Developmental Biology **247,** 335–350 (2002)
doi:10.1006/dbio.2002.0704

# Pattern Formation in a Pentameral Animal: Induction of Early Adult Rudiment Development in Sea Urchins

**Sharon B. Minsuk**[1] **and Rudolf A. Raff**

*Department of Biology and Indiana Molecular Biology Institute, Indiana University, Bloomington, Indiana 47405*

**We investigated adult rudiment induction in the direct-developing sea urchin *Heliocidaris erythrogramma* microsurgically. After removal of the archenteron (which includes presumptive coelomic mesoderm as well as presumptive endoderm) from late gastrulae, larval ectoderm develops properly but obvious rudiments (tube feet, nervous system, and adult skeleton) fail to form, indicating that coelomic mesoderm, endoderm, or both are required for induction of adult development. Recombination of ectoderm and archenteron rescues development. Implanted endoderm alone or left coelom alone each regenerate the full complement of archenteron derivatives; thus, they are uninformative as to the relative inductive potential of the two regions. However, in isolated ectoderm, more limited regeneration gives rise to larvae containing no archenteron derivatives at all, endoderm only, or both endoderm and left coelom. Adult nervous system begins to develop only in the latter, indicating that left coelom is required for the inductive signal. Isolated ectoderm develops a vestibule (the precursor of adult ectoderm) and correctly regulates vestibular expression of the ectodermal territory marker *HeET-1*, indicating that the early phase of vestibule development occurs autonomously; only later development requires the inductive signal. Another ectodermal marker, *HeARS*, is regulated properly in the larval ectoderm region, but not in the vestibule. *HeARS* regulation thus represents an early response to the inducing signal. We compare *HeARS* expression in *H. erythrogramma* with that in indirect developers and discuss its implications for modularity in the evolution of developmental mode.** 

***Key Words:*** **induction; signaling; mesoderm; coelom; hydrocoel; vestibule; rudiment; modularity; skeletal patterning.**

## INTRODUCTION

Adult echinoderms, which possess a pentaradial symmetry, have a completely different organization than their larvae, which are bilaterally symmetric. This pentameral body plan arises in the adult rudiment (precursor of the postmetamorphic juvenile adult) as it develops within the larval body. Echinoderms evolved from a bilaterally symmetric deuterostome ancestor, in common with hemichordates and chordates (Turbeville *et al.*, 1994; Bromham and Degnan, 1999). We wish to know how ancestral deuterostome developmental mechanisms of pattern formation and morphogenesis have evolved to give rise to the novel radial body plan of adult echinoderms. But before we can meaningfully ask such questions, we must broaden our understanding of developmental mechanisms beyond the well-studied vertebrates, to the adult development of other deuterostome phyla. In this study, we pursue this goal by investigating the role of inductive processes in the development of the adult sea urchin.

Inductive signaling is critical in the development of all metazoans. It defines the boundaries of regional identities (e.g., induction of the floorplate of the vertebrate neural tube by the notochord; Tanabe and Jessell, 1996) and coordinates the development of organ components spatially and temporally (e.g., the retina, lens, and cornea of the vertebrate eye; Hay, 1980; Saha *et al.*, 1989). Induction constrains evolution by providing an epigenetic link between developmental stages of increasing complexity, and defines the nature of the phylotypic stage (Raff, 1996). A great deal is known about inductive signaling in the bilateral embryos and pluteus larvae of sea urchins (Hörstadius, 1973; Czihak, 1965; Ettensohn and McClay, 1986; Ransick and Davidson, 1993, 1995; Guss and Ettensohn, 1997;

[1] To whom correspondence should be addressed. Fax: (812) 855-6082. E-mail: sminsuk@bio.indiana.edu.

0012-1606/02 $35.00

Wikramanayake and Klein, 1997; Wikramanayake *et al.*, 1998; Sherwood and McClay, 1999, 2001; Angerer and Angerer, 2000; Angerer *et al.*, 2000; McClay *et al.*, 2000; Vonica *et al.*, 2000). However, less is known about development of the pentameral adult due to the difficulty of studying the small, inaccessible rudiments of typical planktotrophic echinoderm larvae, which develop slowly and are difficult to raise.

The key initial event in the establishment of the adult rudiment within the pluteus larva is the interaction between the vestibule and the hydrocoel, a partition of the mesodermal left coelom (Figs. 1A–1C). Vestibular ectoderm invaginates from the oral ectoderm of the pluteus and comes to lie apposed to the hydrocoel, forming the bilayered rudiment. Adult structures (including the central nerve ring, tube feet, and spines) develop subsequently from these layers (Okazaki, 1975).

Although rudiment morphology is well described (MacBride, 1903; von Ubisch, 1913), only a few studies have addressed developmental mechanisms. The earliest studies focused on cases of ectopic placement of vestibule and/or hydrocoel that occurred spontaneously, or that resulted from experimental starvation. Czihak (1965) performed UV ablations of left coelom, hydrocoel, and vestibule. These studies (reviewed in Hörstadius, 1973) concluded that the initial formation of the vestibule as well as its positioning on the left side occur independently of left coelom or hydrocoel. A second phase of vestibule invagination, in which the vestibule orients toward and approaches the hydrocoel, requires induction by hydrocoel. Likewise, hydrocoel is required for the development of adult structures in the vestibular ectoderm. Development of adult ectodermal structures, including neural development, therefore appears to be induced by signals from the mesoderm, as in vertebrates. An understanding of the echinoderm body plan, its development within a bilaterally symmetric larva, and its evolution from bilaterally symmetric ancestors requires a more detailed characterization of inductive events in rudiment development.

The sea urchin *Heliocidaris erythrogramma* is a direct developer, forming the adult body without generating a feeding pluteus larva. It develops a left coelom in under a day, and a vestibule about 10 h later. Despite a divergent larval form and associated differences in the origin of the vestibule and coeloms, the anatomy and subsequent development of the rudiment from the time the vestibule and hydrocoel come into contact is remarkably similar to that in indirect-developing species (Williams and Anderson, 1975; Haag and Raff, 1998; Ferkowicz and Raff, 2001), suggesting that *H. erythrogramma* rudiment development is representative of echinoids. Its egg is large (430 $\mu$m diameter), and the rudiment represents a large proportion of the larva from the earliest stages, facilitating microsurgical approaches.

The archenteron of *H. erythrogramma* never forms a mouth as in a pluteus, but instead forms a large left coelom at its tip that quickly extends back toward the vegetal pole (Figs. 1D and 1E). A smaller right coelom develops some-

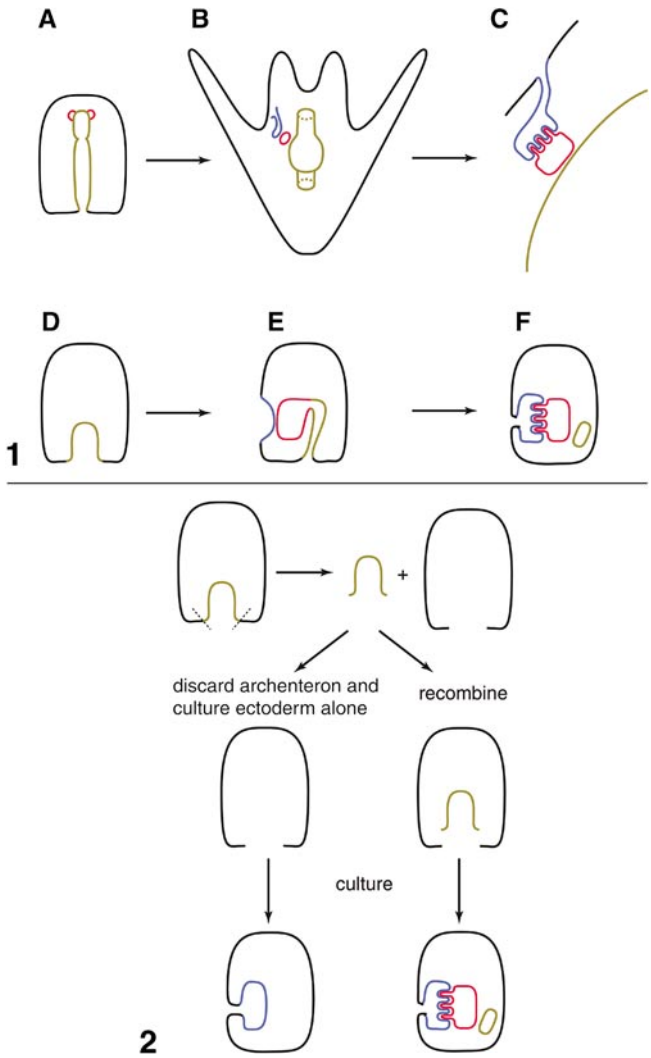



**FIG. 1.** Simplified schematic of rudiment development in indirect-developing sea urchins (A–C) and in *H. erythrogramma* (D–F). Vestibular ectoderm is blue; coeloms and hydrocoel (a later derivative of the left coelom) are red; archenteron/gut is yellow. (A) At the early prism stage, the left and right coeloms form at the tip of the differentiating gut. (B) In a pluteus larva, the hydrocoel lies against the left side of the stomach. (The other coelomic derivatives have been omitted.) The vestibule invaginates from the oral ectoderm and comes to lie next to the hydrocoel. (C) A magnified view of the developing rudiment, showing tube feet developing where the vestibule and hydrocoel interact. (D) In *H. erythrogramma*, the archenteron invaginates only part way. (E) Archenteron gives rise to the left coelom (and a smaller right coelom which has been omitted). The vestibule invaginates from the larval ectoderm. (F) The hydrocoel separates from the nonfunctional gut, and interacts with the vestibule to form the rudiment.

**FIG. 2.** Dissection of *H. erythrogramma* gastrulae, separating archenterons from ectodermal shells. Dashed lines indicate the cut around the base of the archenteron. Shells cultured alone develop into ecto-larvae; recombinants rescue normal rudiment development. Colors as in Fig. 1.

