Commentary on arthropod brain evolution

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When molecules and morphology clash
Analysis of nubbin expression patterns in insects

Hua Li and Aleksandar Popadić*

Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA
*Author for correspondence (email: apopadic@biology.biosci.wayne.edu)

SUMMARY Previous studies have shown that the gene nubbin (nub) exhibits large differences in expression patterns between major groups of arthropods. This led us to hypothesize that nub may have evolved roles that are unique to particular arthropod lineages. However, in insects, nub has been studied only in Drosophila. To further explore its role in insects in general, we analyzed nub expression patterns in three hemimetabolous insect groups: zygentomans (Thermobia domestica, firebrat), dyctiopterans (Periplaneta americana, cockroach), and hemipterans (Oncopeltus fasciatus, milkweed bug). We discovered three major findings. First, observed nub patterns in the ventral central nervous system ectoderm represent a synapomorphy (shared derived feature) that is not present in other arthropods. Furthermore, each of the analyzed insects exhibits a species-specific nub expression in the central nervous system. Second, recruitment of nub for a role in leg segmentation occurred early during insect evolution. Subsequently, in some insect lineages (cockroaches and flies), this original role was expanded to include joints between all the leg segments. Third, the nub expression in the head region shows a coordinated change in association with particular mouthpart morphology. This suggests that nub has also gained an important role in the morphological diversification of insect mouthparts. Overall, the obtained data reveal an extraordinary dynamic and diverse pattern of nub evolution that has not been observed previously for other developmental genes.

INTRODUCTION

Arthropods are the most diverse and successful animal phylum, encompassing a spectacular range of morphological diversity. This extraordinary diversity was made possible by their modular body design, a key feature of which is the division of bodies into separate modules (segments). In terms of macroevolutionary trends, arthropod evolution has been characterized by fusion of segments into discrete functional units. This process, tagmosis, resulted in the establishment of distinct morphological and functional body regions, such as the head, thorax, and abdomen in insects. At the same time, the identity of a particular segment is influenced by the type and function of its appendages. These two processes, the establishment of distinct body regions and appendage diversification, are mutually interdependent and together account for a large portion of the morphological diversity in arthropods. Thus, elucidating the molecular mechanisms that have contributed to these two trends is key to understanding morphological evolution in arthropods.

In the past decade, largely through the evolutionary developmental studies of homeotic (Hox) genes, we have started to gain insight into the molecular basis of tagmosis (Raff 1996; Carroll et al. 2001; Wilkins 2002). Because of their role in establishing segmental identities along the anteroposterior axis, Hox genes can serve as useful molecular markers to study patterns of regional differentiation leading to the formation of distinct body regions (Carroll 1995; Gellon and McGinnis 1998; Popadić et al. 1998a; Hughes and Kaufman 2002). The emerging view from these studies indicates that changes in both the expression patterns and functional domains of Hox genes have played an important role in establishing distinct body plans that characterize the four major extant arthropod groups (Cohen 1993; Carroll et al. 2001). In addition, alterations of homeotic gene expression patterns are also associated with the morphological diversification of specific appendages. For example, changes in proboscipedia patterning are tightly linked with the transformation of chewing to sucking mouthparts in insects (Rogers et al. 2002), whereas Ultrabithorax variations can be correlated with the morphological evolution of the anterior thoracic legs in malacostracan crustaceans (Averof and Cohen 1997). However, much less is known about the role of other nonhomeotic genes in diversification of arthropod appendages. A number of recent studies indicate that nubbin, or pdm-1, may be a good candidate for such a role.

Nubbin (nub) belongs to the POU homeodomain gene family; hence, it is also referred to as a pdm (POU domain protein) (Billin et al. 1991). POU domain genes are developmental regulators involved in a number of different processes such as cell migration, proliferation, lineage, neurogenesis, and the differentiation of specific structures.
(Anderson et al. 1995; de Celis et al. 1995; Yeo et al. 1995; Wang and Way 1996; Ryan and Rosenfeld 1997). Most of our knowledge of nub function is based on Drosophila studies, which were primarily focused on wing development and neurogenesis (Ng et al. 1995, 1996). More recent studies in Drosophila have revealed that nub may also have an important role in leg development and segmentation (Cifuentes and Garcia-Bellido 1997; Rauskolb et al. 1999). This putative function is consistent with its expression in leg discs, where nub is expressed in a series of concentric rings (Anderson et al. 1995; Averof and Cohen 1997; Cifuentes and Garcia-Bellido 1997). However, this Drosophila leg disc pattern is very different from that observed in chelicerate and crustacean embryos and larvae. In these arthropods, nub expression is localized to only one or two rings in the most distal leg segments (Averof and Cohen 1997; Abzhanov and Kaufman 2000). In a similar fashion, whereas nub is required for neuronal lineage specification in the central nervous system (CNS) of Drosophila embryos (Isshiki et al. 2001; Brody and Odendal 2002), its expression has not been observed in the CNS of other arthropods (Gilbert et al. 2002).

The observed differences in nub expression patterns between fly, crustacean, and spider embryos indicate that this gene may have acquired novel functions unique to insects. Furthermore, its involvement in leg development in Drosophila suggests a possible role for nub in the diversification of insect appendages. To further examine these issues, we decided to examine nub expression in the embryos of several hemimetabolous insect species. In this study we report on the results of our analysis of nub in the primitively wingless insect Thermobia domestica (firebrat), Periplaneta americana (cockroach), and Oncopeltus fasciatus (milkweed bug). The first two species represent basal insect lineages, whereas Oncopeltus is a hemipteran, a moderately derived hemimetabolous order. We use our observations to elucidate key trends in the evolution of nub expression patterns and, consequently, to infer its role in the development of specific insect structures.

