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The expression of *pannier* and *achaete-scute* homologues in a mosquito suggests an ancient role of *pannier* as a selector gene in the regulation of the dorsal body pattern

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SUMMARY

The *Drosophila* gene *pannier* (*pnr*) has recently been assigned to a new class of selector genes (Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., Simpson, P. and Morata, G. (2000). *Development* 127, 3971-3980; Mann, R. S. and Morata, G. (2000). *Annu. Rev. Cell Dev. Biol.* 16, 243-271). It specifies pattern in the dorsal body. On the dorsal notum it is expressed in a broad medial domain and directly regulates transcription of the *achaete-scute* (*ac-sc*) genes driving their expression in small discrete clusters within this domain at the sites of each future bristle. This spatial resolution is achieved through modulation of Pnr activity by specific co-factors and by a number of discrete *cis*-regulatory enhancers in the *ac-sc* gene complex. We have isolated homologues of *pnr* and *ac-sc* in *Anopheles gambiae*, a basal species of Diptera that diverged from *Drosophila melanogaster* (*Dm*) about 200 million years ago, and examined their expression patterns. We found that an *ac-sc* homologue of *Anopheles*, *Ag-ASH*, is expressed on the dorsal medial notum at the sites where sensory organs emerge in several domains that are identical to those of the *pnr* homologue, *Ag-pnr*. This suggests that activation of *Ag-ASH* by Ag-Pnr has been conserved. Indeed, when expressed in *Drosophila*, *Ag-pnr* is able to mimic the effects of ectopic expression of *Dm-pnr* and induce ectopic bristles. These results are discussed in the context of the gene duplication events and the acquisition of a modular promoter, that may have occurred at different times in the lineage leading to derived species such as *Drosophila*. The bristle pattern of *Anopheles* correlates in a novel fashion with the expression domains of *Ag-pnr*/*Ag-ASH*. While precursors for the sensory scales can arise anywhere within the expression domains, bristle precursors arise exclusively along the borders. This points to the existence of specific positional information along the borders, and suggests that *Ag-pnr* specifies pattern in the medial, dorsal notum, as in *Drosophila*, but via a different mechanism.

Key words: Diptera, *Anopheles gambiae*, Sensory organ, *achaete-scute*, *pannier*, *Drosophila melanogaster*

INTRODUCTION

During the past two or three decades, investigation into the genetic control of development has shown that, in the model organism, *Drosophila melanogaster*, the body is progressively subdivided into smaller and smaller domains, called compartments (Garcia-Bellido et al., 1973). Patterning within each domain then proceeds relatively independently. Compartments are derived from groups of cells, or polyclones, that are subsequently defined by lineage (Crick and Lawrence, 1975). The process of compartmentalisation and patterning within compartments is regulated by selector genes such as *engrailed*, *apterous* and the genes of the Bithorax Complex (BXC) (Blair, 1993; Diaz-Benjumea and Cohen, 1993; Garcia-Bellido, 1975; Lewis, 1978; Morata and Lawrence, 1975). The boundaries between compartments have been shown to be sources of signalling molecules that coordinate growth and patterning of each compartment (Lawrence and Struhl, 1996). Conservation of this developmental strategy is not clear since compartments in other animals have been difficult to demonstrate. Recently, however, evidence has been obtained for a new class of selector genes whose activity is not confined to lineage-based compartments (Calleja et al., 2000; Mann and Morata, 2000). They behave similarly to classical selector genes and generate morphological differences between different parts of the body plan (Mann and Morata, 2000). Two such genes are *pannier (pnr)* and the genes of the *iroquois* complex (*iro-C*; *araucan*/*caupolican*), that encode transcription factors of the GATA and homeobox-containing protein families respectively (Cavodeassi et al., 2001; Gomez-Skarmeta et al., 1996; Ramain et al., 1993).

pannier is expressed in a longitudinal, dorsal domain extending from the head to the end of the abdomen in both larvae and imagos and is involved in the subdivision of the

dorsal component of each segment (Calleja et al., 2000; Maurel-Zaffran and Treisman, 2000). It acts in combination with *engrailed* and the genes of the BXC to specify the identity of the dorsal, medial domain (Calleja et al., 2000). The lateral domain is specified by the expression of the *iro-C* genes (Calleja et al., 2000; Diez del Corral et al., 1999; Kehl et al., 1998). The most obvious pattern elements of the notum are the large sensory bristles that arise in a stereotyped pattern as a result of the spatially regulated expression of the *achaete-scute* (*ac-sc*) genes (Cubas et al., 1991; Ghysen and Dambly-Chaudiere, 1988; Romani et al., 1989; Skeath and Carroll, 1991). Both Pnr and the *iro-C* gene products have been shown to activate transcription of *ac-sc* in *Drosophila*, and this regulatory function of the *iro-C* proteins appears to have been conserved in *Xenopus* (Garcia-Garcia et al., 1999; Gomez-Skarmeta et al., 1996; Gomez-Skarmeta et al., 1998; Haenlin et al., 1997).

There are about 60,000 species of true flies many of which display species-specific bristle patterns that differ from that of *Drosophila* (McAlpine, 1981; Simpson, 1999). Dipteran flies thus provide a convenient model group in which to investigate evolutionary changes in the regulation of expression of *ac-sc* by the selector genes of the *iro-C* and *pnr*. The more derived species of cyclorraphous Diptera, such as *Drosophila*, *Ceratitis capitata* and *Calliphora vicina*, display stereotyped bristle patterns. These result from the expression of *ac-sc* in discrete proneural clusters or stripes, corresponding to each bristle or bristle row (Cubas et al., 1991; Pistillo et al., 2002; Romani et al., 1989; Simpson et al., 1999; Skeath and Carroll, 1991; Sturtevant, 1970; Wülbeck and Simpson, 2000). In *Drosophila* this complex spatial expression relies on a number of *cis*-regulatory elements scattered throughout the *ac-sc* gene complex (ASC) (Gomez-Skarmeta et al., 1995). These are likely to be conserved in *Ceratitis* and *Calliphora*, together with the function of *pnr*, which is expressed in an identical medial dorsal domain in all three species (Pistillo et al., 2002; Wülbeck and Simpson, 2000).

The Nematocera comprises a group of basal Dipteran species in most of which the bristles are randomly positioned on the notum (McAlpine, 1981; Simpson et al., 1999). A few families, such as the Culicidae, do include species with a simple arrangement of bristles into two or three rows on the notum, most of the body being densely covered with sensory scales (Stone, 1981; McIver, 1975). Here we examine the expression patterns of *pnr* and an *ac-sc* homologue, *Ag-ASH*, on the notum of *Anopheles gambiae* (Culicidae), a vector of the malaria-causing parasite. We find that, on the medial notum, *Ag-ASH* is expressed in very broad domains coincident with domains of expression of *Ag-pnr*. This suggests that activation of *Ag-ASH* by Ag-Pnr has been conserved. Indeed expression of *Ag-pnr* in *Drosophila* mimics the effects of mis-expression of *Dm-pnr*, and causes the development of ectopic bristles. The coincident expression domains of *Ag-pnr* and *Ag-ASH* suggest that activation of *Ag-ASH* may not require the complex modular promoter characteristic of the ASC of *Drosophila*. We hypothesise that duplication of the ASC genes, acquisition of position-specific *cis*-regulatory sequences, and regulatory co-factors for Pnr, may only have been co-opted after the separation of the Nematocera and the Brachycera, some 200 million years ago, and may have allowed the evolution of stereotyped bristle patterns. Interestingly, all of the bristles on the medial notum of *Anopheles* appear to arise along the borders of the *Ag-pnr* (and *Ag-ASH)* expression domains. This indicates that *pnr* may specify the dorsal bristle pattern in both *Drosophila* and *Anopheles*, but in quite different ways.

