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[SHORT COMMUNICATION]

Embryonic Expression of a Hemichordate *distal-less* Gene

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ABSTRACT—Hemichordates occupy a critical phylogenetic position among deuterostomes because they exhibit echinoderm-like larval morphology and chordate-like adult morphology. Analyses of the expression and function of hemichordate developmental genes will therefore provide insight into the evolution of deuterostome body plans. The *distal-less/dlx* gene encodes a homeodomain transcription factor and plays roles in the development of appendages and the brain in a variety of animals. Here we have characterized a *distal-less* gene (*Pf-dlx*) of the hemichordate *Ptychodera flava*. During embryogenesis, *Pf-dlx* is expressed in the whole aboral ectoderm of the blastula and gastrula. Later, its expression appears in several cells in the boundary region between the oral and aboral ectoderm. The tornaria larvae express *Pf-dlx* in some specific cells of the ciliary band. The results are discussed in terms of an ancestral function of the *distal-less/dlx* gene in the formation of the nervous system.

INTRODUCTION

Our present knowledge of the molecular mechanisms of evolutionary developmental processes has mainly been obtained from investigations using model animals such as flies (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*), fish (*Danio rerio*), frogs (*Xenopus laevis*) and mice (*Mus musucurus*). From comparative studies of these animals, a number of conserved regulatory systems have been found to play roles in putatively comparable developmental processes among such model organisms. For example, the HOM-C/Hox complex has been shown to play a central role in the antero-posterior axis formation in a variety of animals (Miller and Miles, 1993).

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Interestingly, such correspondences are seen even in the formation of structures which are not necessarily thought to involve plegiomorphy. For example, the formation of the *Drosophila* 'wing' and the chick 'wing' utilizes some shared developmental regulatory systems (Laufer *et al.*, 1997), although these structures are thought of as a good instance of evolutional convergence (see Hall, 1998). Such similarity of gene expression pattern between vertebrates and insects appears to be surprising for several reasons. One reason is that these two phyla are phylogenetically distant from each other. Another reason is that modes of their development may not have persisted well after evolving from their ancestral condition, but rather they have been altered, since most invertebrate taxa display different modes of development than vertebrate taxa, particularly concerning the initial process of cell specification (reviewed by Davidson, 1991). Therefore, in order to understand the evolution of animal body plans, we need to accumulate much more basic molecular information on the development of animals, including small phyla of marine invertebrates, because they display a major part of the biodiversity of metazoa.

Hemichordates, acorn worms, occupy a unique phylogenetic location because they exhibit echinoderm-like larval morphology as well as chordate-like adult morphology (e.g.,

Brusca and Brusca, 1990). Early embryogenesis of acorn worms is a definitive example of indirect development. Recently several developmental regulatory genes from the acorn worm *Ptychodera flava* have been characterized, and their expression patterns during embryogenesis have been investigated (Tagawa *et al.*, 1998b, 2000; Peterson *et al.*, 1999; Taguchi *et al.*, 2000; Harada *et al.*, 2000). It is obvious that better understanding of the ontogeny of hemichordates will fill a gap in our understanding of the two quite dissimilar developmental modes of echinoderms and chordates and help us to determine the evolutionary scenario of the developmental processes of all deuterostomes.

The *distal-less (dll)/dlx* gene encodes a homeodomain transcription factor (Duboule, 1994). The *distal-less* gene of *D. melanogaster* is required for the development of both appendages and the central nervous system (CNS) (Cohen, 1990). Localization of the *distal-less* gene product in developing appendages has been reported in various taxa (Panganiban *et al.*, 1997; Lowe and Wray, 1997). Vertebrates have multiple copies of *dlx* class genes as a result of tandem and clustered gene duplications (Stock *et al.*, 1996). These *dlx* genes are expressed in a variety of sites, including limb buds and forebrain (e.g., Price *et al.*, 1991; Dollé *et al.*, 1992). A single copy of the amphioxus *distal-less* gene is also expressed in the forebrain (Holland *et al.*, 1996). Thus, the *distal-less/dlx* genes share roles in the formation of the appendages and the anterior nervous system.

In the present study, we characterized the *distal-less/dlx* gene of the acorn worm *P. flava*. We report here the dynamics of its expression pattern during early embryogenesis, which are suggestive about its ancestral function.

