INTRODUCTION

The essential element involved in determining embryonic cleavage patterns is the placement of the cleavage planes (Freeman, 1983). It is widely accepted that the cleavage plane is established by the position and orientation of the mitotic spindle; the cleavage plane is always perpendicular to the spindle and bisects it (Conklin, 1912; Rappaport, 1986; Strome, 1993; White and Strome, 1996). Rappaport (1961) demonstrated that the position of the cleavage plane is determined by the two poles, i.e., centrosomes or microtubule-organizing centers, of the spindle. The position and orientation of the mitotic spindle, therefore, dictates the cleavage plane and determines the cleavage pattern. During early cleavage in the embryos of many animal systems, blastomere spindles are oriented perpendicular to the spindles of parental blastomeres. In embryos exhibiting radial and bilateral cleavage, this alternating pattern is maintained through at least the third cleavage, whereas in spiralian this orthogonal pattern does not occur until the third cleavage. Embryos of the marine shrimp, *Sicyonia ingentis*, appear to follow certain criteria of the spiralian pattern. The spindles of the second metaphase are slightly oblique to the first parental spindle and subsequent spindles undergo a 90° rotation. Other spiralian characteristics, however, are not seen (Hertzler and Clark, 1992).

SUMMARY

During early cleavages of *Sicyonia ingentis* embryos, mitotic spindle orientations differ between blastomeres and change in a predictable manner with each successive mitosis. From 2nd through 7th cleavages, spindles orient at a 90° angle with respect to the spindle of the parent blastomere. Thus, spindle orientation is parallel to the cleavage plane that formed the blastomere. To determine if specific spindle orientations were intrinsic properties of individual blastomeres, we altered blastomere associations and asked how mitotic spindle orientation was affected in successive cleavages using laser scanning confocal microscopy. Linear embryos were constructed by dissociating 4-cell embryos and recombining the blastomeres in a linear array. The ensuing cleavage (3rd embryonic cleavage) of these linear embryos was parallel to the long axis of the embryo, resulting in four parallel pairs of blastomeres which lay in a common plane that was parallel to the substratum. The 4th cleavage produced a linear embryo with the 16 blastomeres arranged in four parallel quartets. Then, in preparation for 5th cleavage, spindles oriented at a 45° angle (not parallel as in normal development) with respect to the previous cleavage plane. When 8-cell linear embryos were separated into linear half-embryos, subsequent spindle orientations were not like those observed for intact 8-cell linear embryos, but rather regressed to the orientation seen in 4-cell linear embryos. We suggest that the reorientation of mitotic spindles during early cleavage of *S. ingentis* is neither an intrinsic property nor age dependent, but rather is cell contact related. Further, these results in conjunction with observations of non-manipulated embryos suggest that spindle poles (centrosomes) avoid cytoplasmic regions adjacent to where there is cell-cell contact during early development.

Key words: *Sicyonia*, Crustacea, Decapoda, mitotic spindle, cleavage pattern, cell contact
spindle orientation in the embryos of the pulmonate mollusks, *Lymnaea stagnalis*, *L. palustris*, *Physa acuta*, *Apaxa hypnorum*, *Coreutus cornex*, *Radix ovata* and *R. pereger*, however, questioned Costello’s ‘centriolar principle’ and suggested that spindle orientation was controlled by regions of cell-cell contact. In the present paper, we have re-examined this question using embryos of the marine shrimp *S. ingentis*. We believe that our results clearly demonstrate that spindle orientation in the shrimp embryo is not an intrinsic property of the blastomeres; but, rather, that spindle poles at early cleavage stages always become positioned in cortical regions where cell-cell contact is lacking.

**MATERIALS AND METHODS**

**Experimental materials**

*Scyonia ingentis* were obtained off the coast of Southern California at a depth of approximately 100 meters using otter trawls. Gravid females were transported to Bodega Marine Laboratory and maintained in 1000-gallon aquaria with running sea water. Spawns were induced as previously described by Pillai et al. (1988). Spawning females were held over finger bowls containing approximately 250 ml artificial sea water (ASW), prepared according to the formula of Cavanaugh (1956). The finger bowls were gently swirled for approximately 1 minute post-spawning to prevent eggs from aggregating or sticking to the substratum. Hatching envelopes (HE) were removed using a modification of a method originally described by Lynn et al. (1993). Eggs were collected and transferred to ASW, containing 0.5 mM 3-amino-1,2,4-triazole, 5 minutes post-spawning to prevent hardening of the HE. The eggs remained in this solution for approximately 50 minutes and then were filtered through 200 μm mesh latex screen to remove the unhardened HE. Eggs were washed three times with ASW subsequent to HE removal.