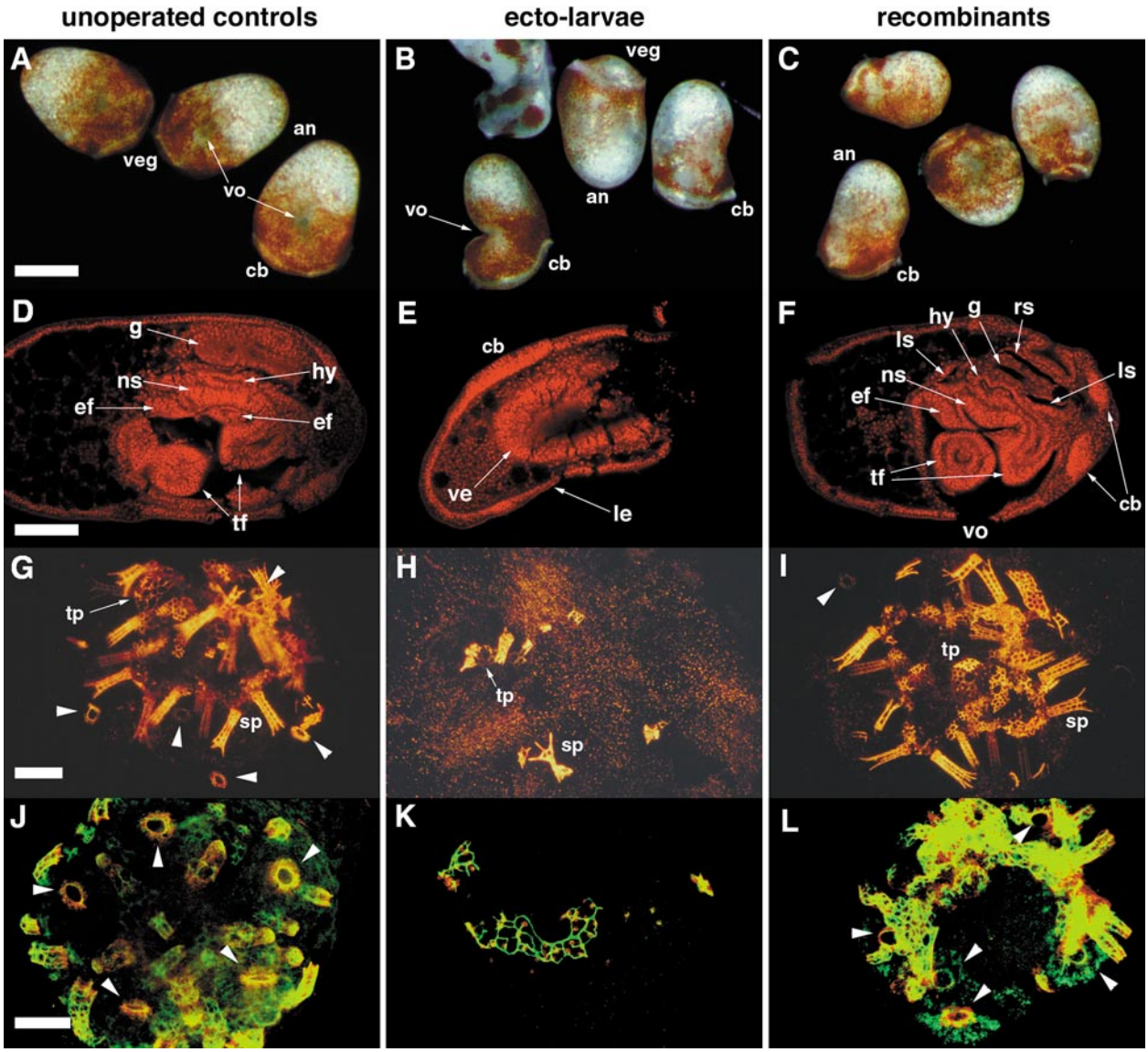



**FIG. 3.** Unoperated larvae (left hand column), ecto-larvae (middle column), and recombinants (right hand column). (A–C) Live larvae, 5 days. The pigment abnormality in the upper-left ecto-larva in (B) occurred at low frequency under laboratory conditions in both ecto-larvae and normal embryos. (D–F) Confocal images of 41-h larvae, nuclei labeled with propidium iodide. (D) Normal larva with a well developed rudiment. (E) Ecto-larva with vestibular ectoderm, but no adult structures. (F) Recombinant showing all the normal adult organs, including tube feet and developing nervous system (the thickened radial nerve and the epineural folds). (G–I) Live larvae, one from each panel (A–C), viewed under polarized light. Flattening of specimens due to cover slip distorts the pentameral pattern somewhat, but the five tube foot skeletal rings (arrow heads) can still be seen in (G); the overall skeletal arrangement in (I) is similar. (J–L) MSP130 (red) and PM27 (green) in 3.5-day embryos. Overlap of the two markers is yellow. The five tube foot skeletal rings in (J) and (L) are indicated by arrow heads. an, animal pole; cb, ciliated bands; ef, epineural folds; g, gut; hy, hydrocoel; le, larval ectoderm; ls, left somatocoel; ns, nervous system; rs, right somatocoel; sp, spine; tf, tube foot; tp, test plate; ve, vestibular ectoderm; veg, vegetal pole; vo, vestibular opening. Bars, each referring to the entire row: (A–C) 250 $\mu$m; (D–L) 100 $\mu$m.

what later. The vestibule forms from a large patch of ectoderm on the left side of the larva, apposed to the left coelom (Williams and Anderson, 1975; Ferkowicz and Raff, 2001; Haag and Raff, 1998). Together, the two tissues develop adult structures as in indirect-developing urchins (Fig. 1F). Tube feet form from pentamerally arranged hydrocoel lobes combined with an external covering of vestibular ectoderm. Epineural folds arise from ectoderm between the

**TABLE 1**
Coelo- and Endo-Recombinants[a]

| | Unoperated controls | Coelo-recombinants | Endo-recombinants | Ecto-larvae |
|---|---|---|---|---|
| *n* (number sectioned) | 22 | 14 | 11 | 20 |
| Rudiment structures: | | | | |
| tube feet | 22 (100%) | 14 (100) | 10 (91) | 2 (10) |
| mean # of tube feet[b] | 5.0 | 4.0 | 3.7 | 1.5 |
| CNS | 22 (100) | 14 (100) | 10 (91) | 7 (35) |
| Archenteron derivatives: | | | | |
| coelomic mesoderm | 22 (100) | 14 (100) | 11 (100) | 10 (50) |
| gut | 22 (100) | 13 (93) | 11 (100) | 18 (90) |
| relative amount of gut[c] | +++ | ++ | +++ | + |

[a] All rows refer to the number (and percentage) of sectioned larvae scoring positive for each tissue type, except where noted.
[b] Mean number per positive scoring larva.
[c] Relative amount per positive scoring larva.

tube feet and fuse, internalizing the presumptive central nervous system (CNS). Large numbers of mesenchyme cells produce the adult skeleton.

There is some evidence that *H. erythrogramma* exhibits mesoderm–ectoderm signaling, consistent with the reports from other sea urchin species. In *H. erythrogramma* embryos bisected along the third cleavage plane at the eight-cell stage, yielding animal and vegetal half embryos (Henry and Raff, 1990), the animal half embryos developed morphologically normal vestibules. In most cases, this occurred in the absence of endoderm and coeloms, indicating autonomy of vestibule development. Tube feet developed only when a coelom was present, suggesting their dependence on a signaling event.

In this study, we used microsurgical experiments to investigate the behavior of *H. erythrogramma* ectoderm in the presence and absence of the archenteron and its separate coelomic and endodermal components. Our results demonstrate that the vestibule arises autonomously, but that further development of vestibule-derived structures requires inductive signals from the left coelom. Development of larval ectoderm is not affected by archenteron removal, indicating that larval and vestibular development are independent developmental modules. We further characterized the expression patterns of *HeET-1* (encoding apextrin, an integral membrane protein expressed at the apical cell surface; Haag and Raff, 1998; Haag *et al.*, 1999) and *HeARS* (arylsulfatase; Haag and Raff, 1998), defining them as markers of complementary ectodermal territories in both intact and surgically modified embryos from the beginning of vestibule development. We also showed that *HeARS* expression demonstrates a downstream molecular response to the inductive signal. In addition, the normal spatiotemporal pattern of *HeARS* expression provides suggestive clues to the cellular mechanisms underlying ectodermal morphogenesis in the formation of tube feet and nervous system.

## MATERIALS AND METHODS

### *Embryos and Microsurgery*

Gametes of *H. erythrogramma* were obtained and fertilized, and embryos cultured, as previously described (Wray and Raff, 1989). Because of their large lipid stores, *H. erythrogramma* gastrulae float. In initial experiments, embryos were centrifuged prior to surgery according to the methods of Emlet and Hoegh-Guldberg (1997) to remove the lipid droplets from the blastocoel, and hence eliminate floating. These embryos successfully develop through metamorphosis. However, with practice we were able to perform the surgery without this step, and operated embryos then developed with fewer abnormalities. Experimental outcomes were the same with or without centrifugation.

Gastrulae were transferred into $Ca^{2+}$-free artificial sea water (0.443 M NaCl, 10 mM KCl, 25 mM $MgCl_2$, 16.8 mM $MgSO_4$, 2.1 mM $NaHCO_3$) a few minutes prior to surgery to loosen epithelial junctions. Surgery was performed by using two eyebrow-hair tools, one to steady the embryo while the other was used as a knife. Archenterons were removed from ectodermal shells by cutting a circle around the base of the archenteron (Fig. 2). The ectoderm was then transferred

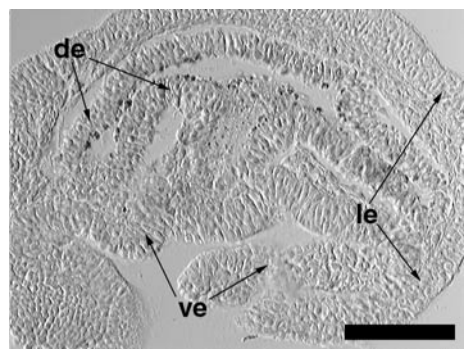



**FIG. 4.** Host ectoderm with implanted donor ectoderm (de) from another embryo. At 2 days, vestibular ectoderm (ve) has invaginated but has not developed tube feet. le, larval ectoderm. Bar, 50 $\mu$m.

back into FSW for healing and culturing. For recombinants, the ectoderm was allowed to begin healing in FSW for a few minutes before an explanted archenteron from a second embryo was placed inside it, in the correct animal–vegetal orientation. (Rotation around the animal–vegetal axis was not controlled.) Surgery was performed between 16 (midgastrula) and 24 h (late gastrula) postfertilization. After 20 h postfertilization, left coelomic pouches were present at the archenteron tips. In additional experiments, the archenteron was removed only after the left coelomic pouch was present; the coelom was then separated from the endoderm (the remaining archenteron), and the two parts were separately implanted into host ectoderms to create left-coelom-only or endoderm-only recombinants. *H. erythrogramma* gastrulae each contain about 2000 mesenchyme cells (Parks *et al.*, 1988), spread around the blastocoel surface in visible clumps. Most of these clumps stayed in place during surgery, associated with either the ectoderm or the archenteron, depending on the stage at which surgery was performed. Thus, large amounts of mesenchyme were present in cultured ectodermal shells as well as in recombinants. Unoperated embryos were raised as controls from each culture used. After culturing, embryos were fixed in 2% paraformaldehyde in FSW at 4°C overnight, then dehydrated to 70% ethanol for storage.

