arranged in longitudinal rows that arise, in part, from longitudinal stripes of *sc* expression (Pistillo *et al.*, 2002). It has been postulated that the proneural clusters of *D. melanogaster* may have arisen from stripe-like expression domains through evolution of corresponding *cis*-regulatory elements (ibid).

In contrast, the second phase of *sc* expression, that gives rise to microchaete precursors, takes place after pupation and does not appear to be spatially complex in *D. melanogaster*. Ubiquitous expression of *sc* precedes segregation of the microchaete precursors in *C. capitata* and *C. vicina* (Pistillo *et al.*, 2002; Wülbeck and Simpson, 2000). *D. melanogaster* bears five rows of acrostichal microchaetes, each heminotum resulting from five stripes of *ac-sc* expression (Simpson *et al.*, 1999). However, it does not appear that each stripe is regulated independently by a specific regulatory element (Ruiz-Gomez and Modolell, 1987), and the

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of the prospective notum that prefigure the sites where the acrostichal (AC) and dorsocentral (DC) macrochaete rows form in *C. vicina*. Despite the widespread absence of macrochaetes from these domains in *P. terranovae*, the expression of *sc* is essentially identical. In the lateral domain, at the sites of the future intra-alar (IA) and supra-alar (SA) rows, *sc* is expressed in both species in groups of cells resembling the proneural clusters of *D. melanogaster*. *scute* expression in both *P. terranovae* and *C. vicina* is interrupted by mediolateral gaps at the sites of the future transverse and scutellar sutures. A band of *sc* expression is also observed in the prospective scutellum of both species. (E–H): *sc* expression in *P. terranovae* at four later stages of pupal development. (E) Seven hours APF: *sc* is expressed in groups of cells in the anterior IA and SA domains where it is not detected earlier (compare E with A and C). (F) Twelve hours APF: the clusters of *sc* expression which give rise to the lateral macrochaete precursors have faded from the IA and SA domains and have been replaced by more uniform expression, which gives rise to the microchaete precursors (indicated by the boxed region). However, in the medial AC and DC regions, *sc* remains expressed in discrete stripes, suggesting that in these domains macrochaete precursors arise simultaneously with those of the lateral microchaetes (this is confirmed by *asense* expression in Fig. 3D). (G) Left heminotum of the pupal thorax at 15 h APF: *sc* is reexpressed in all regions of the pupal thorax where microchaetes are found and is partially refined to individual microchaete precursors in this preparation, particularly in the lateral domain. By 19 h (H, complete pupal notum), the complete microchaete pattern has been established, and *sc* expression begins to fade. SC, scutellear; NP, notopleural.

number of rows is dependent on the size of the individual (Simpson *et al.*, 1999).

Here, we have investigated *sc* expression in *Phormia terranovae*, a species belonging to the family Calliphoridae that is closely related to *C. vicina* (Erzinclioglu, 1996; McAlpine, 1981). We find that the spatial expression pattern is the same in the two species, in spite of the fact that the bristle patterns are different. The timing of expression, however, was found to differ. Unlike those in *C. vicina*, the two periods of *sc* expression in *P. terranovae* overlap in time. Consequently, the last precursors segregating from the first phase of spatially regulated expression arise at the same time as the first precursors from the second phase of ubiquitous expression. They give rise to bristles morphologically indistinguishable from microchaetes. Thus, a noteworthy difference in morphology, an apparent absence of macrochaetes at some positions, is simply due to a shift in the timing of *sc* expression. We speculate that such heterochronic shifts may have enabled changes in bristle patterning to take place more rapidly than those based on changes in spatial regulation. We also discuss the possibility that expression of *sc* prior to the initiation of metamorphosis may have been the mechanism that gave rise to macrochaetes in Diptera.

MATERIALS AND METHODS

Isolation of Genes

RT-PCR. Fragments of *P. terranovae scute* (*sc*) (680 bp); *lethal of scute* (*l'sc*) (120 bp), *asense* (*ase*) (513 bp), *pannier* (*pnr*) (297 bp), and *Delta* (*Dl*) (918 bp) were isolated by RT-PCR using the following degenerated primers (5′ to 3′, forward then reverse): *sc*, AAYGCIMGIGARMGIAAYCG, TAIGGYTCRAAYTTIARYTC; *l'sc*, AAYATGCCITAYGGIGARC, CIGTYTGIGGIARRRTGYTG; *ase*, AARGGIYTICCIYTICCICARGCIG, ARYTGRTAIGTRTTIGTICC; *pnr*, GAYTTYCARTTYGGIGARGG, CCRTGYAAYTTRAARTA; *Dl*, TTYTGYMGICCIMGIGAYG, RCAIGTICCICCRTTIVCRCAIGG. cDNA was generated from mRNA extracted from a 0- to 24-h embryo collection using Superscript II Reverse Transcriptase (Gibco BRL). This was then used as a template. PCR was performed according to the following general scheme: 94°C, 1 min; annealing temperature, 1 min 30 s; 72°C, 2 min; 35 cycles; 10 min, 72°C. PCR products were cloned into pGem T easy vector (Promega).

Construction of a P. terranovae embryonic cDNA library. Construction of a *P. terranovae* embryonic cDNA library was performed by using the ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene) according to the indications of the manufacturer. Total RNA was extracted with TRIZOL (Gibco BRL) from a 0- to 24-h collection of *P. terranovae* embryos according to indications of the manufacturer. mRNA was purified by using the Oligo-dT Beads Kit (Dynall) and reverse transcribed with SuperscriptII-RT (Gibco).

High-stringency screening. To recover the full sequence of *l'sc*, the *P. terranovae* embryonic cDNA was screened at high stringency with the 120-bp fragment recovered by RT-PCR using Amersham Hybond-NX filters and conditions according to the manufacturer.

All sequences were submitted to GenBank (Accession Nos. will follow).

Sequence Analysis

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

Rearing of P. terranovae

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. *P. terranovae* pupal development takes 6 days to be completed, while *C. vicina* takes 9 days.

