Expression of the *labial* group Hox gene *HrHox-1* and its alteration induced by retinoic acid in development of the ascidian *Halocynthia roretzi*

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**SUMMARY**

Ascidian embryogenesis shares several developmental features with vertebrates. Thus, it is presumed that some molecular mechanisms that are critical for vertebrate development may also act in the early development of ascidians. Here, we investigated expression of the ascidian *labial* group Hox gene *HrHox-1* in the development of *Halocynthia roretzi*. *HrHox-1* showed a spatially restricted expression pattern along the anterior-posterior axis, which is remarkably similar to that of the vertebrate gene, *Hoxb-1*. The expression of *HrHox-1*, however, was exclusively in tissues of ectoderm origin unlike its vertebrate counterpart. Exposure of the embryos to 10⁻⁶ M *all-trans* retinoic acid induced a larval phenotype with elimination of the anteriormost structures, the papillae. In this phenotype, the level of *HrHox-1* expression was enhanced and ectopic expression was observed at the anterior terminal epidermis where the papillae are otherwise formed. These observations suggest that there are some conserved mechanisms in the spatial regulation of expression of *labial* group genes in embryogenesis of ascidians and vertebrates.

Key words: ascidian, Hox gene, *labial* group gene, retinoic acid, *moonfaced* phenotype, gene expression

**INTRODUCTION**

Ascidians are classified in the phylum Chordata together with cephalochordates and vertebrates. Adult ascidians, sessile filter-feeding animals, have a body plan apparently dissimilar to other members of chordates. Ascidian morphogenesis can be divided into two distinct processes. First, ascidian embryos develop into motile tadpole larvae. A larva has organs for locomotion, attachment and sensory-input and, in addition, rudimentary tissues for adult organs in its trunk in many solitary ascidians such as *Ciona* and *Halocynthia* (Garstang, 1928; Berrill, 1955; Katz, 1983). During the course of early development, ascidian embryos undergo gastrulation and neurulation to form a hollow neural tube (Conklin, 1905; Scott, 1946) and a notochord (Conklin, 1905; Cloney, 1964; Miyamoto and Crowther, 1985), which are the hallmarks of chordate embryos (Foster, 1869; Garstang, 1928; Berrill, 1955; Romer, 1971; Willmer, 1990). Second, ascidian larvae undergo metamorphosis to form juveniles. Upon metamorphosis, larval organs such as tail muscles and the notochord disintegrate and adult organs differentiate instead (Cloney, 1978). Therefore, it is expected that embryogenesis of ascidians may involve similar morphogenetic mechanisms to that of vertebrates and, by contrast, metamorphosis may involve unique ones.

In recent years, many lines of evidence have been accumulated to show that HOM/Hox genes play a central role in animal development (McGinnis and Krumlauf, 1992). The most remarkable feature of HOM/Hox genes is colinearity between their physical organization on the chromosome(s) and their expression patterns along the anterior-posterior (A-P) axis (McGinnis and Krumlauf, 1992). Colinearity has been well demonstrated with HOM/Hox genes of *Drosophila*, mouse and *Caenorhabditis elegans* (references therein, Krumlauf, 1994; Kenyon, 1994). These observations and other experimental results suggest that HOM/Hox genes are conserved through metazoans and involved in patterning and regionalization of a body along anterior-posterior axis (Slack et al., 1993).

To understand ascidian morphogenesis better, we have chosen to study HOM/Hox genes of an ascidian. Elucidation of the structure and expression of ascidian HOM/Hox genes, comparing them with those of other animals, will shed some light onto the morphogenetic mechanisms underlying the unique way in which ascidians develop.

For this purpose, we isolated Hox gene fragments from the genome of the solitary ascidian, *Halocynthia roretzi*. Here, we report the isolation and expression of an ascidian Hox gene, *HrHox-1*, which belongs to the *labial* group or paralogous subgroup 1 (Bürglin, 1994). Many genes of this group have been isolated from various species of vertebrates and a number of observations have been made on their expression in embryogenesis under normal and experimental conditions. This situation enables us to compare the expression pattern of *HrHox-1* with those of vertebrate counterparts. This may, in turn, allow us to assess the function of *HrHox-1* in the morphogenesis of ascidians.

