The expression of the proximodistal axis patterning genes Distal-less and dachshund in the appendages of Glomeris marginata (Myriapoda: Diplopoda) suggests a special role of these genes in patterning the head appendages

Nikola-Michael Prpic and Diethard Tautz*

Institut für Genetik, Universität zu Köln, Weyertal 121, 50931 Köln, Germany

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Abstract

The genes Distal-less, dachshund, extradenticle, and homothorax have been shown in Drosophila to be among the earliest genes that define positional values along the proximal–distal (PD) axis of the developing legs. In order to study PD axis formation in the appendages of the pill millipede Glomeris marginata, we have isolated homologues of these four genes and have studied their expression patterns. In the trunk legs, there are several differences to Drosophila, but the patterns are nevertheless compatible with a conserved role in defining positional values along the PD axis. However, their role in the head appendages is apparently more complex. Distal-less in the mandible and maxilla is expressed in the forming sensory organs and, thus, does not seem to be involved in PD axis patterning. We could not identify in the mouthparts components that are homologous to the distal parts of the trunk legs and antennae. Interestingly, there is also a transient premorphogenetic expression of Distal-less in the second antennal and second maxillary segment, although no appendages are eventually formed in these segments. The dachshund gene is apparently involved both in PD patterning as well as in sensory organ development in the antenna, maxilla, and mandible. Strong dachshund expression is specifically correlated with the tooth-like part of the mandible, a feature that is shared with other mandibulate arthropods. Homothorax is expressed in the proximal and medial parts of the legs, while extradenticle RNA is only seen in the proximal region. This overlap of expression corresponds to the functional overlap between extradenticle and homothorax in Drosophila.

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Introduction

Axis formation is one of the fundamental processes during embryogenesis of most multicellular animals. The body of higher metazoans is patterned along three axes, namely the anterior–posterior (AP) axis, dorsal–ventral (DV) axis, and left–right (LR) axis. Within this framework, many organs develop along additional, organ-specific axes. A better-known example of the latter is the proximal–distal (PD) axis of the appendages in arthropods. In general, development of the appendages is guided by the coordinate system provided by the AP, DV, and LR axes of the body itself (e.g., Cohen, 1990; Goto and Hayashi, 1997), but the outgrowth away from the body requires an additional guidance system along an axis from the tip of the growing appendage to its root on the body.

In the fruitfly Drosophila genes have been identified that are involved in establishing the PD axis of the appendages. It has been shown that especially four genes set up the first crude positional values on the PD axis of the fly legs. The genes extradenticle (exd) and homothorax (hth) are cofactors which together instruct proximal leg fates (Gonzalez-Crespo and Morata, 1996; Rieckhof et al., 1997; Abu-Shaar et al., 1999; Wu and Cohen, 1999), whereas the gene Distal-less (Dll) is indispensable for the development of distal leg
parts (Sunkel and Whittle, 1987; Cohen et al., 1989). In between these two domains is a third domain, governed by the gene *dachshund* (*dac*), which is required for the development of medial leg parts (Mardon et al., 1994; Dong et al., 2001). Apart from their function during PD axis formation, all four genes have other functions as well. The genes *exd* and *lth* are expressed in most cells of the *Drosophila* embryo and are involved in wing, eye, and salivary gland development, body tagmosis and segmentation, development of the peripheral and central nervous systems (PNS, CNS), and several other developmental processes (Rauskolb et al., 1993, 1995; Rieckhof et al., 1997; Pai et al., 1998; Kurant et al., 1998; Henderson and Andrew, 2000; Casares and Mann, 2000; Azpiazu and Morata, 2000; Pichaud and Casares, 2000; Nagao et al., 2000; Bessa et al., 2002). The *dac* gene is involved in eye development, development of the corpora pedunculata (“mushroom bodies”), and the development of the antennal lobe of the brain (Shen and Mardon, 1997; Chen et al., 1997; Kurusu et al., 2000; Martini et al., 2000; Noveen et al., 2000; Jhaveri et al., 2000). Furthermore, it is expressed in the ventral nerve cord and in the wing discs, but its function in these structures remains to be determined (Mardon et al., 1994). Finally, *Dll* is involved in the development of specific cephalic sensory organs and thoracic mechanosensors (Keilin’s organs) (Sunkel and Whittle, 1987; Cohen and Jürgens, 1989a). It is also expressed in the CNS (optic lobe, glial cells of the ventral nerve cord) and the wing disc, but its functions there are unclear (Gorflinkiel et al., 1997; Campbell and Tomlinson, 1998; Panganiban, 2000; Panganiban and Rubenstein, 2002). Thus, for all four genes, PD axis patterning is not the only function during development. This opens up the possibility that these genes could have additional functions also in the appendages apart of PD axis patterning. In fact, it is known that *flh* is not restricted to PD axis patterning alone. *Dll* also determines the sensory organs associated with the rudiments of labrum, antennae, maxillae, labium, and thoracic legs in the larva (Sunkel and Whittle, 1987; Cohen and Jürgens, 1989a). In addition, it has recently been shown that *Dll* expression is associated with sensory bristles in the appendages in crustaceans, arthropod insects, and chelicerates (Mittmann and Scholtz, 2001; Williams et al., 2002). Thus, the function of the PD axis patterning genes in the appendages appears to be more complex than previously thought, at least in the case of *Dll*.

