INTRODUCTION

Homeotic genes are expressed differentially along the anteroposterior axis of the animal body and specify segment-specific morphological characteristics by regulating sets of target genes (Levis, 1978). How the function of Hox genes have diverged and contributed to diversification of animal morphologies has been a major focus of investigations (Carroll et al., 2001). Genetic alteration of local (Warren et al., 1994) or segmental (Averof and Patel, 1997) patterns of Hox gene expression and changes in selectivity of target genes (Weatherbee et al., 1999) have been proposed as evolutionary mechanisms of segmental diversification of arthropod limbs. Another hypothesis is that structural changes in homeotic proteins have altered their regulatory capabilities and contributed to evolutionary changes in the number of arthropod limbs (Li and McGinnis, 1999).

Crustaceans bear several pairs of thoracic limbs in a variety of sizes as well as branching patterns that are grouped into two major types (Brusca and Brusca, 1990). Larger ones serve as legs for mainly locomotive functions. Another type of the thoracic limb, termed the maxilliped, is smaller and its functions are mainly feeding functions. The number of maxillipeds varies from three to four according to the size and type of the species. Branching is suppressed, resembling head appendages used for feeding. Distal-less homologues in several crustacean species examined, and that the changes correlate well with the borders of transition from maxillipeds to legs. The authors proposed that evolutionary changes in Ubx/abdA expression in the thorax. The expression levels of these and other chimeric constructs were determined with RNA in situ hybridization and/or antibody staining and were found to be two- to fourfold higher.

SUMMARY

Evolutional changes in homeotic gene functions have contributed to segmental diversification of arthropod limbs, but crucial molecular changes have not been identified to date. The first leg of the crustacean Daphnia lacks a prominent ventral branch found in the second to fourth legs. We show here that this phenotype correlates with the loss of Distal-less and consequent expression of Antennapedia in the limb primordium. Unlike its Drosophila counterpart, Daphnia Antennapedia represses Distal-less in Drosophila assays, and the protein region conferring this activity was mapped to the N-terminal region of the protein. The results imply that Daphnia Antennapedia specifies leg morphology by repressing Distal-less, and this activity was acquired through a change in protein structure after separation of crustaceans and insects.

Key words: HOM protein, Antennapedia, Distal-less, Evolution, Crustacean, Limb, Daphnia

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than that of the endogenous *Antp* gene. The *yw* strain was used as a wild-type control. Strain information is available from FlyBase (FlyBase, 1999).

Isolation of *Daphnia* *Antp* cDNA

Homeobox fragments were PCR amplified from the *Daphnia magna* cDNA library (Tokishita et al., 1997) using a set of primers 5′- CGC-GGA TTCAGACSCTGGAGCTGGAGAARGA-3′ and 5′-TCCGG-ATTCCCACTTCA TGCGCCGRTTCTGRAACCA-3′ that corresponds to highly conserved regions of ANTP-type genes. An ANTP-like fragment was used as a probe to screen the same library to identify full-length *Antp* cDNAs.

Antibody staining

Fixation and antibody staining of *Daphnia* embryos were basically carried out according to the protocol by Panganiban et al. (Panganiban et al., 1995) with modifications. Rabbit anti-DapANTP was raised against a recombinant peptide (residue 1 to 539) and affinity purified for immunostaining. Other antibodies used were anti-UBX/ABDA FP6.87 (Kelsh et al., 1994), anti-DmANTP (4C3) (Glicksman and Brower, 1988), anti-DLL (Panganiban et al., 1995) and anti-TSH (Andrew et al., 1994). For DapANTP/DLL double labeling, anti-DapANTP was biotinylated and affinity-purified for detection with an ABC elite kit (Vector Lab) and a TSA direct kit (New England Nuclear). Protocols for *Drosophila* are described in Sullivan et al. (Sullivan et al., 2000).

