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Genetic evidence for the subdivision of the arthropod limb into coxopodite and telopodite

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SUMMARY

Arthropod appendages are thought to have evolved as outgrowths from the body wall of a limbless ancestor. Snodgrass, in his *Principles of Insect Morphology* (1935), proposed that, during evolution, expansion of the body wall would originate the base of the appendages, or coxopodite, upon which the most distal elements that represent the true outer limb, or telopodite, would develop. The homeobox gene *Distal-less (Dll)*, which is required in the *Drosophila* appendages for development of distal regions, has been proposed to promote formation of telopodite structures above the evolutionary ground-state of non-limb or body wall. Here, we present evidence that another homeobox gene, *extradenticle (exd)*, which is required for appropriate development of the trunk and the proximal parts of the appendages, represents a coxopodite gene. We show that *exd* function is eliminated from the distal precursors in the developing limb and remains restricted to proximal precursors throughout development. This elimination is important because, when ectopically expressed, *exd* prevents distal development and gives rise to truncated appendages lacking distal elements. Moreover, the maintenance of *exd* expression during larval stages, contrary to *Dll*, does not require the *hedgehog (hh)* signaling pathway, suggesting that the proximal regions of the appendages develop independently of *hh* function. Finally, we show that in the crustacean *Artemia*, *exd* and *Dll* are expressed in comparable patterns as in *Drosophila*, suggesting a conserved genetic mechanism subdividing the arthropod limb.

Key words: arthropod, limb, coxopodite, telopodite, *Drosophila*, *Distal-less*, *extradenticle*

INTRODUCTION

In *Drosophila*, the homeotic genes of the Antennapedia-Complex (ANT-C) and the Bithorax-Complex (BX-C) (hereafter called *Hox* genes) are responsible for the generation of morphological diversity along the anteroposterior body axis (reviewed in McGinnis and Krumlauf, 1992; Botas, 1993; Lawrence and Morata, 1994). Each body segment is specified by a *Hox* gene or a particular combination of *Hox* genes. As they encode homeodomain proteins, *Hox* genes are supposed to regulate transcription of specific target genes, which are ultimately responsible for the *Hox* function. However, Hox proteins by themselves show little DNA-binding specificity and their association with cofactors has been proposed as a mechanism to modulate their DNA-binding properties (Wilson and Desplan, 1995).

One of these *Hox* cofactors is the product of the gene *extradenticle (exd)* (Peifer and Wieschaus, 1990; Rauskolb et al., 1993). *exd* is a member of the highly conserved *PBC* family of homeobox genes, which also includes the mammalian *pbx* and the *C. elegans ceh-20* genes (Bürglin, 1995). PBC proteins have been shown to act as cofactors of the homeotic function by modulating the DNA-binding affinity and specificity of Hox gene products (reviewed in Mann and Chan, 1996).

However, *exd* is also involved in controlling specific adult subpatterns (González-Crespo and Morata, 1995; Rauskolb et al., 1995), suggesting that some of its functions may be independent of interactions with Hox proteins. This is the case for the legs, where *exd* does not seem to participate in the control of leg identity mediated by differential *Hox* gene activity in each of the three thoracic segments; instead, *exd* is differentially required along the proximodistal (P-D) axis of all six legs (González-Crespo and Morata, 1995; Rauskolb et al., 1995). Other appendages like wings and halteres also show localized requirements for *exd*. These observations suggest an involvement of *exd* with discrete patterning events in imaginal discs which would be independent of *Hox* gene activity, as the latter is concerned with overall segment identity.

The *exd* requirement in the legs is restricted to the proximal regions and this localized requirement is paralleled by a localized distribution of the exd product in the corresponding precursor regions of the leg imaginal discs (González-Crespo and Morata, 1995). Interestingly, another homeobox gene, *Distal-less (Dll)* (Cohen et al., 1989), is required and expressed in the distal regions of the legs (Cohen and Jürgens, 1989; Díaz-Benjumea et al., 1994) in a pattern that appears to be nearly complementary to that of *exd*, suggesting a subdivision of the leg into two distinct regions along the P-D axis.

Such a subdivision of the arthropod leg into proximal and distal components was proposed by Snodgrass (1935) on the

basis of comparative morphology in a model of how arthropod appendages arose in evolution. According to this hypothesis, arthropod appendages would have originated from a primitive limb just formed by a basis, called coxopodite, that would be an expansion of the trunk, and a distal arm, or telopodite, that would represent the limb proper. This primitive appendage would have evolved by acquisition of joints both in the coxopodite and the telopodite, thus giving rise to the present morphology.

In this paper, we present a detailed study of the expression patterns of *exd* and *Dll* throughout development of the limb primordia in the insect *Drosophila* and in the crustacean *Artemia*. We also show that the domains defined by *exd* and *Dll* have distinct functional properties. Altogether, these results provide genetic evidence for the Snodgrass hypothesis and suggest that the coxopodite and the telopodite are genetically represented by the *exd* and *Dll* domains.

MATERIALS AND METHODS

Clonal analysis

exd^{-} clones were generated by X-rays-induced mitotic recombination using the null allele *exd*^{XP11} and the markers *yellow (y)* and *forked*^{36a} (*f*^{36a}) as described (González-Crespo and Morata, 1995).

Drosophila and *Artemia* immunostaining

Drosophila embryos, second and third instar larvae, and pupal leg discs were processed for double immunofluorescence staining as described (Patel, 1994) and analyzed by confocal laser-scanning microscopy. *exd* and *Dll* expression were detected with a rat polyclonal anti-exd antibody (González-Crespo and Morata, 1995) and a mouse monoclonal anti-Dll antibody (Díaz-Benjumea et al., 1994). *teashirt (tsh)* and *optomotor-blind (omb)* expression were detected using corresponding *lacZ* lines (Rauskolb et al., 1995; Grim and Pflugfelder, 1996) and a rabbit polyclonal anti-β-gal antibody (Cappel). Secondary antibodies were an anti-rat biotinylated (Amersham) coupled to lissamine-rhodamine-conjugated streptavidin (Jackson), a FITC-conjugated anti-mouse (Jackson) and a FITC-conjugated anti-rabbit (Jackson).