### *Immunocytochemistry*

For antibody visualization of skeleton and skeletogenic mesenchyme, embryos were double labeled with mouse antibody to MSP 130 (Leaf *et al.*, 1987) and rabbit antibody to PM27 (Harkey *et al.*, 1995; Stander, 1999) and visualized with rhodamine-anti-mouse and fluorescein-anti-rabbit secondaries, respectively, as previously described (Stander, 1999). Embryos were viewed using a laser scanning confocal microscope. Controls exposed to secondary but not to primary antibodies showed no fluorescence (not shown).

### *In Situ Hybridization*

*In situ* hybridization was performed by either of two methods. A method using $^{33}$P-labeled RNA probes (Angerer and Angerer, 1991) was carried out using *HeET-1* and *HeARS* cDNA as previously described (Haag and Raff, 1998).

A whole-mount, nonradioactive method was modified from that of Klingler and Gergen (1993). Probes were transcribed using digoxigenin-labeled UTP. Embryos were rehydrated, rinsed in PBT (PBS + 0.1% Tween 20), treated with 8 $\mu$g/ml proteinase K, washed in 2 mg/ml glycine, hybridized to probe at 45°C for 34 h, and washed to 0.5× SSC at 65°C. Embryos were then blocked in PBT containing 10% sheep serum and 0.1% bovine serum albumin, incubated with preabsorbed, alkaline phosphatase (AP) conjugated, anti-digoxigenin antibody at 4°C overnight, rinsed in PBT, and transferred to AP-buffer (100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 1 mM levamisole). Embryos were then incubated in the dark in AP-buffer containing the enzyme substrates NBT (nitro blue tetrazolium; 4.5 $\mu$l/ml buffer) and BCIP (5-bromo-4-chloro-3-indolyl phosphate; 3.5 $\mu$l/ml buffer). Controls performed without antibody or with sense probes indicated that no significant background was caused by endogenous alkaline phosphatases or by nonspecific probe binding (not shown). Embryos were then postfixed in 2% paraformaldehyde in PBT, dehydrated to ethanol, embedded in Paraplast, and sectioned at 10 $\mu$m without counterstaining.

### *Morphological Analysis of Surgically Altered Embryos*

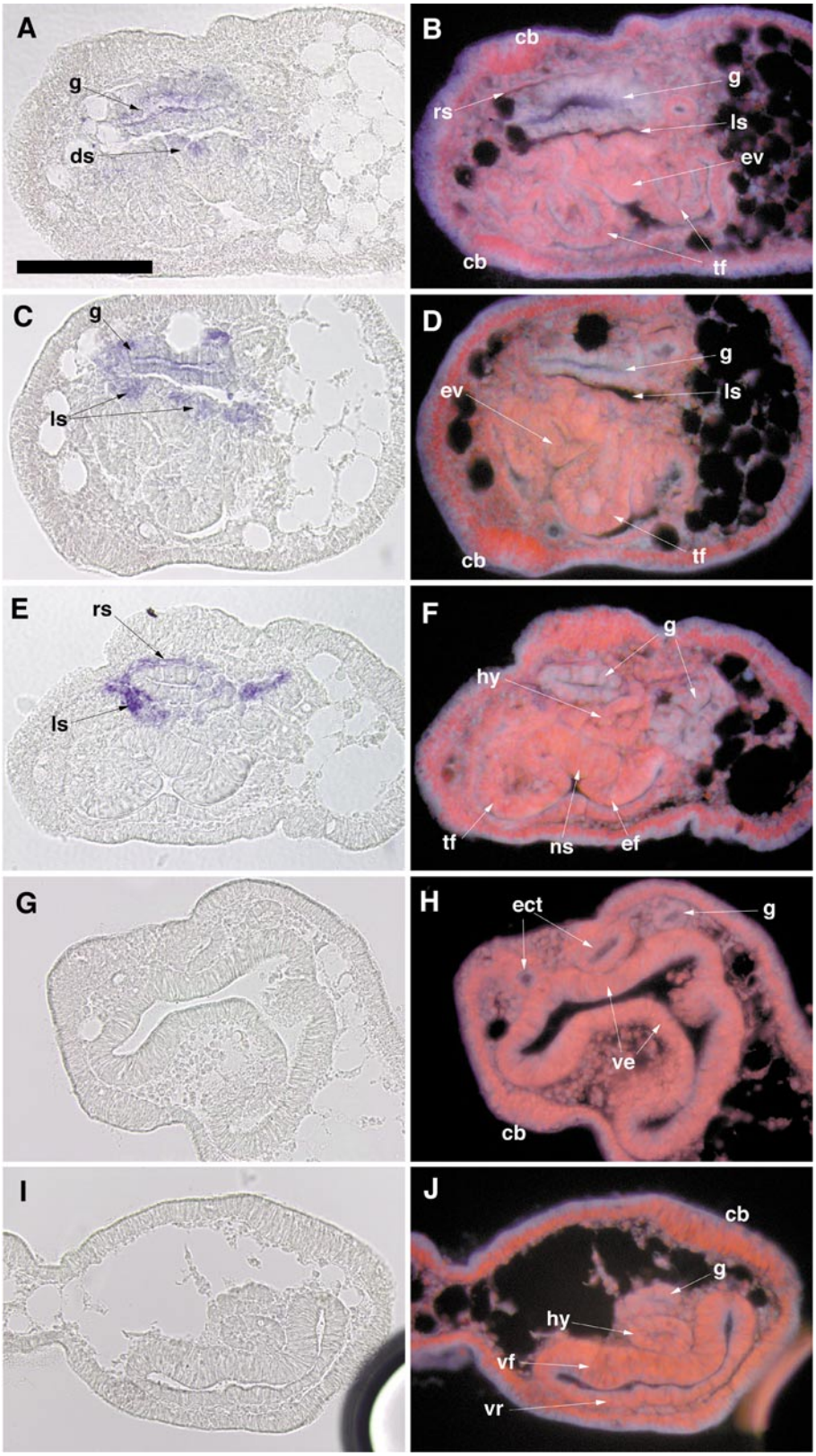
Tissue identities in altered embryos were scored morphologically (by the shape and arrangement of organs, tissues, and cells) and by histochemistry. Endogenous alkaline phosphatase detection did not specifically identify gut as expected, but was also found prevalently in somatocoel, and in much lesser amounts in hydrocoel, complementing morphological features in the identification of these structures. Fixed embryos were rehydrated and transferred to Tris-buffered saline (100 mM Tris, pH 7.4, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20), then into AP buffer (as above but with 8 mM levamisole). The AP reaction was carried out, and the embryos were embedded and sectioned, as described above (but without postfixing). Propidium iodide was used to label nuclei red, and when viewed with a DAPI instead of a rhodamine filter, cytoplasm appeared blue, thus making gut tissue definitively identifiable because its columnar cells, with large areas of nucleus-free blue cytoplasm, stood out distinctly from the more cuboidal, mostly pink cells of other internal tissues. Tube feet were identified by their double-layered structure and counted. The presence of neural ectoderm (a characteristic distinct localized thickening in the vestibule floor) was also noted, and the relative amount of gut in each specimen was assessed.

## RESULTS AND DISCUSSION

### *Archenteron Is Required for Ectodermal and Skeletal Organogenesis, but Not for Initial Development of Vestibule*

To determine whether coelomic signals are required for the development of vestibular and embryonic ectoderm, archenterons were surgically removed from mid to late gastrulae, prior to the development of vestibular ectoderm (Fig. 2; see Materials and Methods). Archenteron at this stage contains the precursors of both the gut and the coelomic mesoderm. The remaining ectodermal shells were then cultured in filtered sea water for 24 h to a few days.

Ectodermal shells healed and developed as "ecto-larvae" with normal exterior larval features. Development was sometimes slightly delayed, never by more than a couple of hours. As in normal embryos (Fig. 3A), dark red pigment cells (derived from mesenchyme) accumulated predominantly in the vegetal halves, and functioning ciliated bands developed with normal morphology and orientation (Fig. 3B). Vestibular ectoderm also developed normally in 95 of 99 cases: the ectoderm on one side of the embryo became thicker, and then a patch of tissue about one-third of the way up from the vegetal pole became flattened. This patch of tissue then turned white as pigment cells migrated away from the field, and it invaginated to form a vestibule as in a normal larva, although sometimes morphogenesis was defective, with partial or complete evagination resulting in an exterior vestibular pouch. Frequently, the invagination appeared to progress further than normal, resulting in a puckered appearance at the vestibular opening (Fig. 3B). Normal larvae usually had identifiable tube feet inside the vestibule by 2 days, but ecto-larvae developed visible tube



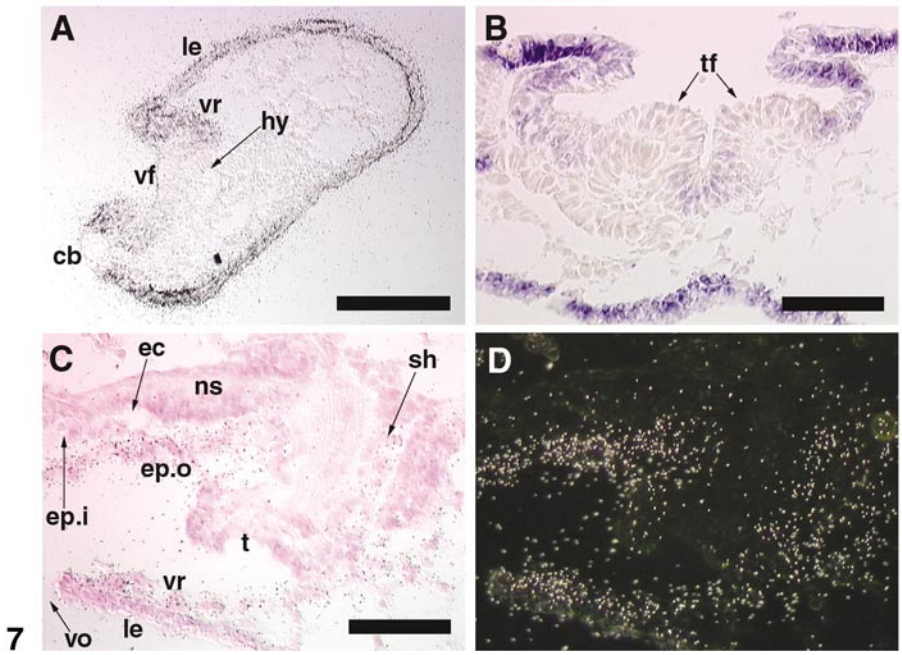




**FIG. 6.** *HeET-1* expression in early vestibule-stage embryos. (A, B) Bright field and dark field, respectively, of a $^{33}$P *in situ* hybridization at 36 h. Signal (bright grains in B) is present only in larval ectoderm (le), not in vestibular ectoderm (ve). (C) Nonradioactive (digoxigenin/alkaline phosphatase) *in situ* hybridization at the earliest formation of vestibular ectoderm. *HeET-1* signal (purple) defines the larval/vestibular boundary prior to invagination. g, gut; hy, hydrocoel; rc, right coelom. Bars, (A) 100 $\mu$m; (C) 50 $\mu$m.

