

Archenteron precursor cells can organize secondary axial structures in the sea urchin embryo

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SUMMARY

Local cell-cell signals play a crucial role in establishing major tissue territories in early embryos. The sea urchin embryo is a useful model system for studying these interactions in deuterostomes. Previous studies showed that ectopically implanted micromeres from the 16-cell embryo can induce ectopic guts and additional skeletal elements in sea urchin embryos. Using a chimeric embryo approach, we show that implanted archenteron precursors differentiate autonomously to produce a correctly proportioned and patterned gut. In addition, the ectopically implanted presumptive archenteron tissue induces ectopic skeletal pat-

ternerng sites within the ectoderm. The ectopic skeletal elements are bilaterally symmetric, and flank the ectopic archenteron, in some cases resulting in mirror-image, symmetric skeletal elements. Since the induced patterned ectoderm and supernumerary skeletal elements are derived from the host, the ectopic presumptive archenteron tissue can act to 'organize' ectopic axial structures in the sea urchin embryo.

Key words: induction, gastrulation, sea urchin, endoderm

INTRODUCTION

Progressive refinement of the embryonic body plan via local inductive interactions is a common theme among animal embryos. Ultimately, such refinement of the body plan results in the appearance of the definitive axial structures of the embryo. A convenient model system for studying the local inductive interactions between blastomeres of the early embryo that result in the production of axial structures is the sea urchin embryo. In the normal sea urchin embryo, the first two radial cleavages bisect the animal vegetal axis and the third occurs orthogonal to the animal vegetal axis, producing an embryo with eight cells of equal size. At the fourth cleavage, the animal tier of cells divides meridionally to produce eight *mesomeres*; the vegetal tier of blastomeres divides equatorially and unequally to produce four large *macromeres* and four small *micromeres*, which lie at the extreme vegetal pole of the embryo (Fig. 1A). The *mesomeres* go on to divide to produce an animal tier of **an1** cells and a vegetal tier of **an2** cells at the next cleavage; by the 64-cell stage, the *macromeres* have produced animal granddaughters known as **veg1** cells and more vegetal granddaughters known as **veg2** cells (actually, due to asynchrony of division, there are 60 cells at this time in *S. purpuratus*; Cameron and Davidson, 1991). The **veg2** tier gives rise to the archenteron and non-skeletogenic (secondary) mesenchyme of the midgastrula embryo, while the **veg1** tier gives rise to lateral ectoderm (Fig. 1A). Recently, more precise fate

mapping studies have shown that **veg1** descendants contribute tissue to the vegetal regions of the archenteron by the late gastrula stage (Logan and McClay, 1997); by this time **veg1** and **veg2** descendants intermingle within the wall of the archenteron (Fig. 1A). The descendants of the micromeres go on to produce skeletogenic, or primary, mesenchyme cells (PMCs), which adopt a highly stereotyped pattern that presages the pattern of the calcareous skeletal rods of the pluteus larva. In particular, skeletogenic mesenchyme cells aggregate into two large clusters (*ventrolateral clusters*) atop thickened ectoderm in the ventrolateral regions of the embryo. Together, the archenteron, ventrolateral ectoderm and associated skeletogenic mesenchyme constitute major axial structures of the sea urchin embryo.

A number of recent and classical experiments have identified several important local cell-cell signaling events, which are thought to be required for the ultimate appearance of specific tissue territories in the sea urchin embryo (reviewed by Hörstadius, 1939, 1973; Davidson, 1989, 1993). Wilt (1987) and Davidson (1989) have persuasively argued that the best interpretation of the available data favors sequential, contact-mediated interactions between blastomeres in the 16- to 64-cell embryo, rather than opposing gradients of animal and vegetal inducers as favored by classical workers (e.g., Hörstadius, 1939). The most important early cell-cell interaction in the early embryo is the ability of micromeres (or their larger daughters) to alter the fate of neighboring cells, an event that

Davidson and colleagues have proposed 'entrains' subsequent contact-mediated inductive interactions (Davidson, 1989, 1993; Ransick and Davidson, 1995).

Two potential effects of micromeres on nearby cells have been investigated (reviewed by Livingston and Wilt, 1990a; Davidson, 1993). First, Wilt and colleagues have shown that mesomeres can produce gut and skeletal structures when in contact with micromeres (Livingston and Wilt, 1990b; Wilt et al., 1995). Second, transplants by Hörstadius (1935) and Ransick and Davidson (1993) have shown that micromeres transplanted to ectopic locations in either 16- or 32-cell embryos can induce nearby tissue to form an archenteron, even though under normal circumstances this tissue is fated to form ectoderm. The induced tissues express appropriate mRNAs and/or proteins consistent with their morphology (Khaner and Wilt, 1990; Ransick and Davidson, 1993; Wilt et al., 1995). Differences in the timing of gastrulation and the morphology of the vegetal plate following removal of the micromeres suggest that contact between micromeres and immediately adjacent macromeres plays a role during normal development in helping to specify macromeres and their descendants (Ransick and Davidson, 1995).

Implantation of ectopic micromeres has an additional consequence: one or more supernumerary skeletal elements are produced (Hörstadius, 1935; Ransick and Davidson, 1993). When Ransick and Davidson (1993) transplanted micromeres to the animal poles of early 16-cell-stage embryos, the resulting embryos possessed a complete, bilateral skeleton formed by the implanted cells. The labeled micromere descendants form the skeleton around the unlabeled, induced archenteron, aligned with the dorsoventral axis of the host (Ransick and Davidson, 1993). Although the models of Davidson (1989, 1993) favor a cascade of sequential inductive interactions, the experiments of Ransick and Davidson and Hörstadius raise several alternative logical possibilities regarding how ventrolateral patterning sites for PMCs arise. Among the simplest are the following: (1) patterning sites for PMCs could arise via inductive signals produced directly by the micromeres, (2) they could arise via a cascade of interactions, requiring induction of presumptive gut tissue followed by the induction of ventrolateral patterning sites via lateral signaling from the presumptive gut tissue or (3) a combination of such signals could be involved (these possibilities are summarized in Fig. 1B).

In order to provide experimental support for one of these alternative mechanisms, we have used a chimeric embryo approach to implant gut progenitors in ectopic locations to assess their inductive capacities. We show here that such ectopic cells generate an additional archenteron via the autonomous differentiation of the implanted material, but they also induce two new bilateral sites of skeleton formation. The ectoderm underneath the new skeletal elements is from the host; this implies that lateral induction of host ectoderm by the incorporated cell or its descendants produces new patterning sites for skeletogenic mesenchyme within the embryo. This result in turn provides support for the sequential signaling model, consistent with the views of Davidson (1989).

