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# Restricted expression of a median Hox gene in the central nervous system of chaetognaths

Daniel Papillon, Yvan Perez, Laurent Fasano, Yannick Le Parco, Xavier Caubit

**Abstract** *Hox* genes encode a set of evolutionarily conserved transcription factors that regulate anterior–posterior patterning. Here we report the first developmental expression of a *Hox* gene from Chaetognatha. These metazoans have been shown recently to be part of the protostome group of bilaterians. We describe the analysis of the *ScMed4* gene (a *Spadella cephaloptera* Median *Hox* gene) including its expression from late stages of egg development to 7 days after hatching. In all of these stages, *ScMed4* is expressed in two lateral stripes in a restricted region of the developing ventral ganglion.

**Keywords** Chaetognatha · *Hox* genes · *ScMed4* · Central nervous system · *Spadella cephaloptera*

## Introduction

Chaetognaths constitute a small marine phylum of approximately 120 species. In the last decade, their relationships within the metazoans have been strongly debated because of embryological and morphological features shared with the

two main branches of Bilateria, the deuterostomes and the protostomes (Nielsen 2001). Until now, and due to long branch attraction artefacts (see Mallatt and Winchell 2002), sequence data, including ribosomal RNA 18S (18S) (Telford and Holland 1993; Wadah and Satoh 1994; Halanych 1996; Littlewood et al. 1998; Zrzàvy et al. 1998; Giribet et al. 2000; Peterson and Eernisse 2001) and intermediate filaments (Erber et al. 1998), did not allow the chaetognaths to be classified precisely, except that they are probably not deuterostomes. The recent use of the mitochondrial genome, a powerful phylogenetic marker, indicates close relationships with the protostomes (Helfenbein et al. 2004; Papillon et al. 2004). This conclusion is supported by the study of the cerebral ganglion (Rehkämper and Welsch 1985) and by cell fate analysis showing that the four-cell stage of chaetognath embryos displays similarities with classic spiralian (Shimotori and Goto 2001). Tree topologies obtained from the sequence of mitochondrial DNA of the chaetognath benthic species *Spadella cephaloptera* also indicate that chaetognaths are distant from arthropods (Papillon et al. 2004). This hypothesis is supported by a recent report in which a specific tissue marker showed that chaetognaths are not ecdysozoans (Haase et al. 2001).

*Hox* genes are characterised by the presence of homeoboxes and are usually organized in clusters encoding for transcription factors that regulate anterior–posterior (A-P) patterning (Carroll et al. 2001). Their order within the cluster correlates with their spatial expression pattern along the A-P axis; called the “colinearity rule.” Comparative analyses of metazoan *Hox* genes have provided much information on both the genetic basis of developmental pattern formation and the evolution of patterning mechanisms. Within protostomes, most analyses showed a colinear expression of *Hox* genes in the developing central nervous system (CNS) (Hirth et al. 1998; Hughes and Kaufman 2002 for arthropods; Nardelli-Haeffliger and Shankland 1992; Kourakis et al. 1997; Irvine and Martindale 2000 for annelids; Giusti et al. 2000; Hinman et al. 2003; Lee et al. 2003 for molluscs). In deuterostomes, *Hox* genes are also involved in the patterning of the CNS (e.g. Keynes and Krumlauf 1994 for vertebrates, Wada et al. 1999 for

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amphioxus, Ikuta et al. 2004 and Seo et al. 2004 for urochordates).

Seven *Hox* genes were isolated in *S. cephaloptera*: one anterior gene (unpublished data), one member of the group of orthology Hox3, four median genes (*SceMed1-4*) and a mosaic gene that shares features of both median and posterior classes (Papillon et al. 2003). Determination of *Hox* gene expression in Chaetognatha is of importance for several reasons. First, molecular aspects of their embryonic development is poorly understood. By examining the spatiotemporal pattern of *Hox* gene expression, one can guess which role(s) the *Hox* genes may be playing in the cellular diversification along the A-P axis in an organism that does not possess iterated structures. Second, this is an organism remotely related to arthropods, probably to ecdysozoans (Papillon et al. 2004; Haase et al. 2001), and studies of *Hox* genes have to be broadened in order to encompass a wide range of bilaterians.

The work presented here is the first *Hox* gene expression profile reported for a chaetognath species. We describe the expression of the *SceMed4* gene (a *S. cephaloptera* Median *Hox* gene) in the developing ventral ganglion of *S. cephaloptera* from late stages of egg development to 7 days after hatching.

