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Original Article

Differential expression of sea urchin Otx isoform ($HpOtx_E$ and $HpOtx_L$) mRNAs during early development

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ABSTRACT Two distinct types of *orthodenticle*-related proteins (early type: $HpOtx_E$, late type: $HpOtx_L$) of the sea urchin, *Hemicentrotus pulcherrimus*, have been implicated as enhancer element binding factors of the aboral ectoderm-specific arylsulfatase *(HpArs)* gene. In order to understand the role of these isoforms during sea urchin development, we have isolated and characterized *HpOtx* gene. Here we describe the spatial expression patterns of $HpOtx_E$ and $HpOtx_L$ mRNAs and effects of overexpression of these mRNAs on embryogenesis. Whole-mount *in situ* hybridization using each isoform-specific probe reveals the complex and dynamic change of expression patterns among three germ layers. $HpOtx_E$ mRNA is maternally stored and exists apparently in a non-localized manner by the blastula stage. After hatching, $HpOtx_E$ transcripts are expressed predominantly in presumptive endoderm cells and gradually decrease during gastrulation. Signals for $HpOtx_L$ mRNA are intense at the vegetal half after hatching and subsequently, its expression is restricted to the micromere-derived cells. After primary mesenchyme cell (PMC) ingression, $HpOtx_L$ transcripts are localized at the vegetal plate and thereafter, concentrated primarily in ectoderm. Eggs injected with $HpOtx_E$ or $HpOtx_L$ mRNA develop into similar radialized structures without PMC ingression and gut invagination, whose oral-aboral axes are disrupted. Overexpression of $HpOtx_E$ induces accumulation of $HpOtx_L$ mRNA at the significantly earlier stages, though $HpOtx_L$ overexpression inhibits the accumulation of $HpOtx_E$ transcripts. Expression patterns of $HpOtx_E$ and $HpOtx_L$ in all three germ layers and dramatic morphological changes observed in the mRNA-injected embryos suggest that each HpOtx isoform has an important role in sea urchin embryogenesis.

KEY WORDS: *orthodenticle (otd)/Otx, sea urchin, gene expression, early development, overexpression*

Introduction

Metazoan organisms undergo complex developmental programs orchestrated by precise temporal and spatial patterns of transcription. Among them, sea urchin embryos provide a useful model system for studying the regulatory mechanisms of gene expression during early development. Sea urchin possesses the simple body plan with structural symmetrical enterocoelous larva having three germ-layers. Cell lineage studies as well as cell-type-specific gene expression analyses have demonstrated that, in the sea urchin embryo, five territories for founder cells, which include the oral ectoderm and the aboral ectoderm, the vegetal plate, the skeletogenic mesenchyme and the small micromeres, can be distinguished (Davidson, 1989; Cameron and Davidson, 1991). Expression of each cell lineage-specific gene is controlled by multiple specific interactions between DNA-binding transcriptional factors including mediators and the target site sequences that they recognize within its *cis*-regulatory domain (Arnone and Davidson, 1997).

One molecular marker for the aboral ectoderm is the arylsulfatase *(HpArs)* gene, which is transcriptionally activated late at the blastula stage and is expressed exclusively in the aboral ectoderm after the gastrula stage (Sasaki *et al.*, 1988; Akasaka *et al.*, 1990). In gene transfer experiments, a 229bp region in the first intron of *HpArs* contains a tandem repeat of core consensus sequences of *orthodenticle (otd/Otx)*-related protein binding sites, which serve

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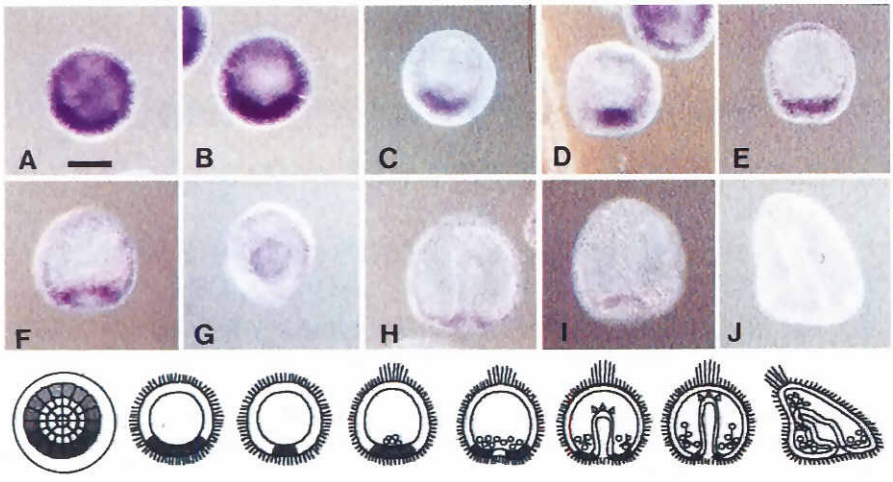