MATERIALS AND METHODS

Cloning and sequence analysis of cDNA fragments

Fresh embryos of T. domestica (firebrat), P. americana (cockroach), and O. fasciatus (milkweed bug) were used for total RNA extraction. RNA was prepared using the TriZol reagent (Life Technologies, Grand Island, NY, USA), according to the manufacturer’s protocol. Total RNA was then Poly(A) selected using the Oligotex Mini mRNA kit (Qiagen, Valencia, CA, USA), and the Reverse Transcription System (Promega, Madison, WI, USA) was used to produce cDNA. For the reverse transcriptase-polymerase chain reaction (PCR), we used previously described primers (Averof and Cohen 1997) targeting the highly conserved amino acid motifs EQFAKT (5’-GGAATTCGARCARTTYG-CIAARAC-3’) and KEKRNP (5’-GCTTAGAGGRTTIATTCYTTYTCTYT-3’). Two rounds of PCR were performed, based on the slightly modified protocol of Rogers et al. (1997). For the first five cycles, the annealing temperature was 58°C, followed by a 1-min ramp to the extension temperature (72°C). The remaining 35 cycles also used an annealing temperature of 50°C but had no extended ramp time. All extension times were 30 sec. The length of amplified fragments ranged from 441 to 444 bp in the firebrat and milkweed bug, respectively.

The fragments generated were cloned into pCR4.0 vector (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s protocol. Twenty copies of each nub/pdm clone were recovered and sequenced. All obtained nucleotide sequences were compared with each other and to previously described nub/pdm homologs in GenBank using MacVector 6.0.1 (Kodak, Rochester, NY, USA) software. No evidence for paralogous copies was found, suggesting the existence of a single copy of nub/pdm-1 in the three insect species examined. All sequences used for phylogenetic analysis were aligned by eye according to the protein alignment shown in Fig. 1A.

Phylogenetic analyses were performed on protein sequences with all characters weighted equally, using the neighbor-joining method of the PHYLIP software package (Felsenstein 1993) and the maximum parsimony method of the PAUP software package (Swofford 1993). Sequence accession numbers were as follows: M81957 (Dm-pdm1), M81958 (Dm-pdm2), Y09993 (Af-nub), AF273262 (St-nub), AJ420131 (Cs-nub), AF273259 (Ps-nub), AF273261 (Ps-nub), L04646 (Sp-Oct), X13403 (Hs-Oct1), CG10037 (Dm-vvl), AF273266 (Ps-vvl), and AF273267 (St-vvlB).

In situ hybridization

All RNA probes were generated using Promega’s Riboprobe kit with digoxigenin-UPT (Roche, Mannheim, Germany), following purification with a Mini Quick RNA column (Roche). The in situ hybridization procedure was developed based on the protocols used by Panganiban et al. (1994) and Rogers et al. (1997), with slight modification. Embryos were first dissected from their chorionic and extraembryonic membranes directly in the fixative (10% formaldehyde in PBT). Following fixation of 1–2 h, embryos were treated with proteinase K (50 μg/ml). The duration of the proteinase step was found to be critical and ranged from 8 min (early germ-band milkweed bug embryos) to 15 min (late-stage cockroach embryos). A postfixation of 20 min was found to be necessary. Hybridization with the riboprobe was done at 55°C for 24–48 h. After the probe was removed, a long soak of 12–36 h in hybridization buffer at 57°C helped to reduce background.

To visualize the RNA pattern generated, anti-digoxigenin antibody conjugated to alkaline phosphatase was used (Roche), with 2- to 3-h incubation at room temperature. The signal was revealed by an NBT/BCIP color reaction (Roche). Stained embryos were then cleaned from residual yolk as much as possible and mounted on a microscope slide in a drop of Aqua Polymount (Polysciences Inc, Warrington, PA, USA). For each species, we examined 150–200 stained embryos of various stages to infer the general expression pattern. Interested researchers are encouraged to contact the authors for a full detailed in situ protocol.
RESULTS

Cloning and analysis of nub/pdm-1 homologs in insects

A monoclonal antibody that recognizes a conserved epitope in the Nub/pdm protein has been generated and used successfully in a number of crustacean and chelicerate species (Averof and Cohen 1997; Abzhanov and Kaufman 2000; Damen et al. 2002). Unfortunately, this antibody does not recognize insect nub orthologs except for that of Drosophila. To examine the expression of nub/pdm, we used reverse transcriptase-PCR with degenerate primers to amplify cDNAs of the nub orthologs from three insects (firebrat, Thermobia; cockroach, Periplaneta; and milkweed bug, Oncopeltus). PCR on these three species yielded a single fragment, ranging from 441 to 444 bp. We sequenced 20 clones from each species and found no evidence for paralogous nub genes. The sequences of cloned portions of Thermobia, Periplaneta, and Oncopeltus pdm genes are shown in Fig. 1A. They have been aligned with orthologs from several other arthropods as well as with those of the related Vvl gene. The positions of the POU domain and homeodomain are indicated above the sequence.

Overall, the cloned Nub/Pdm sequences showed a high degree of conservation with other arthropod nub/pdm orthologs and were quite distinct from sequences of a related Vvl gene. Among insects, nub sequences showed perfect conservation in the POU domain except for a single amino acid replacement. In addition, insect Nub/Pdm sequences showed a high degree of sequence similarity in the homeodomain. Even a variable “linker” region was conserved between firebrats, cockroaches, and milkweed bugs. Conservation was especially noticeable at the beginning of the POU domain and homeodomain. Furthermore, the ladder-like pattern was not uniform.