Artemia cysts (San Francisco Bay Brand) were cultured in reconstituted sea water at 30°C and nauplius larvae were collected, fixed in 4% paraformaldehyde in PBS during 3 hours at 4°C, dehydrated through a methanol series and stored at −20°C. For staining, nauplius were rehydrated to PBS, 0.3% Triton X-100, sonicated four times during 5 seconds at an amplitude of 6 μm with an immersion tip sonicator, blocked in 1% BSA, 0.3% Triton X-100 in PBS during 2 hours and incubated in primary antibodies (a rat polyclonal anti-exd antibody (González-Crespo and Morata, 1995) and a rabbit polyclonal anti-Dll antibody (Panganiban et al., 1995)) overnight at 4°C. After washing and second blocking, nauplius were incubated in corresponding biotinylated secondary antibodies during 2 hours and processed for DAB staining using the Vectastain Elite ABC Kit (Vector). In double staining experiments, both antigens were distinguished by the presence or absence of Ni in the developing reaction. Stained nauplius were dehydrated through an ethanol series, treated with xylenes, mounted in Permount (Fisher) and photographed under Nomarski optics.

Ectopic expression

The UAS/GAL4 system (Brand and Perrimon, 1993) was used to target ectopic *exd* expression. The full-length *exd* cDNA (Rauskolb et al., 1993) was cloned into the pUAST vector (Brand and Perrimon, 1993) to generate transgenic flies by P-element transformation. Six independent UAS-*exd* lines were obtained and assayed for their ability to ectopically deliver immunodetectable exd protein, giving similar results. We used as drivers the E132 line (Halder et al., 1995), a *Dll*-GAL4 (Calleja et al., 1996) and a *decapentaplegic (dpp)*-GAL4 (Wilder and Perrimon, 1995).

TUNEL analysis

The method of TdT-mediated dUTP-biotin nick end labeling (TUNEL) (Gavrieli et al., 1992) was applied to *Drosophila* third instar larvae imaginal discs. Discs were dissected in PBS, fixed in 4% paraformaldehyde in PBS for 20 minutes and in 4% paraformaldehyde, 0.1% Triton X-100 and 0.1% sodium deoxycholate in PBS for 20 minutes, washed in PBS, 0.3% Triton X-100 and incubated in 125 mM sodium cacodylate (pH 7.2), 2.5 mM $CoCl_2$, 0.25 mM 2-mercaptoethanol, 0.3% Triton X-100, 6 μM dATP-biotin and 0.5 u/μl of terminal deoxynucleotidyl transferase (TdT) at 37°C for 3 hours. After washing, discs were processed for DAB staining using the Vectastain Elite ABC Kit (Vector), mounted in 80% glycerol and photographed under Nomarski optics.

hh temperature shift

The temperature-sensitive allele *hh*^{ts2} (Ma et al., 1993) was used to inactivate *hh* function. Embryos and first instar larvae were kept at a permissive temperature (17°C), transferred to the restrictive temperature (29°C) during the second instar and discs from third instar larvae were processed for immunostaining. Mutant larvae were identified by the absence of the dominant marker *Tubby* carried on the TM6B balancer.

RESULTS

exd and *Dll* in the developing *Drosophila* leg

The functional requirements for *exd* in the adult *Drosophila* legs have been described previously (González-Crespo and Morata, 1995; Rauskolb et al., 1995). *exd*$^{-}$ clones differentiate abnormally in proximal regions but, in distal regions, the loss of *exd* has no effect. These results suggested that the leg is subdivided into two regions according to their *exd* functional requirement (González-Crespo and Morata, 1995).

However, Rauskolb et al. (1995) reported that a fraction of *exd*$^{-}$ clones in the intermediate segments femur and tibia exhibit defective patterns. As this may suggest that *exd* is required in a graded fashion along the leg, we have re-examined the question of *exd* requirements in intermediate leg regions by studying a large number of small clones, which were confined within each of these segments. A total of 77 clones were analyzed: 9 in the trochanter, 45 in the femur and 23 in the tibia. In the trochanter all 9 clones exhibited transformation towards a more distal, tibia-like pattern (Fig. 1B). The 45 clones found in the femur were subdivided according their position in proximal (13), medial (13) and distal (19). The 13 proximal clones differentiated inappropriate structures that tended to sort out from the surrounding normal tissue. In most cases, the clone bristles showed an associated bract, a feature corresponding to more distal leg elements (Fig. 1A). Of the 13 medial clones, 3 developed abnormally like those in the proximal femur and the remaining 10 differentiated normally. All 19 distal clones were also normal (Fig. 1C). In the tibia, 22 out of the 23 clones differentiated normally. These results establish a precise boundary for *exd* requirement along the P-D axis of the *Drosophila* leg at the proximal femur. It is of interest that, within the proximal leg regions where the *exd*$^{-}$ clones exhibit a phenotype, those in the more proximal areas like coxa and pleura differentiate aberrant patterns unlike any other leg pattern (Fig. 1A), whereas those in the trochanter and proximal femur show transformation to patterns characteristic of more distal regions of the leg (Fig. 1A,B).

