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Expression Patterns of Smad Family Members during Embryogenesis of the Ascidian *Halocynthia roretzi*

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ABSTRACT—The ascidian embryo has been long thought to show a mosaic mode of development. However, recent studies revealed significance of cell-cell communication during cleavage stages of embryogenesis. FGF and BMP signalings play critical roles in determination of cell types. Little is, however, known about regulation of competence of cells to the signals. Here we report the isolation of ascidian smad genes; *Hrsmad4* which encodes a homolog of smad4 of vertebrates, *Hrsmad6/7* which encodes a homologous gene of smad 6 and smad7 of vertebrates, and *Hrsmad2/3* which encodes a homolog of smad2 and smad3 of vertebrates. The mRNAs of the isolated smad family genes were maternally inherited in egg and early embryos. While *Hrsmad4* and *Hrsmad6/7* RNAs distributed broadly in the early embryos, *Hrsmad2/3* RNA was preferentially accumulated in the animal hemisphere.

INTRODUCTION

Cell-cell communications are a mechanism to make diversification of cell types during embryogenesis. Ascidian eggs are considered as a typical mosaic egg, in which the inheritance of maternal deteminants accounts for cell fate specification of epidermis, muscle and endoderm (Nishida, 1997). However, cell-cell communications are required to form notochord, mesenchyme, trank lateral cells and neural tissues (Nakatani and Nishida, 1994; Kim and Nishida, 1999; Kawaminami and Nishida, 1997; Rose, 1939; Nishida, 1991). Basic fibroblast growth factor (bFGF) and bone morphogenetic factor (BMP) were reported to function in these interactions (Nakatani *et al.*, 1996; Inazawa *et al.*, 1998; Kim *et al.*, 2000; Miya *et al.*, 1997; Darras *et al.*, 2001). Endogenous FGF in the ascidian is not reported, while an FGF receptor-encoding cDNA is isolated (Kamei *et al.*, 2000). On the other hand, molecular biological study on BMP signaling in the ascidian embryo has revealed that *HrBMPa* (BMPs 5–8 homolog) is expressed in presumptive epidermis (Miya *et al.*, 1996), while *HrBMPb* (BMPs 2/4 homolog) is expressed only in the vegetal hemisphere in the gastrula (Miya *et al.*, 1997). Overexpression of the BMPs in the whole embryo by injecting the synthetic RNA results in the neural-to-epidermal transformation in the anterior-most region of the tailbud embryo (Miya *et al.*, 1997). Little is, however, known about regulation of competence of cells to the signals: i. e. which cells receive and properly transduce the signaling molecules that mediate the cellular interactions during normal embryogenesis.

BMP belongs to the transforming growth factor (TGF-β) superfamily. The members of the TGF-β superfamily are secreted signaling molecules that have important roles in the developmental processes (reviewed by Kingsley, 1994). Signalings of TGF-β superfamily members are transduced by smad family members into nuclei in the signal-receiving cells to activate or repress specific gene expression (Heldin *et al.*, 1997). The functions of *smad* homologous gene products have been isolated and analyzed extensively using *Xenopus* embryos. *Smad* homologous gene products isolated from many species are divisible into several groups, and it was suggested that the genes in the same group have similar activities (reviewed by Wrana and Attisano, 1996).

The Smad family members have the ability to bind to type1 and type2 TGF-β superfamily receptors and are involved in a cascade of TGF-β superfamily signal transduction. Smad1 and smad5 transduce BMP signals, whereas smad2 and smad3 transduce TGF-β/activin signals. In the signal transduction, smad1 is directly phosphorylated by an activated type1 BMP receptor, and the phosphorylated smad1 is translocated to the nucleus (Hoodless *et al.*,. 1996; Liu *et al.*, 1996; Kretzschmar *et al.*, 1997), whreas smad2 is phosphorylated by an activated type 1 TGF-β recetor (Eppert *et al.*, 1996; Zhang *et al.*, 1996; Nakao *et al.*, 1997b; Chen *et al.*, 1996). Smad4 forms a heterodimer with other smad molecules and is involved in both signal transductions. On the other hand, smad6 and smad7 inhibit TGF-β and BMP signaling by preventing other smad molecules from being phosphorylated and activated. Thus, signalings of TGF-β superfamily are regulated by smad family proteins both positively and negatively. It has been shown that the *Smad* gene family is preserved among a vari-

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ety of species and is important in animal development (Padgett *et al.*, 1998; Whitman *et al.*, 1998).

We previously reported that ascidian smad gene, *Hrsmad1/5*, which is a homologous gene of smad1 and smad5 of vertebrates is expressed in fate-restricted epidermis cells (Kobayashi *et al.*, 1999). The expression of the gene was observed not only in the anterior-most neural cells of the animal hemisphere of the embryo but also in the presumptive epidermis cells throughout the animal hemisphere, which do not respond to the injected BMP. In contrast, most of neural cells which also possess maternal *Hrsmad1/5* do not respond the signal. These suggested that molecules but smad1/5 restrict the spatial range of competence to BMP signaling in the ascidian embryo. To gain the overall information of the BMP and TGF-β signaling in the ascidan embryo, we isolated each homolog of all smad family members from *Halocynthia roretzi*.

Here we report the isolation of ascidian smad genes; *Hrsmad4* which is a homolog of smad4 of vertebrates, *Hrsmad6/7* which is a homologous gene of smad 6 and smad7 of vertebrates, and *Hrsmad2/3* which is a homolog of smad2 and smad3 of vertebrates.

MATERIALS AND METHODS

Eggs and embryos

Adults of the ascidian *Halocynthia roretzi* were purchased near the Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo, Iwate, Japan. Naturally spawned eggs were fertilized with a suspension of non-self sperm. The fertilized eggs were reared in filtered seawater containing 50 mg/ml streptmycin at 11–13°C. Embryogenesis proceeded with a high degree of synchrony in various batches of eggs. At this temperature, they developed into gastrulae and early tailbud embryos about 12 hr and 24 hr after fertilization, respectively.