MATERIALS AND METHODS

Manual dissection of macromeres

Lytechinus variegatus eggs (obtained from T. Andacht, Beaufort Marine Station, Duke University) were fertilized in 10 mM *p*-

aminobenzoic acid (PABA; sodium salt, Sigma) and demembrated by passage through 73 μ m Nitex mesh. Demembrated embryos were washed twice in artificial sea water (ASW). Embryos were dissociated at the 8-cell stage by settling twice through ice-cold calcium-free sea water (CFSW). Embryos were dissociated by trituration through a long-bore Pasteur pipette. The resulting dissociated blastomeres were cultured in finger bowls at 22°C until they underwent the next cleavage. An aliquot of the dissociated blastomeres was placed in a depression slide and inspected under the dissecting microscope; blastomeres that generated macromere/micromere pairs were mouth pipetted into a second depression slide. The macromere/micromere pairs were then separated by cutting them with a glass needle that had been hand-pulled from glass capillary tubing in an alcohol flame. Isolated macromeres were then mouth pipetted to a depression slide containing ASW and cultured until siblings had reached the prism stage. Embryoids were photographed using Nomarski optics on a Nikon Diaphot II inverted microscope using a Nikon 35 mm photomicrographic attachment and Panatomic X ASA 32 film.

Production of chimeric embryos

Chimeric embryos were produced as described in Wray and McClay (1988) and Hardin (1989). Briefly, *Lytechinus variegatus* eggs were fertilized in 10 mM *p*-aminobenzoic acid and demembrated by passage through 73 μ m Nitex mesh. Demembrated embryos were washed twice in ASW. Host embryos were allowed to develop normally in shallow bowls in ASW; donor embryos were resuspended in CFSW. Rhodamine isothiocyanate (RITC) was prepared as follows. 1 mg RITC (Sigma) was dissolved in 20 μ l dimethyl sulfoxide (DMSO) in a glass tube, 10 ml CFSW was then added and the resulting solution was vortexed. The solution was filtered through a 0.22 μ m syringe filter and 1 ml filtered staining solution was added to 250 ml of embryos; embryos were subsequently cultured in the dark. At the 16-cell stage, donor embryos were dissociated by settling twice through CFSW and a final rinse in ice-cold hyaline extraction medium (HEM; McClay, 1986). Embryos were dissociated by trituration through a long-bore Pasteur pipette. Clumps of undissociated cells were removed by passing the resulting cell suspension through 28 μ m Nitex mesh. In some experiments, blastomeres were separated by size on a 2%/8% v/v 0.75 M sucrose step gradient as described in detail in Wray and McClay (1988). The partially purified macromere fraction was collected from near the bottom of the 8% layer and placed in a small finger bowl. In other experiments, the dissociated, labeled cells were allowed to settle through HEM on ice without size separation and collected. The host embryos were then rendered somewhat adhesive by brief treatment in CFSW for 10 minutes. Finally, donor cells were added to host embryos in medium-sized finger bowls and the donor cells were allowed to settle onto the host embryos for 20 minutes. The majority of the medium was then withdrawn with a Pasteur pipette and replaced with fresh ASW.

Identification and immunostaining of embryos with second axes

At the gastrula stage or later, living chimeric embryos were identified under the dissecting microscope. Clonal derivatives of incorporated blastomeres were scored for macromere or micromere descendant patterns by assessing whether or not they produced gut tissue. Chimeras with ectopic gut tissue were mouth pipetted into a depression slide containing 1 ml ASW in preparation for immunostaining. Chimeric embryos were fixed by adding 100 μ l 37% formaldehyde directly to depression slides containing embryos with supernumerary archenterons; following a 30-60 minute incubation in fixative, embryos were permeabilized in ice-cold acetone for 6 minutes. Fixed embryos were then serially transferred into depression slides containing fresh ASW until all trace of organic odor was removed. Embryos were immunostained for the endodermal marker Endo 1 (Wessel and McClay, 1985) and the primary mesenchyme-specific

marker 1d5 as whole mounts using monoclonal antibody supernatants as described previously (Hardin et al., 1992), using FITC-conjugated goat anti-mouse secondary antibodies (Organon Technika).

Quantification of primary mesenchyme cells and position of incorporation of labeled clones in chimeric embryos

Fixed embryos were immunostained for primary mesenchyme cells (PMCs) and photographed or counted directly under the fluorescence microscope. In cases where an unambiguous ectopic archenteron was observed, PMCs were counted using a handheld counter. To measure the position of incorporation of ectopic archenterons, an arc extending from the blastopore of the host archenteron along a meridian to the blastopore of the donor archenteron was measured manually from prints or negatives using a protractor. Angular statistics (Zar, 1984) were calculated using software written in this laboratory.

RESULTS

En masse isolation of blastomeres and production of chimeras containing labeled gut tissue: technical considerations

It is known from extensive fate mapping studies that macromeres give rise to cells within the archenteron, including endoderm and secondary mesenchyme cells, as well as lateral and anal ectoderm (Hörstadius, 1973; Cameron et al., 1991). However, isolation of the macromere granddaughters that produce only archenteron and secondary mesenchyme, which Hörstadius referred to as **veg2** cells, is technically challenging. This makes manual transplantation of **veg2** cells to new sites to evaluate their inductive capacities extremely difficult. Hörstadius (1935) reported two cases in which he believed that he had successfully transplanted these cells to new locations; however, the time required for their isolation and their adherence to host embryos made it difficult for them to be incorporated sufficiently quickly for them to be present at the time such cells would be hypothesized to send inductive signals to nearby tissue.

In order to produce greater numbers of embryos possessing ectopic gut progenitors, we have used en masse dissociation and purification of *Lytechinus variegatus* blastomeres to obtain enriched populations of macromeres and their descendants for use in making chimeric embryos, using the technique of Wray and McClay (1988). At the 16-cell stage, there are four macromeres and embryos dissociated en masse produce appropriate percentages of embryoids resembling those derived from manually dissected macromeres (cf. Driesch, 1900; Hörstadius, 1936). Embryoids produced via en masse isolation of 16-cell *L. variegatus* embryos can also be recovered that appear identical to those produced by manual dissection of *L. variegatus* macromeres (data not shown), confirming that their behavior is similar to that of other species, and that dissociation does not adversely affect the autonomous differentiation of *L. variegatus* macromeres.

When rhodamine-labeled, dissociated blastomeres are added to unlabeled host embryos, in most cases the labeled blastomeres incorporate homotopically and appear to participate in normal morphogenetic and differentiation events (Wray and McClay, 1988; Hardin, 1989). In other cases, however, ectopic incorporation of the blastomere occurs. In these cases, a labeled clone of cells derived from the donor blastomere can be assessed for its capacity to differentiate, and for its ability

to send inductive signals to surrounding cells. If incorporated macromeres or half macromeres have the capacity to form ectopic clones, they would be expected to produce anal ectoderm and an archenteron, albeit smaller than the host archenteron, since a single macromere only gives rise to one-fourth of the archenteron in the normal embryo and a half macromere only one-eighth (Hörstadius, 1936; Cameron and Davidson, 1991). In addition, Wray and McClay (1988) showed that, in a reproducible percentage of cases, chimeras can be recovered in which the number of labeled cells and the fates of the labeled cells are consistent with the incorporation of a blastomere derived from a 32- or 64-cell embryo, even though the donor cells derive from populations of dissociated 16-cell embryos. If a smaller incorporated clone were derived from the vegetal granddaughter of a macromere (i.e., a **veg2** cell), the clone would be expected to produce only endoderm and mesoderm, based on existing fate maps (Hörstadius, 1973; Cameron and Davidson, 1991; Logan and McClay, 1997; these expected patterns are summarized in Fig. 2).

