Development 124, 13-20 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV5092

Multiple signaling events specify ectoderm and pattern the oral-aboral axis in the sea urchin embryo

Athula H. Wikramanayake and William H. Klein*

Department of Biochemistry and Molecular Biology, University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

*Author for correspondence

SUMMARY

In the sea urchin embryo, the animal-vegetal axis is established during oogenesis and the oral-aboral axis is specified sometime after fertilization. The mechanisms by which either of these axes are specified and patterned during embryogenesis are poorly understood. Here, we investigated the role of cellular interactions in the specification of the ectoderm territories and polarization of the ectoderm along the oral-aboral axis. Isolated animal halves (mesomeres), which are fated to give rise to oral and aboral ectoderm, developed into polarized embryoids that expressed an oral ectoderm-specific marker uniformly. These embryoids also produced neuron-like cells and serotonergic neurons, suggesting that mesomeres are autonomously specified as oral ectoderm. Mesomere-derived embryoids did not express any aboral ectoderm-specific markers, although we previously showed that aboral ectoderm-specific genes can be induced by 25 mM lithium chloride, which also induced endoderm formation (Wikramanayake, A. H., Brandhorst, B. P. and Klein, W. H. (1995). *Development* 121, 1497-1505). To ascertain if endoderm formation is a prerequisite for induction of aboral ectoderm by lithium and for normal ectoderm patterning in animal halves, we modulated the lithium treatment to ensure that no endoderm formed. Remarkably, treating animal halves with 10 mM LiCl at ~7 hours postfertilization resulted in embryoids that displayed oral-aboral axis patterning in the absence of endoderm. Application of 25 mM LiCl to animal halves at ~16 hours postfertilization, which also did not induce endoderm, resulted in polarized expression of the aboral ectoderm-specific LpS1 protein, but global expression of the Ecto V antigen and no induction of the stomodeum or ciliary band. These results suggest that at least two signals, a positive inductive signal to specify the aboral ectoderm and a negative suppressive signal to inactivate oral ectoderm-specific genes in the prospective aboral ectoderm territory, are needed for correct spatial expression of oral and aboral ectoderm-specific genes. Transmission of both these signals may be prerequisite for induction of secondary ectodermal structures such as the ciliary band and stomodeum. Thus, differentiation of ectoderm and polarization of the oral-aboral axis in *Lytechinus pictus* depends on cellular interactions with vegetal blastomeres as well as interactions along the oral-aboral axis.

Key words: sea urchin, ectoderm specification, ectoderm patterning, animal halves, lithium

INTRODUCTION

During early embryonic development, embryos are partitioned into discrete regions that are specified to follow different programs of development. How this regional or territorial specification occurs is a key question in development. In some species, territorial specification occurs predominantly as a result of asymmetric inheritance of cytoplasmic determinants while, in others, territorial specification occurs mostly through cell-cell interactions (Davidson, 1986; Slack, 1991). Embryos from most species, however, rely on a combination of these two mechanisms for early specification (Sternberg and Horvitz, 1986; Priess and Thomson, 1987; Nishida and Satoh, 1989).

The sea urchin embryo is a useful model for studying territorial specification since all five embryonic territories are specified by the 60-cell stage or shortly thereafter (Davidson, 1989). These territories are specified by a combination of invariant cleavages and cell-cell interactions. At the 60-cell stage, the embryo is arranged into distinct tiers along the animal-vegetal axis: an1, an2, veg1, veg2 and the micromeres. Roughly, the an1, an2 and veg1 tiers correspond to the oral and aboral territories, the veg2 tier corresponds to the vegetal plate territory, and the large and small micromeres form the most vegetal tiers and the two remaining territories (Davidson, 1989; Cameron and Davidson, 1991). In a model to account for the animal-to-vegetal and the oral-to-aboral patterning of the sea urchin embryo, Davidson (1989) proposed that the large micromeres, which are autonomously specified by inherited maternal determinants, act as a vegetal signaling center and initiate a cascade of upwardly directed signaling events. Signaling by the large micromeres induces the overlying veg2 tier, specifying the vegetal plate territory. The veg2 tier then induces the tiers of cells above, which are the prospective ectodermal territories. Polarization of the oral-aboral axis was

proposed to be the result of signaling by an autonomously specified prospective oral ectoderm region that specifies the aboral ectoderm. Diversification of ectoderm to form such structures as the ciliary band, the stomodeum and oral hood results from vegetal signaling and interactions between aboral and oral ectoderm cells (Davidson, 1989). This model also proposes that interblastomeric signaling selectively modulates the activity of maternal transcription factors, leading to the unique patterns of gene expression seen in the descendants of the five territories (Davidson, 1989).

We are interested in understanding the mechanisms that mediate territorial specification during sea urchin embryogenesis. In particular, we have focused on the two ectoderm territories that are derived mostly from the animal half of the embryo. Previously, we showed that signaling from vegetal blastomeres was necessary for the normal differentiation of ectoderm derived from the animal halves of 8-cell sea urchin embryos (Wikramanayake et al., 1995). Here, we extend our studies and provide evidence to support the hypothesis that oral ectoderm is autonomously specified in *Lytechinus pictus* embryos. We also show that two distinct vegetal signals are required for the normal specification of the aboral ectoderm territory and that additional signaling between the prospective ectoderm territories may be required for patterning ectoderm along the oral-aboral axis.

MATERIALS AND METHODS

Obtaining gametes and culturing embryos

L. pictus were obtained from Pacific Biomarine (Venice, CA) or Marinus (Long Beach, CA). Gametes were obtained by intraceolomic injection of 0.5 M KCl. Embryos were cultured with stirring in artificial sea water (ASW; pH 8.0) at 15°C.

Isolation and manipulation of animal halves

Animal halves were isolated from 8-cell *L. pictus* embryos as previously described (Wikramanayake et al., 1995). Explants were cultured in 1% noble agar-coated (DIFCO Laboratories) 10 cm Petri dishes (Falcon, Becton Dickinson) in the presence of 50 μg/ml gentamicin (Sigma).