**FIG. 7.** *HeARS* expression. (A) A 36-h embryo with invaginated vestibule. $^{33}$P *in situ* hybridization. Signal (dark grains) is strong enough to see in bright field in both the vestibule roof (vr) and the larval ectoderm (le), but is absent from the vestibule floor (vf) and from the ciliated bands (cb). Part of the hydrocoel (hy) can be seen apposed to the vestibule floor just where the signal is weakest. (B) A 42-h embryo with early tube foot primordia (tf). Nonradioactive *in situ* hybridization. *HeARS* signal (purple) is present in the vestibular ectoderm around and between the tube feet, but not on the tube feet themselves. (C, D) $^{33}$P *in situ*, bright field and dark field, respectively. A 4-day embryo. Signal is present in the tube foot shaft (sh) and the vestibule floor (ep.o: outer layer of the epineural veil), but not in tube foot tips (t), the inner layer of the epineural veil (ep.i), the nervous system (ns), or the larval ectoderm. ec, epineural cavity; vo, vestibular opening. Bars, (A) 100 $\mu$m; (B, C) 50 $\mu$m.

---

**FIG. 5.** Morphological analysis of surgically altered embryos (see Materials and Methods) fixed at 38–49 h: endogenous alkaline phosphatase detection (bright field, left hand column) and propidium iodide counterstaining (viewed through DAPI filter, right hand column). (A, B) Unoperated control. (C, D) Coelo-recombinant, and (E, F) endo-recombinant, both contain gut and coelomic mesoderm; both have developed tube feet and nervous system. (G, H) Class 2 ecto-larva, containing a tiny gut but no coelomic mesoderm. The vestibular ectoderm is of approximately uniform thickness all the way around. (Two additional small vesicles are also ectodermal, opening to the exterior in nearby sections.) (I, J) Class 3 ecto-larva, containing hydrocoel. The vestibule roof is thin, but the vestibule floor, apposed to the hydrocoel, is distinctly thickened and columnar, as in normal nervous system development. cb, ciliated band; ds, incipient dental sac (an evagination of the left somatocoel wall, which tends to show higher levels of alkaline phosphatase expression); ect, ectodermal vesicles; ef, epineural fold; ev, epineural veil (the later, fused stage of epineural folds); g, gut; hy, hydrocoel; ls, left somatocoel; ns, nervous system; rs, right somatocoel; ve, vestibular ectoderm; vf, vestibule floor; vr, vestibule roof. Bar, 100 $\mu$m.

**TABLE 2**

Individual Ecto-Larvae: Correlation of Rudiment Development with Regeneration of Endoderm or Coelomic Mesoderm[a]

| | Class 1 | Class 2 | Class 3 |
|---|---|---|---|
| *n* (20 total) | 2 | 8 | 10 |
| Rudiment structures: | | | |
| tube feet | − | − | 2 |
| CNS | − | − | 7 |
| Archenteron derivatives: | | | |
| coelomic mesoderm | − | − | + |
| gut | − | + | + |

[a] The 20 ecto-larvae from Table 1, broken down into 3 classes based on the presence or absence of gut and coelomic mesoderm.

feet in only 2 out of 99 cases, even when cultured as long as 10 days, and never underwent metamorphosis. Most of these embryos were scored by observing live embryos through a stereoscope; some may have contained small, poorly formed tube feet not visible under these conditions, since in a later experiment (see below), 10% of ecto-larvae revealed such structures when sectioned.

Normal larvae at 41 h (Fig. 3D) have well-developed tube feet, and the central nervous system can be seen developing in the center of the vestibule floor (identifiable as a columnar epithelium with characteristic contours localized to the center of the vestibule floor, and flanked or covered by developing epineural folds or a completed epineural veil, depending on the stage). By contrast, interior views of ecto-larvae reveal a normal vestibule, equivalent to that of a 30- to 35-h embryo prior to rudiment development (Fig. 3E). These vestibules maintain their original simple morphology indefinitely, developing a variety of folds and wrinkles, but no recognizable adult structures even at late stages. Therefore, the ecto-larvae develop their early vestibule invagination and larval ectodermal features autonomously, whereas the development of adult structures requires the presence of archenteron.

Adult skeletal development was observed by viewing live specimens under polarized light. Normal larvae at 5 days (Fig. 3G) contain extensive, pentamerally arranged adult skeletal elements including numerous spines, reticulated plates of the adult test, and small skeletal rings that form at the tips of the tube feet. Ecto-larvae (Fig. 3H) contain a reduced number of skeletal elements. Spines and test plates are identifiable, although they are frequently slightly misshapen; tube foot rings were not seen in any of five ecto-larvae examined. The few skeletal elements are not arranged in any recognizable pattern. In addition to the organized skeletal elements, a bright punctate scatter was seen throughout the ecto-larvae, indicating the presence of numerous tiny spicule particles, unincorporated into any larger structure. This suggests that mesenchyme cells are present and differentiated and biochemically active, but are lacking some required patterning information.

Mesenchyme cells and skeleton were identified by using antibodies to the extracellular matrix proteins MSP130 and PM27, respectively (Leaf *et al.*, 1987; Harkey *et al.*, 1995; Stander, 1999). Normal larvae (Fig. 3J) contain the expected pentameral pattern, with the two colocalized antigens reflecting the association of mesenchyme cells and the skeleton they secrete. In each of six ecto-larvae (Fig. 3K), PM27 identifies a reduced number of skeletal elements, irregularly shaped, and arranged in a nonpentameral pattern, while MSP130 reveals mesenchyme cells sometimes dissociated from skeleton.

### *Recombination of Archenteron and Ectoderm Is Sufficient to Rescue Ectodermal and Skeletal Organogenesis*

Recombinants were made by removing archenterons from their surrounding ectoderm as described above, and then implanting an archenteron from one embryo into the ectoderm of another embryo (Fig. 2). These healed completely within a few hours. Occasionally, the cut edges healed back together, reconstituting an embryo with a blastopore; more often, the ectoderm healed to itself, sealing the archenteron inside.

Recombinants developed all the normal external larval features (Fig. 3C), including the correct pigmentation pattern, ciliated bands, and vestibular invagination. The puckered morphology seen in some ecto-larvae was not seen in the recombinants.

Unlike ecto-larvae, recombinants developed both larval and adult features (visible tube feet in 47 of 50 cases, and occasionally spines). They were sometimes able to metamorphose, everting their vestibules to become juvenile adult urchins with external tube feet. At late stages, even prior to metamorphosis, mature individual tube feet which frequently protruded through the vestibular opening were capable of coordinated muscle function, and their suckers could adhere strongly to the dish bottom.

Interior views (Fig. 3F) reveal normal tube foot morphology, consisting of an internal mesodermal layer and an external ectodermal layer. Central nervous system can also be identified, frequently with remarkably normal morphology, along with other endodermal and mesodermal organs. However, pentamery was frequently defective (quantified more precisely in a later experiment; Table 1).

Polarized light reveals extensive adult skeleton in the recombinants, arranged in a strikingly normal pattern (Fig. 3I). All types of elements, including test plates, spines, and tube foot rings, are present and are morphologically normal. The scattering of tiny spicule particles was significantly less than in the ecto-larvae. Likewise, antibodies to MSP130 and PM27 reveal extensive, normal adult skeletal elements (Fig. 3L).

As a control for the specificity of the inducer, six ectoderm–ectoderm recombinants were made. Host ectodermal shells were obtained as above, and the archenterons were discarded. The ectodermal animal half of a second, donor embryo was cut into smaller pieces and placed inside

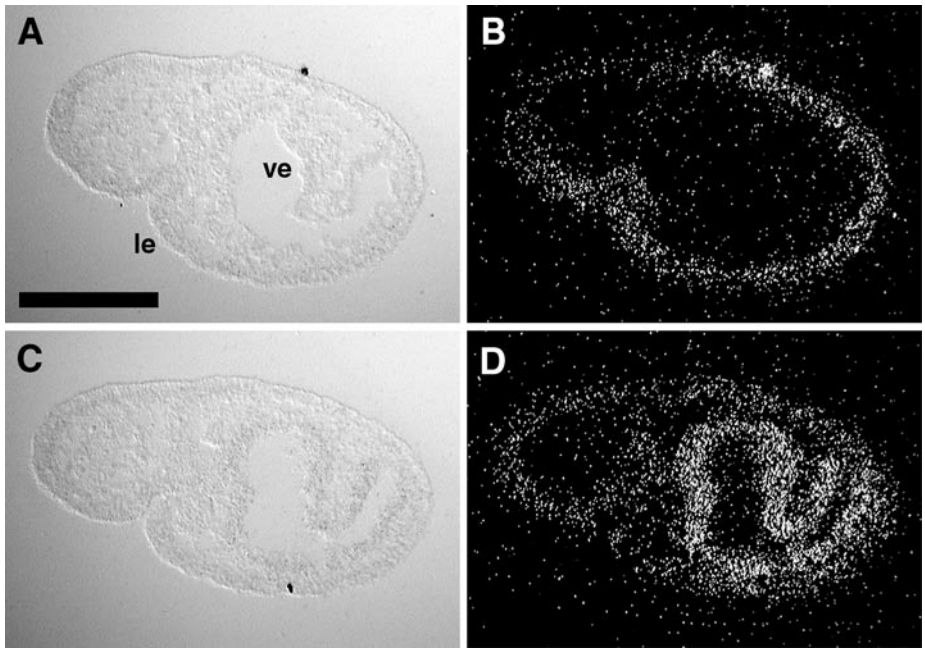



**FIG. 8.** *HeET-1* and *HeARS* expression in a 3.5-day ecto-larva. (A, B) Bright field and dark field, respectively, of a section probed with *HeET-1* mRNA. Signal is present throughout the larval ectoderm but is absent in the vestibule. (C, D) Another section of the same specimen, probed with *HeARS* mRNA. Weak signal is present in the larval ectoderm, and strong signal is present throughout the vestibule, with no region of downregulation. le, larval ectoderm; ve, vestibular ectoderm. Bar, 100 $\mu$m.

the host, which healed around them. All six recombinants developed normal exterior larval features as well as vestibules, but never developed any tube feet or other identifiable adult structure (Fig. 4).