Here, we report the isolation of the genes *lth*, *exd*, *dac*, and *Dll* from the diplopod *Glomeris marginata* (*Gm*), a representative of the myriapods. The myriapods are the only arthropod group from which data on these genes were hitherto almost entirely missing. We show that the temporal and spatial expression patterns in the trunk legs are different from *Drosophila*, but are consistent with a conserved role in PD axis formation. In the head appendages, however, their role appears more complex. It is demonstrated that *Gm-Dll* in the mouthparts is expressed only in the primordia of the sensory organs. This does not support a role in PD axis patterning in the mouthparts, but instead suggests involvement in sensory organ development. *Gm-dac* is also expressed in sensory organ primordia in antennae and mouthparts, but also at a medial PD position in the antenna and probably is important for the specific tooth-like morphology of the mandibles, not only of myriapods, but also of other mandibulate arthropods.

The possibility that some PD axis patterning genes have a complex role during appendage development also has ramifications for the use of these genes in evolutionary developmental comparisons. The expression of the *Dll* gene has been studied in a variety of arthropod species (e.g., Popadic et al., 1998; Scholtz et al., 1998; Williams, 1998; Abzhanov and Kaufman, 2000), employing a cross-reacting antibody (Panganiban et al., 1995). However, these studies have not all taken into account the possibility of additional functions of *Dll*. We have therefore readdressed this particular possibility here and find indeed that some of the homology assessments have to be revised.

### Materials and methods

#### Animals and embryo fixation

*Glomeris marginata* were collected in the city forest of Cologne, Germany, kept in large petri dishes, and supplied regularly with earth, water, and food (decomposing beech leaves). Eggs were removed from the earth covers by hand and dechorionated with DanKlorix (Colgate-Palmolive; equals a 4% sodium hypochloride solution) for 2 min. Eggs were then washed several times in water and fixed [1 ml heptane, 100 µl formaldehyde (37%)] for up to 4 h at room temperature and washed in methanol several times. Vitelline membranes were removed by hand using watchmaker forceps (Dumont 5). Embryos were stored in methanol at −20°C.

#### In-situ hybridisation

Treatment of the embryos followed the protocol by Tautz and Pfeifle (1989), using labeled riboprobes (Klingler and Gergen, 1993). All incubation/washing steps were prolonged, because of the bigger size of the *Glomeris* embryos. To reduce background, embryos were additionally treated with 2.5 µl acetic anhydride in 1 ml of 0.1 M TEA buffer (Sigma). A detailed step-by-step protocol is available upon request.

#### Cloning of cDNA fragments

Total RNA from 60 selected *G. marginata* embryos was extracted by using Trizol (Invitrogen). Messenger RNA was extracted by using the PolyATtract system (Promega) and used to synthesize cDNA using the SuperScript II system.
To amplify a fragment of the Gm-Dll homoeobox, the primers eDP fw (GGN AAR GGN AAR AAR ATN MG) and eDP bw (TTY TGR AAC CAD ATY TTN AC) were used. A nested PCR was performed by using the primers iDP fw (ATN MGN AAR CCN MGN ACN ATH TA) and iDP bw (AAC CAD ATY TTN ACY TGN GTY TG). The resulting fragments were subjected to standard molecular cloning and sequenced on an ABI 377 automated sequencer (Perkin Elmer). Using this sequence, species-specific primers were designed. In a primary PCR, the primer eGSP Gm (CGT GAG ACC CAA AGA AGC TGC C) was used together with the degenerate primer DP dLxm1 (AAR WSN GCN TTY ATN GAR HTN CAR CAR C) directed against the DLX-1 motif (Aspöck and Bürglin, 2001). A nested PCR using the primers iGSP Gm (TTC AGC CTC TGG TAG AGC C) and DP dLxm1 specifically amplified Gm-Dll cDNA fragments.

The primers used for amplification of Gm-dac have been described before (Prpic et al., 2001). For Gm-exd, the primers were exd-fw1 (YTN AAY TGY CAY MGN ATG AAR CC) and exd-bw1 (TTN CCR AAC CAR TTN SWN ACY TG). In a nested PCR, the primers exd-fw2 (GTN YTN TGY GAR ATH AAR GAR AAR AC) and exd-bw2 (GCN ARY TCY TCT TTN GCY TCY TC) were used. For Gm-hth, the primers hth-fw1 (GAY AAR GAY GACN ATH TAY GRN CAY CC) and hth-bw1 (YTG RTC DAT CAT NGG YTG NAC DAT) were used in the initial PCR, hth-fw2 and hth-bw2 (GC RTT DAT RAA CCA RTT RTT NAC YTG) were used in a nested PCR. GenBank Accession nos. are as follows: Gm-Dll (AJ551276), Gm-dac (AJ551277), Gm-exd (AJ551278), Gm-hth (AJ551279).