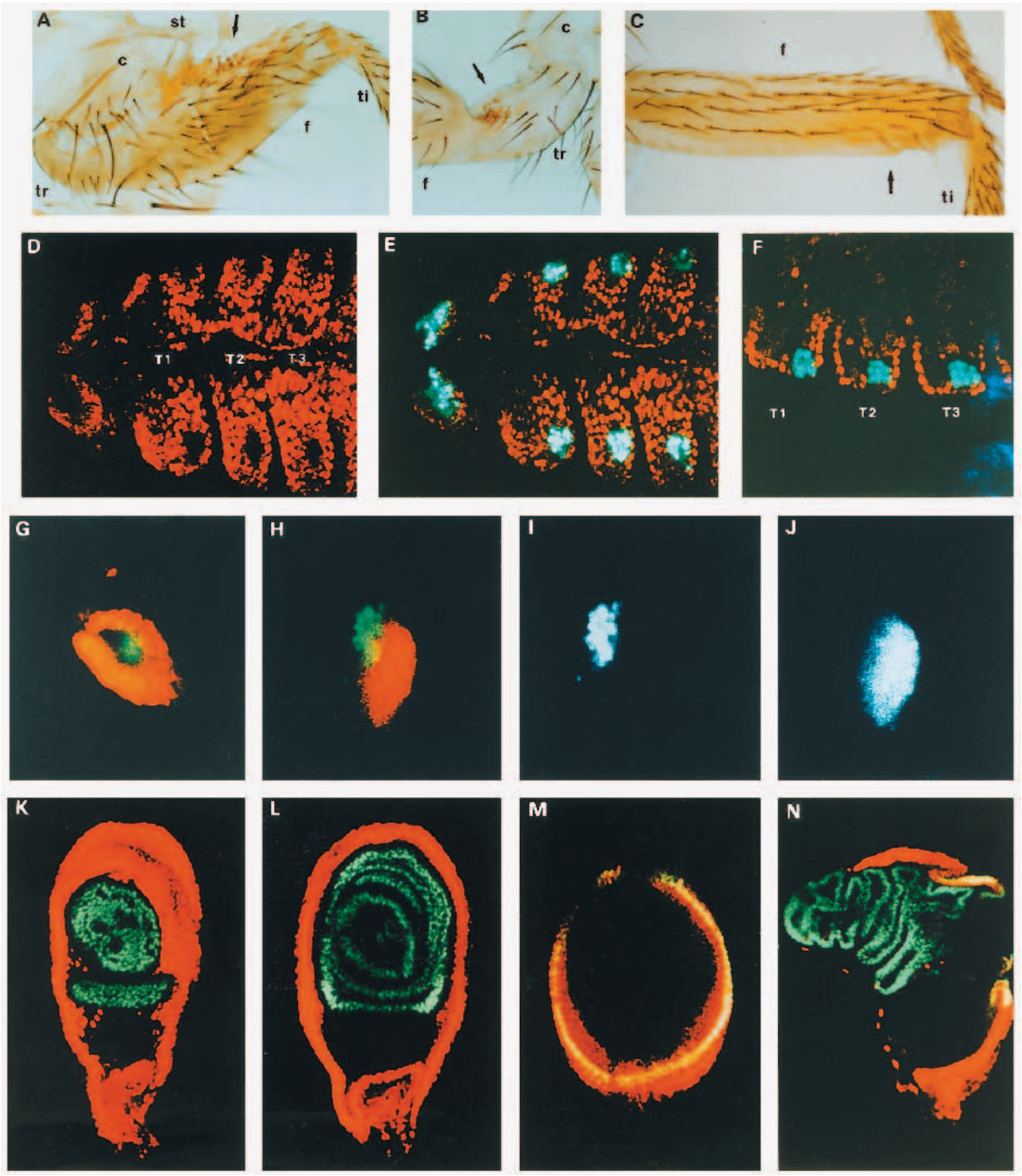

Fig. 1. (A-C) *exd* is required in proximal, but not in distal, leg. (A) *exd*$^{-}$ clone in proximal leg (arrow). The proximal femur is reduced and fused to coxa and ventral body wall or sternopleura. The clone extends to distal femur where it does not produce any abnormality. (B) Small *exd*$^{-}$ clone (arrow) in trochanter forming an outgrowth with bracted bristles which are characteristic of distal leg parts. Only clones in trochanter and proximal femur produce distal patterns, possibly because there is Dll product (see M). (C) *exd*$^{-}$ clone in distal femur (arrow). Removal of *exd* in this and any other more distal region does not affect normal leg development. (D-N) *exd* and *Dll* expression during *Drosophila* leg primordia development. In all cases exd is in red and Dll in green. (D) Ventral view of a stage 14 embryo stained with the exd antibody. Thoracic segments are indicated by T1-T3 in the ventral midline. Six 'holes' of exd staining appear in the ventrolateral epidermis. (E) Same view as in D showing that *exd* expression abuts the *Dll*-expressing imaginal disc primordia. (F) Sagittal view of an embryo in a similar stage as the one in D and E. Cells forming the imaginal disc primordia are stained only with the Dll antibody and invaginate from the outer *exd*-expressing epidermal cells. (G) Frontal view of a second instar leg imaginal disc stained for exd and Dll. The *exd*-expressing cells form a peripheral ring surrounding the central *Dll*-expressing cells. (H) Side view of a second instar disc. Note that there is no overlapping between the *exd* and the *Dll* expression domains. The split channels are shown in I (Dll) and J (exd). (K) Frontal view of a third instar leg disc. (L) Middle optical section of the same disc. **(**M) Basal optical section showing the only region where exd and Dll overlap (yellow). (N) Sagittal optical section of an everting leg disc 2 hours after pupariation. Note the yellow overlapping region**.** Abbreviations: st, sternopleura; c, coxa; tr, trochanter; f, femur; ti, tibia.