### *Rudiment Induction Requires Left-Coelomic Derivatives*

In order both to further dissect the relative roles of endoderm and left coelom in signaling ectodermal development, and to evaluate the role of regeneration of excised tissue in these experiments, we analyzed the internal tissues of larvae from three types of surgical interventions. In addition to ecto-larvae prepared as described above, we made recombinants using as the donor tissue either endoderm alone ("endo-recombinants"), or left coelom alone ("coelo-recombinants"), instead of the entire archenteron. After the left coelom became distinct from the rest of the archenteron (Fig. 1E, and earlier), the two regions could easily be separated from one another and implanted separately. This was prior to the formation of the right coelom, so the right coelomic precursors were presumably present within the archenteron tissue remaining after the left coelom was removed; therefore, the endo-recombinants contained mostly, though not strictly, endodermal donor tissue.

The recombinants, ecto-larvae, and unoperated embryos were cultured at least until tube feet were visible in the recombinants (38–49 h). Surprisingly, the endo-recombinants and coelo-recombinants had equal success at rescuing development. Examination of these larvae in paraffin section (Table 1; Fig. 5) revealed that tube feet were present in all coelo-recombinants (Figs. 5C and 5D) and in all but one endo-recombinant (Figs. 5E and 5F), in similar numbers. Neural ectoderm developed near the base of the tube feet. In addition, both types of recombinant contained both gut and coelomic mesoderm. Furthermore, the coelomic derivatives were frequently well-developed as identifiable radial water canals, hydrocoel, and left- and right-somatocoel (Table 1; Figs. 5C–5F), with the beginnings of dental sac development in the older specimens. Therefore either tissue (gut or left coelom), implanted alone, regenerated the other tissue. This prevents us from drawing conclusions from the respective recombinants about their separate roles in rudiment induction.

However, the ecto-larvae provide evidence that left coelom is required for rudiment development. These ecto-larvae did not contain any tube feet large enough to be visible in the live larvae. But examination in paraffin section revealed the presence of distinct, sharply bounded local thickenings in the vestibular ectoderm in 35% of these larvae. The columnarization and localization of this feature suggest that it is neural ectoderm (Table 1; Figs. 5I and 5J). In addition, 10% contained small, imperfectly formed, tube-foot-like projections into the vestibule (Table 1), consisting of folds of vestibular ectoderm containing an inner epithelial layer. Most of these larvae also contained a small amount of gut (Table 1; Figs. 5G–5J), and 50%

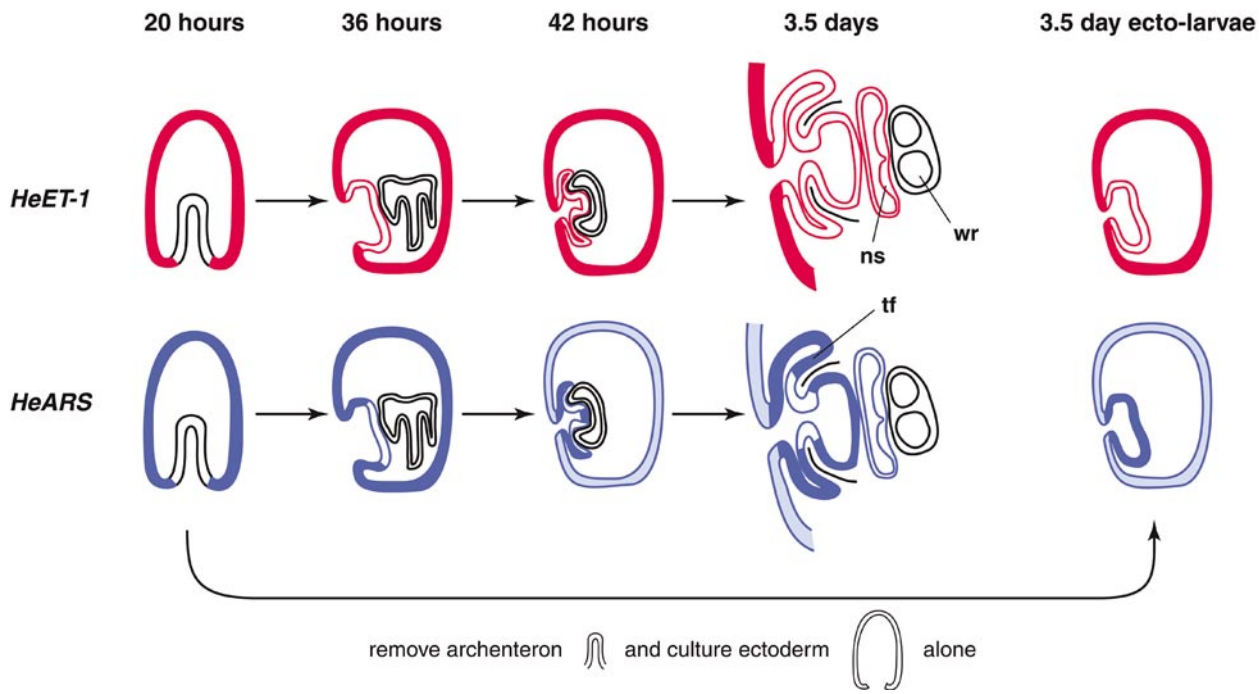



**FIG. 9.** *HeET-1* and *HeARS* expression during normal development, and in 3.5-day ecto-larvae. For clarity, some internal structures have been omitted from the normal 42-h and 3.5-day larvae, and in the 3.5-day larvae, only the rudiment region is shown, magnified to show detail. All ectoderm is shown in color; mesoderm and endoderm are black. Solid fill indicates *HeET-1* and *HeARS* expression. Light blue indicates a reduced level of *HeARS* expression in the larval ectoderm. *HeARS* downregulation in the vestibule floor (36 h, bottom) never occurs in the ecto-larva (far right, bottom), but downregulation in the larval ectoderm (42 h and 3.5 days, bottom) occurs properly. *HeET-1* larval expression and vestibular downregulation (36 h, top) occur properly in ecto-larvae (far right, top). ns, nervous system; tf, tube foot; wr, water ring (hydrocoel).

contained some coelomic mesoderm (Table 1; Figs. 5I and 5J). The presence of each of these tissues in the individual ecto-larvae is correlated in such a way that it supports the requirement for an inductive signal, and further, demonstrates that coelom is required for the transmission of this signal, whereas endoderm alone is not sufficient. Table 2 shows the same 20 ecto-larvae described in Table 1, broken down into three classes. Class 1 comprises those larvae containing no archenteron derivatives at all. Class 2 ecto-larvae comprises those larvae containing a small amount of gut tissue (Figs. 5G and 5H), which may represent either a small amount of regeneration from ectoderm, or a small amount of presumptive endoderm inadvertently left behind during surgery. No rudiment structures were ever seen in any class 1 or class 2 ecto-larva. Class 3 ecto-larvae (Figs. 5I and 5J) comprises those larvae containing a similarly small amount of gut tissue, but in addition, a relatively extensive amount of somatocoel and hydrocoel tissue. The neural-like vestibular thickenings were present in 70% of class 3 ecto-larvae (Table 2), and were always found directly apposed to the coelomic structures (Figs. 5I and 5J), as is normal nervous system. This was accompanied by the tube-foot-like structures in two cases. This correlation demonstrates that the presence of gut alone (at least the small amount that developed in class 2 ecto-larvae) is not sufficient to bring about rudiment development (tube feet and CNS); the presence of left-coelomic derivatives (hydrocoel and/or somatocoel) is required. The absence of the "missing" class 4 (coelomic derivatives without any gut present) demonstrates that mesoderm does not regenerate directly from ectoderm, but arises secondarily by regenerating from gut.

### *Ectodermal Gene Expression Boundaries Arise during Vestibule Invagination in Normal Larvae, and Shift during Rudiment Morphogenesis*

Two ectodermally expressed genes, *HeET-1* and *HeARS*, have been identified in *H. erythrogramma*, and their expression described at several developmental stages (Haag, 1997; Haag and Raff, 1998; Haag *et al.*, 1999). At late gastrula stage (20 h), prior to the development of vestibular ectoderm, both genes are expressed throughout the ectoderm, and are restricted to that tissue. By midrudiment stages, when partially developed tube feet are present (44 h), transcripts of the two genes have resolved into a roughly complimentary pattern, with *HeET-1* mRNA restricted to the larval ectoderm, whereas *HeARS* mRNA is strongly