Results

Cloning and sequence analysis

A PCR strategy combining degenerate and gene-specific primers was used to amplify sequences homologous to Dll/ Dlx genes. Eleven identical clones with similarity to Drosophila Dll were recovered. We designate the gene from which these cDNA fragments derive as Gm-Dll. The alignment of the deduced amino acid sequence of Gm-Dll and all other known arthropod DLL homologues is shown in Fig. 1. Apart from the homeodomain, there are only short stretches of sequence which are conserved to some extent in all species. The Glomeris sequence shows an insertion of an alanin and histidin stretch directly following the DLXM1 motif. The insertion before the DLXM2 motif that is seen in the spider Cupiennius salei (Schoppmeier and Damen, 2001) is not present in Glomeris. The amino acid sequence of the homeodomain part is fully conserved between the different species.

The same strategy was applied to clone Gm-dac, Gm-exd, and Gm-hth. For each gene, a number of clones were sequenced (13 for Gm-dac, 16 for Gm-exd, and 29 for Gm-hth). All clones of each gene contained identical sequences, suggesting that no paralogous genes are present in the Glomeris genome. The fragment of Gm-dac is 940 bp long and encodes a partial protein with high similarity to DAC proteins from other arthropods. The Gm-DAC sequence contains parts of the DD1 and DD2 domains that are conserved in DAC/DACH proteins in nematodes, arthropods, and vertebrates (Hammond et al., 1998; Kozmik et al., 1999; Davis et al., 1999; Caubit et al., 1999).

The fragment of Gm-exd is 529 bp long and encodes a partial protein with very high similarity to EXD proteins from other arthropods. It contains a part of the PBC-A domain, a PBC-B domain, and a partial homeodomain. The amino acid conservation is very high within the PBC-A and PBC-B domains with 96, 89 and 70% sequence identity to Drosophila EXD, mouse PBX1, and nematode CEH-20, respectively. The fragment of Gm-hth is 817 bp long and encodes a partial protein with very high similarity to HTH proteins from other arthropods. It contains a partial MEIS domain and a partial homeodomain. Amino acid sequence conservation within the MEIS domain ranges from 95, 91, and 63% sequence identity to Drosophila EXD, mouse MEIS1, or nematode UNC-62, respectively. The MEIS and PBC domains mediate the heterodimerization of the HTH/MEIS and EXD/PBX proteins, which is necessary for their joint translocation to the nucleus where they bind target DNA sequences (e.g., Abu-Shaar et al., 1999; Berthelsen et al., 1999; Jaw et al., 2000). The high degree of amino acid sequence conservation suggests that, also in Glomeris, the Gm-HTH and Gm-EXD proteins form heterodimers before they are able to enter the nucleus.

Correlation between embryonic and adult appendages in Glomeris marginata

We have studied the expression of these genes in several embryonic structures, focusing on the developing appendages of the head and trunk. Therefore, we begin with a brief description of the correlation of embryonic appendages with the appendages in adult G. marginata in order to introduce the specific terms for myriapod appendage morphology. The trunk legs are simple outgrowths of the body and, thus, the embryonic leg appears to correlate directly to the adult leg (Fig. 2A). The same is true for the antennae (Fig. 2D). In the adult, this appendage has four sensory organs at its tip, the primordia of which can already be discerned in the embryo (Fig. 2D; sc). The mandible in the embryo is a stout structure with two lobes (Fig. 2C; il, ol). These two lobes differentiate into the gnathal parts of the mandibles (Dohle, 1964) (Fig. 2C; lower part): the inner lobe will give rise to the pectinate lamella, intermediate piece, and molar plate. The outer lobe will give rise to both the external and the internal part of the tooth. Finally, the lower lip in adult Glomeris, the gnathochilarium, is a complex structure. It clearly develops from the appendages of the maxillary segment (Fig. 2B; upper part). Already at late embryonic stages.
Fig. 1. Alignment of all known arthropod DLL sequences. Dashes are gaps introduced to improve the alignment. Bars below the alignment denote the conserved regions (DLX1, DLX2; Aspöck and Bürglin, 2001). Bars on top denote newly identified variable regions. Sequence underlaid gray: partial homeodomain. Sequence underlaid black: nonhomologous homopolymeric amino acid stretches. Question marks replace primer sequences. GenBank Accession nos. are as follows: BaDLL (AAL69325), JcDLL (AAK97630), TcDLL (AAG39634), DmDLL (AAB24059), CsDLL (CAC34380), GmDLL (CAD82905). Abbreviations: HPAAS, homopolymeric amino acid stretch; Ba, Bicyclus anynana (butterfly); Jc, Junonia coenia (butterfly); Tc, Tribolium castaneum (beetle); Dm, Drosophila melanogaster (fruitfly); Gm, Glomeris marginata (millipede); Cs, Cupiennius salei (spider).

