Size regulation and morphogenesis: a cellular analysis of skeletogenesis in the sea urchin embryo

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SUMMARY

The formation of the skeleton is a central event in sea urchin morphogenesis. The skeleton serves as a framework for the larval body and is the primary determinant of its shape. Previous studies have shown that the size of the skeleton is invariant despite wide experimentally induced variations in the number of skeleton-forming primary mesenchyme cells (PMCs). In the present study, we have used PMC transplantation, fluorescent cell markers and confocal laser scanning microscopy to analyze cellular aspects of skeletal patterning. Labeling of embryos with 5-bromodeoxyuridine demonstrates that the entire embryonic phase of skeletal morphogenesis occurs in the absence of PMC division. During embryogenesis, skeletal rods elongate by one of two mechanisms; either preceded by a cluster (plug) of PMCs or by extending along an existing PMC filopodial cable. Elongation of skeletal rods occurs exclusively by the addition of new material at the rod tips, although radial growth (increase in rod thickness) occurs along the length of the rods. Photoablation of a distinctive region of ectoderm cells at the arm tip results in an inhibition of skeletal rod elongation, indicating that a local ectoderm-PMC interaction is required for skeletal growth. The regulation of skeletal patterning was also examined in embryos that had been microinjected with additional PMCs and in half-sized larvae derived from blastomeres isolated at the 2-cell stage. Microinjection of 50-100 PMCs into the blastocoel at the mesenchyme blastula stage leads to an increase in the numbers of PMCs along all skeletal rods and a two-fold increase in the number of cells in the plugs, yet no increase in the length of the skeletal rods. The length of the anal rods can, however, be increased by microinjecting developmentally ‘young’ PMCs into the arm tips of late stage embryos. We find that the rate of skeletal rod elongation is independent of both the mode of rod growth (chain or plug) and the number of PMCs in the plug at the growing rod tip. Instead, the rate of elongation appears to be strictly regulated by the quantity of ectodermal tissue present in the embryo. These studies provide new information concerning normal mechanisms of skeletal growth and patterning and lead us to propose a model for the regulation of skeleton size based upon an intrinsic PMC ‘clock’ and an ectoderm-derived signal that regulates the rate of skeletal rod elongation.

Key words: sea urchin embryo, mesoderm, primary mesenchyme, skeletogenesis, morphogenesis, size regulation

INTRODUCTION

Studies of morphogenesis seek to identify the mechanisms that underlie changes in embryonic form (see Trinkaus, 1984; Bard, 1990). In the sea urchin embryo, the formation of the skeleton offers a unique opportunity to combine molecular and cellular studies of a critical morphogenetic process. The synthesis of the skeleton involves an interplay between a small group of skeletogenic cells, rigidly specified early in development with respect to their program of gene expression and developmental fate (Okazaki, 1975a), and a series of important cell interactions that regulate the size and shape of the skeleton (Ettensohn and Ingersoll, 1992; McClay et al., 1992).

The sea urchin larva has a distinctive, angular shape that has been proposed to play a role in orientation and swimming (Pennington and Strathman, 1990). The primary determinant of this angular form is the larval skeleton; in the absence of skeletal elements the embryo retains the spherical shape characteristic of the blastula and gastrula stages (references cited by Hörstadius, 1939 and Gustafson and Wolpert, 1963; see also Fukushi, 1962; Ettensohn and McClay, 1988; Pennington and Strathman, 1990). The embryonic skeleton is produced by the primary mesenchyme cells (PMCs), a population of mesodermal cells that undergo a series of changes in their adhesive properties and motile behavior during development. These cells have been studied extensively and considerable information is available concerning their early determination, migration, program of gene expression and later morphogenesis (see
reviews by Gustafson and Wolpert, 1967; Okazaki, 1975b; Solursh, 1986; Decker and Lennarz, 1988a; Wilt and Benson, 1988; Ettensohn and Ingersoll, 1992).

The crystalline rods (spicules) that constitute the skeleton are composed primarily of CaCO₃ embedded in an organic matrix of several glycoproteins (Wilt and Benson, 1988; Benson and Wilt, 1992). These skeletal rods are partially ensheathed by the syncytial filopodial cables of the PMCs (Okazaki, 1960; Gibbins et al., 1969; Millonig, 1970; Decker et al., 1987). Studies of PMCs cultured in vitro have shown that incorporation of [⁴⁵Ca] into new crystalline spicule material occurs primarily at the growing tips of the skeletal rods (Decker and Lennarz, 1988b), although the possibility of limited intercalary spicule growth (i.e., addition of new crystalline material along the length of the skeletal rods) has not been excluded. Time-lapse cinemicrographic studies of spiculogenesis in vivo demonstrate that the growth of the two pairs of arm rods (anal and oral arm rods) is preceded by a cluster or ‘plug’ of PMCs located at the rod tips (Wolpert and Gustafson, 1961). Recently, it has been shown that PMCs in these plugs and at other sites of skeletal growth are distinctive at the level of gene expression. At late embryonic stages, these cells express higher levels of mRNA encoding the PMC-specific cell surface protein msp130, a putative regulator of calcium transport (Carson et al., 1985; Leaf et al., 1987; Farach-Carson et al., 1989), than do PMCs along the length of the skeletal rods (Harkey et al., 1992). Because there is strong evidence that earlier in their morphogenetic program, all PMCs are equivalent with respect to their spicule-forming potential, migratory behavior and molecular properties (Wessel and McClay, 1985; Leaf et al., 1987; Ettensohn, 1990a; Harkey et al., 1992), the localized regulation of msp130 expression by PMCs at later stages suggests that external signals act to modulate the pattern of gene expression exhibited by these cells.