Animal halves were treated with LiCl for 12-15 hours, removed to fresh ASW, and cultured at 15°C until control embryos reached the pluteus stage (approximately 72 hours postfertilization (PF)).

RNA isolation and RT-PCR analysis

Total RNA was isolated from 15-25 animal halves or control embryos using a single-step method (Chomczynski and Sacchi, 1987). Extracted RNA was suspended in 20 μl of DEPC-treated H_2O. The RNA was used to synthesize cDNA using SuperScript reverse transcriptase (GIBCO, BRL) and random hexamers. PCR reactions contained 0.6 μM concentrations of the appropriate PCR primers, 1 μM of [α^{32}P]dCTP and 2.0 mM $MgCl_2$. PCR amplification conditions were 94°C for 1 minute, 60°C for 1 minute and 72°C for 45 seconds for the *LpS1*, *LpC2* and *LpUSF* primers. For *LpN1.2*, the same conditions applied except that the annealing temperature was dropped to 50°C. All reactions were performed in the linear range of amplification. One fourth of the products were resolved on native polyacrylamide gels. The sequences of the primers used in the study are shown in Table 1.

Immunolocalization of spatial markers

The following antibodies were used for localization of spatial markers. The anti-Spec 1 rabbit polyclonal antibody (Wikramanayake et al., 1995), which cross-reacts with the LpS1 protein (Xiang et al., 1988), was used to localize the protein in the aboral ectoderm of *L. pictus* embryos. The mouse monoclonal antibody (mAb) Ic12, which recognizes the oral ectoderm-specific antigen Ecto V, was used as a marker for oral ectoderm (Coffman and McClay, 1990). The Endo 1 antibody recognizes an antigen in the midgut and hindgut (Wessel and McClay, 1985) and was used as a probe for endoderm. The mAb UH295 recognizes an antigen in the ciliary band (David McClay, personal communication) as well as a subset of embryonic neuron-like cells. An anti-serotonin rat monoclonal antibody (Chemicon) was used to label serotonergic cells in embryos and animal halves.

For localizing the LpS1 protein, embryos were fixed in 1% glutaraldehyde buffered with ASW (pH 8.0) for 30 minutes. Following rinsing with ASW, the embryos were incubated in a 0.02% solution of trypsin (wt/vol) in ASW for 10 minutes. Embryos and animal halves were blocked in phosphate-buffered saline (PBS)/0.5 M NaCl/0.2% Tween-20 and 3 mg/ml bovine serum albumin (BSA) for 1 hour, and then incubated with a 1:75 dilution of the anti-Spec1 antibody for 3 hours. The primary antibody was followed by 1:1000 dilution of a donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Jackson Immunochemicals). The color reaction was obtained using the Zymed AEC staining kit. For staining with the ciliary band antibody and the anti-serotonin antibody, embryos and animal halves were fixed in 4% paraformaldehyde and methanol as described in Malinda et al. (1995). Blocking and antibody dilutions were done in 3 mg/ml BSA/10 mM PBS/0.2% Tween-20. Staining embryos using the mAbs Ig12 and Endo 1 were done on methanol-fixed embryos, using either fluorescein isothiocyanate (FITC) or rhodamine-conjugated secondary antibodies (Cappel).

RESULTS

Morphology of lithium-treated animal halves

In an earlier study, we developed a simple method for making large numbers of animal halves from 8-cell sea urchin embryos

Table 1. Oligonucleotide primers used for the RT-PCR assay in this study

Marker	Nucleotide sequence	References
LpS1	U 5´-GACAACTATGACACAAACAAAGACG-3´ D 5´-ACCATCGCCATCCTTGTCTGCTTCT-3´	Xiang et al. (1988).
LpC2	U 5´-TGTATCACGGATTGGCTCAAAG-3´ D 5´-TCCTGAACCTGCCTCAACTTGG-3´	Fang and Brandhorst (1996)
LpN1.2	U 5´-TACGGTGGCTTGGGACTTTG-3´ D 5´-GGGAATGTGTTTTGGAATCA-3´	Wessel et al. (1989)
LpUSF	U 5´-TCAGGTGGTCAGTTTTAC-3´ D 5´-CGTGGCTCTCCTCCTCTC-5´	Kozlowski et al. (1991)

U and D refer to upstream and downstream primers.

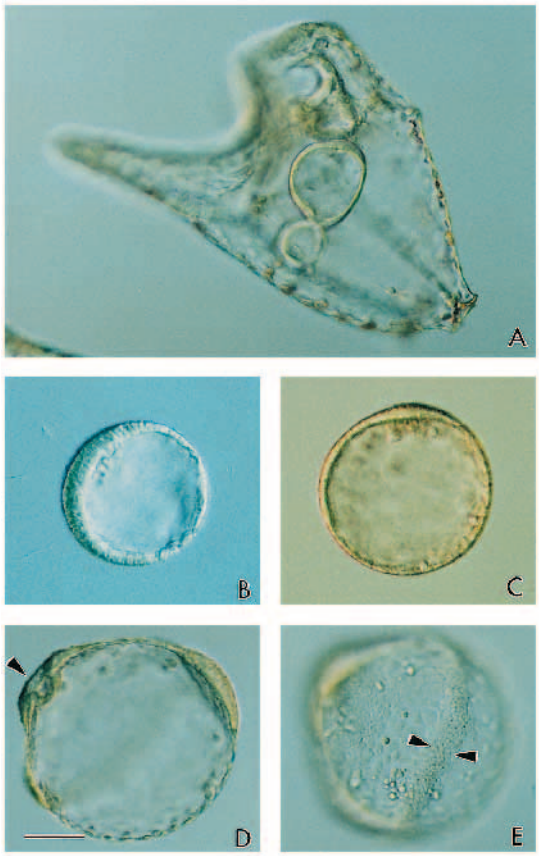

Fig. 1. Morphology of *L. pictus* embryos and animal halves. (A) An embryo at 3 days postfertilization. (B) An untreated animal half isolated from an 8-cell embryo, after 3 days of culture. (C) An animal half that was treated with 25 mM LiCl at 16 hours postfertilization, after 3 days of culture. (D) An animal half treated with 10 mM LiCl at 7 hours postfertilization, after 3 days of culture. The arrowhead points to the stomodeum. (E) The same embryo as in D at a different plane of focus. The arrowheads point to the ciliary band. (Bar, 50 μm.)