Fig. 2. Schematic representation of embryonic and adult appendages of G. marginata. (A) Stage 6 embryonic (top) and adult (bottom) trunk leg. (B) Ventral view of the maxillary segment (light gray) with the developing maxillae. At stage 4, the maxillae are still separate (top). At stage 6, the maxillae have approached each other and the internal tissue contributing to the intermaxillary plate (black) has already fused (middle figure). In the adult, the maxillae form the lower lip (gnathochilarium) (black, intermaxillary plate; dark gray, stipes; medium gray, cardo). (C) Stage 6 embryonic (top) and adult (bottom) mandible. Corresponding tissues have the same shade of gray/black. (D) Stage 6 embryonic (top) and adult (bottom) antenna. Abbreviations: li, lobi interiori; sp, sensory palp; il, inner lobe; ol, outer lobe; ch, cheek; mp, molar plate; ip, intermediate piece; pl, pectinate lamella; i/o, inner/outer; sc, sensory cones.
(stage 6; for staging see Dohle, 1964), the two maxillae are fused along their innermost margins (Fig. 2B; middle part). This fused part will contribute to the so-called intermaxillary plate in the adult gnathochilarium (Fig. 2B; lower part, black filling). Both the external parts of the gnathochilarium (stipes; Fig. 2B; dark gray filling) and the intermaxillary
plate bear sensory organs (Fig. 2B; li, sp). The premandibular segment, which is homologous to the intercalary segment in insects, and the postmaxillary segment, which is homologous to the labial segment in insects, do not develop appendages. Dohle (1964) found no evidence for a contribution of remnants of possible postmaxillary appendages to the gnathochilium, as it is sometimes claimed (Kraus, 2001). This is confirmed by our results described below.

**Gm-Dll expression**

Gm-Dll transcripts are detected in all segments of the head and trunk. At stage 1 (for staging see Dohle, 1964), mRNA expression is seen in segmental pairs of spots in the antennal, premandibular, mandibular, maxillary, and the first three trunk segments (Fig. 3A and B). Except for the premandibular segment, which does not develop appendages, this segmental expression prefigures the sites of appendage formation in later stages of development. It is therefore referred to as premorphogenetic expression. No signal is detected in the postmaxillary segment at this stage. At stage 2, however, expression of Gm-Dll is detected also in this segment at a position that is apparently serially homologous to the remaining segmental spots (Fig. 3C; pmx). In the premandibular segment, the signal has vanished at this point (Fig. 3D; arrows) and a small spot of expression has appeared in the fourth trunk segment (Fig. 3C). Expression in trunk segment 1–3 at this stage extends dorsally. This changes as soon as limb buds appear on these segments at stage 3. Now expression is restricted to the forming buds and does not extend toward the dorsal side anymore (Fig. 3E). Expression in the postmaxillary segment meanwhile has ceased. Coincident with the appearance of limb buds on the mandibular and maxillary segment expression strength of Gm-Dll decreases in these segments.

At stage 4 (Fig. 3F), the homogeneous mRNA expression in the buds of the mandibles and maxillae has been replaced by a more complex pattern, which will develop further complexity at stages 5 and 6 (see below). In the trunk, expression marks primordial and developing trunk legs (Fig. 3F and G). Clearly, the legs on the first three trunk segments develop simultaneously and ahead of the remaining legs, which develop in a regular temporal sequence. This is most obvious at stage 6 when the first three trunk legs have grown considerably, but the other legs are still at the limb bud stage—the oldest on trunk segment 4, the youngest on trunk segment 8 (Fig. 3H and I).

Apart from the segmental expression, Gm-Dll mRNA is also detected in other structures. Throughout development, expression is seen in the labrum and the developing anal valves (Fig. 3; lbr and av, respectively). Expression in the CNS is restricted to the brain. At stage 1, diffuse staining fills the entire anterior part of the germ band (anterior cap; Fig. 3A), similar to what has been reported for Drosophila (Kumar and Moses, 2001). At stage 2, anterior staining is reduced to several spots in the head lobes (Fig. 3D). These spots persist through stages 3 and 4 (Fig. 3E and F). The pattern increases in complexity at stage 5 (Fig. 3G) and dissolves into a rather diffuse staining at stage 6 (Fig. 3H and I). The position of the spots is consistent with the expected location of the optic lobes and the corpora pedunculata, respectively. However, further studies are necessary to corroborate the function of Gm-Dll in the CNS.

**Dynamic expression of Gm-Dll in the mouthparts**

The expression pattern of Gm-Dll is dynamic and changes with the developmental stage (Fig. 4). This is most prominent in the gnathal appendages. In the maxillary segment, premorphogenetic Gm-Dll expression fades as soon as the maxillary limb buds start to form (stage 3; Fig. 3E) and is replaced at stage 4 by expression in the primordia of the two maxillary sensory palps (Fig. 4D; arrows). Subsequently, the maxillae are displaced toward the ventral midline. This, at stage 5, results in the close proximity of the two maxillary appendages. Expression of Gm-Dll is now seen also in the primordium of the lobs interior (Fig. 4E; arrow). At stage 6, the maxillae have started to fuse to form the gnathochilium (lower lip) and have to be torn apart for preparation (Fig. 4F; arrowhead). The lateral (outer) part of each half of the primordial gnathochilium will give rise to cardo and stipes (see Fig. 2B). The primordia of the two sensory palps of the stipes still express Gm-Dll (Fig. 4F).

In the mandibles, the strong premorphogenetic expression at stage 2 (Fig. 3C) decreases at stage 3 with the onset of the formation of mandibular buds (Fig. 3E) and at stage 4 is replaced by expression in three small clusters of cells (Fig. 4G). The innermost two clusters are within the inner lobe, whereas the lateral cluster is in the outer lobe. At stage 5, expression in the outer lobe increases and in the inner lobe three clusters of cells express Gm-Dll (Fig. 4H). At stage 6, expression is weaker and diffuse. Prominent ex-
pression is seen in two fuzzy stripes, one in the outer lobe and one in the inner lobe (Fig. 4I).