## Materials and methods

### Specimens

Adult specimens of the benthic species *S. cephaloptera* Busch 1851 were collected in the bay of La Ciotat near Marseille (South of France) on the *Posidonia oceanica* bed, at a depth of 3–5 m. In the laboratory, samples were kept in aquaria containing natural sea water and placed in a constant temperature at 21±1°C. The specimens used for experiments were at the same stage of sexual maturity (mean length ~4 mm) with well-developed ovaries and were nourished daily with *Artemia salina* nauplii. Twenty-four hours after the isolation in small dishes of mature specimens containing post-vitellogenic oocytes (i.e. fertilized oocytes) the sides and the bottom of each dish were examined. All the newly deposited eggs (measuring approximately 0.25 mm in diameter) were placed in new dishes filled with 100 ml of natural filtered sea water to observe the appearance of early hatchling specimens and their growth.

### In situ hybridizations

Using a combined strategy of PCR amplification and cDNA library screening, *SceMed4* (accession no. AY162323) and other fragments of Hox homeodomains (HDs) of *S. cephaloptera* were identified (Papillon et al. 2003).

Whole-mount in situ hybridization (WMISH) of *SceMed4* were performed at several embryonic stages: late embryos at various stages, newly hatched young (hatchling), 1 day old (1d), 2d, 3d, 4d, 5d, 6d and 7d specimens. All

specimens were fixed in 4% paraformaldehyde (PFA) in 0.1 M MOPS buffer (pH 7.5) and 0.5 M NaCl. Fixed specimens were immersed in 80% ethanol and kept at –20°C until use. Digoxigenin (DIG)-labeled RNA probes were generated using a DIG RNA labeling kit (Boehringer Mannheim). Fixed specimens were rehydrated in phosphate-buffered saline (PBS), 0.1% Tween 20 and then treated with 2 µg/ml proteinase K for 40 min at 37°C. Before proteinase K treatment, eggs of various stages were treated with 20 g/ml sodium thioglycolate (pH about 11) for 20 min as previously described in Takada et al. (2002). After proteinase K treatment, the embryos were postfixed with 4% PFA in PBS for 1 h at room temperature. Embryos were hybridized at 65°C for 16 h with the DIG-labeled probe in the hybridization buffer (50% formamide, 5× SSC, 0.1% Tween 20, 2 mM EDTA, 5 µg/ml yeast tRNA, 50 µg/ml heparin). After hybridization, two 65°C washes were performed with hybridization buffer for 30 min. Specimens were then rinsed in washing buffer (500 mM NaCl, 100 mM Tris, 0.1% Tween 20) and incubated with 100 µg/ml RNase A at 37°C to avoid non-specific staining. After a blocking step using 10% (v/v) sheep serum in TBST [140 mM NaCl, 2.5 mM KCl, 25 mM Tris/HCl (pH 7.5), 0.1% (v/v) Tween 20] including 2 mM levamisol. The specimens were incubated with an alkaline-phosphatase-coupled antibody (1:2000) against DIG for 16 to 18 h at 4°C. The hybridized probe was visualized using NBT/BCIP.

After in situ hybridization, some specimens were post-fixed, cryoprotected in 30% sucrose, embedded in OCT (Tissue-Tek), frozen in isopentane at –40°C and cut at 25 µm on a cryostat.

## Results and discussion

Comparison of partial Hox HDs and flanking sequences of various bilaterians with *SceMed4* is shown in Fig. 1. The *S. cephaloptera* HD shares high sequence identity with several median HDs. The absence of specific residues within the HD sequence of *SceMed4* does not allow a clear orthology assignment. Moreover, no specific parapeptide at the C-terminal end of the HD (as defined in Balavoine et al. 2002) has been identified (e.g. LPNTK in Dfd/Hox4, the spiralian peptide in Lox5 or the UbdA peptide in Ubx/AbdA/Lox2/Lox4). Altogether, this sequence seems to belong to the Scr/Hox5 or Antp orthology groups.

