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Evolving role of Antennapedia protein in arthropod limb patterning

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SUMMARY

Evolutional changes in homeotic gene functions have contributed to segmental diversification of arthropodan 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 concomitant 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 *Dapnia* 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*

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 (Lewis, 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 evolutional mechanisms of segmental diversification of arthropod limbs. Another hypothesis is that structural changes in homeotic proteins have altered their regulatory capabilities and contributed to evolutional 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 branching is suppressed, resembling head appendages used for feeding functions. The number of maxillipeds varies from three pairs in *Homarus* to one in *Mysidium* and none in *Artemia*. Averof and Patel (Averof and Patel, 1997) have reported that the anterior borders of Ubx/abdA expression varied in ten crustacean species examined, and that the changes correlate well with the borders of transition from maxillipeds to legs. The authors proposed that evolutional change in Ubx/abdA expression determines the number of segments bearing maxillipeds. However, what specifies the morphological characteristics of maxillipeds is not known to date. One prediction is that another homeotic gene expressed anterior to Ubx/abdA directly specifies the shape of the maxillipeds.

We have studied the expression and function of the homeotic gene *Antennapedia* of *Daphnia* magna. We first show that *Daphnia Antennapedia* (*DapAntp*) is regulated by a post-transcriptional mechanism that limits its protein expression to a subset of T1 leg primordium in a pattern complementary to the expression of Distal-less (DLL). Using assays in *Drosophila*, we show that *DapAntp* is capable of repressing *Distal-less* (*Dll*) expression and limb development. The protein region responsible for this repressive activity was mapped to the highly divergent N-terminal region of DapANTP, suggesting that functional alteration of homeotic proteins has played a significant role in the evolution of crustacean limb patterns.

MATERIALS AND METHODS

Animals

The *Daphnia magna* strain used in this work was originally isolated in Matsuyama, Japan (Mashiko and Ito, 1951) and has been maintained parthenogenetically. Fly strains used were P{w+mW.hs=GAL4-dpp.blk1}40C.6 (*dpp*-Gal4), Dll^{md23} (*Dll*-Gal4), UAS-*DmAntp* (containing *Antp* cDNA G1100; M. Pettite and M. Scott, unpublished), P{lacZDll.304}(Dll304) and P{lacZDll.305}(Dll305). P{GAL4-Antp.P1.A} (*Antp*-Gal4) and P{GawB}559.1 (ptc-Gal4) were used to drive expression in the thorax. The expression levels of these and other chimeric constructs were detected with RNA in situ hybridization and/or antibody staining and were found to be two- to fourfold higher