Fig. 1. Spatial distribution of *HpOtx$_E$* transcripts during *H. pulcherrimus* embryogenesis analyzed by whole-mount *in situ* hybridization using a specific 500 nt, digoxigenin-labeled antisense probe. *For each developmental stage except early gastrula* **(G)**, *views of optical sections along the animal-vegetal axis are shown.* **(A)** *Unhatched blastula. Transcripts are distributed throughout embryos.* **(B,C)** *Hatched blastula. Hybridization signals become intense at the vegetal half.* **(D)** *Late hatched blastula. Expression is detectable at the vegetal plate, prior to PMC ingression.* **(E)** *Early mesenchyme blastula. Transcripts are distributed in the vegetal plate.* **(F,G)** *Early gastrula. Intense signals are observed as a ring. PMCs in the blastocoel are negative. In* **(G)**, *vegetal pole view shows the non-staining center of the vegetal plate, occupied by the skeletogenic mesenchyme and small micromeres.* **(H)** *Mid-gastrula.* **(I)** *Late-gastrula. During gastrulation, the level of HpOtx$_E$ transcripts declines gradually. The vegetal plate cells especially surrounding the blastopore stain intensely, while the invaginating archenteron does not show signals above the background.* **(J)** *Prism stage. Signals become low as the background level. Bar, 50 μm. Diagram of spatial expression pattern of HpOtx$_E$ is also illustrated under the photographs.*

as the major source of positive control for the *HpArs* gene (Iuchi *et al.*, 1995; Sakamoto *et al.*, 1997). Two isoforms of Otx proteins bind to this enhancer element. The first type of Otx protein, referred to as early type Otx (HpOtx$_E$), appears in the early development and gradually decreases by the gastrula stage. The second type of Otx protein, referred to as late type Otx (HpOtx$_L$), appears at the blastula stage and remains until the gastrula stage. The temporal expression pattern of the *HpOtx$_L$* is similar to that of *HpArs* gene (Sakamoto *et al.*, 1997). The mRNAs of *HpOtx$_E$* and *HpOtx$_L$* are transcribed from a single *HpOtx* gene by altering the transcription start sites and by alternative splicing (Kiyama *et al.*, 1998). The homeobox and downstream regions through to the C-terminal among them are identical, while the N-terminal region has different amino acid sequences. (Sakamoto *et al.*, 1997).

Mao *et al.* (1996) reported that SpOtx, an *HpOtx$_E$* homolog of *Srongylocentrotus purpuratus*, has a key role in the specification of aboral ectoderm during sea urchin embryogenesis. However, recent study of transactivation assay in sea urchin embryos has demonstrated that *HpOtx$_L$* activates the aboral ectoderm-specific *HpArs* promoter, though *HpOtx$_E$* does not under the same condition (Kiyama *et al.*, 1998). These results raise questions about the precise functions of both Otx isoforms during sea urchin embryogenesis.

Otx proteins have been identified in a wide organisms as divergent as *Drosophila* and vertebrates. Expression patterns of *Otx* genes are analyzed by *in situ* hybridization in vertebrate embryos. Mouse cognate *Otx2* expression is reported to occur in the entire epiblast at the prestreak stage. During midstreak stages, the expression domain becomes restricted to the anterior region in all three germ layers (Simeone *et al.*, 1993; Ang *et al.*, 1994). These expression patterns are also confirmed in *Xenopus laevis* (Pannese *et al.*, 1995) and chick embryos (Bally-Cuif *et al.*, 1995). Common expression among these cognates, knockout experiments in the mouse (Acampora *et al.*, 1995; Matsuo *et al.*, 1995; Ang *et al.*, 1996) and gain-of-function experiments performed in *Xenopus* (Blitz and Cho, 1995) have demonstrated an essential role of Otx in the specification of anterior structures, including a role in the initiation and progression of involution movements during gastrulation (Bally-Cuif *et al.*, 1995; Pannese *et al.*, 1995; Wada *et al.*, 1996; Lemaire and Kassel, 1997; Bally-Cuif and Boncinelli, 1997). However, their downstream target genes and mechanisms of action are not known. There is little information about their functions in the early phase of development and in simple deuterostome embryogenesis.

To gain an insight into the role of Otx in early development and ancient function of *Otx* genes, we have analyzed spatial expression patterns of *HpOtx$_E$* and *HpOtx$_L$* during early development of sea urchin and performed overexpression of these isoforms mRNAs.

Results

Spatial expression patterns of HpOtx$_E$ and HpOtx$_L$ mRNAs during embryogenesis

The spatial expression patterns of *HpOtx$_E$* and *HpOtx$_L$* in developing embryos were examined by means of whole-mount *in situ* hybridization. Each isoform-specific probe, derived from the nucleotide sequence in the region upstream of the homeobox, was labeled with digoxigenin and incubated with blastula-, gastrula- and prism-stage embryos.