Mesomeres and macromeres are not sufficiently different in size in *L. variegatus* to cleanly separate them using the chimera technique. However, Wray and McClay (1988) found that in more than 50% of the cases, chimeric embryos derived from donor cells taken from near the top of the 8% sucrose layer produced labeled ectoderm consistent with the incorporation of a mesomere and that cells taken from near the bottom of the 8% layer of the sucrose gradient yielded labeling patterns consistent with the incorporation of a macromere or macromere descendant. Thus although the chimera approach does not permit us to say that a particular clone of cells was definitely derived from a macromere or macromere descendant, the data are consistent with the view that chimeras containing labeled gut tissue do in fact result from the incorporation of such cells.

Chimeras can be recovered with ectopic archenterons

Fig. 3 shows that chimeric embryos can be recovered at low frequency in *L. variegatus* in which the labeled cells produce an ectopic archenteron and associated structures in what appears to be an otherwise normal embryo (i.e., in which the host tissues perform typical morphogenetic movements, with the exception of specific alterations in patterning described below). Depending on the particular experiment, 4-8% of otherwise normal chimeras possessed labeled gut tissue in an ectopic location. The remaining 92-96% of embryos with labeled cells were either grossly abnormal (approximately 50%) or had homotopically incorporated cells. Wray and McClay (1988) previously reported that, in 52% of cases of homotopic incorporation, labeled gut tissue was found in the host archenteron, consistent with the incorporation of a macromere or macromere descendant. 26% had labeled patches of ectoderm consistent with incorporation of a mesomere (it is not possible to determine whether such patches are ectopic, as they have no effects on subsequent patterning), 10% had labeled primary mesenchyme cells, consistent with incorporation of a micromere, and the remainder had ambiguous patterns (see Wray and McClay, 1988, for further details). We obtained similar percentages of homotopically incorporated clones, which are not surprising, based on the purity of the macromere-enriched fraction used in these studies. In the case of homotopically incorporated, labeled gut

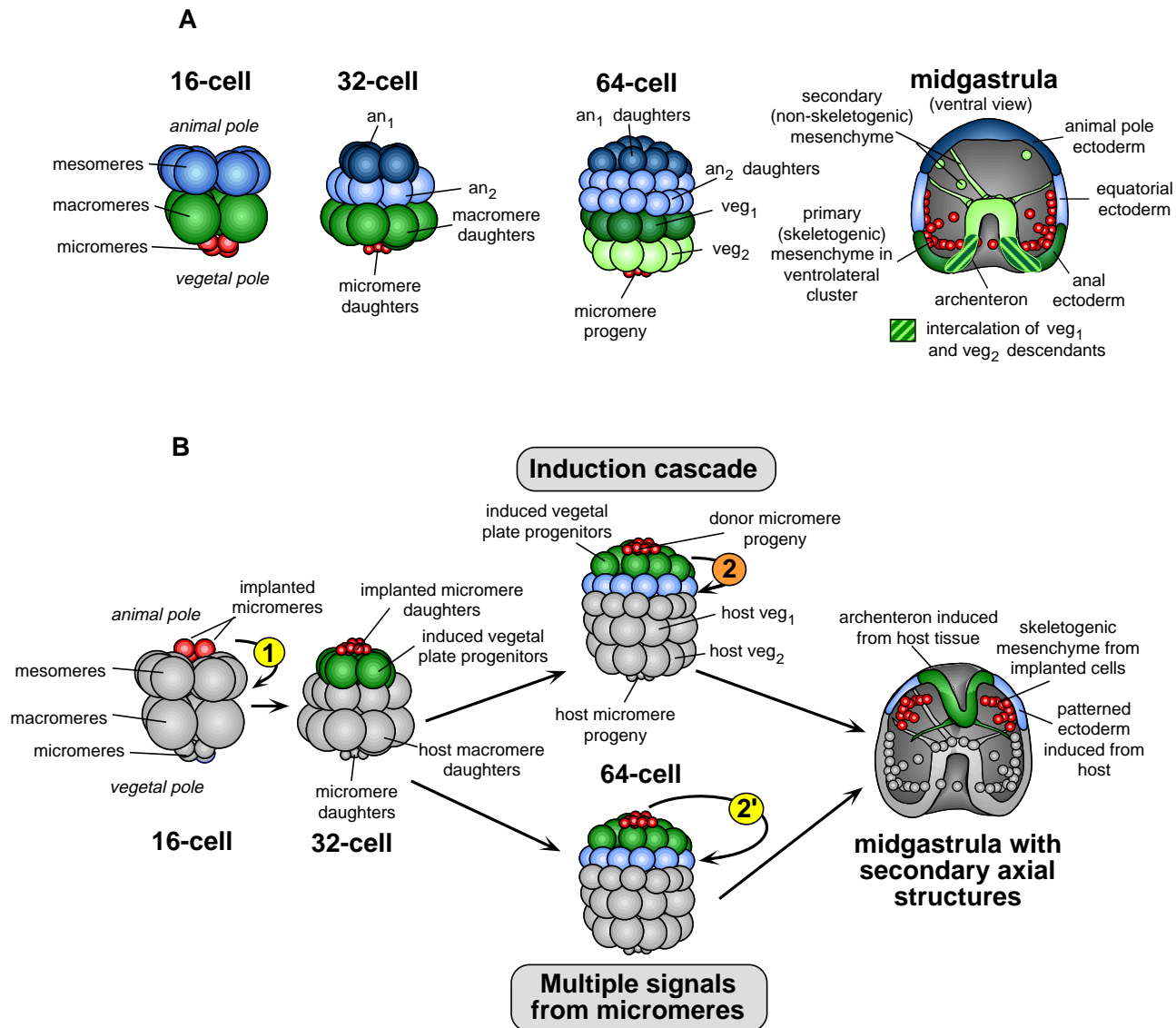
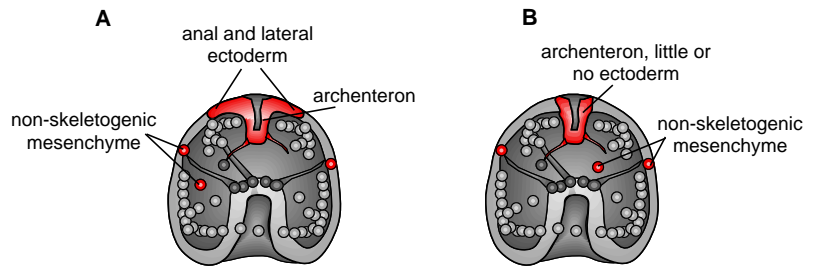