The classical demonstrations of the extensive regulative properties of the sea urchin embryo (Hörstadius, 1939) have been confirmed and extended by recent studies using fluorescent cell-labeling methods and molecular markers of cell phenotypes (Khaner and Wilt, 1991; Ettensohn, 1992; Ransick and Davidson, 1993). The formation of the larval skeleton provides an especially compelling example of the regulative nature of morphogenetic processes in this organism. Cell transplantation experiments have shown that when the number of PMCs in the blastocoel is increased greatly, even to 2-3 times the usual number of cells, the size of the embryonic skeleton is normal (Ettensohn, 1990a). The mechanism by which the embryo regulates the skeletal pattern in response to such wide variations in the number of skeletogenic cells is unknown.

In this study, we have used a combination of light microscopic methods to examine important features of normal skeletal growth and patterning. Confocal laser scanning microscopy has been used in combination with a cell-specific antibody to determine the distribution of PMCs in normal and experimentally manipulated embryos. The possible roles of PMC division and intercalary spicule growth in the formation of the skeletal pattern have been assessed, and fluorescence photoablation and cell transplantation have been used to analyze the role of ectoderm cells in skeletal patterning. These studies provide new information concerning the normal mechanisms of skeletal growth and lead us to propose an explanation for the regulative phenomenon described above.

MATERIALS AND METHODS

Embryo culture

Adult Lytechinus variegatus were obtained from a commercial supplier (Susan Decker, Hollywood, FL). Animals were induced to shed gametes by intracoelomic injection of 0.5 M KCl. Eggs were washed several times with filtered, natural seawater (SW), fertilized with a dilute suspension of sperm and cultured in SW in glass bowls. The developmental rate of the embryos was controlled by maintaining cultures at 18-25°C in constant-temperature water baths. In some experiments, larvae were fed Rhodomonas lens, obtained from the Center for the Culture of Marine Phytoplankton (West Boothbay Harbor, ME). Feeding was carried out according to Leahey (1986) using a simplified algal culture medium consisting of sterile SW supplemented with Alga-Gro Concentrate (Carolina Biological Supply Co.).

Immunofluorescence

Monoclonal antibody (mAb) 6a9b10 (Ettensohn and McClay, 1988) was used as a marker for the skeletogenic phenotype. This mAb recognizes the PMC-specific glycoprotein msp130 (Leaf et al., 1987; Parr et al., 1990) as well as several other PMC-specific cell surface glycoproteins (Ettensohn and Fuhrman, unpublished observations). Immunostaining of embryo whole mounts and mounting of specimens for confocal laser scanning microscopy was carried out as described by Ettensohn and Ruffins (1993), with slight modifications. Fixation of later stage (plateus larva) embryos with methanol usually caused retraction of the ectoderm overlying the arm tips, making it difficult to count accurately the numbers of PMCs in that region. For better morphological preservation, embryos were fixed for 1 hour at room temperature in either 10% formalin or 4% fresh paraformaldehyde (both in SW), followed by a brief rinse in SW and postfixation/dehydration in 100% methanol for 20 minutes at −20°C. Following immunostaining, embryos were resuspended in FITC-Guard mounting medium (Testog, Inc.) and mounted on glass slides with Scotch double-sided tape spacers as previously described (Ettensohn and Ruffins, 1993).

Confocal laser scanning microscopy

Immunostained whole mounts were examined with a Bio-Rad MRC-600 confocal laser scanning microscope (LSM) equipped with a 15 mW krypton-argon laser. Images were collected using a 60× (N.A. 1.3) planapochromat oil immersion lens and were Kalman filtered. The pinhole aperture was at the minimum setting in order to optimize z-axis resolution. In most cases, dual images of the same specimen were obtained simultaneously using one fluorescence channel and a substage fiber optic device to detect transmitted light (for single-labeled specimens) or two fluorescence channels (for double-labeled specimens). Image files were transferred from the LSM to a Macintosh IICl workstation, where they were processed using Adobe Photoshop software.

5-Bromodeoxyuridine labeling

Labeling of 5-phase cells with 5-bromodeoxyuridine (BrdU) was carried out according to Tanaka and Dan (1990) with modifications as previously reported (Ettensohn and Ruffins, 1993).

Fluorescent cell marking, cell transplantation and fluorescence photoablation

Vital fluorescent labeling of cells with rhodamine B isothiocyanate (RITC) and cell transplantations were performed according to

"...normal and experimentally manipulated embryos. The developmental rate of the embryos was controlled by maintaining cultures at 18-25°C in constant-temperature water baths. In some experiments, larvae were fed Rhodomonas lens, obtained from the Center for the Culture of Marine Phytoplankton (West Boothbay Harbor, ME)...."
Ettenson and McClay (1988) and Ettenson (1990a). Photoablation of RITC-labeled ectoderm cells was carried out as previously described (Ettenson, 1990b), with the following modifications. When using a Nikon Diaphot epifluorescence microscope, the size of the photoablated region was controlled by closing the iris diaphragm on the epi-illumination tube. Some ablations were carried out using an upright Zeiss epifluorescence microscope with a pinhole aperture inserted into the epi-illumination tube at the conjugate image plane (a 200 μm pinhole was suitable for single-cell ablations using a 40× objective lens). The cantilevered stage configuration of the latter microscope was preferable to the fixed-stage Diaphot as the image of the pinhole remained sharp regardless of the plane of focus. Although 550 nm excitation light (a standard rhodamine filter set) was used previously for photoablations, it was empirically observed that excitation with UV light (standard DAPI filter set) or with unfiltered mercury arc illumination (a filter set with the excitation filter removed and only an emission filter in position for eye protection during the illumination) was more effective at ablation of RITC-labeled cells. Although the mechanisms of light-induced damage to cells remain poorly defined, we hypothesize that this effect may reflect the cumulative action of UV-induced photooxidation of cellular proteins (McCormick et al., 1976) combined with free radical production associated with fluorescence decay (see Giloh and Sedat, 1982).