In contrast to the patterns observed previously in crustacean and chelicerate embryos (Averof and Cohen 1997; Damen et al. 2002; Gibert et al. 2002), in which nub is never associated with the developing CNS. However, in firebrat embryos there were two square clusters of cells, in the middle of each segment, that expressed nub. These two clusters were separated by a central region in which nub was absent. Although nub was clearly expressed in the head and thoracic mid-ventral regions, its expression in the anterior abdomen (A1–A4) was very weak. This was followed by an increasing gradient of expression from A5 posteriorly (Fig. 2A’). Nub expression was also observed in the ventrolateral region from A8 to A10, with the strongest expression in segment A10. There was no nub expression in any of the head or thoracic appendages (Fig. 2A’). This is in contrast to the patterns previously observed in crustacean and chelicerate embryos (Abzhanov and Kaufman 2000), where nub is expressed in appendages even at the early limb bud stage.

As development progressed, a higher level of nub expression was detected in the mid-ventral CNS region, especially in the posterior part of the abdomen (Fig. 2B). At this stage, nub mRNA also started to accumulate in some appendages (Fig. 2B’). Although there was no expression in the mandibles, there were two weak bands of nub expression in the middle portion of the maxillary appendages (Fig. 2B’). There was also a faint diffuse signal in the proximal portions of the labial appendages and thoracic legs. The mid-ventral CNS expression also became much stronger from segments A5 to A10 (Fig. 2B”). Whereas the two lateral clusters of nub-expressing cells were still separated in segments A5 and A6, they began to fuse in segments A7–A9 and were completely fused in segment A10 (Fig. 2B”).

As embryos began to undergo dorsal closure, nub expression strengthened and the pattern was more resolved (Fig. 2, C and C”). Except for the antennae, expression was now obvious in all appendages: There was a diffuse signal in the proximal regions of the mandibular and labial appendages and a single distal band in each maxillary appendage (Fig. 2C’, arrowhead). There were also three patches of nub-expressing cells in the proximal halves of each leg.
Fig. 1. Sequence comparisons and phylogenetic relationships of the arthropod Pdm/Nub orthologs. The names of sequences that were generated by this study are highlighted in bold. (A) Alignment of amino acid sequences of the Pdm/Nub and Vvl orthologs from different arthropod species. The POU domains and homeodomains of both genes have unique amino acids that can serve as reliable diagnostic markers for homology assignment. The amino acid differences between Pdm and Vvl genes are highlighted in gray. Portions of the POU domain and the homeodomain within the nub and vvl fragments are marked above the sequences. Arrows mark the sequences of primers used in this study. The sequence corresponding to the in situ probe is marked with a bar. (B) Neighbor-joining tree depicting the sequence relationships of the firebrat, cockroach, and milkweed bug Pdm genes to other PDM/NUB/OCT family members (class II POU-homeodomain proteins) and to members of the related Vvl genes (class III POU-homeodomains). This unrooted tree shows a clear assignment of the three insect nub genes to the PDM/NUB/OCT family with a strong bootstrap support (100%). The internal nodes in the PDB/NUB/OCT family are not significantly resolved. All sequences except those of the three insect species (Oncopeltus, Periplaneta, and Thermobia) were acquired from GenBank; for accession numbers see Materials and Methods. Dm, Drosophila melanogaster; Of, the milkweed bug Oncopeltus fasciatus; Pa, the cockroach Periplaneta americana; Td, the firebrat Thermobia domestica; Ps, the woodlouse Porcellio scaber; Pc, the crayfish Procambarus clarkii; Af, the brine shrimp Artemia franciscana; St, the spider Steatoda triangulosa; Cs, the spider Cupiennius salei; Hs, human Homo sapiens; Sp, the sea urchin Strongylocentrotus purpuratus.
In the mid-ventral neuroectoderm, *nub* expression was expanded laterally to include an additional cluster of cells on both sides of the embryo. This expansion was most noticeable in the thoracic region and in the posterior abdomen in segments A5–A8 (Fig. 2C’). In addition, the trend toward fusion of the two mid-ventral regions continued...
in the posterior abdomen. The final modulation of the firebrat pattern occurred toward the end of dorsal closure (Fig. 2D). Although nub continued to be absent in the antennae, it was strongly expressed around the base of the mandibles. It was also expressed in the basal and proximal regions of the maxillae and in most of the labial appendages (Fig. 2D'). In the legs, there were three distinct domains of nub expression: a proximal region corresponding to the future coxal segment, a middle region, and in the distal tip. In the mid-ventral region, nub expression was restricted to cells in the center of each segment; lateral expression completely disappeared (compare Fig. 2C'' and Fig. 2D''). In summary, firebrat embryos were characterized by a distinct and dynamic nub expression pattern quite different from those observed in other arthropods.

**Embryonic expression pattern of nub in Periplaneta americana (cockroach)**

Using a cloned Pa nub cDNA fragment for whole-mount in situ hybridization, we also studied nub mRNA expression patterns in embryos of this species. Cockroaches belong to one of the more basal insect orders (Blattodea) and undergo typical hemimetabolous development. However, compared with those of Thermobia, Periplaneta embryos begin leg segmentation at a much earlier stage. Thermobia embryonic legs become segmented quite late, around the time of dorsal closure, when these appendages have almost reached their final length. In contrast, Periplaneta appendages undergo segmentation much earlier, during germ-band extension, around the time when legs just begin to elongate.