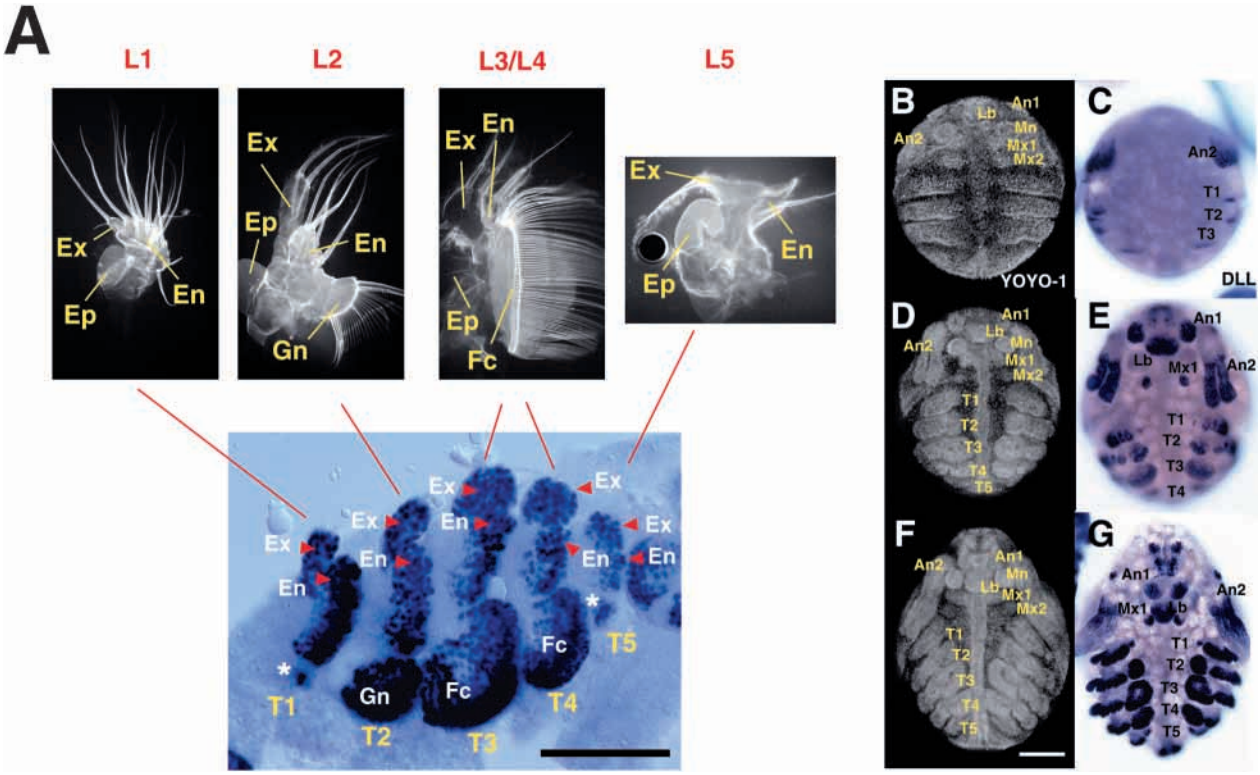

Fig. 1. Limb development in *Daphnia*. (A) First to fifth trunk limbs of *Daphnia* and their relationship to DLL expression. Epipod (Ep), exopod (Ex), endopod (En) and gnathobase (Gn) or filter comb (Fc) are arranged in a dorsal-to-ventral orientation. Endites of second to fourth leg bear prominent comb-like 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 (Obreshkove 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.

than that of the endogenous *Antp* gene. The *y w* 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-GGATTCAGACSCTGGAGCTGGAGAARGA-3′ and 5′-TCCGG-ATCCCACTTCATGCGCCGRTTCTGRAACCA-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).

Construction of mutant *Antp* genes

Mutant *Antp* genes were constructed by a series of PCR based site-directed mutagenesis, and after confirmation by DNA sequencing, cloned into pUAST (Brand and Perrimon, 1993) for germline transformation. Detailed protocols are available upon request.