(Wikramanayake et al., 1995). In culture, these animal halves typically formed the dauerblastulae described by Horstadius and others (Horstadius, 1973). While pluteus-stage embryos formed tripartite guts, skeletons and other mesoderm-and endoderm-derived tissues (Fig. 1A), identically staged untreated animal halves never formed any of these structures. Embryoids derived from animal halves were morphologically polarized and always had a thickened epithelium that tapered off to a squamous epithelium (Fig. 1B). Furthermore, untreated *L. pictus* animal halves made at the 8-cell stage never expressed any aboral ectoderm-specific genes; they did, however, express the oral ectoderm marker Ecto V globally (Wikramanayake et al., 1995). Adding 25 mM LiCl to these animal halves beginning at 7 hours PF resulted in induction of endodermal as well as mesodermal cell types in the animal halves and in the activation of aboral ectoderm-specific genes (Wikramanayake et al., 1995).

We interpreted these results as indication that some endoderm differentiation was essential for aboral ectoderm-specific gene activation. To extend these observations, we asked if expression of aboral ectoderm-specific genes could be induced in animal halves in the absence of prior endoderm induction. We found that animal halves lost their competence to form endoderm in response to lithium over developmental time. We also found that there was a threshold lithium concentration for induction of endoderm in animal halves. Thus, when 25 mM LiCl was added to *L. pictus* animal halves at approximately 16 hours PF and when 10 mM LiCl was added to animal halves at approximately 7 hours PF, there was no apparent endoderm formation, as indicated by the lack of an invaginating gut. However, these treatments resulted in significant and reproducible alterations in the morphology of the treated animal halves.

We examined live, lithium-treated animal halves for morphological alterations using differential interference contrast microscopy (DIC). To facilitate microscopic observation, embryos and animal halves were immobilized on poly-L-lysine-coated glass slides. Animal halves treated with 25 mM LiCl at 16 hours PF showed reduced ciliary length and a distinct apical plate (Fig. 1C). There was some polarization of the epithelium, with the side with the apical plate being thicker than the opposite side. These animal halves did not form a ciliary band or a stomodeum even after 3 days in culture (Fig. 1C).

Animal halves treated with 10 mM LiCl at 7 hours PF underwent more striking morphological alterations. There was a sharp polarization between the thickened epithelium and the thin squamous epithelium (Fig. 1D). Unlike untreated animal halves, where the thickened epithelium gradually tapered off into a squamous epithelium, animal halves treated with 10 mM LiCl at 7 hours PF showed an abrupt transition of the two epithelial types (Fig. 1D). The thickened epithelium contained a prominent apical plate and a distinct stomodeum, which are normally derived from oral ectoderm (Fig. 1D). Between the thickened epithelium and the squamous region was a ciliary band (Fig. 1E), which in the normal embryo forms at the boundary of the aboral and oral ectoderm regions. Thus, in animal halves treated with 10 mM LiCl at 7 hours PF, ectoderm derived from mesomeres appeared to have undergone correct oral-to-aboral patterning as assessed by morphological criteria. This patterning of ectoderm was independent of prior endoderm induction by lithium, as morphologically assessed by gut formation.

Activation of aboral ectoderm-specific genes in animal halves by lithium in the absence of prior endoderm induction

Animal halves exposed to the various lithium treatments were assayed for expression of aboral ectoderm- and endoderm-specific genes using RT-PCR. This analysis showed that when 1 mM LiCl was added at 7 hours PF, there was no expression of aboral ectoderm- and endoderm-specific genes (Fig. 2A). At a 10 mM lithium concentration, there was strong expression of the aboral ectoderm-specific *LpS1* and *LpC2* actin genes, but no expression of the endoderm-specific *LpN1.2* gene (Fig. 2A). At higher LiCl concentrations, isolated animal halves began to express the endoderm-specific marker *LpN1.2* (Fig. 2A), indicating induction of endoderm formation. The results with the *LpN1.2* gene were corroborated using the Endo 1 antibody (data not shown), which stains even the smallest endodermal invagination induced by lithium (Livingston and Wilt, 1990). Staining with the Endo 1 antibody also confirmed that the

invaginations formed in response to 10 mM lithium at 7 hours PF was a stomodeum and not an invaginating gut.

We also found that animal halves lost the competence to form endoderm in response to relatively high concentrations of lithium over developmental time. This was also observed by Livingston and Wilt (1989) with isolated *Strongylocentrotus purpuratus* mesomeres. Thus, while animal halves formed endoderm when treated with 25 mM LiCl at approximately 7 hours PF (Fig. 2B), no endoderm was induced in animal halves exposed to 25 mM LiCl at 16 hours PF as assayed by the two endoderm markers *LpN1.2* (Fig. 2B) and Endo 1 (data not shown). Animal halves treated with lithium at 16 hours did, however, strongly express the aboral ectoderm-specific genes *LpS1* and *LpC2* (Fig. 2B). These data clearly indicated that lithium treatment activated aboral ectoderm-specific genes even though endoderm had not been induced.

Spatial expression of ectoderm markers in lithium-treated animal halves

To assess ectoderm patterning induced by the various treatments, we used immunostaining to determine the expression pattern of ectoderm markers. The oral marker Ecto V was expressed in the oral ectoderm of pluteus-stage embryos (Coffman and McClay, 1990, Fig. 3A,A′). While there was no expression of aboral ectoderm-specific genes in untreated animal halves, there was global expression of the oral ectoderm-specific marker Ecto V (Fig. 3B,B′). Expression of the Ecto V antigen was also global in animal halves treated with 25 mM LiCl at 16 hours PF (Fig. 3D,D′). In sharp contrast, in animal halves treated with 10 mM LiCl at 7 hours PF, the Ecto V antigen was expressed only in the thick epithelium, which by morphological criteria corresponded to the oral ectoderm (Fig. 3C,C′). In these embryoids, there was no expression of Ecto V in the squamous epithelium (Fig. 3C,C′), which expressed an aboral ectoderm marker (see below).