WMISH of *SceMed4* was performed at various stages of the development of *S. Cephaloptera* (Fig. 2). The expression of *SceMed4* was initially detected when embryos are still rolled up in the egg, just before hatching (Fig. 2a). We were not able to detect expression at any earlier egg stages. Figure 2a and b show a staining on each side of the middle of the trunk in late curved embryos and hatchlings. This region corresponds to the developing ventral ganglion. Transversal sections through the region of expression in 2d specimens shows that *SceMed4* mRNA localization is restricted to the bilateral clusters of the ventral ganglion composed of the neuronal cell bodies (Fig. 2j,k). Expression is not detected in the neuropile (Fig. 2j). Although the

DmeAntp	HFNYRLTRRRRIEIAHALCLTERQIKIWFQNRMRKWKKEN	KTKGEPG
Nvidfd	-----	KLPNTKNRLS
Dmedfd	-Y-----T-V-S-----D-	KLPNTKNVRKKTIV
BflHox4	-----S-G-----D-	RLPNTKTRSS
MmuHoxc4	-Y-----S-S-----DH	RLPNTKVRSAAPP
<b>ScMed4</b>	-----G-A-----H	<b>RMAAVHMGFQHGMDCDPLAAAHSQLMWTMQSGW</b>
DmeScr	-----H	KMASMNVPHYMG
LanScr	-----N-----Q	KLHLTKTEAMKLSIDGKPTVGLAET
FcaScr	-----S--FS-----	KMATMNALPHHHGMGPPHHMQ
EscScr	-Y-K-----N-----H	KLSHIAKNMNVCAPLDKGLDI
BflHox5	-----	KLKSL
OlaHoxB5	-----D-	KVKMSLTAPGASHFQP
EruAntp	-----	KAKLEGAGDCLTGL
LanAntp	-----	KGIELSRDSPPGVL
EscAntp	-----	KAEMPGTENGKQPQ
LsaHox7	---K-----KE	KQPVGIVCKEHEEPEP
HroLox5	YS-----S-A-S-----	NVQKLTGP
LanLox5	-Y-----H-G-----	NIPKLTGP
LsaHox6	---K-----G-----	NLQKLTGPNTKLEPQDPDTKSQSPPSD
Aloftz	-----S-G-----A---	IVDN
AkaUbx	-T-H-----M-----L--M	QTIKDLNEQEKK
AkaAbdA	---H-----V-----L--L	RAVKEINEQARR
HroLox2	K-----LS-T-Y-----E--V	QAIRELNEIEKT
LanLox4	Q--H---K---V-----L--R	QQIKELNDTLKK
MmuHoxa6	-----N-----	KLINSTQASGEDSEAKAGE
HsaHoxA7	-----H	KDEGPTAAAPPEGAVPSAAATAADKAD
BflHox8	---K-----G-----L--A	AMLCPPKAETETEKSSDQGSEKSEEV

**Fig. 1** Comparisons of known Hox homeodomains (HDs) and flanking sequences with predicted *S. cephaloptera* *ScMed4* sequence (boxed, accession no. AF458055). Note that outside the HD, *ScMed4* displays short motifs also observed in the orthology group Scr/Hox5 (MA, HGM, HMG). The *Drosophila* Antennapedia HD sequence is shown at the top. Non-identical amino acids are shown, and dashes indicate identity. Diagnostic parapeptides at the C-terminal end of the HD as defined in Balavoine et al. (2002) are highlighted in grey. *Aka*, *Acanthokara kaputensis* (Onychophora); *Alo*, *Archegozetes*

*longisetosus* (Arthropoda); *Bfl*, *Branchiostoma floridae* (Cephalochordata); *Cva*, *Chaetopterus variopedatus* (Annelida); *Dme*, *Drosophila melanogaster* (Arthropoda); *Eru*, *Ethmostigmus rubripes* (Arthropoda); *Esc*, *Euprymna scolopes* (Mollusca); *Fca*, *Folsomia candida* (Arthropoda); *Hro*, *Helobdella robusta* (Annelida); *Hsa*, *Homo sapiens* (Vertebrata); *Lan*, *Lingula anatina* (Brachiopoda); *Lsa*, *Lineus sanguineus*; *Mmu*, *Mus musculus*; *Nvi*, *Nereis virens* (Annelida); *Ola*, *Oryzias latipes* (Vertebrata); *Sca*, *Sacculina carcini* (Arthropoda)

intensity of the signal decreases, the pattern of expression observed at 2d is maintained at 3d (Fig. 2e) and throughout subsequent development (Fig. 2f–i) until 7d (Fig. 2i). At 7d, specimens resemble the adults in all aspects (except for the undeveloped gonads and smaller body size). To summarize, in all stages observed *ScMed4* is expressed in two lateral stripes in the middle of the ventral ganglion. This region always corresponds to the middle of the expanding trunk.

