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Anteroposterior patterning of the epidermis by inductive influences from the vegetal hemisphere cells in the ascidian embryo

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SUMMARY

Patterning along the anteroposterior axis is a critical step during animal embryogenesis. Although mechanisms of anteroposterior patterning in the neural tube have been studied in various chordates, little is known about those of the epidermis. To approach this issue, we investigated patterning mechanisms of the epidermis in the ascidian embryo. First we examined expression of homeobox genes (*Hrdll-1*, *Hroth*, *HrHox-1* and *Hrcad*) in the epidermis. *Hrdll-1* is expressed in the anterior tip of the epidermis that later forms the adhesive papillae, while *Hroth* is expressed in the anterior part of the trunk epidermis. *HrHox-1* and *Hrcad* are expressed in middle and posterior parts of the epidermis, respectively. These data suggested that the epidermis of the ascidian embryo is patterned anteroposteriorly.

In ascidian embryogenesis, the epidermis is exclusively derived from animal hemisphere cells. To investigate regulation of expression of the four homeobox genes in the epidermis by vegetal hemisphere cells, we next performed hemisphere isolation and cell ablation experiments. We showed that removal of the vegetal cells before the late 16-cell stage results in loss of expression of these homeobox genes in the animal hemisphere cells. Expression of *Hrdll-1* and *Hroth* depends on contact with the anterior-vegetal (the A-line) cells, while expression of *HrHox-1* and *Hrcad* requires contact with the posterior-vegetal (the B-line) cells. We also demonstrated that contact with the vegetal cells until the late 32-cell stage is sufficient for animal cells to express *Hrdll-1*, *Hroth* and *Hrcad*, while longer contact is necessary for *HrHox-1* expression. Contact with the A-line cells until the late 32-cell stage is also sufficient for formation of the adhesive papillae.

Our data indicate that the epidermis of the ascidian embryo is patterned along the anteroposterior axis by multiple inductive influences from the vegetal hemisphere cells and provide the first insight into mechanisms of epidermis patterning in the chordate embryos.

Key words: Anteroposterior axis, Ascidian, Epidermis, Inductive influence, Patterning, Vegetal hemisphere cell

INTRODUCTION

Patterning along the anteroposterior axis is a critical step during animal embryogenesis. In the vertebrate central nervous system (CNS), the patterned neural tube is characterized by region-specific expression of many genes including homeobox genes such as *dlx*, *otx*, *pax*, *Hox* and *cdx* cognates (e.g., McGinnis and Krumlauf, 1992; Shimamura et al., 1995). The patterning of the vertebrate neural tube has been shown to be controlled by inductive signals emanating from the organizer tissue (Harland and Gerhalt, 1997; Kessler and Melton, 1994) as well as the anterior endoderm (Beddington and Robertson, 1998) and nonaxial mesoderm (Koshida et al., 1998; Woo and Fraser, 1997).

Recently, comparative studies on regional specification in the CNS among the chordates have revealed that different members of the chordates (the vertebrates, amphioxus and ascidians) exhibit similar expression patterns of homeobox genes in the neural tube (Williams and Holland, 1998). For example, in the ascidian embryo, the ascidian homologue of *orthodenticle*/*otx*, *Hroth* (Wada et al., 1996) is expressed in the anteriormost region of the neural tube (the sensory vesicle) while the *labial* group *Hox* gene, *HrHox-1* (Katsuyama et al., 1995) is expressed in a more posterior region of the neural tube. The single ascidian homologue of the *Pax2*, *Pax5* and *Pax8* genes is expressed in between (Wada et al., 1998). Expression pattern of these genes in the ascidian embryo resembles that of *Otx1* and *Otx2*, *Pax2*, *Pax5* and *Pax8*, and *Hox* genes in the vertebrate neural tube and thus suggests that regional specification of the CNS is conserved, at least in part, between the vertebrates and the ascidians (Katsuyama et al., 1996; Wada et al., 1998).

The CNS patterning of the ascidian embryo also resembles that of the vertebrates with respect to cellular interactions involved in the process. The sensory vesicle of the ascidian embryo is induced by the vegetal hemisphere cells around gastrulation (Nishida, 1991; Nishida and Satoh, 1989; Okado and Takahashi, 1988, 1990; Reverberi et al., 1960; Rose, 1939).

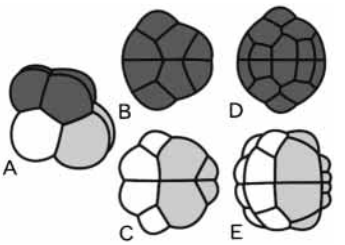

Fig. 1. Schematic representation of structures of 8-, 16- and 32-cell-stage embryos. (A) A lateral view of 8-cell-stage embryo. (B,C) Animal (B) and vegetal (C) views of 16-cell-stage embryo. (D,E) Animal (D) and vegetal (E) views of 32-cell-stage embryo. With all diagrams, anterior is to the left. The epidermis is entirely derived from the animal hemisphere cells (dark gray). The vegetal hemisphere cells consist of the A-line and B-line (light gray) cells.

Formation of neural tissue in the sensory vesicle is inhibited by overexpression of the ascidian homologue of vertebrate *Bmp-2*/*Bmp-4* (Miya et al., 1997) and induced by application of bovine recombinant bFGF (Inazawa et al., 1998). Although details of neural-inducing mechanisms in the ascidian embryo remain to be clarified, these suggest that cellular mechanisms to induce and pattern the neural tube are also conserved between the vertebrates and the ascidians.

In contrast to our knowledge about patterning of the CNS, little is known about mechanisms of anteroposterior patterning of the epidermis in the chordates. Regional specification of the epidermis is best characterized in *Drosophila* embryos, in which identity of each segment is primarily determined through interactions among zygotic genes activated or repressed by actions of localized maternal cytoplasmic factors (St. Johnson and Nüsslein-Volhard, 1992). How then is the epidermis patterned along the anteroposterior axis in the chordate embryos? Do inductive interactions also play important roles in the epidermis patterning? Is there a conserved mechanism among the chordates? To address these questions, we utilized the ascidian embryo.