At early stages of Periplaneta development, two features characterized nub expression pattern in this species. First, there was strong distinct nub expression in the appendages. In firebrat embryos, nub was mostly absent from the appendages (compare Figs. 2A and 3A). Second, there was weak expression in the middle and lateral region of the abdominal segments. The abdominal pattern was characterized by an ascending gradient in the posterior direction from A1 to A10, resulting in the strongest nub expression being in the posterior-most abdominal segments (Fig. 3, A and B). This is reminiscent of abdominal nub expression in firebrat embryos, which was also distinguished by an ascending gradient in the posterior direction (compare Figs. 2A and 3A).

As cockroach development progressed, nub expression continued to be dynamic, with distinctly different patterns in the head and thoracic appendages (Fig. 3C). A high level of nub appeared in the mid-ventral region, starting at the labial segment and ending at the segment A1 (Fig. 3C). The earlier faint expression in the abdomen faded, and only the strong most posterior abdominal expression persisted (Fig. 3D). In later stages of development, when the embryos began dorsal closure, the expression of nub in the appendages continued to change (Fig. 3E). Expression disappeared in the antennae and mouthparts and was restricted to several clusters of nub-expressing cells in the legs. The most distinct feature of this late pattern was strong and elaborate mid-ventral neuroectodermal expression, from the labial to the segment A4 (Fig. 3E). This CNS pattern was not uniform and encompassed a wider mid-ventral segmental region in the thorax and a more narrow area in the abdomen. At this time, posterior abdominal expression was restricted to A11 (Fig. 3E).

In the head region, from early to late stages, nub was absent from antennae. As limb buds began to elongate, nub was expressed throughout the maxillae and labium (Fig. 3F) and in a diffuse spot in the mandible (Fig. 3G). By 50% of embryogenesis, mandibular expression disappeared (Fig. 3H). Maxillary and labial expression continued to be similar and resolved into a distinct “rings and sock” pattern, with two “rings” in the middle and a “sock” at the tip of both maxillary and labial palps (Fig. 3H). In addition, diffuse nub expression was also observed in the proximal portion of the maxillary and labial appendages, corresponding to the future lacinia (glossoa) and galea (paraglossa). As development progressed, only cells in two rings in the maxillary and labial palps continued to express nub (Fig. 3I). Finally, by the end of dorsal closure, nub expression in all head appendages was completely lost (Fig. 3J).

Highly specific nub expression was also observed in the thoracic legs. At early stages, when the leg limb buds just began to elongate, three bands of nub expression were seen in the distal half of each leg (Fig. 3K). In the proximal to distal direction, we labeled these bands as 1 (most proximal, with the narrowest and weakest level of expression), 2 (located in the middle, with the highest level of expression), and 3 (most distal, similar in intensity to 1). As appendages continued to elongate, a new band (4) of nub expression appeared in the distal portion of the leg (Fig. 3L). At about 50% stage, the cockroach legs underwent segmentation, and it was at this time that a fifth band of nub (5) appeared in the distal portion of the appendage (Fig. 3M). At this stage, the spatial and temporal organization of nub expression coincided precisely with the establishment of distinct leg segments: the boundary between body wall and coxa (band 1), between coxa and trochanter (band 2), between trochanter and femur (band 3), between femur and tibia (band 4), and between tibia and tarsus (band 5). As the embryo began dorsal closure, the legs became fully segmented and the coxa became enlarged compared with the other segments (Fig. 3N). Although nub expression in each segment was still detectable, the previously strongly stained bands started to fade in an anterior (band 5) or in posterior direction (bands 3 and 4). The exceptions were bands 1 and 2, which continued to display a strong signal. Toward the late stages of embryogenesis, legs were fully elongated and only clusters of cells continued to express nub (Fig. 3O). On the ventral side of the coxa, there was distinct and strong nub expression in the posterior part and weaker
expression anteriorly. Two additional clusters of nub-expressing cells were observed in the trochanter, although at a very low level. There was also novel nub expression in the tip of each leg.

Embryonic expression patterns of nub in a hemipteran Oncopeltus fasciatus

To study hemipteran nub mRNA expression, we used the cloned Of Pdm cDNA fragment for whole-mount in situ hybridization. Hemipterans represent one of the phylogenetically “younger” hemimetabolous insect orders, compared with Zygentoma (firebrats) and Blattodea (cockroaches). Thus, studies of milkweed bug embryos provide an important data point, allowing us to infer whether the trends in nub expression observed in more basal insect groups extend to hemipterans as well.

Three key features characterized the nub expression in Oncopeltus embryos (Fig. 4, A–E). First, during early development, strong nub expression was localized primarily
in the ocular and brain head regions and in the limb buds of the head and thorax (Fig. 4, A and F). Mid-ventral expression in the thoracic and abdominal segments was faint and concentrated in several clusters of neuroblasts in each segment. Thus, early expression was more reminiscent of the situation observed in cockroach embryos and very different from the firebrat pattern characterized by absence of signal in the appendages at this stage. Second, during the mid-stages of *Oncopeltus* development, *nub* continued to be present in the appendages with distinctly different patterns in the head and thoracic segments (Fig. 4B). In addition, strong mid-ventral CNS expression appeared in the form of two parallel columns of *nub*-expressing cells (Fig. 4B). At the corresponding stage (Fig. 3B), cockroach expression localized to appendages only. Therefore, CNS expression appeared much earlier in milkweed bug embryos than in cockroaches.
The CNS pattern in *Oncopeltus* was also different, with *nub*-expressing cells localized at each side of the central midline (Fig. 4B). As the appendages continued to elongate, there was continuing strong expression in the brain and in the head appendages but weakening of signal in the legs (Fig. 4C). In the mid-ventral region, whereas most of *nub*-expressing cells were still in two mid-lateral columns, some cells started to express *nub* in the central midline of the mandibular segment (Fig. 4C). As a consequence, the CNS expression pattern in this segment took an appearance of the letter “x.” The third key feature of milkweed *nub* expression was observed during late development (Fig. 4D and E), with all segments in the mid-ventral region exhibiting this “x”-like pattern.

