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# Spatio-Temporal Expression Patterns of Eight Epidermis-Specific Genes in the Ascidian Embryo

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**ABSTRACT**—During embryogenesis of the ascidian *Halocynthia roretzi*, exactly eight-hundred epidermal cells are formed in the larva, and the lineage of the cells has been almost completely described. In the present study, we examined the spatio-temporal expression patterns of eight epidermis-specific genes which we already isolated. *In situ* hybridization of whole-mount specimens unambiguously demonstrated that the expression patterns of the eight genes were not identical, and that they were categorizable into several types. Transcripts of seven genes were restricted to presumptive epidermal cells, although transcripts of one gene were evident in the presumptive neural cells in addition to the presumptive epidermal cells. Therefore, most of the epidermis-specific genes in ascidian embryos are expressed in lineage-associated manner. We discuss these results in relation to the question of whether (a) epidermis-specific genes are expressed exclusively in presumptive epidermal cells prior to neural induction, or (b) the genes are first expressed in both epidermal precursors and precursors of the central nervous system, then the gene expression is downregulated in the latter after the completion of neural induction. Interestingly, cells of the anterior-most region as well as the dorsal midline of the tailbud embryo did not express most of the epidermis-specific genes, suggesting regional differences in the embryonic epidermis. Some other genes might be expressed in a complementary pattern in these regions.

## INTRODUCTION

The epidermis of multicellular animals serves several important functions. Because the identity of individuals is achieved by this tissue, sensory machinery as well as defense machinery have evolved in association with epidermis. During embryogenesis of the ascidian *Halocynthia roretzi*, exactly 800 epidermal cells differentiate to cover the outermost part of the embryo (Nishida, 1987; reviewed by Satoh, 1994). The lineage of the epidermal cells has been defined almost completely (Conklin, 1905; Ortolani, 1962; Nishida, 1987; Nicol and Meinertzhagen, 1988). All the epidermal cells originate from the four cells of the animal half of the eight-cell embryo (the anterior a4.2 and the posterior b4.2 pairs of cells). The vegetal four blastomeres are not involved in the formation of epidermis. As early as the 16-cell stage, the developmental fate of a pair of animal blastomeres becomes restricted to epidermis. Five pairs of blastomeres of the 32-cell embryo and eleven pairs of the 64-cell embryo become the primordial epidermal cells (Nishida, 1987). Reflecting the early establishment of their developmental fate, presumptive epidermal cells show high potential for autonomous differentiation when they are isolated from early embryos (reviewed by Satoh, 1994). This autonomy is dependent on prelocalized maternal factors (Nishida, 1994).

We have already isolated and characterized several cDNA clones that correspond to epidermis-specific genes of the ascidian embryo (Ueki *et al.*, 1991, 1994; Ueki and Satoh, 1994, 1995). When cleavage of fertilized eggs of *H. roretzi* was blocked and the blocked eggs were cultured as one-celled embryos for about 30 hr, they developed features of differentiation of the epidermal cells only (e.g., Nishikata *et al.*, 1988). Utilizing these characteristics, we isolated cDNA clones for eight different epidermis-specific genes (Ueki *et al.*, 1991; Ueki and Satoh, 1995). In the present study, using the cDNA as probe, we examined the following questions as to the gene expression leading to epidermis differentiation in ascidian embryos: How is the expression of these epidermis-specific genes regulated spatially and temporally during the differentiation of epidermis? Are these epidermis-specific gene expressions regulated in the same manner, or differentially according to the gene or the region of the embryo? Are these genes really expressed exclusively in cells of epidermal lineage throughout the embryogenesis? Since the finding of the "organizer" by Spemann and Mangold (1924), the differentiation of epidermis in vertebrate embryos has been studied and discussed repeatedly in relation to the neural induction (e.g., Spemann, 1938; Slack, 1991). Transplantation of presumptive chordal mesoderm of an early amphibian embryo into a blastocoel of another embryo causes transdifferentiation of epidermal cells into neural cells. This induction, in turn, results

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in the formation of secondary axis of the embryo. During normal embryogenesis, both the epidermis and the central nervous system (CNS) are derived from ectoderm, and their early territories are continuous. In this context, there is a question as to whether (a) epidermis-specific genes are expressed exclusively in differentiating epidermal cells before neural induction, or (b) the genes are first expressed in cells of presumptive epidermis and in those of CNS, and then the gene expression is downregulated in the latter after neural induction.

## MATERIALS AND METHODS

*Ascidian eggs and embryos*

*Halocynthia roretzi* was collected during the spawning season near the Asamushi Marine Biological Station of Tohoku University, Aomori, Japan. *H. roretzi* is a self-sterile hermaphrodite. Naturally spawned eggs were fertilized with a suspension of non-self sperm, and fertilized eggs were cultured in filtered seawater at about 13°C. Embryogenesis proceeded with a high degree of synchrony in various batches of eggs. At this temperature, the first cleavage took place about 2 hr after insemination, and embryos divided at approximately hourly intervals. They developed into gastrulae, neurulae, and early-tailbud stage embryos about 12, 15, and 20 hr after fertilization, respectively. Tadpole larvae hatched about 35 hr after fertilization.

Eggs and embryos at appropriate stages were collected by low-speed centrifugation and fixed for *in situ* hybridization.

*cDNA probes for* H. roretzi *epidermis-specific genes*

Isolation and characterization of cDNA clones for eight different epidermis-specific genes of *H. roretzi* embryos were reported previously (Ueki *et al.*, 1991, 1994; Ueki and Satoh, 1994, 1995). The cDNAs were inserted into the *Eco*RI site of the plasmid vector pBluescript II SK(+) or (–) (Stratagene, La Jolla, CA, USA). The length of the inserts ranged from 0.9 kb to 2.1 kb. Digoxigenin (DIG)-labeled sense and antisense probes were synthesized following the instructions from the suppliers of the kit (DIG RNA Labeling kit; Boehringer Mannheim, Heidelberg, Germany). Their final sizes were reduced to approximately 150 nucleotides by alkaline hydrolysis.

In situ *hybridization*

Whole-mount specimens were hybridized *in situ* using DIG-labeled antisense and sense RNA probes essentially according to the method described by Yasuo and Satoh (1994). Briefly, specimens were fixed in 4% paraformaldehyde in 0.1M MOPS buffer (pH 7.5), 0.5 M NaCl. After being thoroughly washed with PBT (phosphate-buffered saline containing 0.1% Tween 20), the specimens were treated with 2 μg/ml proteinase K (Sigma, St. Louis, MO, USA) in PBT for 30 min at 37°C, then post-fixed with 4% paraformaldehyde in PBT for 1 hr at room temperature. After a 1-hr period of prehybridization at 42°C, the specimens were allowed to hybridize with the DIG-labeled probes at a concentration of 1 μg/ml for at least 16 hr at 42°C. After hybridization, the hybridization solution was gradually replaced by PBT, then the specimens were digested with 20 μg/ml RNase A (Sigma). Thereafter, the samples were incubated for 1 hr with 500 μl anti-DIG-alkaline phosphatase conjugate, and color was developed according to the Boehringer protocol.