Labelling of RNA Probes

Digoxigenin-labelled RNA probes (DIG-UTP; Roche) were generated by 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, 1992) in a solution of 4% formaldehyde, 5% DMSO in PBS. *In situ* hybridisations were performed by using a protocol adapted from Wülbeck and Campos-Ortega (1997).

Immunostaining. Wing discs and pupal thoraces were dissected in PBS, fixed in 4% formaldehyde/PBS for 20 min, 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 by using a standard ABC kit (Vector Chemicals). All preparations were mounted in 80% glycerol, 1× PBS.

Thoraces. Adult flies were collected 30–90 min 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 min, and mounted under raised coverslips in Euparal (Fisher Chemicals).

Timing of SOP Formation in D. melanogaster Hairy wing Mutants

The following stocks of *D. melanogaster* were generated: Hw^{1} v/FM7i- GFP;neu^{A101} UAS-sc/TM6b Hu Tb; neu^{A101} UAS-sc/TM6b Hu Tb. For description of mutants and markers, see The FlyBase Consortium (1999). Third instar larvae were sexed, and female white pupae carrying neither the *Tb* nor GFP markers were collected and staged at 25°C. Dissection, mounting, and immunostaining protocols were as above, using mouse anti-β-galactosidase as the primary antibody to recognise neu^{A101} expression.

Adult thoraces were mounted as above after the cuticle had tanned.

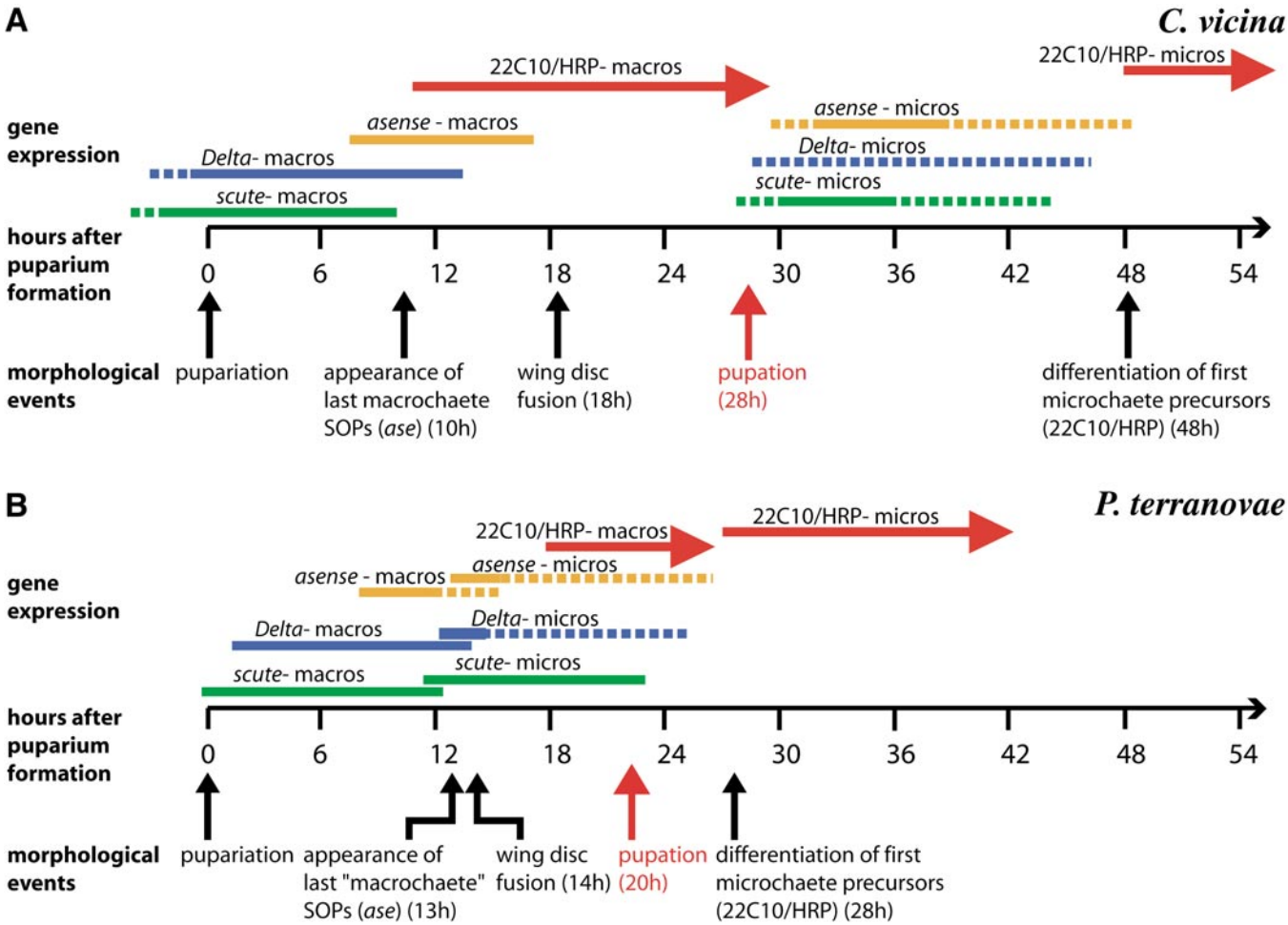

FIG. 3. Temporal comparison of pupal development of *C. vicina* (A) and *P. terranovae* (B). At 25°C, pupal development in *C. vicina* takes 9 days, while that of *P. terranovae* takes 6 days. In *C. vicina*, cursors form before pupation (which occurs at 28 h APF), whereas microchaete precursors form much later. Microchaete precursor formation in *P. terranovae* takes place, by comparison, much earlier and starts before pupation, which in *P. terranovae* occurs at 20 h APF. Thus, in *C. vicina*, *scute* is expressed in two discrete phases separated by a long period devoid of expression. In contrast, the two phases of *scute* expression in *P. terranovae* partially overlap. Dashed lines indicate inferred gene expression.