We also attempted to identify other oral ectoderm territory-derived cell types in *L. pictus* animal halves. One of the larval cell types derived from the oral ectoderm are neurons (Cameron et al., 1987; Bisgrove and Burke, 1987). We used an anti-serotonin antibody to assess animal halves for the presence of serotonergic neurons. This antibody specifically labeled a subset of neurons in the basal region of the oral hood of 3-day pluteus larvae (Fig. 4A,B), consistent with the results of Bisgrove and Burke (1987). Staining for serotonin was seen in some cells of the

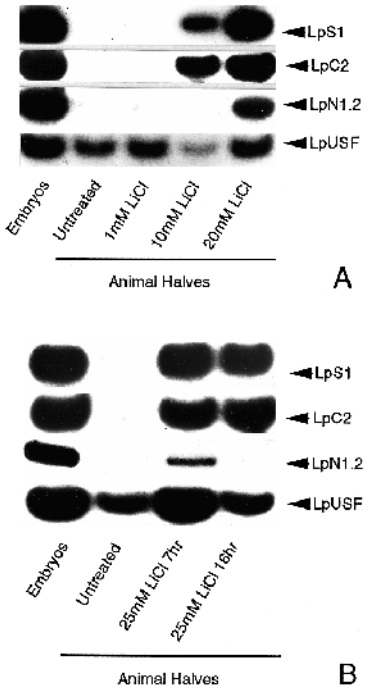

Fig. 2. RT-PCR analysis of gene expression in *L. pictus* embryos and animal halves after the various lithium treatments. (A) Effect of lithium concentration on expression of cell-type-specific markers. (B) Effect of time of lithium application on expression of cell-type-specific markers. PCR products were analyzed using autoradiography

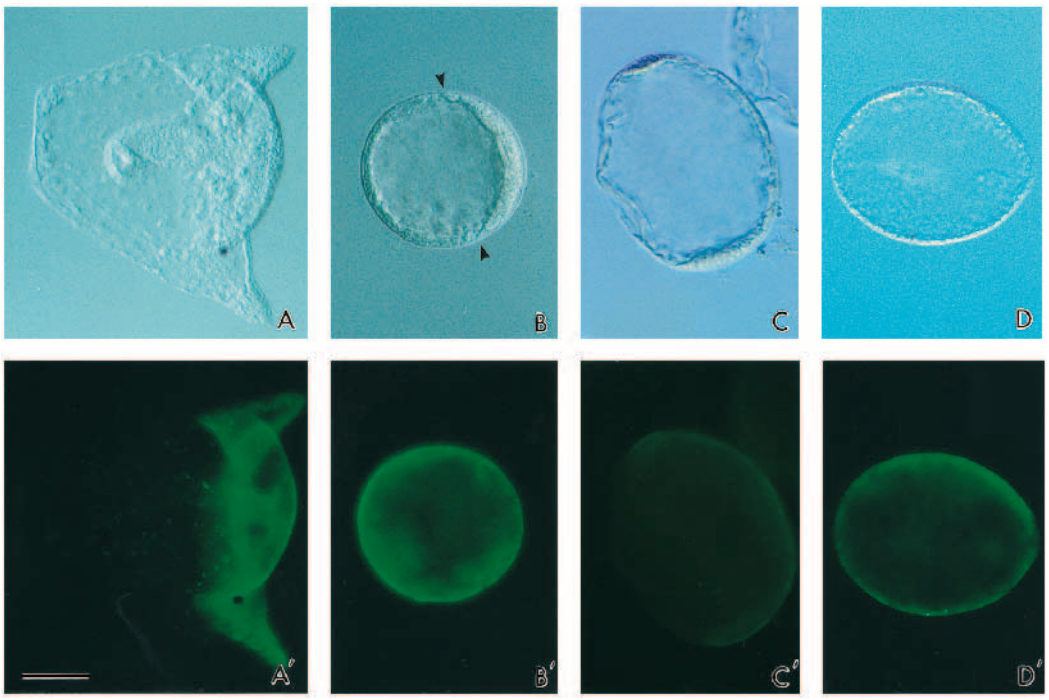

Fig. 3. Localization of Ecto V in 3 day *L. pictus* embryos and animal halves. The antigen was localized using indirect immunofluorescence on methanol-fixed embryos and animal halves. (A-D) Differential interference contrast images of embryos and animal halves; (A′-D′) the respective indirect immunofluorescence images. (A,A′) 3 day embryo showing Ecto V localized to the oral ectoderm. (B,B′) Untreated animal half after 3 days of culture. Ecto V is expressed globally. The arrowheads mark the boundary of the putative oral and aboral regions. (C,C′) Animal half treated with 10 mM LiCl at 7 hours postfertilization. Ecto V is only expressed in the thick epithelium. (D,D′) Animal half treated with 25 mM LiCl at 16 hours postfertilization. Ecto V expression is global as in the untreated animal half (B,B′). Bar, 50 μm.

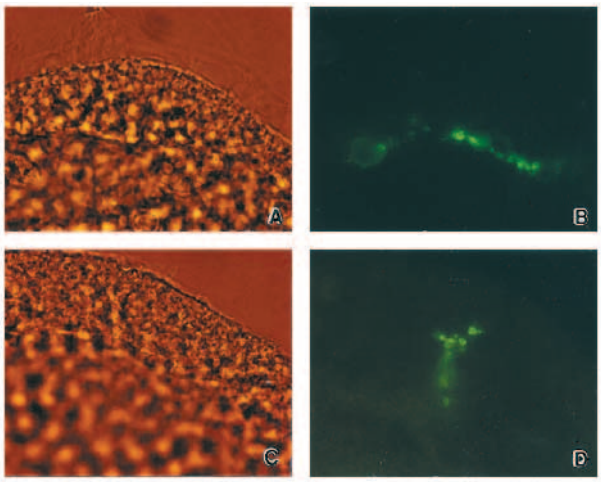

Fig. 4. Localization of serotonin expressing cells in *L. pictus* embryos and animal halves. (A,C) Bright-field images; (B,D) indirect immunofluorescence images. (A,B) A 3-day pluteus-stage embryo stained with an anti-serotonin antibody; (C,D) an untreated animal half stained with the anti-serotonin antibody.

thickened epithelium of untreated animal halves (Fig. 4C,D), but no staining was seen in any cells in the squamous epithelium.

