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The *Hox8* of the hemichordate *Balanoglossus misakiensis*

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ABSTRACT

Deuterostomes comprise a monophyletic group of animals that include chordates, xenoturbellids, and the Ambulacraria, which consists of echinoderms and hemichordates. The ancestral chordate probably had 14 *Hox* genes aligned linearly along the chromosome, with the posterior six genes showing an independent duplication compared to protostomes. In contrast, ambulacrarians are characterized by a duplication of the posterior *Hox* genes, resulting in three genes known as *Hox11/13a*, *Hox11/13b*, and *Hox11/13c*. Here, we isolated 12 *Hox* genes from the hemichordate *Balanoglossus misakiensis*, and found an extra *Hox* gene that has not been reported in hemichordates. The extra *B. misakiensis* gene was suggested to be *Hox8* from paralog-characteristic residues in its hexapeptide motif and homeodomain and a comparison with *Strongylocentrotus purpuratus* *Hox* genes. Our data suggest that the ancestor of echinoderms and hemichordates may have had a full complement of 12 *Hox* genes.

Key words: Ambulacraria, *Balanoglossus misakiensis*, Echinoderm, Hemichordate, *Hox* gene

INTRUDUCTION

Deuterostomes consist of four phyla: Chordata, Echinodermata, Hemichordata, and Xenoturbellida (Bourlat et al. 2006). Studies of molecular phylogeny, larval morphology, and the adult heart/kidney complex suggest that echinoderms and hemichordates are sister taxa, forming a group known as the Ambulacraria (Smith et al. 2004; Swalla and Smith 2008). Since hemichordates share gill slits, an endostyle, and a post-anal tail with chordates, the deuterostome/chordate ancestor has been argued to have been a benthic wormlike creature (Brown et al. 2008).

The *Hox* complex is a duplicated set of genes that frequently occurs in a single cluster on the chromosome and controls spatial patterning mechanisms along the anteroposterior axis in bilateral animals (Carroll 1995). The ancestral chordate probably had 14 *Hox* genes aligned linearly along the chromosome, with the posterior six genes showing an independent duplication compared to protostomes (Amemiya et al. 2008; Kuraku et al. 2008). In the sea urchin *Strongylocentrotus purpuratus*, three anterior *Hox* genes have been translocated in an inverse orientation to the 5' end of the cluster, which lacks *Hox4* (Cameron et al. 2005). The loss of *Hox4*, however, appears to be a derived state in sea urchins, since Long et al. (2003) and Hara et al. (2006) isolated a *Hox* gene encoding Hox4-characteristic residues from the asteroid *Patiriella exigua* and the crinoid *Metacrinus rotundus*, respectively. In contrast, 11 and eight *Hox* genes, respectively,

have been isolated from the hemichordates *Saccoglossus kowalevskii* and *Ptychodera flava* (Aronowicz and Lowe 2006; Peterson 2004). Peterson (2004) found that ambulacrarians have four posterior *Hox* genes, *Hox9/10*, *Hox11/13a*, *Hox11/13b*, and *Hox11/13c*; *Hox9/10* is shared by deuterostomes, whereas the *Hox11/13* groups are specific to hemichordates and echinoderms.

We previously described the development of the hemichordate *Balanoglossus misakiensis* (Urata and Yamaguchi 2004). In this study, we amplified 12 *Hox* genes by PCR and RACE from *B. misakiensis*, and found an extra *Hox* gene that has not been reported in hemichordates. The extra *B. misakiensis* gene was suggested to be *Hox8* from paralog-characteristic residues in its hexapeptide motif and homeodomain and a comparison with *Strongylocentrotus purpuratus* *Hox* genes. Our data suggest that the ancestor of echinoderms and hemichordates may have had a full complement of 12 *Hox* genes.

MATERIALS AND METHODS

Animals

Adult *B. misakiensis* were collected at Masuho-ga-ura Beach, Ishikawa, Japan. After artificial insemination, embryos were cultured at 23-25°C as described previously (Urata and Yamaguchi, 2004).