In the ascidian embryo, about 800 epidermal cells are formed and cover the outermost part of the larva (Nishida, 1987). The epidermis is entirely derived from the animal hemisphere cells (Nishida, 1987; Fig. 1). Differentiation of the epidermis proceeds autonomously through the action of cytoplasmic determinants (Nishida, 1994a). At the anterior tip of the embryo, epidermal cells are specified to form the adhesive papillae that serve to attach the larva to a settlement surface (Katz, 1983).

Interestingly, *Hroth* and *HrHox-1* are expressed in the epidermis of different anteroposterior levels (Katsuyama et al., 1995; Wada et al., 1996). This suggests that anteroposterior patterning occurs in the epidermis of the ascidian embryos and provides us with an opportunity to investigate mechanisms of epidermis patterning. Therefore, in this study, we concentrated on epidermal expression of homeobox genes. First, we examined the expression domains of four homeobox genes (*Hrdll-1*, *Hroth*, *HrHox-1* and *Hrcad*). *Hrdll-1* and *Hrcad* are the ascidian homologues of *Distal-less* and *caudal*, respectively (Y. K. and H. S., unpublished data; Katsuyama et al., 1999). Expression analyses of these genes suggested that the epidermis of the ascidian embryo is subdivided into different regions along anteroposterior axis. Next, to investigate regulation of expression of the four homeobox genes, we carried out hemisphere isolation and cell ablation experiments. We showed that expression of the four homeobox genes in the epidermis is controlled differentially by multiple inductive influences from the A-line and B-line cells. In the light of the present findings, we discuss mechanisms of anteroposterior patterning of the ascidian embryo and compare ectoderm patterning between ascidian and vertebrate embryos.

MATERIALS AND METHODS

Embryos

Adult ascidians, *Halocynthia roretzi*, were obtained from fishermen near Asamushi Marine Biological Station, Tohoku University, Aomori, Japan and Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate, Japan. Naturally spawned eggs were fertilized with a suspension of sperm from other individuals. Fertilized eggs were raised at 11-13°C.

Hemisphere isolation and cell ablation

Fertilized eggs were manually dechorionated using tungsten needles and reared in 0.9% agarose-coated plastic dishes filled with the supernatant of a homogenate of the cleaving eggs prepared according

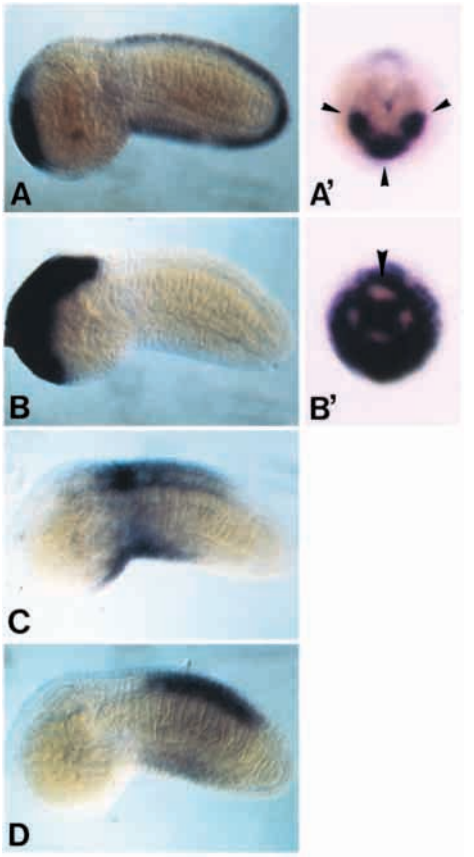

Fig. 2. Expression of *Hrdll-1* (A,A′), *Hroth* (B,B′), *HrHox-1* (C) and *Hrcad* (D) in normal middle tailbud stage embryos detected by whole-mount in situ hybridization. (A-D) Lateral views. Anterior is to the left. (A′) A frontal-dorsal view of the specimen in A. The three spots with intense *Hrdll-1* are the presumptive papillae-forming regions (arrowheads). (B′) A frontal view of the specimen in B. Three out of the four spots where *Hroth* expression is excluded correspond to the future papilla-forming regions. The dorsalmost spot (arrowhead) is around the neuropore, unrelated with papilla formation. (A′,B′) Dorsal is to the top.

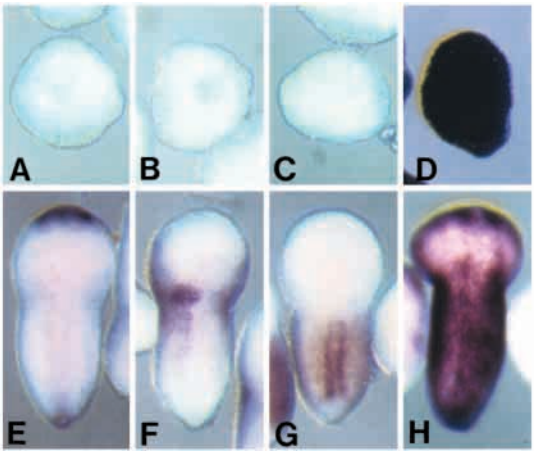

Fig. 3. Expression of three homeobox genes in animal hemisphere explants prepared at the late 8-cell stage. Expression of *Hrdll-1* (A,E), *HrHox-1* (B,F), *Hrcad* (C,G) and *HrEpiD* as control for epidermal differentiation (D,H) at the middle tailbud equivalent stage was detected by whole-mount in situ hybridization. (A-D) Animal hemisphere explants. (E-H) A dorsal view of control normal embryos. Anterior is to the top.

to Nishida and Satoh (1985), supplemented with 100 μg/ml streptomycin and 100 units/ml penicillin. Animal hemisphere was isolated from the 8-cell-stage embryos manually using a fine glass needle under a dissection microscope. As control, dechorionated but non-manipulated siblings were cultured simultaneously. Isolated animal hemispheres were cultured as explants and fixed for in situ hybridization at the middle tailbud equivalent stage, that is, when control embryos reached the middle tailbud stage.