The transcripts of early type *HpOtx$_E$* were distributed throughout the embryos by the blastula stage. During further development after hatching, hybridization signals became gradually intense at the vegetal half (Fig. 1B and C) and detectable at the small central region of the vegetal plate where primary mesenchyme precursors reside (Fig. 1D). At the mesenchyme blastula stage, the *HpOtx$_E$* mRNA was expressed at high levels throughout most of the vegetal plate. Primary mesenchyme cells (PMCs) that had ingressed into the blastocoel did not express detectable levels of *HpOtx$_E$* mRNA (Fig. 1E). As the primary invagination was initiated, intense hybridization signals appeared in a ring of presumptive endodermal cells, though signals were detected neither in invaginating archenteron nor in PMCs in the blastocoel (Fig. 1F and G). *HpOtx$_E$* transcripts gradually decreased by the prism stage, (Sakamoto *et al.*, 1997), a positive signal for *HpOtx$_E$* was retained only by presumptive

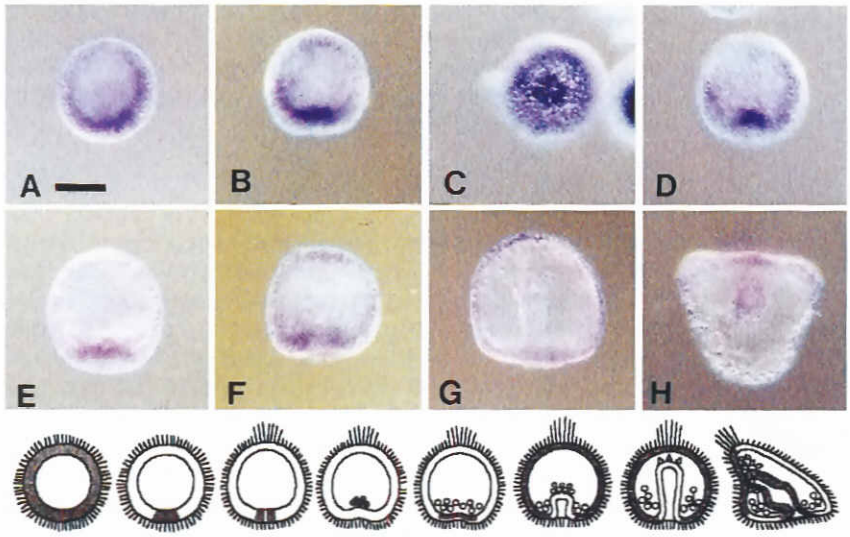

Fig. 2. Localization of *HpOtxL* mRNA detected by whole-mount *in situ* hybridization. *Bright field micrographs of embryos hybridized to digoxygenin-labeled HpOtxL antisense RNA probe (739 nt). For each developmental stage except* **(C)** *and* **(H)**, *views of optical sections along the animal-vegetal axis are shown.* ***(A)*** *Hatched blastula. Transcripts are distributed throughout embryo with intense signals at the vegetal half.* **(B)** *Late blastula. Intense hybridization signals are localized at the vegetal plate.* **(C)** *Vegetal pole view of late blastula. Hybridization signals are concentrated in micromere-derived cells.* **(D)** *Mesenchyme blastula. PMCs that have just ingressed are positive.* **(E)** *Late mesenchyme blastula. HpOtxL transcripts are localized at the vegetal plate cells.* **(F)** *Early gastrula.* **(G)** *Mid-gastrula. During gastrulation, $HpOtx_L$ transcripts are primarily detected in ectoderm. Mesenchyme cells and invaginating archenteron are negative.* **(H)** *Prism. Hybridization signals are detectable in gut and oral ectoderm. Bar, 50 μm. Diagram of spatial expression pattern of $HpOtx_L$ is also illustrated under the photographs.*

endodermal cells surrounding the blastopore (Fig. 1H and I). At the prism stage, expression of $HpOtx_E$ transcripts was under a detectable level (Fig. 1J).

The late type $HpOtx_L$ mRNA was first detectable in hatched blastula stage embryos with the intense signal at the vegetal half (Fig. 2A). Subsequently, $HpOtx_L$ transcripts were intensely distributed at the vegetal plate cells prior to PMC ingression (Fig. 2B). At the late blastula stage, $HpOtx_L$ transcripts were concentrated in micromere-derived cells (Fig. 2C) and were found in PMCs that had just ingressed into the blastocoel (Fig. 2D). Thereafter, hybridization signals were observed at the vegetal plate cells including precursors of secondary mesenchyme cells and endoderm, though PMCs in the blastocoel became negative (Fig. 2E). During gastrulation, $HpOtx_L$ transcripts were exclusively expressed within the ectoderm; appreciable levels of $HpOtx_L$ expression were not detected in the invaginating archenteron or in the mesenchyme cells located in the blastocoel (Fig. 2F and G). At the prism stage, however, signals were detectable at the oral ectoderm and endoderm (Fig. 2H).

