A Complex Role for Distal-less in Crustacean Appendage Development

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The developing leg of Drosophila is initially patterned by subdivision of the leg into proximal and distal domains by the activity of the homeodomain proteins Extradenticle (Exd) and Distal-less (Dll). These early domains of gene expression are postulated to reflect a scenario of limb evolution in which an undifferentiated appendage outgrowth was subdivided into two functional parts, the coxapodite and telopodite. The legs of most arthropods have a more complex morphology than the simple rod-shaped leg of Drosophila. We document the expression of Dll and Exd in two crustacean species with complex branched limbs. We show that in these highly modified limbs there is a Dll domain exclusive of Exd but there is also extensive overlap in Exd and Dll expression. While arthropod limbs all appear to have distinct proximal and distal domains, those domains do not define homologous structures throughout arthropods. In addition, we find a striking correlation throughout the proximal/distal extent of the leg between setal-forming cells and Dll expression. We postulate that this may reflect a pleiomorphic function of Dll in development of the peripheral nervous system. In addition, our results confirm previous observations that branch formation in multiramous arthropod limbs is not regulated by a simple iteration of the proximal/distal patterning module employed in Drosophila limb development. © 2001 Elsevier Science

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INTRODUCTION

Limb diversity is a hallmark of the arthropods. While the activities of many genes that regulate patterning in the Drosophila leg are well known (reviewed in Cohen, 1993; Morata, 2001), less is known about the variation in developmental mechanisms responsible for diversification of limb form among arthropods. Drosophila limb development can be modeled as a simple coordinate system, in which the three axes of the limb—anterior–posterior (A/P), proximal–distal (P/D), and dorsal–ventral (D/V)—are established by three interacting cell signaling systems. Some of the patterning genes that regulate the three axes in Drosophila have been examined in other species to analyze conservation in mechanisms of limb development. Although it is already known that the complete set of regulatory interactions is not conserved even in other insects (e.g., Jockusch et al., 2000), the role of some genes in limb patterning does appear widely conserved. DII, which specifies distal structures and promotes P/D outgrowth of the leg (Cohen and Jurgens, 1989; Cohen et al., 1989), has been found in all other insects examined as well as other arthropods such as spiders and crustaceans (Panganiban et al., 1994, 1995, 1997; Niwa et al., 1997; Popadic et al., 1998; Williams, 1998; Schoppmeier and Damen, 2001). Similarly, Exd, another homeodomain protein, has been found restricted to a proximal domain in other arthropods with unbranched legs: insects, a spider, and a crustacean (Jockusch et al., 2000; Abzhanov and Kaufman, 2000).

Dll and Exd function near the beginning of a regulatory network that patterns the P/D axis of the Drosophila leg. The two expression domains arise independently via distinct upstream regulation. The proximal Exd domain, for example, can develop normally without most distal structures (Gonzalez-Crespo et al., 1998; Abu-Shaar and Mann, 1998). These two domains are demarcated by exclusive expression of DII in the distal limb domain and nuclear Extradenticle (n-Exd) in the proximal domain. The exclusivity of the two domains is present by stage 14: limb primordia show a subdivision into centrally located cells with nuclear DII expression and peripherally located cells
with nuclear Exd expression (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). This exclusivity is presumably maintained throughout development since, by the early third larval stage, the two exclusive domains are present within the leg disc. The Exd/Dll domains remain exclusive while P/D values within the leg are subsequently refined through additional growth and modulation of gene expression (Gonzalez-Crespo and Morata, 1995, 1996; Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). The exception is a small region in the proximal domain of the leg in which both DII and Exd are expressed.