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

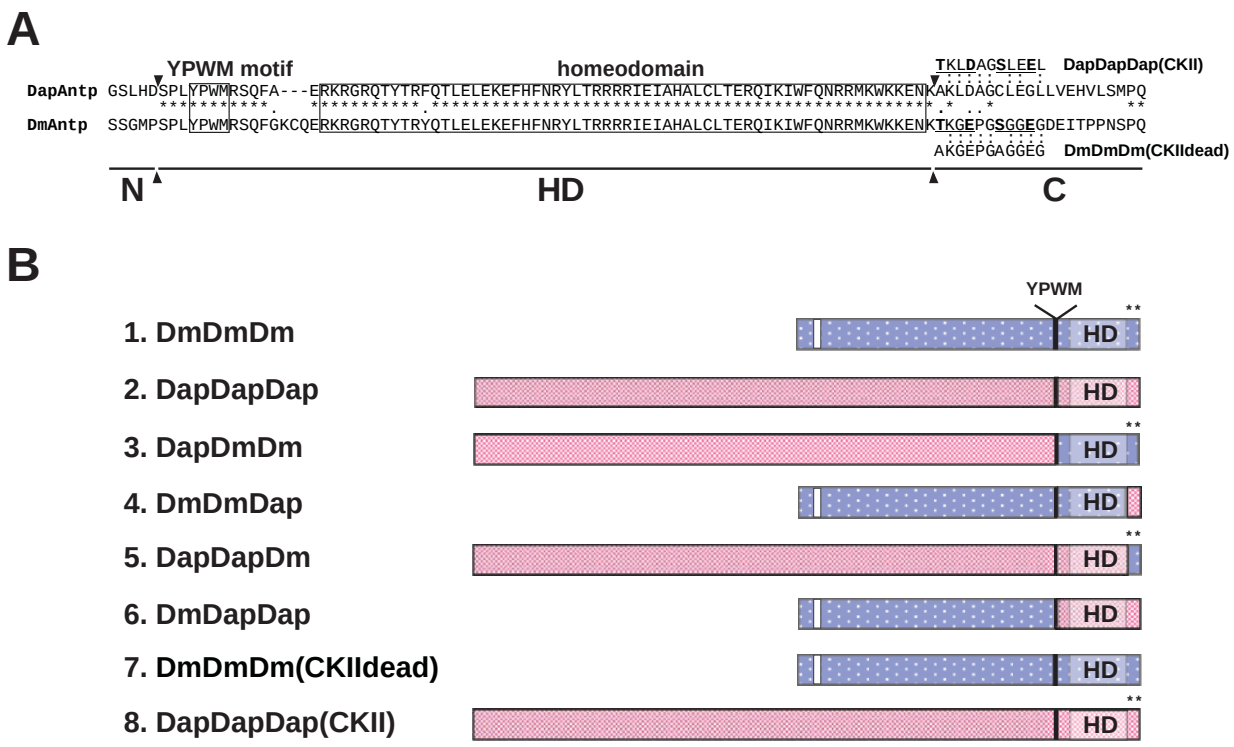

Fig. 2. Structures of ANTP. (A) An alignment of amino acid sequences around YPWM motifs and homeodomains (blocked) of DapANTP (DDBJ Accession Number, AB069680) and DmANTP (cDNA G1100) (Laughon et al., 1986). Identical amino acid residues are indicated by asterisks and conserved ones by dots. Borders of N, HD and C regions are indicated. Amino acid sequences matching the consensus CKII phosphorylation sites are underlined and diagnostic amino acid residues are indicated in bold. (B) Structures of native and mutant ANTP constructs used in this study. Amino acid sequences derived from DapANTP are shown in pink and those from DmANTP in blue. Black bars, blocks and the open box indicate the YPWM motifs, homeodomains and the homology found in several insects, respectively. Asterisks indicate putative CKII sites in the C^{Dm}.

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 does 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

