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Short communication

Presence of *Proboscipedia* and *Caudal* Gene Homologues in a Bivalve Mollusc

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Homeobox genes encode a family of transcription factors that have essential roles in regulating the development of eukaryotes. Although they have been extensively studied in different phyla, relatively little is known about homeobox-containing genes and their function in molluscs. In this study, we used a polymerase chain reaction to investigate homeobox genes in the bivalve mollusc *Pecten maximus*. Four different homeobox sequences were identified; two were homologues of the non-Hox cluster gene *caudal* and the two remaining sequences had a significant homology to the ANT-C gene *proboscipedia*. These sequences represent the first *cad* and *pb* homologues isolated from a member of the class Bivalvia, phylum Mollusca.

Keywords: *Caudal*, Homeobox, Mollusc, *Pecten maximus*, *Proboscipedia*

Introduction

Homeobox genes encode a family of transcription factors characterized by the homeodomain, a DNA-binding motif extensively conserved throughout the Metazoa, and it is encoded by a short 180 bp DNA fragment, the homeobox (Bürglin, 1994; Zanetti *et al*., 2004). The homeotic genes are master control genes that have an essential role in the regulation of bilaterian development and they share this characteristic DNA segment, the homeobox (Gehring, 1987).

Molluscs (excepting the Cephalopoda) display a similar pattern of development despite the rich diversity of adult morphologies (Wilbur, 1983), however, very little is known about the developmental mechanisms in molluscs. The genome of molluscs is not as well described as are those of model bilaterian organisms, although in recent years several homologues of developmental genes have been identified (Wray *et al*., 1995; Lee *et al*., 2001; Callaerts *et al*., 2002). In fact, the DNA-binding motif from homeobox genes has been very useful for isolating gene homologues in different animals on the basis of sequence similarity. Identification of these genes helps in the construction of the history of this conserved gene family, and this allows gene sequences to be analyzed for their evolutionary relationship.

The homeotic gene *proboscipedia* (*pb*) is a member of the Antennapedia Complex (ANT-C) that also contains the genes *labial* (*lab*), *Deformed* (*Dfd*), *Sex combs reduced* (*Src*) and *Antennapedia* (*Antp*). In insects, *pb* is required for the specification of both antennae and mouthparts in adults (Percival-Smith *et al*., 1997). The action of *pb* is unusual because it does not confer segment identity, but rather it acts to modify structures in the segments. Pb is unique among the homeotic proteins, as it possesses Val-47 in place of Ile. One potential target of Pb protein is the *Antp* gene (Bürglin, 1994). *Src* and *Dfd* act as positive regulators of *pb* (Rush and Kaufman, 2000). *Dfd* and *lab* function in the development of the head in both embryonic and adult flies (Diederich *et al*., 1991).

The *caudal* (*cad*) gene is a maternal effect gene that encodes a homeobox-containing transcription factor that has a conserved role in regulating posterior development. The Cad function in *Drosophila* and its expression pattern in vertebrates indicate that this gene family is important in the axial pattern formation (Epstein *et al*., 1997). Homologues of *pb* and *cad* genes have been reported in different taxa including sepiolid squids (*Euprymna scolopes*) (Callaerts *et al*., 2002) or abalones (*Haliotis rufescens*) (Degnan and Morse, 1993), but no *cad* or *pb* gene fragments have been reported in bivalve molluscs.

The aim of the present study was to analyze the homeobox-containing genes of the bivalve mollusc *Pecten maximus* (Linnaeus), and we demonstrated the presence of *proboscipedia* and *caudal* gene homologues using a degenerate polymerase chain reaction.

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Materials and methods

Adult scallops (*Pecten maximus*) were collected from Ría de Arousa, Galicia, Spain. For the polymerase chain reaction, we used a pair of degenerate oligonucleotide primers that correspond to homeodomain consensus sequences from helix 1 and 3: 5'-ATGCGGATCCAGACSYTGGARYTGGARAARGARTTYCWY-3' and 5'-ATGCAAGCTTCATSCKNCGRTTYTGRAACCARAT YTTNAY-3' (Amersham Biosciences, Barcelona, Spain). Genomic DNA was extracted from the muscle mass of ten adult scallops using CTAB (cetyltrimethylammonium bromide) (Ausubel *et al*., 1992). A sample of the DNA (200 ng) was amplified in 50 µl of a total PCR mixture containing 1x PCR buffer, 2 mM $MgCl_2$, 0.5 µM dNTPs (Roche Molecular Biochemicals, Basel, Switzerland), 1 µM of each primer and 2 U Taq DNA Polymerase (GIBCO BRL, Grand Island, USA). The cycling conditions were one cycle at 95ºC (5 min), 30 cycles at 95ºC (1 min), 40ºC (1 min), 70ºC (30 s) and a final 10-min incubation at 70ºC. A PCR amplification technique resulted in two predominant 170- and 130-bp fragments that were excised from a 3% MS-8 agarose (Pronadisa, Spain) gel, and then these fragments were incubated for 10 min at 80ºC in 100 µl of water. A sample aliquot (1 µl) of this mixture was reamplified as described above. The reamplification products were cloned by a pGem-T Easy Vector System II (Promega, Madison, USA) and the DNA of the individual clones was obtained by the alkaline lysis method (Sambrook *et al*., 1989). The DNA was double-strand sequenced using an ABI Prism Rhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA).