Expression of *HrHox-1* was first observed at the neurula stage by northern analysis. Examination by whole-mount in situ hybridization revealed that *HrHox-1* was expressed in...
epidermis and the central nervous system (CNS) in a spatially restricted fashion along A-P axis. To characterize further the expression of \textit{HrHox-1} in ascidian embryogenesis, we examined effects of retinoic acid (RA) on the expression pattern of \textit{HrHox-1}. In larvae treated with RA, ectopic or enhanced expression of \textit{HrHox-1} was observed in the anterior terminal region of the CNS and epidermis. We discuss possible roles of \textit{HrHox-1} in ascidian development in the light of these findings.

**MATERIALS AND METHODS**

**Ascidians**

Adult ascidians, \textit{Halocynthia roretzi}, were purchased from fishermen near Asamushi Marine Biological Station, Tohoku University, Aomori, or Otsuchi Marine Research Center of the Ocean Research Institute, University of Tokyo, Iwate, Japan. Naturally spawned eggs were fertilized with a suspension of non-self sperm, and these eggs were raised in filtered sea water (FSW) at 11-13°C. Batches of eggs in which embryogenesis proceeded synchronously were selected for experiments. For collecting ascidians undergoing metamorphosis, batches were used in which synchronous metamorphosis began naturally or metamorphosis was induced artificially by adding 1% of Nile Blue to sea water.

**PCR amplification**

PCR was carried out using \textit{H. roretzi} genomic DNA as template to amplify \textit{Antennapedia} class homeobox gene fragments including Hox genes that belong to paralogous subgroups 1 to 10. Primers used were Antp US; T(A/G)G(C/G/A)/G(A/G)/G(T/C)/G(T/C)/G(A/G)/AAG. Each primer set was subjected to PCR amplification with an initial denaturation at 94°C, followed by 32 cycles of 94°C for 1 minute, 50°C for 1.5 minutes, and 72°C for 2 minutes. PCR products were subcloned into the \textit{Smal} site of the plasmid pBluescript KS+

**Preparation of poly(A)* RNA**

Total RNA extraction and poly(A)* RNA selection were carried out as described previously (Saiga et al., 1991).

**Northern and Southern analyses**

5 μg of poly(A)* RNA was electrophoresed on a 1% agarose gel containing 6% formaldehyde. After electrophoresis RNAs were transferred to a nylon membrane (Zeta-Probe Blotting Membranes, Bio-Rad) by capillary blotting. 2 μg of genomic DNA digested with EcoRI, HindIII or BamHI was separated on a 1% agarose gel. After electrophoresis, the gel was soaked in 0.5 M NaOH for 15 minutes and subjected to blotting onto a nylon membrane as described previously (Saiga et al., 1991).

Probe DNA fragments were labeled with [α-32P]dCTP using the Random Primer DNA Labeling Kit (Takara). Membranes were hybridized and washed in 0.1x SSC-0.2% SDS at 60°C (or in 2x SSC-0.2% SDS at 42°C in Southern genomic analysis under reduced stringency conditions) for 30 minutes twice. Membranes were exposed on a X-ray film (X-OMAT AR, Kodak) or exposed on an Imaging Plate, followed by analysis using Fujix BAS2000 Bio-imaging Analyzer (Fuji Photo Film).

**cDNA screening and nucleotide sequencing**

A cDNA library from retinoic acid-treated \textit{H. roretzi} larvae was constructed as described previously (Wada et al., 1995). The cDNA library was screened using as probe a subcloned DNA fragment that was isolated by PCR from \textit{Halocynthia} genomic DNA and identified as a part of a \textit{labial} group Hox gene. Two clones, HH-1s and HH-1f, were obtained, which have inserts of 0.6 kb and 1.8 kbp, respectively. These were subcloned in the \textit{Smal} site of the pBluescript KS+ plasmid DNA. The nucleotide sequences of HH-1s and HH-1f insert cDNA fragments were determined using a \textit{Taq} dye primer cycle sequencing kit (Applied Biosystems) and ABI 373A DNA sequencing apparatus (Applied Biosystems). The insert of HH-1s clone was found to be a part of the HH-1f insert cDNA fragment.