During development, the ganglion is generated from the presumptive ectoderm (Doncaster 1902; John 1933). This process starts in 1-day-old eggs, when the body cavities are formed and start to fill up coincident with the embryo curling up in the egg. At the onset of CNS formation, the neuronal progenitors become located in two bilaterally arranged ventral clusters. Later, the body expands but not the ventral ganglion. The ventral ganglion is then proportionally much smaller in adults than in juveniles. The neuropile, which contains the axons, lies under the intestine in the middle of the ventral side.

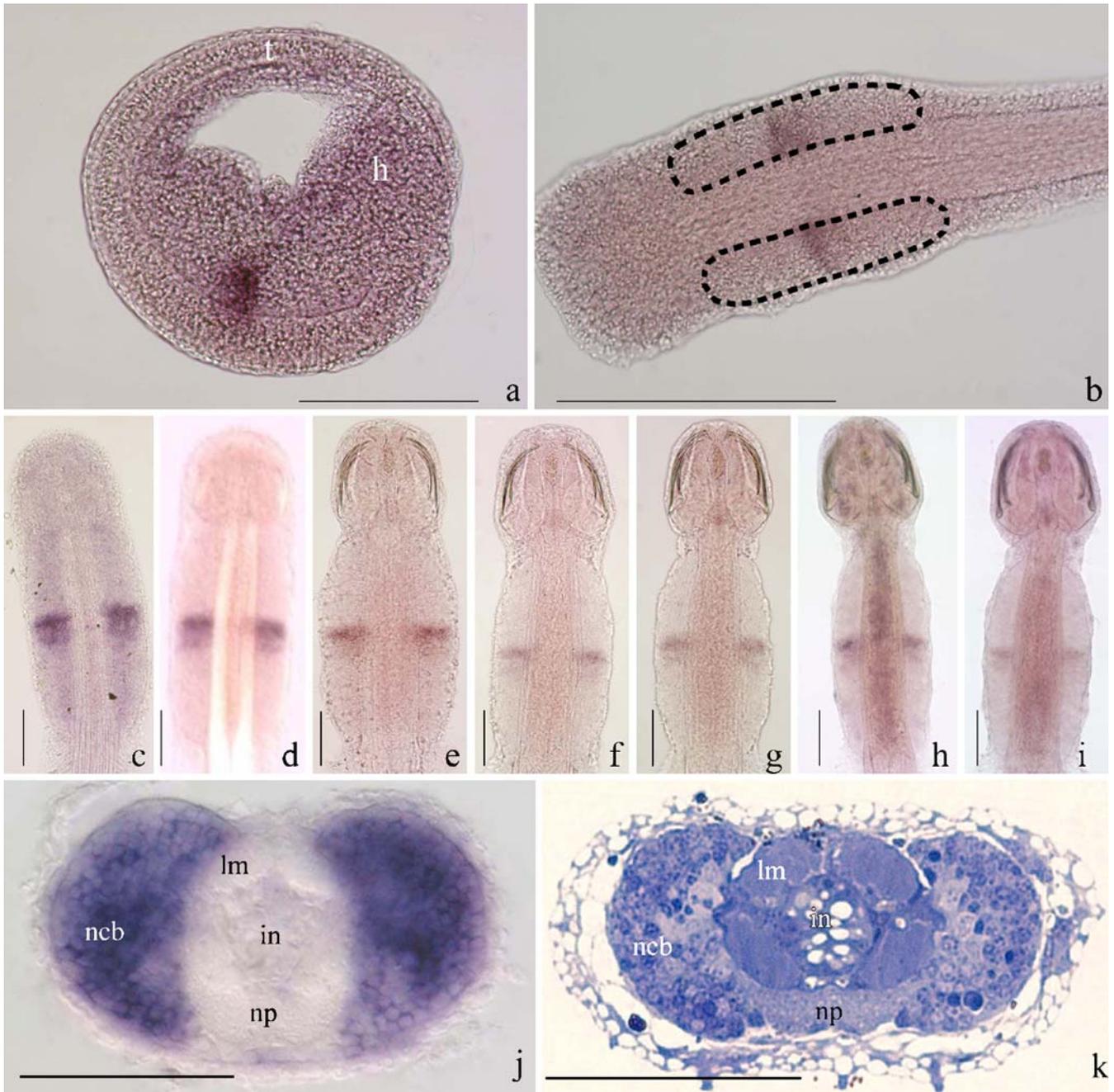
Our analysis shows that, during development, a median *Hox* gene is expressed in the trunk of chaetognaths. Furthermore, the results presented here demonstrate that *ScMed4* expression is restricted to a limited zone of the ventral CNS.