The existence of two genetically defined limb domains has been hypothesized to support a classic scenario for the evolutionary origin of arthropod limbs (Gonzales-Crespo and Morata, 1996) proposed by Snodgrass (1935). In this scenario, the ancestral limb was initially an unjointed outgrowth without stiff cuticle. With increased cuticularization, the first joint or pivot point evolved near the body wall permitting the majority of the stiffened limb a wide arc of swing for locomotion. This critical primary joint (coxa/trochanter) divided the limb into two domains, referred to as the coxapodite and telopodite. Gonzalez-Crespo and Morata (1996) hypothesized that the genetically defined domains of Exd and DII correspond to the coxapodite and telopodite and thus represent a genetic interaction conserved since the pre-Cambrian origin of arthropods. They further hypothesized that the proximal Exd domain, because it is contiguous with Exd expressed in the adjacent body wall, has a more direct derivation and relationship to the body wall than the DII domain, which represents a novel regulatory pathway (see also Morata and Sanchez-Herrero, 1999). Under this scenario, Exd and DII would mark homologous limb structures throughout arthropods and provide a useful tool to simplify the relationships between disparate adult arthropod limb morphologies. However, their presentation of this hypothesis contains a central ambiguity about the correspondence between gene expression data and Snodgrass' evolutionary hypothesis. In their paper, they say the ancestral coxapodite could subsequently be subdivided by joints. Thus, the Exd expression in the Drosophila coxa, trochanter, and proximal femur could equal the ancestral coxapodite (Gonzales-Crespo and Morata, 1996). However, this is not what Snodgrass (1935) claimed since, as mentioned above, the original subdivision of the limb involved formation of the coxa/trochanter joint (Snodgrass, 1935; p. 86). Thus, to support Snodgrass' hypothesis, Exd would have to be expressed exclusively in the coxa. Comparative work on representative segmented legs in insects, crustaceans, and spiders has shown that Exd can be variably expressed in the coxa, trochanter (or crustacean basiopodite), and proximal femur (Abzhanov and Kaufmann, 2000). One could infer from these data that, if ancestral proximal/distal domains exist, they do not somehow define homologous leg segments in uniramous limbs.

In spite of its ambiguities, the Gonzales-Crespo and Morata (1996) hypothesis is intriguing. Even if proximal/distal domains do not map to particular segments, are they still used to set up all limbs? This would be interesting since in Drosophila the Exd and DII domains are controlled by independent regulatory networks. To evaluate this hypothesis, we analyzed nonsegmented limbs that have a highly modified morphology. In this case, in analyzing whether proximal and distal domains can be identified, we are not looking at simply tubular outgrowths with a single P/D axis. Instead, we are analyzing limbs with a flattened paddle-like morphology and multiple outgrowths. We find that the very earliest expression of DII is exclusive of Exd in these limbs. Thus, we hypothesize that arthropods pattern the primary P/D axis of their trunk appendages using reciprocal exclusive domains of DII and Exd, during a narrow window of time at the onset of appendage outgrowth. However, the morphological structures to which these domains correspond are not simply translated into "coxapodite" and "telopodite." Furthermore, in the species we have examined, Exd and DII are often coexpressed later in limb development and their expression domains do not simply correlate with proximal and distal position in the limb. Instead, late DII expression strongly correlates with setal position. Consequently, we hypothesize that DII expression has a dual function in crustacean appendages. In early development, DII defines the distal tip of the P/D axis of the limb and is likely required for distal outgrowth. While serving this function, DII excludes Exd expression. Later, DII expression is required for setal formation. In this role, DII and Exd can be coincidently expressed. We discuss a hypothetical scenario for the dual roles of DII during the evolution of arthropod limbs.

**MATERIALS AND METHODS**

**Animal culture.** Cysts of Triops longicaudatus (Wards) and Thamnocephalus platyurus (a gift from D. Belk) were hydrated in artificial pond water and hatched after 24 h. Larvae of various stages were fixed for 30 m in PEMFA (100 mM Pipes, 2.0 mM EGTA, 1.0 mM MgSO4, 4% formaldehyde; pH 6.9) and stored in MeOH at −20°C.

**Immunostaining.** Specimens were rehydrated, then washed in PBS and PBT (PBS + 0.1% Triton); they were briefly sonicated by using a bath sonicator; blocked (PBT + 10% normal goat serum) for 1 h; then incubated overnight in a 1:10 dilution of mouse anti-Exd (a gift from R. White) and rabbit anti-DII (a gift from G. Pangani-ban). Specimens were washed 10 times in PBT then incubated for 1 h at room temperature with Cy3-conjugated anti-rabbit and Cy2-conjugated anti-mouse secondaries (Jackson); then washed 10 times in PBT, counterstained with Hoescht 22358 (10 ng/ml); washed in PBS and stored in 80% glycerol with 4% propyl gallate. Specimens were viewed on a Bio-Rad confocal microscope.