**Retinoic acid treatment**

Stock 0.01 M \textit{all trans retinoic acid} (RA) (Sigma) was diluted with DMSO at various concentrations and added to FSW in 0.001 volume of FSW. About 15 minutes after insemination, eggs were rinsed with FSW and suspended in fresh FSW. 100 μl of fertilized egg suspension was added to 2 ml FSW with various concentrations of RA prepared in a 24-wall culture plate. Embryos were allowed to develop in the dark at 11°C until the larva stage.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was carried out as described previously (Wada et al., 1995). In brief, the method was originally described for amphioxus embryos by Holland et al. (1992) and modified to fit ascidian embryos, in which digoxigenin-labeled RNA probes and alkaline phosphatase-conjugated antibody were used for detection of mRNA. As for probe preparation, the pBluescript plasmid DNA containing \textit{HrHox-1} cDNA (the insert of HH-1f, see above) was used as template for in vitro RNA synthesis after digestion with either BamHI or HindIII. In the final step of this procedure, specimens were incubated in coloring solution at room temperature for 2 days or 5 days (prolonged staining). For further localization of expression, whole-mount in situ hybridization specimens were dehydrated in Nile Blue to sea water.

**Fig. 1.** Comparison of the homeodomain sequences of the ascidian and other Hox genes. Deduced amino acid sequences of ascidian Hox gene fragments isolated by PCR are aligned with those of representative Hox genes of mouse and \textit{Drosophila}. The identical residues are indicated by a dash (−). Identity to the homeodomain of the ascidian Hox gene is indicated by percentage in the right column.
Ascidian *labial* group Hox gene

Fig. 2. *HrHox-1* sequence, comparison with other *labial* group genes and Southern genomic analysis of *Halocynthia* genomic DNA with *HrHox-1* probes. (A) The nucleotide sequence of a *HrHox-1* cDNA and conceptual translation is indicated by the single letter code. The homeodomain and hexapeptide are indicated by stippling and underlining, respectively. The longest open reading frame is shown: there are three possible initiating methionine residues in frame, but none of them completely fit Kozak’s consensus (Kozak, 1987). The nucleotide sequence has been deposited in the DDBJ database under accession number D49983. (B) Comparison of the amino acid sequence of the *HrHox-1* homeodomain and its flanking regions with those of other *labial* group genes; *Drosophila labial* (Mlodzik et al., 1988), mouse *Hoxa-1*, b-1, d-1 (Bürglin, 1994), amphioxus *Amphihox-1* (Garcia-Fernàndez and Holland, 1994), *C. elegance ceh-13* (Schaller et al., 1990) and hydra *cnox-1* (Schummer et al., 1992). *Drosophila Antennapedia* is also shown as an outer group gene (Schneuwly et al., 1986). Residues that are well conserved among *labial* group genes are indicated by stippling. (C) Southern genomic hybridization of *Halocynthia* genomic DNA with *HrHox-1* probes. 2 µg of DNA from one adult individual was digested with *Eco*RI (E), *Hind*III (H) or *Bam*HI (B), transferred to a nylon membrane and hybridized with a cDNA probe encompassing nucleotides 1-1768 in Fig. 2A (a) or a cDNA fragment (nucleotides 429-1768) which includes a large part of the homeobox (b), respectively. There is a unique *Eco*RI site (nucleotide 429) in the *HrHox-1* cDNA in Fig. 2A. The size marker used was *λ* DNA fragments digested with *Eco*RI and *Hind*III (H).

Fig. 3. Northern blot analysis of *HrHox-1*. Each lane contains 5 µg of poly(A)*⁺* RNA. The blots were hybridized with a *HrHox-1* cDNA probe (nucleotides 1-1768 in Fig. 2A). Heat denatured *Eco*RI and *Hind*III digested *λ* DNA was used as a size marker. (A) Early development up to the swimming larva stage. (B) Later development after metamorphosis. The day when metamorphosis began naturally or artificially was regarded as day 0 (Met. 0 Day). In the two lanes left-side, two larval RNA samples that were identical to those in panel A were loaded.
ethanol and embedded in paraffin. They were sectioned at 7 μm. After rehydration, sections were stained with DAPI, mounted in glycerol and examined with fluorescence microscope.