Isolation of the *B. misakiensis* *Hox* gene fragments

Hox genes from *B. misakiensis* were PCR-amplified using genomic DNA as the template with three degenerate primers: Hox-F1, 5'-CARYTNACNGARYTNGARAA-3' coding for QLTELEK; Hox-F2, 5'-YTNGARYTNGARAARGARTT-3' coding for LELEKEF; and Hox-R, 5'-TTCATNCKNCKRTTYTGRAA-3' coding for FQNRRMK. The *BmHox11/13b* cDNA fragment was amplified by RT-PCR using total RNA from *B. misakiensis* larvae with a Hox11/13b-specific primer and Hox-R. The Hox11/13b-F primer was designed based on the N-terminal flanking region of the homeodomain, the sequence of which (5'-ACNTTYACNACNACC-3') codes for TFTTTP. Total RNA was extracted using Sepasol I Super (Toyobo) from the regenerating bud of an adult or larva (Table 1). The 3' ends of the *BmHox1*, *BmHox2*, *BmHox3*, *BmHox4*, *BmHox9/10*, *BmHox11/13a*, and *BmHox11/13c* cDNAs were isolated using the 3' RACE System for Rapid Amplification of cDNA Ends (Gibco). The 3' ends of *BmHox5*, *BmHox6*, *BmHox7*, *BmHox8*, and *BmHox11/13b*, and the 5' end of *BmHox3*, *BmHox4*, *BmHox5*, *BmHox6*, *BmHox7*, and *BmHox8* were isolated using a GeneRacer Kit (Invitrogen). The primers used were as follows: BmHox1-F, 5'-TCGACGTGTTGAAATAGCCGCCATGTT-3'; BmHox2-F, 5'-GGAGTTTAATTATAACAGATATCTCTGCAG-3'; BmHox3-F, 5'-AACAGATATCTGCAAAAATCACGCCGAGA-3'; BmHox3-R, 5'-ATGTCGTCGTATTTGTACAGTTTCT-3'; BmHox4-F,

5'-TCACTTCAATCGATACTTGACCAGGAGACG-3'; BmHox4-R,

5'-TCTTGTTTTTCGTGTTTGGAAGGTTGTGAT-3'; BmHox5-F, 5'-

TCACTTCAACCGGTACCTCACACGCA-3'; BmHox5-R,

5'-ATTCTTGGGATCCTCCATTAGCTGTGATAT-3'; BmHox6-F, 5'-

TCACTTTAACCGTTACCTGACGCGACG-3'; BmHox6-R,

5'-TGACTTCTGGTGTTTCGTGCGAACGGTTTTGA-3'; BmHox7-F, 5'-

AATCGAACTGTCGCACCTTCTCGGA-3'; BmHox7-R, 5'-CAGTTTCACTGTCATCTTTCTTGCT-3';

BmHox8-F, 5'-ACGGATCGAAATCTCACAGATTGTGGGA-3'; BmHox8-R,

5'-GAAGCTAACACGTCGCCTGTCGTTC-3'; BmHox9/10-F,

5'-ATTTGACACGGGAAAGGAGAGTGGAAATAT-3'; BmHox11/13a-F,

5'-TTTGTACAATATGTACCTGACCAGGGACC-3'; BmHox11/13b-F, 5'-

GCGAACAAAGCGACGTCCGTACTC-3'; and BmHox11/13c-F,

5'-TTCCAACAAAACATGTACTTGACGCGCGA-3'. The sequences have been deposited in the DNA

Data Bank of Japan (DDBJ) under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