After dehydration, some of the specimens were cleared by placing them in a 1:2 mixture (v/v) of benzyl alcohol and benzyl benzoate.

## RESULTS

With the aid of *in situ* hybridization of whole-mount specimens, we carefully examined the distribution of transcripts of each of the eight epidermis-specific genes in *H. roretzi* embryos. In most cases of whole-mount *in situ* hybridization of ascidian embryos, signals are first detected in the nucleus of certain blastomeres, then the signals distribute over the entire cytoplasm (e.g., Yasuo and Satoh, 1993; Satou *et al.*, 1995). This situation enables us to judge unambiguously which blastomeres of early embryos express the gene and which do not. Of the eight different epidermis-specific genes, *HrEpiA, HrEpiB, HrEpiE, HrEpiF, HrEpiG* and *HrEpiH* are exclusively zygotic, whereas *HrEpiC and HrEpiD* are both maternal and zygotic (Ueki *et al.*, 1991; Ueki and Satoh, 1995). In the present study, we examined the zygotic expression of these genes. Controls hybridized with sense probes did not show signals above the background (data not shown).

*Lineage of cells of epidermis and CNS*

In order to help the reader's understanding of the spatial distribution of epidermis-specific gene transcripts, we briefly describe here the lineages of epidermis and CNS of the ascidian embryo. The lineages of epidermis and CNS are well described (Conklin, 1905; Ortolani, 1962; Nishida, 1987; Nicol and Meinertzhagen, 1988). The ascidian embryo is bilaterally symmetrical, the first cleavage plane being only a plane of symmetry. The gene expression is usually detected symmetrically in a pair of right and left blastomeres. For example, in the following description, we used the a8.25 cells instead of a pair of right a8.25 and left a8.25 cells.

As shown in Fig. 1, the epidermis is derived entirely from blastomeres of the animal hemisphere of early embryos. As early as the 16-cell stage, the b5.4 cells have been restricted to give rise to epidermis (Fig. 1A). Through its derivatives of the b6.7 and b6.8 cells of the 32-cell embryo (Fig. 1B), then through the b7.13, b7.14, b7.15 and b7.16 cells of the 64-cell embryo (Fig. 1C), the b5.4 cells give rise to 256 epidermal cells of the ventral part of the tail of the mid-tailbud embryo, because each of the b7.13, b7.14, b7.15 and b7.16 cells give rise to a clone of 64 epidermal cells (Nishida, 1987; Fig. 1H). The a6.6, a6.8 and b6.6 cells become restricted to epidermis at the 32-cell stage. The first two pairs of cells give rise to a total of 256 epidermal cells of the lateral and ventral regions of the head of the mid-tailbud embryo after six subsequent divisions, whereas the b6.6 cells form 128 epidermal cells of the lateral part of the tail (a7.11, a7.12, a7.15, a7.16, b7.11, and b7.12 in Fig. 1H). At the 64-cell stage, the a7.14 cells also become restricted to give rise to 64 epidermal cells of the dorsal part of the head (Fig. 1H). Epidermis of the dorsal part of the trunk and tail is derived from the b8.18 and b8.20 cells (Fig. 1H), which are derivatives of the b5.3, b6.5, b7.9, and b7.10 cells. Each of these cells forms 32 epidermal cells. In addition, 32 epidermal cells of the anterior dorsal part of the head are derived from the a8.26 cells (Fig. 1H). Furthermore, the adhesive organ of the anterior-most region of the embryo is derived from the a8.18 and a8.20 cells (Fig. 1H).

Cells constructing the CNS are derived from a7.9, a7.10, a7.13 (brain lineage), b7.9 (spinal cord lineage) and b7.10 (brain stem and spinal cord lineage) cells at the 64-cell stage (Fig. 1C), and a8.17, a8.19, a8.25 (brain lineage), b8.17 (spinal

cord lineage) and b8.19 (brain stem and spinal cord lineage) at the 110-cell stage (Fig. 1D, E) in addition to A-line-derived neuronal cells.

*Spatial distribution of* HrEpiA *transcripts*

Northern blot analysis showed that the *HrEpiA* transcript is about 3.0 kb, and that *HrEpiA* expression is zygotic (Ueki *et al.*, 1991; Ueki and Satoh, 1995). The transcript is first detected at the gastrula stage. The amount of the mRNAs increases dramatically at the neurula and tailbud stages. However, the transcript is no longer detectable after the beginning of metamorphosis.

Analyses of *in situ* hybridization demonstrated that the first distinct signals were detected at the early gastrula stage (Fig. 2A). At this stage, hybridization signals were evident in nuclei of the b8.25 and b8.27 primordial epidermal cells at the posterior-most region of the embryo (also see Fig. 1D, E). During gastrulation, signals became evident in many cells of the posterior-animal region of the embryo, and at the mid-gastrula stage, most of the b-line primordial epidermal cells showed hybridization signals (Fig. 2B). However, signals were not detected in the b8.17, b8.18, b8.19, b8.20, b9.47, and b9.48 cells at both sides of the embryo, although the b8.18, b8.20, b9.47 and b9.48 cells are of epidermal lineage (the b8.17 cells are of spinal cord and muscle lineages, and the b8.19 cells are of neuronal lineage). At the neural-plate stage, signals were evident in the posterior epidermal cells (Fig. 2C, D). Although hybridization signals were not detected in a-line cells until the neural-plate stage, a pair of clusters consisting of about four cells (presumably descendants of the a7.14 cells) began to show signals at the neurula stage (Fig. 2E). It was noticed that the expression of *HrEpiA* was not detected in presumably one row of epidermal cells located most inside around the neural folds (Fig. 2E; compare with Fig. 4F). These cells seem to form the dorsal midline of the tail at the early-tailbud stage. As development proceeded, signals became evident in many a-line epidermal cells (Fig. 2F, G). However, there were cells without signals in the dorsal midline of the a-derived trunk region and the oblique line alongside it (Fig. 2F). In addition, the transcript began to disappear from epidermal cells at the midline of the ventral side of the embryo (Fig. 2G).