Morphological effects in $HpOtx_E$ or $HpOtx_L$ mRNA-injected embryos

Capped mRNAs were injected into fertilized eggs and embryos were developed for 45 h at 15°C. Overexpression of $HpOtx_E$ or $HpOtx_L$ caused similar morphological effects on sea urchin embryogenesis in spite of difference in their N-terminal regions (Fig. 3). More than 90% of the eggs injected with 11pg mRNA formed radialized spherical structures without PMC ingression and archenteron invagination (Fig. 3A and D). No recognizable stomodea were formed and no ciliary band was evident. These embryos lacked the morphological features that distinguished the oral from aboral ectoderm. About 10% of embryos, however, formed a spherical shape with a reduced gut and mesenchyme like-cells in the blastocoel (partial effect; Fig. 3B,C,E and F). As the amount of injected mRNA decreased to 3 pg/egg, about 35% of embryos developed into embryos with partial effect. The embryos injected with the same amount of control luciferase mRNA (11pg/egg) developed into apparently normal prism embryos (Fig. 3G) as uninjected ones.

Expression of cell-type-specific proteins and genes in $HpOtx_{E/L}$ mRNA-injected embryos

The morphological features mentioned above suggest that specification of positional identity along the oral-aboral axis did not occur in the *HpOtx* mRNAs-injected embryos. To determine the state of differentiation in the $HpOtx_{E/L}$ mRNA-injected embryos, the spatial expression of several cell-type-specific proteins were analyzed. Overexpression of $HpOtx_E$ or $HpOtx_L$ caused both the aboral ectoderm- specific HpArs (Mitsunaga-Nakatsubo *et al.*, 1998; Fig. 4B and C) and the oral ectoderm-specific Hpoe (Yoshikawa, 1997; Fig. 4E and F) to be expressed in all ectoderm cells. Expression of endoderm-specific HpEndo16 (Akasaka *et al.*, 1997; Fig. 4H and I) and PMC-specific protein detected by the

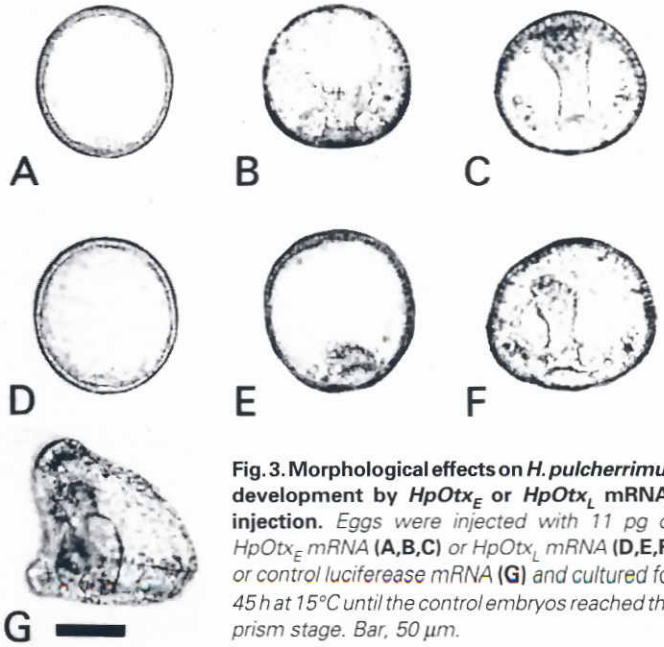

Fig. 3. Morphological effects on *H. pulcherrimus* development by $HpOtx_E$ or $HpOtx_L$ mRNA-injection. *Eggs were injected with 11 pg of $HpOtx_E$ mRNA* **(A,B,C)** *or $HpOtx_L$ mRNA* **(D,E,F)** *or control luciferease mRNA* **(G)** *and cultured for 45 h at 15°C until the control embryos reached the prism stage. Bar, 50 μm.*