(A)

Gene		[accession#]		% identity
vox2		[AJ575210]	FNKYLCRPRRIEIAASLDLTERQ	100
DmHMPB	Pb	[P31264]	----------	100
SsHXB2	Pb	[P09638]	----------V----L-------	91
hHXB2	Pb	[P14652]	----------V----L-------	91
mHXA2	Pb	[P31245]	----------V----L-------	91
mHXA1	Lab	[P09022]	-----T-A--V------Q-N-T-	74
hHXA1	Lab	[P49639]	-----T-A--V------Q-N-T-	74
XlHXA1	Lab	[Q08821]	-----T-A--V----A-Q-N-T-	70
Hrox3			----------	100
Hrox5			-----T-A-------A-G-N-T-	74
vox1		[AJ575209]	----------FAGSHRKT	65

(B)

Gene		[accession#]		% identity
vox5		[AJ575213]	YSRYITIRRKSELAQTLALSERQ	100
DmHMCA	Cad	[P09085]	T----------S-----	91
hCDX2	Cad	[Q99626]	----------A---A--G-----	87
mCDX2	Cad	[P43241]	F----------A--G-----	87
Hrox2			----------A---NQ-Q-----	83
LsCDX	Cad	[P81193]	------MN--A---KS-D-T---	70
vox4		[AJ575212]	------V----------	96

Fig. 1. Alignment of derived amino acid sequences of *Pecten maximus* homeoboxes with other known metazoan sequences. (A) Deduced amino acid translation of vox1 and vox2 against several Pb and Lab proteins. (B) Deduced amino acid translation of vox4 and vox5 against several Cad proteins. Dashes indicate amino acid identity to vox2 or vox5. EMBL-EBI accession numbers follow gene names. Dm, *Drosophila melanogaster*; Hr, *Haliotis rufescens*; h, human; Ls, *Lineus sanguineus*; m, mouse; Sa, *Salmo salar*; Xl, *Xenopus laevis*.

Results and Discussion

Following PCR amplification of the *P. maximus* DNA, we were able to recover 4 unique homeobox gene fragments from a total of 14 informative sequences (designated vox1, vox2, vox4 and vox5 for "vieira" homeobox; EMBL-BI accession numbers AJ575209, AJ575210, AJ575212 and AJ575213 respectively). The homeobox sequence vox5 was found within six clones. Four clones contained an identical homeobox gene fragment (designated vox4) and the other vox sequences were represented twice in the 14 clones sequenced. A direct sequencing of uncloned PCR products was not undertaken because the original product represented a mixture of homeobox sequences. The PCR amplification products yielded 69 bp of novel sequence information after the primers were excluded, corresponding to homeodomain amino acid positions 22 to 44. Two of these sequences appear to be homologues of the non-Hox cluster gene *caudal* (vox4 and 5) and we were also able to identify two sequences with significant homology to *proboscipedia* genes (vox1 and 2).

All the Vox derived amino acid sequences have invariant residues Val, Ala, Leu and Leu at positions 25, 35, 38 and 40 respectively, except for an Ala and a Ser substitution for Leu at positions 38 and 40, respectively, in Vox1. Positions 26, 31 and 34 are also well conserved. The two groups of vox sequences show a low divergence among the members of each group.