The most distinguishing feature of the *Oncopeltus* pattern was its dynamic and complex expression in the head appendages. Very early in development when limb buds were just being formed, *nub* was strongly expressed in the ocular and brain region and in all head appendages (Fig. 4F). For the first time noted in hemimetabolous insect embryos, *nub* was expressed in the antennae. Slightly later, at 20% of development, the signal in the head limb buds became much weaker (compare the staining in head and thoracic limb buds in Fig. 4A). However, the signal in the brain and ocular region remained strong. At 25% of development, antennal expression disappeared completely and the signal in the mandibles became weak and diffuse (Fig. 4G). Maxillary expression remained strong and localized in the central portion of the maxillary limb buds, whereas labial expression differentiated into two diffuse spots (Fig. 4G, arrows). Thus, at this stage, each head appendage exhibited a unique *nub* pattern. By 30% of development, *nub* expression reappeared in antennae, but only in their ventral region (Fig. 4H). Mandibular and maxillary expressions were similar, with a diffuse ventrally localized signal. *nub* expression in the labial appendages was weak at this stage and was restricted to their distal region. At 35–40% of development, antennal expression encompassed the distal portion of these appendages (Fig. 4I).

The mandibular and maxillary appendages exhibited an increase in *nub* expression that extended throughout their ventral portions. In addition, a strong signal appeared toward the dorsolateral sides of the maxillary segment. Expression in the labial appendages also increased and remained localized ventrally in their distal regions. Finally, as the embryo underwent dorsal closure, the *nub* pattern changed again (Fig. 4J). Expression in the antennae disappeared, the mandibles and maxillae retained their strong ventral signal, and the fused labial appendages exhibited weaker expression in their distal region. Overall, milkweed bug embryos were characterized by a complex pattern in the head region that had several unique features. First, *Oncopeltus* exhibited an expression in the antennae (in contrast to firebrats and cockroaches). Second, the antennal pattern was dynamic, with multiple appearances and disappearances of *nub*. Third, in milkweed bugs it was the mandibles and maxillae that showed a similar pattern, whereas in cockroaches and firebrats maxillae and labium were similar.

In *Oncopeltus* embryos, the timing of leg segmentation was intermediate compared with that of firebrat and cockroach embryos: It started later than in the cockroach but earlier than in firebrats. In other words, at comparable mid-developmental stages, milkweed bug embryos have fewer distinct leg segments than cockroach embryos. At the same time, firebrat embryonic legs exhibited no visible segmentation at all. As shown in Fig. 4A, all thoracic leg buds expressed *nub*-during early germ-band extension. As these limb buds elongated, a diffuse spot appeared at the base of the legs nearest the body wall (Fig. 4K, star). There were also three bands of *nub*-expressing cells located at proximal, middle, and distal leg regions (Fig. 4K, arrowheads). Although there were no discernible leg segments at this stage, the proximal and middle bands roughly corresponded to the location of the coxa–trochanter/femur and femur–tibia boundaries. However, as these leg segments became visible (Fig. 4L), *nub* expression subsequently disappeared except for a distal band in the tibial/tarsal segment (Fig. 4L). As leg elongation continued, this band first became restricted to the ventral side (Fig. 4M) and then became diffuse (Fig. 4N). Generally speaking, *nub* expression in milkweed bug legs is distinct, encompassing both conserved and novel aspects. Furthermore, the observed leg pattern was also partially associated with leg segmentation, but not at the level seen in cockroaches. Whereas *nub* was localized at every leg joint in *Periplaneta* embryos, its expression could only be associated with only three leg segments in *Oncopeltus* (consistent with the later completion of leg segmentation in this species).

**DISCUSSION**

From spiders to insects, the global evolution of *nub* expression patterns in arthropod embryos