**RESULTS**

**Branchiopod Limb Morphogenesis**

Because the process of limb development is so different between insects and branchiopod crustaceans, we begin with a brief description of limb morphogenesis. Both the
tadpole shrimp, Triops longicaudatus, and the fairy shrimp, Thamnocephalus platyurus, hatch with three pairs of head appendages and a relatively undifferentiated trunk that shows an anterior to posterior gradient in segment development. As larvae continue to grow, segments differentiate posteriorly while more anterior segments mature and elaborate limb buds (Williams and Müller, 1996). Thoracic limbs of T. longicaudatus and T. platyurus are shown in Fig. 1. In both species, limbs have a flattened, paddle-like morphology with numerous branches arising from a broad central stem. Because these limbs are atypical, an explanation of their morphology follows. In the most general definition of an arthropod limb, lobes arising from the medial side are termed endites; those arising from the lateral side are termed exits (in these species the exits have a more specialized name, epipod, which we use here; Fig. 1). In the typical biramous crustacean limb, the main stem of the limb has two segments followed by the endopod (= the insect telopod) which is composed of five segments. The main outer branch of a typical biramous limb is an exit of the second limb segment, the basis (the insect trochanter), and is called the exopod (McLaughlin, 1982; Snodgrass, 1935). The morphological status of the endites and epipods of the two species T. longicaudatus and T. platyurus and their homologies with other crustacean limbs or other arthropod limbs outside the crustaceans is ambiguous. Most commonly, they are related to the typical biramous crustacean walking limb by designating what appears as the most distal inner and outer branches as the endopod and the exopod (Linder, 1952; Walossek, 1993; Manton, 1977; Fryer, 1988). We use this terminology here but note that alternative explanations exist (see Hessler, 1982), e.g., the endopod may include the whole medial series of endites and adjacent unsegmented limb stem (Snodgrass, 1935). We use the term branches throughout the text in speaking generally, without any intention of indicating homology. Limb morphogenesis is essentially similar in the two species (Williams and Müller, 1996; Williams, 1999). After segments are formed, the limb bud begins to grow out from the body wall. Unlike insects and many other crustaceans, the initial limb bud occupies most of the ventral and ventral lateral surface of the segment. The initial limb bud has a smooth hemispherical shape and then, via an unknown mechanism, the marginal epithelium is folded or subdivided into small protrusions that will eventually form the medial and lateral branches of the adult limb (Williams and Müller, 1996; Williams, 1999).

**Expression ofDll and Exd in Branchiopod Limbs**

**The fairy shrimp T. platyurus.** Nuclear Exd expression is ubiquitous throughout the anterior half of the trunk in the earliest larvae and is expressed much more faintly in the posterior half (Fig. 2A). No evidence of cytoplasmic Exd was found at any stage of development (e.g., see Figs. 2C and 2F). As larvae mature and trunk limbs begin to develop, Dll is first activated in a small cluster of cells in the ventrolateral sector of the limb bud. This coincides with the disappearance of Exd in those cells (Fig. 2C, arrow), i.e., Dll and Exd are never detected coincidentally in this region. Dll is then expressed in the endites. Exd is not down-regulated in this region of the limb bud, so that as Dll expression

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**FIG. 1.** Diagrams of thoracic limbs from juvenile (A) Thamnocephalus platyurus and (B) Triops longicaudatus. Both limbs are flattened and bear numerous branches from the main limb stem. Two branches are located most distally, the endopod and exopod. Medial is to the right; the medial branches are called endites (asterisks). The most proximal endite is specialized for food handling and termed the gnathobase (gn). The lateral branches are called epipods.