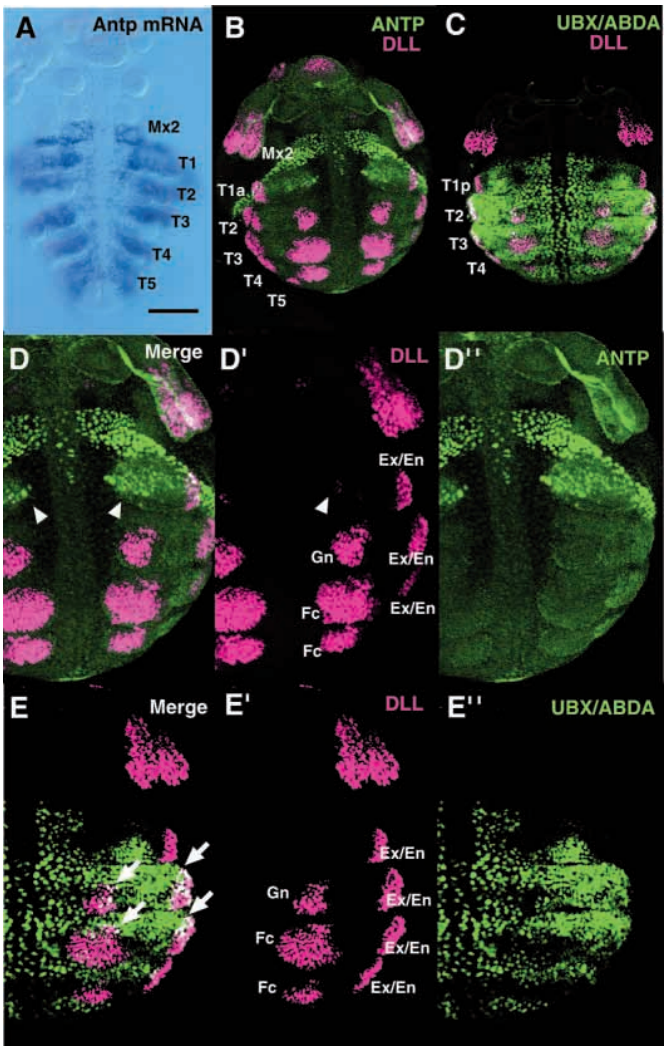

Fig. 3. Expression of ANTP and UBX/ABDA, and their relationship to DLL. (A) Antp mRNA was expressed in Mx2 and more posterior segments. (B-E) Confocal images of embryos double labeled with DLL (purple in B,C,D,D′,E,E′) and ANTP (green in B,D,D″) or UBX/ABDA (green in C,E,E″) (green). (B,D) ANTP expression was limited to Mx2 and anterior of first thoracic segment (T1a). Most of the DLL expression in T1 did not overlap with ANTP. Note that prominent expression of ANTP in ventral T1 corresponds to reduced expression of DLL (arrowheads in D). The dorsal cells expressing DLL are located in a different cell layer expressing ANTP but appear white in some cells because of the projection of multiple confocal sections. (C,E) UBX/ABDA expression was detected in dorsal part of T1 and in posterior segments. In the ventral region, the anterior staining border is at the posterior edge of the first thoracic segment, abutting the posterior border of ANTP. As reported in other crustaceans, millipedes and insects (Panganiban et al., 1995), UBX/ABDA are co-expressed with DLL in a large number of leg cells that appear white in the merged image. Scale bar: 100 μm.

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

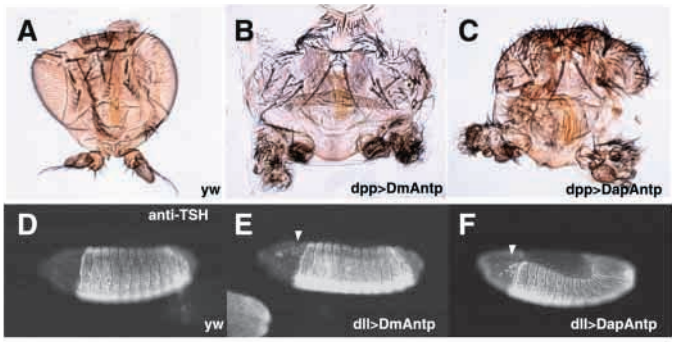

Fig. 4. DapANTP specifies thoracic identities in the *Drosophila* head. (A-C) Transformation of adult heads into thorax by ectopic expression of ANTP. Dorsal head views of (A) wild-type *y w* strain, (B) *dppGal4; UAS-DmAntp* and (C) *dppGal4; UAS-DapAntp* flies. Transformation of antennae to legs characterized 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).

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 the leg and of the dorsal head to the thorax similar to those caused by DmANTP (Schneuwly et al., 1987) (Fig. 4A-C). When misexpressed in embryonic heads, it induced ectopic expression of the trunk-specific gene *teashirt* (*tsh*) (Fasano et al., 1991) (Fig. 4D-F), demonstrating that DapANTP is a functional homolog of DmANTP.