MATERIALS AND METHODS

Embryos

Mature adult *Ptychodera flava* were collected in December at a sand bar in Kaneohe Bay, Oahu, Hawaii. Natural spawning was induced as described by Tagawa *et al.* (1998a). Fertilized eggs were allowed to develop to the desired developmental stages at room temperature in the Kewalo Marine Laboratory, Pacific Biomedical Research Center, University of Hawaii.

Molecular cloning

PCR amplification was carried out using a *P. flava* gastrula cDNA library (Tagawa *et al.*, 1998b) as template DNA. The primer sequences were as follows: DLX-F: 5'-MGNAARCCNMGNACNATHTA-3' and DLX-R: 5'-CKRTTYTGRAACCADATYTT-3' (where D = not C, H = not G, K = G or T, M = A or C, N = any, R = A or G, and Y = C or T). We screened the cDNA library by probing with the PCR fragments obtained. The probes were labelled with [^{32}P]dCTP. Several positive cDNA clones were isolated. We selected a clone which contained the longest insert and a poly A tail. Then the phage cDNA clone was converted into plasmids. Both strands of the cDNA clone were sequenced using an automated DNA sequencer (ABI PRISM, Perkin Elmer).

Sequence comparisons and molecular phylogenetic analysis

The putative amino acid sequence of Pf-Dlx was deduced from its nucleotide sequence. The amino acid sequence was aligned with sequences of related homeobox gene products. The relationship was analyzed molecular phylogenetically by means of the neighbor-joining method using PHYLIP ver. 3.5 (Felsenstein, 1993).

Whole-mount *in situ* hybridization

Whole-mount specimens were hybridized *in situ* basically as described by Tagawa *et al.* (1998b). Embryos were fixed in 4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS, pH 7.5 on ice overnight. Probes for *Pf-dlx* were synthesized following the instructions supplied with the kit (Boehringer Mannheim DIG RNA Labelling kit) and used at 0.5 μg/ml in hybridization buffer. The hybridization signal was detected using NBT and BCIP following the supplier's instructions.

Double *in situ* hybridization was preformed as described in our previous report (Shoguchi *et al.*, 2000). RNA probes for *Pf-dlx* and *Pf-otx* were synthesized, and specimens were simultaneously hybridized with a mixture of the two kinds of riboprobes labelled with digoxigenin and fluorescein, respectively. After washing, coloring reactions were made first to produce a brown *Pf-otx* signal and then to produce a red *Pf-dlx* signal.

RESULTS AND DISCUSSION

Isolation and characterization of a hemichordate *distal-less* gene

The longest cDNA clone we isolated possessed a single open reading frame, which predicted a polypeptide of 320 amino acids. The full-length nucleotide and predicted amino acid sequences of the cDNA clone are available in the database under DDBJ/EMBL/GenBank Accession Number, AB028221. We named the gene *Pf-dlx* (*P. flava dlx*).

Fig. 1. shows a comparison of the amino acid sequences of the homeodomain of Pf-Dlx with those of other *distal-less/dlx* class gene products. Based on a comparison of the amino-acid sequences, we constructed a molecular phylogenetic tree

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acorn worm Pf-Dlx    KMRKPRTIYSSLQLQQLNRRFQRTQYLALPERAELAASLGLTQTQVKIWFQNRRSKYKKVLK
mouse Dlx-1          KIRKPRTIYSSLQLQALNRRFQQTQYLALPERAELAASLGLTQTQVKIWFQNKRSKFKKLMK
mouse Dlx-2          KVRKPRTIYSSFQLAALQRRFQKTQYLALPERAELAASLGLTQTQVKIWFQNRRSKFKKMWK
mouse Dlx-3          KVRKPRTIYSSYQLAALQRRFQKAQYLALPERAELAAQLGLTQTQVKIWFQNRRSKFKKLYK
mouse Dlx-5          KVRKPRTIYSSFQLAALQRRFQKTQYLALPERAELAASLGLTQTQVKIWFQNKRSKIKKIMK
mouse Dlx-6          KIRKPRTIYSSLQLQALNHRFQQTQYLALPERAELAASLGLTQTQVKIWFQNKRSKFKKLLK
mouse Dlx-7          KLRKPRTIYSSLQLQHLDQRFQHTQYLALPERAQLAAQLGLTQTQVKIWFQNKRSKYKKLLK
amphioxus AmphiDll   KMRKPRTIYTSFQLQQLNRRFQRTQYLALPERAELAAQLGLTQTQVKIWFQNRRSKYKKLMK
Drosophila Dll       KMRKPRTIYSSLQLQQLNRRFQRTQYLALPERAELAASLGLTQTQVKIWFQNRRSKYKKMMK
                     *.*******.* **  * .***..**********.*** **************.*** **. *
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Fig. 1. Alignment of amino acid sequences of the homeobox region of Pf-Dlx with those of other Dlx orthologs. Asterisks indicate amino acids identical among all members, while dots indicate similar amino acids.