In the antenna and trunk legs 1–3, expression of Gm-Dll is found in the distal portion of the appendages.Expression in the antenna is very strong (Fig. 4J) but decreases at stage 6 (Fig. 4K). Expression in the trunk legs is also strong (Fig. 4A) but is getting slightly heterogeneous at stage 6 (Fig. 4B). In embryos shortly before the secretion of the embryonic cuticle (late stage 6, the upper age limit accessible for in situ hybridization), expression of Gm-Dll has decreased in terminal and medial cells but is still strong in supraproximal and subterminal cells (Fig. 4C; arrowheads).

**Gm-dac expression**

Expression of Gm-dac is seen in a number of different organ primordia. The most prominent expression occurs in the central nervous system, which is also highly dynamic. The earliest CNS expression is in the brain (stage 2) in a spot roughly corresponding to the prospective visual centers (Fig. 5A; oc). Expression in the brain rapidly becomes more complex (Fig. 5E), and at later stages, many proto- and deutocerebral cells express Gm-dac (Fig. 5H and I). In the ventral nerve cord, expression starts in the mandibular and premandibular segment at early stage 3 (Fig. 5C and D), but soon thereafter is initiated also in the remaining head and first three trunk segments (Fig. 5E), with the exception of the postmaxillary segment, in which expression is delayed until stage 4 (Fig. 5G). Each of the trunk segments as it matures goes through the same temporal–spatial sequence of Gm-dac activation in a steadily increasing number of neuroectodermal cells, that is reminiscent of the discrete pulses of neuroblast formation in Drosophila (Goodman and Doe, 1993). However, the nature of these neuroectodermal cells is not directly comparable to neuroblasts (Dove and Stollewerk, 2003).

Prominent expression is also seen in the anal valves (Fig. 5A; av) and, in later stages, extending from there along the lateral edges of the posterior zone (Fig. 5E). This expression may be correlated with the formation of the proctodeal part of the digestive system. Weaker expression is seen laterally in the postmaxillary segment and all trunk segments as soon as they are separated from the posterior zone (Fig. 5C). At stage 6, however, this lateral expression is restricted to the primordium of the heart (Fig. 5J). Finally, expression is seen also in the labrum with increasing intensity from stage 3 to 6 (Fig. 5D; lbr).

**Differential Gm-dac expression in the trunk legs and mouthparts**

Similar to Gm-Dll, premorphogenetic expression prefiguring the location of the appendage buds is also observed for Gm-dac. However, this only applies to the antennal, mandibular, and maxillary segment (Fig. 5A and B). No premorphogenetic signal is detected in nonappendage bearing head segments or any of the trunk segments. In the legs on trunk segments 1–3, Gm-dac is first expressed at stage 3 when the limb buds start to form (Fig. 5E). At this stage, expression encircles the leg buds slightly above the limb base. At stage 4, Gm-dac is clearly confined to medial parts of the PD axis of the legs. However, at stage 5, expression starts to spread proximally (Fig. 6A) and at stage 6 Gm-dac is also expressed in the proximal leg, albeit visibly weaker than in the medial leg (Fig. 6E). Additionally, a small cluster of dorsal PNS cells near the leg tips expresses Gm-dac at stage 6 (Fig. 6E; arrowhead).

In the antenna, Gm-dac is also expressed in a ring at a medial level on the PD axis. At first, this ring is narrow (stage 3) (Fig. 5F), but soon broadens (stage 4) and even starts to separate into two incompletely separated rings (stages 5 and 6) (Fig. 6D and H). In contrast to the trunk legs, proximal parts of the antenna always remain free from Gm-dac expression. Similar to the trunk legs, Gm-dac is expressed in the PNS in the antenna. Starting at the end of stage 3, prominent expression of Gm-dac is found in the primordia of the four sensory cones on the tip of the antennae (Fig. 6D and H; arrows).

In contrast to the trunk legs and the antennae, in the mouthparts, Gm-dac is never expressed in a fashion that would suggest a role in the definition of medial fates on their PD axis. At stage 3, expression is strong in the buds of mandibles and maxillae (Fig. 5F). At stage 4, this homogeneous expression is replaced by a more complex pattern in both appendages. In the maxilla, Gm-dac is expressed strongly but diffusely in the primordial stipes, and a separate expression domain is present in the tissue that will contribute to the intermaxillary plate (not shown). At stage 5, the expression in the stipes becomes slightly bipartite (Fig. 6B) and at stage 6 the entire expression pattern resolves into several smaller clusters of cells within the primordia of the sensory palps of the stipes and the lobi interiori of the intermaxillary plate (Fig. 6F). In the mandibles, expression at stages 4 and 5 is very strong in the outer lobe. In the inner lobe, several smaller clusters of cells show expression (Fig. 6C). The strong expression in the outer lobe persists through stages 5 and 6, while expression in the inner lobe becomes increasingly more diffuse toward stage 6 (Fig. 6G).