Fig. 1. Early blastomeres and the origins of axial structures in the sea urchin embryo. (A) Normal fates of early blastomeres in the sea urchin embryo, with special reference to axial structures of the gastrula/larva. Data are based on work from several species (Hörstadius, 1935; Cameron and Davidson, 1991; Logan and McClay, 1997). At the 16-cell stage, eight *mesomeres* (blue) lie at the animal pole of the embryo; below these are four large *macromeres* (green) and at the extreme vegetal pole, four small *micromeres* (red). At the next cleavage, the mesomeres divide to form the **an₁** and **an₂** tiers, the macromeres divide meridionally to produce eight macromere daughters and the micromeres divide to produce micromere daughters. At the 64-cell stage, the macromere daughters divide equatorially to produce the **veg₁** and **veg₂** tiers. In the gastrula, the archenteron and its associated secondary mesenchyme cells are produced by **veg₂** descendants (light green), some archenteron tissue as well as anal and lateral ectoderm are produced by **veg₁** descendants (dark green; the zone of overlap between **veg₁** and **veg₂** descendants within the archenteron is indicated as a striped region), and the rest of the ectoderm arises from **an₁** and **an₂** descendants (dark blue and light blue, respectively). The skeletogenic, or primary, mesenchyme arises from the large micromeres of the 32-cell embryo (red). Two large ventrolateral clusters of primary mesenchyme cells lie above thickened regions of the ectoderm approximately at the boundary between **veg₁** and **an₂** descendants. (B) Possible means by which micromeres could induce axial structures. In the normal embryo, the **veg₂** cells, which are granddaughters of the macromeres of the 16-cell embryo, generate the archenteron. Two ectodermal sites adjacent to the archenteron in the ventrolateral region of the embryo thicken prior to gastrulation, and these sites correspond to the two bilateral sites where skeletogenic mesenchyme localize into clusters. At least three mechanisms involving inductive signals from specific blastomeres or their descendants are possible. (i) *A cascade of inductive signals*. In this model, micromeres (red) send signals to adjacent cells (signal 1) which differentiate as macromere-like cells (green). These cells or their descendants then send signals to adjacent cells in the ectoderm (signal 2), which go on to produce pattern information required by primary mesenchyme cells as they form the larval skeleton (blue). (ii) *Two inductive signals from the micromeres*. In this scenario, micromeres send out a signal to adjacent cells to adopt a macromere-like fate, and a second signal to cells somewhat further away (signal 2'), which induces them to form ectodermal patterning sites for skeletogenic mesenchyme. The molecular nature of the two signals need not be distinct. (iii) *Combinatorial signals* (not shown). In this model, micromeres induce adjacent cells, which adopt a macromere-like fate. Then both the micromeres and the induced macromere-like cells send inductive signals to nearby tissue, thereby inducing skeletal patterning sites (i.e., a combination of signals 2 and 2').

Fig. 2. Expected patterns of ectopic tissue derived from macromeres or macromere descendants. Data are based on the fate-mapping studies of Hörstadius (1935), Cameron and colleagues (reviewed in Cameron and Davidson, 1991), and Logan and McClay (1997). (A) Ectopically incorporated macromeres or macromere daughters would be expected to produce labeled gut, non-skeletogenic mesenchyme and adjacent anal/lateral ectoderm. (B) In contrast, ectopic *veg2* cells would be expected to produce only gut and non-skeletogenic mesenchyme, with little or no ectoderm.



tissue, both Wray and McClay (1988), using *L. variegatus*, and Hardin (1989), using *L. pictus*, reported that no disruption of axial patterning was obtained and we obtained similar results in the present study. We did not see situations in which both labeled gut tissue and labeled primary mesenchyme cells were obtained in a single chimera. Such patterns could conceivably result from the incorporation of a vegetal blastomere from an 8-cell embryo (which gives rise to a micromere and a macromere at the next division), or from simultaneous incorporation of a micromere and a macromere from a 16-cell embryo. Only chimeras with labeled, ectopic archenterons had skeletal pattern defects and thus such chimeras were the focus of study. In subsequent sections, data are only reported for chimeras with such ectopic archenterons.

We first screened chimeric embryos for labeled clones that only produced ectopic archenterons and a small amount of adjacent thickened ectoderm, which would suggest the autonomous differentiation of a macromere or half macromere. The donor cells adhere rapidly and, by the end of the 64-cell-stage, blastomeres are completely integrated into the host tissue (Fig. 3A). The labeled, clonal descendants of the incorporated blastomere are easily distinguished from the unlabeled host tissue in such chimeras, and there is a sharp boundary between labeled and unlabeled cells. The labeled cells in such cases generate tissues that are morphologically indistinguishable from typical anal and lateral ectoderm. In addition, they produce an obvious archenteron. The labeled tissue appears to perform the morphogenetic movements of gastrulation on roughly the same schedule as the host vegetal plate, based on (i) the timing of the onset of invagination of the archenteron, (ii) the time of appearance of migratory non-skeletogenic mesenchyme emanating from the tips of such archenterons and (iii) the timing of subsequent differentiation of the gut into its characteristic three parts. The archenteron is often small and somewhat short, presumably because it contains one-fourth the usual number of cells (Fig. 3B). One important difference between normal and ectopic vegetal plates is that, since the labeled clone is presumably derived from an incorporated macromere or macromere descendant, no primary mesenchyme cells are present within the ectopic vegetal plate. Thus there is no ingression of skeletogenic mesenchyme preceding the invagination of the

vegetal plate in the labeled tissue. The ectopic archenteron is capable of generating pigment cells and other non-skeletogenic mesenchyme cells, which migrate within the host blastocoel (Fig. 3B). Thus these archenterons are apparently capable of normal differentiation and morphogenesis despite their ectopic location.

Ectopic archenteron progenitors produce patterned guts and induce new bilateral skeletal elements

The results of the previous section indicate that ectopic archenteron progenitors differentiate autonomously in their new location. We next asked whether ectopic archenteron progenitors exert inductive influences on adjacent tissue. In normal embryos, two bilateral clusters of skeletogenic mesenchyme aggregate in the ventrolateral region of the embryo as the larval