Embryos at the early-tailbud stage showed a characteristic distribution pattern of *HrEpiA* transcripts (Fig. 2H, I). Signals were evident in almost all of epidermal cells except for the following three regions; the anterior-most region and about a line of cells at the boundary between the cells that started to express *HrEpiA* earlier among the a-line cells at the neurula stage and those that began to express the gene later; secondly, the dorsal midline of the trunk and tail region, which are

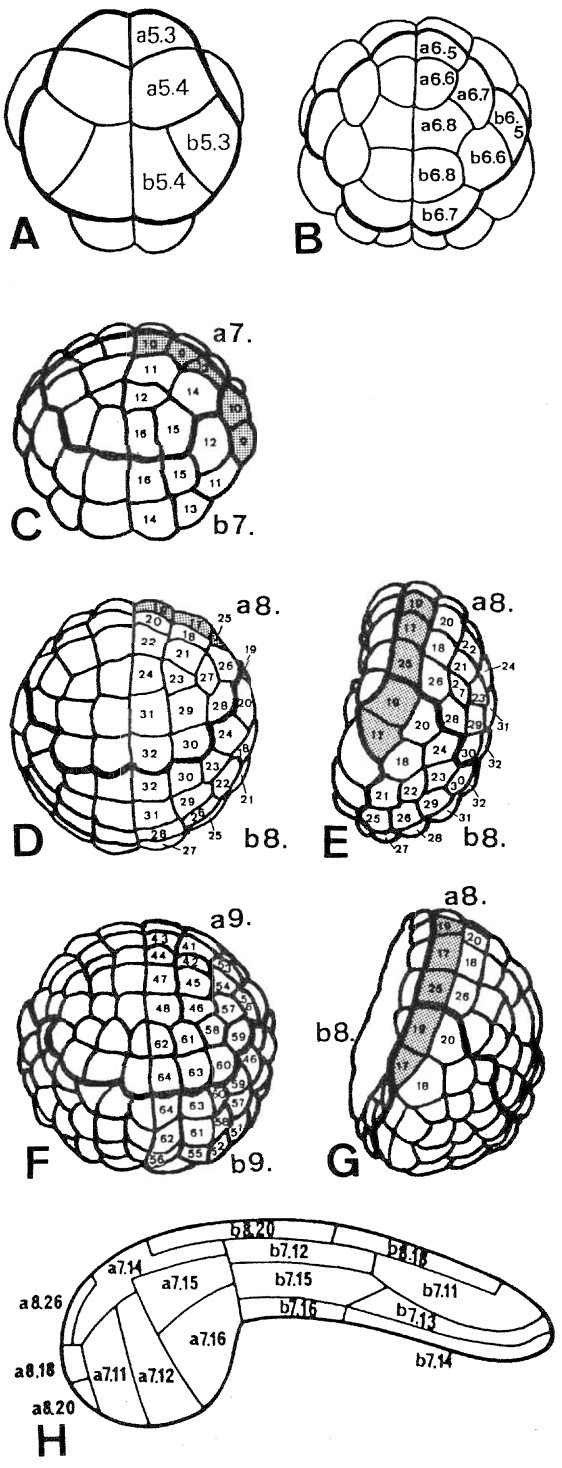



Fig. 1. Diagram illustrating the lineage (A-G) and clonal organization (H) of epidermal cells during embryogenesis of the ascidian *Halocynthia roretzi*. (A) A 16-cell stage embryo, animal pole view; (B) a 32-cell stage embryo, animal pole view; (C) a 76-cell stage embryo, animal pole view (the pattern of the animal blastomeres of the 76-cell stage embryo is the same as that of the 64-cell stage embryo); (D, E) a 110-cell stage embryo, viewed from (D) the animal pole and (E) right side; and (F, G) a mid-gastrula, viewed from (F) the animal pole and (G) right side. Anterior is up. Blastomeres of epidermal, neuronal (shaded) and other lineages are shown by their nomenclatures. (H) The clonal organization of the epidermal cells of a tailbud embryo, with domains of cells derived from epidermis-restricted blastomeres at the 64-cell and 110-cell stages. The domains derived from the a8.18 and a8.20 cells develop into palps of the larva. See the text for details. Based on description by Nishida (1987).