RESULTS

The Bristle Patterns of C. vicina and P. terranovae Are Different

C. vicina bears four longitudinal rows of macrochaetes on the scutum named acrostichal (AC), dorsocentral (DC), intra-alar (IA), and supra-alar (SA) (Figs. 1D and 1E). In addition, most of the scutum is covered with microchaetes. There is a distinct size difference between the two classes of bristles. The notum of *P. terranovae* displays not only macro- and microchaetes, but in addition, bristles of intermediate size. For the purposes of discussion, we have grouped the macrochaetes of *P. terranovae* into three categories: large, intermediate, and small. The large ("true") macrochaetes are invariant in number and position and are therefore morphologically the equivalent of the macrochaetes of *C. vicina*. The intermediate macrochaetes are also invariant in number and position, but are significantly smaller than the "true" macrochaetes. Finally the small macrochaetes, while still considerably bigger than the microchaetes, are variable in number and position. The three categories, as well as the microchaetes, are distinguished by colour in the schematic drawings in Fig. 1. The basic Schizophoran scutal pattern of four macrochaete rows (McAlpine, 1981; Simpson *et al.*, 1999) is still apparent in *P. terranovae*, and macrochaetes of all three categories are only found in these four rows. *P. terranovae* bears rows of IA and SA macrochaetes that are virtually identical to those of *C. vicina* but has a reduced number of macrochaetes in the AC and DC rows (Fig. 1). The AC row generally consists

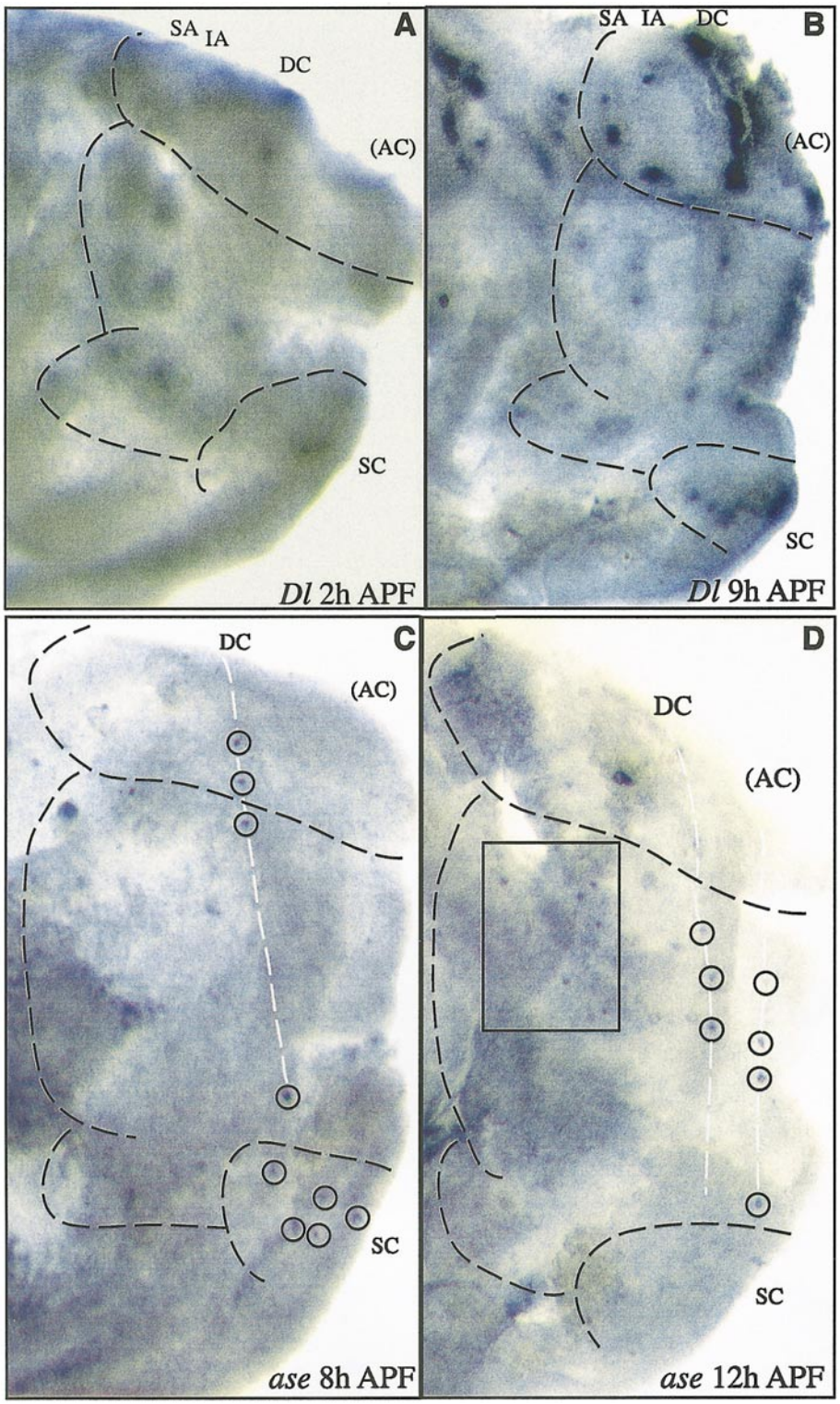

FIG. 4. Delta (*Dl*) and *asense* (*ase*) expression in the thoracic region of wing discs of *P. terranovae*. (A) *Dl* expression at 2 h after puparium formation (APF) and (B) at 9 h APF. *Dl* starts to be expressed in the wing disc after pupariation following *sc* expression and reflects the order in which macrochaete precursors are determined. Expression is first detectable in the intra-alar (IA) and supra-alar (SA) domains (A) and later on in the dorsocentral (DC), acrostichal (AC), and scutellar (SC) domains (B). *ase* expression is transient, but follows *sc* and *Dl* expression and also reflects the order of precursor formation. By 8 h APF (C), *ase* is expressed in the SC precursors and in some of those of

of a single posterior intermediate macrochaete named the prescutellar; occasionally, there is a second, small macrochaete in this row. The DC row displays macrochaetes of all three types, the central postsutural ones are mostly small or intermediate. As in *C. vicina*, microchaetes, the smallest bristles, are of uniform size and cover the notum in a regular, spaced array. A transverse row of scutellar (SC) bristles is found on the scutellum of both species.