**Sucrose-swelling and blastomere separations**

Embryos with enlarged blastocoels were produced using the osmotic swelling method of Moore (1940). Embryos were fertilized in SW, then transferred after 5-10 minutes to a solution consisting of 30% 0.76 M sucrose (aqueous), 70% SW (vol/vol). Embryos were returned to SW at the mesenchyme blastula stage, when the wall of the blastocoel is no longer permeable to sucrose. Blastomere separations were carried out by fertilizing eggs in 10 mM paraaminobenzoic acid (PABA) in SW, then gently replacing the PABA-SW with calcium-free artificial seawater supplemented with 50 μM EGTA (CF-ASW). The eggs were subsequently washed several times with CF-ASW. At the 2-cell stage, embryos were demembranated and dissociated by pouring them twice through 76 μm Nitex cloth. Separated blastomeres were cultured sparsely in SW.

**RESULTS**

**Normal patterns of PMC distribution and skeletal rod growth**

Because there is no universally accepted nomenclature for the skeletal rods of the embryo and larva, we have adopted a combination of the different terminologies used by Hörstadius (1939), Gustafson and Wolpert (1967) and Okazaki (1975b) (Fig. 1). This nomenclature was chosen for simplicity and completeness. We favor the terms ‘oral rods’ and ‘anal rods’ for the two pairs of embryonic arm rods, rather than the more descriptive but cumbersome ‘anterolateral’ and ‘postoral’ rods (Okazaki, 1975b). The anonymous rods are identified as distinct rods (Gustafson and Wolpert, 1967) and the dorsoventral connecting rods are considered to be distinct from the oral rods since the angle between the two is often sharp (Okazaki, 1975b). We have adopted Okazaki’s terminology for the two pairs of arm rods that arise after feeding begins: the pre-oral arms (which arise from the dorsal arch) and the posterodorsal arms.

The distribution of PMCs at different developmental stages was analyzed by confocal laser scanning microscopy (Fig. 2). These data, shown in Table 1, reveal several important features of skeletal growth. As noted by Wolpert and Gustafson (1961), there are two distinct ways in which skeletal rods are formed. The growth of the oral and anal rods, which arise as branches of the dorsoventral connecting rods and anonymous rods, respectively, is not preceded by an existing syncytial PMC cable. Instead, these rods elongate by means of a cluster, or ‘plug’ of PMCs at the tip and extend the body of the embryo as they grow, giving rise to the characteristic larval arms. The body rods, in contrast, extend along an existing syncytial chain of PMCs; the former dorsal aspect of the subequatorial PMC ring.

In the case of those rods that grow by a plug mechanism (the oral and anal rods), the number of PMCs in the plug decreases as the rod elongates (Table 1). This decrease is significant when analyzed by two sided t-tests (P>99%). Therefore, unless cell death occurs, PMCs must leave the plug during rod growth and become deposited along the length of the spicule. Even at the late pluteus stage, however, when the larval arms have reached their maximum length in the absence of feeding, 1-2 PMCs (more commonly 2) remain associated with the tips of the anal and oral rods, suggesting that the length of these rods is not simply regulated by the supply of PMCs in the plug (see below).

PMCs that are aligned along the skeletal rods are not immobile and can redistribute themselves considerably. This is indicated by a significant increase in the distances that separate neighboring PMCs along skeletal rods. The average distance between the first and second PMCs along the anal rod (the first cell being the one closest to the anonymous rod) at the early pluteus stage is 18.3 μm (s.d.=6.3 μm), while the average distance between the second and third cells is 10.5 μm (s.d.=3.8 μm). By the late pluteus stage, these distances have increased significantly, to 40.8 μm (s.d.=7.8 μm) and 39.3 μm (s.d.=4.5 μm), respectively. In the absence of intercalary rod growth (see below), this increased separation can only be explained by the movement of cell bodies along the rods. PMC redistribution clearly takes place during body rod elongation, as well. Between the
prism and pluteus stages, although the overall length of the body rod does not change, the number of PMCs along the rod (excluding the scheitel) decreases significantly from 6-7 cells to only 2-3 cells (Table 1). Based upon their time-lapse studies of living embryos, Wolpert and Gustafson (1961) reported that, at the start of arm formation, PMCs migrate along the skeletal rods to become concentrated in the plugs. The changes in PMC distribution during later embryogen-

<table>
<thead>
<tr>
<th>Skeletal rod</th>
<th>Length (µm)†</th>
<th>No. PMCs on rod (total)</th>
<th>No. PMCs at rod tip‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prism Pluteus</td>
<td>Feeding Pluteus larva</td>
<td>Feeding Pluteus larva</td>
</tr>
<tr>
<td>Oral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>29±5 88±12</td>
<td>415±30</td>
<td>2.3±0.6 3.6±0.7</td>
</tr>
<tr>
<td>Right</td>
<td>27±4 82±11</td>
<td>247±21</td>
<td>2.4±0.6 3.5±0.8</td>
</tr>
<tr>
<td>Anal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>68±11 247±21</td>
<td>503±23</td>
<td>6.5±0.8 8.8±1.3</td>
</tr>
<tr>
<td>Right</td>
<td>67±12 242±22</td>
<td>503±23</td>
<td>6.4±1.0 9.1±1.1</td>
</tr>
<tr>
<td>Body</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>104±8 107±5</td>
<td>115±4</td>
<td>6.6±1.2 2.8±0.8</td>
</tr>
<tr>
<td>Right</td>
<td>105±10 107±4</td>
<td>115±4</td>
<td>6.5±1.1 2.6±0.9</td>
</tr>
</tbody>
</table>

*All data are from embryo cultures raised in natural seawater at 22°C. Embryos were fixed sequentially in paraformaldehyde and methanol and immunostained with mAb 6a9. Three developmental stages were analyzed: prism, late prism stage embryos (27.5 hours); pluteus, late pre-feeding pluteus larvae (40 hours); feeding larva, larvae fed with Rhodomonas lens and cultured to the six-armed stage. For each developmental stage, 20 embryos were scored. For feeding larva, no distinction was made between left and right rods, and the numbers of PMCs at the rod tips were not determined. Values shown represent means±standard deviations.