The mAb UH295 recognizes an epitope in the ciliary band, which is a feeding structure that includes neurons (Fig. 5A, arrows). We found that this antibody also stained other embryonic neuron-like cells (Fig. 5A, arrowheads). When we probed untreated animal halves with mAb UH295, the antibody stained many neuron-like cells with long processes emanating from the thickened epithelium, whereas there was no staining in the squamous epithelium (Fig. 5B). This indicated that only the cells in the thickened epithelium were competent to form these neuron-like cells. The observation that a smaller number of cells in the animal halves stained for serotonin than with mAb UH295 indicates that more than one neuron type forms in animal halves. We have been unable to determine what type of neurons these cells may represent. These experiments demonstrate that oral ectoderm differentiation occurs in isolated animal halves and support the hypothesis that oral ectoderm differentiation occurs autonomously in *L. pictus* animal halves.

Since the oral marker was expressed in the thickened epithelium of animal halves that morphologically polarized after 10 mM LiCl treatment, we asked if the aboral ectoderm-specific LpS1 protein was selectively expressed by the squamous epithelium in the same animal halves. The Spec 1 antibody, which cross-reacts with the LpS1 protein (Xiang et al., 1988), showed specific staining in the aboral ectoderm but not in the oral ectoderm or the gut (Fig. 6A). Thus, we used this antibody to assess spatial expression of the LpS1 protein in animal halves. Animal halves treated with 10 mM LiCl at 7 hours PF showed LpS1 staining in the squamous epithelium, but not in the thickened epithelium of the embryoids (Fig. 6B). Interestingly, in animal halves that were treated with 25 mM LiCl at 16 hours PF, there was also polarized expression of the LpS1 protein in the squamous epithelium (Fig. 6C). Thus, in those embryoids that expressed the oral marker Ecto V globally (Fig. 3B,B′), a subset of cells expressed markers for both oral and aboral ectoderm.

In undisturbed embryos, the ciliary band forms at the border between the oral and aboral ectoderm. We asked if the ciliary band formed in control and lithium-treated animal halves. The mAb UH295 labeled the ciliary band in pluteus-stage *L. pictus* embryos (Fig. 7A). In 10 mM LiCl-treated animal halves, this antibody labeled the ciliary band at the border between the thickened oral ectoderm region and the squamous aboral ectoderm region (Fig. 7B). This result confirmed the morphological observation that a ciliary band had formed in these animal halves. Thus, after 10 mM LiCl treatment at 7 hours PF, *L. pictus* animal halves were patterned along the oral-aboral axis, with an oral epithelium composed of most structures normally derived from the oral ectoderm separated from the squamous aboral epithelium by the ciliary band. No ciliary band formed in untreated animal halves or animal halves treated with 25 mM LiCl as assessed morphologically (Fig. 1B,C) and by using mAb UH295 (data not shown). These results lend strong support to the interpretation of Wikramanayake et al. (1995) that the polarization seen in animal halves is along the oral-aboral axis rather than along the animal-vegetal axis.

DISCUSSION

While the role of micromere signaling in the specification of the vegetal plate territory has served as a paradigm for inductive events in the sea urchin embryo, much less is known about the role of vegetal signaling in specification of the animal pole-derived ectodermal territories or the role of signaling in the polarization of the oral-aboral axis. Our results provide some insight into the signaling mechanisms operating in the early embryo that specify the ectoderm territories and pattern the oral-aboral axis.

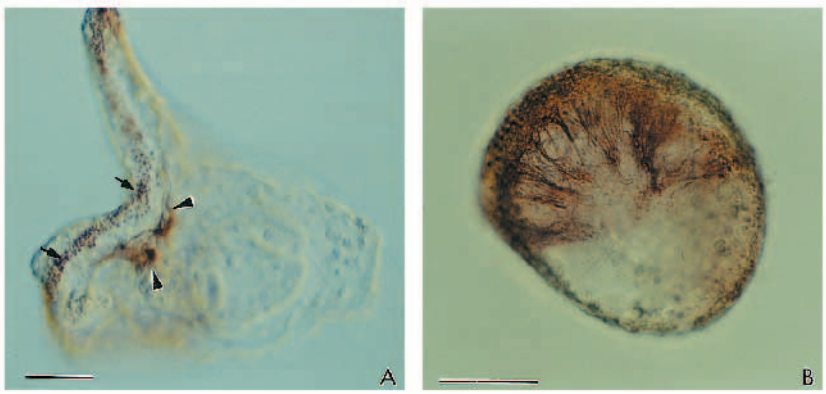

Fig. 5. Localization of neuron-like cells in *L. pictus* embryos and untreated animal halves. (A) A 3-day-old embryo stained with the mAb UH295. Staining is seen in the ciliary band (arrows) as well as a cluster of neuron-like cells (arrowheads). (B) A 3-day-old untreated animal half stained with the mAb UH295. The antibody stains numerous neuron-like cells that only form in the thickened epithelium of untreated animal halves. Bar, 50 μm.