**Gm-exd and Gm-hth expression**

Both Gm-exd and Gm-hth are expressed in most cells of the germ band (Fig. 7). In general, judging from the in situ signal, Gm-exd seems to be expressed significantly weaker than Gm-hth; however, no quantitative comparative measurements have been performed. Gm-exd is expressed only weakly in many areas of the developing brain (Fig. 7B–F), whereas Gm-hth is expressed strongly in most cells of the proto- and deutocerebrum (Fig. 7H–L). Gm-hth is strongly expressed in the lateral plates of the segments (Fig. 7I and K), while expression of Gm-exd in the lateral plates is weak.
during early stages (Fig. 7B–D) and absent towards stage 6 (Fig. 7F). Expression of Gm-exd appears evenly distributed, whereas expression strength of Gm-hth is clearly heterogeneous, indicating tight spatial regulation of the expression of this gene.

In the trunk legs, expression of Gm-exd is restricted to proximal cells (Fig. 8A), whereas Cs-hth is expressed in proximal and medial cells (Fig. 8E). The tip of the legs is free from expression in both cases. In the antenna, expression of Gm-hth appears uniformly distributed in the entire appendage (Fig. 8H). In contrast, Gm-exd in the antenna is expressed in a more complex pattern consisting of high expression in proximal cells followed distally by two narrow rings of low and high expression, respectively (Figs. 7E and 8D). Cells at the tip of the antenna do not express Gm-exd.

In the mouthparts, expression of Gm-hth and Gm-exd is ubiquitous, but nonhomogeneous (Figs. 7E, 8B and C, and 8F and G).

**Discussion**

In order to study proximodistal axis formation in the appendages of the pill millipede G. marginata (Myriapoda: Diplopoda), we have isolated homologues of four genes known from *Drosophila* to be most relevant in this process. *Dll*, *dac*, and *hth/exd* in the fruitfly set up the first crude positional values along the PD axis of the legs: distal, medial, and proximal, respectively (Abu-Shaar and Mann, 1998; Dong et al., 2001). Although the expression of the homologous *Glomeris* genes differs from the *Drosophila* patterns in several aspects, they are expressed in a similar proximal-to-distal order, which suggests that their function of subdividing the developing leg into three distinct units is conserved. In the head appendages, however, their function is apparently more complex. The Gm-dac gene is expressed in two distinct domains in the antenna: at a medial level on the PD axis and in the sensory cones, suggestive of a role in PD axis patterning as well as sensory organ development. The strong expression of Gm-dac in the part of the mandible that will develop a tooth-like morphology suggests a third role for Gm-dac, that of specifying mandible identity. In the mouthparts, expression of Gm-Dll is correlated with forming sensory organs, but not with distal elements.

**Premorphogenetic expression of Gm-Dll is independent of later appendage development**

In *Drosophila* embryos, *Dll* prefigures the sites where the imaginal disc primordia will arise (Cohen et al., 1991, 1993). Similar to the situation in *Drosophila*, also in *Glomeris*, expression of Gm-Dll prefigures the sites where head...
and trunk appendages will form. However, in contrast to Drosophila, we also detect expression of Gm-Dll in segments which do not develop appendages, namely in the premandibular and the postmaxillary segment. The location of the Gm-Dll expression in these segments nevertheless suggests that they are serially homologous to those in the appendage-bearing segments. This demonstrates that premorphogenetic Gm-Dll expression is maintained irrespective of whether a segment will develop appendages or not. Given that there is no premorphogenetic expression of Dll in the nonappendage-bearing segments in either Drosophila or the more basal insect Tribolium (Beermann et al., 2001), premorphogenetic expression may represent a significant difference between myriapods and insects. However, because Gm-Dll expression in the premandibulary and postmaxillary segment is only transient and short-lived, it may be a nonfunctional rudiment of an ancestral state.

Gm-dac shows also premorphogenetic expression, but only in the antennal, mandibular, and maxillary segment. Premorphogenetic expression of dac has not been found in other arthropods so far. Rather, reports from Drosophila, Tribolium, and Cupiennius (Abu-Shaar and Mann, 1998; Prpic et al., 2001; unpublished observations) had suggested that the temporal sequence of dac activation following after the activation of Dll is a conserved feature in the arthropods.

In fact, this temporal sequence is also found in the trunk legs...
of *Glomeris*. The fact that in antennae, mandibles, and maxillae this temporal sequence is broken and *Gm-Dll* and *Gm-dac* are activated more or less simultaneously even before limb buds are formed suggests that their regulation in these appendages may be different from the regulation in the trunk legs.

**Proximodistal patterning functions in the trunk legs resemble those in other arthropods**

Results from *Drosophila* indicate that the early leg is subdivided into two fundamental compartment-like units, governed by two independent genetic pathways. The proximal unit expresses both *hth* and *exd*, whereas the distal unit expresses *Dll* (Gonzalez-Crespo and Morata, 1996; Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). Subsequently, a third unit is introduced between these first two, which is determined by the activity of the *dac* gene (Mardon et al., 1994; Dong et al., 2001). Thus, the leg at this stage comprises three distinct and antagonistic units, which form the basis for the subsequent subdivision of the PD axis into finer positional values.