**RESULTS AND DISCUSSION**

**Segmental difference in *Daphnia* leg patterns correlates with a change in Distal-less expression**

The water flea, *Daphnia magna* (Cladocera, Crustacea) has five pairs of multiply branched thoracic limbs that differ from each other, with the exception of the third (L3) and the fourth legs (L4), which have essentially the same morphology (Fig. 1A). The second to fourth legs are characterized by prominent comb-like feeding structures (gnathobase, Gn; filter comb, Fc) associated with endites and are significantly larger than L1 and L5. Although all the legs are covered with carapace and are not used directly for locomotive functions, the morphological

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**Fig. 1.** Limb development in *Daphnia*. (A) First to fifth trunk limbs of *Daphnia* and their relationship to DLL expression. Epipod (Ep), exopod (Ex), endopod (En) and gnathobase (Gn) or filter comb (Fc) are arranged in a dorsal-to-ventral orientation. Endites of second to fourth leg bear prominent comb-like structures (Gn and Fc) that correspond to strong expression of DLL. No such branches or DLL expression are present in T1 or T5 (asterisks). (B,D,F) *Daphnia* embryos stained with nuclear marker YOYO-1. (C,E,G) DLL expression. Ventral view, anterior is upwards. (B,C) At 18 hours (Obreschkov and Fraser, 1940), with visible furrows separating thoracic segments, DLL expression begins in a dorsal cluster of cells in the first thoracic leg primordia, and in dorsal and ventral clusters in the second and the third primordia. Ventral DLL-expressing clusters in T2 and dorsal clusters in T3 are out of focus in this image. (D,E) At 21 hours, the DLL-expressing domain is expanded and clear nuclear localized signals are detected in future internal branches of developing thoracic legs and in first antenna, labrum, first maxilla, but not in mandibles and second maxilla. (F,G) Later stage embryos (24-27 hours) show strong DLL signals in limb branches, which show characteristic segment-specific morphologies. An1, first antennae; An2, second antennae; Lb, labrum; Mn, mandible; Mx1, first maxillae; Mx2, second maxillae; T1-T5, first to fifth thoracic segment; L1-L5, first to fifth thoracic limb. Scale bars: 100 μm.
difference between L1 and L2-L4 is analogous to the maxilliped/leg difference in other crustaceans. We focused our analyses on the difference between L1 and L2-4.

We studied the early stages of Daphnia limb development by following the expression of the homeodomain protein DLL (Fig. 1) that is implicated in the development of distal parts of appendages of several taxa (Panganiban et al., 1997). DLL expression starts early in limb development (Fig. 1C) and persists until a late stage when each limb primordium acquires branched morphology characteristics of each segment, allowing for the correlation of each domain of DLL expression to specific limb branches (Fig. 1A). Thoracic DLL expression starts in the prospective exopod/endopod region of the leg primordium (Fig. 1C). As development progressed, several clusters of DLL expression emerged ventrally, corresponding to future endites in T2-T4 (Fig. 1E). Finally, additional DLL expression emerged in an intermediate region. In T1 and T5, where the comb-like structures from endites are not formed, ventral DLL expression was reduced to a few cells. Those early expression patterns of Dll that prefigure the pattern of distal branching suggest that a failure to activate DLL expression in the endites resulted in reduced formation of ventral limb branches.

A post-transcriptional mechanism limits Daphnia ANTP expression to Mx2 and anterior L1

To address the mechanism for regulating segmental differences of thoracic limbs, we examined homeotic gene expression in the trunk. We cloned cDNAs encoding Daphnia Antennapedia (DapAntp) and found that the encoded protein is highly homologous to Drosophila Antennapedia (DmAntp) in the region spanning the YPWM motif and the homeodomain, but the remaining protein-coding region was highly divergent (Fig. 2A,B). DapAntp mRNA was expressed broadly from the second maxilla (Mx2) to the post-thoracic segments (Fig. 3A), which was similar to the pattern reported for other crustaceans (Abzhanov and Kaufman, 2000; Averof and Akam, 1995). By contrast, DapANTP protein was detectable only in Mx2 and the anterior region of the first thoracic segment (T1a), including L1 (Fig. 3B), suggesting that DapANTP expression is regulated post-transcriptionally.