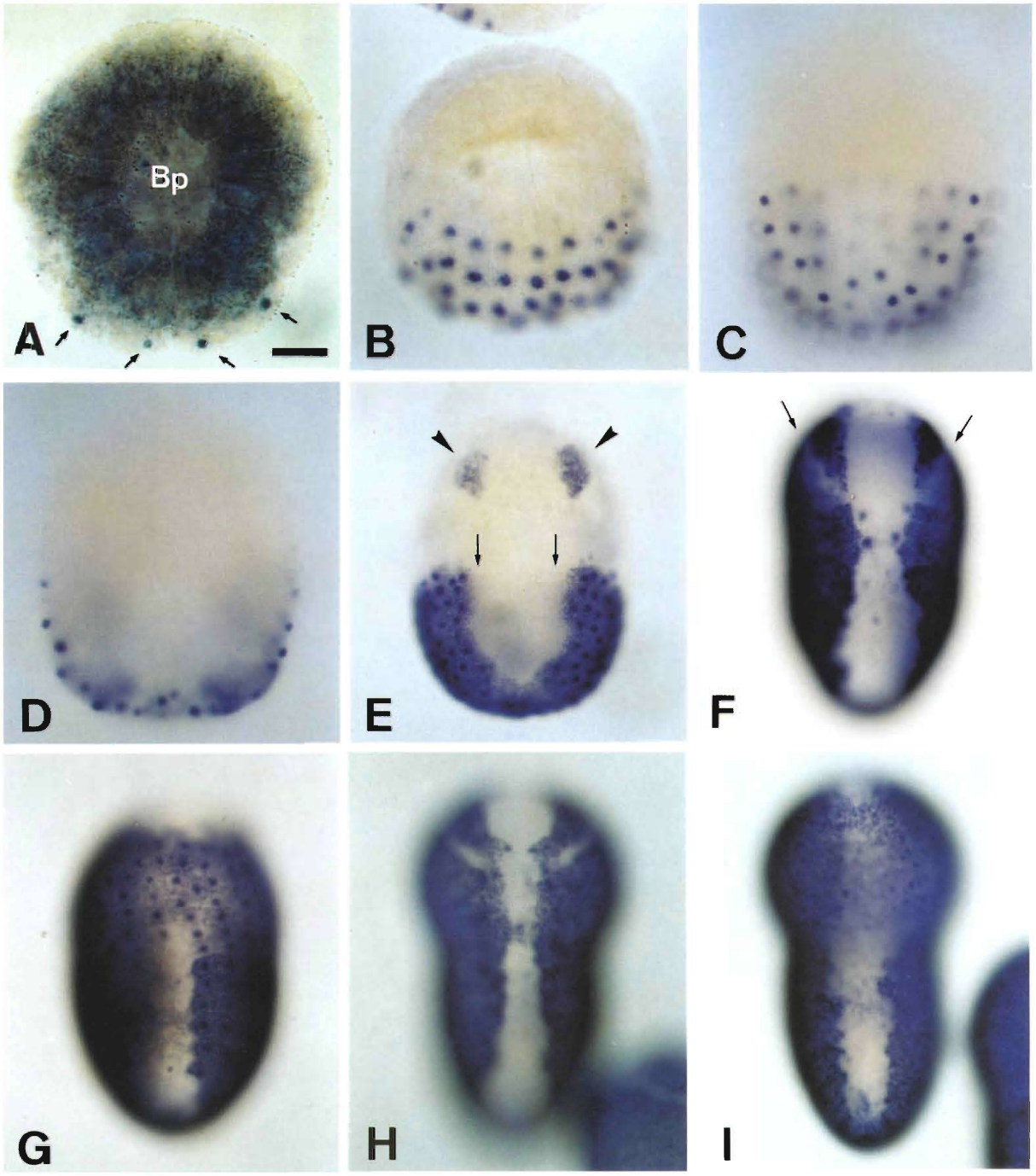



Fig. 2. Spatial distribution of *HrEpiA* transcripts, as revealed by whole-mount *in situ* hybridization with a digoxigenin-labeled antisense probe. (A) An early gastrula viewed from the vegetal pole (future dorsal side of the embryo). Hybridization signals are seen in nuclei of two pairs (b8.25 and b8.27) of the primordial epidermal cells (arrows) at the posterior-most region of the embryo. Bp, blastopore. Scale bar represents 50 μm for all panels. Anterior is up for all panels. (B) A mid-gastrula viewed from the animal pole. Signals are found in many primordial epidermal cells of the posterior region of the embryo. (C, D) Neural-plate stage embryos, animal side view. Focus is on the ventral surface (C) and the dorsal surface (D). Signals are seen in the posterior epidermal cells. (E) A neurula, dorsal view. A pair of clusters of a-line epidermal cells began to express the gene at the anterior side of the embryo (arrowheads). Signals cannot be seen in presumably one row of epidermal cells located most inside around the neural folds (arrows). The transcripts begin to disappear from epidermal cells at the posterior midline of the embryo. (F, G) An initial-tailbud stage embryo viewed from (F) the dorsal and (G) ventral side. A characteristic pattern of signal distribution is evident; signals are seen in the epidermal cells except those of the midline and the anterior-most regions of the embryo as well as the anterior ring (arrows in F). (H, I) An early-tailbud stage embryo viewed from (H) the dorsal and (I) ventral side. Distribution of signals is essentially identical to that of the initial tailbud embryo.

descendants of the a7.14 and a8.26 (both are the trunk region, see also Fig. 1F), and the b8.18 and b8.20 cells (both are the tail region); thirdly, the ventral midline of the tail region, which are descendants of the b7.14 and b7.16 cells. This distribution pattern of *HrEpiA* transcripts was retained at the middle-tailbud stage. However, signals of the cells in the ventral midline of the trunk region disappeared, and two patches of signals became distinct which were located horizontally below the ring of signals around the neuropore in the anterior-most region (data not shown).

Therefore, *HrEpiA* is expressed in cells of differentiating epidermis but not in cells of the CNS. However, the gene was not expressed in epidermal cells of the midline of the tailbud embryo.

*Spatial distribution of* HrEpiB *transcripts*

*HrEpiB* shows a sequence similarity to UDP-glucose-4-epimerases and 3β-hydroxysteroid dehydrogenase/isomerases (Ueki *et al.*, 1994). The transcript is about 1.2 kb. The activity of *HrEpiB* is also zygotic, and *HrEpiB* transcript is retained after metamorphosis by juveniles.

*HrEpiB* transcript was first detected at the mid-gastrula stage (Fig. 3A). At this stage, hybridization signals were evident in many b-line primordial epidermal cells except for the b8.17, b8.18, b8.19, b8.20, b9.47 and b9.48 cells, although signals were weak in the b9.59, b9.60, b9.63 and b9.64 cells (Fig. 3A; see also Fig. 1F, G). At the neurula stage, the expression was evident in b-line epidermal cells at the posterior part of the embryo. As in the case of *HrEpiA,* the expression of *HrEpiB* was undetectable in presumably one row of epidermal cells located most inside around the neural folds (Fig. 3B). After the neurula stage, in addition to b-line epidermal cells, a-line epidermal cells began to express the gene. In contrast to *HrEpiA*, however, all of the a-line lateral epidermal cells expressed *HrEpiB* (Fig. 3C). At the early-tailbud stage, the *HrEpiB* transcript was evident in the cells that occupied most of the outer surface, except for two regions; one was the anterior-most region of the embryo, descendants of the a8.18 and a8.20 cells, and the other was the anterior dorsal midline of the embryo, descendants of the a7.14, a8.26 and b8.20 cells (Fig. 3C). This distribution pattern of *HrEpiB* transcripts was retained at the middle-tailbud stage, except that signals of the cells in the ring around the neuropore disappeared discontinuously (data not shown).

Therefore, *HrEpiB* was expressed in cells of differentiating epidermis but not in cells of the CNS, although the gene was not expressed later in cells of the anterior region and anterior midline of the tailbud embryo.

*Spatial distribution of* HrEpiC *transcripts*

The *HrEpiC* transcript is about 1.9 kb (Ueki *et al.*, 1991; Ueki and Satoh, 1995). The gene expression is both maternal and zygotic, although *HrEpiC* mRNA becomes undetectable soon after the beginning of metamorphosis.

The pattern of appearance and distribution of *HrEpiC* transcripts differs from that of *HrEpiA* and *HrEpiB. In situ* hybridization demonstrated that hybridization signals were first detected at the 64-cell stage in all of the animal (a-line and b-line) blastomeres except for the a7.9, a7.10, b7.9 and b7.10 cells (Fig. 4A). The a7.9 cells give rise to brain and palps, a7.10 to brain, primordial pharynx and palps, b7.9 to epidermis, spinal cord, muscle and endodermal strand, and b7.10 to epidermis, brain stem, spinal cord and muscle, respectively (Nishida, 1987). The a7.13 cells expressed *HrEpiC,* although its developmental fate was yet not restricted to epidermis. At the 110-cell stage, the a7.13 cells divide into the a8.25 and a8.26 cells. The former gives rise to brain and pigment cells, and the latter is restricted to give rise to epidermis. The expression of *HrEpiC* was evident in the a8.26 cells but not in the a8.25 cells.