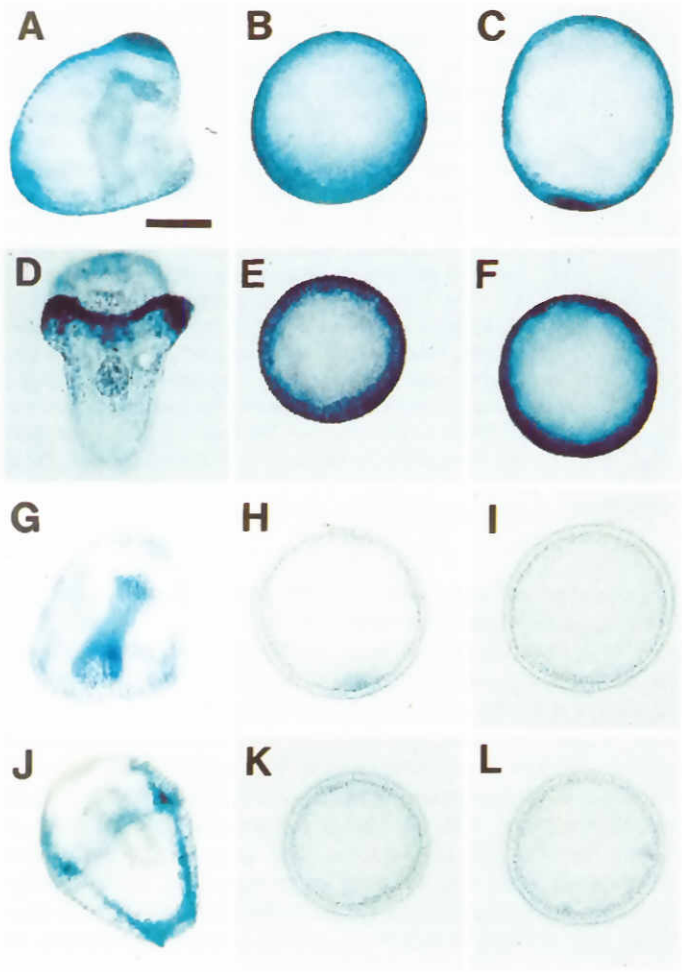

Fig. 4. Expression of cell-type-specific proteins in *HpOtx*$_{E/L}$ mRNA-injected *H. pulcherrimus* embryos. *Control embryo* **(A,D,G,J)**, *HpOtxE mRNA-injected embryo* **(B,E,H,K)** *and HpOtx*$_L$ *mRNA injected-embryo* **(C,F,I,L).** *Expression of HpArs* **(A,B,C)**, *Hpoe* **(D,E,F)**, *HpEndo16* **(G,H,I)** *and PMC-specific protein detected by the monoclonal antibody PMCA4* **(J,K,L)** . *Bar, 50 μm.*

monoclonal antibody PMCA4 (Fig. 4K and L) were markedly reduced compared to the uninjected control (Fig. 4G and J).

Expression of cell-type-specific genes in *HpOtx*$_{E/L}$ mRNA-injected embryos were also examined by quantitative RT-PCR using specific primers. As shown in Figure 5B, excessive expression of *HpOtx*$_E$ and *HpOtx*$_L$ was detected in each of the mRNA-injected embryos at the prism stage. The embryos injected with *HpOtx*$_{E/L}$-mRNA showed significantly lower levels of *HpEndo16* (a marker for endoderm; Akasaka *et al.*, 1997) and *HpSM50* (PMC; Katoh-Fukui *et al.*, 1992) mRNAs in comparison to the uninjected ones. In contrast, the expression of aboral ectoderm-specific *HpArs* (Yang *et al.*, 1989; Akasaka *et al.*, 1990) and *HpHbox1* (Angerer *et al.*, 1989) were slightly affected by injections of *HpOtx*$_{E/L}$ mRNA.

At the unhatched blastula stage, expression of these cell-type-specific genes were too low to determine the effect of HpOtx$_{E/L}$-mRNA injections (data not shown). However, mRNA injection of each isoform affected another isoform-mRNA expression. As shown in Figure 5A, *HpOtx*$_E$-mRNA injection showed a significant amount of *HpOtx*$_L$ as compared with uninjected embryos, while only slight signals were observed in uninjected controls. When *HpOtx*$_L$ was injected, *HpOtx*$_E$ expression was declined under the level of the control embryo.

Discussion

We have performed whole-mount *in situ* hybridization to analyze the expression patterns of *HpOtx*$_E$ and *HpOtx*$_L$ mRNAs using each isoform-specific probe. Present results show that they accumulate in a distinct, complex and dynamic manner during sea urchin embryogenesis. Previously characterized cell-lineage-specific markers, such as *Endo 1, LvN1.2, HpArs* and *Endo 16* genes, are reported to exhibit a gradual restriction of their expression domains (Wessel and McClay, 1985; Wessel *et al.*, 1989; Akasaka *et al.*, 1990; Ransick *et al.*, 1993), suggesting a common mode of gene regulation in the sea urchin embryos (Kingsley *et al.*, 1993). Spatial expression patterns of two *HpOtx* isoforms are distinct from these markers. They do not show a gradual restriction of expression domains to specific cell-lineage sectors alone, but instead, their expression domains change from one germ layer to the other as development proceeds. These dramatic changes occur after the specification of the five embryonic territories; the oral ectoderm, the aboral ectoderm, the vegetal plate, the skeletogenic mesenchyme, and the small micromreres (Davidson, 1989; Cameron and Davidson, 1991). Overexpression of *HpOtx*$_{E/L}$ mRNA in sea urchin embryos, which disturbed the expression pattern of each HpOtx isoform, inhibited PMC ingression and gut invagination, and caused disruption of the oral-aboral axis. However, stimulation of expression of any cell-lineage-specific genes used as markers in the present study, were not detected. Taken together, these results suggest that complex expression pattern of each *HpOtx* has an important role in regulation of sea urchin early development, though they are not implicated in the process of specific cell fate determination.