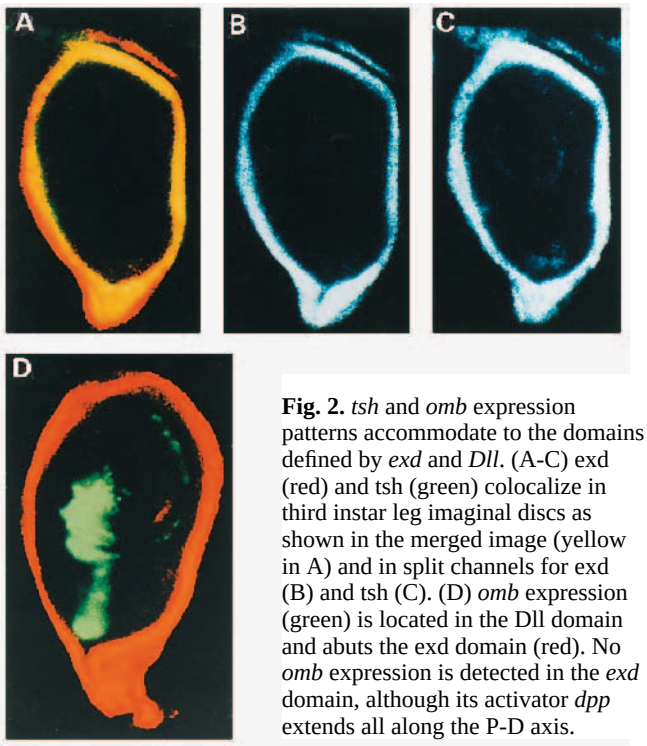

Fig. 2. *tsh* and *omb* expression patterns accommodate to the domains defined by *exd* and *Dll*. (A-C) exd (red) and tsh (green) colocalize in third instar leg imaginal discs as shown in the merged image (yellow in A) and in split channels for exd (B) and tsh (C). (D) *omb* expression (green) is located in the Dll domain and abuts the exd domain (red). No *omb* expression is detected in the *exd* domain, although its activator *dpp* extends all along the P-D axis.

The functional requirements for *Dll* have already been described (Cohen and Jürgens, 1989). Cells homozygous for null *Dll* mutations fail to develop in distal regions, from the trochanter to the tarsus, while they develop normally in the body wall and coxa. Thus it appears that *exd* and *Dll* are required in near complementary domains along the leg, with the exception of the trochanter and proximal femur where both gene functions are needed.

To determine if these complementary domains of function correlate with a localized protein distribution, we analyzed *exd* and *Dll* expression patterns during leg development by immunofluorescence and confocal laser-scanning microscopy. In early embryogenesis, exd is broadly distributed throughout the embryo and colocalizes with Dll in the limb primordia, where *Dll* expression is initially turned on (data not shown). However, by stage 14, *exd* is excluded from the 20-25 *Dll*-expressing cells that form the thoracic imaginal disc primordia (Cohen et al., 1993) and remains restricted to the nuclei of the surrounding cells in the ventrolateral epidermis of the embryo (Fig. 1D-F). During the second larval instar, exd localizes to a peripheral ring of the leg disc (Fig. 1G-J) surrounding the central *Dll*-expressing cells (Díaz-Benjumea et al., 1994), with no detectable overlap. In the third instar (Fig. 1K-M) and early pupal discs (Fig. 1N), *Dll* and *exd* also show reciprocal expression patterns, except for a proximal ring of *Dll* expression (Fig. 1M,N) (Díaz-Benjumea et al., 1994). According to the fate map of the third instar leg imaginal disc (Fristrom and Fristrom, 1993), the peripheral *exd*-expressing cells will give rise to the proximal parts of the adult leg, while central *Dll*-expressing regions are precursors of the distal elements. Therefore, the reciprocity between *exd* and *Dll* genetic requirements correlates with their product distribution during leg development.

This restriction of exd protein to the peripheral parts of the disc is in contrast with its reported uniform mRNA distribution (Rauskolb et al., 1995). We have found that the exd mRNA accumulates preferentially in the periphery of the leg disc, although lower levels are also detected in the central regions (N. Azpiazu and G. Morata, unpublished data). This *exd* mRNA in central regions may be responsible for the low levels of exd protein detected in the cytoplasm (Mann and Abu-Shaar, 1996), suggesting that the restriction of *exd* function to proximal leg parts may be controlled not only transcriptionally but also at the level of nuclear transport.

Altogether, these functional and expression data suggest an early subdivision of the leg primordium into two different regions that correspond with the *exd* and *Dll* domains. This suggestion is reinforced by the observation that other developmental genes are expressed within these two domains. For example, the product of the gene *teashirt (tsh)*, involved in the specification of thorax and abdomen (Röder et al., 1992), colocalizes with *exd* in the mature leg imaginal discs (Fig. 2A-C) and, like *exd*, is absent from the embryonic thoracic disc primordia (data not shown). Conversely, the limb patterning gene *optomotor-blind (omb)*, a *dpp* target (Grim and Pflugfelder, 1996), is expressed only within the *Dll* domain and abuts the *exd* domain (Fig. 2D).

exd expression is confined to proximal precursors in *Artemia* appendages

We also studied whether the reciprocity between *exd* and *Dll* domains is evolutionary conserved by looking at *exd* expression in the crustacean *Artemia*. The expression of *Dll* is restricted to the appendages (Panganiban et al., 1995), in close parallelism to *Drosophila*. We have found that our anti-exd antibody, made against the *Drosophila* exd homeodomain, is able to immunoreact in *Artemia* embryos, which is not surprising since the sequence similarity between members of the PBC family is very high (up to 95 % identity between the exd and the pbx homeodomains).