For cell ablation, a target blastomere was identified and destroyed by piercing it using a fine glass needle at the 8-, 16- or 32-cell stage. Debris of destroyed blastomeres were usually extruded, remaining on the surface of the embryos and were removed using a fine glass needle at the next developmental stage. Manipulated embryos were cultured as partial embryos and fixed for in situ hybridization at the middle tailbud equivalent stage. Formation of the adhesive papillae was examined by morphology at the swimming larva equivalent stage. Isolation and ablation were carried out during the late period of each developmental stage.

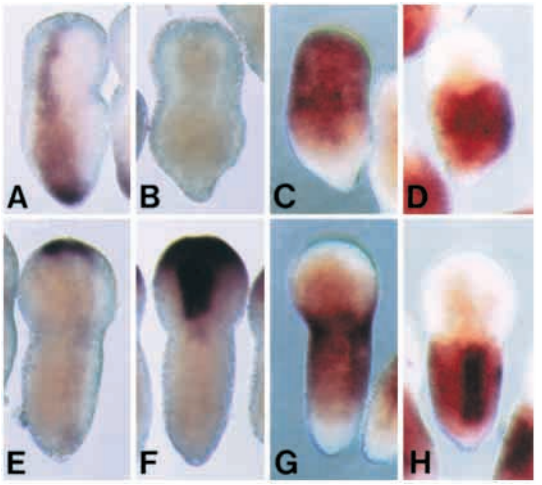

Fig. 4. Expression of four homeobox genes in A4.1 pair-ablated embryos. Expression of *Hrdll-1* (A,E), *Hroth* (B,F), *HrHox-1* (C,G) and *Hrcad* (D,H) at the middle tailbud equivalent stage was detected by whole-mount in situ hybridization. With all specimens, anterior is to the top. (A-D) A dorsal view of A4.1 pair-ablated embryos. (E-H) A dorsal view of control normal embryos.

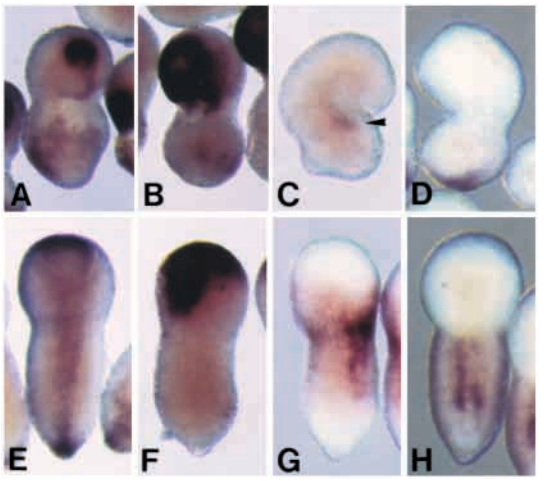

Fig. 5. Expression of four homeobox genes in B4.1 pair-ablated embryos. Expression of *Hrdll-1* (A,E), *Hroth* (B,F), *HrHox-1* (C,G) and *Hrcad* (D,H) at the middle tailbud equivalent stage was detected by whole-mount in situ hybridization. With all specimens, anterior is to the top. (A-D) A lateral view of B4.1 pair-ablated embryos. (E-H) A dorsal view of control normal embryos. Arrowhead in C indicates *HrHox-1* expression that is retained in the neural tube of B4.1 pair-ablated embryo.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as described previously (Wada et al., 1995).

RESULTS

Hrdll-1, *Hroth*, *HrHox-1* and *Hrcad* are expressed in the epidermis with distinct domains along the anteroposterior axis

Previously we showed that *Hroth* and *HrHox-1* are expressed in the epidermis (Katsuyama et al., 1995; Wada et al., 1996). To understand epidermal patterning in the ascidian embryo more deeply, we added two more homeobox genes, *Hrdll-1* and *Hrcad* (the ascidian homologues of *Distal-less* and *caudal*, respectively; Y. K. and H. S., unpublished data; Katsuyama et

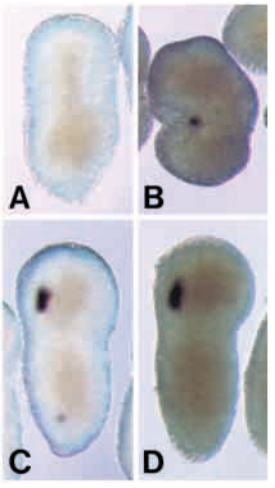

Fig. 6. Expression of the tyrosinase gene in A4.1 pair- or B4.1 pair-ablated embryos at the middle tailbud equivalent stage detected by whole-mount in situ hybridization. With all specimens, anterior is to the top. (A) A dorsal view of A4.1 pair-ablated embryo. (B) A lateral view of B4.1 pair-ablated embryo. (C,D) A dorsal view of control normal embryos.

al., 1999) for this study. Although expression patterns of *Hrdll-1* and *Hrcad* in detail will be reported elsewhere, we made here a brief description of expression of the four homeobox genes to show epidermal subdivision demarcated by their expression. The four genes are expressed exclusively in the ectodermal lineage except that *Hroth* is also expressed in the mesendoderm lineage before and during gastrulation (Wada et al., 1996). As described below, we found that they are expressed in the epidermis of different anteroposterior levels.

Region specificity of expression domains of these genes was most clearly visible at the middle tailbud stage (Fig. 2). At this stage, *Hrdll-1* is expressed in the anterior tip of the epidermis, which consists of the a-line (anterior-animal) cells (Fig. 2A). The expression domain is v-shaped, with three spots (two dorsal and one ventral) of high level expression (arrowheads in Fig. 2A′). Judging from their positions, each spot seems to correspond to the point where a cone of the adhesive papilla forms later. *Hrdll-1* is also expressed in a narrow region of the epidermis along the midline at a lower level (Fig. 2A). Expression of *Hrdll-1* in the adhesive papillae and the midline region begins at the neurula stage and gastrula stage, respectively, and continues up to the late tailbud stage (not shown).