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FIG. 2. Exd/Dll double label in T. platyurus. (A) Hatchling larva showing head and relatively undifferentiated trunk. Nuclear Exd (green nuclei) is found in only the anterior half of the trunk (arrow). Dll (red nuclei) is found in the labrum, eye and distal parts of the head appendages (A2, antenna). (B) Later stage larva with developing trunk limbs. Maturing limbs show both regions of exclusive Dll expression and Dll/Exd coexpression (yellow nuclei). (C) Dissected ventral body wall showing the earliest Dll expression (arrow). Initial Dll expression is exclusive of Exd. (D–F) Exd/Dll double label in dissected limbs of T. platyurus. (D) Early limb bud showing incipient branches. The endopod (en) and exopod (ex) show Dll expression exclusive of Exd. Subsequent Dll expression in the medial branches overlaps with Exd. (E, F) Maturing stages of limb development illustrating the maintenance of the initial exclusivity between Exd and Dll in the endopod/exopod branches and the continued broad overlap of Exd/Dll medially.
Exd/Dll double label in T. longicaudatus. (A–C) Whole-mount larvae, ventral view. (A) Second-stage larva showing head and developing trunk. Nuclear Exd (green nuclei) is found throughout most of the body but fades posteriorly (arrow). Dll expression exclusive of Exd is found in some head structures, the distal regions of the head appendages and in the posterior developing furcae (red nuclei). (B) Higher magnification of (A). Initial Dll expression in the developing limbs is located laterally on the trunk and is exclusive of Exd (arrow). As Dll then is expressed in the medially developing branches, it is often, although not always, coexpressed with Exd (yellow nuclei). (C) Older larva; the coexpression is maintained in some but not all cells of the medial branches (arrow). Only the most medial branch, the gnathobase (gn), shows a segregation of Exd- and Dll-expressing cells. However, it is not along the proximal/distal axis but is medial/lateral. (D–F) Dissected limbs of T. longicaudatus. (D) Early limb bud showing the incipient branches of the adult limb. Dll is expressed exclusive of Exd in the lateral sector of the limb bud, endopod (en) and exopod (ex). More medial expression shows some cells with overlapping Dll/Exd expression (arrow). (E, F) Maturing stages of limb development illustrating the maintenance of the initial exclusivity between Exd and Dll in the endopod/exopod branches and the continued overlap of Exd/Dll medially (arrows). In (E), the most medial branch, the gnathobase, shows a medial/lateral segregation of Exd- and Dll-expressing cells. (F) Older limb with elaborated branches showing numerous Dll-only-expressing cells mixed with Exd/Dll expressing cells.
accumulate there is a broad region of Dll/Exd overlap (Figs. 2B, 2D–2F, yellow nuclei). There are a few isolated nuclei that express Dll alone (red), implying that in a few cells Exd is down-regulated in response to Dll expression. The details of the expression are more apparent in dissected limbs. In the early limb bud, DII is expressed exclusive of Exd in cells that will apparently become the two distalmost branches (endopod and exopod; Fig. 2D). Cells in this region maintain the exclusivity between Dll and Exd expression throughout the development of the limb (Figs. 2D–2F). DII expression is then activated medially but, in this case, Exd expression is maintained, creating a broad region of Dll/Exd overlap in expression (Fig. 2D). This basic pattern is maintained as the limbs grow and mature with the addition of exclusive DII expression in the epipods (Fig. 2F). In later stages, the DII expression in the proximal epipod is maintained while DII is lost from the more distal epipod (see Fig. 4A).

**The tadpole shrimp T. longicaudatus.** DII expression in Triops has already been described (Williams, 1998). Here, we emphasize its relation to Exd expression and differences from the fairy shrimp. As in the fairy shrimp, Exd is virtually ubiquitous in early larvae although it fades posteriorly (Fig. 3A). DII is first expressed in the lateral sector of the limb bud to the exclusion of Exd (Fig. 3B) although later more medial expression shows considerable overlap with Exd (Fig. 3C). An early limb bud dissected free of the body shows the expression of DII exclusive to Exd in the endopod and exopod (Fig. 3D). When DII expression is first detected medially in the endites there is coexpression with Exd, although, unlike in the fairy shrimp, there are also numerous cells showing exclusive DII expression (Fig. 3E). However, with one exception these do not form discrete domains within the branches. The exception is the most medial endite (gnathobase) where Exd and DII form exclusive domains, although not along the P/D axis of the branch but medially/laterally (Figs. 3C and 3E; gn). In contrast to the fairy shrimp, DII is never strongly expressed lateral to the exopod (Figs. 3D–3F).