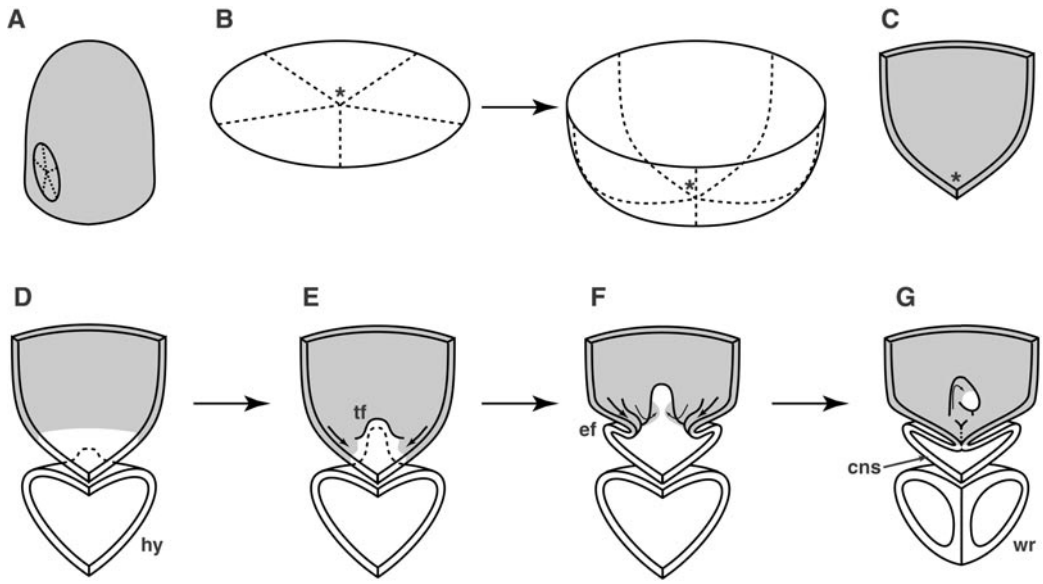



**FIG. 10.** A model of vestibular morphogenesis suggested by the pattern of *HeARS* expression (shaded). (A) The vestibule field is shown in a late gastrula, divided into the presumptive five sectors of the adult pentameral body plan. The entire ectoderm is shaded because at stages prior to vestibule invagination, *HeARS* is expressed in all ectoderm. (B) Schematic diagram of the vestibule field before and after invagination. One tube foot will arise from each sector, and the epineural folds will arise along the dashed lines between the tube feet. The adult mouth will form in the center of the field (asterisk). One of the sectors (containing the asterisk) is represented in (C–G). (C) One sector of the vestibule of an ecto-larva after invagination. Asterisk indicates the same point as in B. In the absence of left coelom, the entire vestibular ectoderm continues to express *HeARS*. (D–G) The same sector of a normal larva as it develops in the presence of hydrocoel (hy). (D) *HeARS* expression ceases in the center of the vestibule floor, in proximity to the coelomic signal. A radial water canal extends from the hydrocoel; this is shown at a later stage in (E), projecting into the tube foot ectoderm, but is omitted from (F) and (G) for clarity. (E–G) Continued morphogenesis of the ectoderm. In this model, *HeARS* expression undergoes no further regulation within vestibular cells, so the moving expression boundaries represent cell movements (arrows). (E) As a tube foot (tf) begins to form, cell rearrangements bring *HeARS*-expressing cells between the tube feet (compare Fig. 7B). The tube foot itself is initially formed from nonexpressing cells. (F) An intermediate stage showing a hypothesized *HeARS* expression pattern that would explain the transition between the patterns seen in (E) and (G). As the epineural folds (ef) rise from the vestibular ectoderm between the tube feet (MacBride, 1903; von Ubisch, 1913), *HeARS*-expressing cells give rise to the outer layer and nonexpressing cells give rise to the inner layer. As the tube foot elongates, newly recruited cells at the base of the tube foot come from *HeARS*-expressing territory, spreading around the tube foot shaft from the aboral toward the oral face of the shaft. (G) The epineural folds fuse along the *HeARS* expression boundary, leaving a *HeARS*-expressing vestibule floor and a nonexpressing nervous system (cns, presumptive nerve ring and radial nerves). More *HeARS*-expressing cells are recruited into the base of the growing tube foot, leaving only the tip nonexpressing. This divides the nonexpressing territory into isolated regions (compare Figs. 7C and 7D). By this time, the hydrocoel has become ring shaped (wr, water ring).

expressed in the vestibular ectoderm and is expressed only at lower levels in larval ectoderm. To determine whether these genes would be suitable markers for distinguishing ectodermal territorial identities in very early or prerudiment stage larvae, and hence also in ecto-larvae and recombinants, we further characterized their expression patterns at intermediate stages, when the vestibule is just beginning to form. Older rudiment stages were also further examined.

*HeET-1* expression was examined in embryos fixed during the process of vestibule invagination. As in older larvae, *HeET-1* mRNA was present throughout the larval ectoderm but was not detectable in vestibular ectoderm (Fig. 6). The downregulation of *HeET-1* expression in vestibular ectoderm, distinguishing the vestibular from the larval territories, therefore occurs at or before the time that vestibular ectoderm begins to invaginate.

*HeARS* expression was examined at similar stages. By the time the vestibule has invaginated (but before any tube feet have formed), the *HeARS* expression pattern subdivides the vestibular ectoderm (Fig. 7A). Expression is no longer detectable in the floor of the vestibule (the region most distal from the vestibular opening and apposed to the hydrocoel). It is still strong in the vestibule roof (the region proximal to the opening), and the expression boundary between the two regions is sharp. Expression is also strong in the larval ectoderm, continuous with the vestibule roof around the lip of the opening. *HeARS* expression thus provides an early marker of regionalization within the vestibular ectoderm.

In only slightly older embryos, in which tube feet are just beginning to form (Fig. 7B), the subdivision between *HeARS*-expressing and nonexpressing vestibular ectoderm cells persists, but with a more complex boundary. Signal is

present in the vestibule roof all the way down to the base of the tube feet, and in some sections, can be seen in the ectoderm between the tube feet as well, but is not detectable in the tube foot ectoderm itself.

In older larvae, as noted in Haag and Raff (1998), *HeARS* expression is reduced to relatively low levels in most of the larval ectoderm (Figs. 7C and 7D). In addition, *HeARS*-expressing and nonexpressing regions continue to exist in the vestibular ectoderm and its derivatives (Figs. 7C and 7D). Signal is present at this stage throughout the vestibule floor and in the ectodermal walls of the tube foot shaft but is frequently absent from the ectoderm at the tube foot tip, where the sucker is differentiating. The CNS and the roof of the epineural cavity are derived from the floor of the vestibular ectoderm, and no *HeARS* mRNA is detected here. This pattern of changes in *HeARS* expression during rudiment development (summarized in Fig. 9) suggests that the boundary (prior to tube foot development) between *HeARS*-expressing vestibule roof and nonexpressing vestibule floor may mark the boundary between the inner and outer faces of the presumptive epineural folds, ultimately becoming the site of fusion between neighboring folds (see Fig. 10).

### *Downregulation of HeARS Expression within the Vestibule Is an Early Response to Inductive Signaling*

Autonomous development of vestibular ectoderm in the absence of inducing tissue suggests that ectodermal markers should be expressed in ecto-larvae in their normal patterns. On the other hand, downregulation of *HeARS* in the vestibule floor of intact embryos takes place in close apposition to the left coelom (Fig. 7A), raising the possibility that *HeARS* downregulation in the vestibule floor is an early response to this inductive event. *HeET-1* downregulation (in the entire vestibular ectoderm) likewise could be an even earlier response to induction. To distinguish these possibilities, the expression patterns of both these genes were examined in ecto-larvae by *in situ* hybridization.

In 3.5-day ecto-larvae with well developed vestibules, *HeET-1* is expressed throughout the larval ectoderm but cannot be detected anywhere in the vestibular ectoderm (Figs. 8A and 8B), indicating that it downregulates correctly with or without the presence of archenteron.

In those same ecto-larvae, *HeARS* (Figs. 8C and 8D) is expressed much less strongly in larval ectoderm than in vestibular ectoderm. However, the signal is strong throughout the vestibular ectoderm, showing no vestibular regions of downregulation. Therefore, the later event in *HeARS* regulation (the subdivision between larval and vestibular territories) takes place correctly in ecto-larvae, even though the earlier event (the subdivision within the vestibule) has failed. Specifically, the changes that normally take place in the vestibule floor, in apposition to left coelom, do not occur. Therefore, the downregulation of *HeARS* in vestibule floor is an early response to inductive signals, whereas the later downregulation of *HeARS* in larval ectoderm is autonomous. These patterns are summarized in Fig. 9.

## CONCLUSION

### *Formation of the Adult Rudiment Requires Inductive Signals from the Left Coelom, but Larval Features Develop Autonomously*

Our results reveal a fundamental property of the early development of the pentameral adult sea urchin from its bilateral larva. Ectodermal development in the *H. erythrogramma* larva and adult rudiment consists of two sets of processes, those that are autonomous to the ectoderm, and those that are dependent on signals from the coelomic mesoderm. Regulation of *HeARS* in the vestibular ectoderm is an early response to these signals.

The subdivision of the embryonic ectoderm into larval and vestibular fields takes place autonomously. The vestibule field then autonomously invaginates to form the vestibule, and *HeET-1* is downregulated there, remaining on only in the larval field. The larval field autonomously develops exterior larval features (ciliated band and pigment cell distribution), and eventually downregulates *HeARS*, leaving it expressed primarily in the vestibule. All these processes take place properly when the archenteron is removed from a late gastrula, and therefore are not dependent on the endoderm or coelomic mesoderm for any type of signaling or mechanical interaction.

Although the initial invagination of the vestibule occurs autonomously, further development of this tissue requires signals from the left coelomic pouch or its derivatives. In response to these signals, the vestibule is partitioned into roof and floor territories, turning off *HeARS* expression in the latter. In the absence of left coelom, *HeARS* expression was never turned off, indicating failure of the vestibule to partition into roof and floor. This reflects the early arrest of rudiment development in the absence of coelomic signal, indicating that partition of the vestibule, including normal regulation of vestibular *HeARS* expression, is an early response to induction by the coelomic mesoderm.

Likewise, the subsequent development of rudiment structures (tube foot and spine ectoderm, epineural folds and CNS) depends on the presence of the left coelom. Nervous system development is induced by coelomic signaling. This may be the same signal that triggers *HeARS* downregulation, or it may be an indirect effect downstream of the early response to this signal, or it may be a separate, later signal from the coelom. In the case of tube foot development, the coelomic influence may be either inductive or mechanical or both, since the hydrocoel lobes themselves form the inner layer of the tube feet. However, even if the direct influence is mechanical, signaling likely plays some role here as well, because the earlier downregulation of *HeARS* across the entire rudiment field suggests that an early coelomic influence is triggering development

of the whole rudiment as a module, rather than exerting only later, piecemeal effects on individual adult organs.

The interpretation that left coelom is required for development of the rudiment structures depends on proper consideration of postsurgical tissue regeneration. In coelo- and endo-recombinants, left coelom fully regenerated from endoderm, and endoderm from left coelom. This demonstrates that even after the formation of a morphologically distinct left coelom, coelomic and endodermal territories are still labile: mesoderm can repattern itself to generate endoderm, and vice versa. This is consistent with studies in indirect-developing sea urchin species. Partially UV-ablated left coelom was able to regenerate fully and produce a hydrocoel (Czihak, 1965). Ablated hydrocoels also regenerated. McClay and Logan (1996) surgically removed partial archenterons from *Lytechinus variegatus* embryos, and found a strong potency to regenerate a complete new archenteron and develop into a normal pluteus.

Because of this full potency of the coelomic and endodermal territories to regenerate the full complement of archenteron derivatives, the data on the relative inductive potential of these regions came not from the recombinants but from the ecto-larvae. Regeneration of archenteron derivatives from ectoderm also occurred, but not to the same extent. Only very tiny guts developed in the ecto-larvae, and coelomic mesoderm was produced in only half of them. This reduced potency of ectoderm to regenerate archenteron derivatives is in contrast to that of *L. variegatus*. When McClay and Logan (1996) surgically removed entire archenterons or vegetal plates, they found the same strong regeneration potential as when they removed only partial archenterons. The reduced regeneration potential of *H. erythrogramma* ectoderm is consistent with the evolution of its precocious adult development (Williams and Anderson, 1975; Raff, 1987; Parks *et al.,* 1988). This change has been accompanied by the earlier determination of embryonic axes and restriction of cell fates (Henry and Raff, 1990; Henry *et al.,* 1990).