We have previously demonstrated by the transactivation assay that HpOtx$_L$ activates the *HpArs* promoter, while HpOtx$_E$ does not under the same condition (Kiyama *et al.*, 1998). Given these findings, we might expect that the *HpArs* expression in the aboral ectoderm would be carried out by switching of two HpOtx isoforms. However, the present study suggests a more complex picture of tissue specificity of *HpArs* expression. Expression patterns of two *HpOtx* isoforms are far distinct from that of *HpArs* (Akasaka *et al.*, 1990), a target gene of these isoforms, suggesting that HpOtx isoforms by themselves are not sufficient for the aboral ectoderm specificity of *HpArs* expression and postulating the necessity of involvement of other factors that negatively regulate this gene in non-aboral ectoderm tissue. In addition, the present result that overexpression of HpOtx only slightly affects the *HpArs* expression also suggests the requirement of another cofactors for correct enhancer activity for aboral ectoderm-specific *HpArs* expression, though Mao *et al.* (1996) demonstrated that SpOtx, an *HpOtx*$_E$ homolog in *S. purpuratus*, functions in differentiation of aboral ectoderm by activating aboral ectoderm-specific gene.

Expression domains of *Otx* genes have been analyzed in wide organisms. Especially, their expression patterns in vertebrate organogenesis have been studied in detail and have demonstrated the essential roles of Otx in the specification of anterior structures

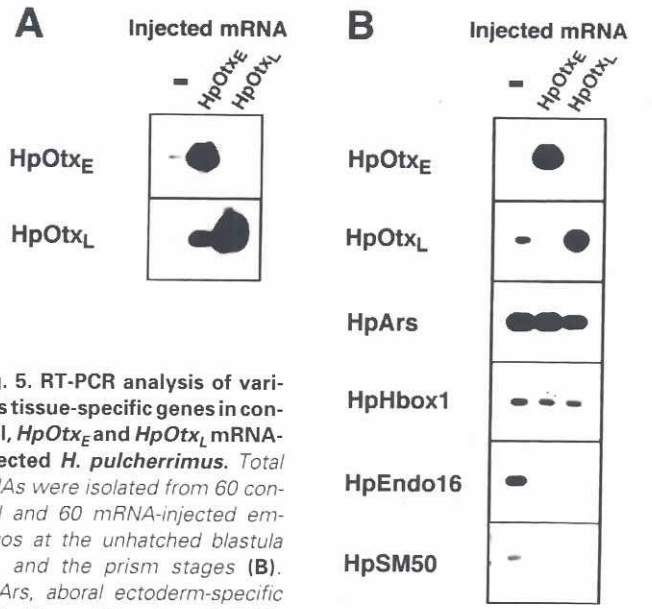

Fig. 5. RT-PCR analysis of various tissue-specific genes in control, *HpOtx*$_E$ and *HpOtx*$_L$ mRNA-injected *H. pulcherrimus*. *Total RNAs were isolated from 60 control and 60 mRNA-injected embryos at the unhatched blastula* **(A)** *and the prism stages* **(B)**. *HpArs, aboral ectoderm-specific arylsulfatase (Akasaka et al. 1990); HpHbox1, aboral ectoderm-specific Antennapedia class homeobox gene (Angerer et al., 1989); HpEndo 16, endoderm-specific extracellular matrix protein (Akasaka et al., 1997) and HpSM50, skeletogenic mesenchyme-specific matrix protein (Katoh-Fukui et al., 1992).*

(Finkelstein and Boncinelli, 1994). *Otx* cognates of vertebrates are also reported to be expressed in all three germ layers at the early stages of embryogenesis. Since patterns of *HpOtx* expressions are similar to those of vertebrates, the present result will provide a new insight of Otx functions during early development.

Previously, we have reported that two distinct types of *HpOtx* mRNAs are produced from a single gene by altering the transcription start sites and by alternative splicing (Kiyama *et al.*, 1998). In the present study, overexpression of HpOtx$_E$ induces the accumulation of *HpOtx*$_L$ mRNA at the significantly earlier stage, though HpOtx$_L$ overexpression inhibits the accumulation of *HpOtx*$_E$ transcripts. There are four consensus sequences of Otx binding sites in the 5' UTR region of *HpOtx*$_L$ and at least one binding site in the first intron (Kiyama *et al.*, 1998). Generation of two structurally diverse isoforms from a single gene may be autoregulated by bindings of each isoform to these elements. The result of the significant induction of *HpOtx*$_L$ transcripts may also explain the present finding that overexpression of each isoform causes the similar morphological effects and gene expressions.