Isolation of cDNA clone for ascidian smad genes and sequencing

PCR fragments of smad4 and smad6/7 were amplified from poly(A)+RNA of the early gastrulae and the 64-cell embryos, respectively, using degenerated primers which correspond to the conserved MH2 regions of the smad family proteins: 5'-TGGTGYVWIR-TIGCITAYTGGGA-3' and 5'-CCCCANCCYTTNACRAARCTIAT-3'. PCR conditions were 30 cycles of 94°C for 1 min, 37°C for 2 min, 94°C for 2 min. The obtained 434 bp fragments encoding smad6/7 and smad4 were used to screen a cDNA library as probes.

Smad2/3 was found in a cDNA project in which arrayed clones of *H. roretzi* fertilized egg-cDNA library were sequenced in order, then the sequences were used to search homology (Makabe *et al.*, in preparation). The sequences of the clones were determined by an automated DNA sequencer (ABI PRISM377, PE Biosystems Japan, Chiba). Because the clone did not contain a full-length smad2/3 cDNA, the full length cDNA was obtained by 5'RACE using SMART RACE cDNA amplification kit (Clontech, USA). The cDNA was digested by some restriction enzymes and subcloned to be determined the entire sequence.

Sequence comparisons and molecular phylogenetic analysis

We introduced gaps between the amino acid sequences of smad family to align them. Molecular phylogenetic relationships of the smad family gene products were estimated by means of neighbor-joining method (Saitou and Nei, 1987) using the PHYLIP ver. 3.5c package (Felsenstein, 1993). The distance matrix was constructed according to the Dayhoff model (Dayhoff *et al.*, 1978). Confidence in the phylogeny was assessed by bootstrap resampling of the data (Felsenstein, 1985).

Whole-mount *in situ* hybridization

Digoxigenin (DIG)-labelled antisense RNA probe was synthesized following instructions from the suppliers of the kit (DIG RNA Labeling Kit, Roche Diagnostics, Tokyo). Its final size was reduced to about 500 nucleotides by limited alkaline hydrolysis. Whole-mount specimens were fixed in 4% paraformaldehyde in 0.1 M MOPS buffer (pH 7.5), 0.5 M NaCl for 12h at 4°C. The method of whole mount *in situ* hybridization was carried out basically as described previously (Miya *et al.*, 1994; Kobayashi *et al.*, 1999; Satou, 1999). Photo images of stained samples were captured by digital cameras: for *Hrsmad4* and *Hrsmad6/7*, Olympus OP50-CAM-SP on a microscope (Olympus BX60), for *Hrsmad2/3*, Olympus HC-300Z/OL on a dissecting microscope (Olympus SZH10).

RESULTS AND DISCUSSION

Isolation and sequence analysis of the ascidian homologs of smad family members

Molecular phylogenetic analysis suggested that smad family consist of three subfamilies: a common smad, a signal-specific smad and an inhibitory smad subfamily (Fig. 1A). Furthermore, the signal-specific smad subfamily members are divided into a smad1 and 5 subclass involved in BMP signaling and a smad2 and 3 subclass involved in TGF-β/activin signaling. The inhibitory smad contains smad6 and 7. Of these, we previously reported on the ascidian smad1/5 homolog (Kobayashi *et al.*, 1999). Here we tried to isolate and characterize all the others.

Smad4 is a common inevitable partner of signal-specific smad proteins (Zhang *et al.*, 1996; Kretzschmar *et al.*, 1997; Lagna *et al.*, 1996). When BMP or TGF-β/activin signal is transduced, signal-specific smad such as smad1, 5, 2 and 3 activated by the receptors associate with smad4 to translocate into the nucleus. On the other hand, smad6 and smad7 proteins are known to function as inhibitors of BMP signaling and TGF-β/activin signalings (Imamura *et al.*, 1997; Nakao *et al.*, 1997a). These share two molecular characteristics: they lack an SSXS phosphorylation domain in the carboxyl terminus conserved in the signal-specific smad proteins. Also, an MH1 domain of the inhibitory smad proteins is less conserved among the smad family. To isolate homologs of these from the ascidian *H. roretzi*, we designed the degenerate oligonucleotide primers correspoding to the conserved MH2 domains of smad proteins for PCR. Poly(A)+RNA were purified from embryos in several developmental stages and provided for RT-PCR using the primers. A band of the expected 434 bp in length in each lane in a gel was cloned into a plasmid and sequenced. As the results, cDNA obtained from the gastrulae encoded smad4, while cDNA from the 64-cell embryos encoded smad6/7. The PCR fragment from the gastrulae was used as a probe to obtain full-length cDNAs from cDNA libraries and we cloned the full-length cDNA encoding smad4. It was 2923 bp long and its deduced amino acid was 514 residues. In Fig. 2, the putative amino acid sequence deduced from the full-length cDNA showed high similarity to mouse