In early nauplius larvae, exd immunoreactivity is detected along the trunk and also in the limb primordia (Fig. 3A), but as development proceeds the exd product disappears from the most distal tip of the limb bud (Fig. 3B). In older nauplius limbs, exd staining becomes restricted to the gnathobase and the endites in the proximal regions and no staining is detected in the growing exopodite and endopodite that will form the most distal elements of the crustacean natatory biramous appendage (Fig. 3C) (Brusca and Brusca, 1990). Double staining for exd and Dll shows that Dll, but not exd, is present in these most distal precursors (Fig. 3D,E) (Panganiban et al., 1995). In conclusion, the exd product in *Artemia*, just like in *Drosophila*, is confined to the proximal limb precursors.

Ectopic *exd* expression prevents distal leg development

This parallelism between *Drosophila* and *Artemia* concerning the restriction of the exd product to proximal regions suggests that its elimination from distal precursors is a requisite for arthropod limb development. To test this idea, we ectopically expressed *exd* in distal regions of the *Drosophila* leg using the GAL4/UAS system (Brand and Perrimon, 1993).

One driver that we used is the GAL4 line E132 (Halder et al., 1995), which directs gene expression in the central region of the leg disc (Fig. 4A). Ectopic *exd* expression in this region (Fig. 4B) causes deformations in the central folds mainly due

to ring fusions, as visualized by Dll staining (Fig. 4C). The phenotypic effects in the adult leg are shown in Fig. 4D, where the basitarsus is fused to the proximal femur due to disappearance of distal femur and tibia.

Using a *Dll*-GAL4 driver (Fig. 4E) (Calleja et al., 1996), we expressed *exd* all over the *Dll* domain (Fig. 4F). This results in a complete disappearance of the central rings and a severe reduction in the disc size (Fig. 4G). The adult legs appear truncated, only containing coxa, trochanter and proximal femur (Fig. 4H,I), a phenotype very similar to that observed in strong *Dll* hypomorphic alleles (Cohen at al., 1989).

We also targeted *exd* expression all along the P-D axis of the leg in the dorsal anterior regions of both *exd* and *Dll* domains using a *dpp*-GAL4 driver (Wilder and Perrimon, 1995). In this case, *exd* overexpression produces similar morphological disturbances as described above, but the effects are precisely restricted to the distal leg (Fig. 4J). The excess of *exd* product in the proximal regions does not have any consequence (Fig. 4J).

Altogether, these results indicate that distal structures cannot develop in the presence of *exd*. Therefore, the restriction of *exd* expression to proximal precursors is a requisite during limb development.

Although ectopic *exd* expression in the *Dll* domain gives rise to a phenotype similar to that of *Dll* mutants, exd does not repress *Dll* expression. In E132/UAS-*exd* and *Dll*-GAL4/UAS-*exd* leg discs both products are present in the corresponding central cells (Fig. 4C,G). Instead, ectopic *exd* seems to interfere with cell proliferation in the central region of the disc which, in the wild-type situation, grows exponentially during larval stages (Bryant and Schneiderman, 1969). We applied the TUNEL technique (Gavrieli et al., 1992), which stains apoptotic nuclei, to detect programmed cell death in imaginal discs. In wild-type discs, very few cells undergo apoptosis (Fig. 4K) but, when *exd* is ectopically expressed, a large number of nuclei appear stained in the central region (Fig. 4L), indicating that ectopic *exd* results in cell death induction in the distal domain of the leg.

Normal *exd* expression in the absence of *hh* function

The *hh* gene plays a major role in controlling pattern formation along the P-D axis of the leg (Campbell et al., 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994; Díaz-Benjumea et al., 1994). In the absence of *hh* function, *Dll* expression is totally abolished in the central regions of the leg disc, although the proximal ring is unaffected (Díaz-Benjumea et al., 1994). We analyzed whether *exd* expression is dependent on *hh* function using a temperature-sensitive *hh* allele (Ma et al., 1993). As expected, *Dll* expression in the hh^{-} discs was confined to the femur ring (Fig. 5A) (Díaz-Benjumea et al., 1994), thus confirming the loss of *hh* function. However, *exd* expression remained completely normal (Fig. 5A). This is particularly clear in a sagittal view (Fig. 5B) as compared to a wild-type disc (Fig. 5C). This result, together with the fact that *hh* null clones in the leg only disturb distal regions (Mohler, 1988), indicates that the proximal leg is specified independently of *hh* function.

DISCUSSION

The *exd* and *Dll* domains in the arthropod leg

The results presented here and those in Cohen and Jürgens (1989) indicate the existence in the *Drosophila* leg of two domains along the P-D axis with distinct genetic regulation.