Li *et al.* (1997) also analyzed the expression patterns of Otx isoforms: *SpOtx* (α) and (β) in *S. purpuratus* at the several stages by means of autoradiographic *in situ* hybridization. These authors noted that *HpOtx*$_E$ homolog, *SpOtx* (α), transcripts are accumulated in all cells during cleavage and are gradually concentrated in oral ectoderm and vegetal plate territories during gastrulation. *HpOtx*$_L$ homolog, *SpOtx* (β), transcripts are reported to begin to accumulate at mesenchyme blastula stage primarily in ectoderm and then later are largely restricted to oral ectoderm and endoderm. The different results of our findings on spatial expression patterns from Li *et al.* (1997) could be due to difference of sea urchin species, since number of late type Otx and their temporal accumulation patterns are different between two urchins (Sakamoto *et al.*, 1997; Kiyama *et al.*, 1998). In addition, axis determination in sea urchins is different among the species (Jeffery, 1992). The difference of aboral and oral ectoderm-specific gene expression in Otx isoform-overexpressed embryos between *H. pulcherrimus* and *S. purpuratus* (Mao *et al.*, 1996; Li *et al.*, 1997) is also described to the species difference.

Materials and Methods

Embryo culture

Gametes of the sea urchin, *Hemicentrotus pulcherrimus*, were obtained by intracoelomic injection of 0.55 M KCl. The eggs were washed with artificial sea water (ASW) three times and inseminated. Embryos were cultured at 15°C with gentle aeration.

Whole-mount in situ hybridization

The whole-mount *in situ* hybridization was performed basically according to the procedure originally described by Harkey *et al.* (1992) and modified by Ransick *et al.* (1993). Digoxigenin (DIG)-labeled antisense RNA probe was prepared with an Ambion's MEGAscript™ T7 kit using digoxygenin-11-UTP (Boehringer Mannheim, Germany). The original HpOtx cDNA (HpOtx$_E$ and HpOtx$_L$) was isolated from *H. pulcherrimus* (Sakamoto *et al.*, 1997). To prepare RNA antisense probe specific to *HpOtx*$_E$, 5' region upstream of the homeobox was generated by PCR using following primers: *HpOtx*$_E$S primer, 5'-CGGGATCCGCACATGATTACTCACTC-3' and HpOtx$_E$A primer, 5'-CCCAAGCTTTTCACTGGTCATTGGACC-3'. HpOtx$_E$S and HpOtx$_E$A primers contain *Bam*HI and *Hin*dIII cleavage sites at their 5' ends, respectively. The product was subcloned into the pBluescript SK- vector (Stratagene Cloning System, CA, USA) and was linearized with *Xba*I, which makes an *in vitro* transcription product of 500 nt. The 5'-terminal specific region of HpOtx$_L$ cDNA, obtained by the 5' RACE method and subcloned into pBluescript SK- vector (Sakamoto *et al.*, 1997), was linearized with *Bam*HI to produce a transcription product of 739 nt. Riboprobes were partially alkaline hydrolyzed to sizes of about 150-400 nucleotides according to the procedure described by Cox *et al.* (1984). Embryos fixed in glutaraldehyde were incubated for 5 min with 5 μg/ml Proteinase K in PBS containing 0.1% polyoxyethylenesorbitan monolaurate (Tween 20) (PBST). Following post fixation in 4% paraformaldehyde in PBST and prehybridization at 48°C for 3 h, embryos were mixed with riboprobes (100 ng/ml) in hybridization buffer (50% formamide, 10% PEG#6000, 0.6 M NaCl, 5 mM EDTA, 20 mM Tris-HCl at pH 7.5, 500 μg/ml yeast tRNA, 2xDenhardt's solution, 0.1% Tween 20). Hybridization was carried out at 48°C for 16 h. Following washes with PBST (once at room temperature and several times at 48°C) and finally with 0.5xSSC-0.1% Tween (three times for 30 min each at 60°C), embryos were treated with 50 μg/ml RNase A for 30 min at 37°C. After blocking with 5% sheep serum in PBST, the probe location was visualized using alkaline phosphate-conjugated anti-digoxigenin Fab fragments and the substrate, 4-Nitro blue terazolium chloride (NBT)/5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) (Boehringer Mannheim, Germany), following the supplier's instructions. The embryos were dehydrated in a graded series of ethanol solution and cleared by placing them in terpineol.

RNA preparation and microinjections into sea urchin eggs

Capped mRNAs were prepared from RNA transcribed *in vitro* according to the manufacture's instructions (MEGAscript™, Ambion, USA) using as the templates the cDNAs of HpOtx$_E$ and HpOtx$_L$, whose 5' untranslated regions were truncated (Sakamoto *et al.*, 1997), and fire fly luciferase. The cDNAs for HpOtx$_E$ and HpOtx$_L$ were linearized with *Hin*dIII and each sense mRNA was synthesized using T3 polymerase. The RNA was precipitated with isopropanol to separate unincorporated cap analogs and free nucleotides, and the RNA pellet was resuspended in diethylpyrocarbonate (DEPC)-treated H_2O. The RNA was mixed with 20% glycerol to a final concentration of 5.5 μg/μl. About 2 pl of the solution was injected into the egg cytoplasm as described by Gan *et al.* (1990a,b).