Daphnia ANTP has novel activities in thoracic segments of *Drosophila*

Unexpectedly, expression in the thoracic region revealed activities unique to DapANTP. When Antp P1 promoter was used to drive expression, DapANTP inhibited development of the ventral thorax (Fig. 5; Ant*P*>DapAntp). Larvae showed various defects in ventral epidermis, including reduction of ventral denticle belts, loss of Keilin's organ and loss of ventral cuticle (Fig. 5C). Interestingly, the defects were biased toward the ventral side, even when another driver (*ptc-Gal4*) that promoted equal levels of expression in both sides was used (data not shown). Dorsal landmarks such as dorsal hairs and dorsal black dots formed normally. An identical defect was observed in a DmANTP null mutant background (data not shown), and DmANTP expressed in an identical condition

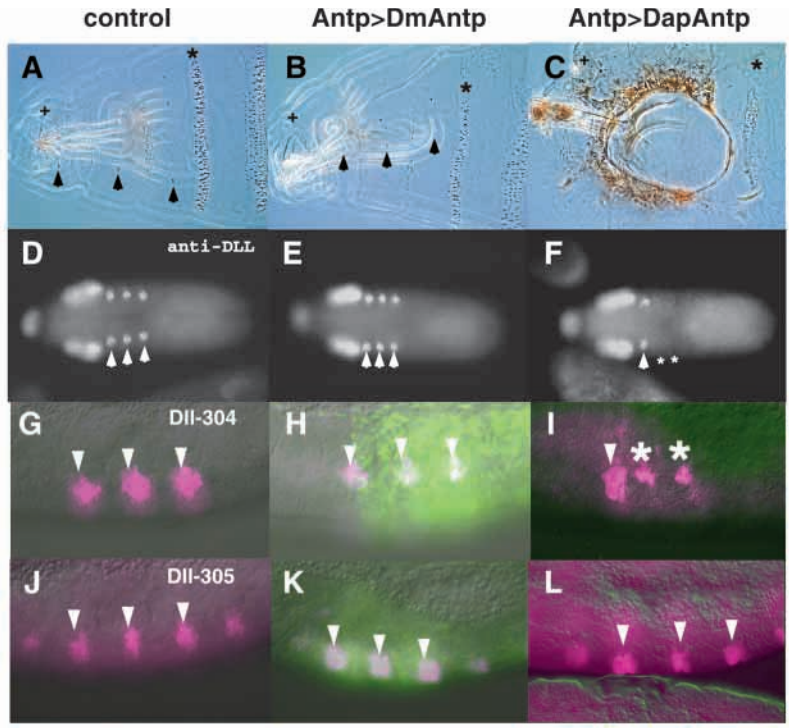

Fig. 5. Repression of *Drosophila* limb development by DapANTP. Genotypes (B,E,H,K) *AntpGal4; UAS-DmAntp*; (C,F,I,L) *AntpGal4; UAS-DapAntp*. (A-C) Larval cuticle phenotypes. (B) Mild type II phenotype (see Table 1 legend), (C) Severe type V phenotype. Positions of Keilin's organ, T1 and A1 denticle belts are indicated by arrowhead, cross and asterisk, respectively. (D-F) DLL expression (arrowheads). DapANTP eliminated DLL in T2 and T3 (asterisks in F). (G-L) DapANTP represses *Dll* through a DNA element that is a target for *Drosophila* UBX and ABDA. (G-I) Expression pattern of *Dll-304* (purple). It reproduces DLL expression in the thorax (G) and is repressed by DapANTP (I, asterisks). (J-L) Expression of *Dll-305* was not affected by either DmANTP (K) or DapANTP (L). In H and K, DmANTP was stained green to indicate the domain of ANTP expression that overlaps with T2 and T3. Leg primordia are indicated by 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>DapAntp 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 N^{Dm} with N^{Dap} conferred the *Daphnia*-specific activity (compare constructs 1 and 3 in Table 1), and a reciprocal replacement of N^{Dap} 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 (N^{Dap}). Analyses also revealed a negative effect of C^{Dm} on *Daphnia*-specific

Table 1. Functional analyses of ANTP proteins

Construct	TSH induction	Antenna-to-leg transformation	Cuticle pattern*	DLL repression†
DmDmDm	+	+++	II	–
DapDapDap	+	++	V	+++
DapDmDm	+	++	IV	++
DmDmDap	+	++	II	+
DapDapDm	+	+	I	–
DmDapDap	+	++	III	+
DmDmDm (CKII dead)	+	++	II	–
DapDapDap (CKII)	+	++	III	++