RESULTS

Isolation of DNA fragments of ascidian Hox genes
To isolate HOM/Hox type genes from the ascidian Halocynthia roretzi, we carried out PCR using degenerate primers designed for Antennapedia class homeobox genes. PCR products derived from genomic DNA preparation from one adult individual were separated by gel electrophoresis, which gave 6 bands (data not shown). After analysis of the nucleotide sequence of approximately 100 clones, eight types of homeobox fragment were found (data not shown). Five out of these were identified as parts of HOM/Hox type genes (Fig. 1). We designated these HrHox (Halocynthia roretzi Hox) genes.

Fig. 4. HrHox-1 expression in embryos of various stages as visualized by whole-mount in situ hybridization. (A) Lateral view of an early neurula stage embryo. (B) Lateral view of a mid neurula stage embryo. Specimen was subjected to prolonged staining of 5 days. (C) Ventral view of an early tailbud stage embryo. (D) Dorsal view of an early tailbud stage embryo. (E) Dorsal view of an early tailbud stage embryo. The stage of this embryo is more progressed than that in C or D. (F) Lateral view of a mid tailbud stage embryo. Specimen was subjected to prolonged staining. (G) Dorsal view of a mid tailbud stage embryo. (H) Dorsal view of a late tailbud stage embryo. For A through H, anterior is to the top. For A and B, ventral is to the right. Magnification of all pictures is the same. Scale bar indicates 100 μm.

Fig. 5. Spatial expression of HrHox-1 at the larva stage. (A) Lateral view of whole-mount in situ hybridization specimen of a swimming larva. Anterior to the right. Two black spots are the ocellus (left) and the otolith (right). Tunic was nonspecifically stained. (A') Control whole-mount in situ hybridization specimen of A using a sense probe. Only tunic was nonspecifically stained, but to lesser extent than in A. Magnification is the same as A. (B) Sagittal section of whole-mount in situ hybridization specimen that was subjected to prolonged staining. (B') The same section as in B stained with DAPI and observed using a fluorescence microscope. Four nuclei are seen in HrHox-1 positive region (indicated by arrowheads). Other nuclei are out of focus. (C,D) Serial sections of the same specimen as shown in B. Arrows indicate signals around pigment cells. (E) Cross section of a whole-mount in situ hybridization specimen at the mid-brain stem level. ep; epidermis, bs; brain stem, nc; notochord, es; endodermal strand, me; mesenchymal cells. Also this section reveals that expression of HrHox-1 in the CNS is restricted in dorsal side. For A-D anterior is to the right, and dorsal is to the top. For E, dorsal is to the top. Scale bars indicate 100 μm.
Based on the characteristic conservation of amino acid residues, these HrHox genes were further classified as such that HrHox-1, HrHox-2, HrHox-10 correspond to paralogous subgroup 1, 2 and 10, respectively. The other two HrHox gene fragments exhibited the same degree of similarity to members of paralogous subgroups 4 through 7 and were therefore tentatively designated HrHox-4/7A and HrHox-4/7B.

In the rest of this study, we focus on the characterization of HrHox-1.

**Nucleotide sequence of HrHox-1**

Fig. 2A shows the nucleotide sequence of the HrHox-1 cDNA. Conceptual translation of the cDNA shows that HrHox-1 encodes a protein of 318 amino acid residues containing a homeodomain in the middle. 5'-upstream of the homeodomain, a hexapeptide is present which is a characteristic amino acid sequence conserved among the homeoproteins of the Antennapedia class (Bürglin, 1994). The C-terminal third of HrHox-1 protein is serine rich. The homeodomain of HrHox-1 contains seven amino acid residues that are well conserved among members of the labial group Hox genes (see legend) as shown in Fig 2B. Flanking regions of the homeodomain also show significant similarity, especially in the region between the hexapeptide and the homeodomain that is designated labial domain (Sive and Chen, 1991). In this region, HrHox-1 shows