Mutually exclusive expression of ANTP and DLL in Daphnia L1

To understand the molecular basis for the L1-specific morphological characteristics, we compared the expression pattern of DLL and DapANTP in L1. Double labeling of DLL and DapANTP demonstrated that their expressions do not overlap in T1 (Fig. 3D) or in Mx2 (not shown). To examine whether the non-overlap of ANTP with DLL expression domain is a common property of thoracic homeotic genes, we examined the expression of posterior homeotic genes Ultrabithorax (Ubx) and abdominal A (abdA) with monoclonal antibody FP6.87, which recognizes an epitope (UBX/ABDA) common to UBX and ABDA. Expression of UBX/ABDA was
strong in posterior T1-T4, and weak in T5 and post-thoracic segments (Fig. 3C), suggesting that the borders of strong UBX/ABDA expression correlate with the change in leg patterns. UBX/ABDA expression extensively overlapped with DLL (Fig. 3E), as has been reported for other crustaceans, millipedes and insects (Panganiban et al., 1995; Grenier et al., 1997). Therefore, the expression pattern complementary to that of DLL is a unique feature of ANTP. One prediction would be that DapANTP represses ventral DLL expression in L1 to modify limb morphology to a maxillipeds-like morphology.

Daphnia ANTP specifies thoracic identities in the Drosophila head

The proposed role of DapANTP in modifying DLL expression and limb morphologies may have arisen through changes in transcriptional enhancers of its target genes such as Dll. An alternative, but non-exclusive possibility, is that a change occurred in the protein-coding region of DapANTP to alter its target specificity. To test the latter possibility, we compared the activities of DapANTP and DmANTP by using assays in Drosophila, where DmANTP is compatible with limb development. Misexpression of DapANTP in eye-antennal discs caused transformation of the antennae to legs characterized by bracted bristles, dorsal thoracic cuticle in place of dorsal head and compound eyes were observed in B,C. (D-F) Induction of an ANTP target gene Teashirt (TSH). (D) y w, (E) DllGal4; UAS-DmAntp, (F) DllGal4; UAS-DapAntp embryos. Ectopic head expression of TSH was observed in E,F (arrowheads).
caused only minor defects (Fig. 5B). It is therefore unlikely that defects caused by DapANTP are due to a dominant-negative effect on endogenous DmANTP or to titration of general transcriptional factors. It instead suggests that DapANTP possesses a novel activity not present in DmANTP.

**Daphnia ANTP, but not Drosophila ANTP, represses DLL expression in Drosophila**

We examined embryos stained with various marker genes expressed differently along the DV axis. We noted that Antp>gapDap embryos in late stages showed massive cell death in the ventral half of affected segments and eliminated rhomboid mRNA in the ventral midline and tracheal pits (data not shown). The ventrally pronounced phenotype was also reproduced by the ptc-Gal4 driver, suggesting that DapANTP activity is more potent in the ventral ectoderm of the embryo. We focused our analyses on embryos earlier than stage 12 before cell death took place. Strikingly DapANTP eliminated DLL expression in T2 and T3 (Fig. 5F), suggesting that DapANTP inhibits limb development in Drosophila. By contrast, Dll was not affected by DmANTP (Fig. 5E). We also examined ectodermal expression of wg, dpp and UBX/ABDA, all of which are known to regulate Dll, but DapANTP had no effect prior to stage 12 (data not shown).

**Daphnia ANTP regulates Dll enhancer through the region normally mediating repression by UBx and ABDA**

To elucidate the mechanism by which DapANTP represses DLL, we examined the transcriptional enhancer of Dll that reproduces Dll expression in the limb primordium (Vachon et al., 1992) (Dll304; Fig. 5G). While ectopic DmANTP has no effect on Dll304, DapANTP strongly repressed Dll304 expression, leaving only a small number of Dll304-expressing cells remaining (Fig. 5H, I). It has been shown that Dll304 is induced at the ventral edge of limb primordia and those cells migrate in the dorsal direction (Goto and Hayashi, 1997). Therefore, the remaining Dll304-positive cells in DapANTP expressing embryos may be the earliest born limb primordial cells that have yet to receive the repressive effect of DapANTP. The enhancer fragment used to construct Dll304 contains multiple binding sites for UBx and ABDA. Deletion of all but one of those sites in the construct Dll305 caused de-repression in abdominal segments (Vachon et al., 1992) (Fig. 5J). DapANTP failed to repress Dll305 effectively (Fig. 5L), suggesting that DapANTP regulates Dll enhancer through the region normally mediating repression by UBx and ABDA. It should be noted that in Drosophila embryos, the expression domain of DmANTP covers those of DLL (Casares and Mann, 1998) (Fig. 5H). Therefore, DmANTP does not repress DLL in this stage of development.