Thus, in both species exclusive DII expression is found in a region of the limb bud that will eventually become most distal. This region corresponds to the mature endopod/exopod. Also common to each species is overlap in medial branches although the degree of overlap differs in the two species.

**DII Expression in Both Species Is Strongly Correlated with Patterns of Setal Growth**

Both T. longicaudatus and T. platyurus have highly setose limbs with a complex range of setal morphologies that function in swimming, feeding, grooming, and sensory reception (Fryer, 1988; see also Fig. 1). The setose regions of the limb correlate with the regions expressing DII.

**The fairy shrimp.** In T. platyurus, DII staining of a juvenile stage limb with well-developed setae shows that every setae has cells at its base that strongly express DII (Fig. 4A). Although crustacean setae are not well characterized in comparison to insect bristles (Hallberg and Hansson, 1999), we assume that these cells are involved in setal formation. The most setose regions of the limb are the medial endites that will form the filter for gathering food particles from the water (Fig. 1). Setae are apparent in linear arrays near the margin of the endites where DII expression is robust and Exd/DII overlap is extensive (Figs. 4B and 4C). In addition, the antennae, with broadly spaced swimming setae, show nuclei at the base and extending into each seta that clearly express DII (Fig. 4D). In later stage limbs, the epipods show exclusive DII staining although as the limb develops and adds setae, this expression is down-regulated in the distal epipod while maintained in the proximal one (Fig. 4A). In the most proximal epipod, there are clear although small marginal setae. The more distal epipod has no apparent setae.

**The tadpole shrimp.** Unlike in the fairy shrimp, T. longicaudatus is benthic and uses varied feeding modes which presumably requires a high degree of sensory input. Correspondingly, its limbs have a large array of setal types (Fryer, 1988). Thus, although we still see a correlation between setae and DII-expressing cells it is more complex than in the fairy shrimp. The five endites on T. longicaudatus form finger-like projections. DII expression and therefore DII/Exd overlap differs in these endites (Figs. 3D–3F). In the first, most proximal, endite DII is restricted to the distal tip. This correlates directly with the position and extent of setal development (Fig. 1B). In the other endites, DII is expressed along approximately three-fourths of the length of these branches. Again, this correlates to setal development. Unlike T. platyurus with its very precise array of marginally arranged setae on the endites, the numerous setae on the endites of T. longicaudatus are not restricted to the tips or the margins but correspond to approximately the same extent of the branches as DII expression. In addition, these branches have fine bristle arrays (data not shown). In contrast to T. platyurus, the epipod, in which DII is not expressed, also has no setae, or any sensory structures (Fig. 1B).

In both species, where we find setae, we find DII expression. However, there is some DII expression not restricted to either the distal limb or setal formation. In the epipods of T. platyurus, we see DII in the distal epipod that is subsequently down-regulated. Also, we see low levels of DII in the proximal epipod not associated with the bristles at the margin of the limb.

**DISCUSSION**

We have undertaken a comparative analysis of Exd/DII expression in multibranched crustacean appendages. Through a careful comparison of two branchiopod species, with small variations in their limb morphologies, we have identified both general similarities and subtle differences in
the observed patterns of Exd/Dll expression patterns. We conclude that genetically defined proximal and distal domains do exist, although Exd and Dll do not correspond to homologous subdivisions within the arthropod limb. Rather, in their early expression domains, they can be inferred to specify “distal” and “not-distal” leg positional information. Our data are inconsistent with the idea that the genes that pattern the P/D axis might provide a molecular map to morphological subunits within the limbs and thereby provide another criterion for establishing limb homologies. This supports previous work dealing with comparisons between uniramous walking limbs (Abzhanov and Kaufmann, 2000). In addition, we see an unexpected degree of overlap in the expression of Dll and Exd in the legs of two crustacean species. This would not be predicted based on the model for leg patterning in Drosophila. We postulate that much of the late Dll expression has no direct role in establishing the P/D axis of the leg but is involved with setal development.