We amplified 12 *Hox* gene fragments by PCR from *B. misakiensis*, and the 3' end of each cDNA as well as the 5' end of five medial *Hox* plus *Hox3* cDNAs were isolated by RACE (Table 1). Figure 1 shows alignments of the deduced homeodomain sequences and C-terminal flanking regions of *Hox1*, *Hox2*, *Hox3*, *Hox4*, *Hox5*, *Hox6/Hox6/8*, *Hox7*, *Hox9/10*, *Hox11/13a*, *Hox11/13b*, and *Hox11/13c* from the hemichordates *S. kowalevskii* and/or *P. flava* with six full and five partial sequences of the *B. misakiensis* *Hox* genes. The boxes in Figure 1 indicate paralog-characteristic residues conserved between *Drosophila* and vertebrate Hox members (Sharkey et al. 1997). From the alignment, the 11 *B. misakiensis* *Hox* genes were inferred to correspond to *Hox1*, *Hox2*, *Hox3*, *Hox4*, *Hox5*, *Hox6/Hox6/8*, *Hox7*, *Hox9/10*, *Hox11/13a*, *Hox11/13b*, and *Hox11/13c* of *S. kowalevskii* and/or *P. flava*, respectively. When compared to *P. flava*, the putative *B. misakiensis* *Hox1*, *Hox4*, *Hox5*, *Hox6/Hox6/8*, *Hox9/10*, *Hox11/13a*, *Hox11/13b*, and *Hox11/13c* were nearly identical to the *P. flava* Hox proteins, in terms of both their homeodomain sequences and their C-terminal flanking regions. Most of the sequences of the homeodomains and/or C-terminal flanking regions were conserved in the *S. kowalevskii* Hox proteins, although *S. kowalevskii* *Hox11/13b* sequence was fairly diverged (Fig. 1). When compared to *S. kowalevskii* *Hox2*, *Hox3*, and *Hox7*, a *B. misakiensis* Hox similar to *S. kowalevskii* *Hox2* was identified with two *Hox2*-characteristic residues in

its homeodomain, whereas another *B. misakiensis* Hox with a Hox3-related homeodomain sequence shared a Hox3-specific glycine residue in its C-terminal flanking region of the homeodomain. The other *B. misakiensis* Hox was identical to *S. kowalevskii* Hox7 in its homeodomain sequence and the C-terminal flanking four residues. Therefore, we tentatively assigned the 11 *B. misakiensis* genes to *BmHox1*, *BmHox2*, *BmHox3*, *BmHox4*, *BmHox5*, *BmHox6*, *BmHox7*, *BmHox9/10*, *BmHox11/13a*, *BmHox11/13b*, and *BmHox11/13c*, respectively, although their order on the chromosome is unknown.

We isolated an extra medial *Hox* gene fragment from *B. misakiensis* in addition to the 11 assigned *Hox* genes. Because Hox6, Hox7, and Hox8 include almost no paralog-characteristic residues in either their homeodomains or flanking regions, it is generally difficult to infer the paralogous groups to which these three *Hox* genes belong based on their sequences. Indeed, when analyzed by a neighbor-joining method (CLUSTAL W) using the homeodomain sequences plus both the N-terminal and the C-terminal flanking six residues (a total of 72 residues) of medial Hox members from chordates, echinoderms, hemichordates, and *Drosophila*, Hox6, Hox7, or Hox8 members, including supposed *B. misakiensis* Hox8, did not form a paralog-specific clade even in deuterostomes, although *Hox4* and *Hox5* members constituted a monophyletic and a paraphyletic bilaterian clades, respectively (Fig. 2). However, in the present case, the ambulacrarian Hox proteins included residues that implied orthology. Figure 3 shows alignments of the