Our search for related sequences in the public databases was performed using the Blast option with the EBI server. A comparison of the vox1 and 2-deduced homeodomains with previously reported Pb proteins reveals a high level of conservation within this homeodomain throughout the Metazoa (Fig. 1a). Vox1 shows limited similarity to Pb and Lab proteins (a 65% similarity to the *Drosophila* Pb protein and a 52% similarity to the mouse or human Lab protein). Vox2 shows a higher level of homology with *Drosophila*, mouse or human Pb homeodomains (they diverge by 0% and 9% respectively). The identification of *proboscipedia* orthologs from molluscs has proven difficult (Callaerts *et al*., 2002) although a study of the gastropod *Haliotis* suggested the presence of a Hox2 paralog (Degnan and Morse, 1993); in fact, Vox1 and Vox2 display significant similarity to the Hrox3 and 5 sequences. Alignment of the Vox4 and Vox5-derived amino acid sequences reveals the greatest similarity to members of the Cad protein family (Fig. 1b). Vox4 shows a higher divergence (13%) than the Vox5 sequence (9%), as compared with the *Drosophila* Cad protein. Vox4 and 5 show a high similarity to *Haliotis* Hrox2 (Degnan and Morse, 1993), and they represent the first *caudal* gene fragments reported in the class Bivalvia, phylum Mollusca.

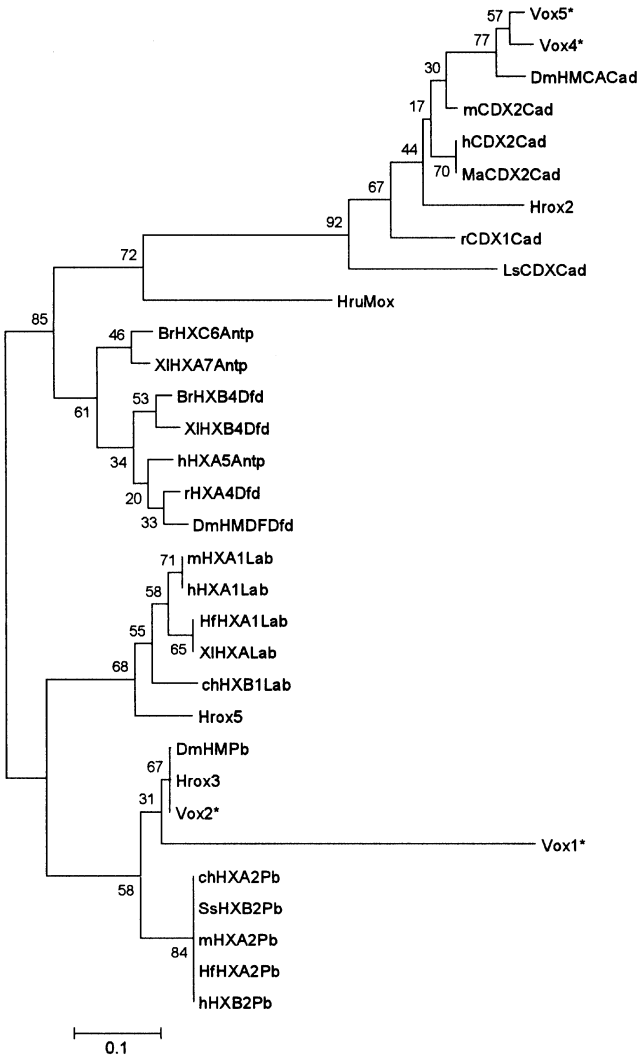

Fig. 2. Phylogenetic analysis of *P. maximus* homeobox fragments using aneighbour-joining analysis. Existing representative sequences from Deuterostome and Protostome clades were selected for comparison. The scale shows the number of amino acid substitutions per site. The Vox sequences are marked by asterisks. Taxa abbreviations are as in Fig. 1; Br, *Brachydanio rerio*; ch, chick; Hf, *Heterodontus francisci*; Ma, *Mesocricetus auratus*; r, rat.

A multiple alignment of selected sequences was constructed by Clustal W and a phylogenetic analysis of *Pecten* sequences was carried out by the neighbour-joining method (Saitou and Nei, 1987). The reliability of the resulting evolutionary tree was tested by a bootstrap analysis using 100 replications. The unrooted tree (Fig. 2) supports the finding that Vox4 and Vox5 belong to the Cad class of homeotic proteins and this assigned Vox2 to the same clade as Pb proteins. Vox1 could be included in either pb or lab clades. A Parsimony analysis yielded similar groupings, as did a distance analysis (data not shown).

The conservation of *pb* and *cad* genes in molluscs is surprising given the evolutionary distance of (Mollusca) from the other Bilaterian phyla. The identification of homeobox-containing genes and their targets, which are involved in the formation and function of animal body plans, will contribute to our understanding of the basic mechanisms of development, and this will permit a detailed study of the role of these genes in the morphological diversification within the phylum Mollusca.

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