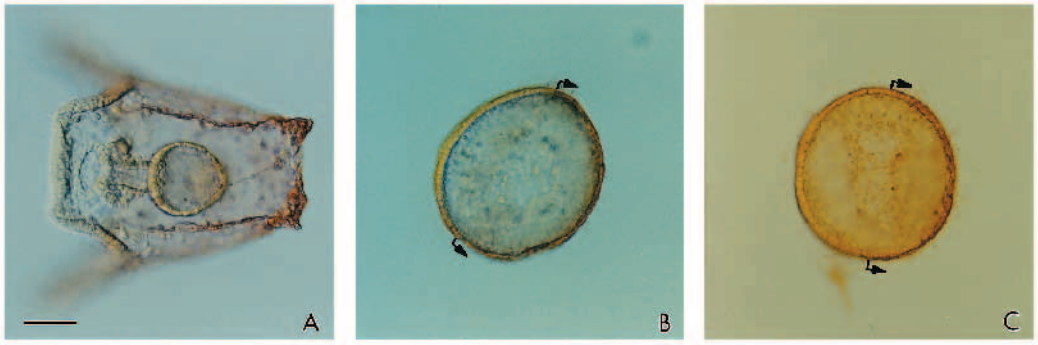

Fig. 6. Localization of LpS1 protein in 3-day *L. pictus* embryos and animal halves. LpS1 was localized on glutaraldehyde-fixed embryos and animal halves using immunohistochemistry. (A) 3-day embryo stained with the LpS1 antibody. (B) 3-day animal half treated with 10 mM LiCl at 7 hours postfertilization stained with the LpS1 antibody. (C) 3-day animal half treated with 25 mM LiCl at 16 hours postfertilization stained with the LpS1 antibody. Staining in B and C is seen in the squamous epithelium indicated by the bent arrows. Bar, 50 μm.

Oral ectoderm specification in the absence of vegetal signaling

Several lines of evidence indicated that the oral ectoderm territory is autonomously specified in *L. pictus* embryos. Animal halves isolated from 8-cell *L. pictus* embryos expressed Ecto V, a marker for oral ectoderm, but did not express the aboral ectoderm-specific markers LpS1 and LpC2. These untreated animal halves also produced serotonergic cells, which are known to be derived from the oral ectoderm territory (Bisgrove and Burke, 1987). Most neurons in the sea urchin embryo originate from the oral ectoderm territory, and untreated animal halves also formed numerous neuron-like cells that were localized to the thickened epithelium. However, untreated animal halves did not form oral ectoderm-derived structures such as the stomodeum and also did not form the ciliary band which is partly derived from the oral ectoderm territory (Cameron et al., 1993). Based on these observations, we suggest that the oral ectoderm is autonomously specified and produces at least one terminally differentiated cell type derived from this territory. Vegetal signaling may be necessary for the induction of the stomodeum and the ciliary band. Consistent with this idea is the observation of Horstadius (1939) that when animal halves are recombined with the veg1 tier the stomodeum and ciliary band are sometimes induced.

In our experiments, we observed that expression of the oral ectoderm marker Ecto V was ubiquitous in untreated *L. pictus* animal halves. Expression of this marker, however, was restricted to the thickened epithelium when animal halves were patterned along the oral-aboral axis by the 10 mM lithium treatment. Restriction of this marker to the thickened epithelium occurred concomitant with the induction of oral-specific structures such as the stomodeum and a distinct apical plate in this epithelium. Coffman and McClay (1990) showed that the Ecto V antigen was not found on the surface of unfertilized eggs and appeared on the egg surface following fertilization, presumably from stored maternal protein sources. At the blastula stage, the maternal protein is barely detectable by immunofluorescence (Coffman and McClay, 1990. Wikramanayake et al., 1995). Zygotic synthesis begins at around the mesenchyme blastula stage and, by the prism stage, strong expression of the antigen is seen in the oral ectoderm (Coffman and McClay, 1990). Therefore, the high level of expression seen on all cells of untreated animal halves, as detected by immunofluorescence, is most likely due to zygotic synthesis of the antigen. Additionally, the Ecto V expression that we observed is not likely due to maternal protein because during isolation of animal halves 8-cell embryos are washed with hyaline extraction medium which removes the hyaline layer on the embryo surface. This will also remove the maternal Ecto V antigen since it closely associates with the hyaline layer (Coffman and McClay, 1990). In a previous study, Livingston and Wilt (1990) noted that induction of archenterons in isolated *L. pictus* mesomeres using lithium led to the suppression of Ecto V expression in the region around the induced gut. Based on their studies Livingston and Wilt (1990) concluded that normal Ecto V expression depended on multiple signals and that the signals necessary for the correct spatial restriction of Ecto V was lacking in isolated mesomeres. We favor the possibility that misexpression of the Ecto V antigen in the squamous cells is due to the lack of a suppressive signal from the vegetal blastomeres and suggest that oral ectoderm differentiation occurs in a stepwise manner. In this view, oral ectoderm differentiation may have an autonomous as well as a non-autonomous component. The autonomous component would consist of localized maternal determinants that are somehow localized to the prospective oral ectoderm, and the non-autonomous component would consist of the restriction of expression of other oral-specific genes, which initially have the potential to be active in all mesomeres, to the oral ectoderm by vegetal cell interactions. Thus, the squamous epithelium in untreated animal halves may not be a true oral ectoderm but rather prospective aboral ectoderm that aberrantly expresses oral ectoderm genes due to the lack of vegetal signaling.

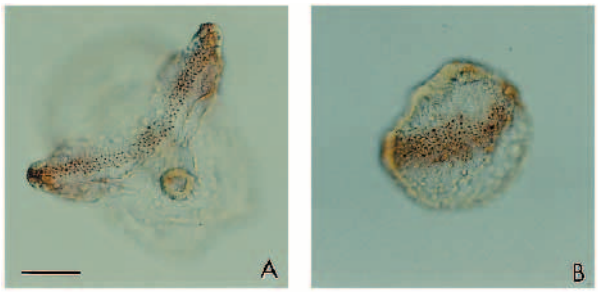

Fig. 7. Localization of the ciliary band in *L. pictus* embryos and animal halves. (A) 3-day embryo and (B) animal half treated with 10 mM LiCl at 7 hours PF stained with the mAb UH295. Bar, 50 μm.