Hroth is expressed in the anterior half of the trunk epidermis at the middle tailbud stage (Fig. 2B). Expression of *Hroth* is excluded from three spots (two dorsal and one ventral) at the anterior tip (Fig. 2B′) which correspond to the three spots of high-level *Hrdll-1* expression and from the cells around the neuropore (arrowhead in Fig. 2B′). *Hroth* is also expressed in the sensory vesicle (Fig. 2B). Expression of *Hroth* in the anterior epidermis and the sensory vesicle begins at the neural plate stage and the 32-cell stage, respectively, and persists by the swimming larva stage (Wada et al., 1996).

HrHox-1 is expressed more posteriorly in the epidermis and the neural tube at the middle tailbud stage (Fig. 2C). Expression is detected in the epidermis of the posterior half of the trunk, which is of a-line origin, and that of anterior part of the tail, which is of b-line (posterior-animal) origin. Expression in the neural tube is restricted to the posterior part of the visceral ganglion (Fig. 2C). Expression of *HrHox-1* in the epidermis and the neural tube begins at the early neurula stage and the early tailbud stage, respectively, and persists up to the swimming larva stage (Katsuyama et al., 1995).

Expression of *Hrcad* at the middle tailbud stage is detected in the epidermis of the tail, which is of b-line origin, except for the tip of the tail (Fig. 2D). It is also detected in lateral walls of the caudal neural tube (Fig. 2D). The anterior border of *HrHox-1* and *Hrcad* expression lies close to the posterior border of *Hroth* expression (Fig. 2B,C) and at the junction of the trunk and tail (Fig. 2D), respectively. Expression of *Hrcad* in the posterior epidermis and the caudal neural tube begins at the early neurula stage and the middle gastrula stage, respectively, and continues by the larva stage (Katsuyama et al., 1999).

Noteworthy, epidermal expression of each of the four genes starts at the site where it finally locates and sift of expression domains during development was never observed. Therefore we utilized their expression at the middle tailbud stage as markers for epidermal patterning in the following experiments. Expression domains of the four genes at the middle tailbud stage are summarized in Fig. 10A.

Table 1. Marker gene expression in animal hemisphere explants

Specimens examined	Genes examined	Specimens that express the gene (%)	Total number of specimens
Animal explants (8-cell stage)[a]	*Hrdll-1*	0	26
	HrHox-1	0	20
	Hrcad	0	24
	HrEpiD	100	10
Animal explants (16-cell stage)[a]	*Hrdll-1*	0	8
	Hroth	0	9
	HrHox-1	0	8
	Hrcad	0	8
Animal explants (32-cell stage)[a]	*Hrdll-1*	64	14
	Hroth	92	13
	HrHox-1	6	16
	Hrcad	82	11
	tyrosinase	0	14
Control embryos	*Hrdll-1*	100	27
	Hroth	100	31
	HrHox-1	100	24
	Hrcad	100	31
	HrEpiD	100	10
	tyrosinase	100	15

[a]Stage in parentheses indicates when animal explants were prepared.

Contact with vegetal cells after the 8-cell stage is required for animal cells to express *Hrdll-1*, *HrHox-1* and *Hrcad*

The epidermis of the ascidian embryo is entirely derived from animal hemisphere cells (Nishida, 1987; Fig. 1). Previously, we demonstrated that *Hroth* expression in animal cells requires contact with vegetal cells after the 8-cell stage (Wada and Saiga, 1999). Thus we examined initially whether this holds true for *Hrdll-1*, *HrHox-1* and *Hrcad*.

Animal hemispheres were isolated from late 8-cell-stage embryos, cultured as explants until control embryos reached the middle tailbud stage and examined for gene expression by whole-mount in situ hybridization (Fig. 3; Table 1). We found no expression of *Hrdll-1*, *HrHox-1* and *Hrcad* in animal hemisphere explants (Fig. 3A-C), although expression of these genes was detected in normal embryos (Fig. 3E-G). As a positive control, we examined expression of an epidermis marker *HrEpiD* (Ishida et al., 1996; Ueki and Satoh, 1994) and found that it was expressed in the explants (Fig. 3D,H). Together with our previous observations, these results indicate that animal hemisphere cells require inductive influences from vegetal cells after the 8-cell stage to express *Hrdll-1*, *HrHox-1* and *Hrcad* as well as *Hroth*.

Expression of *Hrdll-1* and *Hroth* is dependent on contact with the A-line cells, while contact with the B-line cells is required for expression of *HrHox-1* and *Hrcad*

The vegetal hemisphere of the 8-cell-stage ascidian embryo consists of anterior (the A4.1) and posterior (the B4.1) pairs (Fig. 1). We next tested which pair is responsible for inducing

Table 2. Marker gene expression in vegetal cell-ablated embryos

Specimens examined	Genes examined	Expression[a]			Total number of specimens
		Normal[b] (%)	Reduced[c] (%)	Not detected[d] (%)	
A4.1 pair-ablated embryos (8-cell stage)[f]	*Hrdll-1*	0	0	100	15
	Hroth	0	1	99	71
	HrHox-1	100[e]	0	0	24
	Hrcad	100	0	0	13
	tyrosinase	0	0	100	11
B4.1 pair-ablated embryos (8-cell stage)[f]	*Hrdll-1*	100	0	0	21
	Hroth	100	0	0	20
	HrHox-1	0	30	70	27
	Hrcad	0	97	3	30
	tyrosinase	35	41	24	17
A-line cells-ablated embryos (32-cell stage)[f]	*Hrdll-1*	85	0	15	13
	Hroth	91	0	9	11
	HrEpiD	100	0	0	14
Control embryos	*Hrdll-1*	100	0	0	82
	Hroth	100	0	0	98
	HrHox-1	100	0	0	25
	Hrcad	100	0	0	28
	tyrosinase	100	0	0	17
	HrEpiD	100	0	0	20

[a]In respect of four homeobox genes, only expression in the epidermis was considered. With *Hrdll-1*, only epidermal expression in the anterior tip was counted.
[b]Percentage of embryos that showed the normal level of expression of a given gene.
[c]Percentage of embryos that showed the reduced level of expression of a given gene.
[d]Percentage of embryos that showed no expression of a given gene.
[e]In 58% of cases, expression domain was expanded anteriorly.
[f]Stage in parentheses indicates when vegetal cells were ablated.

expression of the four homeobox genes. For this, we ablated the A4.1 or B4.1 pair at the late 8-cell stage and examined resultant embryos for the gene expression at the middle tailbud equivalent stage.