*nub* is one of the few developmental genes for which extensive comparative data are available. Among arthropods, *nub* expression has been examined in chelicerates, including spiders and horseshoe crabs (Abzhanov and Kaufman 2000; Damen et al. 2002), and in several crustacean species (Averof and Cohen 1997; Abzhanov and Kaufman 2000; Gilbert et al. 2002). Within insects, this gene was studied only in *Drosophila* (Lloyd and Sakonju 1991; Anderson et al. 1995; Isshiki et al. 2001). Because higher flies have a highly derived mode of development and a relatively recent phylogenetic origin, it is unclear whether the pattern observed in *Drosophila* is representative of all insects. With our analysis of an apertygote and two hemimetabolous insects, it is now possible to consider the evolution of *nub* expression in arthropods in general.
As shown in Fig. 5, nub expression is class specific and sometimes even species specific. Nonetheless, all examined arthropods share a common expression pattern in appendages, indicating that nub was originally an “appendage” gene. In chelicerates, generally thought to be basal arthropods, nub is localized exclusively to the walking legs and other leg-derived structures (Abzhanov and Kaufman 2000; Damen et al. 2002). In the spider Steatoda triangulosa (Fig. 5A), a single band of nub expression was detected in the tarsus of all prosomal legs. This basic chelicerate pattern was substantially altered in the crustacean Porcelio scaber (Fig. 5B). First, nub expression spread anteriorly into the head region. Note that this head expression is incomplete, encompassing some but not all the segments. Second, although nub is still restricted to the distal leg segments, it is expressed in a set of rings (instead of in a single band as in spider embryos). This refinement of the nub expression continues even further in insect embryos. In firebrat and cockroach embryos (Fig. 5, C and D), there was no expression in the antennal and mandibular appendages. However, milkweed bug embryos exhibited a strong antennal and mandibular staining (Fig. 5E). Thus, the spread of nub expression in the anterior direction is complete in insects and now includes all the head segments. In addition, insect embryos also exhibit a further proximal expansion of nub expression in the legs (Fig. 5, C–E). Whereas nub is localized only in the distal leg segments in spiders and crustaceans, its expression in insects encompasses proximal leg regions as well. This is particularly pronounced in cockroaches (Fig. 5D), in which nub is expressed in all leg segments.
The other key trend in the evolution of nub expression in arthropods is in its expansion from the periphery of the embryo (appendages) to the mid-ventral region. In both chelicerates and crustaceans, there is complete absence of nub in the center of the embryo (Fig. 5, A and B). In insects, however, mid-ventral expression in neuroectoderm is one of the most noticeable aspects of nub expression. Intriguingly, each insect species exhibits a unique species-specific CNS pattern (Fig. 5, C–E). Functional studies in Drosophila show that nub is part of a gene regulation cascade specifying the identity of developing neuroblasts in the CNS (Yeo et al. 1995; Brody and Odenwald 2000; Ishikii et al. 2001). These findings, combined with our observations, suggest that nub expression in the CNS is unique to the insects and has continued to evolve toward more elaborate spatial and temporal patterns that coincide with diversification of insect morphology.

**Expression patterns within insects are dynamic and species specific**

As depicted in Fig. 5, nub expression patterns exhibited substantial differences between and within the major arthropod classes. However, in groups such as spiders and crustaceans, these differences are rather static (Fig. 5, A and B). More specifically, once the pattern of expression is established, it does not change during development. In contrast, our data clearly indicate that variation is a key feature of nub expression within insect embryos. The best illustration of this variation is provided by dynamic temporal and spatial changes of nub expression in the CNS and antennae.

If one compares the timing of its expression in the CNS versus appendages, it is evident that the onset of nub expression within the CNS is highly variable. In firebrat embryos, nub is first expressed in the CNS and is subsequently detected in the appendages. In cockroach embryos, the earliest expression of nub is detected in the appendages followed by the CNS. And in milkweed bug embryos, expression of nub in appendages and CNS occurs simultaneously at a very early stage. In addition to this temporal variation, nub also exhibits spatial variation in its pattern. nub expression in the CNS was found to have a unique species-specific pattern for each insect species studied. In firebrats, nub is expressed in groups of cells aligned along the midline of each segment in a uniform pattern that persists throughout development. In the cockroach, nub expression appears in an irregular triangular pattern encompassing a wider region in the thoracic than in the abdominal segments. Once established, this pattern does not change from early to late development. In contrast, CNS expression of nub in milkweed bugs is very dynamic. In early developmental stages, nub is expressed in cells aligned along either side of the midline, with expression excluded from any of those cells within the midline. As development proceeds, cells within the midline form an “x” pattern of expression in each segment, beginning in T1 and ending at the posterior abdominal segment.

Antennal expression represents another example of a highly variable nub pattern. In embryos of basal insect groups such as firebrats and cockroaches, nub is absent in antennae (Fig. 5, C and D). This observation is also consistent with those on spiders and crustaceans. No nub expression is found in the first two prosomal segments (cheliceral and pedipalpal) in spiders. Similarly, nub is absent from the antennal 1 segment of crustaceans (Abzhanov and Kaufman 2000). These observations indicate that lack of nub expression in the anterior-most appendages is an ancestral arthropod feature. However, in more derived hemimetabolous insects such as milkweed bugs, we observed nub in this new domain. Moreover, its expression undergoes dramatic temporal and spatial change during development (Fig. 4, F–J). Early on, nub is strongly expressed throughout the antennae. Then, as the appendages start to elongate, its expression is lost. As development of appendages continues, nub accumulation reappears again in a cluster of cells at the posterior-distal portion of antennae and then shifts to their most distal part and continues to be strongly expressed during dorsal closure. Toward the end of development, its expression disappears again. nub accumulation has also been detected in the eye/antennal disc of Drosophila larvae (Dick et al. 1991; Rodriguez Dd Ddel et al. 2002), further suggesting this novel pattern to be of relatively recent origin and to be shared among phylogenetically more derived insect groups.

This study revealed that nub function may be evolving more rapidly than that of homoeotic or other leg patterning genes such as Dil and dac (Panganiban et al. 1995; Popadic et al. 1998b; Prpic et al. 2001; Hughes and Kaufman 2002). Also, the most dynamic temporal and spatial changes in expression occurred in milkweed bugs, a relatively derived hemimetabolous insect. Additional dynamic changes in nub pattern were observed in firebrat and cockroach embryos, especially in legs and mouthparts. These observations pose an intriguing question as to what level of developmental variation actually exists in nature. Whereas traditional views support relatively high levels of conservation in developmental pathways (Raff 1996), the present analysis of nub suggests that variation may be more prevalent than previously thought.