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**Fig. 6.** Effect of RA on larval morphology and expression of HrHox-1. (A) Appearance of moonfaced larva induced by RA. Embryos were cultured in filtered sea water (FSW) in the presence of 10⁻⁶ M RA from 1-cell stage. Under these conditions, all embryos developed into moonfaced phenotype larvae. In this larva, the papillae (indicated by arrowheads in B) are absent. An arrow indicates position of the sensory vesicle containing pigment cells in the CNS. (B) A control larva cultured in FSW containing 0.1% DMSO that shows normal appearance. An arrow indicates position of the sensory vesicle. Scale bars in A and B indicate 100 µm. (C) Northern blot analysis of poly(A)* RNAs from RA-treated and control larvae. Cont.: 5 µg of poly(A)* RNA prepared from larvae reared in 0.1% DMSO in FSW was loaded. RA: 5 µg of poly(A)* RNA prepared from larvae reared in 10⁻⁶ M RA in FSW was loaded. Upper: Expression of HrHox-1. Lower: The same membrane was examined for expression of HrHox-10 after removal of the HrHox-1 probe.

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**Fig. 7.** Effect of RA on expression of HrHox-1 examined by whole-mount in situ hybridization. Specimens shown here were prepared from the same batch of embryos used for northern analysis shown in Fig. 7C. (A) An early larva reared in 10⁻⁶ M RA in filtered sea water (FSW). A white arrow indicates ectopic expression at the anterior terminal epidermis. An arrowhead indicates highly enhanced expression at the anterior terminus of the CNS. (B) A control larva reared in 0.1% DMSO in FSW. Conditions for in situ hybridization and staining were the same as in A. Magnification is the same as A. (C-E) Serial parasagittal sections of the whole-mount in situ hybridization specimen of a larva treated with RA. Direction of specimen was slightly slanted rightward and plane of the section is slightly slanted to the left. The progression of the sectioning (C-E) is from left to right side of the larva. Arrows indicate ectopic expression of HrHox-1 in epidermis and an arrowhead indicates enhanced signal in CNS. Anterior is to the left, and dorsal is to the top. ot, otolith. Scale bars indicate 100 µm.
a high level of similarity to the three murine labial group genes as shown in Fig. 2B. These structural features indicate that HrHox-1 is a genuine ascidian labial group gene. When genomic Southern analysis was carried out using HrHox-1 cDNA fragments as probes, simple band patterns were observed as shown in Fig. 2C. These correspond to the HrHox-1 gene. When hybridization was carried out under reduced stringency conditions, no significant band were observed other than these. Thus, HrHox-1 is assumed to be a single copy gene in Halocynthia genome.

**Temporal expression pattern of HrHox-1**

Upon northern analysis, expression of HrHox-1 was first detected at the neurula stage (Fig. 3A). Two species of HrHox-1 transcript were observed, a major species of 2.4 kb and a minor species of 1.7 kb. This observation is consistent with those of labial group genes of vertebrates, which have been reported to produce several forms of transcript (Frohman et al., 1990; Barou et al., 1987). Expression of HrHox-1 peaks once at the tailbud stage and decreases at the stage of larva before hatching (Fig. 3A). Expression increases again, reaching a maximum at the swimming larva stage (Fig. 3A,B). After onset of metamorphosis, HrHox-1 expression appeared to be kept at a lower level (Fig. 3B).

**Spatial expression pattern of HrHox-1**

The spatial expression pattern of HrHox-1 was examined by whole-mount in situ hybridization. Throughout Halocynthia embryogenesis, expression of HrHox-1 is restricted to ectodermal tissues, epidermis and central nervous system (CNS).