In annelids, insects and vertebrates (segmented animals) median *Hox* genes are expressed, among other locations, in the mesoderm, suggesting a function for these genes in providing segmental identity (McGinnis and Krumlauf

1992; Lawrence and Morata 1994; Irvine and Martindale 2000). In chaetognaths as well as in gastropods (Hinman et al. 2003), *Hox* expression is restricted to the developing CNS (a derivative of the ectoderm). Absence of *Hox* expression in the mesoderm of these animals could be linked to the absence of segmentally repeated mesodermal structures in adults.

The expression of median *Hox* genes, such as *Scr* and *Antp* orthologues, in the developing CNS has been reported in various bilaterian groups (e.g. Kourakis et al. 1997; Irvine and Martindale 2000 for annelids; Hinman et al. 2003; Lee et al. 2003 for molluscs; Hirth et al. 1998 for insects; Keynes and Krumlauf 1994 for vertebrates; Wada et al. 1999 for amphioxus; Ikuta et al. 2004; Seo et al. 2004 for urochordates). In these animals, *Hox* genes seem to be involved in the neuronal specification along the A-P axis.

During chaetognath development, the dynamics of cell division and differentiation of the ventral ganglion is poorly documented. However, the timing of appearance of specific neuronal cell types has been analysed during CNS formation in *Paraspadella gotoi* using immunocytochemistry (Goto et al. 1992). They identified a single serotonin-like immunoreactive (5HTLI) neuron and numerous Rfamid-like immunoreactive (RFaLI) neurons. During development, the 5HTLI neuron is first detected in the head at the 2–3d stage. In the ventral ganglion, no RFaLI neuron is detected before 1d. *P. gotoi* is a benthic species that belongs to the Spadellidae family and is therefore phylogenetically close to *S. cephaloptera*. One can consider that the devel-



**Fig. 2** Expression of *SceMed4* at various stages of *S. cephaloptera* development. **a** Embryo curved in late eggs. **b** Hatchling. *Dashed line* indicates the two bilateral clusters of the ventral ganglion. **c** One-day-old (1d), **d** 2d, **e** 3d, **f** 4d, **g** 5d, **h** 6d, **i** 7d specimens. **j** Transversal section through the region of expression in the trunk of a 2d specimen. **k** Light micrograph from transversal section through the trunk region of a 2d animal, showing the ventral ganglion com-

posed of the two bilateral masses of the neuronal cell bodies (*ncb*) and their axons that run longitudinally in the neuropile (*np*). Note the dramatic change of morphology between 2d (**d**) and 3d (**e**) specimens. *h* head, *in* intestines, *lm* longitudinal muscles, *ncb* neuron cell bodies, *np* neuropile, *t* tail. Orientation: **b** anterior is left; **c**–**i** anterior is up; **j**–**k** dorsal is up. *Scales*: **a** 0.15, **b** 0.4, **c**, **d** 0.1, **e**–**g** 0.13, **h**, **i** 0.15, **j**, **k** 0.12 mm

omplemental timing of these two species is roughly similar (e.g. hooks appear at 3d stage in both species; see Fig. 2e and Goto et al. 1992). We therefore conclude that *SceMed4* expression starts significantly earlier (before hatching) than the detection of 5HTLI and RFaLI neurons.

The position-specific expression pattern of *SceMed4* in chaetognath is typical of the coordinated expression of *Hox*

genes observed in Bilateria (Carroll et al. 2001) and suggests a potential role in regionalisation of the CNS. Consistent with such an activity is the presence of distinct neuronal populations in the ventral ganglion according to the topographic organisation of neuronal projections (Bone and Pulsford 1984; Duvert et al. 1997). Although the position of the neuronal cell bodies in the ventral ganglion in

relation to their peripheral targets needs more investigation, it is possible that *SceMed4* contributes to the diversity of neuronal subpopulations and to establishment of distinct axon projection patterns.

Our data provide the first evidence for a *Hox* gene expression in Chaetognatha. WMISH of the other *Hox* genes have to be performed in order to determine their putative implication in the morphogenesis of the chaetognaths.

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