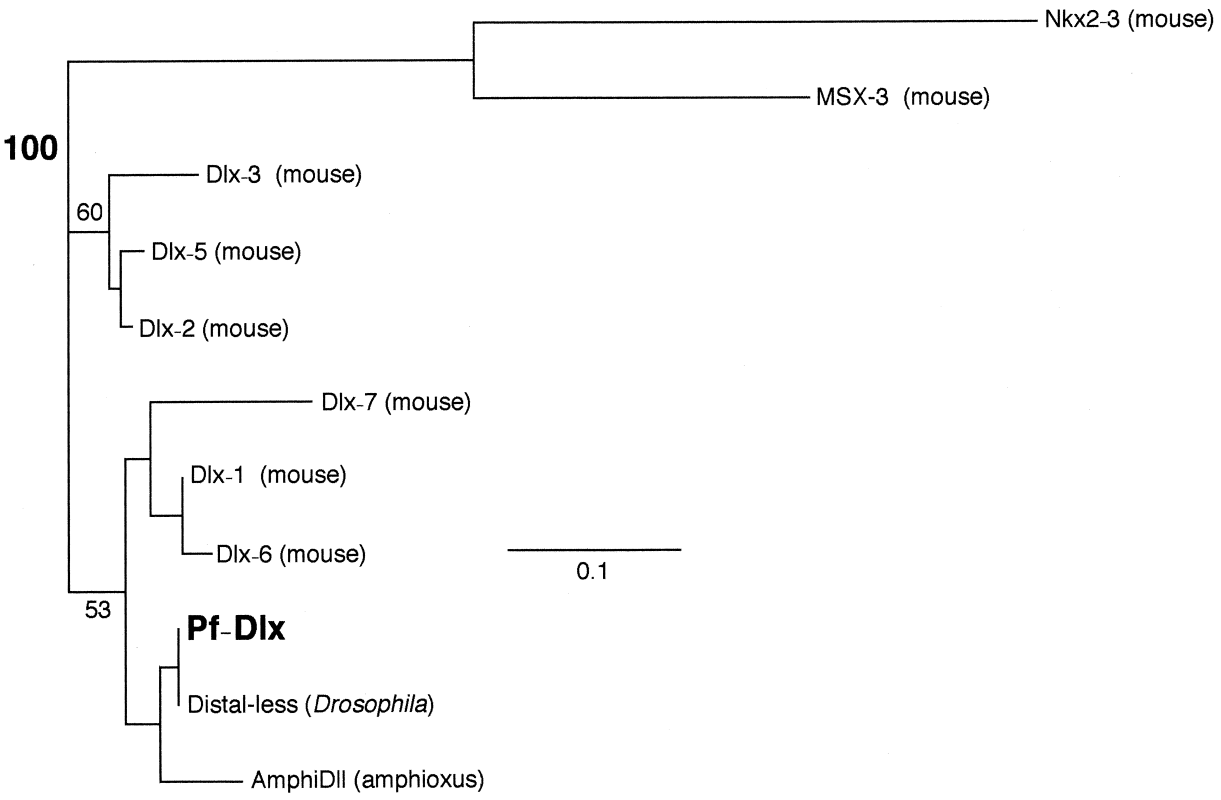

Fig. 2. Molecular phylogenetic analysis of relationships of *distal-less/dlx* subclass and related-class homeobox gene products using the homeodomain amino acid sequences. Branch lengths are proportional to evolutionary distance corrected for multiple substitutions with the scale denoting 0.1 amino acid substitutions per site. The numbers indicate the relative robustness of each node as assessed by bootstrap analysis (100 replications).

of the related homeobox gene products by using mouse *Nkx2-3* and *Msx-3* gene products as outgroups. As shown in Fig. 2, the clade of Dlx proteins, including Pf-Dlx, was supported with a 100% bootstrap value. Thus, we concluded that *Pf-dlx* is a hemichordate ortholog of the *distal-less/dlx* gene. The vertebrate *dlx* family is divided into two major subgroups as a consequence of tandem gene duplication, and each subgroup contains several subclass members (Fig. 2; Stock *et al*., 1996). However, *Pf-dlx* does not show any affinity to a specific subclass, in contrast to amphioxus *AmphiDll* and *distal-less* of *Drosophila*.