Exd and Dll Set Up Early Generic P/D Domains within Limbs

Do our data support genetically defined P/D domains? We can give a qualified “yes.” The timing and position of coexpression of Exd and Dll behave as predicted and correspond with the Drosophila leg. Dll is restricted to what will presumably become the most distal parts of the limb, Exd is downregulated in that region, and Exd is expressed proximally. However, this pattern is found in only the earliest limb bud and is soon obscured by subsequent development, in part by the extensive overlap of Exd and Dll expression domains. We therefore postulate that the initial Dll expression involved in P/D outgrowth is parallel to its well described function in P/D outgrowth in the Drosophila disc and may reflect a generic, genetically-defined P/D domain found in all arthropod limbs. This conservation is intriguing if, as Gonzales-Crespo and Morata (1996) suggest, the proximal and distal limb have not only distinct regulatory pathways but also distinct evolutionary histories. Numerous theories of limb evolution based on adult comparative morphology invoke lability between body and proximal limb structures, i.e., that there is not a fixed and inviolable boundary between the two. Theories like the evolution of wings via the recruitment of a proximal limb branch to the dorsal body wall (Wigglesworth, 1973; Kukalova-Peck, 1983) or the origins of a biramous limb (Budd, 1996) depend explicitly on such variability.

P/D Patterning Genes Do Not Map Directly onto Adult Morphology

Despite this conservation of generic, exclusive P/D domains for Exd and Dll in early limb buds, the combined analysis of these two genes in multiple species suggests they are best viewed as functional domains which in no way map onto specific structures of adult morphology. In this sense, they cannot provide direct data for drawing homologies between limbs. Indeed, even the original definition of the proximal Exd domain as “coxapodite” after Snodgrass (Gonzales-Crespo and Morata, 1996) is misleading since Drosophila Exd clearly functions in the coxa, trochanter, and proximal femur. By contrast, the “coxapodite” defined by Snodgrass (1935) was homologous to the coxa only or if a secondary subdivision had occurred, the coxa and subcoxa. In general, calling the domain of Exd expression the “coxapodite” and the domain of Dll expression the “telopodite” in distantly related taxa implies an unwarranted extrapolation. Both Dll and Exd expression vary greatly depending on leg morphology, stage of development, and species (see also, Abzhanov and Kaufmann, 2000; Williams, 1998). Although the Exd/Dll expression boundaries do not define homologous structures across taxa, it seems clear that they do define some kind of proximal and distal limb region. Therefore, instead of expecting a direct mapping onto adult limb structure, we believe it is likely that early, exclusive Exd and Dll expression domains are used as developmental patterning tools. As with the gap gene expression domains in the early embryo (reviewed in Akam, 1987), they set up developmental domains without a one-to-one relation to adult morphological structures.

Our data support conclusions drawn in a recent study of Exd, dachsund (dac), and Dll in crustacean and insect legs. Abzhanov and Kaufman (2000) found that none of these molecules have boundaries that consistently map to segment boundaries in uniramous limbs in three taxa. This is true in Drosophila as well, where the boundaries that segregate the disc into P/D domains do not map onto the boundaries between leg segments (see Nagy and Williams, 2001). The general picture emerging from comparative data are that although some of the molecules of P/D outgrowth are conserved across taxa—Exd, Dll, and perhaps dac—none of the interesting details of morphological diversification of limb structure are simply correlated with changes in their expression pattern. The control of morphologically relevant features like limb segmentation may lie in the regulation of patterning genes downstream of these molecules, or in a parallel but independent set of gene interactions.