In these ecto-larvae, tube foot and CNS development occurred only in the presence of regenerated coelomic mesoderm; therefore, inductive signaling requires coelom. Gut alone is not sufficient. We therefore believe the coelom is very likely the source of the inducing signal. However, the absence of the fourth possible class of ecto-larva (which would have contained coelom but no gut whatsoever) makes it impossible to know whether coelom is *sufficient* as well as necessary, or whether gut is *unnecessary* as well as insufficient. Therefore, it is conceivable that the source of the signal could actually be the endoderm, but requiring a permissive interaction with coelom. However, this interpretation seems needlessly complex to us, especially since the left coelom and its derivatives—in all echinoid species regardless of developmental mode—are positioned directly between the gut and the ectoderm, and form a close association with the ectoderm.

Our results do not rule out the possibility of signals from vestibular ectoderm that induce or pattern development of the coelomic mesoderm, with reciprocal feedback between the tissues regulating the development of rudiment structures. Di Bernardo *et al.* (1999) found ectodermal expression of *Otp* not only in close apposition to the active sites of skeletal growth which it patterns, but also, suggestively, in close apposition to the incipient left and right coelomic sacs. We are investigating the possibility of such reciprocal signals in experiments currently in progress.

### *Developmental Modules*

*H. erythrogramma* larval and rudiment ectodermal development are highly modular. Larval ectodermal features develop as one functional unit, independent of coelomic signals, and rudiment features develop as another functional unit, this one requiring coelomic signaling. Removal of this signal "unplugs" rudiment development as a whole from larval ectodermal development.

This mosaic of processes is reflected in the expression patterns of *HeARS* and *HeET-1* (Fig. 9). The downregulation of *HeET-1* expression in the vestibule is part of the autonomous early differentiation of the vestibule field and its initial invagination. The initial pan-ectodermal expression of *HeARS* becomes restricted along two boundaries, one within the vestibule and one at the vestibular/larval interface. The former is regulated along with other rudiment features, dependent on coelomic signaling, and the latter occurs autonomously, along with other larval features.

Despite being organized in two functionally independent regulatory domains, *HeARS* expression has the appearance of taking place in one unified territory and during a single continuous period. *H. erythrogramma* inherits its modularity from its indirect-developing ancestors. In its closest relative, *H. tuberculata*, *ARS* is expressed at two different times: early in aboral ectoderm, and then much later in vestibular ectoderm (Raff and Sly, 2000). These two domains are also separated spatially, since the vestibule develops within the oral territory. But the evolution of direct development in *H. erythrogramma* has involved a heterochronic shift of adult development into the late gastrula stage (Raff, 1987; Parks *et al.,* 1988). Furthermore, the oral territory has been lost, with the concomitant spread of aboral *HeARS* expression to the entire larval ectoderm (Haag and Raff, 1998; Nielsen *et al.,* 2000; Raff and Sly, 2000). Thus *HeARS* expression in the two domains now occurs simultaneously, and in direct contact, creating the appearance of a unified territory. But its organization reveals the evolutionary histories of the two modules.

### *Coelom Is Required for Normal Adult Skeletal Patterning*

In indirect-developing sea urchins, the formation of larval skeleton by primary mesenchyme cells (PMCs) is patterned by signals from the ectoderm. The PMCs are intrinsically able to migrate and to make $CaCO_3$ spicules even *in vitro* (Harkey and Whiteley, 1980), and are probably prepro-

grammed, in a lineage-dependent manner, to express an early set of genes that in normal embryos are expressed uniformly in all PMCs (Guss and Ettensohn, 1997). However, signals from the ectoderm direct the timing of PMC migration in the blastocoel and their targeting to the correct sites of skeletal formation, induce subsequent signal-dependent modulation of early PMC genes and additional later-expressed genes to create regionalized expression patterns, and control the rate of skeletal growth, probably by the induction of PMC genes that control rate-limiting processes (Ettensohn and McClay, 1986; Armstrong *et al.*, 1993; Ettensohn and Malinda, 1993; Guss and Ettensohn, 1997; Di Bernardo *et al.*, 1999). Experimental perturbation of either the patterning or the size of the ectoderm modifies these signals and the resulting skeletal development, whereas perturbation of the number of PMCs results in regulation of normal skeleton formation. No role has been demonstrated for coelomic mesoderm in the patterning of larval skeleton, which is as expected since the skeleton is initiated prior to coelom formation, and develops to advanced stages while the coeloms are still rudimentary.

The adult skeleton forms later, well after the coeloms have formed and the rudiment has been established, but much less is known about its development. Several lines of evidence suggest that adult skeleton is formed at least in part by the descendants of PMCs. The mesenchyme cells that form the adult and larval skeletons exhibit similar behaviors and express some of the same genes involved in biomineralization (Parks *et al.*, 1988; Richardson *et al.*, 1989; Drager *et al.*, 1989). Some of the juvenile test plates arise directly from the bases of larval arm rods and have common crystallographic axes, indicating continuity (Emlet, 1985), and in *Lytechinus pictus*, PMCs associated with larval skeleton contribute to pedicellaria, the earliest forming adult skeletal structures (Burke, 1980). Yet nothing is known about the patterning mechanisms involved in these later events.

*H. erythrogramma* produces thousands of mesenchyme cells early in development (Parks *et al.*, 1988), but they produce only vestigial larval skeleton (Emlet, 1995). These cells immediately go on to produce the adult skeleton during rudiment development. As in indirect developers, some test plates form from the larval spicules (Emlet, 1995), and the cells express the same genes (Parks *et al.*, 1988; Stander, 1999). We do not know how the limited larval skeleton of *H. erythrogramma* may be patterned, but the experiments reported here demonstrate that the adult skeleton is patterned quite differently from the larval skeleton of indirect developers. In ecto-larvae, only a few poorly formed adult skeletal elements develop. This deficiency may simply be due to a loss of mesenchyme cells during archenteron removal; however, the presence of scattered particles of calcite unincorporated into any spine or plate implies that mesenchyme cell number is not a limiting factor. Adult skeletal development is rescued by recombination with archenteron. Given the lack of normal skeletal organization in the ecto-larvae and its rescue in recombinants, we conclude that the coelom is required for transmission of instructive patterning information necessary for normal adult skeletal development. This does not preclude a role for ectodermal signaling, and the fact that any skeletal elements form at all in ecto-larvae may in fact reflect such a role. Alternatively, it may simply reflect a limited autonomous ability of mesenchyme cells to build individual adult skeletal elements, similar to the ability of PMCs of indirect developers to form individual larval spicules (Harkey and Whiteley, 1980). The coelom may signal the mesenchyme directly, or it may exert its influence indirectly, by interacting with the ectoderm to induce the production of an ectodermal signal.

We do not know whether this patterning mechanism is specific to *H. erythrogramma*; if so, it could represent an evolutionary change correlated with developmental mode. However, we consider it more likely that adult skeleton is patterned by this same mechanism in other sea urchins; patterning of adult skeleton may simply be distinct from that of larval skeleton. In all sea urchins, regardless of developmental mode, adult skeletal development must be coordinated with the development of other adult structures. These structures contain both ectoderm- and coelom-derived components and are significantly more complex structurally than larval structures such as pluteus arms, so ectodermal signaling alone may not be sufficient to regulate their development. Elucidation of adult skeletal patterning mechanisms in indirect developers would provide further insight into this problem.

### *HeARS Expression and Rudiment Morphogenesis*

During normal development, the pattern of vestibular *HeARS* expression boundaries exhibits increasing complexity. Beginning as a single nonexpressing region in the center of the vestibule floor, by the end of rudiment morphogenesis it has been transformed into multiple nonexpressing regions separated by intervening *HeARS* expression (Fig. 9, bottom). Two possible mechanisms (not mutually exclusive) can explain this dynamic pattern. The simpler explanation is that during the extensive morphogenetic deformation of the rudiment ectoderm to form tube feet, spines, and nervous system, the changing pattern of *HeARS*-expressing cells reflects the movement of those cells and the rearrangement of expressing and nonexpressing regions (Fig. 10). In this model, there is no further change in *HeARS* expression level within individual cells once the initial induced down-regulation has taken place in the vestibule floor. Nothing is known about the cellular mechanisms of morphogenesis during rudiment development, but such extensive deformation almost certainly involves enough cell rearrangement to account for the observed changes in the *HeARS* pattern.

In this model, *HeARS* would serve as a lineage marker, and its expression pattern would reflect patterns of morphogenesis (Fig. 10). Cells from the *HeARS*-expressing vestibule roof would push inward toward the center of the vestibule floor, between the tube foot primordia, giving rise

to the outer surface of the epineural folds. The boundary between *HeARS*-expressing roof and nonexpressing floor would give rise to the boundary between the inner and outer surfaces of the epineural folds, so that when the folds fuse, creating the separate inner and outer layers of the epineural veil, the outer layer would still contain *HeARS* message, whereas the inner layer would not. This is reminiscent of ectodermal differentiation in chick, in which *BMP7* is expressed in the epidermis but not in the neural plate, its expression boundary identifying the line of neural fold fusion (Liem *et al.*, 1995). The *HeARS* expression pattern in the advanced rudiment ectoderm would represent a fate map of the two regions of the vestibule: the *HeARS*-expressing roof giving rise to the main body wall and to the tube foot shafts, and the nonexpressing floor giving rise to the tube foot tips and to the CNS.

On the other hand, individual cells may continue to regulate *HeARS* expression as development proceeds. *HeARS*-downregulated cells in the vestibule floor would subsequently restart *HeARS* expression. This would be more analogous to *BMP4* in the chick, which starts out, like *BMP7*, expressed only in the epidermis adjacent to the neural plate, but subsequently becomes expressed also in the dorsal neural tube (Liem *et al.*, 1995). In the most extreme version of this model, tissue deformations during morphogenesis could occur in such a way as not to greatly disturb the boundaries between expressing and nonexpressing regions, and the changing expression pattern then would be explained entirely by intracellular *HeARS* regulation.

## ACKNOWLEDGMENTS

We thank the Sydney Aquarium and the School of Biological Sciences, University of Sydney for making our work in Australia possible. E. Raff, E. Popodi, M. Nielsen, J. Villinski, B. Sly, and the anonymous reviewers gave helpful comments on the manuscript. New South Wales Fisheries provided permits for collecting sea urchins. This work was funded by an NIH Postdoctoral Fellowship (to S.B.M.) and an NSF Research Grant (to R.A.R.).