Pf-dlx is expressed in the aboral ectoderm of the blastula and gastrula and in cells of the oral/aboral boundary of the tornaria larva

The expression of *Pf-dlx* was detected as early as the blastula stage. It was expressed in half of the ectodermal region of the blastula and early gastrula (Fig. 3A, B). The *Pf-dlx*-positive half corresponds to what will become the aboral side, and *in situ* hybridization signal was stronger in the aboral side than in the oral/aboral boundary side. This expression pattern suggests that the ectoderm of the acorn worm blastula can be subdivided into oral and aboral halves, as in the case of the ectoderm of the sea urchin blastula (reviewed by Davidson *et al*., 1998). Since the tornaria larva of acorn worms and the larva of echinoderms show remarkable morphological similarity to each other, it will be interesting to examine whether the ectoderm of acorn worm embryos is specified by mechanisms similar to those for the ectoderm of echinoderm embryos (discussed in Davidson *et al*., 1998).

The expression of *Pf-dlx* in the aboral ectoderm decreased during gastrulation, except in cells on the edge of the expression domain, or else expression appeared in this region *de novo*. At the late gastrula stage, cells with strong *Pf-dl*x expression were observed in the oral/aboral boundary (Fig. 3C). Figure 3D-G shows *Pf-dlx* expression in the tornaria larva. Judging from specimens viewed from the animal pole, the aboral ectodermal expression continued at this stage (Fig. 3E), although it became weaker. *Pf-dlx* signals were evident in cells located discontinuously in the line of the oral/aboral boundary (Fig. 3D, F). This boundary may correspond to the aboral part of the post-oral ciliary band, which contains cells of the nervous system, by analogy to the sea urchin embryo (Nakajima, 1986; Cameron *et al*., 1993). Figure 3D shows an aboral view of the early tornaria; *Pf-dlx*-positive cells are buried in the epidermis, and distribution of cells with *Pf-dlx* expression in the larva is not left-and-right symmetrical (Fig. 3D). This pattern of *Pf-dlx* expression persisted in the 6-day-old larva, the last stage we observed.

The *distal-less/dlx* gene is expressed in the appendages of animals in many phyla (e.g., Panganiban *et al*., 1997). However, it is thought that the *distal-less/dlx* gene is older than the