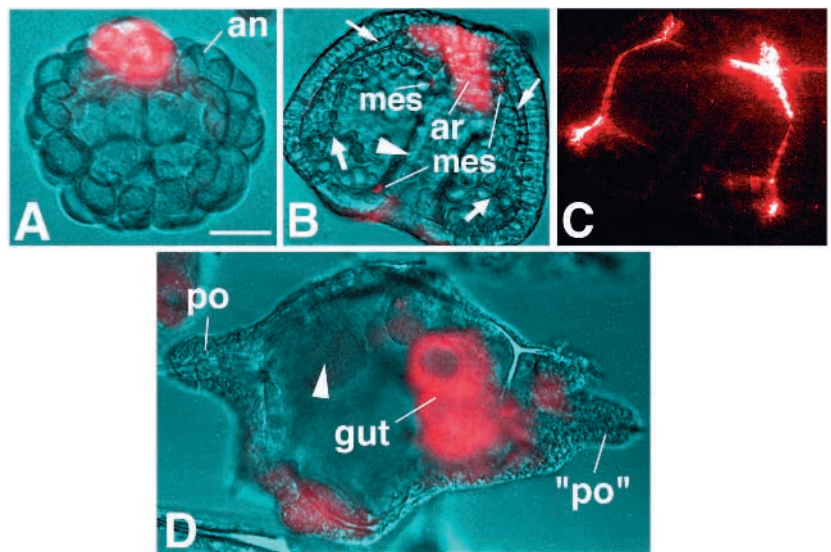


Fig. 3. Stages in the development of embryos containing ectopic archenteron precursors in *Lytechinus variegatus*. (A) A 64-cell embryo containing a labeled clone of cells near the animal pole (an). (B) A late gastrula with an incorporated gut progenitor at the animal pole. The incorporated cell has generated an ectopic archenteron (ar). In addition, two supernumerary skeletal elements are present (small arrows), atop unlabeled host ectoderm. The host archenteron (arrowhead) and the host skeletal elements (large arrows) are also visible in the same plane of focus. Non-skeletogenic mesenchyme derived from the labeled clone are also visible (mes). (C) Polarized light view of the chimera in B. Two sets of bilateral skeletal elements are visible in mirror-image symmetry. (D) A pluteus-stage chimera containing secondary axial structures. The labeled gut tissue is clearly visible (gut); the host gut rudiment is slightly out of focus (arrowhead). Note that postoral-like rods ('po') have been produced in a mirror-image symmetrical pattern with respect to the host postoral rods (po). The ectopic arms are from unlabeled (i.e., host) tissue. Bar, 20 μ m.

skeleton begins to form. These clusters ultimately generate the bilaterally symmetric skeletal spicules of the larva. These two sites flank the archenteron in normal embryos and are associated with local thickenings in the ventrolateral ectoderm (Okazaki et al., 1962). We therefore asked whether or not bilateral skeletal elements formed flanking ectopic archenterons in chimeric embryos. Fig. 3C shows the typical result, in which ectopic skeletal elements form (19 of 20 chimeras with ectopic guts had additional skeletal elements). If the incorporated archenteron is sufficiently distant from the host archenteron (e.g., at the extreme animal pole of the embryo) these skeletal elements are completely distinct from the aggregation of skeletogenic mesenchyme associated with the host archenteron, and can produce an embryo with mirror-image skeletal elements and two guts (Fig. 3D). In previous studies (Hardin, 1989), homotopically incorporated archenteron progenitors did not result in ectopic skeletal elements in *L. pictus*. This suggests that two spatially separated primordia are required for the production of ectopic skeletal elements and that such pattern defects are not simply the result of the excess archenteron precursor cells irrespective of their position. The primary mesenchyme cells that form such ectopic skeletal elements are not labeled, i.e., they derive from the host. Thus the ectopic skeletal elements form as a result of alterations in pattern formation among the host skeletogenic mesenchyme. Such mirror-image pattern defects look strikingly similar to those obtained by Ransick and Davidson (1993), with the important difference that the donor tissue does not produce any skeletogenic mesenchyme.

It is formally possible that the additional skeletal elements result from overproduction of skeletogenic mesenchyme by the host embryos. To determine if overproduction of skeletogenic mesenchyme occurred in the chimeras, we immunostained embryos with the monoclonal antibody 1d5, which recognizes an epitope on the surfaces of primary mesenchyme cells, and then counted the number of primary mesenchyme cells in chimeric embryos. Chimeric *L. variegatus* embryos possessed 63.3 ± 0.8 primary mesenchyme cells (mean \pm s.e.m.; $n=4$ embryos), which is essentially identical to the expected 64 primary mesenchyme in *L. variegatus* (Ettensohn and McClay, 1988).

The sharp boundary formed by the incorporated clone of cells also allows an unambiguous determination of whether the ectoderm over which the new skeletal elements form is derived from donor or host tissue. In all cases, although some ectoderm flanking the ectopic gut was labeled, the thickened ectoderm immediately underlying the additional skeletal elements was derived from the host. The thickened ectoderm was disposed as two bilateral patches; in vegetal views, these ectopic patches of ectoderm are especially clear (Fig. 4A). Pigmented mesenchyme often invade the two ventrolateral regions of thickened ectoderm in normal embryos (Gibson and Burke, 1985; J. H., unpublished observations). Likewise, non-skeletogenic mesenchyme derived from the implanted tissue can be found in the thickened ectoderm induced near the implant (Fig. 4A). At present, no molecular markers exist for this type of ectoderm; however, the morphological appearance of such thickened ectoderm provides clear evidence that the differentiation of ectodermal tissue near the implanted clone has been altered. Clusters of PMCs form in conjunction with the thickened ectoderm (Fig. 4B) and these PMCs secrete spicules

(Fig. 4C). Since new ectodermal patterning sites derive from host tissue and since no additional PMCs are formed in chimeric embryos, we conclude that the additional skeletal elements result from the induction of new, ectopic patterning sites within the host ectoderm. Some time after this induction, PMCs presumably migrate to these new sites.

Axial structures induced by ectopic gut progenitors incorporated at various axial positions

To determine if ectopic archenterons differentiate properly regardless of their location and to determine if the location of the ectopic archenteron affects its ability to induce axial structures, we examined chimeric embryos with secondary axes at various positions with respect to the animal vegetal axis of the host. Ectopically incorporated gut progenitors can incorporate at any axial position. The angle of incorporation ranged from 5° to 180° from the host vegetal pole; the distribution of incorporation angles was not significantly different from random at the 95% confidence level, using Rayleigh's test of circular uniformity (95% confidence limits on the mean angle = $124 \pm 27^\circ$; $n=14$ chimeras measured). Fig. 5 shows that ectopic macromere descendants can incorporate at any position along the animal vegetal axis and still induce axial structures in *L. variegatus*. As previously described, if the blastomere incorporates at the extreme animal pole, then mirror-image symmetric skeletal structures form (Fig. 2E). Blastomeres incorporated at lateral locations can also induce two new skeletal patterning sites, but only if the ectopic archenteron is sufficiently far away from the host archenteron (ectopic guts incorporated at $>86^\circ$ from the host blastopore induced two new, distinct patterning sites). Fig. 5A shows an embryo in which an ectopic archenteron formed approximately 30° from the animal pole (i.e., 150° from the host archenteron). This embryo was subsequently immunostained for the Endo1 antigen, which is normally expressed in the midgut and hindgut of the larva (Wessel and McClay, 1985). Both the endogenous gut rudiment and the rhodamine-labeled, ectopic gut rudiment are positive for the Endo1 antigen. Furthermore, the antigen is autonomously expressed in the correct position in the ectopic archenteron; staining appears at the base of the archenteron and extends two-thirds of the length of the archenteron, corresponding to the midgut and hindgut regions of the ectopic tissue. Identical results were obtained for ectopic archenterons incorporated at other axial positions (total of 4 embryos immunostained). This result indicates that, regardless of the axial position of incorporation, the archenteron differentiates autonomously, both in terms of gross morphology and in terms of at least one molecular marker.