†The oral rod was measured from its distal tip to the junction with the dorsoventral connecting rod, and the anal rod from its distal tip to the junction with the anonymous rod. The length of the body rod was defined as the distance between the anonymous rod and the first major branch near the tip of the body rod.

‡PMCs with at least 50% of the cell body located within 25 µm of the tip of the skeletal rod.

§It was not possible to count accurately the numbers of PMCs at the branched tips of the body rods at late stages.

Fig. 2. Immunostained whole mounts examined by confocal laser scanning microscopy. (A) Anal arm of a 2-day (late prefeeding) pluteus larva. Scale bar (A and C), 45 µm. (B) Posterior-dorsal arm rudiment of a 4-day feeding pluteus larva. Scale bar, 20 µm. (C) Dorsal arch of a 4-day feeding pluteus larva.
esis provide evidence that these cells are capable of considerable movements along the skeletal rods during this period as well.

Comparisons between left and right rods of the same type show that bilateral symmetry with respect to the skeletal pattern is strict. The body rods show almost perfect symmetry; the mean lengths of the left and right rods differ by less than 1%. In the case of the anal and oral rods, the average lengths of the right and left rods vary by only 1-7%. When comparisons are made between left and right rods within the same embryo (rather than considering population averages), the body rods again show almost perfect symmetry (2-3% variation) while the anal and oral rods consistently show more variation (10-12%). Symmetry in the number of PMCs along the rods is also strict; in 90% of 120 right/left comparisons (40 for each of the three rod types), the number of PMCs associated with the two rods was identical or differed by only one cell. This is not unexpected given the overall symmetry of the original PMC subequatorial ring pattern (Ettensohn, 1990a).

After feeding begins, the total number of skeletogenic (6a9-positive) cells increases, approximately doubling to 105-110 cells by five days postfertilization. Some existing skeletal rods, most notably the oral rods, increase in length, while others, such as the dorsoventral connecting rods and body rods, do not (Table 1). Although the embryonic skeleton forms a single network of interconnected rods, skeletal elements initiated after feeding (the dorsal arch and postero-dorsal rods) are unassociated with this network (Fig. 2).

**PMC distribution and skeletal rod growth in PMC(+) embryos**

Previous work has shown that the overall size of the embryonic skeleton is regulated in response to greatly increased numbers of PMCs (Ettensohn, 1990a). To determine whether specific skeletal rods increase in length in PMC(+) embryos and to determine whether PMC transplantation results in the presence of increased numbers of cells in the plugs at the growing arm tips, immunostained whole mounts were analyzed by confocal microscopy. As shown in Fig. 3 and Table 2, microinjection of PMCs results in increased numbers of cells along the skeletal rods, including an increased number of cells in the plugs at the tips of the anal and oral rods. For example, the mean number of PMCs associated with the anal rods increases from 9 to 21 cells, while the number of cells along the body rod (excluding the scheitel) increases from 2-3 cells to 10-11 cells. Similarly, the number of PMCs in the anal and oral rod plugs in PMC(+) embryos is more than twice that observed in control embryos. Despite the increased number of cells along the rods and in the plugs, the length of individual skeletal rods is not increased in PMC(+) embryos at the end of embryonic skeletal growth (Table 2). The body rods of control and PMC(+) embryos are the same in length while, for reasons that are unclear, the arm rods are slightly (6-16%) shorter in PMC(+) embryos.

**Rates of skeletal rod elongation**

Rates of skeletal rod elongation were determined by col-

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**Fig. 3.** Immunostained whole mounts of control and PMC(+) embryos at the late, prefeeding pluteus stage, examined by confocal laser scanning microscopy. (A) Tip of the anal arm of a control larva. An average of 1-2 PMCs are found in the cluster (plug) at the arm tip at this stage. Scale bar (A, B and D), 10 µm. (B) Tip of the anal arm of a PMC(+) larva, showing an increased number of PMCs in the plug. (C) Oral hood of a control larva, showing the two oral rods and associated PMCs. Scale bar, 30 µm. (D) Tip of an oral rod of a PMC(+) embryo, with an increased number of PMCs (at least 5) present in the plug.
Table 2. PMC(+) embryos: skeletal rod lengths and PMC distribution*

<table>
<thead>
<tr>
<th>Skeletal rod</th>
<th>Length (µm)†</th>
<th>No. PMCs on rod (total)</th>
<th>No. PMCs at rod tip‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral rod</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>98±13 (117±12)§</td>
<td>11.2±4.1</td>
<td>3.8±1.6</td>
</tr>
<tr>
<td>Right</td>
<td>97±9 (108±10)</td>
<td>11.0±2.6</td>
<td>4.0±0.8</td>
</tr>
<tr>
<td>Anal rod</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>253±33 (280±22)</td>
<td>21.8±3.1</td>
<td>3.2±1.5</td>
</tr>
<tr>
<td>Right</td>
<td>250±36 (268±20)</td>
<td>21.0±3.9</td>
<td>3.3±1.5</td>
</tr>
<tr>
<td>Body rod</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>107±5 (108±7)</td>
<td>11.0±2.8</td>
<td>N.D.</td>
</tr>
<tr>
<td>Right</td>
<td>106±9 (108±7)</td>
<td>10.2±1.2</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

*60-80 PMCs were microinjected into mesenchyme blastula stage embryos (n=6). The embryos were allowed to develop for an additional 24 hours at 25°C, then were fixed as whole mounts and immunostained with mAb 6a9. At the time of fixation the embryos were late (prefeeding) pluteus larvae with maximally elongated arms, comparable to 40 hour embryos in Table 1.

†Skeletal rod lengths were determined as described in Table 1.

‡PMC's with at least 50% of the cell body located within 25 µm of the tip of the skeletal rod.

§Values in parentheses show skeletal rod lengths of control embryos (n=10) from the same batch that were allowed to develop in parallel with PMC(+) embryos.