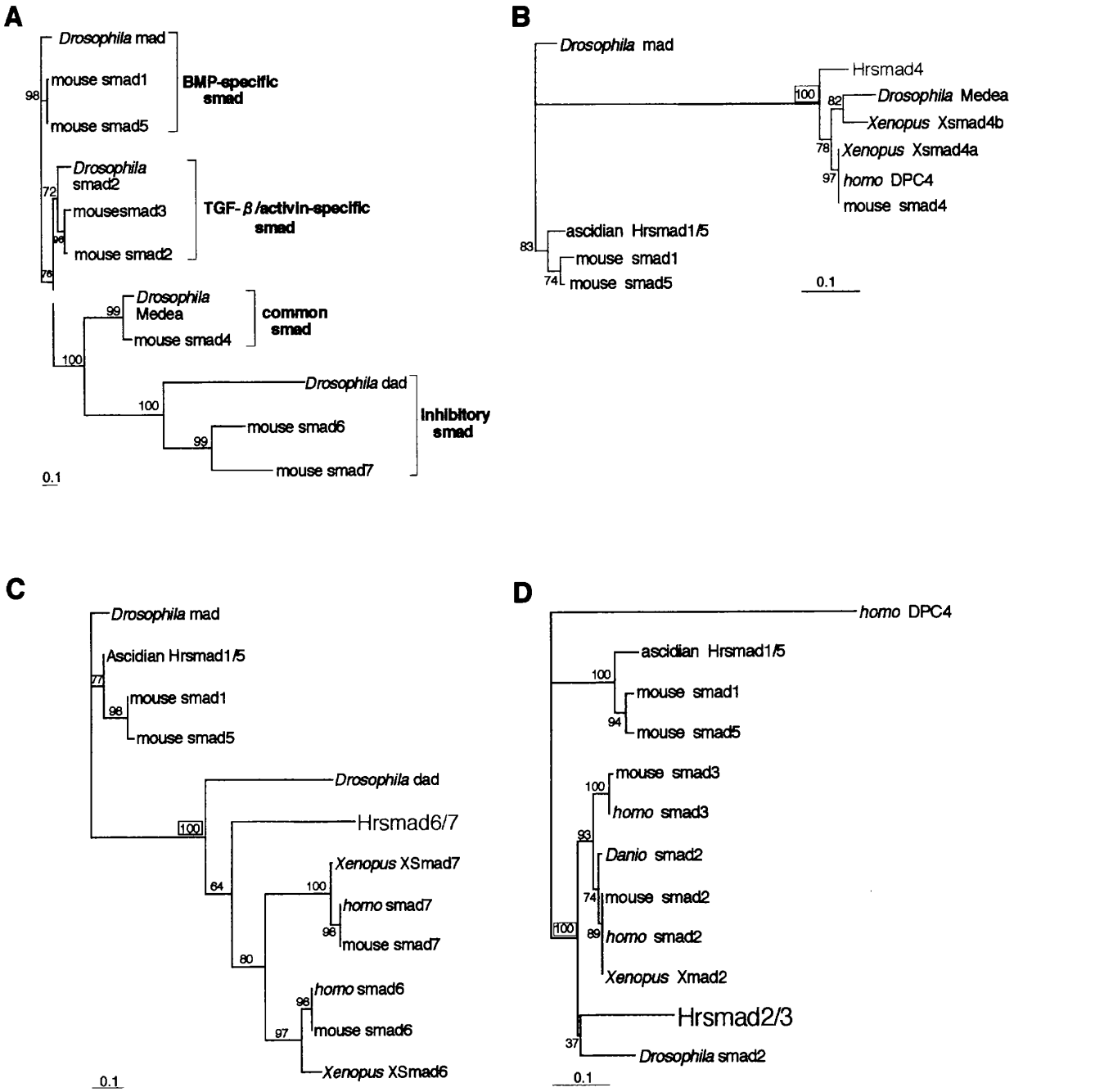

Fig. 1. Phylogenetic tree of smad family genes constructed by the neighbor-joining method, based on the comparison of amino acid sequences of the MH1 domain in the N-terminus and the MH2 domain in the C- terminus. **A,** this tree was constructed from 134 amino acid residues. Mouse smad1 (Yingling *et al.*, 1996; accession no. U58992), mouse smad2 (Baker and Harland, 1996; accession no. NP_034884), mouse smad3 (Yang *et al.*, unpulished; accession no. NP_058049), mouse smad4 (Anna and Devereux, 1997; accession no. NP_032566), mouse smad5 (Yingling *et al.*, 1996; accession no.U58993), mouse smad6 (Imamura *et al.*, 1997; accession no. NP_032568), mouse smad7 (Nakao *et al.*, 1997a; accession no. 2460040), *Drosophila* mad (Sekelsky *et al.*, 1995; accession no.P42003), *Drosophila* smad2 (Brummel *et al.*, 1999; accession no. AAD11458), *Drosophila* Medea (Wisotzkey *et al.*, 1998; accession no. AAC38971) and *Drosophila* dad (Tsuneizumi *et al.*, 1997; accession no. 2541864) were included. The numbers indicate the relative robustness of each node as assessed by bootstrap analysis. **B,** this tree was constructed from 205 amino acid residues. In addition of those used in A, *Xenopus* Xsmad4 α (Masuyama *et al.*, 1999; accession no.BAA77514), *Xenopus* Xsmad4 β (Masuyama *et al.*, 1999; accession no.BAA77515), ascidain Hrsmad1/5 (Kobayashi *et al*, 1999; accession no.AB018106) were included. **C,** this tree was constructed from 65 amino acid residues of the MH2 domain in the C-terminus. In addition of those used in A and B, human smad6 (Hata *et al.*, 1998; accession no.AAB94137), human smad7 (Nakao *et al.*, 1997a; accession no.2460042), *Xenopus* XSmad6 (Nakayama *et al.*, 1998; accession no.AF041839) and *Xenopus* XSmad7 (Casellas and Hemmati-Brivanlou, 1998; accession no.2921581) were included. **D,** this tree was constructed from 217 amino acid residues. In addition of those used in A and C, *Xenopus* Xmad2 (Graff *et al.*, 1996; accession no.AAB39329), human smad2 (Eppert *et al.*, 1996; accession no.U65019) and human smad3 (Arai *et al.*, 1998; accession no.BAA22032) were included. Bootstrap confidence level is based on 100 replications. Bar, evolutionary distance of 0.1 amino acid substitutions per position in the sequence.