We next assessed the effects of axial position of incorporation of the ectopic tissue on the induction of new skeletal patterning sites. To do this, we examined skeletal morphology using polarized light, or performed immunostaining at earlier stages using a monoclonal antibody specific for primary mesenchyme cells, whose pattern presages the bona fide skeletal elements. In the embryo shown in Fig. 5A, a partially mirror-image set of skeletal elements is evident, although the ectopic spicule closest to one of the two normal sites of skeleton formation is somewhat indistinct (Fig. 5B). If the incorporated blastomere is closer to the host archenteron (e.g., incorporation occurs at slightly less than 90° from the animal pole), then an ectopic skeletal patterning site forms on the side furthest from

the host archenteron. The two archenterons then share a skeletal patterning center between them, which is often aberrant in organization. The result is three distinct skeletal patterning sites (Fig. 5C,D). As with archenteron precursor cells incorporated at other axial positions, such clones produce non-skeletogenic mesenchyme that migrate away from the donor archenteron. Some of these cells eventually invade the ectoderm (Fig. 5C). In some cases, the ectopic blastomere incorporates immediately adjacent to the host. In this case, distinct skeletal elements are not found, although the ectopic gut rudiment appears normal (Fig. 5E). Overall, 13 of 14 chimeras with macromere-type ectopic clones possessed supernumerary skeletal elements. In every case, thickened ectoderm was present underneath the supernumerary skeletal elements, indicating that the ectoderm in these regions had undergone a change in differentiation compared with the same tissue in normal embryos. These results indicate that (1) endodermal founder cells can autonomously differentiate as gut when incorporated at any axial position and (2) that the ability to 'organize' distinct axial structures depends on the position of incorporation of the ectopic blastomere with respect to the host archenteron.

Incorporated cells that make only mesoderm and endoderm can induce new bilateral skeletal elements

Macromeres and half-macromeres normally give rise to both mesoderm and endoderm derived from the archenteron, as well as anal ectoderm. Many of the chimeras with ectopic guts that we identified also contained both labeled gut tissue and labeled ectoderm flanking the ectopic gut (Fig. 3), consistent with the view that these ectopic guts arose from macromeres or macromere daughters. However, it is the **veg2** cells that are the definitive founder cells of the early embryo that give rise exclusively to archenteron tissue. In order to determine if cells that give rise exclusively to gut tissue (and the mesenchyme cells that normally migrate away from the archenteron) are capable of inducing axial structures, we next scored for chimeric embryos in which the boundaries of the ectopically incorporated tissue were restricted to the gut and associated non-skeletogenic mesenchyme. If such embryos also have ectopic skeletal patterning sites, then this would indicate that precursors of the archenteron are capable of signaling adjacent tissue to differentiate as ectodermal patterning sites for skeletogenic mesenchyme. Chimeras with labeled gut tissue but no labeled ectoderm can be recovered, although they are rare. Fig. 6 shows that ectopic tissue composed exclusively of archenteron with no ectodermal component can induce adjacent skeletal patterning sites. The ectopic, bilaterally symmetric skeletal sites in embryos with exclusively endodermal clones are indistinguishable from those derived from clones containing both ectopic endoderm and anal ectoderm (Fig. 6A; cf. Fig. 3). Indeed, in some cases, mirror-image bilateral skeletal elements are produced, again as a result of alterations in the patterning of the host skeletogenic mesenchyme (Fig. 6B). All of the 6 chimeras examined with this type of labeling possessed supernumerary skeletal elements. As with the chimeras analyzed in the preceding section, in every case the supernumerary skeletal elements were accompanied by the thickened ectoderm characteristic of skeletal patterning sites in the normal embryo. This result indicates that the ectopic tissue does not need to

produce ectodermal tissue in order to induce supernumerary axial structures.

DISCUSSION

Usefulness of chimeric embryos for the study of blastomere interactions in early sea urchin embryos

The best experiment to test the inductive capacities of the gut progenitors of the early sea urchin embryo would be a direct transplantation of a macromere, a half macromere, or a **veg2** cell to an ectopic location, followed by an assay later in development for the presence of ectopic skeletal patterning sites. However, Hörstadius could not perform such transplants reliably (Hörstadius, 1935), so it is unremarkable that we were unable to do so as well (J. H., unpublished observations). The chimeric embryo technique that we have used is a sensible compromise approach until direct transplantation experiments can be successfully performed. We have shown clearly that archenteron progenitors can organize secondary axial structures in the early sea urchin embryo, since chimeras containing only labeled gut tissue also have ectopic skeletal patterning sites in a high percentage of cases. However, the chimera approach is limited by the inability to show unambiguously that labeled gut tissue derives from a macromere or **veg2** cell; we must infer the origin of the parent blastomere by its pattern of differentiation. We can only state that such clones generate patterns consistent with their being derived from a macromere, a half macromere, or a **veg2** cell.

There are at least two possible explanations for how the more restricted patterns that we have observed arise: (1) inhomogeneity in the age of the embryos, such that there were actually some 32-cell or 64-cell embryos in the dissociated population, or (2) a blastomere from a dissociated embryo may have attached, but only a portion of the original clone remained adherent to the host embryo. We believe that these are equally likely explanations, because even in highly synchronous batches of embryos, restricted labeling patterns appear (J. Hardin and G. Wray, unpublished observations). In either case, a reasonable interpretation would be that a **veg2** cell or the **veg2** portion of a macromere-derived clone adhered to the host embryo.

A second concern that arises regarding the chimera technique is that, since we have screened for ectopic guts, which form at a low percentage compared with homotopically incorporated gut tissue, we have biased our results relative to the 'typical' behavior of a dissociated macromere or macromere descendant, which may default to the production of ectoderm in an ectopic location. However, a review of classical and more recent work involving macromeres or their descendants provides no evidence that macromeres default to producing ectoderm, either in isolation, in combination with other blastomeres, or when transplanted to a new site in an otherwise intact host. First, macromeres and half-macromeres (Driesch, 1900; Hörstadius, 1936), as well as isolated **veg2** quartets (Hörstadius, 1935) make guts and some ectoderm consistently in isolation. Second, whether in reaggregating embryoids (Bernacki and McClay, 1988), in recombinants of half-macromeres and animal cap cells (von Ubisch, 1932), or when macromeres are added to aggregates of mesomeres