*Definition of cuticle phenotypes is as follows: type I, poor development of Keilin's organs (KOs); type II, more than one KO hair is lost and T2/T3 denticle belts are slightly reduced (see Fig. 5B); type III, loss of KO formed and great reduction of T2/T3 denticle belts; type IV, small cuticular holes observed in ventral thorax; type V, large hole in ventral thorax (see Fig. 5C). Note that even with type V phenotype in the ventral thorax, dorsal structures (including dorsal black dots and dorsal hairs) were normal.

†Strength of the phenotypes is expressed from – (normal DLL expression) to +++ (complete elimination of DLL in T2 and T3).

activity when combined with the rest of DapANTP (construct 5). In C^{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^{Dap} to create CKII sites compromised DapANTP activity (construct 8), suggesting that CKII phosphorylation inhibits DapANTP activity. However, addition of C^{Dap} to DmANTP, or mutations of C^{Dm} to disrupt CKII sites failed to provide *Daphnia*-specific activity to DmANTP (constructs 4, 7). Taken together, the results suggest that N^{Dap} is a major determinant of *Daphnia* specific activity of ANTP to repress Dll, and C^{Dm} can interfere with this activity, possibly through phosphorylation of CKII sites. N^{Dap} is two times as long as N^{Dm} and does not contain blocks of obvious sequence homology, except for a short region at the N terminus. In two places, HD^{Dap} differs from HD^{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 evolutional 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 evolutional alterations that have generated segmental diversities of the crustacean limb.

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REFERENCES

Abzhanov, A. and Kaufman, T. C. (2000). Crustacean (malacostracan) Hox genes and the evolution of the arthropod trunk. *Development* **127**, 2239-2249.

Andrew, D. J., Horner, M. A., Petitt, M. G., Smolik, S. M. and Scott, M. P. (1994). Setting limits on homeotic gene function: restraint of Sex combs reduced activity by teashirt and other homeotic genes. *EMBO J.* **13**, 1132-1144.

Averof, M. and Akam, M. (1995). Hox genes and the diversification of insect and crustacean body plans. *Nature* **376**, 420-423.

Averof, M. and Patel, N. H. (1997). Crustacean appendage evolution associated with changes in Hox gene expression. *Nature* **388**, 682-686.

Bermingham, J. R. J. and Scott, M. P. (1988). Developmentally regulated alternative splicing of transcripts from the Drosophila homeotic gene Antennapedia can produce four different proteins. *EMBO J.* **7**, 3211-3222.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brusca, R. C. and Brusca, G. J. (1990). *Invertebrates*. Sunderland, MA: Sinauer Associates.

Carroll, S., Grenier, J. and Weatherbee, S. (2001). From DNA to Diversity-Molecular Genetics and the evolution of anima design. Oxford: Blackwell Science.

Casares, F. and Mann, R. S. (1998). Control of antennal versus leg development in Drosophila. *Nature* **392**, 723-726.

Estrada, B. and Sanchez-Herrero, E. (2001). The Hox gene Abdominal-B antagonizes appendage development in the genital disc of Drosophila. *Development* **128**, 331-339.

Fasano, L., Roden, L., Core, N., Alexandre, E., Vola, C., Jacq, B. and Kerridge, S. (1991). The gene teashirt is required for the development of Drosophila embryonic trunk segments and encodes a protein with widely spaced zinc finger motifs. *Cell* **64**, 63-79.

FlyBase (1999). The FlyBase database of the Drosophila genome projects and community literature. *Nucleic Acids Res.* **27**, 85-88.

Galant, R. and Carroll, S. B. (2002). Evolution of a transcriptional repression domain in an insect Hox protein. *Nature* **415**, 910-913.