deduced homeodomain sequences and flanking regions encoded by *Hox6*, *Hox7*, and/or *Hox8* from the mouse *Mus musculus*, the lancelet *Branchiostoma floridae*, the sea urchin *S. purpuratus*, the sea lily *M. rotundus*, and the hemichordates *S. kowalevskii* and *P. flava* with the sequences of the *B. misakiensis* *Hox* genes. When compared to *S. purpuratus* *Hox6*, *Hox7*, and *Hox8*, which are ordered linearly on the chromosome (Cameron et al. 2005), the ambulacrarian *Hox7* and supposed *Hox8* genes, respectively, encoded several residues that were shared by paralogous ambulacrarian groups, but were not conserved in chordates, in their hexapeptide motifs and/or homeodomains (boxes in Fig. 3). Based on these ambulacrarian-characteristic residues and a comparison with the *S. purpuratus* *Hox* genes, we designated the extra *B. misakiensis* gene *BmHox8*, although the gene number may not reflect the gene order. Pendleton et al. (1993) reported an unidentified *S. kowalevskii* *Hox* sequence (accession number Q26523; homeodomain positions 21–47) that included two substitutions shared by the supposed ambulacrarian *Hox8* (Fig. 3). From these observations, we propose that hemichordates have a complete set of medial *Hox* genes comparable to chordates, at least in number, together with three anterior and four posterior *Hox* genes (Fig. 4). Based on the *Hox* complements from sea lily, feather star, star fish, and sea cucumber, the common ancestor of echinoderms is parsimoniously inferred to have had a complete set of anteromedial *Hox* genes (Hara et al. 2006; Long and Byrne 2001; Long et al. 2003). Thus, the ambulacrarian ancestor may have had

a *Hox* cluster similar to one of the hemichordates in the complement. The *Saccoglossus kowalevskii* Genome Project being undertaken by Genamics GenomeSeek (<http://genamics.com/>) will reveal the *Hox* gene order and illuminate the ancestral *Hox* cluster of the deuterostome ancestor as well as its chordate origins.

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FIGURE LEGENDS

Fig. 1. Alignments of the deduced homeodomain sequences and C-terminal flanking regions of *Hox* genes from the hemichordates *S. kowalevskii* (Sk) and/or *P. flava* (Pf) with six full and five partial sequences of the *B. misakiensis* (Bm) *Hox* genes. Dots indicate conserved residues between the indicated organism and *S. kowalevskii*, whereas the dash indicates a gap (inserted for Hox9/10 alignment). Asterisks denote stop codons. Boxes indicate paralog-characteristic residues conserved between *Drosophila* and the vertebrate Hox members (Sharkey et al., 1997). The accession numbers are as follows: *SkHox1*, AAP79296; *SkHox2*, ABK00018; *SkHox3*, AAP79286; *SkHox4*, AAP79297; *SkHox5*, ABK00019; *SkHox6*, ABK00020; *SkHox7*, AAP79287; *SkHox9/10*, ABK00021; *SkHox11/13a*, ABK00022; *SkHox11/13b*, ABK00023; *SkHox11/13c*, AAP79288; *PfHox1*, AAR07634; *PfHox4*, AAR07635; *PfHox5*, AAR07636; *PfHox6/8*, AAR07637; *PfHox9/10*, AAR07638; *PfHox11/13a*, AAR07639; *PfHox11/13b*, AAR07640; and *PfHox11/13c*, AAR07641.

Fig. 2. Neighbor-joining tree constructed from the homeodomain sequences plus both the N-terminal and the C-terminal flanking six residues (a total of 72 residues) of medial Hox members from chordates,

echinoderms, hemichordates, and *Drosophila*. Hox3 genes act as outgroups. The numbers on the branches are bootstrap values higher than 50% over 1,000 replications. Hox6, Hox7, or Hox8 members did not form a paralog-specific clade even in deuterostomes, whereas Hox4 and Hox5 members constituted a monophyletic and paraphyletic bilaterian clades, respectively. The accession numbers are as follows: *MmHoxa6*, NP_034584; *MmaHox7*, NP_034585; *MmbHox8*, NP_034591; *BfHox6*, ABX39490; *BfHox7*, ABX39491; *BfHox8*, ABX39492; *SpHox6*, GLEAN3_05171; *SpHox7*, GLEAN3_02634 and GLEAN3_05170; *SpHox8*, GLEAN3_21309; *MrHox7*, BAF43725; and *MrHox8*, BAF43726.