Table 3. Rates of skeletal rod elongation*

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Skeletal rod</th>
<th>Average rate of growth (µm/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anal rod</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>Anal rod</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Body rod</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Anal rod</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Body rod</td>
<td>20</td>
</tr>
</tbody>
</table>

*Rates of skeletal rod elongation were determined by measuring the mean lengths of rods in populations of fixed embryos at different time points. The difference between the means divided by the time interval was the average rate of growth. Intervals between measurements were no less than 2 hours and were chosen to ensure that rods were elongating throughout the entire interval. Prism stage embryos were used for measurements of body rod elongation and early-mid pluteus stage embryos for measurements of anal rod elongation, except for trial 3 (below). Each trial represents a different embryo batch and 20 rods of each type were measured at each time point. All embryos were cultured in temperature-controlled water baths at 25°C.

Because the elongation of the body rods is completed earlier than that of the arm rods (see Table 1) and because the body rods extend along an existing chain of PMCs, we considered the possibility that their rate of elongation might be greater than that of either the oral or anal rods. This was not the case, however. As shown in Table 3, the average rate of rod elongation was 17-20 µm/hour (at 25°C) for all three rod types tested. In addition, in one trial (Trial 3, Table 3), the entire period of anal rod elongation, from early to late pluteus stage, was divided into four separate intervals, each 2-5 hours in duration. The rate of rod elongation was similar during these intervals.

Because the number of PMCs at the tips of the arm rods decreases during development (Table 1) while the rate of rod elongation remains constant (Table 3, Trial 3), the elongation rate must be independent of the number of PMCs in the plug. In support of this conclusion, the rate of rod elongation in PMC(+) embryos is the same as in control embryos although the number of PMCs in the plugs is doubled (Table 2). Spiculogenesis is initiated at the same time in control and PMC(+) embryos (Ettensohn, 1990a) and the final lengths of the rods are similar in the two cases (Table 2). In one experiment, direct measurements of rod elongation rates in PMC(+) embryos were made by culturing the embryos in individual wells of depression slides and measuring the lengths of the anal rods at the early pluteus stage and 7 hours later, at the mid-pluteus stage. In no case was an anal rod observed to elongate at a rate greater than the average control value of 20 µm/hour during this interval (n=10). These observations demonstrate that the rate of skeletal rod elongation in vivo is the same whatever rods grow by either a 'chain' or 'plug' mechanism and is independent of the number of PMCs at the growing tip over a wide range.

Because it has been recently reported that the size of the skeleton is reduced in larvae derived from blastomeres isolated at the 2- or 4-cell stage regardless of the number of PMCs present (McClay et al., 1992), we examined whether rates of rod elongation, determined from mean rod lengths as described above, might be altered in such embryos. As shown in Fig. 4, the rates of anal rod elongation in half-sized larvae were found to be significantly decreased when compared to control embryos from the same batch. In three independent trials, the average rates of anal rod elongation in control larvae were 22.4, 18.3 and 19.9 µm/hr, while corresponding values in half-larvae were 12.2, 10.7 and 12.9 µm/hour (54%, 58% and 65% of control values, respectively).

Intercalary growth

The autoradiographic studies of Decker and Lennarz (1988b) indicate that addition of new spicule material occurs primarily at the tips of the growing spicules. In an earlier
study of $[^{45}\text{Ca}]-$uptake by whole embryos, however, Nakano et al. (1963) showed that labeling of pluteus stage embryos for 2 hours resulted in incorporation of label along the entire length of the skeletal rods, although at higher levels near the rod tips. Because there appears to be little free exchange of calcium ions between the seawater and the spicule (Decker and Lennarz, 1988b), incorporation of $[^{45}\text{Ca}]$ along the length of the skeletal rods indicates that intercalary growth takes place. Such intercalary growth could be radial (i.e., result in an increase in the thickness of the skeletal rods) or could reflect growth along the long axis of the spicule, increasing its overall length.

Measurements of skeletal rod thickness provide evidence for radial intercalary growth. For example, between the early and late pluteus stages, the average thickness of the anal rod (measured near its junction with the anonymous rod) almost doubles, increasing from 3.5 µm (s.d.=0.3 µm, n=20) to 6.3 µm (s.d.=0.5 µm, n=20), a statistically significant increase ($P>99\%$). There is no indication of intercalary growth along the long axis of the rods, however. For example, the average length of the anonymous rod, which lacks a free end and is defined by the position of the anal rod branch point, remains constant at 15 µm throughout later embryogenesis. We also used the spike-like projections that form along the rods as landmarks to test for elongation by intercalary growth. Distances between projections along the anal arms were first measured at the early pluteus stage. Embryos were then cultured individually in glass depression slides and recovered at the late pluteus stage, when distances between the same pairs of projections were again measured. During the intervening 24 hour period, although the anal rods more than doubled in length, 85% of the cases (n=100) showed no measurable increase in the distance between projections, while 15% appeared to show a slight increase (10-15%), a change that might be accounted for by experimental error in the measurements. These data support the view that all, or nearly all, rod elongation occurs at the tips.

**PMC division**

To determine whether the formation of the embryonic skeleton takes place in the absence of PMC division, we used the BrdU labeling method of Tanaka and Dan (1990) in combination with PMC-specific immunolabeling. Confocal laser scanning microscopy was required in order to visualize the labeled nuclei of PMCs in the interior of the embryo and to distinguish them from the labeled nuclei of surrounding cells. Continuous labeling of embryos from fertilization to the pluteus larva stage with 1 µM BrdU resulted in distinct labeling of PMC nuclei (Fig. 5A). When embryos were labeled from the early gastrula stage to the late, prefeeding pluteus stage, however, no labeling of PMCs was evident, even at the growing tips of the skeletal rods (Fig. 5B). Numerous other cells incorporated label at these stages, especially those in the ciliary band. Control experiments showed that a labeling period of 60 minutes or less was required in order to detect incorporation of the label. As shown in Fig. 5 (C,D), a 1 hour exposure to BrdU at even lower levels (0.5 µM) resulted in distinct nuclear labeling. Based upon estimates of cell cycle times in other sea urchin species (Dan et al., 1980), it seems highly probable that 1 hour corresponds to less than a single S-phase in these cells. We therefore conclude that PMCs do not undergo DNA synthesis, and therefore do not divide, during the entire period of embryonic skeletogenesis.