**N-terminal region of Daphnia ANTP contributes to the major functional differences**

To map the region responsible for the functional differences in the two proteins, we constructed chimeric proteins and repeated all analyses. We divided ANTP proteins into three regions: the diverged N-terminal region (N), highly conserved YPWM motif and homeodomain (HD) and C-terminal tails (C, Fig. 2, Table 1). All constructs retained the activities to transform the head to the thorax, and to induce TSH expression in the head. Replacement of NDm with N Dap conferred the Daphnia-specific activity (compare constructs 1 and 3 in Table 1), and a reciprocal replacement of NDap with N Dm greatly compromised DapANTP activity (constructs 2, 6). Those results suggest that the majority of Daphnia-specific activity resides in the N-terminal region of DapANTP (NDap). Analyses also revealed a negative effect of C Dm on Daphnia-specific...
activity when combined with the rest of DapANTP (construct 5). In C\textsuperscript{Dm}, there are two casein kinase II (CKII) phosphorylation consensus sites that are conserved in several insect species, but are not present in DapANTP. These sites are required to modulate ANTP activity in Drosophila (Jaffe et al., 1997). Mutation of C\textsuperscript{Dm} to create CKII sites compromised DapANTP activity (construct 8), suggesting that CKII phosphorylation inhibits DapANTP activity. However, addition of C\textsuperscript{Dm} to DmDap, or mutations of C\textsuperscript{Dm} to disrupt CKII sites failed to produce Daphnia-specific activity to DmDapANTP (constructs 4, 7). Taken together, the results suggest that N\textsuperscript{Dap} is a major determinant of Daphnia specific activity of ANTP to repressDll, and C\textsuperscript{Dm} can interfere with this activity, possibly through phosphorylation of CKII sites. N\textsuperscript{Dap} is two times as long as N\textsuperscript{Dm} and does not contain blocks of obvious sequence homology, except for a short region at the N terminus. In two places, HDD\textsuperscript{Dap} differs from HD\textsuperscript{Dm}, one is conservative F to Y substitution in the homeodomain and the other in the region connecting YPWM motif and homeodomain, the latter being affected by alternative use of splicing acceptor sites (Bermingham and Scott, 1988). The significance of these differences remains to be determined.

### Evolutional implications

The highly restricted expression of DapANTP in L1 of Daphnia suggests a model that ANTP modifies the morphology of the T1 leg to a smaller one by repressing DLL, although a causal relationship between the expression of ANTP and DLL in Daphnia remains to be tested by genetic approaches. This idea would explain the observation that crustacean legs anterior to the domain of UBX/ABDA are, in general, small and resemble feeding appendages when compared with more posterior limbs specialized for locomotive functions (Averof and Patel, 1997). Given the strong limb suppressing activity of DapANTP observed in the Drosophila assays, expression of ANTP seems to be tightly regulated in Daphnia, and the post-transcriptional regulation of Antp expression observed in this study is one mechanism assuring limited expression of ANTP. Modification of T5 limbs may be due to activities of the posterior Hox gene AbdB that has been shown to repress limb development in Drosophila (Estrada and Sanchez-Herrero, 2001).

We have shown here that diversification of the ANTP protein outside the homeodomain contributed to its functional variation in modifying limb patterns. The region responsible for Daphnia-specific activity was mapped to the N terminal region of ANTP that is highly diverged. Two recently works on Ubx proteins (Galant and Carroll, 2002; Ronshaugen et al., 2002) reported that functional alteration of homeotic proteins played a significant role in restricting the number of insect limbs. This work demonstrates that an evolutionary change in Antennapedia protein has contributed to a micro-evolutionary event that has produced the difference in the shape of T1 leg and T2-4 legs of Daphnia. Taken together, homeotic proteins have undergone a number of alterations in regions outside the homeodomain to change their target specificity and the way they control limb development. More importantly, Daphnia-specific ANTP activity and the pattern of its expression account for segment-specific limb morphology of Daphnia, suggesting that protein-coding regions of Hox genes serve as rich substrates for evolutionary alterations that have generated segmental diversities of the crustacean limbs.

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### REFERENCES