**Blastomere manipulation, fixation and immunofluorescence staining**

Blastomere manipulations were performed under a dissecting microscope using glass needles. For manipulation, embryos were maintained in ASW in 60 mm disposable Petri dishes coated with agarose (Sigma, type III, 1% in ASW).

Manipulated embryos were fixed at different stages with cold 90% methanol containing 50 mM EGTA at pH 6.0 (Harris, 1987) for 30 minutes. Fixed samples were washed with Tris-buffered saline (TBS) at pH 7.0 and embedded in agarose (Sigma, type IX, 1% in TBS) to prevent disassociation and blastomere damage during processing. Embedded samples were blocked with TBS containing 1% bovine serum albumin (BSA) for 1 hour, incubated with a monoclonal antibody to yeast α-tubulin (Sera-lab, MAS 077) diluted 5× with TBS containing 1% BSA for 1.5 hours, washed with TBS three times, blocked again with BSA for 1 hour, incubated with fluorescein or rhodamine-conjugated anti-IgG antibodies (Jackson Immuno Research Laboratories, Inc., # 715-016-150 or #712-086-153) for 1.5 hours and washed three times with TBS.

**Confocal microscopy**

Prior to microscopic examination, samples were dehydrated through a graded ethanol series and mounted in methyl salicylate (Summers et al., 1991). Cleared samples were examined with an Olympus BH-2 inverted microscope interfaced to a Bio-Rad MRC-600 laser scanning confocal system equipped with a krypton/argon laser. Optical sections were collected at 4-5 μm intervals and projected through the area of the mitotic spindles. In some instances, images were Kalman averaged. Collected image files were printed with a Lexmark laser printer or a Focus Dye Sublimation printer.

**RESULTS**

**Development with and without hatching envelopes**

During normal development in *Scyonia ingentis*, the first mitotic spindle is oriented obliquely to the polar axis (Fig. 1a; see also Hertzler and Clark, 1992; Hertzler et al., 1994). The first cleavage gives rise to two blastomeres, AB and CD; CD is slightly larger than AB. During the second metaphase the two spindles are oriented slightly oblique to each other (Fig. 1b; see also Hertzler and Clark, 1992). The resultant blastomeres (A, B, C and D) are arranged in a rotational-like pattern (Fig. 1c). The D blastomere is slightly larger than A, B and C, which are indistinguishable in size from each other.

Early blastomere arrangements in *S. ingentis* embryos are altered by the absence of the hatching envelope (HE). Fig. 1e depicts a 4-cell embryo in which the HE was removed prior to...
the first cleavage (at 50-55 minutes post-spawning). The first two cleavages resulted in four blastomeres that were aligned in a single plane, parallel to the substratum (>90% in each of 80 spawns; the number of spawns is high since early cleavages were used as an index to determine normal fertilization). The third cleavage resulted in an embryo composed of two super-imposed quartets resembling the blastomere arrangement of a typical radial cleavage (Fig. 1f). Fig. 1c shows a 4-cell-stage embryo in which the HE was not removed until after the first cleavage (95-100 minutes post-spawning), the arrangement of blastomeres at the 4-cell stage resembles a rotational cleavage pattern (>90% in each of 2 spawns). The third cleavage gave rise to embryos composed of two tiers of intercalating blastomeres (Fig. 1d) as previously described for HE intact *S. ingeritis* embryos (Hertzler and Clark, 1992). Regardless of whether a HE was present or not, embryos formed normal blastulae, gastrulated normally and developed into swimming larvae.