In the course of labeling experiments using gastrula stage embryos, we observed that short-term exposure to BrdU always resulted in discrete patches of labeled cells (Fig. 5C,D). These patches varied in size from as few as 10 cells to more than 50 cells. They represent domains of cell cycle (S-phase) synchrony within the gastrula, and may be analogous to the mitotic domains described in other organisms (Foe, 1989), although we have not yet determined whether the domains in the sea urchin are consistent from embryo to embryo. As shown in Fig. 5D, labeling was often observed to be fainter at the periphery of a patch. This was not the result of the curvature of the specimen and the narrow focal plane, but reflected a reduced incorporation of label, indicating that synchrony breaks down gradually at the edges of domains.

**A local PMC-ectoderm interaction is required for skeletal rod elongation**

To test the hypothesis that a local interaction between ectoderm cells and PMCs at the arm tip might be required for skeletal growth, ectoderm cells at the tips of the growing anal arms were specifically eliminated using fluorescence-based photoablation. Cells were ablated by irradiating the apical regions of cells in a symmetrical zone near the rod tip (see Fig. 6A). Of 157 embryos photoablated at the early-mid pluteus stage, 140 (89%) showed an inhibition of arm elongation on the ablated side. The mean difference between the ablated and contralateral arm was 75 µm. An example of arm truncation following photoablation of the tip ectoderm is shown in Fig. 6B. In the remaining 11% of the cases, the difference in the length of the two arms was <10 µm. In most cases, it was apparent that limited elongation of the anal rod took place even after photoablation. In 30% of the photoablated embryos, the tip of the anal rod was observed to project through the ectoderm either immediately after the ablation or at the end of the experiment, while, in the remaining 70% of the cases, the only indication of the
photoablation was the disorganization and granular appearance of the ectoderm cells. This marked inhibition of arm elongation was seen only when ectoderm at the distal tip of the arm was ablated. Irradiation of an ectodermal region of similar size but located 25-50 µm from the tip did not result in a detectable decrease in the length of the ablated arm relative to the contralateral control (59/64 cases).

To control for the possibility that irradiation of the distal tip of the arm might ablate PMCs as well as ectoderm cells, some embryos were fixed immediately after photoablation, immunostained with mAb 6a9 and examined by confocal microscopy. As shown in Fig. 7A, photoablation changed the normally smooth appearance of the ectoderm cells, but the PMCs remained rounded and smooth, as in control embryos. When the irradiated region was deliberately extended proximally to include PMCs, however, the PMCs appeared highly fragmented and were clearly dissociated from the skeletal rod (Fig. 7B). The zone of irradiation could therefore be precisely controlled and PMCs were not ablated under standard experimental conditions.

**Regulation of skeletal rod length**

Transplantation of PMCs from mesenchyme blastula stage donors into recipients of the same stage does not result in an increase in the size of the embryonic skeleton (Ettensohn, 1990a, and above). To test whether the developmental stage of the PMCs might play a role in PMC patterning, RITC-labeled PMCs were isolated from mesenchyme blastula stage embryos and microinjected into the tip of the anal arms of early-mid pluteus stage recipient embryos (Fig. 8A). The number of PMCs injected ranged from 7 to 20 cells, and resulted in 2-6 labeled cells incorporated into the plug, with the remaining donor cells scattered along the arm rod. This operation resulted in an increase in both skeletal rod and arm length. Of 118 recipient embryos, 58% showed an increase in the length of the experimental arm relative to the contralateral control (average increase=38 µm), 34% showed no difference (<10 µm) and in 8% of the cases the experimental arm was shorter. An example of a recipient embryo at the late pluteus stage is shown in Fig. 8B. This observation suggests that the length of the anal rod prior to feeding is limited, at least in part, by the developmental stage of the PMCs.

The strict symmetry of the right and left body rods suggests that a single factor might regulate the length of both rods in a given embryo. Because the body rods grow along an existing chain of PMCs, one possibility is that the length of the rod is determined by the length of the PMC chain. This region of the PMC cable represents the dorsal aspect of the subequatorial PMC ring that forms along the blastocoel wall and its size is determined by the overall diameter of the blastocoel cavity. We tested whether an increase in the blastocoel diameter was sufficient to increase the length of the body rods. Embryos were raised in 30% sucrose until the mesenchyme blastula stage, when they were swollen osmotically by replacing the sucrose solution with SW (Moore, 1940). This treatment resulted in an average increase in the diameter of the blastocoel at the mid-gastrula stage from 135 µm (s.d.=4.5 µm, n=12) to 188 µm (s.d.=11 µm, n=12), an average increase of 39%.
Size regulation and morphogenesis

Blastocoel resulted in an increase in the average length of the body rods, from 133 µm in control embryos (s.d.=6.5 µm, n=40) to 152 µm in sucrose-treated embryos (s.d.=9.5 µm, n=42), an average increase of 14%. This difference was readily apparent upon microscopic examination of the embryos and was highly significant by a two sided t-test (P>99%). The average length of the anal arms was not increased in sucrose-treated embryos; in fact, it was slightly reduced (controls: mean=264 µm, s.d.=24.3 µm, n=40; sucrose-treated: mean=212 µm, s.d.=21.8 µm, n=40).