Aboral ectoderm specification requires vegetal signaling

Previously, we showed that specification of aboral ectoderm was conditional in animal halves made from 8-cell *L. pictus* embryos (Wikramanayake et al., 1995). Aboral ectoderm-specific genes were activated by replacing vegetal blastomeres or by adding 25 mM LiCl to animal halves, which also induced endoderm in these explants. In the current study, we show that prior endoderm induction is not required for lithium activation of aboral ectoderm-specific genes. This result was unexpected since many studies have shown that applying lithium to cleavage-stage embryos suppresses development of the ectoderm lineages (Horstadius, 1973; Ghiglione et al., 1993). Clearly, lithium treatment of isolated mesomeres under appropriate conditions has a powerful morphogenetic effect on patterning ectoderm. It is interesting that at lithium concentrations sufficient for animal halves to begin to form small archenterons, these always formed at the boundary between the thickened oral ectoderm and the squamous aboral ectoderm opposite to the apical tuft (A. Wikramanayake, unpublished observations). Since the apical tuft forms the animal-most part of the embryo, this observation suggested that lithium had a differential effect on the vegetal-most cytoplasm of the mesomeres. A differential effect of lithium on the vegetal-most cytoplasm of animal halves was also shown by Horstadius (1936). When Horstadius removed the cells inheriting the vegetal-most cytoplasm of a lithium-treated animal half and recombined them with an untreated animal half, the untreated animal half gave rise to a pluteus larva. The cells inheriting the animal-most cytoplasm of a lithium-treated animal half recombined with an untreated animal half had no effect (Horstadius, 1936). Due to the threshold effects of lithium on induction of more vegetal structures in mesomeres (Fig. 2; Livingston and Wilt, 1990), it is possible that the oral-aboral patterning that we observed when animal halves were treated with 10 mM LiCl was due to the induction of a veg1 equivalent cell type from an1/an2 cells. In the current study, delaying lithium addition resulted in no endoderm induction, the activation of aboral ectoderm genes and the global expression of the oral marker Ecto V, suggesting that the signal to activate the aboral ectoderm was different from the signal to restrict Ecto V expression.

Surprisingly, the spatial pattern of LpS1 gene expression was similar in animal halves treated with 25 mM LiCl at 16 hours and with 10 mM lithium at 7 hours PF. In both cases, the LpS1 protein was expressed in a polarized manner in the squamous epithelium of the embryoids. When animal halves are treated with lithium, it is likely that the ion enters all mesomeres non selectively. It is known that lithium inhibits IP_3-dependent signal transduction pathways in cells (Berridge et al, 1989). However, it has also been shown that lithium can stimulate protein kinase C activity (Nishizuka, 1986), and lithium and TPA, a PKC stimulator, have additive effects on sea urchin embryos (Livingston and Wilt, 1992). Recently, Klein and Melton (1996) showed that the dorsalization of *Xenopus laevis* embryos by lithium is not mediated by inhibition of the IP_3-dependent pathways, but rather, by inhibition of the activity of GSK-3β. Regardless of the signaling mechanisms affected by the lithium treatment in sea urchin embryos, it is clear that the LpS1 gene is silent in the oral ectoderm. It is possible, therefore, that the prospective oral region inherits maternal components that makes it refractory to the vegetal signals to activate the aboral ectoderm genes. Our data clearly demonstrate that the mesomeres are not an equivalence group based on the morphological asymmetry generated in animal halves and the observations that only the thickened epithelium produces serotonergic cells and neuron-like cells, while only the squamous epithelium expresses the LpS1 gene.

Polarization of the oral-aboral axis

Our results suggest that multiple signaling events control the polarization and patterning of the oral-aboral axis in the sea urchin embryo. It is known that combinatorial signaling is necessary for establishing the embryonic axes in vertebrates as well (Cornell and Kimelman, 1994). A possible model for specifying the ectoderm and patterning the oral-aboral axis is shown in Fig. 8. During embryogenesis, a signaling cascade initiated by the micromeres specifies the vegetal plate (Davidson, 1989; Ransick and Davidson, 1995). Signaling from the vegetal plate may induce the veg1 tier to signal to the animal pole derived tiers as envisaged by Davidson (1989). A positive inductive signal may activate aboral ectoderm-specific genes, while a negative suppressive signal inactivates the Ecto V gene in the prospective aboral ectoderm territory. Prospective oral ectoderm cells may autonomously produce the neuron-like cells and the serotonergic cells due to inherited maternal determinants. These same determinants may make these cells refractory to the signaling from the vegetal blastomeres that suppresses oral differentiation and may also make these cells refractory to signals that activate aboral ectoderm-specific gene expression. Evidence supporting an early segregation of oral blastomeres comes from classical studies, which showed that, by the 16-cell stage, the future oral blastomeres already manifest metabolic differences from the future aboral blastomeres (Child, 1936; Czihak, 1963).

It has been suggested that isolated 2-cell and 4-cell blaso-

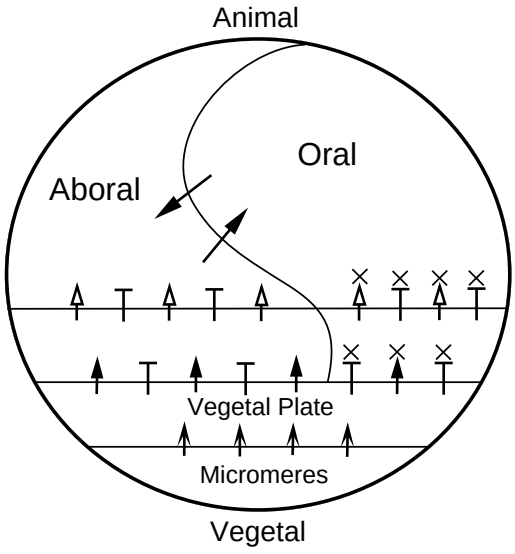

Fig. 8. Model for specification and patterning of ectoderm in sea urchins. The arrows represent positive inductive interactions, while the bars represent suppressive interactions. The Xs over the arrows and bars represent components in the oral ectoderm that make this territory refractory to vegetal signals. See text for details.

tomeres from sea urchin embryos can generate whole embryos. This would contradict our model for an autonomous specification of oral ectoderm through inherited determinants. However, as Wilt (1987) has pointed out, blastomeres isolated from the same embryo have not been carefully examined for normal development. Furthermore, twins derived from a 2-cell embryo will often have defects in development (Marcus, 1979).