A4.1 pair-ablated embryos (A4.1$^-$ embryos) underwent gastrulation but not neural tube formation (not shown) and developed into embryos that were entirely covered with epidermal cells. In these embryos, a tail tip-like structure formed at one end and the muscle-specific actin gene was expressed near this structure (not shown). We therefore used this structure as a marker for the posterior end of A4.1$^-$ embryos. In A4.1$^-$ embryos, expression of *Hrdll-1* and *Hroth* was severely affected (Fig. 4A,B,E,F; Table 2). *Hrdll-1* expression in the anterior epidermis was lost, although expression along the midline was evident (Fig. 4A), and expression of *Hroth* was undetectable in the A4.1$^-$ embryos (Fig. 4B). By contrast, expression of *HrHox-1* and *Hrcad* was evident in the A4.1$^-$ embryos (Fig. 4C,D,G,H; Table 2). In 60% of specimens, the domain of *HrHox-1* expression expanded into the anterior region of the embryo (Fig. 4C), while such expansion of expression domain was not observed in the case of *Hrcad* (Fig. 4D).

B4.1 pair-ablated embryos (B4.1$^-$ embryos) underwent both gastrulation and neurulation (not shown), although neural tube closure was incomplete at the middle tailbud equivalent stage. Like A4.1$^-$ embryos, they formed a tail tip-like structure at one end and expressed *As-T* (the ascidian homologue of mouse *brachyury*; Yasuo and Satoh, 1993), a marker for notochord, near this structure (not shown). We defined the posterior end of B4.1$^-$ embryos by identifying this structure. In B4.1$^-$ embryos, expression of *HrHox-1* and *Hrcad* was affected (Fig. 5C,D,G,H; Table 2). In 70% of the B4.1$^-$ embryos, expression of *HrHox-1* was lost except for a group of cells at the dorsal side (arrowhead in Fig. 5C). Judging from the position, the expression in the neural tube seemed to be retained. The level of *Hrcad* expression in the epidermis was decreased in most of the B4.1$^-$ embryos (Fig. 5D,H). In contrast, expression of *Hrdll-1* and *Hroth* appeared normal in the B4.1$^-$ embryos (Fig. 5A,B,E,F; Table 2).

As a control, we examined expression of the tyrosinase gene, a marker for formation of the sensory pigment cells (Sato et al., 1997). Consistent with the previous reports demonstrating that sensory vesicle-inducing activity is present in the A-line cells (Nishida, 1991; Nishida and Satoh, 1989; Okado and Takahashi, 1988, 1990; Reverberi et al., 1960; Rose, 1939), expression of the tyrosinase gene was detected in the B4.1$^-$ embryos, but not in the A4.1$^-$ embryos (Fig. 6A-D; Table 2). These results suggest that, first, an inductive influence from the A-line cells is required for expression of *Hrdll-1* and *Hroth* in the anterior epidermis as well as sensory vesicle formation and, second, an inductive signal from the B-line cells is required for epidermal expression of *HrHox-1* and *Hrcad*.

Contact with vegetal cells until the late 32-cell stage is sufficient for expression of *Hrdll-1*, *Hroth* and *Hrcad*, but not *HrHox-1*, in animal cells

Next we asked when induction by vegetal cells takes place to allow expression of *Hrdll-1*, *Hroth*, *HrHox-1* and *Hrcad* in the animal cells. We prepared animal hemisphere explants by killing all vegetal cells at the late 16- or late 32-cell stage and examined the explants at the middle tailbud equivalent stage for the gene expression (Fig. 7; Table 1). We found that explants prepared at

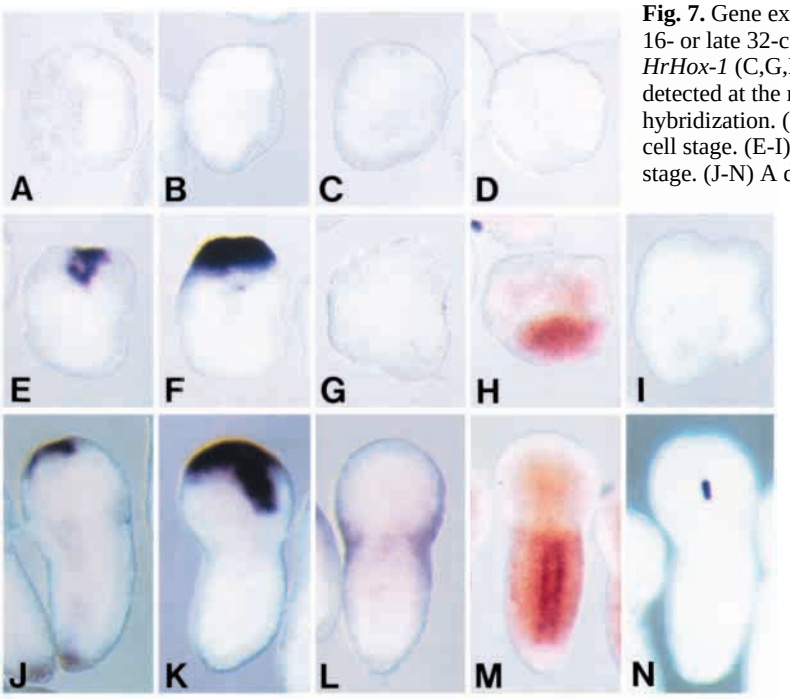

Fig. 7. Gene expression in animal hemisphere explants prepared at the late 16- or late 32-cell stage. Expression of *Hrdll-1* (A,E,J), *Hroth* (B,F,K), *HrHox-1* (C,G,L), *Hrcad* (D,H,M) and the tyrosinase gene (I,N) was detected at the middle tailbud equivalent stage by whole-mount in situ hybridization. (A-D) Animal hemisphere explants prepared at the late 16-cell stage. (E-I) Animal hemisphere explants prepared at the late 32-cell stage. (J-N) A dorsal view of control normal embryos. Anterior is to the top.