MATERIALS AND METHODS

Cloning and sequencing of *achaete-scute* and *pannier* homologues in *Anopheles*

Screening for homologues of the *achaete-scute* (*ac-sc*) and *pannier* (*pnr*) genes was performed under low stringency at 42°C with 20% formamide containing standard hybridisation buffer. Washes were carried out at 50°C with 2× SSC, 1% SDS. A genomic *Anopheles* library and a λZap cDNA library of non-infected fourth instar larvae (from Drs Larry Zwiebel and Fotis Kafatos, EMBL-Heidelberg) was plated and nylon replica filters (PALL, Biodyne A) screened with probes for *ac-sc* or *pnr* respectively, as described previously (Wülbeck and Simpson, 2000). Two overlapping genomic phages (AA2J1 and AA2J2) were isolated, and a 2.8 kb *Xho*I fragment containing the transcription unit of the entire *ac-sc* homologue, *Ag-ASH*, was subcloned in pBluescript SK+. To isolate an *Ag-ASH* cDNA, the fourth larval instar cDNA library was re-screened by using a probe specific to the *Ag-ASH* bHLH-encoding domain, which was generated using degenerated primers as described previously (Wülbeck and Simpson, 2000). Two phages were isolated and in vivo excised; one of them (pBS-AC3K1: 2116 bp) contained the entire protein encoding region. For *pnr*, five phages were isolated and in vivo excised but sequence analysis showed that only one of them (pBS-PAC3F1: 3285 bp) encoded a *pnr* homologue, *Ag-pnr*. Sequence analysis was performed as described previously (Wülbeck and Simpson, 2000) using the GCG programme. All sequences have been submitted to GenBank (*Ag-ASH*: AF395079, *Ag-pnr*: AF395080).

Mosquito cultures

Mosquito larvae were kindly provided by members of the laboratory of Professor Fotis Kafatos, at the EMBL. Larvae were reared in humid chambers at room temperature and fed with cat food. Newly eclosed *Anopheles* adults were dehydrated and mounted in Euparal for microscopic analysis.

Labelling of RNA probes

Digoxigenin-labelled RNA probes (DIG-UTP; Boehringer Mannheim) were generated using the standard protocol of Boehringer Mannheim. The resulting RNA was resuspended in 100 μl preHyb solution (50% formamide, 5× SSC, 0,1% Tween 20, pH 6.0). RNA was transcribed from linearized DNA templates: *Ag-pnr* pBS-PAC3F1 (T3 sense, T7 antisense), *Ag-ASH* pBS-AC3K1 (T3 sense, T7 antisense).

RNA in situ hybridisation

In situ hybridisation was performed (Wülbeck and Simpson, 2000) with some modifications. Incubation with proteinase K was for 5 minutes at room temperature and incubation with anti-digoxigenin alkaline phosphatase-coupled antibody (Boehringer Mannheim) was performed overnight at 4°C instead of for 2 hours at room temperature.

Tissue preparation and antibody staining

Larvae and pupae for RNA in situ hybridisation were dissected in ice cold PBS and fixed as described previously (Wülbeck and Simpson, 2000), then stored in 100% methanol at –20°C. For antibody staining *Anopheles* larvae and pupae were boiled for 5 minutes in PBS and the cuticle removed when possible. Staining was performed immediately afterwards using standard procedures and dilutions of 1/200 in 10% foetal calf serum (FCS) for the primary antibody (rabbit anti-

horseradish peroxidase (HRP); Jackson) and 1/200 in 10% FCS for the secondary antibody (anti-rabbit coupled with HRP; Jackson). DAB staining was performed using standard protocols.

Transformation in *Drosophila*

A full-length cDNA *Eco*RI-*Xba*I fragment of *Ag-ASH* (pBS-AC3K1) and a full-length cDNA *Spe*I-*Kpn*I fragment of *Ag-pnr* (PAC3F1) were each subcloned into the corresponding restriction sites of the pUAST vector under the control of the *Drosophila* HSP70 minimal promoter. Germline transformants were obtained as described previously (Rubin and Spradling, 1982). Three independent lines were established. Expression of *UAS-Ag-ASH* was driven by *GAL4-pnr^{MD237}* and that of *UAS-Ag-pnr* by *Gal4-ap*, C765, *sca^{534}*, *pnr^{MD237}*, MD455 and MD410 (Brand and Perrimon, 1993; Calleja et al., 1996; Gorfinkiel et al., 1997; Garcia-Garcia et al., 1997). Standard procedures were used for X-gal staining. Flies were dehydrated and mounted in Euparal for microscopic analysis.

RESULTS

Isolation of *achaete-scute* and *pannier* homologues in *Anopheles*

The *ac-sc* complex (ASC) of *Drosophila* comprises four genes: *ac*, *sc*, *lethal of scute* (*l'sc*) and *asense* (*ase*), which encode transcription factors of the basic helix-loop-helix (bHLH) family of proteins (Alonso and Cabrera, 1988; Gonzalez et al., 1989; Villares and Cabrera, 1987). Screening of a genomic library under moderately stringent conditions (see Materials and Methods) with a PCR-generated fragment containing the bHLH domain of the *ac* gene of *Drosophila virilis* uncovered two overlapping genomic phages encoding a single *ac-sc* homologue *(Ag-ASH)*. Re-screening of a cDNA library of fourth instar larvae, with a PCR-generated probe from the *Ag-ASH* under moderately stringent conditions, allowed only the recovery of the same sequences.

Ag-ASH appears closest to *Drosophila l'sc* (*l(1)sc – FlyBase*). The complete protein sequence of *Ag-ASH* is compared with that of *l'sc* from *Drosophila melanogaster* and an *ac-sc* homologue from the butterfly *Precis coenia*, in Fig. 1A. Sequence analysis revealed that 81% of the amino acids in the bHLH domain are identical to those of the *Drosophila l'sc* protein. Outside of this functional domain, amino acid sequence conservation is low (ranging from 20-27% for the amino (N)-terminal portion to 25-38% for the carboxy (C)-terminal part). A single stretch of 15 conserved amino acids, which appears to be restricted to insect *ac-sc* proteins, can be seen at the C terminus (shaded blue box). The central tyrosine of this sequence has changed in the butterfly *Precis coenia* (Galant et al., 1998).