**Expression in epidermis**

A significant signal was first detected at the early neurula stage. This signal was observed on the ventral surface of the central third of embryo (Fig. 4A). In the later neurula stage, this expression extended dorsally within the epidermis, hence forming a wide belt of HrHox-1 expression (Fig. 4B-D). Anterior and posterior borders of expression were not clear, but anterior and posterior terminal regions of embryo were not stained even under prolonged staining conditions (Fig. 4B). At the late tailbud stage, HrHox-1 was expressed broadly in trunk and tail, while terminal regions were continuously negative for expression (Fig. 4F). At the stage of larva before hatching, staining of HrHox-1 expression was less evident in the epidermis as well as in the CNS (Fig. 7B). This lowering of expression of HrHox-1 seen by in situ hybridization was consistent with the observation by northern analysis (Fig. 3A). At the swimming larva stage, expression was observed in the posterior half of the trunk, showing a gradient reducing towards the anterior, and there was no HrHox-1 expression at the anterior terminal (Fig. 5A,B). Expression of HrHox-1 was not evident in the tail. In larvae, prolonged staining was not successful because of strong nonspecific staining due to tunic that is secreted by the epidermal cells at this stage. Tunic also interfered with analysis of juveniles by whole-mount in situ hybridization. In addition to the nonspecific staining, it seems that the tough tunic of juvenile prevents RNA probes from penetrating into the juvenile body.

**Expression in the central nervous system**

The first distinct signal in the CNS was observed at the early tailbud stage and it became intense as expression in the epidermis extended dorsally (Fig. 4D). After this, the signal was divided into two spots anterior-posteriorly (Fig. 4E). The anterior spot remains at the level of the junction of trunk and tail (Fig. 4G), and it is finally localized to the brain stem in a swimming larva (Fig. 5A,B). The posterior spot extends to the posterior along the neural tube (Fig. 4G,H). However, the spinal cord of a swimming larva was not stained (Fig. 5A,B).

Sections of whole-mount hybridization specimens showed clearly that expression of HrHox-1 was restricted to the CNS and epidermis (Fig. 5B-E). In the HrHox-1 positive area of the brain stem of swimming larvae, we found that there were approximately 7 cells aligned in a line (compare Fig. 5B with 5B’ counter-stained with DAPI). These cells were ascribed to those of dorsal side of neural tube (Fig. 5E). Prolonged staining revealed that there were two additional signals in the CNS; one at the ventral and left side of the ocellus, a photoreceptor, (Fig. 5C) and the other at the left side of the otolith, a gravity sensory organ (Fig. 5D). The former signal can be traced back even to the late tailbud stage when pigment formation does not yet take place (data not shown). On closer examination the latter signal was sometimes found to possess a thin connection to the thick staining in the brain stem (data not shown).

**Effects of retinoic acid on HrHox-1 expression**

It is well known that expression of Hox genes in vertebrate is affected by retinoic acid (RA) (Boncinelli et al., 1991; Kessel and Gruss, 1991; Marshall et al., 1992). In the compound ascidian Polyandrocarpa misakiensis, RA is known to affect morphogenesis, often inducing a secondary body axis in bud development (Hara et al., 1992; Kawamura et al., 1993). Thus it was expected that RA may affect embryogenesis of ascidians and expression of HrHox-1.

When all trans-RA was applied to Halocynthia embryos from the 1-cell stage, it provoked some peculiar morphological defects in the embryos, though morphological abnormalities were not evident until the larva stage. Embryos treated with 10⁻⁵ M and 10⁻⁶ M all trans-RA developed a phenotype with a round-shaped trunk and we therefore designated this phenotype moonfaced (Fig. 6A). In this phenotype, the papillae do not develop and the two pigment cells normally located in the middle of the trunk are shifted posteriorly (Fig. 6A,B). Detailed description of the effects of RA on embryogenesis of Halocynthia will be reported elsewhere (Katsuyama et al., unpublished data).

Effect of RA on expression of HrHox-1 was examined by RNA blot analysis. When 10⁻⁶ M RA was applied to the embryos, expression of HrHox-1 was much enhanced in moonfaced larvae before hatching (Fig. 6C). For comparison, HrHox-10 was also tested for expression using the same blotting membrane. Expression of HrHox-10 appeared to be scarcely increased in RA-treated larvae (Fig. 6C).