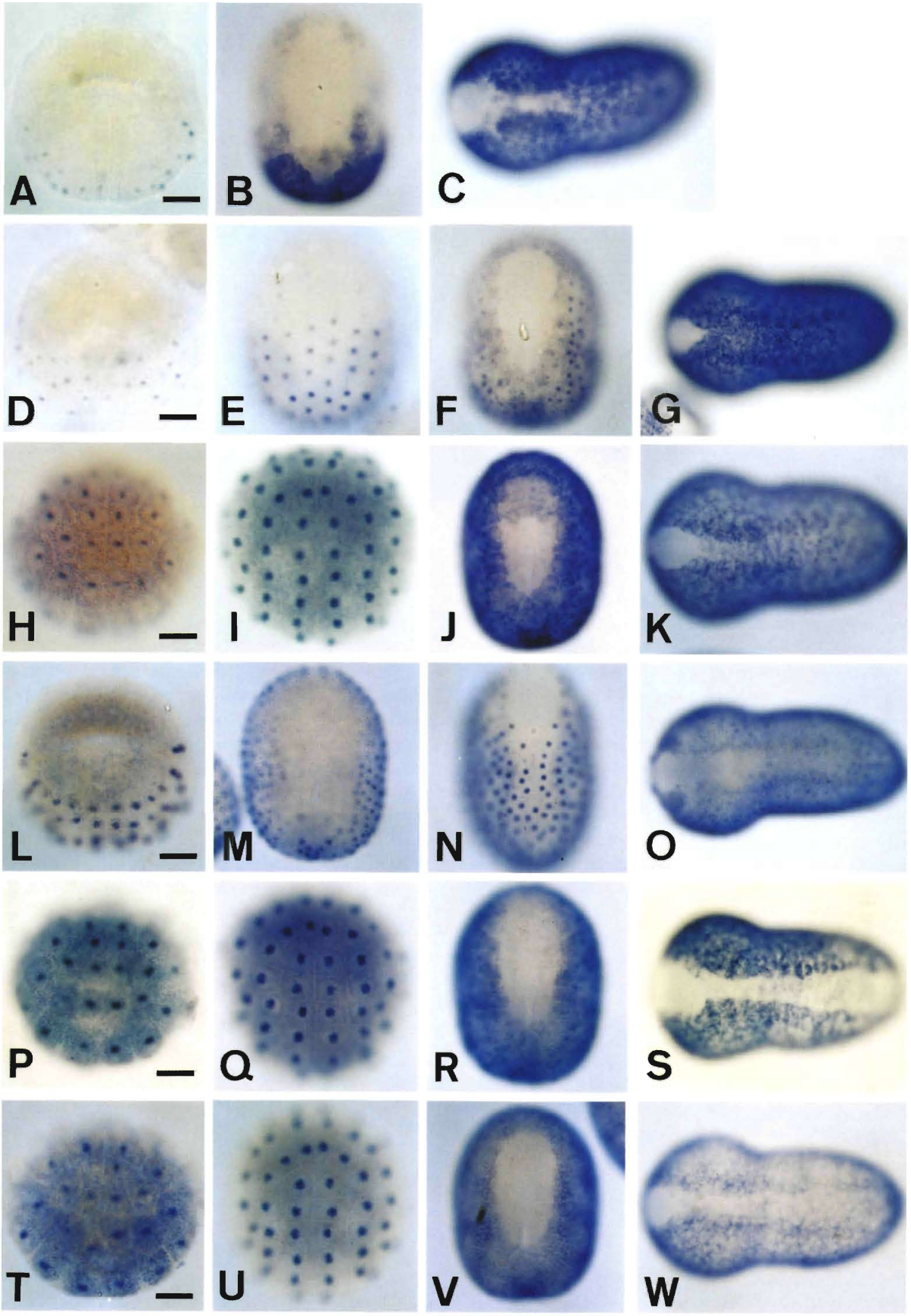
At the 110-cell and mid-gastrula stages, most of the a-line cells showed signals, except for the a8.17-20 and a8.25 cells (the neural lineage and the palps lineage) and most of the b-line cells except for the b8.17-20 cells (Fig. 4B, C). During neurulation, *HrEpiC*-expressing epidermal cells of the ventral side extended to cover the dorsal side (Fig. 4D-F), but the signals were not detected in cells of the anterior-dorsal midline and anterior-most region of the embryo (Fig. 4G). At the early-tailbud stage, signals were evident in all of the epidermal cells except for those of the anterior-most region and anterior dorsal midline of the embryo (Fig. 4H, I). At the middle-tailbud stage, signals of cells in the dorsal midline became evident, although signals in the anterior-most region were still missing (data not shown).

Therefore, *HrEpiC* is expressed in cells of differentiating epidermis but not in cells of the CNS. Although the a7.13 cells (containing both the epidermal and neuronal lineage) express the gene, the transcript was evident only in its epidermal descendants, not in its neural descendants. In addition, the gene expression was not detected in cells of the anterior-most region and anterior-dorsal midline of the tailbud embryo.

*Spatial distribution of* HrEpiD *transcripts*

*HrEpiD* is an ascidian homologue of mammalian and yeast *SEC61* (Ueki and Satoh, 1994). Northern blot analysis shows that the *HrEpiD* transcript is about 2.3 kb. The *HrEpiD* expression is both maternal and zygotic, and a considerable amount of *HrEpiD* transcripts are retained by larvae and metamorphosed juveniles.

As shown in Fig. 3D-G, the spatial expression pattern of *HrEpiD* resembles that of *HrEpiB,* although signals were very weak in gastrulae (Fig. 3D). *HrEpiD* transcript was first detected at the mid-gastrula stage in many b-line primordial epidermal cells with the possible exception of the b8.17-20, b9.47 and b9.48 cells (Fig. 3D). At the neurula stage, in addition to b-line epidermal cells, a-line epidermal cells began to express the gene (Fig. 3F). In contrast to *HrEpiC*, however, cells of the anterior-most region also showed signals (Fig. 3F). At the early-tailbud stage, *HrEpiD* transcript was evident in cells that occupied most of the outer surface, except for the cranial, neuropore-like region of the embryo (Fig. 3G). This distribution pattern of *HrEpiD* transcripts was retained at the middle-tailbud



stage, i.e., signals were evident in almost all the epidermal cells except for the small neuropore region (data not shown).

Therefore, *HrEpiD* was also not expressed in cells of the CNS.

*Spatial distribution of* HrEpiE *transcripts*

*HrEpiE* exhibits two different sizes of transcripts, about 2.3 and 3.0 kb in length, respectively (Ueki and Satoh, 1995). The *HrEpiE* expression is zygotic, and may no longer be detectable after the beginning of metamorphosis.

An early phase of the appearance of *HrEpiE* transcripts resembles that of *HrEpiC* (Fig. 3H, I). Hybridization signals were first detected at the 76-cell stage in all of the animal (a-line and b-line) blastomeres (Fig. 3H), including the a7.9, a7.10 and a7.13 cells, these three containing the neural lineage. In contrast to *HrEpiC*, *HrEpiE* transcripts were evident in cells of the neural lineage and the palps lineage (the a8.17-20 and a8.25 cells) at the 110-cell stage. During gastrulation and neurulation, *HrEpiE*-expressing epidermal cells of the ventral side extended over the dorsal side (Fig. 3I, J). At the early-tailbud stage, signals were evident in all of the epidermal cells except for those of the anterior, neuropore-like region and dorsal midline of the embryo (Fig. 3K). The signals in cells of the brain lineage disappeared by this stage. At the middle-tailbud stage, signals in almost all the epidermal cells including the dorsal midline of the embryo were evident although the signals were still missing in the small neuropore region (data not shown).

Therefore, *HrEpiE* is expressed in cells of differentiating epidermis as well as presumptive brain cells.

*Spatial distribution of* HrEpiF *transcripts*

Northern blot analysis shows that the *HrEpiF* transcripts are about 4.8 and 10.0 kb in length (Ueki and Satoh, 1995). The *HrEpiF* expression is zygotic.