The Spatial Pattern of Expression of scute on the Notum of P. terranovae Is Identical to That of C. vicina

We have isolated sequences specific to *scute* (*sc*), *lethal of scute* (*l'sc*), and *asense* (*ase*) from *P. terranovae*, but as for *C. capitata* and *C. vicina*, we were unable to recover sequences specific to *achaete* (*ac*). Percentage identity with the *D. melanogaster* orthologues is as follows (overall/bHLH only): *sc*, 51/95%; *l'sc*, 61/90%; *ase*, 74/87%. Comparisons with *D. melanogaster ac* were also made (overall/bHLH only): *sc*, 36/73%; *l'sc*, 32/78%; *ase*, 41/69%. Comparisons with the *C. vicina* orthologues returned the following shared homologies (overall/bHLH only): *sc*, 87/100%; *l'sc*, 91/95%; *ase*, 86/85% We have examined the expression patterns of these three genes in the embryo by *in situ* hybridisation and confirmed that they are conserved with those of other species of higher Diptera examined to date (data not shown; Cabrera *et al.*, 1987; Martin-Bermudo *et al.*, 1991; Pistillo *et al.*, 2002; Wülbeck and Simpson, 2000).

The imaginal wing disc of *P. terranovae* is similar in size and shape to that of *C. vicina*. Hybridisation *in situ* revealed that *sc* is expressed in the presumptive notum in an identical spatial pattern to that previously described for *C. vicina* (Pistillo *et al.*, 2002) (Figs. 2A–2D). There are two distinct longitudinal stripes of expression, aligned with the dorsal midline, at the positions of the future AC and DC bristle rows. In the lateral half of the notum, expression appears in several broad clusters at the sites of the future IA and SA bristles. At the positions of the prospective sutures, expression is lacking. A single stripe of expression along the posterior medial edge prefigures the row of SC bristles.

As in *C. vicina*, a second period of ubiquitous *sc* expression is observed on the scutum of *P. terranovae*; it results in the segregation of the microchaete precursors (Figs. 2E–2H).

The Temporal Pattern of Expression of scute Differs between P. terranovae and C. vicina

The early, spatially regulated stripes and clusters of *sc* expression on the notum start at pupariation in *P. terranovae* and have faded by about 12–13 h after puparium formation (APF). In *C. vicina*, expression starts just before pupariation and also finishes by about 12–13 h APF (Pistillo *et al.*, 2002). The timing of the second phase of ubiquitous *sc* expression, corresponding to the formation of the microchaete precursors, differs between *C. vicina* and *P. terranovae*. We have timed these events with reference to five morphological markers, beginning with formation of the puparium (Fig. 3). The second phase of *sc* expression in *P. terranovae* begins just before the first one fades, at about 11–12 h APF (Fig. 2F). By 14 h APF, as the two halves of the notum fuse along the dorsal midline, expression is ubiquitous, and 1 h later, it begins to be restricted to segregating microchaete precursors (Fig. 2G). By the time of the pupal moult (pupation) at 20 h APF, this second phase of *sc* expression has begun to fade (Fig. 2H). There is thus a period of overlap in *P. terranovae* between the two phases of *sc* expression. In contrast, the second phase of *sc* expression in *C. vicina* does not start until just after pupation, which in this animal occurs at 28 h APF. It continues for at least 10 h, and probably ends at about 43 h APF. The two phases of *sc* expression are thus separated in *C. vicina* by a period of about 15 h which is free of any expression (Pistillo *et al.*, 2002). A similar time gap, during which the pupal moult takes place, also separates the two phases in both *C. capitata* and *D. melanogaster* (Cubas *et al.*, 1991; Simpson *et al.*, 1999; Skeath and Carroll, 1991; Wülbeck and Simpson, 2000).

The Time of Segregation of Precursor Cells, Revealed by Expression of asense and Delta and Antibodies to 22C10 and HRP, Correlates with Bristle Size

We examined bristle precursor segregation in *P. terranovae* to see whether it reflected the dynamics of *sc* expression. *P. terranovae asense* (*ase*), as in all other Diptera examined so far, is expressed in single, spaced cells within the stripes and clusters of *sc*-expressing cells (Figs. 4C and 4D) (Brand *et al.*, 1993; Dominguez and Campuzano, 1993; Gonzalez *et al.*, 1989; Jarman *et al.*, 1993; Pistillo *et al.*, 2002; Wülbeck and Simpson, 2000). Cells at similar posi-

the DC domain, but has already largely faded from the lateral precursors. Circles indicate the position of the bristle precursors: in the DC row, the first precursors to form are the ones that correspond to the presutural bristles and to the most posterior bristle just above the scutellar suture. By 12 h APF (D), *ase* expression has faded from these precursors but is now visible in the DC row in the more anterior postsutural bristle precursors and in some precursors that emerge from the AC stripe of *sc* expression. Simultaneously, expression in microchaete precursors can be seen in the lateral region (indicated by a window). White dashed lines indicate the position of the future bristle rows, while black dashed lines indicate the morphology of the future adult thorax and the position of the transverse suture and the scutellar suture.

tions also express higher levels of *sc* (Fig. 2). They correspond to the macrochaete precursors as indicated by their positions and later expression of 22C10 and HRP (Fig. 5) (Zipursky *et al.*, 1984). Single *ase*-expressing cells also appear at nondefined positions over most of the scutum; after the second phase of *sc* expression, they correspond to the microchaete precursors (Fig. 4D).

The last precursors to segregate from the first phase of spatially regulated *sc* expression form at 10 and 13 h APF in *C. vicina* and *P. terranovae*, respectively (Fig. 3). From the second phase of ubiquitous *sc* expression, the first precursors arise at 13 h in *P. terranovae*, but not until after 30 h APF in *C. vicina*. Thus, in *P. terranovae* alone, there is a small overlap between the last precursors forming from the first phase of regulated *sc* expression and the first precursors from the second phase of ubiquitous *sc* expression.

