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

Corinna Wülbeck* and Pat Simpson†,‡

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, 67404 Illkirch Cedex, CU de Strasbourg, France and Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK

*Present address: Institut für Zoologie, Lehrstuhl für Entwicklungsbiologie, Universität Regensburg, 93040 Regensburg, Germany
†Present address: Department of Zoology, Downing Street, Cambridge CB2 3EJ, UK
‡Author for correspondence (e-mail: pas49@cam.ac.uk)

Accepted 30 April 2002

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
The evolution of stereotyped bristle patterns. Interestingly, all of the some 200 million years ago, and may have allowed the after the separation of the Nematocera and the Brachycera, cis-acquisition of position-specific Drosophila complex modular promoter characteristic of the ASC of 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 (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 gambiæ (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 misexpression 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 panner homologues in Anopheles**

Screening for homologues of the achaete-scute (ac-sc) and panner (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 XhoI 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 phases 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 EcoRI-XhoI fragment of Ag-ASH (pBS-AC3K1) and a full-length cDNA SpeI-KpnI 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; Gorfinkeil 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., 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., 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.
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<sup>MD237</sup>, 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; Gorflnklei 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).

**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. 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,
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-pannier 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-pannier 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.
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 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.

**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.
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.

**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

Cyclorrhaphous 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 (Balcels 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 aense 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-Pännier

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 primordia in the lateral notum. Further understanding of the structure and regulation 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 ird-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 cyclorrhaphous 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 cyclorrhaphous 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; Cubada 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 cyclorrhaphous Schizophora in contrast, have very defined rows in which the number and position of bristles varies little if at all. The stereotyped natal 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 natal pattern at a later evolutionary stage, although our results suggest that Ag-Pnr has the requisite domains for association with these proteins (Cubada 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.

We acknowledge the financial support of the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Hôpital Universitaire de Strasbourg, as well as the Programme of the European Community (contract no. FMRX CT 96 0065), the Programme Génôme du CNRS and the Wellcome Trust (grant number 29156). C. W. is the recipient of a fellowship from the DFG. We are very grateful to Professor Fotis Kafatos and the members of his laboratory for their generous supply of mosquitoes and for DNA libraries from *Anopheles* and for hosting C. Wülbeck as a visiting scientist at the outset of this work. We thank Nadia Masdeq for her help with sectioned material, Annie Bauer and the members of the sequencing facility of the IGBMC for technical help, Margit Pal, Daniela Pistillo and Claire Chapman for their help, Margit Pal, Daniela Pistillo and Claire Chapman for their help, and the members of the sequencing facility of the IGBMC for technical help. We thank Carlos Wülbeck as a visiting scientist at the outset of this work. We thank Carlos Wülbeck as a visiting scientist at the outset of this work. We thank Nadia Masdeq for her help with sectioned material, Annie Bauer and the members of the sequencing facility of the IGBMC for technical help, Margit Pal, Daniela Pistillo and Claire Chapman for their help, Margit Pal, Daniela Pistillo and Claire Chapman for their help, and the members of the sequencing facility of the IGBMC for technical help. We thank Carlos Wülbeck as a visiting scientist at the outset of this work. We thank Nadia Masdeq for her help with sectioned material, Annie Bauer and the members of the sequencing facility of the IGBMC for technical help, Margit Pal, Daniela Pistillo and Claire Chapman for their help, Margit Pal, Daniela Pistillo and Claire Chapman for their help, and the members of the sequencing facility of the IGBMC for technical help.
mirror encodes a novel PBX-class homeoprotein that functions in the definition of the dorsal-ventral border in the Drosophila eye. *Genes Dev.* 11, 1073-1082.