**Linear embryos**

To determine if spindle orientation was an intrinsic property of blastomeres, ‘linear embryos’ were formed by dissociating AB from CD blastomeres at anaphase of 2nd cleavage and reassociating them such that they formed a linear array at the completion of the 2nd cleavage that was either A-B-C-D or B-A-C-D; A and B blastomeres are indistinguishable from one another, however, the larger D blastomere is distinguishable from C, as is CD from AB (Fig. 2-1). The term ‘linear embryos’ is to indicate both the linear arrangement of the 4-cell-stage embryos and their later developmental stages. During first metaphase after linear embryo formation (third embryonic metaphase), mitotic spindles were always arranged parallel to regions of cell-cell (blastomere-blastomere) contact (Fig. 3a). In some linear embryos, approximately 10% (17 of 160 samples from 6 spawns), one or two spindles were seen oriented perpendicular to other spindles (Fig. 3a’), however, these too were always parallel to regions where blastomeres were in contact; i.e., the spindle poles were positioned in regions where there were no cell-cell contacts. In the majority of embryos (141 of 160 from 6 spawns), spindles formed parallel to each other (Fig. 3a). The result of the ensuing cleavage (third embryonic cleavage) was four parallel pairs (also as two parallel linear arrays) of blastomeres lying in a single plane parallel to the substratum (Fig. 2c). At the next metaphase (fourth embryonic metaphase), the spindles of the eight blastomeres were rotated 90° with respect to the orientation of their parental spindles. All eight spindles aligned parallel to each other and perpendicular to the substratum (Fig. 3b)
Again, poles of the spindles were located in cortical regions where there was no direct contact with a neighboring blastomere. This cleavage resulted in a linear embryo composed of four parallel quartets (also as four parallel linear arrays) of blastomeres (100% of the above 141 embryos that formed four blastomere pairs) that exhibited parallel spindle orientation (Fig. 2d). The members of each quartet were descended from the same blastomere in the original 4-cell linear embryo. In preparation for the fifth embryonic cleavage, the spindle alignment was rotated approximately 45° with respect to the orientation of parental spindles, not 90° (Figs 3d-g, 4a). In other words, spindle axes were not parallel to the previous cleavage plane. In addition, depending on the arrangement of blastomeres in a linear embryo, one of the inner quartets could be derived from either A or B blastomere; thus, the exact orientation of the spindle axis was dictated by the blastomere’s position within the embryo rather than the lineage of the cell. The spindles in blastomeres of the quartets at either end of the 16-cell-stage linear embryos were situated oblique to the long axis of the embryo (Figs 3d-f, 4a). The resultant cleavage of the blastomeres in these outer quartets produced octets of closely packed cells. In contrast, the 45° angle rotation of spindles in blastomeres from the two inner quartets was such that they formed a ring encircling the long axis of the embryo (Figs 3d-g, 4a). The resultant cleavage of the blastomeres of each of the two inner quartets produced a ring of eight cells around a hollow core. The result of these changes in spindle orientations at the fifth cleavage in linear embryos was the initiation of a spherically shaped blastula in 100% of the blastomeres that exhibited parallel spindle orientation (all 141 of 160 from 6 spawns; Fig. 2e). Seventeen of the remaining embryos (17 of 160 from 6 spawns), though not examined for spindle orientation, also developed into spherical blastulae. 100% of observed blastulae became swimming larvae. Two embryos were lost during the above trials.

**Linear half-embryos**

To determine if spindle orientation is stage specific, i.e., if there is a cleavage clock, 8-cell-stage linear half-embryos were bisected through their third cleavage plane. This resulted in two ‘linear half-embryos’ resembling 4-cell-stage linear embryos;
however, they were one cell cycle older (Fig. 2f). The term ‘linear half-embryos’ is used to describe both the linear blastomere arrangement of the 8-cell-stage half-embryo and their later developmental stages. Spindles for the first cleavage (fourth embryonic cleavage) of the linear half-embryos were oriented exactly as were their parental spindles one cycle earlier. That is, spindles were parallel to one another and to the substratum in 100% of 70 manipulated embryos from 5 spawns (Fig. 5a). Cytokinesis produced the four linear aligned blastomeres along a common plane and resulted in four parallel pairs of blastomeres reminiscent of the blastomere arrangement of an 8-cell-stage linear embryo (Fig. 2g). At the next metaphase (for fifth embryonic cleavage), instead of the 45° adjustment that was observed for intact linear embryos, spindle orientations and cleavage were as had occurred in the fourth cleavage of linear embryos (compare panels i and ii in Fig. 2). Then, in preparation for the next cleavage (sixth embryonic cleavage) of these linear half-embryos, the 45° spindle adjustment was observed in 100% of 70 manipulated embryos (Figs 5d-g, 4b), an orientation seen one embryonic cell cycle earlier in linear embryos.