In *Glomeris*, the expression patterns in the trunk legs are compatible with conserved functions in PD axis patterning. Expression of *Gm-Dll* in a domain comprising the entire distal leg is consistent with a function of this gene in determining distal leg structures. Expression of *Gm-dac* in a medial domain is consistent with a function of *Gm-dac* as instructor of medial leg fates. The genes *hth* and *exd* encode cofactors and thus have to be regarded together. In *Drosophila*, EXD is expressed in the entire leg, but *exd* function is restricted to those parts of the legs where the protein can...
bind its cofactor HTH, i.e., the proximal leg parts where the hth gene is active (Rauskolb et al., 1995; Rieckhof et al., 1997). In Glomeris, expression of Gm-exd and Gm-hth is very different from Drosophila, but coexpression is also restricted to the proximal leg. Thus, the PD sequence of exd/hth, dac, and Dll expression is similar to Drosophila and suggests that also in Glomeris the developing leg is separated into three units that are governed by the activities of hth/exd, dac, and Dll, respectively.

In addition to their role in establishing distinct developmental domains along the PD axis of the Drosophila leg, hth, dac, and Dll, also collectively termed “leg gap genes,” have been shown to be also involved in leg segmentation (Rauskolb and Irvine, 1999; Rauskolb, 2001). Their expression profiles are dynamic and these dynamics are responsible for the formation of the leg joints in a complex temporal sequence (Rauskolb, 2001). Thus, modifications in the dynamics likely will change the number and location of leg joints. In noninsect arthropods, dynamic expression profiles of the leg gap genes have been found in the woodlouse Porcellio scaber (Abzhanov and Kaufman, 2000) and the spider Cupiennius salei (unpublished observations). However, the expression dynamics in the woodlouse and the spider are different from each other and those in Drosophila. Assuming a conserved relevance of the dynamics for the number and positioning of the leg joints, the differences observed between Porcellio, Cupiennius, and Drosophila could explain the different segment composition of their legs (Drosophila: six primary leg segments plus the tarsal segments; Porcellio and Cupiennius: seven leg segments). The present study shows dynamic expression profiles for the leg gap genes also in the myriapod Glomeris. The dynamics in Glomeris are different from those in Drosophila and Porcellio, indicating that the seven leg segments in Glomeris are not directly comparable to the leg segments in the fly and the woodlouse. Interestingly, the pattern dynamics in Glomeris are virtually identical to those in Cupiennius. This indicates that the number and location of joints in spiders

Fig. 8. Expression of Gm-exd and Gm-hth in the developing appendages of Glomeris. (A–D) Expression of Gm-exd in stage 6 embryos in first trunk leg (A), maxilla (B), mandible (C), and antenna (D). Arrow and arrowhead in (D) point to the two rings of weak and strong Gm-exd expression, respectively, that follow distal to the proximal expression domain of Gm-exd in the antenna. (E–H) Expression of Gm-hth in stage 6 embryos in first trunk leg (E), maxilla (F), mandible (G), and antenna (H). For abbreviations, see Fig. 4.
and diplopods may be directly comparable and suggests that the leg segments in these arthropod groups are homologous.

**Gm-Dll and Gm-dac apparently play a complex role in the cephalic appendages**

In the *Drosophila* antenna, Dll is involved in the formation of the PD axis and the development of the larval antennal sensory organ (Cohen and Jürgens, 1989a). It is also a selector gene for antennal identity. Partial loss of Dll function leads to transformation of antennal tissue into leg tissue (Sunkel and Whittle, 1987; Cohen and Jürgens, 1989b; Dong et al., 2000). In the antenna of *Glomeris*, Gm-Dll is expressed in the entire distal part including the primordia of the sensory cones. This hints to a role in distal development, but makes it impossible to discriminate between a function in PD axis formation and a possible role in sensory organ development. In addition, a role of Gm-Dll in specifying antennal identity cannot be substantiated without functional tests, which are not possible to date. In the maxilla, however, expression of Gm-Dll relates exclusively to the primordia of maxillary sensory organs. Therefore, a role of Gm-Dll in establishing the PD axis of the maxilla of *Glomeris* is unlikely. Also in the mandible, expression of Gm-Dll does not suggest a role of this gene in PD axis formation. Rather, like in the maxilla, Gm-Dll expression appears to correlate with mandibular sensory organs. These data suggest that Gm-Dll in the cephalic appendages has at least two distinct functions, that of instructing distal fates on the PD axis and that of guiding the development of sensory organs in the mouthparts. A function of Dll in sensory organ development has also been proposed for other arthropods, including chelicerates, crustaceans, and arachnids (Mittmann and Scholtz, 2001; Williams et al., 2002), and our results support this notion.