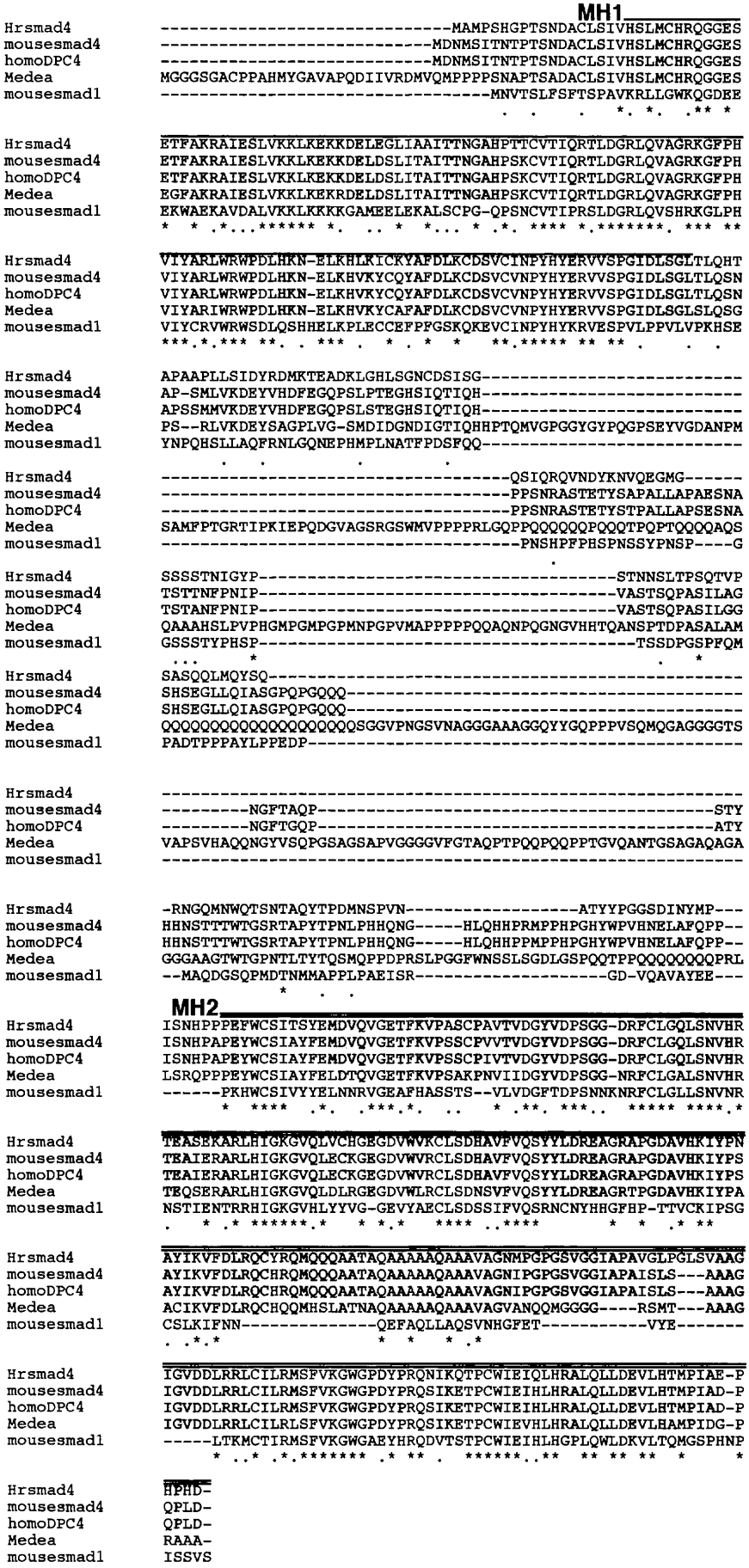

Fig. 2. Comparison of the amino acid sequence of the ascidian Hrsmad4 with mouse smad4, human DPC4 (Hahn *et al.*, 1996; accession no. NP_005350), *Drosophila* Medea and mouse smad1. The asterisks show amino acids conserved in all of these proteins and the dots show those partially conserved in the proteins. Amino acids specifically conserved in a smad4 subfamily are shaded. The hyphens are inserted for alignment. MH1, MH1 domain; MH2, MH2 domain.

smad4 (Anna and Devereux, 1997), human smad4 (Hahn *et al.*, 1996) and *Drosophila* Medea (Wisotzkey *et al.*, 1998) and low similarity to mouse smad1 (Yingling *et al.*, 1996). In contrast, unfortunately, any positive signals were not detected in the cDNA-library screen or 5' and 3'RACE experiments using the PCR fragment from the 64-cell embryos for a long cDNA encoding smad6/7, suggesting low prevalence of this mRNA in the ascidian embryo (see below). Comparisons of the putative amino acid sequences deduced from these sequences with other smad proteins are shown in Figs. 2 and 3. Similarly, as shown in Fig. 3, the deduced amino acid sequence of the PCR fragment had high similarity to mouse smad6 (Imamura *et al.*, 1997), smad7 (Nakao *et al.*, 1997a) and *Drosophila* Dad (Tsuneizumi *et al.*, 1997), while it showed low similarity to mouse smad1. This suggested that this belongs to the smad6/7 subclass. To confirm the smad subclasses these two genes belong to, we constructed molecular phylogenetic trees by the neighboring-joining method (Saitou and Nei, 1987). Fig. 1B and C indicated these are the ascidian homologs of smad4 and smad6/7, respectively. It is also suggested that vertebrate *smad6* and *smad7* diverged after separation of the ascidian gene from the common ancestral gene. We therefore designated these genes *Hrsmad4* and *Hrsmad6/7*, respectively.

Smad2 and smad3 proteins are transducers for TGF-β signaling (Chen *et al.*, 1996). In the process of an EST project