The *pnr* gene of *Drosophila* comprises two zinc fingers characteristic of the GATA family of transcription proteins, and a C-terminal domain bearing two α helices (Ramain et al.,

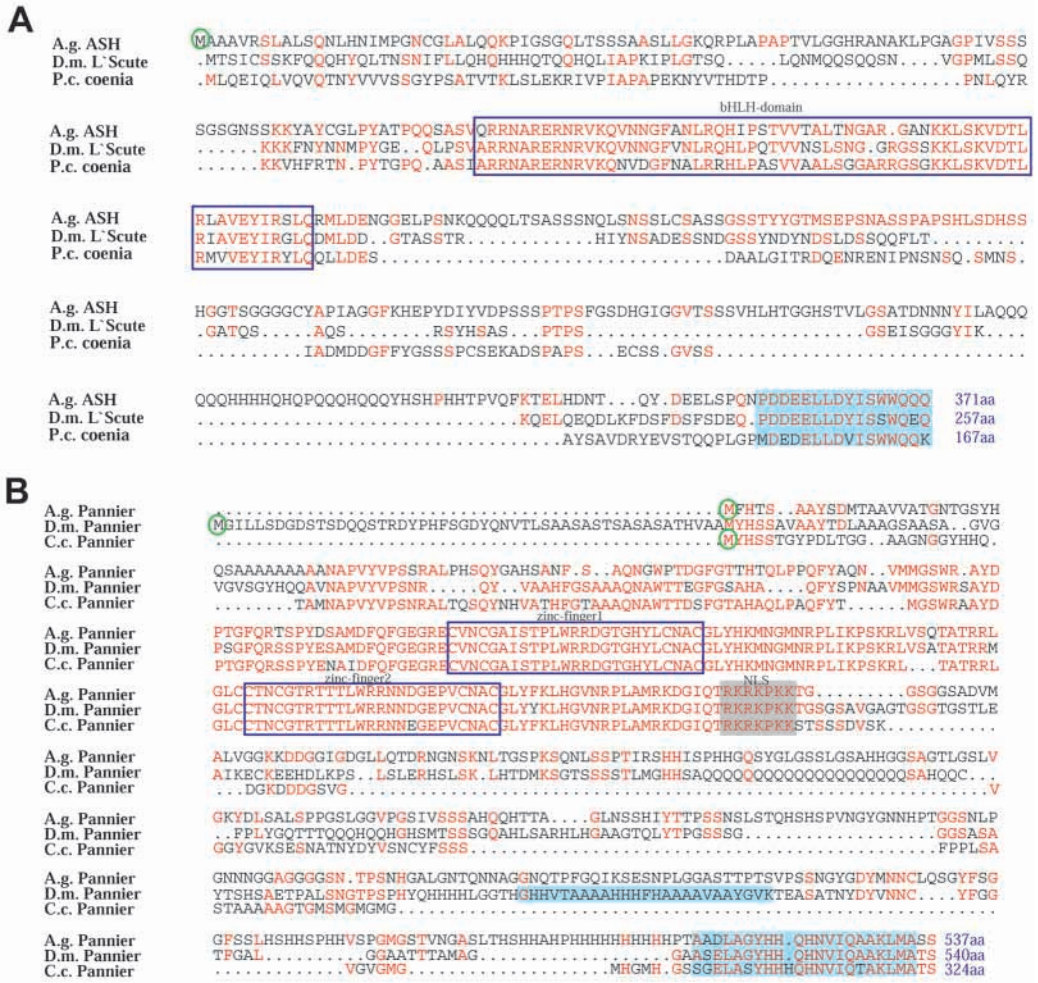


Fig. 1. (A) The complete amino acid sequence of *Ag-ASH* is compared with that of the *achaete-scute* homologue of the butterfly *Precis coenia* (Galant et al., 1998) and with *lethal of scute* from *Drosophila* (D.m. L'scute) (Villares and Cabrera, 1987). Differences are shown in black. The start site is indicated by a green circle. Conservation is highest in the bHLH domain, which is boxed. The shaded blue box indicates a conserved stretch in the C-terminal domain. (B) The complete amino acid sequence of Ag-Pnr is compared with that of the Pannier protein of *Drosophila melanogaster* and *Ceratitis capitata*. Differences are shown in black. The start site is indicated by a green circle. The two zinc fingers are boxed. The α helical structures are shown as shaded blue boxes. The nuclear localisation signal (NLS) is indicated by the shaded grey box.

1993). The 537 amino acid sequence of the *Ag-pnr* protein is shown in Fig. 1B, together with Pnr from *Drosophila* and *Ceratitis capitata* (Wülbeck and Simpson, 2000). It contains two zinc fingers that are very strongly conserved. The proteins are, however, quite divergent in the C-terminal domain. The proteins of *Ceratitis* and *Anopheles* carry a single α helix, in contrast to the two in *Drosophila*.

Ag-pnr and *Ag-ASH* mimic the effects of ectopic expression of *Dm-pnr* and *DmSc* in *Drosophila*

Ag-ASH displays strong proneural activity when expressed in *Drosophila*. We made use of the GAL4-UAS system and the driver pnr^{MD237}, to express *Ag-ASH* in the medial half of the notum. This leads to the formation of an excess of bristles in this region (Fig. 2A,B). These bristles are characteristic of the large bristles, or macrochaetes, of *Drosophila*.

In order to examine the effects of expression of *Ag-pnr* in *Drosophila*, three *Drosophila* lines carrying the *UAS-Ag-pnr* were established. Two of these exhibited mild phenotypes known to be associated with gain of function of *Dm-pnr*, such as a slight midline cleft and additional DC and SC bristles (Haenlin et al., 1997; Heitzler et al., 1996). Each of these lines was crossed to several *Gal4* drivers (see Materials and Methods). Over-expression of either *Dm-pnr* or *Ag-pnr* in the medial notum where the endogenous *Dm-pnr* gene is expressed, using *Gal4pnr*, was without effect on the bristle pattern, other than a slight cleft and an occasional additional scutellar bristle (Calleja et al., 2000), not shown. Two of the lines, C765 and *Gal4apterous*, drive expression in the entire notum (Calleja et al., 1996; Gorfinkiel et al., 1997). The C765 driver resulted in flies of a uniform phenotype: a tuft of additional DC bristles situated laterally on the notum (Fig. 2C). This phenotype is very similar to that seen after ectopic expression of *Dm-pnr* in C765/*UAS-Dm-pnr* flies (Garcia-Garcia et al., 1999). The *Gal4ap* driver gave rise to flies with a range of phenotypes depending on the *UAS-Ag-pnr* line. These ranged from a loss of just one or both DC bristles with a deformed scutellum to loss of most of the notum. Flies from one of the lines were devoid of structures present on the lateral notum and developed only structures typical of the medial pattern that appeared normal apart from an excess of DC bristles (Fig. 2D). This phenotype is almost identical to that seen after ubiquitous expression of *Dm-pnr* in *Gal4-ap/UAS-Dm-pnr* flies (Calleja et al., 2000).