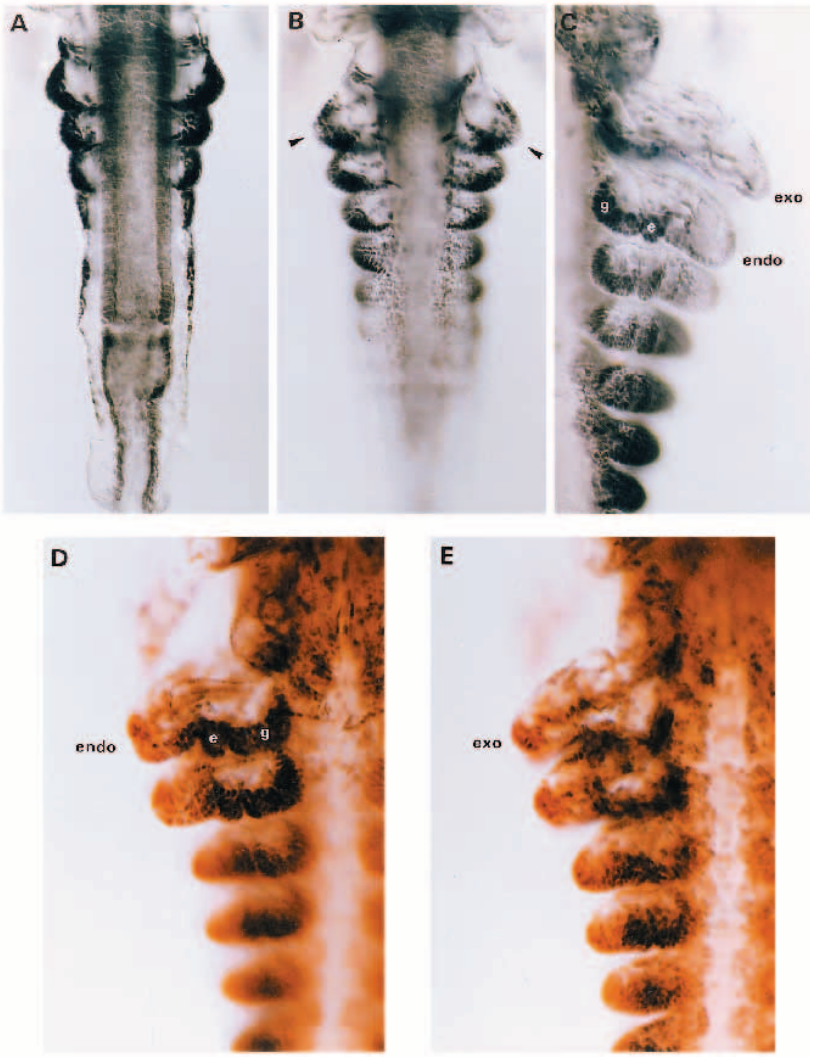

Fig. 3. *exd* and *Dll* expression in a crustacean biramous appendage. In all cases, anterior is to the top and thoracic segments are shown beginning with the first one. Note that the more anterior limb primordia are more developmentally advanced. (A) Young *Artemia* nauplius larvae showing exd staining along the trunk and the limb primordia (black). (B) Slightly older nauplius. exd staining disappears from the distal tip of the most advanced limb primordia (arrowheads). (C) Ventral view of an older nauplius. exd staining is restricted to proximal parts such as the gnathobase (g) and the endites (e) shown in the second thoracic segment. No *exd* staining is detected in the distal endopod ('endo' in the second thoracic segment) and exopod ('exo' in the first thoracic segment in a lower focal plane). (D,E) Double staining for exd and Dll. Dll (brown), but not exd (black), is present in the distal endopod ('endo' in D) and exopod ('exo' in E; lower focal plane than in D).