Whole-mount in situ hybridization revealed that the level of HrHox-1 expression was increased in moonfaced larvae (Fig. 7A) as compared to controls (Fig. 7B). In particular, highly enhanced expression was observed at the anterior terminal region of the CNS as indicated by an arrowhead in Fig. 7A. In addition, ectopic expression of HrHox-1 was observed in epidermis of anterior terminal of the trunk as indicated by an arrow in Fig. 7A. Serial sections of in situ hybridization
specimens from a RA-treated larva show dense staining at the anterior terminal of the CNS as indicated by an arrowhead in Fig. 7C and three dense staining spots in the epidermis at the anteriormost region of trunk (indicated by arrows, one in Fig. 7C and the other two in Fig. 7E). Location of the three spots of ectopic expression in epidermis coincides with the positions where three papillae are formed in a normally developing larva. Furthermore, cells of the papillae in normal embryos are distinguishable because they are taller than other surrounding epidermal cells. By contrast, the cells that expressed HrHox-1 ectopically in the corresponding region in a moonfaced larva could not be discriminated by cell shape from the neighboring cells (data not shown). These observations point to the possibility that ectopic expression of HrHox-1 in epidermis is involved in elimination of the papillae in a moonfaced larva.

DISCUSSION

Ascidian Hox genes may form a cluster

Vertebrates possess four Hox gene clusters, which are assumed to arise from an ancestral complex by twice duplication (Kappen et al., 1989). Recently it has been demonstrated that amphioxus possesses a single Hox gene cluster (Garciá-Fernández and Holland, 1994). Considering the phylogenetic position of ascidians, the organization of their Hox genes is of particular interest. Our efforts in isolation of homeobox clones yielded 5 species of HOM/Hox type gene fragments. Recently, we have isolated two genomic clones that contain HrHox-1 and HrHox-2 homeobox sequences in one clone and HrHox-4/7A homeobox sequences in the other (unpublished results). These suggest that ascidian Hox genes may form a cluster.

Spatial expression pattern of HrHox-1 and that of vertebrate counterparts

Dynamic changes in the expression pattern of HrHox-1 were observed in the CNS. HrHox-1 expression first appeared as a faint signal in the neural tube, this then extended toward the posterior, and was later restricted to the brain stem. This expression pattern is consistent with the assumption that the HrHox-1 gene is involved in patterning mechanisms as has been shown for vertebrate Hox genes, rather than in determination of cell types.

In vertebrates, Hox genes belonging to the same paralogous subgroup show a similar expression pattern (Hunt et al., 1991). By contrast, labial group genes, Hoxa-1, Hoxb-1 and Hoxd-1, exhibit domains of expression distinct from one another (Hunt et al., 1991; Murphy and Hill, 1991). Expression of murine Hoxa-1 is first detected in primitive streak, extends on neurectoderm posteriorly keeping a defined anterior border of expression at rhombomere 2/3 and then retreats posteriorly (Murphy et al., 1989). Hoxb-1 has a similar expression pattern to that of Hoxa-1 initially, but it persists in rhombomere 4 (Wilkinson et al., 1989; Murphy and Hill 1991). Hoxd-1 expression is observed in surface ectoderm (Hunt et al., 1991).

The expression pattern of HrHox-1 in the CNS is similar to that of the Hoxb-1 genes and the expression in epidermis is reminiscent of murine Hoxd-1 expression. Especially, restricted expression of Hoxb-1 in rhombomere 4 and of HrHox-1 in the brain stem strikingly resembles each other.

Although structural features such as rhombomeres in vertebrates have not been reported in ascidians, restricted expression of HrHox-1 suggests that certain functional differentiation occurs along the CNS of ascidians. Furthermore, it is also suggested that the regulatory elements/factors of Hoxb-1 and Hoxd-1 are at least partially preserved in HrHox-1 gene regulation, while those of Hoxa-1 are less evident.

HrHox-1 is expressed exclusively in ectodermal tissues in early embryogenesis. In vertebrates, expression of Hox genes is observed not only in ectodermal tissues but also in endodermal and mesodermal tissues along the A-P axis or distal-proximal axis at embryonic stage (McGinnis and Krumlauf, 1992). Furthermore, it is known that the amphioxus Hox gene Amphihox-3 shows an expression pattern with A-P limitation only in ectoderm, but not in mesoderm (Holland et al., 1992). Expression of HrHox-1 appears to follow the A-P limitation rule. Expression of Hox genes in ectoderm may reflect an archetypal expression pattern.