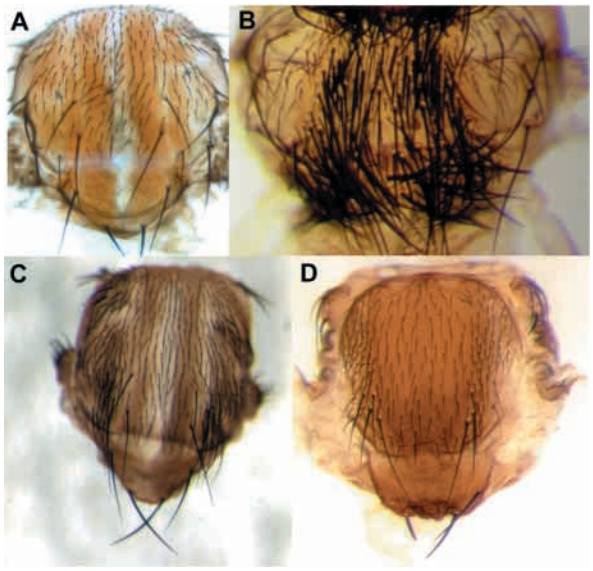


Fig. 2. Expression of *Ag-ASH* and *Ag-pnr* in *Drosophila*. (A) A wild-type notum. (B) The GAL4 line pnr^{MD237} was used to drive *Ag-ASH* expression in the medial notum of *Drosophila*; this leads to the segregation of many ectopic bristles in the medial domain of the notum. Note that the slight midline cleft is a mutant phenotype associated with the pnr^{MD237} insert. (C) The results of ectopic expression of *Ag-pnr* in the lateral notum with the driver C765. Note the presence of additional, large, (dorso-central) bristles on the lateral posterior scutum. (D) The notum of a fly in which early ubiquitous expression of *Ag-pnr* over the entire dorsal notum was driven by *Gal4ap*. Elements of the lateral pattern are absent, but the medial pattern is formed normally apart from some extra bristles along the lateral margin.

Ontogeny of the imaginal notum in *Anopheles*

Many features of the life cycle of *Anopheles* are ancestral and characteristic of Nematocera (Clements, 1992). There are four larval instars after which the animal moults to a free-swimming pupa. The duration of larval development was variable under our laboratory conditions, but most animals pupated after about 12 days at room temperature. As in most Nematocera, the appendages develop from simple imaginal discs that are little more than invaginated pouches attached to the body wall (Clements, 1992). These can be seen at the second larval instar (Fig. 3A). Although the discs are enclosed in a peripodial membrane, their stalks do not close. The wing buds are situated laterally on either side of the larval mesothorax. By the fourth instar, the adult appendages have evaginated into a space outside the larval epidermis and lie flat against the body wall (Fig. 3C). The trunk of much of the adult body does not arise from imaginal discs. The abdomen of the larva, pupa and imago is made from the same epithelium that secretes successive cuticles at each moult (Clements, 1992). Thus, in *Anopheles*, the outline of the imaginal body is already present at pupation. Consequently the pupal period is short, lasting little more than 24 hours. This contrasts with cyclorraphous Schizophora, such as *Drosophila*, where it may last 5 days or more, during which time the larval body is destroyed and an entirely new adult body constructed from the imaginal discs and histoblasts.

We have investigated the origin of the dorsal mesonotum of *Anopheles*. It is not derived from the wing imaginal discs, but from a small epidermal thickening at the junction between the larval body wall and the wing pouch (Fig. 3B). Throughout the third and fourth larval instars, the cells of this region progressively grow across the dorsal part of the body just beneath the larval epidermis. The two edges eventually meet and close at the dorsal midline (Fig. 3C). Closure is always complete before the larval-pupal moult, but the rate of growth and the time of closure vary between individuals. The larval epidermis is gradually destroyed as the imaginal one expands (Fig. 3D). During larval stages, the epithelium of the future notum is very compact and the cells are tall and columnar. At pupation, the cells flatten out leading to an increase in surface area before secretion of the pupal cuticle.

The adult notum of *Anopheles* displays large sensory bristles as well as many small sensory scales (McIver, 1975; Stone,

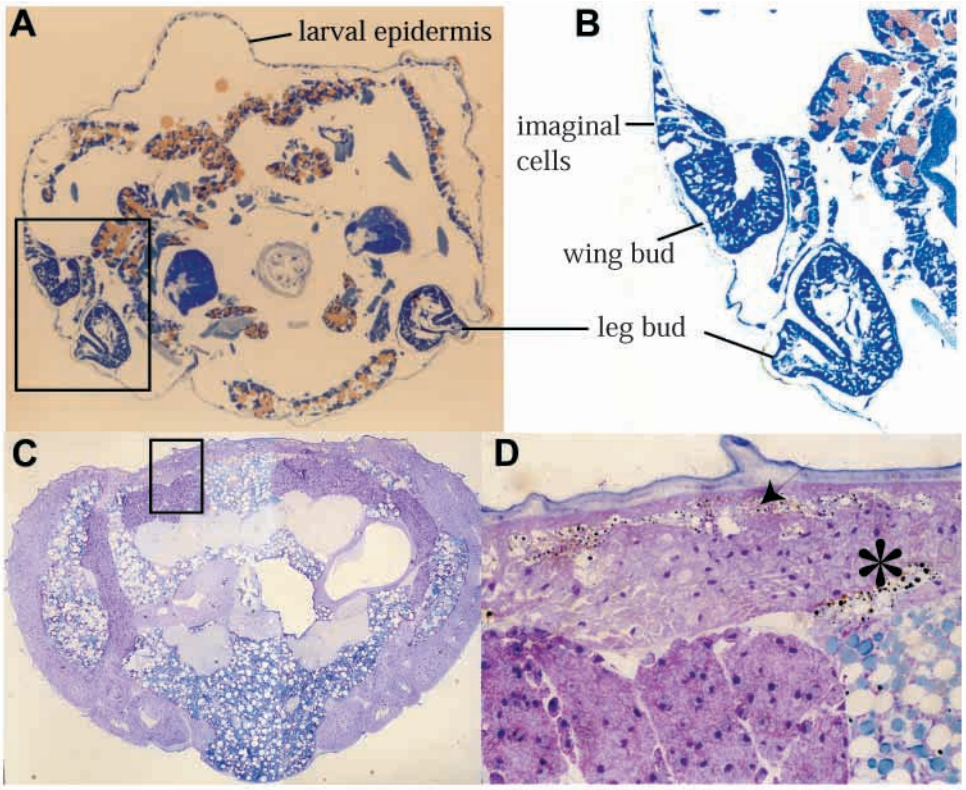
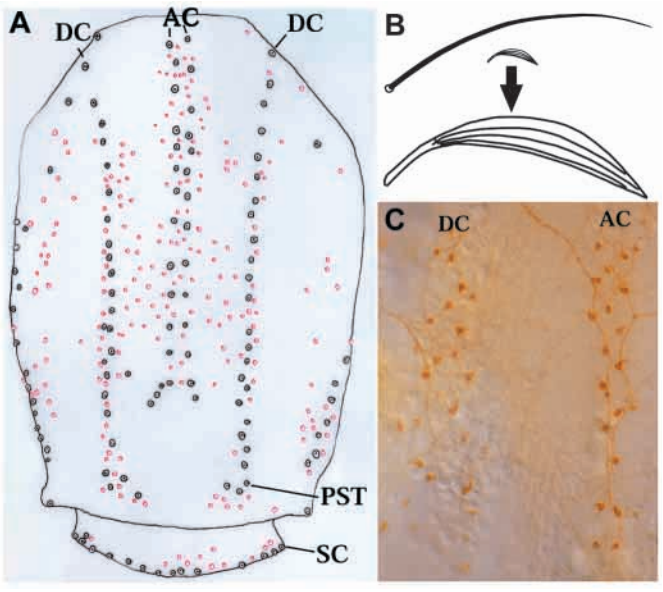


Fig. 3. Sections of larvae illustrating the development of the imaginal notum. (A) A section through a second instar larva, part of which is enlarged in B, showing the leg and wing buds and a small patch of thickened epithelial cells (imaginal cells) at the junction between the larval epithelium and the wing bud. The notum is derived from this structure. (C) Section through a fourth instar larva. The wing and leg buds have evaginated and lie flat against the body wall. (D) An enlargement of the dorsal area of this section at the level indicated. The imaginal epidermis (star) has replaced the larval one and dorsal closure is complete. Remnants of the degenerating cells of the larval epidermis can be seen as small black spots (arrowhead).