**Quarter embryos isolated from 8-cell-stage linear embryos**

To further determine if spindle orientation is affected by cell-cell contact, quarter-embryos were used. These quarter-embryos were obtained by transecting 8-cell-stage linear embryos...
embryos perpendicular to their long axes; yielding four pairs of blastomeres, each pair descended from one of the first four blastomeres, A, B, C and D (Fig. 6). At the next metaphase (fourth embryonic metaphase), parallel spindles formed in paired blastomeres that were parallel to the plane of the previous cleavage in 100% of the embryos (8 of 8 from 3 spawns). The resultant cleavage produced four blastomeres similar to the quartets formed in an intact linear embryo of the same age. Synchronous orientation of spindles continued at the next metaphase (fifth embryonic metaphase); spindles formed parallel to each other and to the previous cleavage. Spindle poles were positioned in cortical regions where there were no direct cell-cell contacts. These spindles exhibited a 90° difference in orientation when compared with those of the inner two quartets of an intact 16-cell linear embryo and a 45° difference when compared with the outer two quartets. The resultant cleavage produced an octet consisting of two quartets of cells, one on top of the other in 100% of the embryos (above 8 of 8 from 3 spawns). This is in contrast to the donut-shaped arrangement of the inner quartets observed with intact linear embryos.

DISCUSSION

The present study with the marine shrimp *Sicyonia ingentis*, has demonstrated that: (i) the arrangement of early blastomeres is not critical for normal development; (ii) during early development, the orientation of a mitotic spindle is not an intrinsic property of the blastomere; (iii) spindle orientations and subsequent cleavage planes are related to cell-cell contacts and (iv) spindle orientation is independent of a blastomere's age.

By the 2-cell stage, individual *S. ingentis* blastomeres are incapable of developing into a complete organism (Hertzler et al., 1994); however, the association or arrangement of blastomeres during early cleavage is not critical to embryonic development. At the 4-cell stage, the arrangement of blastomeres is governed by the presence or absence of the hatching envelope. In embryos with an intact hatching envelope, the blastomeres were arranged similar to those found in a rotational cleavage pattern (Gulyas, 1975; Hertzler and Clark, 1992). Embryos later become planar, however, with all four blastomeres touching the substratum if the hatching envelope was removed before first cleavage (Fig. 1e). Regardless of the different blastomere arrangements in early embryos, spindle orientation adjusted and normal development ensued. A similar phenomenon of blastomere rearrangements has been reported in marsupial embryos that have had their zona pellucida removed; however, these embryos do not proceed through normal development (Selwood, 1989).

The fact that linear embryos developed into swimming larvae further supports the supposition that blastomere arrangements at the 4-cell stage are not critical to early development. This is probably facilitated by the ability of spindles to ‘re-orient’ with respect to the previous cleavage plane. The ability of spindles to ‘re-orient’ was clearly demonstrated in the linear embryo experiments. At the second metaphase after the linear embryos were formed (fourth embryonic metaphase), the daughter spindles rotated 90° with respect to the orientations of their parental spindles (compare Fig. 3a with b, c). At the third metaphase (fifth embryonic metaphase), spindle rotation was altered resulting in a 45° rotation (instead of 90°) with respect to the orientations of their parental spindles (Figs 3d-g, 4a). The result was the formation of a blastula (Fig. 2e). If the 45° rotation had not replaced the 90° one, the spindle poles would have been located in cortical regions adjacent to areas of cell-cell contact and a double layer of blastomeres or an elongated linear quadrille would have resulted (Fig. 7). This 45° adjustment of the spindles is inconsistent with Costello’s ‘centriolic principles.’ We thus hypothesize that spindle orientation is based on the topological positions of the blastomeres. The present results, indicating that spindle poles always locate in cortical regions of the cell not associated with a neighboring cell, are consistent with our hypothesis. The experiments with quarter embryos have further substantiated the significance of the relationship between cell-cell contact and spindle orientation.

The notion that cell-substratum interaction may play an important role in spindle orientation cannot be ignored; however, data presented are not consistent with this supposition, especially once embryos reach the 16-cell stage. While the spindles of most 4-cell-stage linear embryos were parallel to each other and to the substratum (Fig. 3a), spindles were seen that were perpendicular to the substratum (Fig. 3a’). Most convincing, however, is the fact that spindles of a 16-cell-stage linear embryo can be oriented at 45°, 135°, 225° or 315° to the substratum (Figs 3d-g, 4a), while quarter embryos at the 16-cell stage exhibit spindles that are perpendicular to the substratum (Fig. 6). These data strongly suggest that cell-substratum interaction does not play a role in spindle orientation.