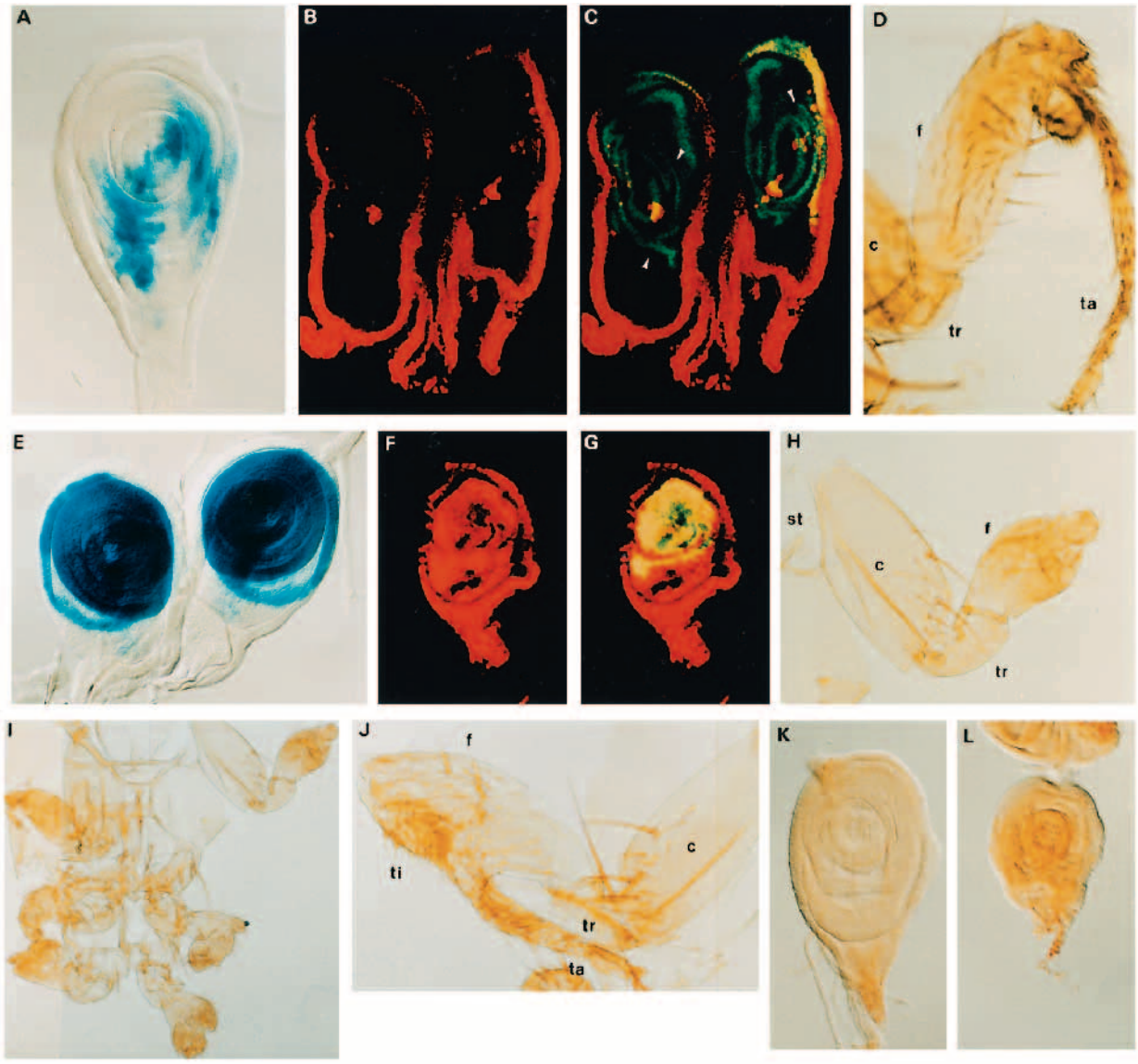

Fig. 4. *exd* prevents distal development in the *Drosophila* leg. In all cases, exd is in red and Dll in green. (A) Gene expression driven by the GAL4 line E132 (Halder et al., 1995) as revealed by X-gal staining using an UAS-*lacZ* strain. (B) *exd* expression in a pair of leg discs from a cross E132 × UAS-*exd*. Endogenous *exd* expression is localized to the periphery, while ectopic exd is in the center. (C) Merged channels of the discs in B. Cells expressing both genes appear yellow. The internal rings are not concentric and most of them are fused to the contiguous (white arrowheads). (D) Adult leg resulting after expressing *exd* with the E132 driver. (E) Gene expression driven by a GAL4 insertion in the *Dll* locus which is identical to the endogenous *Dll* expression pattern (Calleja et al., 1996). (F) *exd* expression in a third instar larval leg disc from a cross *Dll*-GAL4 × UAS-*exd*. exd is present all over the disc, which is shown at 1.5 times the magnification for the ones in E, thus reflecting its great reduction in size after ectopic *exd* expression. (G) Merged channels of the disc in F. The center of the disc (yellow) is dramatically reduced. No internal rings are observed and the remaining *Dll*-expressing cells (which also contain exd) form a round flat structure in the center. (H) Adult leg resulting after expressing *exd* in the *Dll* domain. (I) Lower magnification of the specimen in H showing the complete set of legs. (J) Adult leg after expressing *exd* with a *dpp*-GAL4 driver all along the P-D axis (Wilder and Perrimon, 1995). Coxa, trochanter and proximal femur are not affected by *exd* overexpression, but all distal structures are reduced. (K) TUNEL staining of a wild-type leg disc. Very few randomly distributed apoptotic nuclei are detected. (L) TUNEL staining of a leg disc from a cross *Dll*-GAL4 × UAS-*exd*. Note the large reduction in disc size (same magnification as in K) and the presence of multiple apoptotic nuclei located in the center of the disc where *exd* is ectopically expressed. Abbreviations: st, sternopleura; c, coxa; tr, trochanter; f, femur; ti, tibia; ta, tarsi.

The proximal domain is defined by the identical expression of *exd* and *tsh* in body wall, coxa, trochanter and proximal femur precursors. Although the functional requirements for *tsh* have not yet been described, *exd* is clearly required for normal patterning in these proximal leg parts (González-Crespo and Morata, 1995; Rauskolb et al., 1995). Another common feature of *exd* and *tsh* is that they are also expressed in the embryonic trunk, where they play an important role in specifying segment identity (Peifer and Wieschaus, 1990; Röder et al., 1992).

The distal domain is defined by the expression and requirement of *Dll*, which extends from trochanter to tarsus (Cohen and Jürgens, 1989). Since removal of *Dll* function results in

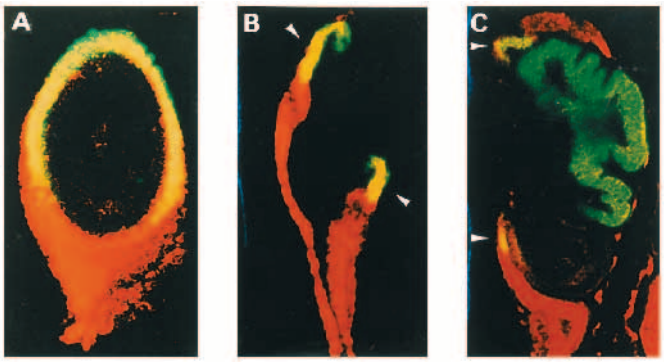

Fig. 5. Normal *exd* expression in the absence of *hh* function. (A) Frontal view of a *hh*$^{-}$ leg disc stained for exd (red) and Dll (green). The yellow ring corresponds to the *Dll* expression in the trochanter and proximal femur which is the only one that remains and overlaps with the *exd* expression domain which, instead, appears normal. (B) Sagittal view of another *hh*$^{-}$ leg disc stained as in A. Note the complete absence of the central folds that correspond to the distal parts and are clearly viewed in the wild-type disc shown in C. The *exd* expression pattern remains unaffected. The yellow ring is indicated by arrowheads. (C) Sagittal view of a wild-type disc stained as the two previous ones. Also, the yellow ring is indicated by arrowheads. The *hh*$^{-}$ discs in A, B have been magnified 1.5 times more than this wild-type disc, illustrating the reduction of disc size in the absence of *hh* function.