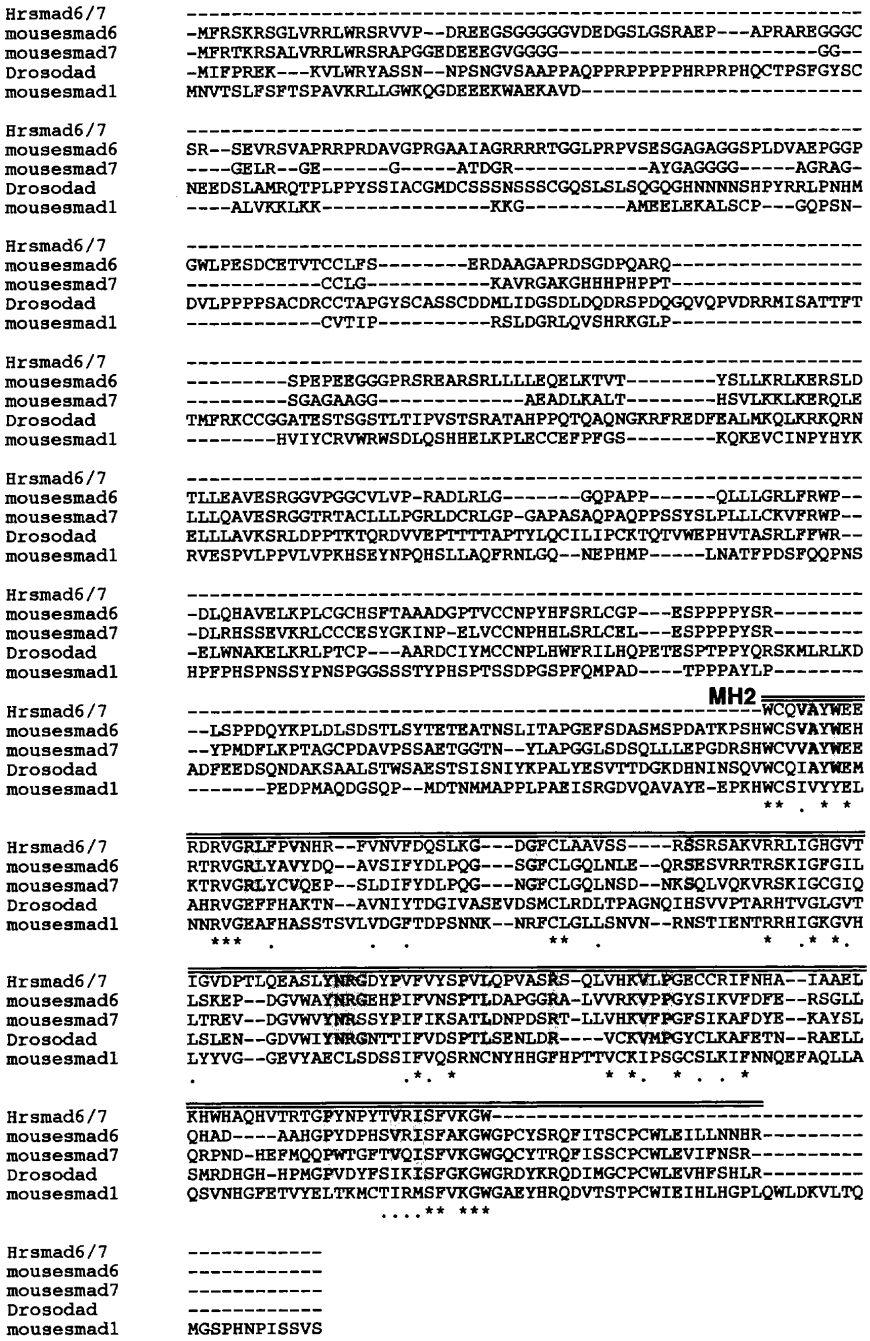

Fig. 3. Comparison of the amino acid sequence of the ascidian Hrsmad6/7 with mouse smad6, mouse smad7, *Drosophila* dad and mouse smad1. The asterisks show amino acids conserved in all of these proteins and the dots show those partially conserved in the proteins. Amino acids specifically conserved in the inhibitory smad subfamily are shaded.

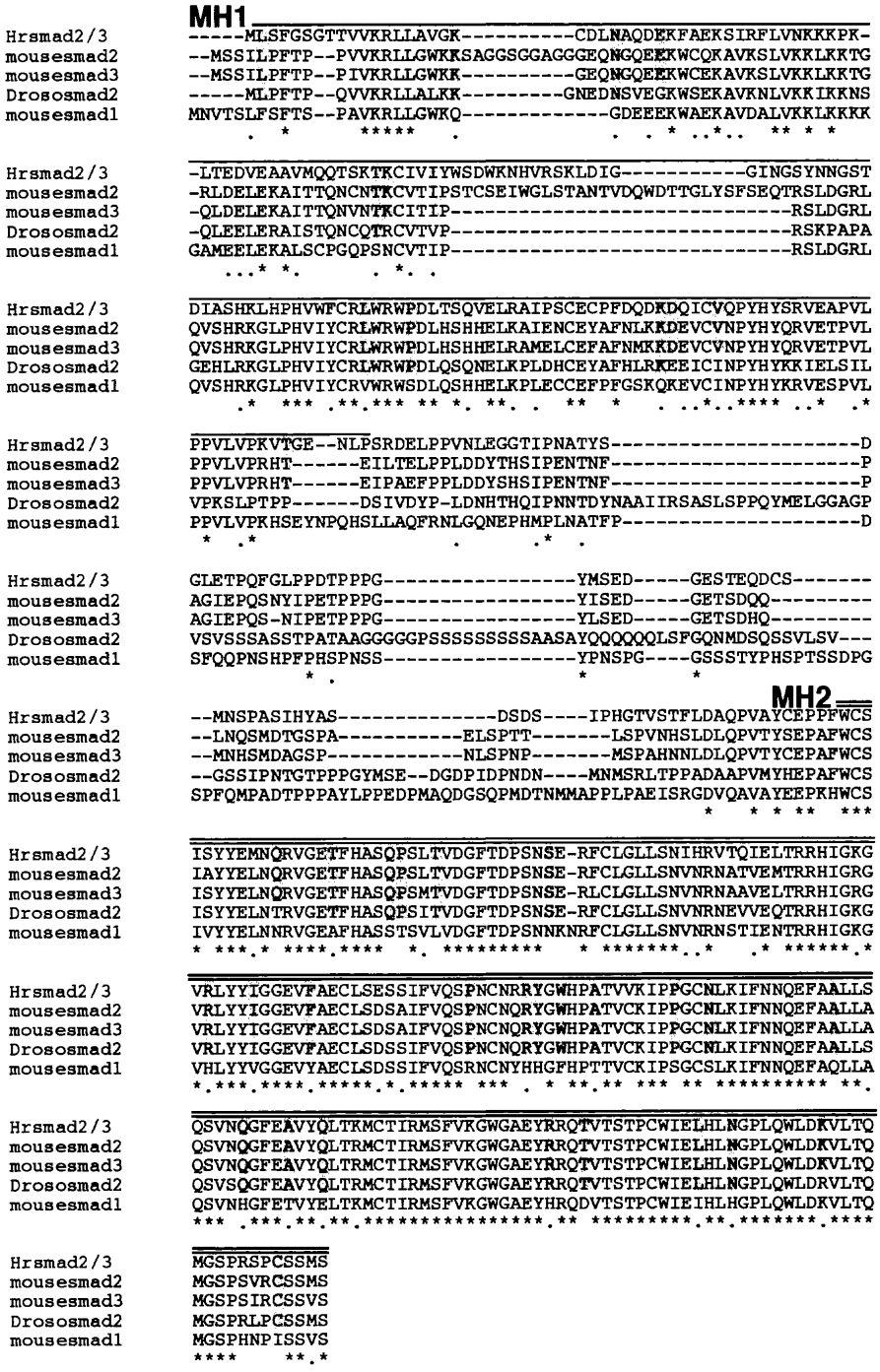

Fig. 4. Comparison of the amino acid sequence of the ascidian Hrsmad2/3 with mouse smad2, mouse smad3, *Drosophila* smad2, and mouse smad1. The asterisks show amino acids conserved in all of these proteins and the dots show those partially conserved in the proteins. Amino acids specifically conserved in a smad2/3 subfamily are shaded.