1981) (Fig. 4A). The medial half of each heminotum bears two rows of bristles named the acrostichal (AC) and dorsocentral (DC) rows. In addition there is a small transverse row of prescutellar (PST) bristles. The lateral part of each heminotum bears a band of antealar bristles and the scutellum a row of scutellar (SC) bristles. The number of bristles in each row varies considerably between individuals (Simpson et al., 1999). Numbers and positions of scales are quite variable between individuals. However, scales do not cover the entire notum and are consistently found in specific regions: between the AC and DC bristle rows, around the positions of the PST and SC bristles and close to the lateral intercalary bristles (Fig. 4A). They are more or less absent from the area between the DC and antealar bristles. Scales are much smaller than bristles, each scale is composed of a socket and a short pedicel followed by a flattened blade (Fig. 4B). Both bristles and scales are innervated (Fig. 4C). It has been suggested, on the basis of expression of an *ac-sc* homologue, that the scales of butterflies are analogous to the bristles of flies (Galant et al., 1998).



Domains of expression of *Ag-pannier* and *Ag-ASH* are co-incident

The domains of expression of *Ag-pnr* and *Ag-ASH* were examined by means of in situ hybridisation. Both genes are expressed on the notum, but only *Ag-ASH* is expressed in the wing pouch and on the legs (not shown). We found that dorsal closure and patterning of the notal epithelium, proceed at different rates in different individuals, such that expression of *Ag-pnr* and *Ag-ASH*, as well as the appearance of sensory organ precursors, may start in the fourth larval instar or only after the pupal moult. Thus patterning of the notum could not be timed with respect to external larval morphology. We therefore looked at a large number of fourth instar larvae and pupae and staged them with respect to the expression domains

Fig. 4. The bristle pattern of the imaginal notum. (A) A camera lucida drawing. The large sensory bristles are indicated by black circles and the sensory scales by red circles. The acrostichal, dorsocentral, prescutellar and scutellar bristles are indicated as AC, DC, PST, SC respectively. Their number varies considerably from one animal to another. (B) A camera lucida drawing of a bristle and a scale illustrating the difference in size and shape. (C) Part of a pupal epithelium stained with anti-HRP antibody that labels neurons. Neurons and axons of the AC and DC bristles can be seen.