As in *C. vicina*, precursors segregate from all of the *sc*-expressing domains present at the first phase of regulated expression in *P. terranovae*. Thus, precursors do arise along the entire length of the AC stripe and they are aligned at birth (Figs. 5D and 5E). AC precursors, particularly the anterior ones, are the last precursors to form from the first phase of *sc* expression, but more precursors arise in the AC area during the second phase (Figs. 2H and 5F). On the adult notum, no macrochaetes are seen at these positions, suggesting that these precursors differentiate into small bristles indistinguishable from microchaetes. Precursors in the centre of the DC stripe, aligned with the anterior and posterior ones, are also among the last to arise (Figs. 5C–5E). It is at these positions that small and intermediate macrochaetes are found on the differentiated cuticle. Note that there are more macrochaetes in the DC than in the IA and SA rows, and that they are closer together (Figs. 1 and 5). This would be consistent with a later time of birth, since less time would be left for growth and division of the intervening epidermal cells, and suggests that the last DC precursors to form do indeed give rise to macrochaetes of smaller size. By the time the last precursors are segregating from the AC and DC stripes, microchaete precursors have started to appear (Fig. 5F).

It is noteworthy that the microchaetes of *C. vicina* are smaller and closer to one another than are those of *P. terranovae* (Fig. 1). This difference in size is likely to reflect the earlier origin of microchaete precursors in *P. terranovae*.

asense expression is maintained in the precursors until cell division, which is the first indication of differentiation. Division of most of the macrochaete precursors takes place around the time of pupation in *P. terranovae*, earlier than that of microchaete precursors, and at this time they can also be visualised with antibodies against 22C10 and HRP (Fig. 5). Division of the last precursors that form from the AC stripe takes place at a similar time to that of the microchaetes, and from this point on, these bristles become morphologically indistinguishable from microchaetes. Later, the alignment of AC bristles is lost (Fig. 5F). Division of the last DC precursors occurs just before that of the microchaetes; the small and intermediate DC macrochaetes do remain aligned, albeit rather more haphazardly.

We also obtained sequences specific to the *Delta* (*Dl*) homologue of *P. terranovae*. *Delta* is known to be regulated by Ac-Sc in *D. melanogaster* (Kunisch *et al.*, 1994), and therefore its expression is similar to that of *sc*. *In situ* hybridisation with a *Dl* probe in *P. terranovae* revealed a similar spatial distribution to that of *sc*. A higher level of *Dl* can also be seen in the presumptive precursor cells and confirms the order in which macrochaete precursors segregate (Figs. 4A and 4B).

The Size of Bristles Correlates with the Time of Precursor Segregation in D. melanogaster

Bristle size correlates with time of appearance of the precursors in *D. melanogaster*: macrochaetes arise earlier and are bigger; the last one to form, the posterior postalar (pPA), is the smallest (Huang *et al.*, 1991). *Hairy wing* (*Hw*) mutants have extra dorsocentral bristles due to generalised overexpression of *ac* and *sc* (Balcells *et al.*, 1988), some of which are smaller than macrochaetes but larger than microchaetes (Fig. 6E). The timing of precursor formation of these intermediate bristles was examined in pupal wing discs and thoraces by using the *neuA101* line which carries a *lacZ* insert under the control of the *neuralised* (*neu*) promoter, a gene expressed early in the precursor lineage. By 2 h APF, precursors of all the macrochaetes, except the posterior postalar, have arisen in the notal domain of the wing imaginal discs (Figs. 6A and 6B). However, by this time, in Hw^1 flies, ectopic precursors have already begun to form in the dorsocentral region of the disc (Fig. 6A). By 6 h APF, several ectopic precursors are visible in the dorsocentral region (Fig. 6C). Precursors of the microchaetes do not appear until 8 or 9 h APF.

DISCUSSION

Bristle Size Correlates with the Time of Precursor Segregation

Bristles on the notum of Diptera are mechanosensory organs responding to tactile stimuli that induce movement of a bristle within its socket (McIver, 1975). Bristles are spaced apart from one another, and it may be necessary to respect a minimum distance in order to minimise domino effects that would prevent stimulation of a single bristle. It is a general rule that large bristles, which are fewer in number, are separated from one another by a greater distance than are the more numerous small bristles.

In species of higher Diptera with two distinct classes of bristles of different sizes, precursors for the macrochaetes arise considerably earlier than those for the microchaetes (Cubas *et al.*, 1991; Huang *et al.*, 1991; Pistillo *et al.*, 2002; Simpson *et al.*, 1999; Skeath and Carroll, 1991; Usui and Kimura, 1993; Wülbeck and Simpson, 2000). Consequently, the earlier arising bristles become separated by a greater