In the antenna, Gm-dac has two distinct expression domains. First, there is a ring-like expression at a medial PD level. At early stages, expression forms a single ring, but later this ring divides into two separate rings linked by residual expression. A similar separation of the dac expression domain during PD axis formation has been shown to occur in the thoracic legs of the cricket *Gryllus bimaculatus* (Inoue et al., 2002), suggesting that the dynamic expression in *Glomeris* may play a similar role in PD axis formation. Second, Gm-dac shows prominent expression in the sensory cone primordia starting at late stage 3, clearly arguing that it is involved in the development of the antennal sensory cones. Because of the complex role of Gm-dac in the antenna, a tentative assessment of its role in the remaining head appendages is difficult to make without functional tests. In the mandible, the expression in small clusters of cells in the inner lobe probably correlates with the formation of sensory organs of this appendage. The very strong expression in the outer lobe, which will give rise to the tooth, is similar to mandibular dac expression in the crustacean *Porcellio scaber* and the insect *Tribolium castaneum* (Abzhanov and Kaufman, 2000; Prpic et al., 2001). It is interesting to note that very strong mandibular dac expression in all three cases correlates with the tooth-like morphology of the mandibular appendage. Thus, it is possible that dac has an additional function in the mandible, that of specifying mandible identity—in analogy to the selector gene function of Dll in the *Drosophila* antenna. It will be interesting to test this hypothesis in an arthropod that is amenable to functional testing (e.g., *Tribolium*). Expression of Gm-dac in the maxilla is at first distributed in the entire primordium of the stipes and the tissue contributing to the intermaxillary plate, but later is restricted to cell clusters in the primordia of the maxillary sensory organs. Thus, Gm-dac is apparently involved in sensory organ development in the maxilla. The role of the earlier ubiquitous expression in the stipes is unclear. Neither in the mandible nor in the maxilla, is expression of Gm-dac found in a pattern that would suggest its involvement in instructing medial fates on the PD axis of these appendages. In summary, Gm-dac expression implicates this gene in at least two distinct processes in the cephalic appendages: PD axis formation in the antenna and sensory organ development in antenna and mouthparts. In addition, Gm-dac may have a third role, that of specifying mandible identity in *Glomeris* and other mandibulate arthropods.

**Phylogenetic implications: serial homologies among mouthparts in insects, crustaceans and myriapods**

In order to reconstruct the evolution of the gene expression pattern, the expression of Dll homologues has been studied in a great number of different arthropod species. The present study is the first detailed account for Dll expression in a myriapod and the first report on expression of dac, hth, and *exd* in a myriapod. The data on Dll presented here show that, in contrast to the antenna and trunk legs, in the two gnathal appendages, mandible and maxilla, no Gm-Dll expression can be found that can be confidently correlated with PD axis formation. This implies that the *Glomeris* mouthparts lack elements serially homologous to the distal Gm-Dll-expressing parts of the antenna and legs. Thus, both mandible and maxilla are gnathobasic in nature and lack distal parts (telopodite, palp). A gnathobasic mandible traditionally is considered as a synapomorphy of crustaceans, insects, and myriapods, uniting them into a monophyletic Mandibulata (Snodgrass, 1938; see review by Koch, 2001). A gnathobasic maxilla, however, is found only in myriapods [apart from *Glomeris* (diplopod) also the maxilla of chilopods, symphylans, and pauropods apparently lacks a telopodite judging from external morphology] and adults of several crustacean species. The maxilla in insects and most crustaceans is not gnathobasic and has a palp (telopodite). This demonstrates that the gnathobasic condition of an appendage can evolve independently by convergence and thus
questions the homology of the mandibular gnathobasic in crustaceans, insects, and myriapods. Indeed, recent molecular phylogenies corroborate two monophyletic groups comprising chelicerates and myriapods on the one hand and crustaceans and insects on the other hand (Hwang et al., 2001; Friedrich and Tautz, 2001). This would argue for an independent origin of the gnathobasic mandible in myriapods and the so-called Tetraconata (insects and crustaceans; Dohle, 2001). However, the results with \textit{Gm-dac} presented here support the homology of the gnathobasic in the mandibles of crustaceans, insects, and myriapods. It has been shown previously that expression of \textit{dac} is very strong in the mandibles of crustaceans and insects (Abzhanov and Kaufman, 2000; Prpic et al., 2001). Similarly, strong expression of \textit{Gm-dac} is seen in the mandible of \textit{Glomeris}. The role of the strong \textit{dac} expression in the development of the mandible is unclear, but it seems possible to correlate it with the specific tooth-like morphology of this appendage (see discussion above). Thus, a common genetic mechanism appears to underlie the gnathobasic nature of the mandibles in myriapods, crustaceans, and insects. In contrast to this, no comparable \textit{dac} expression has been found in the first locomotory leg (L1) in chelicerates (Abzhanov and Kaufman, 2000; unpublished observations). The L1 segment in chelicerates corresponds to the mandibular segment in the other arthropods (Damen et al., 1998; Telford and Thomas, 1998; Mittmann and Scholtz, 2003).

In summary, the results presented here support earlier reports that the mandible in myriapods is indeed gnathobasic (Popadic et al., 1998; Scholtz et al., 1998). Moreover, our results indicate that also the maxilla is gnathobasic, which is different from insects and most crustaceans and also from chelicerates which do not possess a single gnathobasic appendage. Strong expression of \textit{dac} in the mandible in crustaceans, insects, and myriapods suggests that the mandibular morphology is produced by a homologous mechanism and supports the homology of the mandible in all mandibulate arthropods. Although a homologous mandible in crustaceans, insects, and myriapods would support the monophyly of the Mandibulata, it has to be pointed out that it is also compatible with recent molecular phylogenies (Hwang et al., 2001; Friedrich and Tautz, 2001) on the assumption that a true mandible already existed in the last common ancestor of all extant arthropod classes and has been lost secondarily in the chelicerates.

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