**Role of nub in insect leg segmentation**

Mainly based on insights from leg development in Drosophila, it is now known that the Notch (N) signaling pathway plays a fundamental role in the process of segmental boundary formation in legs (de Celis et al. 1998; Rauskolb et al. 1999; Casares and Mann 2001). In flies, the actual joint formation is
mediated by establishing distinct rings of \( N \) expression in these regions of the leg. \textit{nubbin} is also expressed in a series of concentric rings in third instar \textit{Drosophila} leg discs (Anderson et al. 1995; Averof and Cohen 1997), and its mutant expression results in shortened and gnarled legs (Cifuentes and Garcia-Bellido 1997). Mutant clones of \textit{Notch} also cause loss of \textit{nub} expression in \textit{Drosophila} legs. Conversely, ectopic expression of \textit{nub} is induced within clones of cells expressing activated \textit{Notch} (Rauskolb et al. 1999). Thus, in \textit{Drosophila} legs, \textit{nub} is positively regulated downstream of \textit{Notch}. Whereas this information indicates that \textit{nub} is also involved in leg patterning in \textit{Drosophila}, in addition to its previously described roles in CNS and wing development, the question remains as to when this acquisition of leg function occurred.

As shown in Fig. 5A, in spider embryos \textit{nub} is localized to a single distal band on all walking legs. In crustacean embryos with joint-less trunk appendages such as \textit{Artemia franciscana}, no expression of \textit{nub} has been detected (Averof and Cohen 1997). However, in crustaceans with segmented endopods (main leg branches), \textit{nub} is localized to a series of rings that correspond to joints in the distal leg region (Abzhanov and Kaufman 2000; Damen et al. 2002). These findings indicate that \textit{nub} may have a partial role in leg segmentation in chelicerates and crustaceans, mainly during the establishment of distal leg segments. However, as is obvious from Fig. 5, A and B, formation of most leg joints in those groups has to be under the control of other genes.

As summarized in Fig. 5C, there are two dominant bands of \textit{nub} expression in firebrat legs, a proximal band and a mid-distal band. The locations of these bands roughly correspond to the positions of the future coxa–trochanter and femur–tibia boundaries, respectively. But leg segmentation in firebrats occurs very late in development when it is technically very difficult to perform the in situ experiments (due to the deposition of embryonic cuticle). This prevents us from inferring the complete pattern of \textit{nub} in this species and to relate it to the formation of joints. Leg development in \textit{Periplaneta} embryos is quite different, and clearly identifiable leg segments can be recognized by mid-stages of embryogenesis. In this species, the appearance of five \textit{nub} stripes precedes any visible demarcation of distinct leg regions (Fig. 5D). Only afterward does continuing \textit{nub} expression begin to coincide with the formation of segmental boundaries in the legs (Fig. 3, M and N). This coordination between the precise patterning of \textit{nub} expression and leg segmentation in the cockroach embryos is highly indicative of a role in formation of joints. The observed pattern in \textit{Periplaneta} is also reminiscent of \textit{Notch} signaling-regulated \textit{nub} expression in \textit{Drosophila} leg development. These similarities suggest that regulation of leg segmentation in two widely divergent insect species may be homologous.

The timing of leg segmentation in milkweed bug embryos is intermediate compared with firebrats and cockroaches. Proximal leg segments in \textit{Oncopeltus} are established relatively early during germ-band extension, but segmentation of distal leg regions does not occur until late development. As in \textit{Periplaneta}, the leg patterns of \textit{nub} expression in \textit{Oncopeltus} roughly correspond to the position of future joints and its appearance precedes formation of segments. There are also two main differences between observed \textit{nub} patterns between milkweed bugs and cockroaches. First, \textit{nub} expression in \textit{Oncopeltus} legs is composed of only three bands (Fig. 5E) versus five bands in \textit{Periplaneta}. Second, two of these bands in \textit{Oncopeltus} begin to fade much earlier (compared with cockroach). The possible explanation for the first discrepancy is that milkweed bug \textit{nub} may be involved in formation of other legs, but not all, leg segments. The likely explanation for the second discrepancy is that \textit{nub} may play a role in initiating the formation of some leg joints in \textit{Oncopeltus} but is not required for its maintenance.

Overall, our analysis suggests that recruitment of \textit{nub} for a role in leg segmentation may have occurred early in insect evolution. In both insects and crustaceans, \textit{nub} expression is associated with establishment of some but not all leg segments. In insects, the primary association is with proximal and mid-leg segments. However, only the two most distal segments may be involved in crustaceans. These findings indicate that \textit{nub} cannot be a necessary element in the regulation of overall leg segmentation in arthropods. Rather, the observed expression patterns suggest that this gene was independently recruited in insects and crustaceans for a possible role in development of specific leg joints. Subsequently, in some insect lineages (cockroaches and flies) this original role was expanded to include joints between all leg segments.

**Role of \textit{nub} in differential development of head appendages**

The insect head has a modular organization and is composed of the three pregnastral (ocular, antennal, and intercalary) and three gnathal segments (mandibular, maxillary, and labial). A pair of appendages grows from each gnathal segment and forms the future mouthparts (Brusca and Brusca 1990). Diversity in organization of the insect head is best represented by the variation in structure and morphology of the mouthparts. There are two basic types of mouthparts: mandibulate, specialized for chewing and biting, and haustellate, specialized for piercing and sucking (Matsuda 1965). The mandibular type represents the ancestral form and is characteristic of members of most basal hexapod lineages (Zygentoma, Orthoptera, Blattoidea, etc.). Its main feature is that the mandibles function as “jaws,” whereas the maxillary and labial limbs form branched appendages that are structurally identical until the midline fusion of the latter (Fig. 6A, left). In the haustellate type, it is the mandibular and
maxillary appendages that are similar, whereas the labial appendages exhibit a completely different structure (Fig. 6A, right).