*In situ* hybridization signals were detected in all of the b-line blastomeres except the b8.17-20 cells of the mid-gastrula (Fig. 3L). At the neurula stage, signals were evident in a-line epidermal cells although the signals in cells at the anterior-most region disappeared later (Fig. 3M). As in the case of *HrEpiA,* the expression of *HrEpiF* was not detected in one row of epidermal cells located most inside around the neural folds (Fig. 3M). These cells seem to form the future dorsal midline. At the initial-tailbud stage, signals were evident in cells of the dorsal midline (Fig. 3N), although the signals soon disappeared (Fig. 3O). At the early-tailbud stage, signals were detected in all epidermal cells except for cells of the anterior-most region, neuropore-like region and dorsal midline of the embryo (Fig. 3O). The pattern of *HrEpiF* expression at this stage resembles that of *HrEpiA* at the anterior part and the dorsal side of the embryo. This distribution pattern of *HrEpiF* transcripts was retained, except that the signals of the cells in the posterior-dorsal midline became evident (data not shown).

Therefore, *HrEpiF* is expressed exclusively in cells of differentiating epidermal cells.

*Spatial distribution of* HrEpiG *transcripts*

*HrEpiG* encodes two different sizes of transcripts, about 2.9 and 3.1 kb in length, respectively (Ueki and Satoh, 1995). The *HrEpiG* expression is zygotic, and a considerable amount of *HrEpiG* transcripts may be retained by larvae and metamorphosed juveniles.

In a previous study (Ueki and Satoh, 1995), we detected *HrEpiG* transcripts by faint Northern blot at the 64-cell stage. In the present study, however, first hybridization signals of *HrEpiG* were detected at the 76-cell stage in a-line blastomeres, except for the a7.9, a7.10 and a7.13 cells, and in b-line blastomeres except for the b7.9 and b7.10 cells (Fig. 3P). At the 110-cell stage, signals were found in a-line blastomeres except for the a8.17, a8.19 and a8.25 cells and in b-line blastomeres except for the b8.17 and b8.19 cells (Fig. 3Q). At the neurula stage, signals were evident in all of the epidermal cells (Fig. 3R), although signals in cells at the anterior-most region later disappeared. At the early-tailbud stage, signals were found in epidermal cells except for cells of the anterior-most region and dorsal midline of the embryo (Fig. 3S). This distribution pattern of *HrEpiG* transcripts was retained also at the middle-tailbud stage (data not shown).

Therefore, *HrEpiG* is expressed in cells of differentiating epidermis but not in cells of the CNS.

*Spatial distribution of* HrEpiH *transcripts*

The *HrEpiH* transcripts are about 1.1 and 1.5 kb in length (Ueki and Satoh, 1995). The gene expression is zygotic, and a considerable amount of *HrEpiH* transcripts may be retained by larvae and metamorphosed juveniles.

The distribution pattern of *HrEpiH* transcripts resembled that of *HrEpiG.* In the previous study (Ueki and Satoh, 1995), we detected the transcripts at the 64-cell stage faintly by Northern blot analysis. In the present study, however, first hybridization signals were detected at the 76-cell stage in a-line blastomeres except for the a7.9, a7.10 and a7.13 cells

Fig. 3. Spatial distribution of transcripts of six other epidermis-specific genes in ascidian embryos, as revealed by whole-mount *in situ* hybridization. Scale bar represents 50 μm for all panels. Anterior is up for all panels except C, G, K, O, S and W in which anterior is left. (A-C) *HrEpiB.* (A) A mid-gastrula, animal pole view. (B) A neurula, dorsal view. (C) An early-tailbud stage embryo, dorsal view. (D-G) *HrEpiD* (D) A mid-gastrula, animal pole view. (E) A neural-plate stage embryo, ventral view. (F) A neurula, dorsal view . (G) An early-tailbud stage embryo, dorsal view. (H-K) *HrEpiE.* (H) A 76-cell embryo, animal pole view. (I) A 110-cell embryo, animal pole view. (J) A neurula, dorsal view. (K) An early-tailbud stage embryo, dorsal view. (L-O) *HrEpiF.* (L) A mid-gastrula, animal pole view. (M) A neurula, dorsal view. (N) An initial-tailbud stage embryo, dorsal view. (O) An early-tailbud stage embryo, dorsal view. (P-S) *HrEpiG.* (P) A 76-cell embryo, animal pole view. (Q) A 110-cell embryo, animal pole view. (R) A neurula, dorsal view. (S) An early-tailbud stage embryo, dorsal view. (T-W) *HrEpiH.* (T) A 76-cell embryo, animal pole view. (U) A 110-cell embryo, animal pole view. (V) A neurula, dorsal view. (W) An early-tailbud stage embryo, dorsal view.