Fig. 3. Alignments of the homeodomain sequences and flanking regions encoded by *Hox6*, *Hox7*, and/or *Hox8* from the mouse *Mus musculus* (Mm), the lancelet *Branchiostoma floridae* (Bf), the sea urchin *S. purpuratus* (Sp), the sea lily *M. rotundus* (Mr), *S. kowalevskii* (Sk), and *P. flava* (Pf) with the sequences of the *B. misakiensis* (Bm) *Hox* genes. The sequences between the homeodomains and hexapeptide motifs have been omitted. Identical residues based on the *M. musculus* sequences are indicated by dots. Compared to *M. musculus* and *B. floridae*, the ambulacrarian Hox7 and supposed Hox8 proteins include several paralog-characteristic residues shared by ambulacrarians, but not conserved between chordates and ambulacrarians, in their hexapeptide motifs and/or homeodomains (boxes). An unidentified *S. kowalevskii*

Hox sequence (accession number Q26523) included two ambulacrarian-Hox8-characteristic residues. The

accession numbers not listed in Figs. 1 and 2 are as follows: *MmHoxb6*, NP_032295; *MmHoxc6*,

NP_034595; *MmbHox7*, NP_034590; *MmcHox8*, NP_034596; *MmdHox8*, NP_032302.

Fig. 4. Deuterostome taxonomy and *Hox* phylogeny. A phylogenetic tree for three higher taxa and three hemichordate species is shown on the left. The known (solid line) or presumed *Hox* clusters for each taxon or species are shown to the right of the taxonomic tree.

Table 1. *Hox* genes isolated from *B. misakiensis*

Gene	Template for 3'RACE (5'RACE)	DDBJ accession no.
<i>BmHox1</i>	regenerating bud of adult	AB426592
<i>BmHox2</i>	regenerating bud of adult	AB426593
<i>BmHox3</i>	regenerating bud of adult (larva)	AB426594
<i>BmHox4</i>	regenerating bud of adult (larva)	AB426595
<i>BmHox5</i>	larva (larva)	AB426596
<i>BmHox6</i>	larva (larva)	AB426597
<i>BmHox7</i>	larva (larva)	AB426598
<i>BmHox8</i>	larva (larva)	AB426599
<i>BmHox9/10</i>	regenerating bud of adult	AB426600
<i>BmHox11/13a</i>	regenerating bud of adult	AB426601
<i>BmHox11/13b</i>	larva	AB426602
<i>BmHox11/13c</i>	regenerating bud of adult	AB426603