Studies in firebrats, crickets, milkweed bugs, and flies have shown that the basic programming and identity of the gnathal segments are determined by three
\textit{Hox} genes: \textit{proboscipedia} (\textit{pb}), \textit{Deformed} (\textit{Dfd}), and \textit{Sex combs reduced} (\textit{Scr}) (Hughes and Kaufman 2002). Changes in \textit{pb} expression have been directly associated with changes in mouthpart structure (Randazzo et al. 1991; Beeman et al. 1993; Rogers et al. 2002). In insects with biting and chewing mouthparts, \textit{pb} is expressed in the labium and maxillae but not in the

\begin{figure}[h]
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\caption{Evolving pattern of \textit{nub} expression in insect mouthparts. (A) Diagrams of two basic types of mouthparts in insects. In mandibulate type (left), the mandibles function as jaws and are structurally different from both maxillae and labium. The latter two segments are structurally and functionally very similar, but the labium appendages fuse to form a lower lip. In the haustellate type (right), the mandibles and maxillary laciniae form wire-like stylets. In contrast, the labium forms a stylet-enclosing sheath. (B) Differential expression of \textit{nub} (dark blue) in embryonic insect head appendages is associated with particular mouthpart morphology. In embryos of biting and chewing insects (\textit{Thermobia} and \textit{Periplaneta}), \textit{nub} expression is similar in the maxillary and labial appendages. \textit{nub} is generally not expressed in the mandibles except transiently. However, in the haustellate type (\textit{Oncopeltus}), mandibular expression is similar to the maxillary pattern. Expression in the labium occurs only in the distal region of the appendage. Light blue denotes transient \textit{nub} expression in the mandibular segment. Stripped ovals depict species-specific modulations of the labial and maxillary expressions in \textit{Thermobia} and \textit{Periplaneta}, respectively.}
\end{figure}
mandibles. However, in insects with piercing and sucking mouthparts, such as milkweed bugs, *pb* expression is mainly detected in the labial appendages (Rogers and Kaufman 1997). Thus, it has been proposed that loss of *pb* expression in the maxillae was responsible for the transformation of the mandibulate to the haustellate mouth type (Rogers et al. 2002). More specifically, the loss of *pb* expression in milkweed bug embryos would "free" the maxillary appendages to diverge from their original labium-like phenotype. However, such change in *pb* regulation cannot account for the development of stylets in both maxillary and mandibular segments. This suggests that other nonhomeotic genes are involved in the latter process.

Consistent with the morphological diversification of head appendages, the sharpest difference of *nub* expression is observed in this region. Patterns of expression show a coordinated change in association with particular mouthpart morphology. In firebrat and cockroach embryos, which have typical mandibulate mouthparts, *nub* is localized in the maxillary and labial appendages in a similar pattern (Fig. 6B). This is particularly pronounced in *Periplaneta* embryos, which exhibit highly coordinated patterns of expression in the labium and maxillae from early to late development. Such an observation is consistent with the fact that these segments are structurally very similar and differ from the mandibular segment. Second, *nub* is generally not expressed in the mandibles except locally and transiently in cockroaches (Fig. 6B). This suggests that in insects with biting and chewing mouthparts, mandibles were originally devoid of *nub* expression.

In milkweed bug embryos, which have the stylate—haustellate (sucking) mouthparts, *nub* is expressed in a very different pattern. First, there is novel expression along the mid-ventral region of the mandibular appendages. Second, this mandibular expression is similar to the maxillary pattern (Fig. 6B). Expression in the labium, however, is localized only in the distal portion of the appendage and exhibits a completely different timing and pattern. This differential expression correlates with a change in the morphology of the maxillary and labial segments that is characteristic for piercing and sucking mouthparts: Development and morphology of the maxillary segment parallels that of the mandibular segment. These observations provide the first nonhomeotic gene example of a linkage between a change in expression pattern and a corresponding change in the morphology of insect mouthparts. They also provide a strong indication that *nub* may function in the development of haustellate mouthparts.

The emerging insight from this and other recent studies suggests that the evolution of the maxillae and mandibles in *Oncopeltus* and other hemipterans was governed by regulatory changes at both higher (*Hox* genes such as *pb* and *Dfd*) and lower levels (genes such as *nub*) in the developmental hierarchy. This underlines the critical importance of understanding the functional roles and relationships between these two classes of genes. For example, *Dfd* is expressed in the mandibular segments in both *Tribolium* (which has mandibulate mouthparts) and in *Oncopeltus* (which has haustellate mouthparts). Functional studies have shown that in the absence of *Dfd*, mandibles are transformed into antenna-like appendages in these two insects (Brown et al. 2000; Hughes and Kaufman 2000). These findings show that *Dfd* orthologs control mandibular identity in both species. Yet the actual morphology of mandibles in *Tribolium* and *Oncopeltus* is very different, a result of distinct mandibulate and haustellate modifications. Additional factors, either acting in parallel with *Dfd*, or downstream targets, or independently of *Dfd*, must be involved in the establishment of these distinct mandibular morphologies. Based on this analysis of its expression patterns, *nub* is a good candidate for being such a target gene in the mandibular segment (Fig. 6B). Further studies of other genes at lower levels in the developmental hierarchy will be necessary to fully understand the mechanisms underlying the morphological diversification of insect mouthparts.

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