3 In the original formulation of conserved genetically defined P/D domains, Gonzales-Crespo and Morata (1996) claim that the proximal domain originated from body wall and the distal domain was a novel outgrowth. This hypothesis led to the idea that Exd expression could define coxal homologues throughout the arthropods. The argument was based in part on Snodgrass’ theories of limb origins (see Introduction). However, it is important to note that Snodgrass (1936) never claimed that the telopod was a “novel” outgrowth. Instead, he said that the coxa, in some derived cases, becomes incorporated into the body wall as a series of plates or sclerites. The entire limb is derived from the body wall. The salient point is not the origins of telopod versus coxopod but rather to consider how much lability may exist in both developmental and evolutionary terms between the proximal limb and the body wall.
The Roles of Dll in Limb Branching in Branchiopods

Because of the relatively simple experimental manipulations required to form branch-like structures in the Drosophila leg (Campbell and Tomlinson, 1998), it was originally hypothesized that naturally occurring branches might be a reiteration of the patterning module that forms the whole limb in Drosophila. Instead, Exd/Dll appear to define a single axis in both Drosophila and multibranched crustacean limbs. However, at the level of branching, there appears to be no common denominator for patterning all limb branches within a multibranched limb. Patterns of Exd/Dll expression within branches are highly dependent on branch position within the leg, stage of limb development, and species. Thus, although it would have been predicted that each branch would express Dll at the tip and Exd at the base, this is clearly not the case. Even the very earliest expression of Dll in the medial branches of the T. platyurus occurs in nuclei coexpressing Exd. Based on the fact that morphological branching of the proximal branches appears substantially earlier than onset of detectable Dll expression, Williams (1998) has previously hypothesized that the Dll expression in the proximal branches might not be required for distal outgrowth. Our present observations provide an alternative function for Dll in these branches, although leave unknown what might in fact might regulate outgrowth in these branches.

The lack of developmental identity between the limb branches is further supported by previously published wg expression data in T. longicaudatus (Nulsen and Nagy, 1999). Instead of all branches expressing a ventrally located wg stripe—as would be predicted if each branch was patterned like the whole Drosophila limb—some branches express wg ventrally as expected, while some express wg dorsally or dorsally and ventrally. It is clear that limb branches do not constitute a secondary round of generic outgrowth repeated upon the primary limb axis.

Potential Alterations in the Regulatory Interactions Used to Establish Exclusive Exd and Dll Domains

These generic, exclusive P/D domains of Exd/Dll, observed now in several species of arthropods, appear however to be established by a slightly variant regulatory network in the branchiopods. In Drosophila, Exd is functional only when transported into the nucleus. This is accomplished via the activity of the transcription factor, homothorax (hth). High concentrations of Wg and Dpp activate Dll and also repress hth such that Exd is cytoplasmic in the distal domain (Lecuit and Cohen, 1997; Gonzalez-Crespo et al., 1998; Wu and Cohen, 1999). However, we fail to detect cytoplasmic Exd expression at any stage in either of the crustaceans we analyzed; Exd expression is entirely nuclear even prior to the onset of Dll expression. Although this may reflect an inability of the antibody to detect the cytoplasmic form of Exd in these species, we think this unlikely as the same antibody detects both forms readily in insects (Jockusch et al., 2000). Thus, we speculate that the regulatory interactions between Wg and Dpp and Dll/Hth and Exd, which lead to the functional separation of proximal and distal leg domains in Drosophila, are unlikely to be entirely conserved in crustaceans. Whether this means that the domains are patterned by independent regulatory pathways, as in Drosophila, is unknown at this point. It is also interesting to note that we do not observe a region in branchiopod limbs that is not expressing either Dll or Exd. Either dac will not be expressed in branchiopod limbs, dac. Either a region would be expected in a limb expressing the third gene important in patterning the P/D axis of the Drosophila, Tribolium and other crustacean limbs, dac. Either dac will not be expressed in branchiopod limbs, or its expression will overlap in some manner with Exd and Dll. Such insights into potential changes in regulation are one of the strengths of following coexpression. Indeed, the high degree of overlap in Dll and Exd expression—which would not be permitted under the regulatory interactions known...
FIG. 5. A hypothetical evolutionary scenario for the role of Dll function in appendages (A). Dll is initially involved in patterning structures of the peripheral nervous system. Selection occurs for structures to protrude from the body wall to better sample the environment. Some such protrusions could evolve into appendages. Dll, because of its pre-existing association with the protrusions, would be a good candidate for co-optation into the P/D axis patterning process. Thus, primitively Dll would have at least two roles during development, one in the formation of peripheral sensory structures (like setae) and the other in P/D axis formation. In Drosophila (C), because of the extreme modifications for metamorphosis, the P/D axis patterning function of Dll predominates in larval development while setal development is collapsed into the pupal stage. In gradual, direct developers like Triops and Thamnocephalus (B), P/D axis patterning and setal formation would reflect the pleiomorphic condition and remain more overlapping in time. (Red represents Dll expression in both the developmental and evolutionary scenarios. Note that setae would not appear in Drosophila until the final stage of leg development represented in the diagram.)
in Drosophila leg development—led us to speculate that DII had a secondary role in the limb.