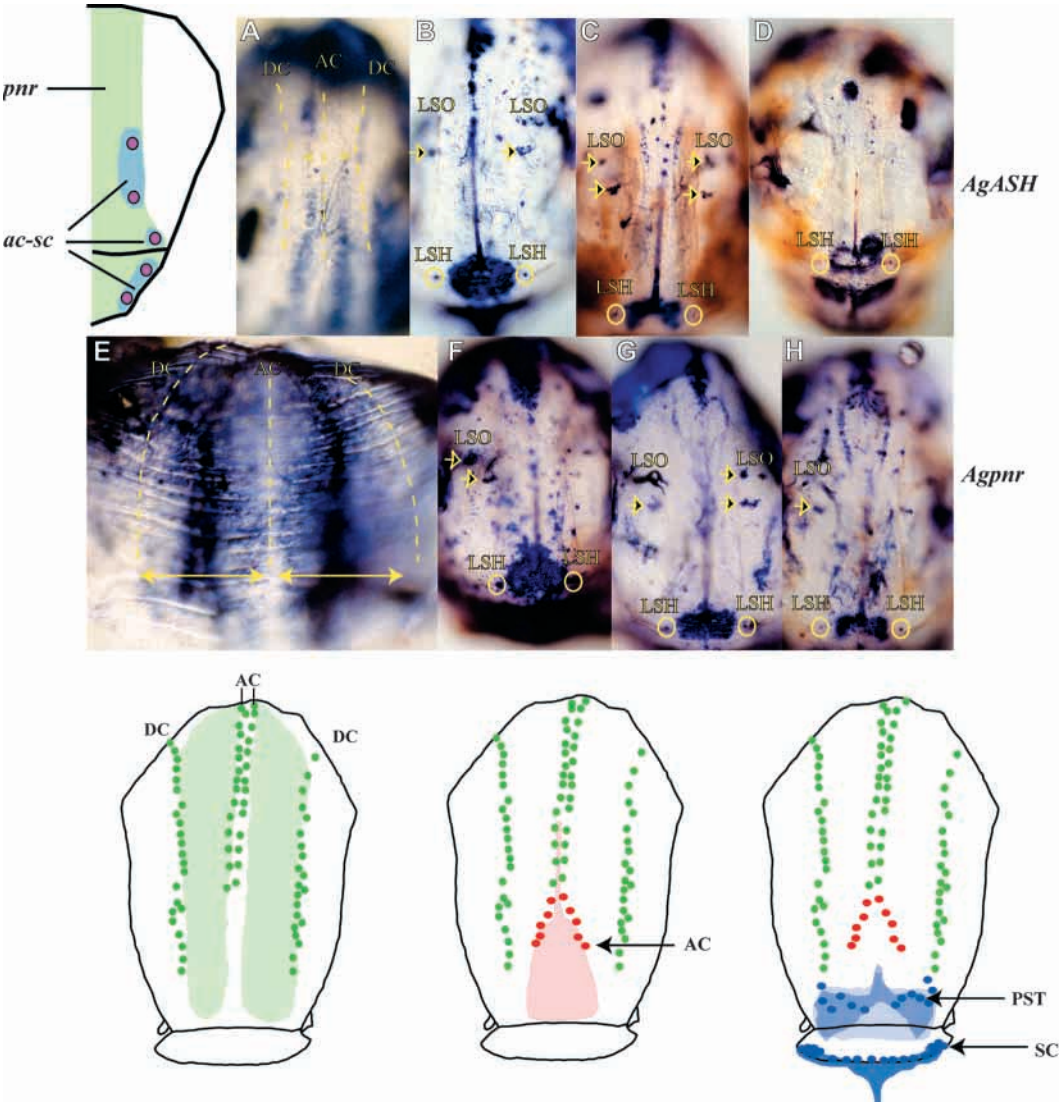


Fig. 5. Domains of expression on the developing *Drosophila* notum. (Top left) A schematic diagram of the *Drosophila* notum, indicating the extent of the expression domain of *pannier* (green), *achaete-scute* (blue) and the positions of the macrochaetes (red). (A-D) *Ag-ASH* and (E-H) *Ag-pnr* expression. The spatial expression of the two genes in the medial half of the notum is identical. Expression of both genes starts in two broad bands, one on either side of the midline. This is best seen in the late fourth instar larva in E, where staining is seen beneath the larval cuticle which has a ridged appearance. The cuticle was removed for all other preparations shown. The two early bands are just becoming visible in A, where the positions of the future acrostichal (AC) and dorsocentral (DC) bristle rows are indicated by dashed lines. Stained areas round the edges of this and other preparations are artefacts due to the folded cut edge of the epithelium. Although the larval epidermis is lost, a number of larval sense organs (LSO, arrows) and larval sensory hairs (LSH, circles) persist and remain just above the imaginal epithelium. They are out of focus in some of the photographs. The larval sensory hair provides a useful positional marker with respect to which the anterior limit of the succeeding triangular and kidney-shaped domains can be measured. The full extent of the triangular domain of staining on the posterior scutum just above the scutellar suture can be seen in F. In this image, and others, some of the acrostichal and dorsocentral bristle precursors can be distinguished. The triangle has already started to shrink from its anterior limit in B and is progressively transformed to the kidney-shaped domain in C, G and H. The crescent-shaped domain on the scutellum can be seen below the LSH in D. (Note some damage to this preparation that distorts the picture of the kidney-shaped domain). A patch of staining on the midline at the anterior edge of the notum is seen in B,C,F,G and H (unlabelled). It is a consistent feature but we have been unable to find a morphological correlate to this domain of expression. The correspondence between the expression domains and the positions of the future bristles is indicated in the schematic drawings below. The acrostichal (AC) and dorsocentral (DC) rows are drawn in green and red, the prescutellar (PST) and scutellar (SC) bristles are shown in blue.

of *Ag-pnr, Ag-ASH* and to HRP staining, that are seen after completion of dorsal closure. We were able to observe a clear progression of patterning events.

We have concentrated on the pattern in the medial notum where *Ag-pnr* and *Ag-ASH* are expressed in domains that appear to be identical. Expression is first evident in two longitudinal bands, one on either side of the dorsal midline that is itself devoid of expression (Fig. 5E). When viewed through

the cuticle of fourth instar larvae, these domains appear to be quite broad (Fig. 5E), but after pupation and the subsequent cell shape changes of the epithelium, they appear as longer, thinner bands (Fig. 5A). These bands extend from the anterior border of the notum to almost the level of the future scutal-scutellar suture. They subsequently fade and three other expression domains appear in rapid succession: a small, posterior, triangle that straddles the dorsal midline (Fig. 5F) and that gradually transforms to a kidney-shaped domain (Fig. 5B,C,G) and a crescent-shaped domain along the future scutellum (Fig. 5D). *Ag-ASH*, but not *Ag-pnr*, is also expressed in another broad longitudinal domain on the lateral part of the notum (not shown).

As the expression domains fade, single, stained cells appear in their wake (Fig. 6A-C). These are also characterised by the expression of both *Ag-pnr* and *Ag-ASH*. We believe these correspond to the sensory organ precursors for the following reasons. The stained cells are always spaced apart from one another. They are of two sizes, large and small. The large cells are positioned at the sites of the future bristles; this is particularly evident for the AC and DC precursors whose numbers correspond roughly to the numbers of bristles found in imagos. The small, stained cells are only found in the areas corresponding to the expression domains of *Ag-ASH* (and *Ag-pnr*). These domains correlate with the areas covered by scales in the imago. No stained cells are found outside the domains of *Ag-ASH* expression, which are also devoid of scales and bristles in the imago. We also looked at neural differentiation with the anti-HRP antibody that labels neurons and axons (Jan and Jan, 1982). Staining was apparent in cells at similar positions to the presumed precursors and the imaginal sensory organs (Fig. 4C and Fig. 6D). Within any specific domain, sensory organ precursors appear to arise in a stochastic fashion, no particular order was apparent. Precursors of bristles and scales also appear to arise simultaneously, but in some preparations stained with anti-HRP, only bristle neurons are labelled, suggesting that neural differentiation may start earlier in bristles than in scales (Fig. 6D). Shortly after segregation of the precursors, both *Ag-pnr* and *Ag-ASH* stainings disappear.

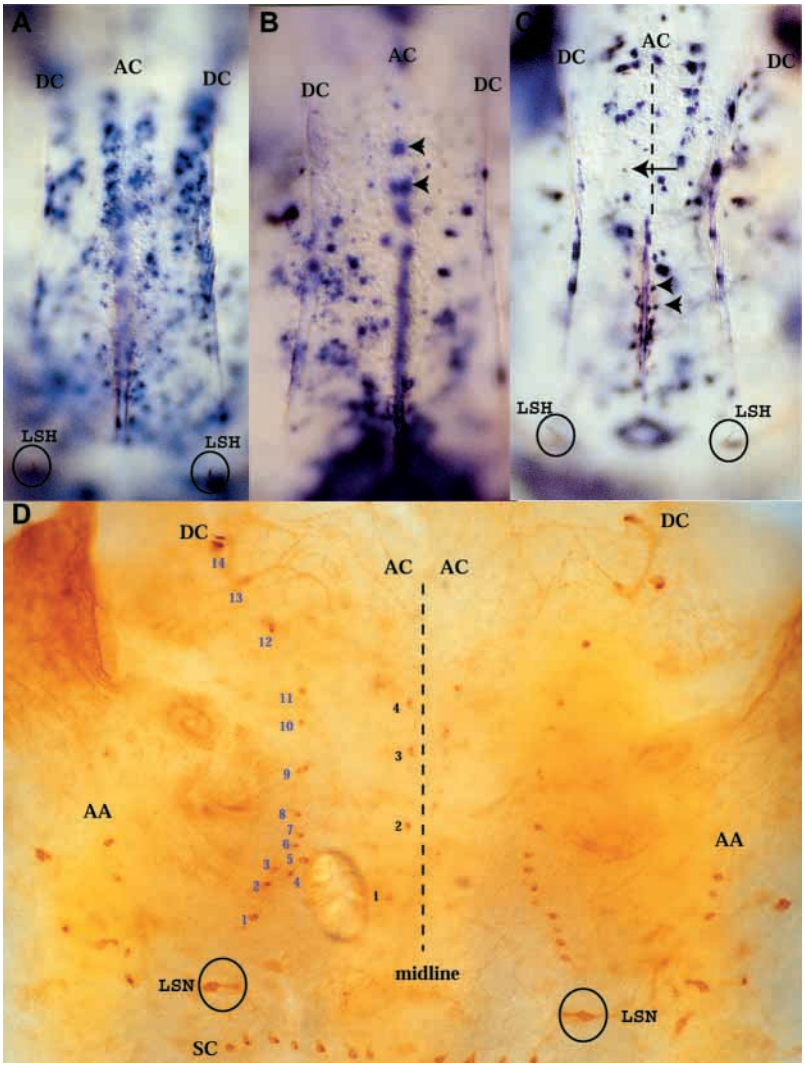


Fig. 6. Expression of (A,B) *Ag-pnr* and (C) *Ag-ASH* in segregating sensory organ precursors. Precursors arise as the domains of expression fade and can be seen as spaced single cells. Precursors of bristles of the acrostichal (AC) and dorsocentral (DC) rows arise first, indicated by arrowheads; they are large and arranged in poorly defined rows. Scale precursors in the vicinity of the AC and DC rows arise at the same time, indicated by the arrow in C. The scale precursors are smaller. The triangular domain of expression is visible at the bottom in B. The epithelium is somewhat creased at these stages distorting the spatial arrangements. Precursors of bristles and scales at other positions arise slightly later. Within the rows and at different spatial locations, there is no apparent order to precursor segregation: the distribution of stained cells is variable between individuals. LSH, persistent larval sensory hair. (D) A fourth instar larva stained with anti-HRP to visualise the neurons. (Precursor segregation is accomplished before pupation in some individuals). Neurons corresponding to some of the AC and DC bristles have appeared in this individual, as well as some of the lateral antealar (AA) neurons. LSN, larval sensory neurons.