in which randomly selected clones of cDNA library from fertilized eggs of *H. roretzi* were sequenced their 5' and 3' termini and homology search of the sequences to databases was carried out, a clone which has high similarity to smad2 and 3 was isolated. Because this cDNA was 2972 bp long and did not contain the first methione, the specific nucleotide primers were synthesized to carry out 5'RACE experiment using the nucleotide sequence of this cDNA. The obtained clone was sequenced and found to be 3170 nucleotides in length and encodes a 450 amino acid polypeptide. The deduced amino acid sequence was compared with smad proteins (Fig. 4). The sequence had high similarity to mouse smad2 (Baker and Harland, 1996), smad3 (Yang *et al.*, unpublished) and *Drosophila* smad2 (Brummel *et al.*, 1999), while it showed low similarity to mouse smad1. The molecular phylogenetic tree indicated that vertebrate *smad2* and *smad3* diverged after separation of this gene from the common ancestral gene and this gene belongs to smad2/3 subclass (Fig. 1D). We named this *Hrsmad2/3*.

Spatial and temporal expression patterns of the ascidian smad genes

Kobayashi *et al.* (1999) demonstrated that *Hrsmad1/5* is expressed entirely in the presumptive epidermis in the animal

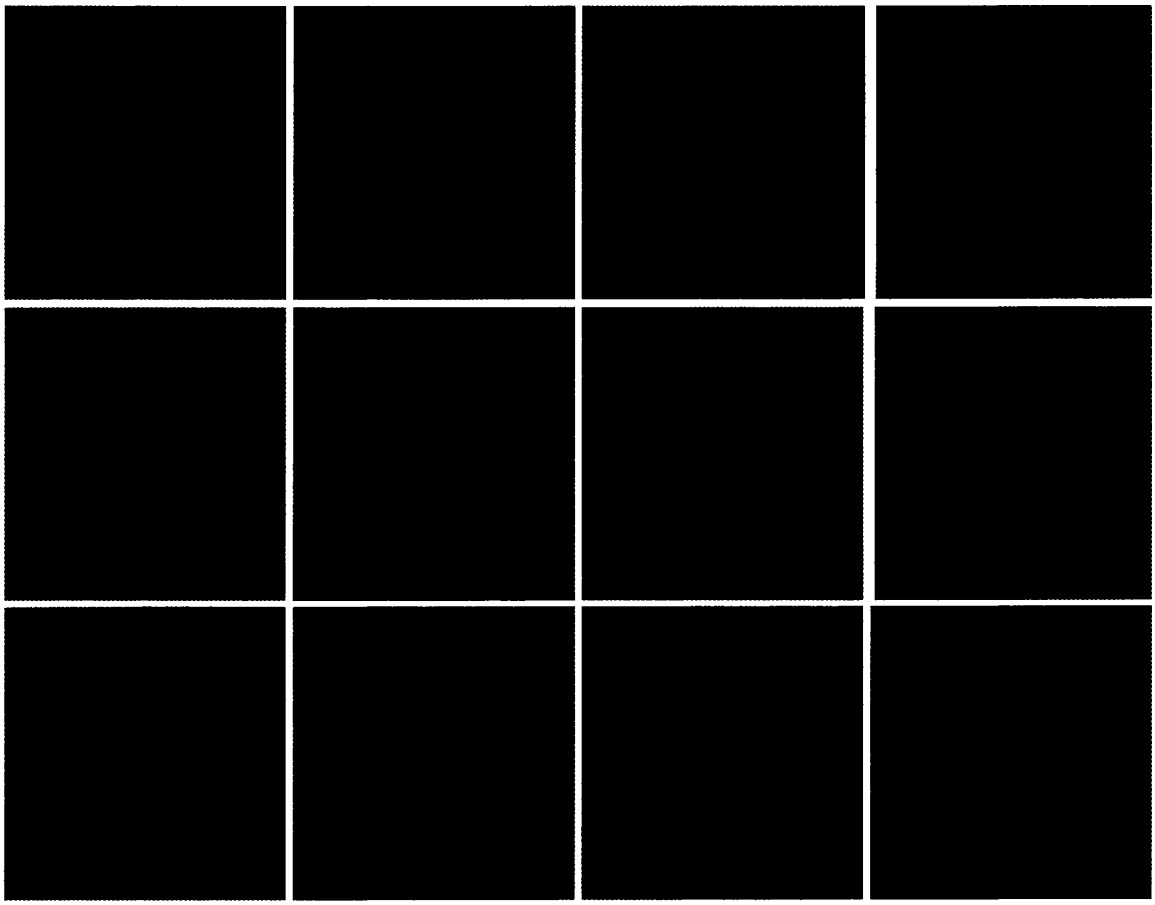

Fig. 5. Spatial expression of *Hrsmad4* revealed by whole-mount *in situ* hybridization with digoxigenin-labeled antisense RNA probe. **A, B,** Fertilized eggs, laterral view. **C, D,** 8-cell stage embryos, lateral view. **E, F**, 16-cell embryos, animal view. **G, H,** 64-cell embryos, animal view. **I, J,** Neurulae, lateral view. **K, L,** Middle tailbud embryos, lateral view. Controls by the sense RNA probe were represented in **B, D, F,** H, J, L. Scale bar, 100 μm.