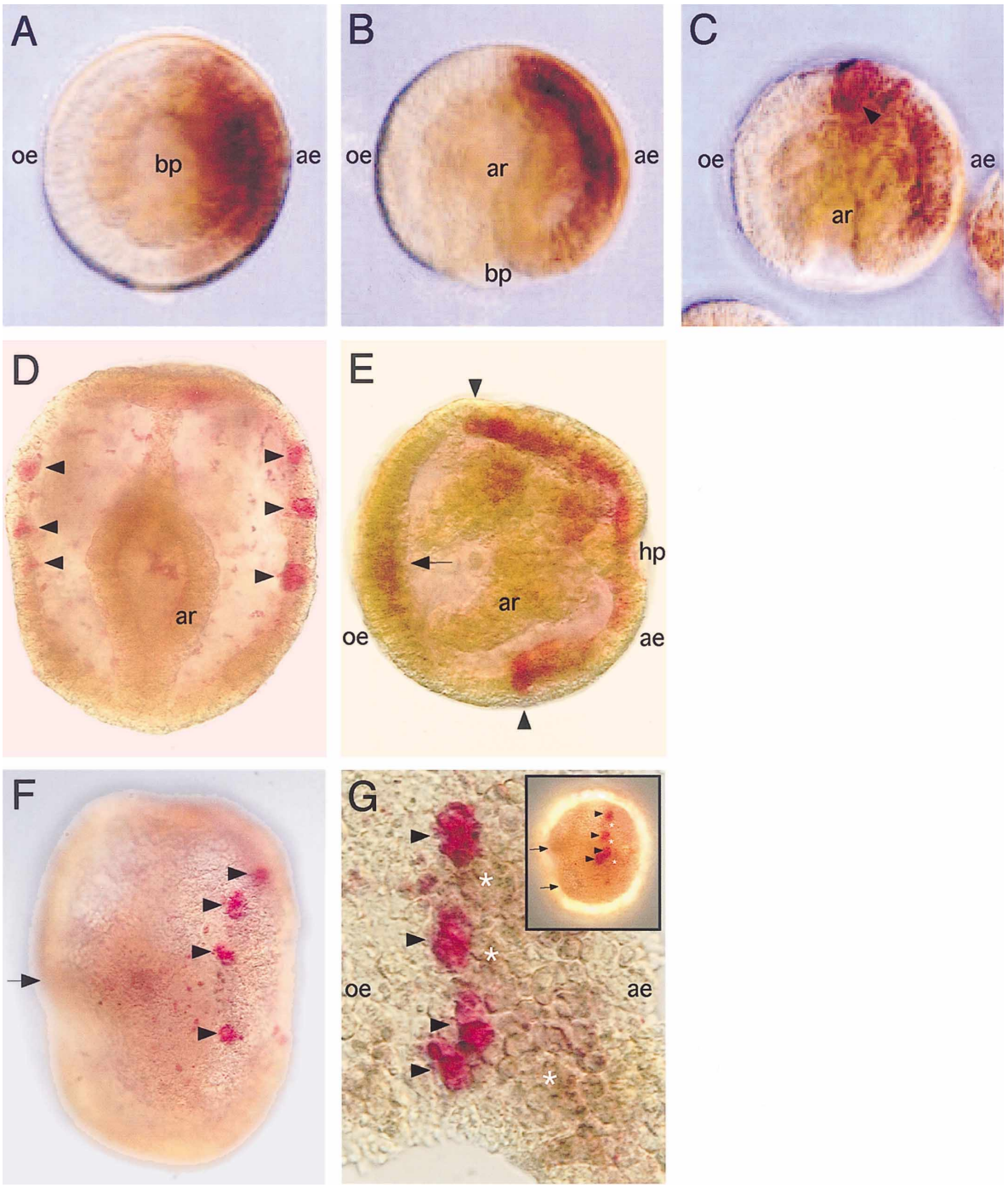

Fig. 3. Spatial expression of *Pf-dlx*. (**A, B**) An early gastrula (18 hr of development), vegetal pole view (**A**) and lateral view (**B**). ae, aboral ectoderm; ar, archenteron; bp, blastopore; oe, oral ectoderm. (**C**) A late gastrula (24 hr of development). The intensity of the signal in the aboral ectoderm decreases, while a distinct signal is detected in the oral/aboral boundary (arrowhead). (**D–F**) Simultaneous detection of *Pf-dlx* (red signals, arrowheads) and *Pf-otx* expression (brown signals, arrows) in early tornaria larvae (3 days of development), viewed from the aboral side (**D**), animal pole (**E**) and lateral side (**F**), respectively. **D** and **E** show the same larva. hp, hydropore. (**G**) An enlargement of the left lateral ectoderm region of a tornaria larva shown in the insert. Red signals of *Pf-dlx* mRNA are detected in several cells at the oral/aboral boundary, which partially overlap the region with brown signals of *Pf-otx* expression (white asterisks).

putative outgrowth-bearing ancestor. In addition, the genes are expressed in the CNS and peripheral nervous system, including parts of the brain involved in optic function (Price *et al.*, 1991; Kaphingst and Kunes, 1994). The *distal-less/dlx* gene is also expressed in the CNS of nematode embryos (Panganiban *et al.*, 1997). Therefore, it has been suggested that the ancestral function of the gene is associated with the CNS formation. Although the function of *Pf-dlx* in the ciliary band remains to be elucidated, the pattern of its expression suggests its role in the nervous system formation. We recently

found that the hemichordate *otx* gene (*Pf-otx*) is expressed along the ciliary band (Harada *et al*., 2000). To determine whether the expression domains of *Pf-dlx* and *Pf-otx* are overlapping, larvae were double-hybridized with two probes, one for *Pf-dlx* and the other for *Pf-otx*, and the relative locations of the regions showing expression of these genes were monitored simultaneously. As shown in Fig. 3G, cells with *Pf-dlx* expression were located within the region of cells with *Pf-otx* expression. However, it was not determined whether individual cells express both *Pf-dlx* and *Pf-otx* simultaneously. These molecular probes can be used as tools for examining further a putative relationship between the ciliary bands of the tornaria larva and the CNS of chordates, an issue which has long been debated by numerous researchers (Garstang, 1928; Crowther and Whittaker, 1992; Nielsen, 1999).

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