Bristles arise along the borders of the domains of *Ag-pnr* expression

The positions of bristles appear to coincide with the borders of the expression domains of *Ag-pnr* and *Ag-ASH*. We could not observe simultaneous staining of the broad domains of expression as well as the precursors, since the latter only appear as the domains are fading. However the positions of all bristle precursors correlate with the edges of the successive domains. The AC and DC bristles are situated along the medial and lateral borders of the first longitudinal band of expression. The posterior transverse AC precursors are positioned along the anterior edge of the triangle, the PST precursors along the anterior edge of the kidney-shaped domain and the SC precursors along the anterior edge of the scutellar crescent (Fig. 5).

Precursors of the sensory scales arise both on the borders and inside the *Ag-ASH* expression domains.

DISCUSSION

Duplication of *achaete-scute* genes in Diptera

Cyclorraphous flies of the Schizophora, *Calliphora vicina*, *Ceratitis capitata* and *Drosophila* spp., possess three or four ASC genes (Alonso and Cabrera, 1988; Gonzalez et al., 1989; Pistillo et al., 2002; Villares and Cabrera, 1987; Wülbeck and Simpson, 2000). Each of the ASC genes of *Drosophila* is regulated independently and expressed in different, albeit overlapping domains of the developing nervous system (Martin-Bermudo et al., 1991; Romani et al., 1989; Ruiz-Gomez and Ghysen, 1993). Their products remain, however, largely functionally redundant (Balcells et al., 1988; Brand et al., 1993; Dominguez and Campuzano, 1993; Hinz et al., 1994; Rodriguez et al., 1990; Skeath and Doe, 1996). *Anopheles* belongs to the sub-order Nematocera, composed of basal species of Diptera that display a number of ancestral features (McAlpine, 1981). Our screening procedure allowed the isolation of a single *Anopheles ASC* homologue, *Ag-ASH*, but examination of the recently published genome of this species reveals the existence of an *asense* gene. *Ag-ASH* is closest to *Drosophila l'sc*, but may representative of an ancestral gene, which was present prior to the duplication events that gave rise to *l'sc*, *sc* and *ac* (Skaer et al., 2002). This may have taken place after separation of the Nematocera (including the mosquitoes) and Brachycera (including *Drosophila* and *Ceratitis*), two lineages that diverged about 200 million years ago. A single ASC homologue has been described in the butterfly *Precis coenia* (Galant et al., 1998).

When expressed in *Drosophila*, *Ag-ASH* has a conserved and strong, proneural function.

Regulation of *Ag-ASH* by Ag-Pannier

Several observations argue in favour of a role for Ag-Pnr in the activation of *Ag-ASH*. First, the two genes are expressed in what appear to be identical domains in the medial notum. Secondly, both genes are also expressed in sensory organ precursors. In the cyclorraphous flies examined to date, *pnr* is not expressed in bristle precursors (Pistillo et al., 2002; Ramain et al., 1993; Wülbeck and Simpson, 2000). Thirdly, expression of *Ag-pnr* is able to mimic the effects of mis-expression of *Dm-pnr* in *Drosophila*. Thus, when expressed in the lateral notum *Ag-pnr* elicits the development of ectopic DC bristles, strongly suggesting that it can activate the *Drosophila ac-sc* genes. Therefore we think it probable that regulation of *ac-sc* genes by Pnr has been conserved throughout the Diptera.

In *Drosophila*, *pnr* is expressed in a conserved broad medial domain but activates *ac* and *sc* in discrete proneural clusters within this domain (Cubas et al., 1991; Garcia-Garcia et al., 1999; Ramain et al., 1993; Romani et al., 1989; Skeath and Carroll, 1991) The *ac-sc* genes of *Drosophila* are organised into a complex containing multiple enhancer regions, each of which independently regulates expression in one or a small number of proneural clusters (Gomez-Skarmeta et al., 1995; Ruiz-Gomez and Modolell, 1987). In this species three proneural clusters arise in the domain of *pnr* expression and Pnr has been shown to directly activate *ac-sc* in the dorso-central cluster, through binding to a *cis*-regulatory sequence just upstream of *ac* (Garcia-Garcia et al., 1999; Haenlin et al., 1997). It is not entirely understood how the broad domain of Pnr is translated into the small clusters of *ac-sc* expression, but this is at least in part achieved through interaction of Pnr with regulatory co-factors (Cubadda et al., 1997; Haenlin et al., 1997; Ramain et al., 2000). The spatially complex expression of *sc* in *Calliphora* and *Ceratitis* suggests that the ASC genes of these species may also have modular promoters (Pistillo et al., 2002; Wülbeck and Simpson, 2000). Furthermore, the expression domain of *pnr* in these species is conserved with that of *Drosophila* (ibid).

In contrast, the regulatory interactions between the two genes appear to have diverged in *Anopheles* since *Ag-ASH* is expressed in all *Ag-pnr*-expressing cells. The common domains of expression suggest that Ag-Pnr may activate Ag-*ASH* in every cell in which it is expressed, in a simple straightforward fashion. This observation raises two possibilities. First, for the regulation of *Ag-ASH*, Ag-Pnr may not associate with the various co-factors known to modulate its activity in *Drosophila*. Second, in order to be activated in all *Ag-pnr*-expressing cells, *Ag-ASH* would not need to have a modular promoter structure like that of the *Drosophila* locus, and could have a less complex organisation. If so, the acquisition of position-specific enhancers may have occurred after the separation of Nematocera and Brachycera, at a time when further gene duplication events appear to have taken place (Skaer et al., 2002). In addition, modulation of Pnr activity through the use of different co-factors may have accompanied the acquisition of *cis*-regulatory enhancer sequences in the lineage leading to *Drosophila*.

Despite the inferred simple regulatory interaction between Ag-Pnr and *Ag-ASH*, it is remarkable that the effects of mis-expression of *Ag-pnr* in *Drosophila* are almost identical to those caused by mis-expression of *Dm-pnr*. For example, ectopic expression of either *Dm-pnr* or *Ag-pnr* on the lateral notum, causes the development of a tuft of ectopic dorso-central bristles. This is due to an expansion of the activity of the dorso-central enhancer element known to be regulated by Dm-Pnr (Garcia-Garcia et al., 1999). This result suggests that Ag-Pnr is able to recognise the relevant regulatory modules of the *Drosophila* ASC promoter which may indicate that these enhancers are derived from an ancestral regulatory sequence also present in *Anopheles*. Alternatively, a number of regulatory modules may in fact be present in the *Anopheles* promoter and govern expression in the various domains on the notum. Further understanding of the structure and regulation of *Ag-ASH* will require investigation of regulatory sequences from this organism. The ectopic expression assay also indicates that Ag-Pnr is probably able to associate with *Drosophila* co-factors such as U-shaped and Chip (Cubadda et al., 1997; Ramain et al., 2000). It has been shown that the N-terminal zinc finger of Dm-Pnr associates with U-shaped, while two C-terminal helical structures are components mediating association with Chip (Haenlin et al., 1997; Ramain et al., 2000). The two zinc fingers are strongly conserved in Ag-Pnr, and there is a single α helix. Thus Ag-Pnr appears to contain the relevant binding regions for these two factors. This complexity of the *Ag-pnr* protein may indicate association with endogenous co-factors, perhaps in a different tissue.