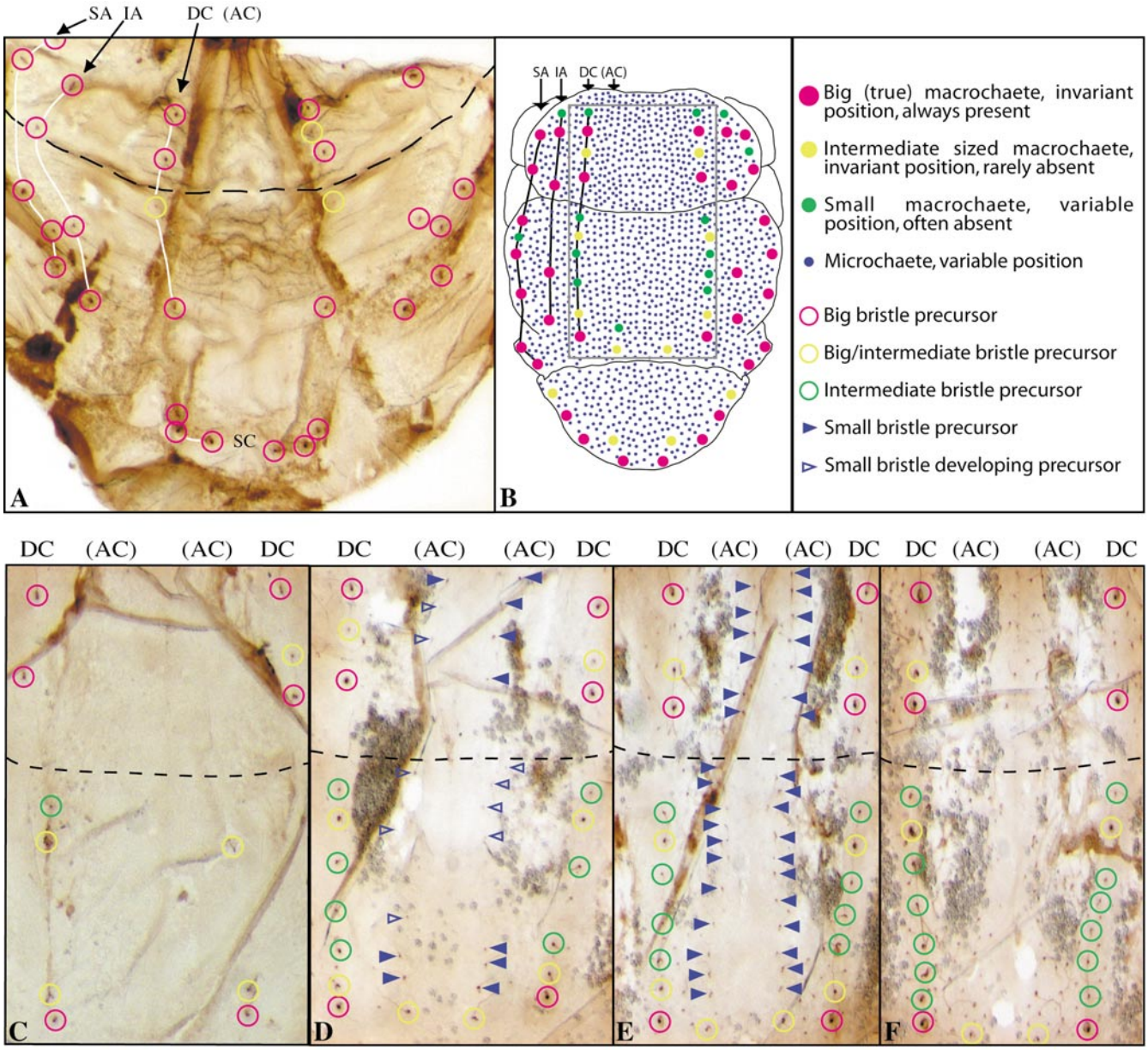

FIG. 5. Timing of emergence of bristle precursors in the developing thorax of *P. terranovae* visualised with the 22C10 antibody. Bristle types (dots in the schematic drawing in B) and their corresponding precursors (circles and triangles in A, C–F) are indicated by the following colour scheme: true macrochaetes are in pink, intermediate macrochaetes rarely absent are in yellow, small macrochaetes often absent are in green, microchaetes are in blue. Dashed black lines indicate the position of the future transverse suture. (A) At 19 h after puparium formation (APF), all the precursors for the true macrochaetes are formed: the intra-alar (IA) and supra-alar (SA) rows are complete and the true macrochaetes of the scutellar row (SC) have formed. In the dorsocentral (DC) row, the presutural precursors, the most posterior precursor, and the precursor of the bristles located directly below the transverse suture (yellow) can be seen. (C–F) are enlargements of the medial region of the developing notum (boxed region in B). (C) At 23 h APF, the precursors of the intermediate size bristles begin to appear (yellow and green) in the DC row, and by 27 APF (D), they have all formed. At 27 h APF, precursors of the bristles in the AC "row" also begin to develop, starting from the posterior region (yellow circles and blue triangles in D); their neurons have clear axonal projections. By 29 h APF (E), the precursors of the two AC "rows" are completely formed, and true microchaete precursors begin to appear in a haphazard manner. By 32 h APF (F), it is impossible to distinguish between these and the precursors derived from the AC stripe of *scute* expression.