Onset of expression of HrHox-1 and development of ascidians

Vertebrate labial group genes, Xlab-2 of Xenopus and Hoxa-1 and Hoxb-1 of mouse or chick, begin to be expressed prior to other Hox genes from the gastrula stage in each animal (Sive and Cheng, 1991; Frohman et al., 1990; Sundin et al., 1990; Murphy and Hill, 1991). Likewise, it appears that HrHox-1 expression also starts prior to other ascidian Hox genes, most of which are expressed from around the larva stage (data not shown). However, the onset of HrHox-1 expression is later than that of vertebrate counterparts. Similarly, in vertebrates, restriction of Hoxb-1 expression to rhombomere 4 occurs at the tailbud stage (Slack et al., 1993), when the other Hox genes are coordinately expressed in the CNS (Wilkinson et al., 1989; Duboule and Dolle, 1989; Krumlauf, 1993). Restriction of HrHox-1 expression to a certain narrow region in the CNS is observed at the larva stage, but not in the tailbud stage. Thus, from the view point of labial group gene expression, the developmental stage after gastrulation of the ascidian may not correspond to that of vertebrates. It is envisaged that the phylotypic stage of ascidian in such a sense as has been defined by Slack et al. (1993) may be later than that of vertebrates. In this context, it may be interesting to examine expression of labial group genes together with other Hox genes in other species of ascidian, since the state of organogenesis in a larva is different from species to species. For example, many colonial ascidians form larvae with well-developed adult organs (Millar, 1971) and, in the most extreme case, some species develop into adults directly without the larva stage (Jeffery and Swalla, 1992). Elucidation of expression of labial group genes as well as other Hox genes in various ascidians will show the relationship between the state of organogenesis and Hox gene expression, and in turn the phylotypic stage of ascidians.

Effect of RA on HrHox-1 expression

In this study, we showed evidence for the first time to indicate that RA affects HOM/Hox gene expression in invertebrates. It has been demonstrated that a conserved RA response element exists in Hoxb-1 genes among vertebrates (Rolland et al., 1994; Studer et al., 1994). The enhancing effect of RA on HrHox-1 but not HrHox-10 expression suggests that an
element analogous to that of vertebrates may also be present in ascidians to regulate HrHox-1 expression.

In moonfaced larvae, clear ectopic signals were observed at the anterior terminal epidermis as three staining spots and expression at the anterior terminus of the CNS is highly enhanced. If ascidian Hox genes are assumed to be responsible for regional identities as reported in other animals, elimination of the papillae might be interpreted as respecification of identity of the terminal epidermis caused by the ectopic expression of HrHox-1. This RA-induced expression of HrHox-1 might be consistent with the assumption that the Hox code is shifted anteriorly by RA in vertebrates (Kessel and Gruss, 1991; Dekker et al., 1992). There are, however, differences in response to RA between HrHox-1 and Hoxb-1. Hoxb-1 expression is restricted to rhombomere 4 in 9.5 dpc mouse embryo. When mouse embryos are exposed to RA, the Hoxb-1 expression domain expands anteriorly and a restricted expression domain is duplicated at the level of rhombomere 2 at 9.5 dpc (Conlon and Rossant, 1992). This ectopic expression of Hoxb-1 is accompanied by transformation of rhombomere identity (Marshall et al., 1992). In the present study, such duplication was not observed. It is also unclear whether a posterior shift of pigment cells may correlate with the enhanced expression of HrHox-1 at the anterior terminus of the CNS. It is necessary to use an appropriate marker such as Krox-20 in vertebrates (Papalopulu et al., 1991; Marshall et al., 1992) for further comparison of the response to RA between ascidians and vertebrates. We have recently isolated an ascidian Orthodenticle homologue, Hroth and found that it is expressed in the sensory vesicle surrounding pigment cells (unpublished data). Expression of the murine Orthodenticle homologue, Otx2 is known to be affected by RA (Ang et al., 1994). Hroth may serve as a marker to further investigate the moonfaced phenotype of the ascidian embryos.

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