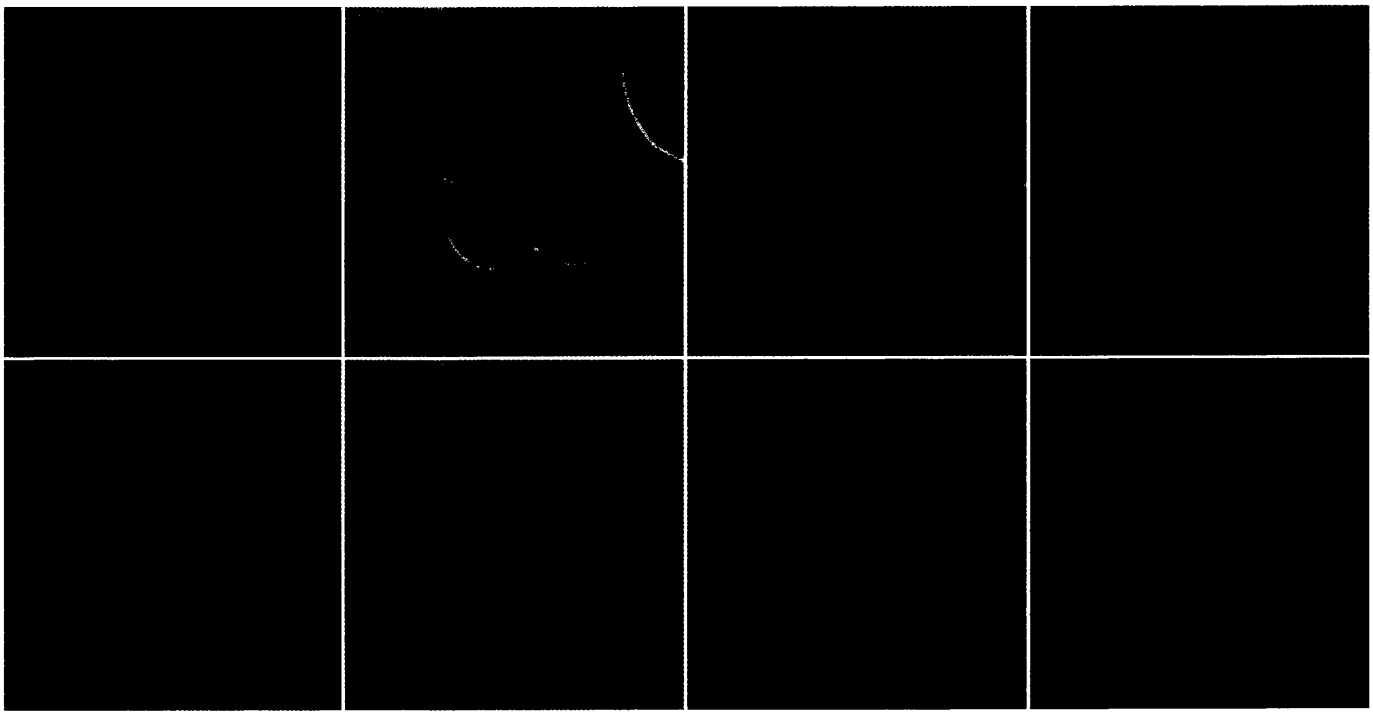

Fig. 6. Spational expression of *Hrsmad6/7*. **A,** A fertilized egg, lateral view. **B,** An 8-cell embryo, lateral view. **C,** A 16-cell embryo, animal view. **D, E,** 64-cell embryos, animal view. **F,** A middle gastrula, animal view. **G,** A neurula, lateral view. H, A middle tailbud embryo, lateral view. Control by the sense RNA probe was represented in D. Scale bar, 100 μm.

hemisphere, irrespective of the effect in the limited anterior region by BMP overexpression (Miya *et al.*, 1997). The incompetence of the epidermis precursors to BMP signaling can be explained in several ways. They may lack smad4, lack the receptor or express the inhibitory smad, or the endogenous BMP signaling is already transduced, for example. On the other hand, it is also unsolved how the competence in the presumptive neural cells is restricted. To investigate the molecular mechanisms involved in cell-cell communication mediated by TGF-β superfamily signalings during ascidian embryogenesis, we analyzed the spatial and temporal expression patterns of the ascidian smad genes by whole-mount *in situ* hybridization to the staged embryos.

Maternal *Hrsmad4* transcripts were detected ubiquitouly in the fertilized egg (Fig. 5A). During cleavage stages, the maternal mRNA remained periphery of cytoplasm in every blastomeres (Fig. 5C, E, G). In addition, there was nuclear staining in B-line (poterior-vegetal) blastomeres in the 16-cell embryo (Fig. 5E). From the neurula stage onward, the staining was mainly observed in the epidermis (Fig. 5I). In the tailbud embryo, the intense staining was seen in the trunk and the tail epidermis (Fig. 5K). To verify that these stainings were due to endogenous mRNA, hybridization experiments using the sense probe were simultaneously carried out to exclude a possibility of high background (Fig. 5B, D, F, H, J, L). This was the same case as reported in various animals, in which smad4 is expressed throughout in the embryos (Wisotzkey *et al.*, 1998; Anna and Devereux, 1997).