Indirect immunostaining

The following antibodies were used for localization of spatial markers. The anti-HpArs rabbit polyclonal antibody was used as a probe for aboral ectoderm (Mitsunaga-Nakatsubo *et al.*, 1998). The anti-Hpoe mouse monoclonal antibody (Yoshikawa, 1997), which recognizes the oral ectoderm-specific antigen Hpoe of *H. pulcherrimus*, was used as a marker for oral ectoderm. The anti-HpEndo16 rabbit polyclonal antibody, which was directed against the recombinant HpEndo16 (Akasaka *et al.*, 1997), was used to localize the protein in the endoderm. The mouse monoclonal antibody PMCA4, which recognizes an antigen in PMCs of *H. pulcherrimus*, was used as a probe for PMCs.

Embryos were fixed in cold methanol (-20°C) for 20 min and rinsed with 80% ASW. For staining with anti-HpArs, anti-HpEndo16, and PMCA4, embryos were blocked in tris-buffered saline/0.1% Triton X-100 (TBST) containing 4% goat serum for 2 h at room temperature. Then, the anti-HpArs 1/1000, anti-HpEndo16 1/1000 and PMCA 1/1000 were used at the indicated dilutions in the presence of 4% goat serum for 1 h at room temperature. Following washes with TBST five times, embryos were incubated with peroxidase-conjugated goat antibody to 1/1000 rabbit IgG (Kirkegaard & Perry, Lab. Inc., MD, USA) or to 1/1000 mouse IgG (CAPPEL™, NC, USA) for 1 h at room temperature. For localizing the Hpoe, embryos were blocked in phosphate-buffered saline/0.1% Tween 20 (TPBS) and 10 mg/ml bovine serum albumin (BSA) at 4°C for 24 h, and then incubated with a 1/1000 dilution of the anti-Hpoe antibody in the presence of 4% normal goat serum at 4°C for 12 h. Following washes with TPBS four times, embryos were incubated with 1/1000 dilution of a peroxidase-conjugated goat anti-mouse IgM (μ chain) (CAPPEL™, NC, USA). The processed embryos mentioned above were rinsed with TBST six times. The TrueBlue™ Peroxidase Substrate (Kirkegaard & Perry, Lab. Inc., MD 20879, USA) was used as the chromogen.

RT-PCR analysis

Sixty control and 60 mRNA-injected embryos were harvested by gentle centrifugation at the unhatched (9 h post fertilization) and the prism stages (42 h), respectively. Total RNA was obtained using ISOGEN (Nippon Gene, Japan). The extracted RNAs were suspended in DEPC-treated H_2O and then were used for reverse transcription by RNA PCR kit (AMV) (TaKaRa, Kyoto, Japan).

One-fifteenth volume of the reverse transcription reaction was used for each PCR reactions. PCR reactions contained 0.2 μM concentrations of appropriate primers and 2.5 mM $MgCl_2$. The oligonucleotides used as primers were 5'-GCTAGTCGTGAAATCAAG-3' and 5'-TTCACTGGTCATTGGACC-3' for $HpOtx_E$, 5'-GTTCGTGAGGCGGTTCG-3' and 5'-TCTATATGACATAGCGGGAT-3' for $HpOtx_L$, 5'-CGACGACATGGGATCTGGC-3' and 5'-CTGTTGCGTAACCGGCCTC-3' for HpArs, 5'-TCCGAATGTTGGTC-3' and 5'-TCCCCCGAGCCTCCTTCCT-3' for HpHbox1, 5'-TACGCCCACGACTTCAACG-3' and 5'-CAGCATTTGGTATAGGCGG-3' for HpEndo16 and 5'-CAGAAGCAACCATGAAGGG-3' and 5'-GACGAGACTGAAGCTAGAG-3' for HpSM50.

The reaction mixture was preheated at 94°C for 2 min. The PCR amplification conditions were 94°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec. All reactions were performed in the linear range of amplification. The products were resolved on 2% agarose gels and then transferred to a Nytran membrane (Schleicher & Schuell, Germany). To visualize the PCR products, hybridization with digoxygenin-labeled RNA probes was followed by commercial Fab fragments of antibody to digoxygenin conjugated to alkaline phosphatase (Boehringer Mannheim, Germany). The chemiluminescent signal produced by enzymatic dephosphorylation of CSPD (TROPIX, MA, USA) by alkaline phosphatase was detected by X-ray film (Fuji, Japan).

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