## REFERENCES

Angerer, L. M., and Angerer, R. C. (1991). Localization of mRNAs by *in situ* hybridization. *Methods Cell Biol.* **35,** 37–71.

Angerer, L. M., and Angerer, R. C. (2000). Animal–vegetal axis patterning mechanisms in the early sea urchin embryo. *Dev. Biol.* **218,** 1–12.

Angerer, L. M., Oleksyn, D. W., Logan, C. Y., McClay, D. R., Dale, L., and Angerer, R. C. (2000). A BMP pathway regulates cell fate allocation along the sea urchin animal–vegetal embryonic axis. *Development* **127,** 1105–1114.

Armstrong, N., Hardin, J., and McClay, D. R. (1993). Cell–cell interactions regulate skeleton formation in the sea urchin embryo. *Development* **119,** 833–840.

Bromham, L. D., and Degnan, B. M. (1999). Hemichordates and deuterostome evolution: robust molecular phylogenetic support for a hemichordate + echinoderm clade. *Evol. Dev.* **1,** 166–171.

Burke, R. D. (1980). Development of pedicellariae in the pluteus larva of *Lytechinus pictus* (Echinodermata: Echinoidea). *Can. J. Zool.* **58,** 1674–1682.

Czihak, G. (1965). Entwicklungsphysiologische Untersuchungen an Echiniden (experimentelle Analyse der Coelomentwicklung). *Roux's Arch. Entw. Org.* **155,** 709–729.

Di Bernardo, M., Castagnetti, S., Bellomonte, D., Oliveri, P., Melfi, R., Palla, F., and Spinelli, G. (1999). Spatially restricted expression of *PlOtp*, a *Paracentrotus lividus Orthopedia*-related homeobox gene, is correlated with oral ectodermal patterning and skeletal morphogenesis in late-cleavage sea urchin embryos. *Development* **126,** 2171–2179.

Drager, B. J., Harkey, M. A., Iwata, M., and Whiteley, A. H. (1989). The expression of embryonic primary mesenchyme genes of the sea urchin, *Strongylocentrotus purpuratus*, in the adult skeletogenic tissues of this and other species of echinoderms. *Dev. Biol.* **133,** 14–23.

Emlet, R. B. (1985). Crystal axes in recent and fossil adult echinoids indicate trophic mode in larval development. *Science* **230,** 937–940.

Emlet, R. B. (1995). Larval spicules, cilia, and symmetry as remnants of indirect development in the direct developing sea urchin *Heliocidaris erythrogramma*. *Dev. Biol.* **167,** 405–415.

Emlet, R. B., and Hoegh-Guldberg, O. (1997). Effects of egg size on postlarval performance: Experimental evidence from a sea urchin. *Evolution* **51,** 141–152.

Ettensohn, C. A., and Malinda, K. M. (1993). Size regulation and morphogenesis: A cellular analysis of skeletogenesis in the sea urchin embryo. *Development* **119,** 155–167.

Ettensohn, C. A., and McClay, D. R. (1986). The regulation of primary mesenchyme cell migration in the sea urchin embryo: Transplantations of cells and latex beads. *Dev. Biol.* **117,** 380–391.

Ferkowicz, M. J., and Raff, R. A. (2001). Wnt gene expression in sea urchin development: Heterochronies associated with the evolution of developmental mode. *Evol. Dev.* **3,** 24–33.

Guss, K. A., and Ettensohn, C. A. (1997). Skeletal morphogenesis in the sea urchin embryo: Regulation of primary mesenchyme gene expression and skeletal rod growth by ectoderm-derived cues. *Development* **124,** 1899–1908.

Haag, E. S. (1997). "Modification of Gene Expression During the Evolution of a Direct-Developing Sea Urchin." Ph.D. thesis, Indiana University.

Haag, E. S., and Raff, R. A. (1998). Isolation and characterization of three mRNAs enriched in embryos of the direct-developing sea urchin *Heliocidaris erythrogramma*: Evolution of larval ectoderm. *Dev. Genes Evol.* **208,** 188–204.

Haag, E. S., Sly, B. J., Andrews, M. E., and Raff, R. A. (1999). Apextrin, a novel extracellular protein associated with larval ectoderm evolution in *Heliocidaris erythrogramma*. *Dev. Biol.* **211**, 77–87.

Harkey, M. A., Klueg, K., Sheppard, P., and Raff, R. A. (1995). Structure, expression, and extracellular targeting of PM27, a skeletal protein associated specifically with growth of the sea urchin larval spicule. *Dev. Biol.* **168,** 549–566.

Harkey, M. A., and Whiteley, A. H. (1980). Isolation, culture, and differentiation of echinoid primary mesenchyme cells. *Roux's Arch.* **189,** 111–122.

Hay, E. D. (1980). Development of the vertebrate cornea. *Int. Rev. Cytol.* **63,** 263–322.

Henry, J. J., and Raff, R. A. (1990). Evolutionary change in the process of dorsoventral axis determination in the direct develop-

ing sea urchin *Heliocidaris erythrogramma*. *Dev. Biol.* **141,** 55–69.

Henry, J. J., Wray, G. A., and Raff, R. A. (1990). The dorsoventral axis is specified prior to first cleavage in the direct developing sea urchin *Heliocidaris erythrogramma*. *Development* **110,** 875–884.

Hörstadius, S. (1973). "Experimental Embryology of Echinoderms." Clarendon Press, Oxford.

Klingler, M., and Gergen, J. P. (1993). Regulation of *runt* transcription by *Drosophila* segmentation genes. *Mech. Dev.* **43,** 3–19.

Leaf, D. S., Anstrom, J. A., Chin, J. E., Harkey, M. A., Showman, R. M., and Raff, R. A. (1987). Antibodies to a fusion protein identify a cDNA clone encoding msp130, a primary mesenchyme-specific cell surface protein of the sea urchin embryo. *Dev. Biol.* **121,** 29–40.

Liem, Jr., K. F., Tremml, G., Roelink, H., and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82,** 969–979.

MacBride, E. W. (1903). The development of *Echinus esculentus*, together with some points in the development of *E. miliaris* and *E. acutus*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **195,** 285–327.

McClay, D. R., and Logan, C. Y. (1996). Regulative capacity of the archenteron during gastrulation in the sea urchin. *Development* **122,** 607–616.

McClay, D. R., Peterson, R. E., Range, R. C., Winter-Vann, A. M., and Ferkowicz, M. J. (2000). A micromere induction signal is activated by β-catenin and acts through Notch to initiate specification of secondary mesenchyme cells in the sea urchin embryo. *Development* **127,** 5113–5122.

Nielsen, M. G., Wilson, K. A., Raff, E. C., and Raff, R. A. (2000). Novel gene expression patterns in hybrid embryos between species with different modes of development. *Evol. Dev.* **2,** 133–144.

Okazaki, K. (1975). Normal development to metamorphosis. In "The Sea Urchin Embryo" (G. Czihak, Ed.), pp. 177–232. Springer-Verlag, New York.

Parks, A. L., Parr, B. A., Chin, J.-E., Leaf, D. S., and Raff, R. A. (1988). Molecular analysis of heterochronic changes in the evolution of direct developing sea urchins. *J. Evol. Biol.* **1,** 27–44.

Raff, R. A. (1987). Constraint, flexibility, and phylogenetic history in the evolution of direct development in sea urchins. *Dev. Biol.* **119,** 6–19.

Raff, R. A. (1996). "The Shape of Life." The University of Chicago Press, Chicago.

Raff, R. A., and Sly, B. J. (2000). Modularity and dissociation in the evolution of gene expression territories in development. *Evol. Dev.* **2,** 102–113.

Ransick, A., and Davidson, E. H. (1993). A complete second gut induced by transplanted micromeres in the sea urchin embryo. *Science* **259,** 1134–1138.

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

Richardson, W., Kitajima, T., Wilt, F., and Benson, S. (1989). Expression of an embryonic spicule matrix gene in calcified tissues of adult sea urchins. *Dev. Biol.* **132,** 266–269.

Saha, M. S., Spann, C. L., and Grainger, R. M. (1989). Embryonic lens induction: more than meets the optic vesicle. *Cell Differ. Dev.* **28,** 153–172.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sherwood, D. R., and McClay, D. R. (1999). LvNotch signaling mediates secondary mesenchyme specification in the sea urchin embryo. *Development* **126,** 1703–1713.

Sherwood, D. R., and McClay, D. R. (2001). LvNotch signaling plays a dual role in regulating the position of the ectoderm–endoderm boundary in the sea urchin embryo. *Development* **128,** 2221–2232.

Stander, M. C. (1999). "Regulation and Evolution of Skeletogenesis in Sea Urchin Development." Master's thesis, Indiana University.

Tanabe, Y., and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274,** 1115–1123.

Turbeville, J. M., Schulz, J. R., and Raff, R. A. (1994). Deuterostome phylogeny and the sister group of the chordates: Evidence from molecules and morphology. *Mol. Biol. Evol.* **11,** 648–655.

von Ubisch, L. (1913). Die Entwicklung von *Strongylocentrotus lividus*. (*Echinus microtuberculatus*. *Arbacia pustulosa*). *Zeitschrift. Zoöl.* **106,** 409–448.

Vonica, A., Weng, W., Gumbiner, B. M., and Venuti, J. M. (2000). TCF is the nuclear effector of the β-catenin signal that patterns the sea urchin animal–vegetal axis. *Dev. Biol.* **217,** 230–243.

Wikramanayake, A. H., Huang, L., and Klein, W. H. (1998). β-Catenin is essential for patterning the maternally specified animal–vegetal axis in the sea urchin embryo. *Proc. Natl. Acad. Sci. USA* **95,** 9343–9348.

Wikramanayake, A. H., and Klein, W. H. (1997). Multiple signaling events specify ectoderm and pattern the oral–aboral axis in the sea urchin embryo. *Development* **124,** 13–20.

Williams, D. H. C., and Anderson, D. T. (1975). The reproductive system, embryonic development, larval development and metamorphosis of the sea urchin *Heliocidaris erythrogramma* (Val.) (Echinoidea: Echinometridae). *Aust. J. Zool.* **23,** 371–403.

Wray, G. A., and Raff, R. A. (1989). Evolutionary modification of cell lineage in the direct-developing sea urchin *Heliocidaris erythrogramma*. *Dev. Biol.* **132,** 458–470.

Received for publication November 13, 2001
Revised April 15, 2002
Accepted April 15, 2002
Published online June 7, 2002