DISCUSSION

Morphogenesis and regulation

Wolpert and Gustafson (1961) noted that, despite considerable variability in some of the events that lead to skeleton formation (for example, in the pathways of PMC migration), the end result is relatively precise and constant. The ability of the embryo to regulate its skeletal pattern in response to wide variations in the number of skeletogenic cells is another indication of the robustness or buffering of morphogenetic processes (Bard, 1990). In this context it is useful to distinguish two separate kinds of embryonic regulation: ‘morphogenetic regulation,’ which involves the establishment of normal morphogenetic patterns in response to an experimental perturbation but without cell fate respecification, and ‘cell-type regulation,’ which has the additional characteristic of involving a respecification of cell fates through cell-cell interactions. In this study, we are concerned with the former mode of regulation, although sea urchin skeletogenesis provides an example of the second type as well (Ettensohn, 1992).

Normal parameters of skeletogenesis

To understand how the size of the skeletal pattern is...
regulated in response to variations in the number of skele-
togenic cells, it is necessary to understand several key
aspects of normal skeletal growth. First, it is important to
determine where growth occurs. Although addition of new
spicule material occurs primarily at the tips of the growing
rods, the location of the PMC plugs (Decker and Lennarz,
1988b), labeling of whole embryos with $^{45}$Ca indicates that
intercalary skeletal growth also takes place (Nakano et al.,
1963). This intercalary growth is restricted to radial growth;
i.e., the addition of material around the periphery of the
skeletal rod to increase its thickness, and there is little, if
any, increase in the length of the skeletal rods as a result of
intercalary growth.

Skeletal rod elongation occurs at a constant rate of
approximately 20 $\mu$m/hour (at 25°C), regardless of whether
elongation is by a ‘plug’ or ‘chain’ mechanism. Surpris-
ingly, for those rods that elongate by a plug mechanism, the
rate of elongation is independent of the number of PMCs in
the plug. Evidence for this comes from the observation that
the rate of anal rod elongation is independent of the number of PMCs at the
growing tip of the rod over a wide range. In addition, rod
eelongation requires a local interaction with ectoderm cells at the
arm tip. For simplicity, we postulate the existence of a single,
local ectoderm-derived signal that is required for and controls the
rate of rod elongation. See the Discussion for details.

Fig. 8. (A) Diagram of the heterochronic PMC transplantation
experiment. Rhodamine-labeled PMCs were removed from
mesenchyme blastula stage donor embryos and microinjected into
the tips of the anal arms of early-mid pluteus stage recipients.
(B) A recipient larva that was allowed to develop to the late
pluteus stage (24 hours after PMC transplantation). The arm that
was injected with PMCs (arrow) is longer than the contralateral
(control) arm. Scale bar, 70 $\mu$m.

Fig. 9. Model of skeletal size regulation. The length of an arm rod
is postulated to depend upon two factors: (1) the rate of rod
elongation, and (2) an internal PMC clock that limits the length of
time a given cell can engage in skeletogenesis. The rate of skeletal
rod elongation is dependent upon the amount of ectoderm present
in the embryo, but is independent of the number of PMCs at the
growing tip of the rod over a wide range. In addition, rod
eelongation requires a local interaction with ectoderm cells at the
arm tip. For simplicity, we postulate the existence of a single,
local ectoderm-derived signal that is required for and controls the
rate of rod elongation. See the Discussion for details.

Whether PMCs divide following ingression has been
unclear. Some workers have reported the appearance of
mitotic figures in mesenchyme cells (Agrell, 1953; Nislow
and Morrill, 1988), while others have argued against the
likelihood of cell division because of the relatively constant
number of mAb 6a9-reactive cells during later embryogen-
esis (Ettensohn and McClay, 1988) and the lack of evidence
of PMC division in time-lapse recordings of developing
embryos (Gustafson and Wolpert, 1961). Stephens et al.
(1986) showed that late blastula stage embryos treated with
aphidicolin, an inhibitor of DNA synthesis, develop almost
normal larval skeletons, indicating that cell division is not
essential for prefeeding skeletogenesis.

In the present study, we have used BrdU labeling to show
that the number of PMCs normally remains constant
throughout the entire period of embryonic skeletogenesis.
Our studies indicate that this probe penetrates rapidly into
dividing cells and is a highly sensitive indicator of DNA
synthesis. Time-lapse videorecordings show that, in L.
variegatus, a round of PMC division occurs shortly after
ingression (Malinda, unpublished observations). Nislow
and Morrill (1988) observed mitotic figures in PMCs of L.
variegatus at the mesenchyme blastula stage and concluded
that the number of PMCs doubles soon after ingression.
Similar observations have been made in other species
(Agrell, 1953), suggesting that this may be a common pattern. From these observations, it can be concluded that the final round of PMC division occurs shortly after ingress and that no further cell division takes place during embryonic stages.

**Cell interactions and skeletal patterning**

Although the PMCs are rigidly specified early in development to differentiate as skeletogenic cells (Okazaki, 1975a), the morphogenesis of the embryonic skeleton is intimately associated with a series of critical cell-cell interactions. Except for the fine structure of the spicule (simple vs. fenestrated), which is determined by intrinsic properties of the PMCs, most other key aspects of morphogenesis, including the pattern of PMC migration and localization, the positions of the two initial skeletal rudiments and the subsequent branching pattern of the skeleton, all are regulated by cell interactions (reviewed by Decker and Lennarz, 1988a; Ettenson and Ingersoll, 1992; McClay et al., 1992). Moreover, the ectodermal cues that govern PMC migration and patterning themselves arise as a result of cell interactions that take place earlier in embryogenesis, as shown by the observation that transplanted micromeres induce the formation of ectopic PMC target sites (Hörstadius, 1939; Ransick et al., 1993).