Cell-cell contact related spindle orientation in early
cleavages has been proposed in pulmonate mollusks (Meshcheryakov and Belousov, 1975; Meshcheryakov, 1978a,b; Meshcheryakov and Veryasova, 1979). Meshcheryakov (1978a,b) speculated that cell contact precipitated changes in cell shape and that spindles oriented parallel to the long axis of the blastomere; as such, this suggestion supports Hertwig’s rule. In S. ingentis, however, spindles do not appear to require orientation along the long axes of blastomeres. This is certainly true starting at the 16-cell stage when the longest axes of the blastomeres is the apical-basal axis; the spindles are always perpendicular to this axis and parallel to the apical membrane (Figs 3g, 5g and figures 1H, 1I in Hertzler and Clark, 1992). A functional explanation for cell-cell contact induced cell polarization in the early development of the mouse embryo has been reported (Ziomek and Johnson, 1980; Johnson and Ziomek, 1981). Plasma-membrane-associated cytoskeletal elements of a polarized mouse blastocyst exhibit distinct differences between the apical and cell-cell contact regions (Ducibella and Anderson, 1975; Ducibella et al., 1977; Pratt and George, 1989). These cytoplasmic-membrane-related elements are believed to be ultimately responsible for the movement of centrosomes (Euteneuer and Schliwa, 1985; Inoue, 1990). This work is strongly supported by studies on centrosome localization in different types of cells (Bacallao et al., 1989; Rizzolo and Joshi, 1993; Zwaal et al., 1996). It is tempting to speculate that similar cell-membrane-related molecules are responsible for positioning the centrosomes and ultimately determine the spindle orientation in the blastomeres of S. ingentis embryos. While such speculation is obviously premature, the blastomeres of S. ingentis embryos may present an exciting system for such studies, especially when one considers the work being done with Caenorhabditis elegans.

Studies on C. elegans have demonstrated that the cortical region of cell-cell contact between specific cells can generate a centrosome attraction site, thus directing the spindle orientation and cleavage plane (Hyman and White, 1987; Hyman, 1989; Goldstein, 1995, White and Strome, 1996; Zwaal et al., 1996). The spindle orientations of S. ingentis blastomeres, up to the 32-cell stage, do not follow the pattern found in C. elegans. Instead, centrosomes are localized at the apical regions of blastomeres where there is no direct cell contact. This cell-cell contact avoidance property of the centrosomes, however, is in part lost during gastrulation and a pattern similar to that seen in C. elegans is established. During the initial phase of gastrulation (62-cell stage), a pair of mitotically arrested cells (ME cells) invaginate into the blastocoel. One spindle pole of each cell, which has direct contact with the ME cells, becomes localized to the cytoplasmic region of ME contact (Hertzler and Clark, 1992 and unpublished data).

Another intrinsic property of spindle orientation, demonstrated with echinoderms, is the ‘cleavage clock’ (Horstadius, 1973; Freeman, 1976, 1983). This principle suggests that the spindle orientations are programmed according to the age of the embryo. For testing the existence of the cleavage clock in S. ingentis development, linear half-embryos were generated. If a cleavage clock was the overriding control in S. ingentis early development, linear half-embryos spindles should have oriented 90° different from their parental spindles in the first cleavage (fourth embryonic), then exhibited a 45° rotation at the next. The 45° spindle adjustment, however, did not occur until the linear half-embryos were composed of 16 cells in a rectangular configuration (32-cell stage; Figs 4b, 5d-g). The orientations of the spindles were dependent on the numbers and the configurations of the blastomeres (compare panels i and ii in Fig. 2). That is, a linear half-embryo cleaved the same as a linear embryo that was one cell cycle younger. S. ingentis spindle orientations and thus the cleavage planes are related to the blastomere configurations, but not the age of the embryos.

In conclusion, we were able to demonstrate that in S. ingentis, the orientations of the mitotic spindles in early development are related to cell-cell contact and that the blastomere re-associated embryos were able to form blastulae by adjusting their spindle orientations. We suggest that this ability to adjust is not restricted to S. ingentis. For example, in the mouse, the establishment of blastocyst polarity is cell contact related (Ziomek and Johnson, 1980; Johnson and Ziomek, 1981). In sea urchins, although the majority of publications support the concept that spindle orientation is an intrinsic property of the blastomere (reviewed by Horstadius, 1973 and Freeman, 1983), we do know that blastomeres separated at the 2-cell stage can each form a blastula and give rise to a complete embryo. Is the adjustment of spindle orientations involved?

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REFERENCES


and archenteron formation in isolated blastomeres from the shrimp *Sicyonia ingentis*. *Dev. Biol.* 164, 333-344.


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