the late 16-cell stage expressed none of the four homeobox genes examined (Fig. 7A-D,J-M). In contrast, explants prepared at the late 32-cell stage largely expressed *Hrdll-1*, *Hroth* and *Hrcad* (about 65, 80 and 90% of specimens examined, respectively; Fig. 7E,F,H), but rarely expressed *HrHox-1* (6% of specimens examined; Fig. 7G).

We also examined expression of the tyrosinase gene as a marker for sensory vesicle formation and found that it was undetectable in the explants prepared at the late 32-cell stage (Fig. 7I,N). These results suggest that inductive influences from the vegetal cells until the late 32-cell, but not the late 16-cell stage, are sufficient for expression of *Hrdll-1*, *Hroth* and *Hrcad* in the animal cells. In contrast, as shown previously (Nishida and Satoh, 1989; Okado and Takahashi, 1990), an inductive influence from the vegetal cells until the late 32-cell stage is insufficient for sensory vesicle formation. It is also suggested that an inductive influence from the vegetal cells after the 32-cell stage is required for expression of *HrHox-1*.

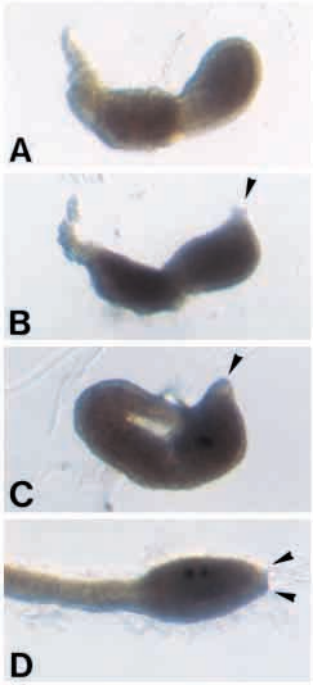

Fig. 8. Formation of the adhesive papillae in cell-ablated embryos examined for the morphology at the swimming larva equivalent stage. (A) Embryo whose A4.1 pair was ablated at the late 8-cell stage. (B) Embryo whose A-line cells were all ablated at the late 32-cell stage. (C) Embryo whose B4.1 pair was ablated at the late 8-cell stage. (D) Control normal embryo. Arrowheads in B-D indicate protrusions that are judged as adhesive papillae from morphology. With all specimens, anterior is to the right.

Contact with the A-line cells until the late 32-cell stage is sufficient for adhesive papilla formation

In normal embryos, the three spots where the adhesive papillae are formed overlap the expression domains of *Hrdll-1* and are surrounded by the *Hroth*-expressing domain (Fig. 2A′,B′), suggesting a close association of expression of *Hrdll-1* and *Hroth* with formation of the adhesive papillae. Thus, we tested this in the manipulated embryos.

First, we examined embryos of which A4.1 or B4.1 pair was ablated at the late 8-cell stage for adhesive papilla formation by morphology at the swimming larva equivalent stage (Fig. 8; Table 3). In about 90% of control embryos, three cone-shaped protrusions were formed at the anterior end (Fig. 8D). Protrusions also formed in the B4.1 pair-ablated embryos (about 70% of cases; Fig. 8C) but not in the A4.1 pair-ablated embryos (1% of cases; Fig. 8A). Because *Hrdll-1* and *Hroth* were expressed in the B4.1 pair- but not A4.1 pair-ablated embryos as described above (see Figs 4A,B, 5A,B), both formation of the adhesive papillae and expression of *Hrdll-1* and *Hroth* seem to be dependent on contact with the A-line cells after the 8-cell stage.

We next examined embryos of which A-line cells were all ablated at the late 32-cell stage for both gene expression at the middle tailbud equivalent stage (Fig. 9; Table 2) and adhesive papilla formation at the swimming larva equivalent stage (Fig. 8; Table 3). As expected, *Hrdll-1* was expressed in a small area of the anterior surface of these embryos (85% of cases; Fig. 9A,D). *Hroth* was expressed in the anterior surface of the embryos except for the small area where *Hrdll-1* was expressed

Table 3. Formation of the adhesive papillae in vegetal cell-ablated embryos

Specimens examined	Specimens that formed the adhesive papillae (%)	Total number of specimens
A4.1 pair-ablated embryos (8-cell stage)[a]	1	70
B4.1 pair-ablated embryos (8-cell stage)[a]	71	21
A-line cells-ablated embryos (32-cell stage)[a]	78	27
Control embryos	91	134

[a]Stage in parentheses indicates when vegetal cells were ablated.

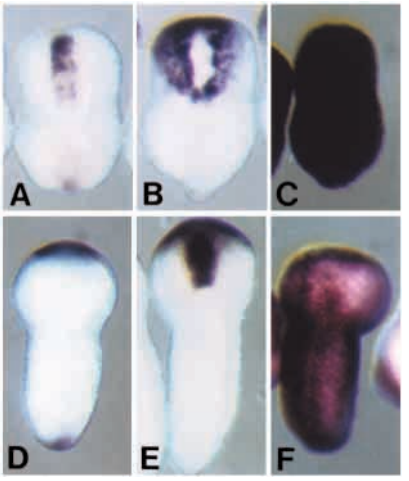

Fig. 9. Gene expression in embryos ablated with all A-line cells at the late 32-cell stage. Expression of *Hrdll-1* (A,D), *Hroth* (B,E) and *HrEpiD* (C,F) was detected at the middle tailbud equivalent stage by whole-mount in situ hybridization. With all specimens, anterior is to the top. (A-C) A dorsal view of embryos whose A-line cells were all ablated at the late 32-cell stage. (D-F) A dorsal view of control normal embryos.