The localized expression of msp130 mRNA in PMCs at the growing tips of the skeletal rods strongly suggests that external signals modulate patterns of gene expression in these cells (Harkey et al., 1992). Photoablation experiments support the view that elongation (at least of the anal rods) is dependent upon a local interaction with ectodermal cells at the arm tip. Control experiments show that the region of photoablation can be precisely controlled and that PMC cell bodies are not damaged by the procedure. One potential limitation of these experiments, however, is that we have no way to assess the possibility of photodamage to regions of PMC cytoplasm that might be distal to the plug of PMC cell bodies. It seems unlikely that the truncation of the anal arm following photoablation is simply the result of a mechanical impediment caused by ablated ectodermal tissue. During normal arm elongation, it is the extension of the skeletal rods that generates force. This force is transmitted to the overlying ectoderm via the PMC plugs and results in a passive extension of the ectoderm (Gustafson and Wolpert, 1963). Such mechanical force would still be produced if rod elongation continued after photoablation and would be expected to deform the overlying ectoderm unless the rigidity of the photoablated cells was significantly increased.

It is not yet known whether tip ectoderm cells are uniquely able to express the putative signal, as we have not yet been successful in attempts to microsurgically replace tip ectoderm with ectoderm cells from other locations. The ectoderm cells at the arm tip, however, are known to be distinct with respect to their pattern of gene expression. The product of the EGF II gene, which is predicted to encode a secreted, EGF-like protein, is expressed primarily by ectoderm cells overlying regions of skeletal rod growth (Grimwade et al., 1991).

Micromere and PMC cultures have provided a valuable model system for the study of skeletogenesis and in vitro studies provide a basis for comparison with our present results. PMCs cultured in vitro in the absence of other cell types form skeletal rods that grow primarily at their tips, despite the absence of any spatially localized cues (Decker and Lennarz, 1988b). From this observation, it is apparent that a spatially localized ectodermal signal is not required for the restriction of skeletal rod elongation to the tip, which must be the result of some other property of the spicule. However, spiculogenesis in vitro takes place only in the presence of serum (Okazaki, 1975a) or EHS matrix (Benson and Chuppa, 1990), which must be added to the cultures for a specific period of time prior to skeletogenesis (Page and Benson, 1992). The essential serum- or matrix-derived factors have not been identified. These in vivo and in vitro results may not be inconsistent if one hypothesizes that ectoderm at the arm tip provides a permissive signal, analogous to the factor(s) supplied by serum or EHS matrix, which is required for rod elongation. This signal might act in concert with other, unidentified properties of the spicule that are responsible for restricting elongation specifically to the tip.

**Regulation of skeleton size**

Addition of PMCs results in morphogenetic regulation and the formation of a skeleton of normal size and pattern. (It should be noted that ‘size’ here refers to the lengths of the various skeletal rods and not to their mass. The overall mass of the skeleton might be increased in a PMC(+) embryo but may not be evident except as a small increase in average rod thickness, a possibility we have not explored.) Several simple explanations for the regulation of skeleton size in PMC(+) embryos can be ruled out. First, all donor cells in PMC(+) embryos continue to express a skeletogenic phenotype and are associated with the skeletal rods, ruling out the possibility that the cells adopt alternative fates or fail to migrate to the proper sites in the blastocoel (Ettenson, 1990a). A different explanation for regulation would be that, although more PMCs are present in a PMC(+) embryo, there is no overall increase in the number of cells at the critical sites of skeletal growth; i.e., at the tips of the growing rods. Examination of PMC(+) embryos by confocal microscopy demonstrates that this is not the case.

We propose that the size of the embryonic skeleton is determined by two factors: (1) the rate of rod elongation and (2) an internal PMC ‘clock’ that limits the length of time a given cell can engage in skeletogenesis (Fig. 9). Given these two parameters, a rate of growth and a duration of growth, the length of a skeletal rod is specified. Evidence for an internal PMC clock comes from cell transplantation experiments demonstrating that the length of anal arm rods can be increased by microinjecting PMCs from mesenchyme blastula stage donors into the arm tips of pluteus stage recipients. These experiments indicate that the developmental stage of the PMCs in the plug, rather than their number, is a key determinant of rod length. The internal clock may simply reflect the period of time that a PMC is capable of biosynthetic activities in the absence of feeding. Once feeding begins, in addition to the formation of new skeletal elements, existing arm rods increase considerably in length. The increase in anal rod length following the transplantation of developmentally ‘young’ PMCs also argues against the possibility that rod length is restricted by an inactivation of...
the local ectodermal signal, or by mechanical factors such as a limit of the extent to which the ectoderm can be passively stretched by the elongating skeletal rod.

The size of the skeleton appears to be regulated by the quantity of ectoderm present in the embryo. Separation of 2- or 4-cell embryos into individual blastomeres results in the formation of half- or quarter-sized larvae with correspondingly miniaturized skeletons (Driesch, 1893). As in the case of normal-sized embryos (Ettensohn, 1990a), PMC transplantation experiments show that the size of the skeleton in half- or quarter-larvae is independent of the number of PMCs and therefore the result of a reduction in the other tissues of the embryo (Armstrong and McClay, unpublished observations, cited in McClay et al., 1992). Our observations indicate that the effect of decreasing the quantity of other tissues is to reduce the average rate of skeletal rod elongation. It is therefore not necessary to assume that the internal PMC clock is altered in reduced larvae, as the decreased rate of rod elongation alone could be sufficient to account for the difference in skeletal size. The explanation for the dependence of rod elongation rate on the amount of ectoderm is unknown. It could, for example, reflect a decrease in the concentration of generally distributed, ectoderm-derived growth factors required for spiculogenesis, or might be a result of reducing the level of the putative local ectodermal signal at the arm tips. In the model presented in Fig. 9, we have assumed the simplest case; i.e., that a single ectodermal signal derived locally from the arm tip is required for and regulates the rate of rod elongation.

The above considerations apply principally to the elongation of the arm rods; in fact, several mechanisms of size regulation probably operate during embryogenesis and different skeletal rods may rely on these mechanisms to varying extents. It seems highly probable that the lengths of those rods that form by a ‘chain’ mechanism are more highly regulated because the interactions of the rods result in spatially complex, dynamic structures (Decker and Lennarz, 1985). As in the case of normal-sized embryos (Ettensohn, 1990a), PMC micromeres on extracellular matrix in the absence of serum. J. Exp. Zool. 256, 222-226.

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