A conserved role for *pannier* in the specification of the dorsal pattern

It has been demonstrated, that, in *Drosophila*, *pnr* and the *iro-C* genes are selector genes involved in the subdivision of the

dorsal component of segments of the head, thorax and abdomen of the adult into medial and lateral domains (Calleja et al., 2000; Mann and Morata, 2000; Maurel-Zaffran and Treisman, 2000). While *pnr* regulates the pattern of the medial domain of the dorsal mesonotum, patterning of the lateral half is regulated by the *iro-C* genes (Gomez-Skarmeta et al., 1996; Calleja et al., 2000; Cavodeassi et al., 2001; Diez del Corral et al., 1999; Kehl et al., 1998). Thus, when either *Dm-pnr* or *Ag-pnr* is expressed from an early stage in the entire notum of *Drosophila*, only structures corresponding to the medial notum are formed, the lateral region fails to develop (Calleja et al., 2000). Ubiquitous expression specifies a single medial domain thought to include cells originally destined to form the lateral region (Calleja et al., 2000). In addition we find that *Ag-pnr* is expressed in the medial, but not the lateral, mesonotum of *Anopheles*, consistent with a conserved function in the medial domain. Thus the selector gene function of *pnr* may have been conserved. The function of proteins of other selector genes of *Anopheles*, such as *engrailed*, has been shown to be conserved (Whiteley and Kassis, 1997).

The precursors of the sensory scales on the notum of *Anopheles* are distributed in a random fashion within the domains of expression of *Ag-pnr/Ag-ASH*. In some respects the sensory scales resemble the small bristles or microchaetes of cyclorraphous Diptera, which are often randomly distributed although sometimes lined up into rows (McAlpine, 1981; Simpson et al., 1999). However, in the latter species they arise later than the large bristles or macrochaetes, from a second period of *ac-sc* expression, and are consequently positioned closer to one another than are the macrochaetes (Simpson et al., 1999; Wülbeck and Simpson, 2000; Pistillo et al., 2002). In contrast, the precursors of scales and bristles appear to arise simultaneously in *Anopheles*, which is consistent with the fact that they are equidistant from each other in the imago. In cyclorraphous flies, the macrochaete pattern is the result of spatially complex *sc (ac)* expression: one (or a very small number) of bristle(s) develops from each small cluster (or stripe) of *sc (ac)* expression. In *Anopheles*, however, the patterning mechanism is different: remarkably, the precursors of the bristles are exclusively positioned along the borders of the expression domains. Thus the positions of the rows of AC and DC bristles, as well as the PST and SC bristles, are coincident with the borders of the four domains of *Ag-pnr/Ag-ASH* expression. This suggests that the boundaries of *Ag-ASH*/*Ag-pnr* expression convey specific positional information causing neural precursors to develop into bristles rather than sensory scales.

Two observations in *Drosophila* may be relevant to this phenomenon. First, some of the macrochaete precursors arise from the edges of the corresponding proneural clusters of *ac-sc* expression, an observation that has been linked to distance from the source of the signalling molecules Wingless and Decapentaplegic (Cubas et al., 1991; Phillips et al., 1999). The expression pattern of these molecules in *Anopheles* is not yet known. Second, it has been demonstrated that the border between *pnr*-expressing and non-expressing cells does in fact display special properties. Cells of the medial domain manifest unique adhesive characteristics that prevent them from mixing with cells of the lateral domain (Calleja et al., 2000). So, as for compartment boundaries, this interface between cells expressing *pnr* and those expressing *iro* may be an important patterning boundary (Dahmann and Basler, 1999; Lawrence and Struhl, 1996; Mann and Morata, 2000). It has indeed been shown to be required for the growth and patterning of the *Drosophila* eye (Cavodeassi et al., 1999; Cavodeassi et al., 2000; McNeill et al., 1997; Yang et al., 1999). Interestingly, the five macrochaetes on the medial notum of *Drosophila* are *pnr*-dependent, and they are all positioned on the lateral border of the domain of *pnr* expression (Fig. 5). Experimentally contrived expression of *ac-sc* inside the *pnr* domain, however, results in the formation of ectopic macrochaetes, indicating that macrochaete formation in *Drosophila*, is not dependent on special properties at the border (Balcells et al., 1988; Cubadda et al., 1997; Haenlin et al., 1997; Rodriguez et al., 1990). Furthermore the prescutellar bristle of *Ceratitis* and the AC row of bristles in *Calliphora*, arise from *sc*-expressing cells situated inside the *pnr* expression domain (Pistillo et al., 2002; Wülbeck and Simpson, 2000).

Although the bristles on the notum of *Anopheles* are aligned into rows, the number and position of bristles within a row varies greatly between individuals, a feature that is thought to be ancestral (McAlpine, 1981; Simpson et al., 1999). Species of cyclorraphous Schizophora in contrast, have very defined rows in which the number and position of bristles varies little if at all. The stereotyped notal bristle patterns of species such as *Drosophila* are thought to be derived from an ancestral pattern of four longitudinal rows of bristles, still present in many extant species of Schizophora (Simpson et al., 1999; Pistillo et al., 2002). These include the AC and DC bristle rows that are in the medial domain of the notum. So, for example, the two DC bristles of *Drosophila* would be vestiges of the DC row. Whether the rows of bristles seen in some families of Nematocera such as the Culicidae, are in any way related by ancestry to the four rows of Schizophoran flies, is more difficult to assess. Nevertheless the DC row of *Anopheles* is positioned on the lateral border of the *Ag-pnr* expression domain, as in *Ceratitis*, *Calliphora* and *Drosophila*, which may indicate a common origin for this row. If so, this would mean that an ancestral pattern of bristle rows was already present in a common ancestor of the Brachycera and at least some families of Nematocera.

Conclusions

Our results indicate a conserved function for *pnr* in the regulation of the bristle pattern on the medial notum. This argues in favour of an ancient role for *pnr* as a selector gene specifying the dorsal medial pattern. The nature of the regulatory interactions between Pnr and its target genes *ac-sc* appears to have changed, however, over evolutionary time. We hypothesise that in Culicid mosquitoes, which have fewer *ac-sc* genes, the regulatory regions of this locus may not be organised in a modular fashion. Evolution of the stereotyped bristle patterns characteristic of species such as *Drosophila* and *Ceratitis* may have entailed the acquisition of a number of additional factors. These would include gene duplication within the ASC and the co-option of *cis*-regulatory sequences. Co-factors for Pnr, such as Ush and Chip, are also likely to have been co-opted for use in constructing the notal pattern at a later evolutionary stage, although our results suggest that Ag-Pnr has the requisite domains for association with these proteins (Cubadda et al., 1997; Haenlin et al., 1997; Ramain et al., 2000). In the lineage leading to *Drosophila*, these different levels of regulation might have been superimposed onto an

ancestral patterning mechanism, similar to that of *Anopheles*, at different times in the 200 million years separating *Drosophila* from the Nematocera.

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