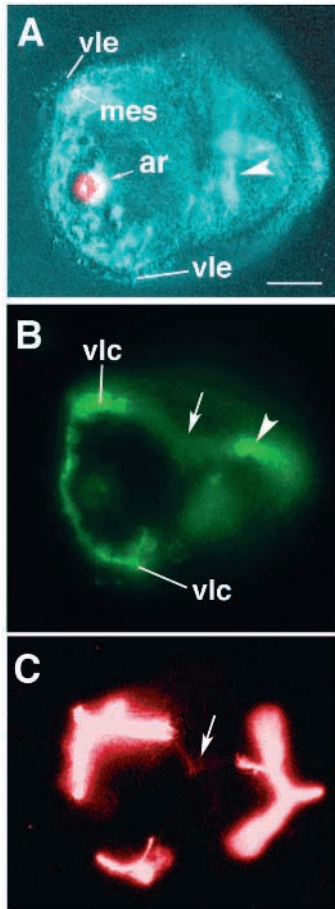


Fig. 4. Induction of thickened ventrolateral-like ectoderm in chimeras containing ectopic archenterons. (A) Vegetal view of a chimera containing a labeled, ectopic archenteron (ar). On either side of the ectopic archenteron are two sites of thickened ectoderm resembling normal ventrolateral ectoderm (vle). The host archenteron, whose base is in focus, is also visible (arrowhead). Non-skeletogenic mesenchyme has invaded the induced thickened ectoderm (mes). (B) Immunostaining for skeletogenic (primary) mesenchyme in the chimera shown in A. Ventrolateral-like clusters of skeletogenic mesenchyme (vlc) are clearly visible. One normally positioned ventrolateral cluster is visible out of the plane of focus (arrowhead). (C) Polarized light view of the same chimera. Notice that this chimera produced three major sites of skeletogenesis, rather than the normal two, as a result of the implanted tissue. However, there is an additional site of somewhat aberrant skeleton formation (arrows in B and C). Bar, 20 μ m.

(Khaner and Wilt, 1991), the gut tissue always derives from the macromere or its descendants. Third, **veg2** cells always produce an archenteron when transplanted to an ectopic location (Hörstadius, 1935). Finally, if the ability of endogenous macromeres or their descendants to produce gut tissue depends on their proximity to the normal vegetal pole of the embryo (where they are presumably induced by the micromeres or large micromere daughters; see Hörstadius, 1935; Ransick and Davidson, 1993), then we would expect that ectopic guts could be recovered relatively easily from positions close to the vegetal pole, but that chimeras with ectopic guts would be found progressively more rarely in locations further from the vegetal pole. However, we did not find this to be the

case (present Results). All of these studies argue against any loss of gut-forming potential by macromeres in ectopic locations and make it unlikely that we have observed the behavior of a subpopulation of extraordinary blastomeres. A reasonable conclusion that can be drawn from all of these studies is that macromeres (and their daughters and grand-daughters) produce an archenteron in all circumstances and that we are justified in supposing that by screening for clones that make gut tissue, we are examining typical macromeres or their progeny.

Which cells engage in inductive interactions leading to skeletal patterning sites?

We screened for two types of chimeric embryos with ectopic archenterons: (i) those containing labeled gut tissue and a small amount of flanking ectoderm (consistent with being derived from a macromere or half-macromere) and (ii) those only containing labeled gut tissue with no ectoderm (consistent with being derived from a **veg2** cell). Since the latter possess ectopic, bilateral skeletal elements overlying unlabeled host ectoderm, presumptive archenteron cells are capable of inducing such sites. Although the chimera technique does not permit us to conclude that **veg2** cells or their progeny perform such signaling, this result does suggest that the **veg2** cells of the normal embryo or their progeny can induce neighboring tissue to differentiate as skeletal patterning sites.

Although Hörstadius only reported results for two viable embryos, the chimeras that we have described containing ectopically incorporated clones that produce only archenteron behave in a manner similar to the implanted **veg2** cells described by Hörstadius (1935). Hörstadius did not report the induction of bilateral axial structures that we have reported here. There are several possible explanations for this discrepancy. First, the **veg2** cells in Hörstadius's experiments could have suffered damage during their isolation. Second, Hörstadius isolated **veg2** cells microsurgically by isolating vegetal halves of 16-cell embryos, allowing the vegetal halves to proceed through two more cleavage divisions, and finally peeling away the adjacent overlying and underlying tiers of blastomeres. The significant time required to perform this isolation may have resulted in **veg2** cells that were relatively advanced in age by the time they were added to host embryos. It is possible that the signals passed from **veg2** cells to surrounding tissue are passed soon after the **veg2** tier is born; in that case Hörstadius's procedure would bias against induction of axial structures. Similar age-dependent effects have been noted by Ransick and colleagues in the case of micromeres (Ransick and Davidson, 1993, 1995). Finally, although Hörstadius did not report the induction of new, bilateral skeletal structures, he did report that aberrant, additional skeletal elements were produced (Hörstadius, 1935); although not as dramatic as our results, these results are consistent with the interpretation that **veg2** cells or their progenitors are capable of inducing adjacent tissue to support skeletal patterning.

Evidence for sequential inductive interactions in the establishment of axial structures in the sea urchin embryo

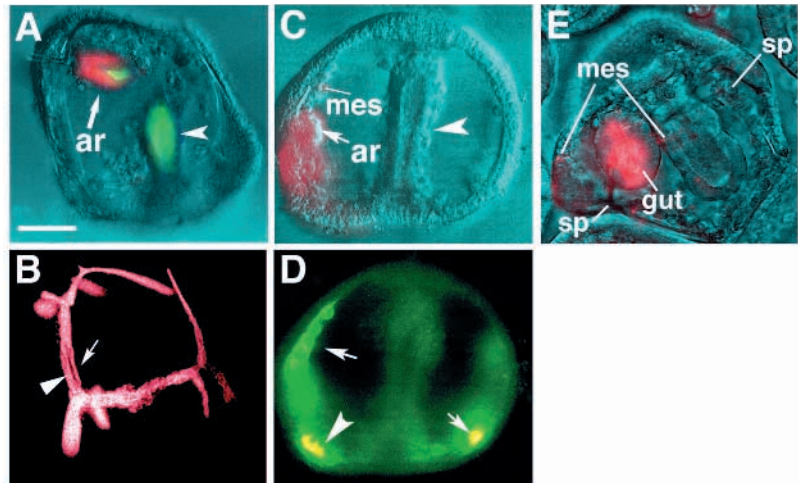
Experiments by Hörstadius (1935) and Ransick and Davidson (1993) indicate that micromeres of the 16-cell sea urchin

Fig. 5. The relationship between production of supernumerary skeletal elements and the position of incorporation of archenteron progenitor cells.

(A) Composite Nomarski and epifluorescence view of a *L. variegatus* chimera at the prism stage. The labeled archenteron (ar) is visible in red, incorporated $\sim 130^\circ$ from the vegetal pole of the host. Immunostaining for the Endo 1 antigen (green) reveals that both the host (arrowhead) and ectopic archenteron (ar) express the antigen.