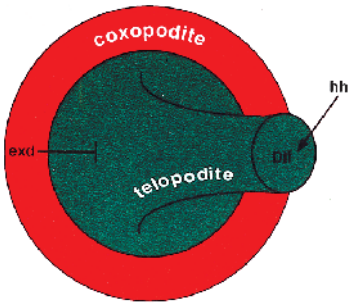

Fig. 6. The two major genetic domains along the P-D axis of the arthropod limb. The base of the appendages, or coxopodite (red), would be controlled by genes like *exd*. The distal part of the appendages, or telopodite (green), would correspond to the *Dll* domain and represents the true limb. This distal region requires the function of the *hh* pathway as well as the elimination of trunk genes like *exd* to avoid their inhibitory effects on distal growth.

elimination of distal leg structures, it has been proposed that *Dll* activates the formation of limb structures above a ground-state of non-limb or body wall (Cohen and Jürgens, 1989). The distinction between 'limb' and 'body wall' fates is observed already during embryogenesis when *Dll* is expressed in the invaginating thoracic imaginal disc primordia while *exd* and *tsh* are confined to the surrounding epidermal cells. It is worth mentioning that *Dll* also has an embryonic function, which is the formation of the rudimentary larval appendages called Keilin's organs (Cohen et al., 1989), in which *exd* and *tsh* expression is eliminated.

Additional evidence supporting this two-domain model is the finding that *exd* and *Dll* are regulated differently. The expression of *Dll* in the leg requires *hh* function, which through *dpp* and *wingless (wg)* activates *Dll* (Díaz-Benjumea et al., 1994), whereas *exd* is unaffected by the loss of *hh* function. This distinct gene regulation is also reflected in the expression of *omb*, a target gene of *dpp* in the leg (Grim and Pflugfelder, 1996). Although *dpp* is expressed all along the P-D axis, *omb* expression is only activated in the *Dll* domain, indicating a control mechanism that discriminates between the two domains.

The fact that, in the trochanter and proximal femur, the expression of *exd* and *Dll* overlap argues in principle against a two-domain model. This region is represented in the mature imaginal disc by a proximal *Dll* ring separated from the main body of *Dll* expression. However, this *Dll* ring, which is independent of *hh*, seems to be a late expression, because we do not observe any overlap between the *exd* and *Dll* domains during the second larval instar. Therefore, we propose an original non-overlapping distribution of both products (as suggested by the expression in the younger discs), which would have been secondarily modified by the late expression of *Dll* in the proximal ring. This idea is also supported by transplantation experiments (Schubiger, 1974) indicating that proximal and distal leg regions are determined first and the middle regions (trochanter, femur and tibia) are determined later, perhaps through interactions between proximal and distal cells.

The two-domain model is also supported by the fact that *exd* and *Dll* show comparable expression patterns in *Drosophila* and *Artemia*. *Dll* homologs have been isolated in vertebrates and *Artemia* (Bürglin, 1995; Panganiban et al., 1995), and exd shows a high level of sequence conservation in *Drosophila*, humans and nematodes (Rauskolb et al., 1993; Bürglin, 1995). Our results indicate that, at least in the arthropods, their respective expression patterns in the limb are also conserved.

The negative effects of ectopic *exd* expression in the leg distal region strongly suggests that the parallelism between *Drosophila* and *Artemia* concerning the elimination of the exd product from the distal precursors is a general phenomenon during appendage formation in arthropods. This process probably involved the singling out of specific groups of cells recruited from the body wall that have to undergo additional proliferation and change of identity. In both *Drosophila* and *Artemia*, the gene *Dll* is specifically activated in those cells and, later, the exd product disappears from the *Dll*-expressing cells. We do not know why *exd* function has to be eliminated from those cells, but the *Dll*-like mutant phenotype caused by ectopic *exd* expression in the telopodite suggests that *exd* may be interfering with *Dll* function. The elimination of distal components associated with localized cell death in central regions of imaginal discs observed after ectopic *exd* expression in the *Dll* domain has also been observed in mutations reducing *dpp* activity (Bryant, 1988), which acts downstream of *hh* in controlling *Dll* expression and pattern formation in imaginal discs. In fact, the *Dll*-GAL4/UAS-*exd* leg discs are morphologically similar to the ones in which *hh* function was eliminated in early second larval instar. Even in the proximal ring corresponding to the trochanter and proximal femur where the exd and Dll products coincide, the presence of exd appears to interfere with *Dll* function since *exd*$^{-}$ clones in this area develop with a distal identity, as if, in the absence of *exd* function, the Dll product is able to perform its role in determining distal development. We might speculate that *exd* suppresses *Dll* functionally, but not transcriptionally, as in other cases of interactions between *Hox* gene products (González-Reyes and Morata, 1990).

Genetic evidence in support of the Snodgrass hypothesis

Based on comparative morphology, Snodgrass in 1935

proposed that primitive arthropod appendages were originally formed by two segments, a proximal coxopodite derived from the trunk, and a distal telopodite or outer limb. This primitive appendage would have evolved by acquisition of joints both in the coxopodite and the telopodite (Snodgrass, 1935).

This view was reinforced by recent analysis of Lower Permian Diaphanopterodea fossils, which represent the most primitive known pterygote order. These primitive insects had abdominal appendages in which the free limb was formed by distal segments from prefemur to tarsus, while the proximal segments were included in the ventral trunk (reviewed in Kukalová-Peck, 1991).

Our results provide genetic evidence for the Snodgrass hypothesis (Fig. 6). On the one hand, the fact that trunk-related genes such as *exd* and *tsh* are associated with the development of proximal leg parts strongly supports that these originated as an expansion of the body wall and provides a genetic definition of the coxopodite. On the other hand, the true outer limb or telopodite would correspond to the *Dll* domain (Cohen and Jürgens, 1989) which development relies on the *hh* signalling pathway and requires the elimination of trunk genes like *exd* to avoid their inhibitory effects on distal growth.

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Note added in proof

In agreement with recent results of Mann and Abu-Shaar (*Nature*, in press), we have also detected low levels of exd staining in the cytoplasm of distal cells of third instar larval leg discs.