(about 90% of cases; Fig. 9B,E). The identity of these expression domains as parts of the epidermis was verified by expression of *HrEpiD* throughout the surface of these embryos (Fig. 9C,F). Therefore, expression patterns of *Hrdll-1* and *Hroth* in these embryos were reminiscent of those in normal embryos, though the shape of the domain marked by the presence of *Hrdll-1* expression and the absence of *Hroth* expression was different from that observed in normal embryos. Consistent with this, adhesive papillae were found to form in these embryos, although the shape of the protrusions was abnormal in these embryos (about 80% of cases; Fig. 8B). These results suggest that patterned expression of *Hrdll-1* and *Hroth* in the anterior epidermis correlates with the formation of the adhesive papillae even under the present experimental conditions and that contact with the A-line cells until the late 32-cell stage is sufficient for adhesive papilla formation in the anterior animal hemisphere cells.

DISCUSSION

The epidermis of the ascidian embryo is subdivided into multiple distinct domains along the anteroposterior axis

In this study, we investigated expression of homeobox genes *Hrdll-1*, *Hroth*, *HrHox-1* and *Hrcad* in the epidermis and its regulation by vegetal hemisphere cells in the ascidian embryo. Analyses of expression of these genes provide evidence for regional specification along the anteroposterior axis in the epidermis of the ascidian embryo. Based on the expression pattern of the four homeobox genes, the epidermis of the middle tailbud stage embryo can be subdivided into at least eight different regions along the anteroposterior axis (Fig. 10A): (1) three spots at the anterior tip of the trunk defined by *Hrdll-1*-only expression; (2) the v-shaped region surrounding the above region, that is defined by co-expression of *Hrdll-1* and *Hroth*; (3) the anterior part of the trunk defined by *Hroth* expression only; (4) the region between *Hroth*-expressing and *HrHox-1*-expressing domains; (5) the posterior part of the trunk defined by *HrHox-1* expression only; (6) the anterior part of the tail defined by co-expression of *HrHox-1* and *Hrcad*; (7) a posterior part of the tail defined by *Hrcad* expression only; (8) the tip of the tail expressing none of the four homeobox genes.

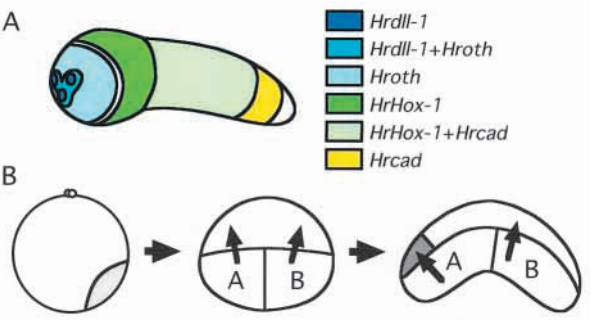

Fig. 10. (A) Patterning of the epidermis along anteroposterior axis as defined by expression of *Hrdll-1*, *Hroth*, *HrHox-1* and *Hrcad* in the ascidian embryo. Shown is a schematic drawing of expression patterns of the four homeobox genes in the epidermis at the middle tailbud stage. (B) Model of anteroposterior specification in the ascidian embryo. (Left) Posterior-vegetal region of a fertilized egg is marked by the localization of specific cytoplasm that confer posterior feature on cells (light gray). The posterior-vegetal cytoplasm is inherited by the B-line cells, resulting in establishment of an initial anteroposterior axis in the vegetal hemisphere (Nishida, 1994b). (Middle) Until the late 32-cell stage, the A-line cells emit inductive signals for the anterior epidermal cells to express *Hrdll-1* and *Hroth*, while the B-line cells emit inductive signals for the posterior epidermis to express *Hrcad*. (Right) Later in development, the A-line cells induce sensory vesicle formation in the a-line precursor cells (dark gray) and the B-line cells specify middle part of the epidermis to express *HrHox-1*.

Previously, Ishida et al. (1996) reported expression patterns of eight distinct epidermis-specific genes and suggested that the epidermis can be subdivided into six regions based on sets of gene expression. However, all of the eight specific genes are expressed in both of the trunk and the tail. The present subdivision of the epidermis is completely different from that suggested by Ishida et al. (1996) except that the adhesive papillae-forming region is commonly marked by both subdivisions. Takahashi et al. (1997) reported cloning and expression of genes that are predominantly expressed in the tail region of the ascidian embryo, some of which are expressed in the epidermis of the tail. It will be interesting to examine in future studies whether the expression of these genes fit with the subdivision of the epidermis observed here.

Specification of the anterior epidermis by the A-line cells

We have shown that expression of *Hrdll-1* and *Hroth* in the anterior epidermis depends on contact with the A-line cells after the 8-cell stage. This suggests that specification of anterior fate in the epidermis depends on an inductive influence from the A-line cells. The induction seems to occur by the late 32-cell stage because the contact until this stage is sufficient for expression of *Hrdll-1* and *Hroth* in the anterior epidermis. This is unexpectedly early, because expression of *Hrdll-1* and *Hroth* in the anterior epidermis begins to be detected at the neurula stage and neural plate stage, respectively. It is likely that the a-line cells are fated to express *Hrdll-1* and *Hroth* long before they express these genes.