(B) Polarized light view of the chimera shown in A. Skeletal rods from the host (arrowhead) and one induced by the donor tissue (arrow) lie parallel to one another (that the two rods are distinct was confirmed by through-focus under polarization optics). (C) Composite Nomarski and epifluorescence view of a *L. variegatus* chimera at the late gastrula stage. The labeled archenteron (ar) is visible in red, incorporated $\sim 90^\circ$ from the vegetal pole of the host. The host archenteron is also visible (arrowhead). Non-skeletogenic mesenchyme derived from the labeled tissue has migrated away from the archenteron (mes).

(D) Immunostaining of the chimera shown in C for a primary mesenchyme-specific antigen using monoclonal antibody 1D5. Notice that three large clusters of skeletogenic mesenchyme are visible. One of these appears to result from the fusion of clusters flanking the ectopic archenteron and the host archenteron (arrowhead); the other two clusters lie exterior to the host and donor archenterons (arrows). (E) Composite Nomarski and epifluorescence view of a *L. variegatus* chimera at the early pluteus stage. The labeled gut is visible in red, incorporated immediately adjacent to the host gut rudiment (gut). Non-skeletogenic mesenchyme cells derived from the ectopic tissue are also visible (mes). Only two spicules (sp) are present in this chimera, presumably because the site of incorporation of the ectopic tissue was so close to the site of the host gut progenitors. Bar, 25 μm .



embryo can induce mesomere progeny to differentiate as vegetal plate cells. In addition, ectopic skeletal elements are formed in such embryos. The experiments that we have performed indicate that ectopic macromeres or later vegetal plate progenitors are sufficient to induce new patterning sites, providing direct support for a model in which sequential signals organize axial structures in the vegetal region of the sea urchin embryo. Our experiments do not rule out additional signals emanating from the micromeres, but they do indicate that such signals are not necessary in the immediate vicinity of vegetal plate progenitors for them to induce ectopic bilateral patterning sites for skeletogenic mesenchyme. Such a sequential model is consistent with the proposals of Davidson (1989). In Davidson's model, 'membrane-tethered' ligands present on the surfaces of the large daughters of the micromeres induce immediately adjacent, overlying cells (i.e. the macromeres or their vegetal descendants, the **veg₂** cells) to adopt a gut progenitor fate. These cells in turn, via contact-mediated inductive signals, would induce the overlying cells (**veg₁** cells) to adopt a specialized fate, and so on. Davidson proposed additional interactions along the oral-aboral (dorsoventral) axis; our experiments do not directly address differentiation along this axis.

Developmental plasticity of ectoderm cells in the sea urchin embryo

Ectopic skeletal patterning sites can be produced throughout the host ectoderm in our experiments. This result suggests that, at the time of the inductive signaling, the presumptive ectoderm has not become committed to a particular, more regional fate. These results are consistent with the models of Davidson (1989), who has proposed that, while membrane-tethered ligands may be present on the surfaces of blastomeres with inductive capacities, all cells within the presumptive ectoderm contain receptors for such inductive signals. Such a result is consistent with other experiments examining the time of com-

mitment of ectoderm to produce skeletal patterning sites. Hardin and Armstrong (1997) have shown in the case of oral ectoderm near the animal pole that cell-cell interactions can generate new skeletal patterning sites until the early gastrula stage. Likewise, Hardin et al. (1992) have shown that the period of sensitivity to treatment with NiCl_2 , which results in conversion of dorsal (aboral) ectoderm to oral (ventral) ectoderm, extends to the mesenchyme blastula stage.

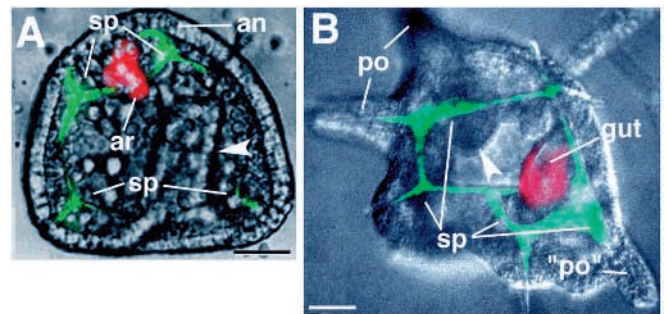


Fig. 6. Production of supernumerary axial structures in chimeras containing clones that produce only mesoderm and endoderm.

(A) Composite micrograph of a chimera at the early prism stage showing ectopically incorporated tissue that has formed an archenteron (ar) in red. Skeletal spicules are shown in green (sp). The ectopic tissue has incorporated near the animal pole (an), resulting in a second set of bilateral skeletal elements produced by host skeletogenic mesenchyme. Notice that the archenteron is labeled, but the surrounding ectoderm is not. (B) Composite Nomarski and epifluorescence view of a *L. variegatus* chimera at the pluteus stage. The labeled donor tissue (red) forms a gut rudiment (gut); skeletal spicules (sp) produced by host mesenchyme are shown in green. The host's postoral arms are visible (po); the incorporated tissue, which has produced only endodermal and non-skeletogenic mesenchymal derivatives, has resulted in the production of additional postoral-like rods ('po'). Bars, 25 μm .

The nature of inductive signals arising from archenteron precursors

Based on knowledge gained about local inductive interactions between blastomeres since Davidson's original proposal (Davidson, 1989), either membrane-tethered signals or highly local activities of growth factors could account for a sequential inductive cascade. The fusion of patterning sites for skeletogenic mesenchyme that occurs when the incorporated blastomere is close to the host vegetal pole suggests that the distance from, or intensity of the inductive signal(s) produced by *veg2* cells or their progeny is important for the placement of patterning sites for skeletogenic mesenchyme. Presumably when the host and ectopic vegetal plates are in close proximity, the intervening ectoderm experiences high levels of inductive signals, resulting in a single skeletal patterning site. Thus our results are consistent with the operation of short-range diffusible signals. Our results are similar to those obtained by Yamada et al. (1991) in the case of the vertebrate floor plate. In their studies, ectopically implanted notochords resulted in both the induction of floor plate neuroepithelium and two adjacent pools of ventral-type motoneurons. However, when the ectopic notochord is sufficiently close to the endogenous notochord, a single expanded floor plate is induced above them. Yamada et al. (1991) suggested that the reason for this behavior is that the cells destined to become floor plate in the normal embryo experience high levels of a diffusible signal emanating from the notochord and that, in the extreme case in which two notochords are close together, a single large region of neuroepithelium experiences elevated levels of this signal. Recently, *sonic hedgehog* (*shh*) has been implicated as the major inductive signal resulting in floor plate and ventral motor neurons (Roelink et al., 1995). Several homologues of growth factors have recently been identified in the sea urchin embryo as well (Sidow, 1992; Stenzel et al., 1994). If a similar mechanism is operating in the case of presumptive archenteron cells of the sea urchin embryo, then a cascade of growth-factor-mediated, short-range signals may also operate in this simple deuterostome.

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