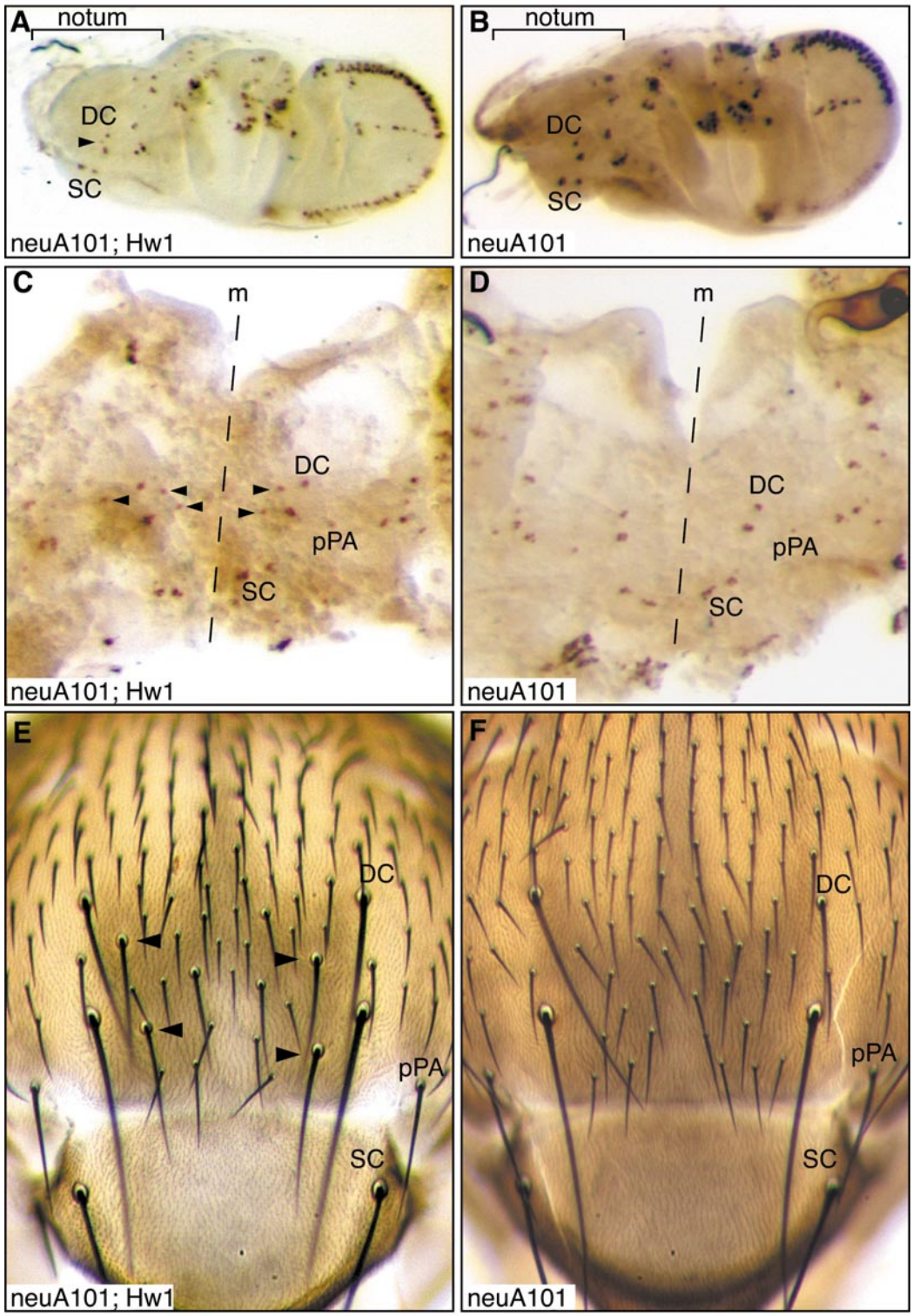

FIG. 6. Timing of formation of bristle precursors in *D. melanogaster* with the *Hairy wing* (Hw^1) chromosome rearrangement visualised with anti-β-galactosidase. Hw^1 flies display bristles of a size intermediate between macrochaetes and microchaetes in the dorsocentral (DC) region of the notum (E) which are not present in controls (F). Bristle precursors are labelled with the *neuA101* line. The images in (A) and (B), taken 2 h after pupariation, show the presence of precursors of all the macrochaetes except the posterior postalar (pPA). The dorsocentral and scutellar precursors are indicated (DC, SC). In Hw^1 flies (A, C), ectopic precursors can be seen in the DC region of the disc (indicated by arrows). Bristle sizes in the adult can be seen in (E) and (F). m, midline.

distance since there is more time left for growth and division of intervening epidermal cells (Lawrence and Hayward, 1971). In some species, the distinction between macro- and microchaetes is blurred by the presence of bristles of intermediate size. Here, we have shown that bristle size in *P. terranovae* correlates with the time of precursor formation. The first precursors to form give rise to large bristles, those segregating in between to bristles of intermediate size, and those forming last to small bristles. Bristle precursors become polyploid and increase in size prior to division and differentiation of the bristle organ (Lees and Waddington, 1942), so differences in size could be attributable to the longer time period available for the early-forming precursors before division. By itself, this mechanism is unlikely to account for the huge difference between macro- and microchaetes in a species like *D. melanogaster*, where the last macrochaete precursor forms only a few hours before the first microchaete precursor (Fristrom and Fristrom, 1993; Huang *et al.*, 1991; Usui and Kimura, 1993). Nevertheless, in the Hw^1 mutant, we have observed the presence of bristles of intermediate size that differentiate from precursors segregating only about 2 h before those of the microchaetes.

Bristle morphology is likely to reflect the time of precursor formation with respect to other events in the life cycle. Pupariation and pupation are crucial events in the life cycle of cyclorraphous flies, and during this period, the hormonal milieu is undergoing rapid changes. The onset of metamorphosis is characterised by two consecutive pulses of 20-hydroxyecdysone, each of which results in the expression of overlapping, but nonidentical, sets of "early" genes thought to regulate a large number of "late" response genes (Ashburner *et al.*, 1974; Fristrom and Fristrom, 1993; Richards, 1997; Riddiford, 1993; Russell and Ashburner, 1996). In at least one case, the "early" response genes of the *Broad-Complex* have been shown to control target genes indirectly through their effects on tissue-specific transcriptional regulators (Renault *et al.*, 2001). Thus, although precursor formation is a response to the activity of Sc, the presence or absence of other factors will determine the precise outcome in terms of bristle morphology. This is shown by the observation that the response of the notal epithelium to the effects of an exogenous supply of *sc* in *D. melanogaster* differs with time. Before pupation, it results in the formation of macrochaete precursors; after pupation, it only results in formation of microchaete precursors (Rodriguez *et al.*, 1990). The two sets of precursors in *D. melanogaster*, *C. capitata* and *C. vicina*, do arise, respectively, before and after the pupal moult. Notably, all of the precursors in *P. terranovae* are formed prior to the pupal moult. Nevertheless precursors of the macrochaete are born before those of the microchaetes, although there is a period of overlap. The crucial factor for the morphology is probably the fact that macrochaete precursors are born nearer to the time of pupation, when the hormonal milieu is changing rapidly. This is because the macrochaete precursors are born later than in *C. vicina*, and because, importantly, pupation takes place earlier in *P. terranovae*. The microchaete precursors form earlier than those of *C. vicina* and are correspondingly larger and spaced farther apart.

Little is known about the physiological functions of individual bristles, but it is likely that the functions of macro- and microchaetes are distinct (Vandervorst and Ghysen, 1980; Usui-Ishihara *et al.*, 1995; Walker *et al.*, 2000). Macrochaetes are invariably arranged into defined species-specific patterns, many of which are phylogenetically ancient (Grimaldi, 1987; Kitching, 1980; McAlpine, 1981). This suggests that the precise positions of individual macrochaetes may be of importance for behaviour and are under strong selective pressure (Mackay, 1995, 1996; Skaer and Simpson, 2000). In contrast, in most species, microchaetes are randomly distributed, and, although they may sometimes be arranged into rows, these patterns are not conserved (Grimaldi, 1990; McAlpine, 1981).