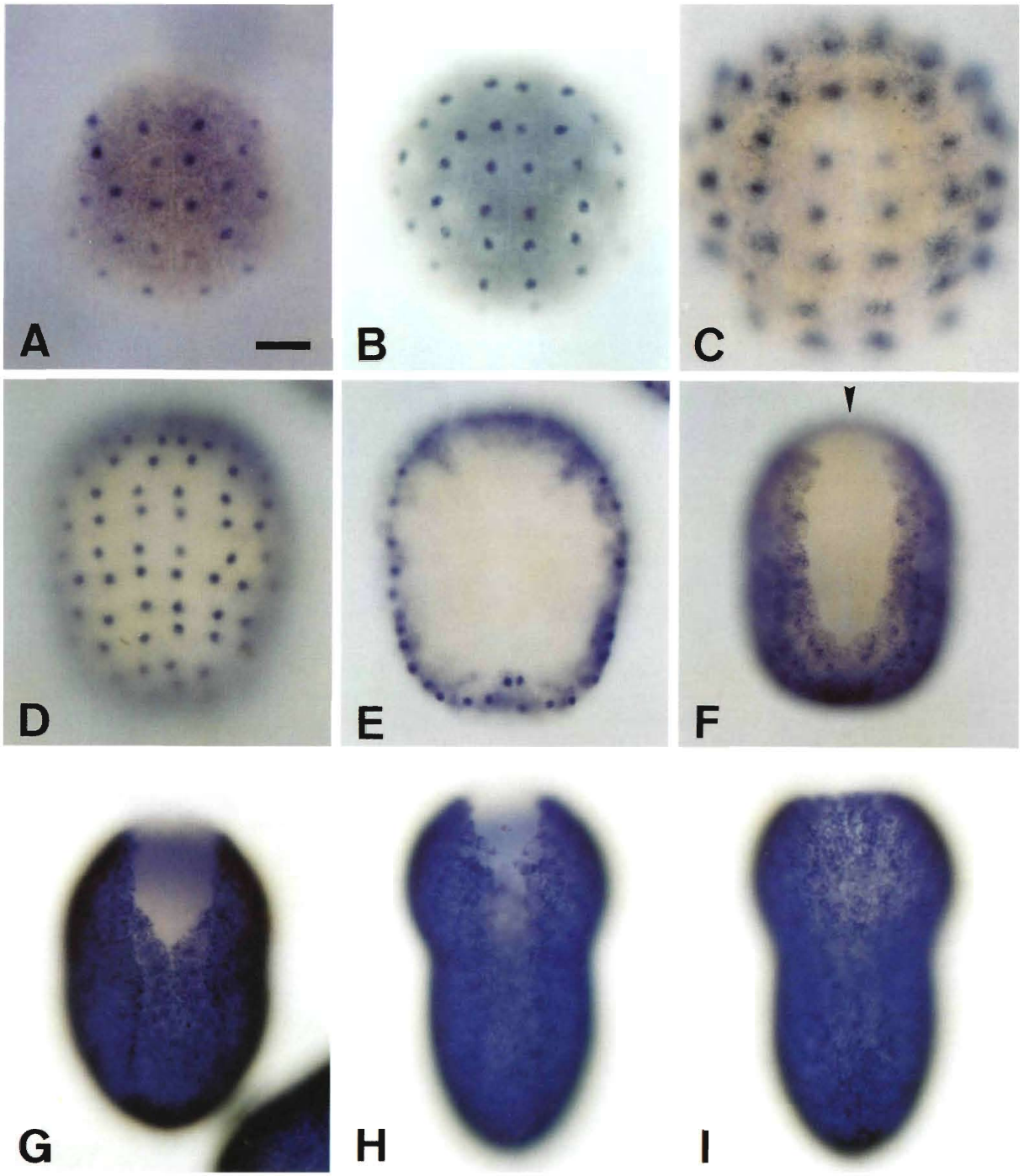



Fig. 4. Spatial distribution of *HrEpiC* transcripts, as revealed by whole-mount *in situ* hybridization with a digoxigenin-labeled antisense probe. (A) A 64-cell and (B) 110-cell stage embryo viewed from the animal pole (future ventral side). Hybridization signals are seen in nuclei of the presumptive as well as primordial epidermal cells at the anterior (a-line) and posterior (b-line) animal hemisphere. Scale bar represents 50 μm for all panels. Anterior is up for all panels. (C) An early gastrula viewed from the animal pole. Signals are evident in all of the primordial epidermal cells. (D, E) A neural-plate stage embryo, viewed from (D) the ventral and (E) dorsal side. Signals are seen in most of the epidermal cells. (F) A neurula, dorsal view. Epidermal cells exhibiting *HrEpiC* expression begin to cover the dorsal side of the embryo. *HrEpiC* expression is downregulated in cells of the anterior-most region at this stage (arrowhead). (G) An initial-tailbud stage embryo, dorsal viewed. Signals are seen in all the epidermal cells except those of the anterior-most region and anterior-dorsal midline of the embryo. (H, I) An early-tailbud embryo viewed from (H) the dorsal and (I) ventral side. Signals are seen in all of the epidermal cells except those of the anterior-most region and anterior-dorsal midline of the embryo.

and in b-line blastomeres except for the b7.9 and b7.10 cells (Fig. 3T). At the 110-cell stage, signals were found in a-line blastomeres except for the a8.17, a8.19 and a8.25 cells and in b-line blastomeres except for the b8.17 and b8.19 cells (Fig. 3U). At the neurula stage, signals were evident in all of the epidermal cells (Fig. 3V), and the signals in the cells of the anterior-most region were retained. At the early-tailbud stage, signals were found in epidermal cells except those of the cranial, neuropore-like region and the dorsal midline of the embryo (Fig. 3W). At the middle-tailbud stage, signals were evident in almost all of the epidermal cells except for the anterior-most region of the embryo (data not shown).

Therefore, *HrEpiH* is also expressed in cells of differentiating epidermis but not in cells of the CNS.

## DISCUSSION

*Lineage-associated expression of epidermis-specific genes*

As shown in this study, most of these epidermis-specific genes are expressed exclusively in cells of epidermal lineage but not in cells of other lineages. Therefore, it is likely that the differentiation of epidermal cells in the ascidian embryo is achieved by the lineage-associated expression of the specific genes. However, one gene (*HrEpiE*) is expressed transiently in cells of the presumptive nervous system in addition to the cells of the presumptive epidermis.

The eight epidermis-specific genes are categorized into two major groups based on the spatio-temporal expression patterns; the first group (*HrEpiA*, *HrEpiB*, *HrEpiD*, and *HrEpiF*) initiates their expression in b-line cells around the mid-gastrula stage and later in a-line cells around the neurula stage. In contrast, the second group (*HrEpiC*, *HrEpiE*, *HrEpiG*, and *HrEpiH*) starts their expression in almost all the a- and b-line cells of the animal hemisphere at the 76-cell stage.

*The expression patterns of the first group; differential regulation in epidermal lineage*

The first four genes, *HrEpiA*, *HrEpiB*, *HrEpiD* and *HrEpiF,* are expressed around the mid-gastrula stage only in b-line epidermal lineage cells. Their expression pattern seems to be regulated dependently upon their origins of blastomeres and simultaneously under a lineage-associated manner. These genes start their expression also in the a-line cells around the neurula stage. Thus, the temporal expression of these genes is differentially regulated between a- and b-line presumptive epidermal cells. It is an intriguing research subject to examine how the timing of the gene expression in certain blastomeres is determined dependently upon the origin of the blastomere. Although the significance of such differential expression is not known, a-line epidermal cells contribute exclusively to the epidermis of the trunk region, and b-line cells to that of the tail region.

*The expression pattern of the second group in relation to neural induction*

The four genes of the second group start their expression around the 76-cell stage in most of the cells in the animal hemisphere. At the 76-cell stage, the a7.9, a7.10, and a7.13 cells have both epidermal and neuronal fates (Fig. 1A), and at the 110-cell stage, the a8.17, a8.19, and a8.25 cells inherit the neuronal fate, whereas the a8.18, a8.20, and a8.26 cells inherit the epidermal and palps fate (Fig. 1B, C). As shown in this study, the distributions of *HrEpiG* and *HrEpiH* transcripts are restricted to cells of epidermal lineage at the 76-cell stage, and so is the distribution of *HrEpiC* transcripts, except for the distribution to the a7.13 cells. At the 110-cell stage, these three transcripts are evident exclusively in cells of epidermal lineage. Neural induction, at the least, has not been completed yet at the 110-cell stage (Nishida, 1991). Therefore, the expressions of these three genes are restricted entirely to epidermal lineage prior to the completion of neural induction. This implies that the presumptive epidermal cells and the presumptive neuronal cells have intrinsic differences already at the time before the completion of the neural induction. This is consistent with the observation that the brain-precursor cells isolated at the 110-cell stage failed to develop epidermis-specific features (Nishida, 1991).

In contrast to the three genes mentioned above, *HrEpiE* is expressed in both cells of the epidermal and neuronal lineages from the 76-cell stage up to the neurula stage. This mode of gene expression is not interpreted as the lineage-associated mode of gene expression, as was observed in *HrEpiC*, *HrEpiG* and *HrEpiH*. The expression pattern of *HrEpiE* implies that the presumptive epidermal cells and the presumptive neuronal cells share some common characteristics around the 110-cell stage, and after neural induction the cells of neuronal lineage lose such characteristics. However, it is not known whether the loss of such characteristics is caused by the neural induction itself or by the intrinsic difference between cells of the neuronal and epidermal lineages that has already been established at the 110-cell stage, or by both.