To date, two distinct inductive roles of the A-line cells have been suggested. One is induction of the sensory vesicle

formation (Nishida, 1991; Nishida and Satoh, 1989; Okado and Takahashi, 1988, 1990; Okamura et al., 1994; Reverberi et al., 1960; Rose, 1939) and the other is induction of adhesive papilla formation (Ortolani and Patricolo, 1984). Considering the timing of sensory vesicle induction, suggested by previous work (Nishida and Satoh, 1989; Okado and Takahashi, 1990), which is between the 64-cell to late gastrula stage, the induction of anterior epidermis specification demonstrated in the present study seems to be a distinct event from the sensory vesicle induction. Consistent with this, we observed that contact with the A-line cells until the late 32-cell stage was insufficient for the sensory vesicle formation (Fig. 7I).

On the contrary, adhesive papilla formation seems to be a visible manifestation of anterior epidermis specification, because patterned expression of *Hrdll-1* and *Hroth* induced by the A-line cells is accompanied by adhesive papilla formation. Induction of adhesive papilla formation by the A-line cells has also been demonstrated in *Ascidia malaca* and *Phallusia mammillata* by Ortolani and Patricolo (1984). However, the mode of induction that they suggested seems to be different from that suggested in the present study because they showed that adhesive papilla formation requires the presence of at least one endoderm precursor of the archenteric roof after the 32-cell stage. At present, the reason for this discrepancy is unknown.

Specification of the middle and posterior parts of epidermis

We have shown that expression of *HrHox-1* and *Hrcad* in the epidermis depends on contact with the B-line cells after the late 8-cell stage. This suggests that inductive influences from the B-line cells are required for specification of the middle and posterior parts of the epidermis. We have also shown that the capability of the epidermis to express *Hrcad* is acquired by the late 32-cell stage. Therefore, similar to the case of the anterior epidermis, the posterior epidermis seems to be determined to express *Hrcad* long before the onset of expression of this gene. One of the important conclusions in this study is that patterning of the epidermis of the ascidian embryo anteroposteriorly begins as early stage as the late 32-cell stage.

In contrast, the capability of the epidermis to express *HrHox-1* appears to be acquired after the late 32-cell stage. This suggests that specification of the middle part of the epidermis may occur at a later stage of embryogenesis. During ascidian embryogenesis, the B-line cells contact the b-line but not a-line cells until the gastrula stage and, after the neural plate stage, they contact the a-line and b-line cells that consist of the middle part of the epidermis. Expression of *HrHox-1* is first detectable at the early neurula stage. Therefore, it may be at the neural plate stage that the B-line cells specify the middle part of the epidermis to express *HrHox-1*.

Posterior epidermal specification may be controlled partially by the A-line cells as well, because expression of *Hrcad* was lost in animal hemisphere explants prepared at the 8-cell stage, while it was retained in A4.1^{-} embryos and, to a lesser extent, in B4.1^{-} embryos. It is possible that *Hrcad*-inducing activity exists mainly in the B-line cells and weaker activity exists in the A-line cells, though the importance of the latter activity in normal development is unknown at present.

In summary, our present findings indicate that anteroposterior patterning of the epidermis depends on multiple inductive influences from both the A-line and B-line cells, in contrast to autonomous differentiation of this tissue as suggested by Nishida (1994a).

Model of anteroposterior specification in the ascidian embryo

An ascidian egg before fertilization is radially symmetrical along the animal-vegetal axis. Ooplasmic segregation that occurs after fertilization results in an embryo with bilateral symmetry, in which the posterovegetal region is marked by the presence of a specific form of cytoplasm called myoplasm. Nishida (1994b) has demonstrated that cytoplasm of the posterior-vegetal region of the fertilized eggs after segregation is necessary and sufficient for conferring posterior fate on cells. The cytoplasm also suppresses anterior fate, which seems to be the default state. Thus, it has been suggested that the B-line cells inherit the posterior-vegetal determinants to develop posterior characteristics, while the A-line cells acquire anterior characteristics through the absence of the determinants during normal development (Nishida, 1994b).

Taking this into account, we propose a hypothetical model, in which the anteroposterior axis of the ascidian embryo is primarily established in the vegetal hemisphere by the cytoplasmic determinants as suggested by Nishida (1994b) and this axis is in turn reflected in the animal hemisphere by distinct inducing activities of the A-line and B-line cells (Fig. 10B). Until the late 32-cell stage, the A-line cells induce anterior fate in the a-line epidermal cells, while the B-line cells direct posterior fate in the b-line epidermis. Later, the A-line cells further induce sensory vesicle formation in the a-line precursor cells and the B-line cells specify the middle part of the epidermis (Fig. 10B).

There are some points to be noted in the mechanisms of epidermal patterning suggested here. First, the epidermis seems to be patterned in a different way from the neural tube, as discussed above. Furthermore, anterior and posterior fates of the epidermis seem to be induced independently, rather than by transforming from anterior to posterior fates. Second, the timing of the induction is relatively early in development and cells that are responsible for the induction are undifferentiated cells that contain multiple developmental fates and develop into both the axial and nonaxial tissues. Taken together, the mechanisms of epidermis patterning suggested here may represent a novel mode of anteroposterior patterning in animal embryos.

Comparison of ectoderm patterning between the ascidians and the vertebrates

There is considerable accumulation of evidence to show that sensory vesicle formation by the A-line cells in the ascidian embryo is homologous to neural induction in the vertebrates (Inazawa et al., 1998; Miya et al., 1997; Okamura et al., 1993). It is unknown whether the vertebrate embryos employ a mechanism of epidermal specification that is homologous to that suggested here in the ascidian embryo. However, some of the features of epidermal patterning discussed above seem to be shared by the recently proposed mechanism for posterior patterning in vertebrate embryo. This comes from studies in zebrafish which show that nonaxial mesendoderm induces a posterior fate in the epiblast by modulating the competence of the epiblast cells to respond the neural inducers before the onset of gastrulation (Koshida et al., 1998; Woo and Fraser, 1997). Thus, there seem to be intriguing parallels in timing and inducers

of posterior fate specification between ascidian and zebrafish embryos. Although at present it remains unclear whether this similarity implies a homologous mechanism or not, the present study provides important clues to investigate conservation and diversification of mechanisms of anteroposterior patterning between ascidian and vertebrate embryos.

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