This work was supported by NIH grant (HD22619) and a Robert A. Welch grant (G-1210) to W. H. K. We thank Jay Vivian for helpful discussions on the RT-PCR procedures. We also thank Dr Gary Wessel, Brown University, and Dr David McClay, Duke University, for gifts of antibodies and Dr Bruce Brandhorst, Simon Fraser University, for critical reading of the manuscript.

REFERENCES

Berridge, M. J., Downes, C. P. and Hanley, M. R. (1989). Neural and developmental actions of lithium: A unifying hypothesis. *Cell* **59**, 411-419.

Bisgrove, B. W and Burke, R. D. (1987). Development of the nervous system of the pluteus larva of *Strongylocentrotus drobachiensis*. *Cell Tiss. Res.* **248,** 335-343.

Cameron, R. A., Hough-Evans, B. R., Britten, R. J. and Davidson, E. H. (1987). Lineage and fate of each blastomere of the eight-cell sea urchin embryo. *Genes Dev*. **1**, 75-84.

Cameron, R. A. and Davidson, E. H. (1991). Cell type specification during sea urchin development. *Trends Genetics* **7**, 212-218.

Cameron, R. A., Britten, R. J. and Davidson, E. H. (1993). The embryonic ciliated band of the sea urchin, *Strongylocentrotus purpuratus* derives from both oral and aboral ectoderm. *Dev. Biol.* **160**, 369-376.

Child, C. M. (1936). Differential reduction of vital dyes in the early development of echinoderms. *Wilhelm Roux' Arch. EntwMech. Org*. **135**, 426-456.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156-159.

Coffman, J. A. and McClay, D. R. (1990). A hyaline layer protein that becomes localized to the oral ectoderm and foregut of sea urchin embryos. *Dev. Biol.* **140**, 93-104.

Cornell, R. A. and Kimelman, D. (1994). Combinatorial signaling in development. *BioEssays* **16,** 577-581.

Czihak, G. (1963). Entwicklungsphysiologische untersuchungen an echiniden (verteilung und be deutung der cytochromeoxydase). *Wilhelm Roux' Arch EntwMech. Org.* **154**, 272-292.

Davidson, E. H. (1986). *Gene Activity in Early Development.* Third edition. New York: Academic Press, Inc.

Davidson, E. H. (1989). Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: A proposed mechanism. *Development* **105**, 421-445.

Ghiglione, C., Lhomond, G., Lepage, T. and Gashe, C. (1993). Cell-autonomous expression and position-dependent repression by Li^+ of two zygotic genes during sea urchin early development. *EMBO J*. **12**, 87-96.

Horstadius, S. (1936). Weitere studien uber die determination im verlaufe der eiachse bei seeigeln. *Wilhelm Roux' Arch. EntwMech. Org.* **135**, 40-68.

Horstadius, S. (1939). The mechanisms of sea urchin development studied by operative methods. *Biol. Rev. Cambridge Phil. Soc*. **14,** 132-179.

Horstadius, S. (1973). *Experimental Embryology of Echinoderms.* Oxford: Clarendon Press.

Klein, P. S. and Melton, D. A. (1996). A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. USA* **93,** 8455-8459.

Livingston, B. T. and Wilt, F. H. (1989). Lithium evokes expression of vegetal specific molecules in the animal blastomeres of sea urchin embryos. *Proc. Natl. Acad. Sci. USA* **86**, 3669-3673.

Livingston, B. T. and Wilt, F. H. (1990). Range and stability of cell fate determination in isolated sea urchin blastomeres. *Development* **108,** 403-410.

Livingston, B. T. and Wilt, F. H. (1992). Phorbol esters alter cell fate during development of sea urchin embryos. *J. Cell Biol.* **119,** 1641-1648.

Malinda, K. M., Fisher, G. W. and Ettensohn, C. A. (1995). Four-dimensional microscopic analysis of the filopodial behavior of primary mesenchyme cells during gastrulation in the sea urchin embryo. *Dev. Biol*. **172**, 552-566.

Marcus, N. H. (1979). Developmental aberrations associated with twinning in laboratory-reared sea urchins. *Dev. Biol.* **70,** 274-277.

Nishida, H. and Satoh, N. (1989). Determination and regulation in the pigment cell lineage of the ascidian embryo. *Dev. Biol.* **132**, 355-367.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science* **233,** 305-316.

Priess, R. A. and Thomson, J. N. (1987). Cellular interactions in early *C. elegans* embryos. *Cell* **48**, 241-250.

Ransick, A. and Davidson, E. H. (1995). Micromeres are required for normal vegetal plate specification in sea urchin embryos. *Development* **121,** 3215-3222.

Slack, J. M. W. (1991). *From Egg to Embryo. Regional Specification in Early Development*. Cambridge: Cambridge Univ. Press.

Sternberg, P. W. and Horvitz, H. R. (1986). Pattern formation during vulval development in *C. elegans. Cell* **44**, 761-772.

Wessel, G. M. and McClay, D. R. (1985). Sequential expression of germ layer specific molecules in the sea urchin embryo. *Dev. Biol*. **111**, 451-463.

Wikramanayake, A. H., Brandhorst, B. P. and Klein, W. H. (1995). Autonomous and non-autonomous differentiation of ectoderm in different sea urchin species. *Development* **121,** 1497-1505.

Wilt, F. H. (1987). Determination and morphogenesis in the sea urchin embryo. *Development* **100,** 559-575.

Xiang, M., Bedard, P., Wessel, G., Filon, M., Brandhorst, B. and Klein, W. H. (1988). Tandem duplication and divergence of a sea urchin protein belonging to the troponin C superfamily. *J. Biol. Chem.* **263,** 17173-17180.

(Accepted 23 September 1996)