The isolated and cleavage-arrested a4.2 blastomere develops epidermal features, but in contact with A4.1 blastomere or treated with subtilisin, it develops neural features as a consequence of neural induction (reviewed in Okamura *et al.,*1993). Under such conditions, the inward-rectifier $K^+$ channel, which seems to be linked with epidermal differentiation in the above-mentioned system, is first expressed in a4.2 but thereafter suppressed by neural induction (Okamura and Takahashi, 1993). Therefore, the temporal expression pattern of this $K^+$ channel appears to be similar to that of *HrEpiE,* although the results of isolated and cleavage-arrested blastomeres do not always reflect the correct internal state of the cells in normal embryos.

*Spatial distribution pattern of epidermis-specific genes does not always coincide with temporal expression pattern*

On the basis of patterns of their temporal expression, the eight epidermis-specific genes of *Halocynthia* embryos are divided into four types (Table 1; Ueki and Satoh, 1995). Expression of the type I (*HrEpiA, HrEpiE* and *HrEpiF*) and type II (*HrEpiB, HrEpiG* and *HrEpiH*) is zygotic. In the case of

Table 1. Summary of the spatial expression patterns of eight epidermis-specific genes in the early-tailbud embryo

| Genes | Type* | Spatial distribution regions+ 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| *HrEpiD* | IV | ● | ● | ● | ● | ● | ● |
| *HrEpiB* | II | | ● | | ● | ● | ● |
| *HrEpiC* | III | | | | | | |
| *HrEpiE* | I | | | ● | ● | ● | ● |
| *HrEpiH* | II | | | | | | |
| *HrEpiG* | II | | | | ● | ● | ● |
| *HrEpiF* | I | | | | | ● | ● |
| *HrEpiA* | I | | | | | | ● |

* The Roman numerals represent the type of temporal expression patterns (Ueki *et al.* 1995; see the text for details).
\+ See Fig. 5 for six regions of the early-tailbud embryo.

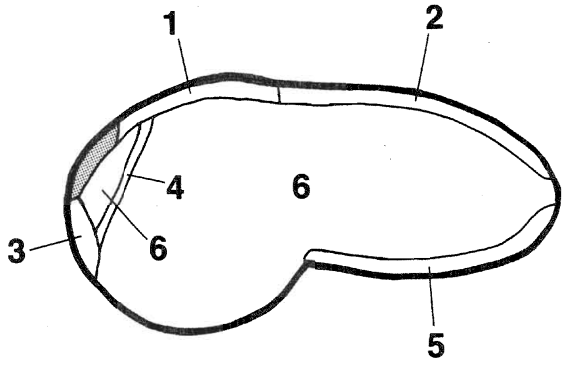



Fig. 5. Six regions in the epidermis of the early-tailbud embryo. The neuropore does not close completely at this stage, so the anterior-upper region (shaded area) is not covered with the epidermis yet. See Table 1.

the types III (*HrEpiC*) and IV (*HrEpiD*), their expression is controlled both maternally and zygotically. In the case of the types I and III, transcripts become undetectable soon after the metamorphosis begins. Therefore, the activity of these genes is required for the formation of larval epidermis. In contrast to types I and III, mRNAs of the types II and IV are retained throughout metamorphosis and by newly formed juveniles. Therefore, the activity of these genes is required not only for the formation of larval epidermis but also for the formation of juvenile epidermis.

In another mode of classification, as mentioned before, the eight epidermis-specific genes are subdivided into two groups based on the spatio-temporal distribution patterns; four genes of the first group (*HrEpiA*, *HrEpiB*, *HrEpiD*, and *HrEpiF*) initiate their express in b-line cells around the mid-gastrula stage and later in a-line cells around the neurula stage. The other four genes of the second group (*HrEpiC*, *HrEpiE*, *HrEpiG*, and *HrEpiH*) start to express in almost all the a- and b-line cells of animal hemisphere. When we compared these spatio-temporal expression patterns with the temporal expression patterns (Table 1), we could not find any direct relationship between these two patterns.

Furthermore, the spatial expression patterns of these eight genes at the early-tailbud stage are also divisible into six groups, as discussed below (Table 1). In this case, again, we could not find any relationship among these three modes of classification.

*Regional difference in epidermal cells*

One of the characteristic features of the eight different epidermis-specific genes in the ascidian embryo, shown in this study, is that many of the genes are not expressed in the anterior-most region as well as in the dorsal midline of the tailbud embryo. In previous studies (Ueki *et al.*, 1991, 1994), we did not detect an exact spatial expression pattern of the epidermis-specific genes, as revealed in the present study; that is, in the previous studies, hybridization signals were detected all over the outer surface of the tailbud embryo. This difference is mainly caused by probes we used, because in the previous studies we used a cDNA probe containing both strands (Ueki *et al.*, 1991, 1994; Ueki and Satoh, 1994), although we used the antisense RNA probe to detect the transcripts of experimental embryos (Ueki *et al.*, 1994). Thus, in the present study we could obtain precise expression patterns of the eight epidermis-specific genes and detect regional differences in epidermal cells based on these distribution patterns.

Based on their spatial expression patterns at the early-tailbud stage, the eight genes can be divided into six groups

(Table 1). In other words, the epidermis of early-tailbud embryos is partitioned into six regions according to the set of gene expression (Table 1; Fig. 5). These six regions are thought to have intrinsic differences from one another. Then, what structural differences would be there in these six regions? In the ascidian tadpole, several structures are known to be derived from epidermis. The adhesive organ is formed from region 3 (Fig. 5). This organ consists of triangular papillae which are enveloped by a single layer of flattened epithelial cells, except at the anterior tips. Regions 2 and 5 may form the dorsal and ventral fins, respectively. Furthermore, it is reported in several ascidian species that the epidermal sensory neurons lie within these regions and that their axons run in grooves in the bases of epidermal cells along the dorsal and ventral midline of the tail (Torrence and Cloney, 1982). However, these epidermal sensory neurons are located pairwise at somewhat irregular intervals. Therefore, it seems unreasonable to relate this structure directly with regions 2 and 5. In region 1, the existence of the epidermal sensory neurons are reported in *Ciona intestinalis* (Takamura, 1995). However, they occupy only a small part of region 1, suggesting no direct relationship between this structure and the region. In region 4, no specific structure is found as yet, although it might be possible that some novel structure will be found in this region in future.

As shown in our present study, most of the epidermis-specific genes are not expressed in the cells of the dorsal midline and the anterior-most region of the early-tailbud embryo. This implies that some other genes, which may confer some characteristics on the cells of this region, are expressed in a complementary pattern there. Recently, Miya *et al.* (1996) isolated an ascidian homologue (*HrBMPa*) of vertebrate *BMPs5-8*, and they showed that the gene is expressed in the adhesive organ, midline of the anterior dorsal neuroectoderm and midline of both ventral and dorsal ectoderm. Therefore, the spatial expression of *HrBMPa* seems to compensate for the expression of epidermis-specific genes. These expression patterns should be focused on in future studies.

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