Although the *Hrsmad6/7* cDNA we cloned was not a full-length but 434 bp in length as described, it is long enough to detect the specific signal in whole-mount *in situ* hybridization (Satou *et al.*, 1995). In the intensive staining after long incubation, the maternally derived transcripts were observed throughout embryogenesis. They were present in the entire cytoplasm in the egg, and more or less succeeded by almost all blastomeres (Fig. 6A, B). In the cleavage-stage embryos, the signals were seen in the periphery of cytoplasm (Fig. 6B-E). In the 16-cell embryo, the signal intensity was relatively

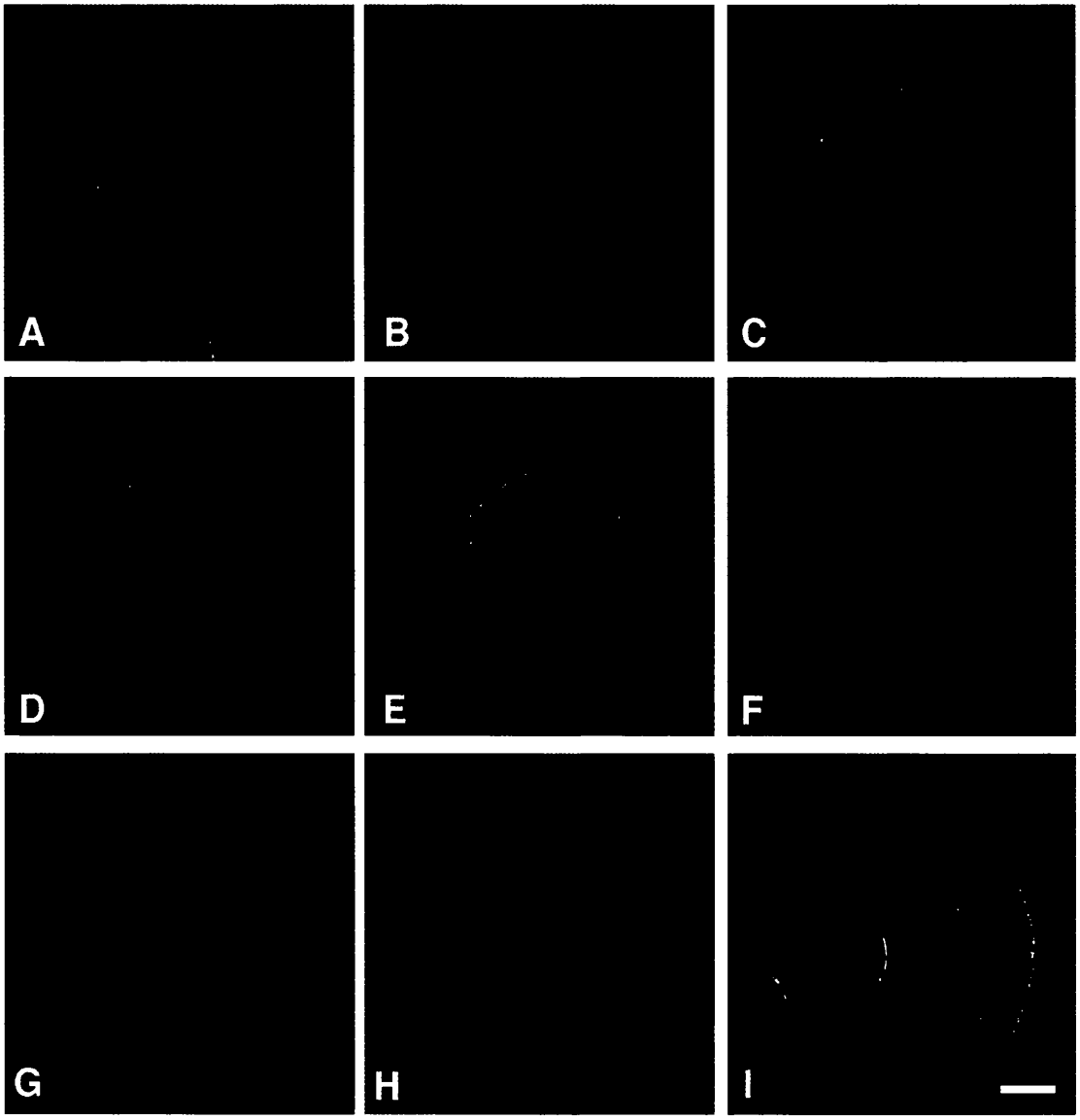

Fig. 7. Spational expression of *Hrsmad2/3*. **A,** A fertilized egg, lateral view. **B,** An 8-cell embryo, lateral view. **C,** A 16-cell embryo, animal view. **D,** A 16-cell embryo, vegetal view. **E,** A 64-cell embryo, animal view. **F,** A 110-cell embryo, animal view. **G,** A 110-cell embryo, vegetal view. **H,** A neurula, dorsal view. **I,** A middle tailbud embryo, lateral view. Scale bar, 100 μm.

high in the animal hemisphere, middle in A-line (anterior-vegetal) blastomeres and low in the B-line blastomeres (Fig. 6C). The control simultaneously carried out using the sense probe verified that these were not due to background by the intensive staining (Fig. 6D). The signals were detected in the entire embryos until the tailbud stage (Fig. 6E-I). Northern blot also showed no signal throughout embryogenesis suggested that *Hrsmad6/7* RNA is rare in the embryo (data not shown).

Smad2 and 3 are known to mediate TGF-β/activin signals. Although the TGF-β/activin signalings are not reported to function during ascidian embryogenesis, finding of the ascidian homolog of smad2/3 in the egg as a maternal stock suggested that TGF-β/activin signalings will be occurring in early development. In order to obtain clues of the putative functions of the signalings, we investigated the expression of *Hrsmad2/3*. The maternal messages were seen ubiquitously in the egg (Fig. 7A). From the 8-cell stage onward, the mRNA appeared to be partitioned mainly into the animal blastomeres (Fig. 7B). *Hrsmad2/3* showed the preferential sequestration of the mRNA in the periphery of the animal blastomeres and little partition in the vegetal blastomeres all through the cleavage stages from the 16-cell embryos (Fig. 7C, D), the 64-cell embryo (Fig. 7E) to the 110-cell embryos (Fig. 7F, G). Reflecting this, stainings were observed in the epidermis on the entire surface of the embryos from the neurula to tailbud stages (Fig. 7H, I).

In conclusion, of TGF-β superfamily tranduction, the molecules involved in the BMP signaling we analyzed in this study broadly distributed in the early embryos unlike *Hrsmad1/5*, whereas the component of the TGF-β/activin signaling showed the similar expression pattern as *Hrsmad1/5* whose RNA is detected only in the presumptive epidermis. In order to resolve the question of what molecule(s) determine the spatio-temporal range of competence of signal-receiving cells to TGF-β superfamily signalings, investigation at the protein level using the specific antibodies against activated forms of smad proteins and investigation of ascidian homologs of BMP/TGF-β receptors are in progress.

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