	homeodomain	c-terminal
SkHox1	PANGRTNFTNKQLTELEKEFHFNKYLTRRRVEIAAMLGLNETQVKIWFQNRRMKKKRF	KDGPSYSHS
PfHox1	.N.....	.EPAFGQGI
BmHox1		.EPTFGQGI
SkHox2	HRRVRTAFTNTQLLELEKEFHFNKYLTRRRRIEIASMLDLSERQVKVWFQNRRMKHKRQI	MKAAVSGSP
BmHox2	N..R.....E.....V	L.VGSGETS
SkHox3	IKRMRTNFCTAQLVELEKEFRFNRYLQKQRTELSVLNLTERRQIKVWFQNRRMKFKKEM	KDKGLVDPY
BmHox3	P...S..S.....S..E...VI.....V.....V	RER...SEKL
SkHox4	HKRTRTAYTRYQVLELEKEFHFNRYLTRRRRIEIAHALGLTERQIKIWFQNRRMKWKDHD	NLPNTKTKS
PfHox4	V.....	
BmHox4	V.....	
SkHox5	AKRSRTAYTRYQVLELEKEFHFNRYLTRRRRIEIAHALGLSERQIKIWFQNRRMKWKKEH	NVKSISQIM
PfHox5		L.
BmHox5		L.
SkHox6	QRRGRQTYTRYQVLELEKEFHFNRYLTRRRRIEIAHTLGLTERQIKIWFQNRRMKWKKEQ	KTGGAPSPK
PfHox6/8	A.....	.MTDSKPF
BmHox6	A.....	.MTDSKPF
SkHox7	KKRCRQTYTRYQVLELEKEFHFNRYLTRRRRIELSHLLGLTERQIKIWFQNRRMKYKES	KKDDGENSN
BmHox7		...S.TGS
SkHox9/10	GRKKRCPTYKFQVLELEKEFLFNMYLTRRRVDIARLLNLTERQVKIWFQNRRMKLKKQN	QRNAT-MLH*
PfHox9/10	E.S.....	H..TIC.I.*
BmHox9/10	E.S.....	H..TMA.I.*
SkHox11/13a	NRKKRKPYTKYQVLELEKEFLFNMYLTRDRRTDIARALNLSEKQIKIWFQNRRMKLKKMR	LREENERKQ
PfHox11/13a	S....T...V.....	
BmHox11/13a	S....T...V.....	
SkHox11/13b	RRTKRKPYTKMQUIFELEQAFQNNMYLTRRRRTKLSQQLSLSEKQIKIWFQNRRMKLKKMT	EREKLEEKE
PfHox11/13b	R..S....Y...K...Q.A.....Q.Y...N.T...V.....S..QV	...M....
BmHox11/13b	R..S....Y...K...Q.A.....Q.Y...N.T...V.....S..L	...M....
SkHox11/13c	RRTKRPPYTKLQIFELEKEFQNNMYLTRDRRSRLSQALNLSEKQIKIWFQNRRMKLKKMT	DRERNEQDM
PfHox11/13c	T..A.T.....V.....	E..HS..EV
BmHox11/13c	T...T.....V.....	E..HS..EV

Fig. 1

Fig. 2

	hexapeptide	homeodomain	c-terminal
MmHoxa6	VYPWMQ	GRRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIANALCLTERQIKIWFQNRRMKWKKEN	KLINSTQAS
MmHoxb6		Y.....H.....	..LSAS..L.
MmHoxc6	I.....	R.....I.S.....	N.TSTLSGG
BfHox6	.F...R	KK.....K...K.....HL.G.....	.IPSLNATT
SpHox6	F...K	.K.....Q.....S.V...F...QS.G.S.....	R.H GSNC.MTNQ
SkHox6	I...R	Q.....L.....HT.G.....	Q .TGGAPSPK
PfHox6/8	I...R	Q.....L.....H.G.....	Q .MTD.KPFD
BmHox6	I...R	Q.....H.G.....	Q .MTD.KPFD
MmHoxa7	IYPWMR	RKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEH	KDESQAPTA
MmHoxb7		Y.....T.....	N .TSGPGT.G
BfHox7		K.....	N .L..LKQQP
SpHox7	G...P	...C.....LS.L.G.....Y...S	.NKEEGGSG
MrHox7	A...N	...C.....LS.L.G.....Y...N	.KDGVTDE
SkHox7	L...N	K..C.....Y.....LS.L.G.....Y...S	.KDDGENSN
BmHox7	L...VN	K..C.....Y.....LS.L.G.....Y...S	.KDDSETGS
MmHoxb8	LFPWMR	RRRGRQTYTRYQTLELEKEFLFNRYLTRRRRIEIVSHALGLTERQVKIWFQNRRMKWKKEN	NKDKFPSSK
MmHoxc8	M.....	..S.....	...L.GAR
MmHoxd8	M.....	F.....T.A.....	A.R
BfHox8	FY....	H..K...R...IA.....I.....L...A	AMLCP.KAE
SpHox8	VYN..K	.K.....T.A.....HY.R.....IAQ.VC.S..I.....R	VR.GAGDDE
MrHox8	VYN..K	KK.....T.....H..R...R...IAQ.VC.S..I.....A...T	SR.ADE.AD
BmHox8	MYG..K	K.....F.....H..Q.....I.QIV..S..I.....Q...G	K.ENLNTNI
Sk Q26523		HY.Q.....IAQMN.....I..	

Fig. 3

Fig. 4