**DII and Setal Formation**

The focus of comparative analyses of DII function in arthropods and diverse metazoans has been its important role in establishing the P/D axis of appendages. However, DII, like many other regulatory genes, performs different roles at different times and places within the developing organism. The lethal embryonic phenotype of DII null mutants is difficult to attribute solely to DII’s function in P/D outgrowth of the limbs (reviewed in Panganiban, 2000). Some variations in DII function are known. For example, contrary to the exclusive DII/Exd domains found in the leg, the two genes are co-expressed in the antennae. In contrast to their function as determinants of proximal position in the leg and wing, Exd/hth function as selector genes in the antennae (Dong et al., 2000). More importantly, DII expression is not restricted to developing limbs; it can be detected in both the central and peripheral nervous system (Cohen et al., 1989). In the peripheral nervous system, genetic studies in Drosophila suggest a role for DII in bristle development. Exd clones make ectopic bristles, DII clones in the femur disrupt bristle formation, and DII regulates expression of the proneural gene achaete in a subset of DII-expressing cells in the wing (Campbell and Tomlinson, 1998). Functionally disentangling bristle development and limb outgrowth can be difficult. For example, scoring of DII clones is often tied to the presence or absence of bristles. Indeed, it is controversial whether the initial DII expression in the embryo may function solely in the development of the Keilin’s organs, larval sensory structures, homologous to legs, or whether these cells also contribute to the leg imaginal disc (Panganiban, personal communication).

Within branchiopod trunk limbs, we find a correlation between setal outgrowth and DII expression. The endites of both species, which bear numerous setae, strongly express DII and the extent of DII staining is directly correlated with the position of the setae. This is particularly straightforward in older T. platyurus limbs in which the DII resolves to the setal bearing margin. This role of DII for setal outgrowth would also explain otherwise anomalous DII expression in a nonbranchiopod crustacean, Nebalia, which shows strong DII along the proximal/medial margin of the limb (Williams, 1998). This margin will eventually bear an extensive setal comb for filtering food. Recently, a correlation between DII and setal formation has also been shown in an arthropod insect and a horseshoe crab (Mittmann and Scholtz, 2001). In those species, patterns of DII expression in the mouthparts, labrum, and trunk limbs correlate with mechanos- and chemoreceptors of the peripheral nervous system.

Our data support the conclusions of Mittman and Scholtz (2001) that DII may have evolved a dual role of both promoting peripheral sensory structures and appendage outgrowth. We schematize this in Fig. 5, which provides a speculative evolutionary scenario that relates the sensory and P/D outgrowth roles of DII. The ancestral role of DII may have been in neural development, including peripheral sensory structures. In at least some cases, there would have been selection for those structures to extend away from the body wall. In this way, DII would have been associated with P/D outgrowth—either causally from the beginning or later through co-option (Fig. 5A).

These two roles of DII persisted into the present. In Drosophila, due to its highly specialized and derived mode of development where much of the patterning is shifted into early development in preparation for the rapid emergence of the adult during metamorphosis, a greater degree of temporal dissociation of these two functions may have occurred (Fig. 5B). By contrast, in the gradually developing branchiopod crustaceans (as well as an arthropod and horseshoe crab; Mittmann and Scholtz, 2001), the two roles remain more temporally intertwined (Fig. 5C).

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