Spatial Regulation of scute Expression and Evolution of Bristle Patterns in Higher Diptera

Stereotyped patterns, such as those of *D. melanogaster* and *C. capitata*, result from complex, spatially regulated expression of *sc* in proneural clusters at the sites of each bristle (Cubas *et al.*, 1991; Skeath and Carroll, 1991; Wülbeck and Simpson, 2000). Spatial regulation in *D. melanogaster* is known to result from the activity of a number of discrete *cis*-regulatory elements within the AS-C (Gomez-Skarmeta *et al.*, 1995; Ruiz-Gomez and Modolell, 1987). Stereotyped patterns on the scutum of cyclorraphous flies may be derived from an ancestral pattern of four longitudinal rows of bristles, similar to the one present in *C. vicina* (Simpson *et al.*, 1999). Two of the rows in *C. vicina* are controlled by two stripes of *sc* expression, suggesting that expression of *sc* in stripes may have been the first spatially regulated transcription event during the evolution of macrochaete patterns and may have preceded the advent of proneural clusters (Pistillo *et al.*, 2002). If so, then the *cis*-regulatory elements of *D. melanogaster* could be of ancient origin and may have driven *sc* in a stripe-like pattern in an ancestor. This hypothesis implies that changes in the spatial regulation of *sc* are the basis of evolution of Dipteran bristle patterns.

Here, we demonstrate that the spatial expression of *sc* on the notum of *P. terranovae* is identical to that of *C. vicina*, in spite of their different bristle patterns. These two species are both members of the Calliphoridae family and are thought to be closely related, although it is not known how long ago they diverged. *P. terranovae* bears a reduced pattern of macrochaetes on the medial notum, in particular the AC row includes a single, or at most two, small posterior macrochaete(s). Our results indicate that loss of anterior AC macrochaetes in *P. terranovae*, as well as of central DC bristles, is not attributable to changes in the spatial regulation of *sc* or to that of its probable upstream regulator *pannier* (unpublished observations). Instead it may be due to a change in the timing of expression, relative

to the hormonal events of metamorphosis. Thus, alternative mechanisms exist that allow the bristle patterns to evolve without altering the spatial pattern of transcription and its underlying regulation.

Temporal Regulation of scute Expression and Bristle Patterns

We show that the temporal regulation of the two phases of *sc* expression in *P. terranovae* differs from that of *C. vicina*: the first phase of expression is slightly delayed, and the second phase remarkably accelerated, such that there is a temporal overlap. The result is that bristle precursors segregating during the period of overlap, even if they arise from the specific expression domains characteristic of the first regulated phase of *sc* expression, may give rise to very small macrochaetes or even to microchaetes. These precursors are born around the time of the major hormonal changes, leading to pupation which takes place earlier in *P. terranovae*. This appears to be the reason for the loss of anterior AC and central DC macrochaetes.

The presence of a single posterior AC bristle, the prescutellar bristle, is thought to be due to secondary loss of anterior bristles (Grimaldi, 1990). This feature is characteristic of many different families scattered throughout the phylogenetic tree of Schizophoran Diptera, suggesting that loss of anterior AC bristles may have occurred independently several times (McAlpine, 1981). The prescutellar bristle has also been secondarily recovered after complete loss of the AC row in at least one genus of Drosophilidae (Grimaldi, 1990). These observations suggest that the AC row of bristles may be subject to frequent change, and one possibility is that this could be due to transcriptional heterochrony of *sc*. It is not known how temporal transcription at the AS-C is regulated, but it is tempting to speculate that it may be able to evolve more rapidly.

An example of developmental heterochrony that appears to have a number of features in common with bristles of Diptera is that of pigment patterns in butterfly wings. The pigments are synthesised in the scales as they develop (Nijhout, 1991). Scales are thought to be homologous to bristles and they depend on *ac-sc* expression for their development (Galant *et al.*, 1998). Delay in the formation of scale precursor cells or in the maturation of scales, caused by microcautery or mutation, correlates with a change in pigmentation, suggesting that timing of precursor segregation and/or scale maturation is essential (Koch *et al.*, 2000; Takayama and Yoshida, 1997). The colour development of scales takes place late in pupal development, and a link with the declining titre of ecdysteroid hormones has been established (Koch, 1995).

Our observations indicate that timing of precursor formation is crucial for the development of precisely positioned macrochaetes in Diptera. Delay may not only lead to a loss of macrochaetes but may also be associated with a decreased precision in the positioning of the bristles. The number, as well as the size, of the small and intermediate macrochaetes in the centre of the DC row of *P. terranovae* is somewhat variable and they are poorly aligned. The microchaetes in the AC area are not aligned at all. One consequence of this loss of stereotyped patterns may be a relaxation of the selective pressures that normally maintain macrochaete positioning (Mackay, 1995, 1996; Skaer and Simpson, 2000). This in turn could lead to a later loss of spatially regulated transcription. Thus, the modifications introduced by transcriptional heterochrony of *sc* may be a first step leading to changes in the spatial regulation of *sc* expression.

One interesting evolutionary perspective on these results is that an earlier expression of *sc* during prepupal stages in higher Diptera may have been the event leading to the origin of macrochaetes. Thus, the generation of patterns composed of two distinct classes of bristles could have been a simple consequence of changes in the temporal expression of *sc*. Higher flies display two temporally separate phases of *sc* expression, but the prediction would be that basal species, with a single size of bristle, will be found to have only one phase of expression. In this respect, it is interesting to note that ancestral patterns of randomly positioned bristles of uniform size are found throughout the phylogenetic tree of the Diptera, even within the derived clades. This means that either the macrochaetes have appeared independently several times in different lineages during the history of the Diptera or they arose once and have been lost many times since. If the appearance of macrochaetes is a consequence of an additional, earlier phase of *sc* expression, then secondary loss of this early period of expression would be sufficient to cause the pattern to revert to an ancestral one.

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