Glicksman, M. A. and Brower, D. L. (1988). Expression of the Sex combs reduced protein in Drosophila larvae. *Dev. Biol.* **127**, 113-118.

Goto, S. and Hayashi, S. (1997). Cell migration within the embryonic limb primordium of Drosophila revealed by a novel fluorescent method to visualize mRNA and protein. *Dev. Genes Evol.* **207**, 194-198.

Grenier, J. K., Garber, T. L., Warren, R., Whitington, P. M. and Carroll, S. (1997). Evolution of the entire arthropod Hox gene set predated the origin and radiation of the onychophoran/arthropod clade. *Curr. Biol.* **7**, 547-553.

Jaffe, L., Ryoo, H. D. and Mann, R. S. (1997). A role for phosphorylation by casein kinase II in modulating Antennapedia activity in Drosophila. *Genes Dev.* **11**, 1327-1340.

Kelsh, R., Weinzierl, R. O., White, R. A. and Akam, M. (1994). Homeotic gene expression in the locust Schistocerca: an antibody that detects conserved epitopes in Ultrabithorax and abdominal-A proteins. *Dev. Genet.* **15**, 19-31.

Laughon, A., Boulet, A. M., Bermingham, J. R. J., Laymon, R. A. and Scott, M. P. (1986). Structure of transcripts from the homeotic Antennapedia gene of Drosophila melanogaster: two promoters control the major protein-coding region. *Mol. Cell. Biol.* **6**, 4676-4689.

Lewis, E. B. (1978). A gene complex controlling segmentation in Drosophila. *Nature* **276**, 565-570.

Li, X. and McGinnis, W. (1999). Activity regulation of Hox proteins, a mechanism for altering functional specificity in development and evolution. *Proc. Natl. Acad. Sci. USA* **96**, 6802-6807.

Mashiko, K. and Ito, T. (1951). Occurrence of Daphnia magna Straus in Japan. *Jpn. J. Limnol.* **15**, 88-91.

Obreshkove, V. and Fraser, A. W. (1940). Growth and differentiation of Daphnia magna eggs in vitro. *Biol. Bull.* **78**, 428-436.

Panganiban, G., Irvine, S. M., Lowe, C., Roehl, H., Corley, L. S., Sherbon, B., Grenier, J. K., Fallon, J. F., Kimble, J., Walker, M. et al. (1997). The origin and evolution of animal appendages. *Proc. Natl. Acad. Sci. USA* **94**, 5162-5166.

Panganiban, G., Sebring, A., Nagy, L. and Carroll, S. (1995). The Development of crustacean limbs and the evolution of arthropods. *Science* **270**, 1363-1366.

Ronshaugen, M., McGinnis, N. and McGinnis, W. (2002). Hox protein mutation and macroevolution of the insect body plan. *Nature* **415**, 914-917.

Schneuwly, S., Klemenz, R. and Gehring, W. J. (1987). Redesigning the body plan of Drosophila by ectopic expression of the homoeotic gene Antennapedia. *Nature* **325**, 816-818.

Sullivan, W., Ashburner, M. and Hawley, R. S. (2000). Drosophila *Protocols*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Tokishita, S., Shiga, Y., Kimura, S., Ohta, T., Kobayashi, M., Hanazato, T. and Yamagata, H. (1997). Cloning and analysis of a cDNA encoding a two-domain hemoglobin chain from the water flea Daphnia magna. *Gene* **189**, 73-78.

Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M. E., Botas, J. and Cohen, S. M. (1992). Homeotic genes in of the bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene Distal-less. *Cell* **71**, 437-450.

Warren, R. W., Nagy, L., Selegue, J., Gates, J. and Carroll, S. (1994). Evolution of homeotic gene regulation and function in flies and butterflies. *Nature* **372**, 458-461.

Weatherbee, S. D., Nijhout, H. F., Grunert, L. W., Halder, G., Galant, R., Selegue, J. and Carroll, S. (1999). Ultrabithorax function in butterfly wings and the evolution of insect wing patterns. *Curr. Biol.* **9**, 109-115.