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The sperm entry point defines the orientation of the calcium-induced contraction wave that directs the first phase of cytoplasmic reorganization in the ascidian egg

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SUMMARY

Ascidians eggs are spawned with their cytoskeleton and organelles organized along a preexisting animal-vegetal axis. Fertilization triggers a spectacular microfilament-dependant cortical contraction that causes the relocalization of preexisting cytoplasmic domains and the creation of new domains in the lower part of the vegetal hemisphere. We have investigated the relationship between fertilization, the cortical contraction and the localization of cytoplasmic domains in eggs of the ascidian *Phallusia mammillata*. We have also examined the link between this first phase of ooplasmic segregation and the site of gastrulation.

The cortical contraction was found to be initiated on the side of the egg where intracellular calcium is first released either by the entering sperm or by photolysis of caged *InsP₃*. The cortical contraction carries the sperm nucleus towards the vegetal hemisphere along with a subcortical mitochondria-rich domain (the myoplasm). If the sperm enters close to the animal or vegetal poles the cortical contraction is symmetrical, travelling along the animal-vegetal axis. If the sperm enters closer to the equator, the contraction is asymmetrical and its direction does not coincide

with the animal-vegetal axis. The direction of contraction defines an axis along which preexisting (such as the myoplasm) or newly created cytoplasmic domains are relocalized. Two microfilament-rich surface constrictions, the 'contraction pole' and the 'vegetal button' (which forms 20 minutes later), appear along that axis approximately opposite the site where the contraction is initiated. The contraction pole can be situated as much as 55° from the vegetal pole, and its location predicts the site of gastrulation. It thus appears that in ascidian eggs, the organization of the egg before fertilization defines a 110° cone centered around the vegetal pole in which the future site of gastrulation of the embryo will lie. The calcium wave and cortical contraction triggered by the entering sperm adjust the location of cytoplasmic domains along an axis within that permissive zone. We discuss the relation between that axis and the establishment of the dorsoventral axis in the ascidian embryo.

Key words: fertilization, calcium, actomyosin-based contraction, cortex, dorsoventral axis, ascidian

INTRODUCTION

When eggs are fertilized, a cascade of ionic and biochemical signals trigger precisely timed spatial reorganizations of their organelles and cytoskeletal components. The cell cortex and cytoplasm transform. Microvilli grow and rearrange, the cortical and cytoplasmic actin cytoskeletons reorganize, asters made of dynamic microtubules grow and move within the egg, and pronuclei migrate. Successive rounds of mitosis and cytokinesis further restructure the cytoplasm and may partition cytoplasmic and cortical domains to different blastomeres with distinct developmental fates (see books edited by Dale, 1990; Schatten and Schatten, 1989a,b and reviews by Elinson, 1990; Sardet et al., 1994).

In ascidians, cortical and cytoplasmic domains consisting of distinct arrangements of organelles and cytoskeletal constituents are already present in the unfertilized egg. These domains are relocalized after fertilization in two distinct phases (Conklin,

1905; Jeffery and Bates, 1989; Sardet et al., 1994, 1989; Satoh, 1994; Sawada, 1988; Speksnijder et al., 1993). In eggs of the ascidian *Phallusia mammillata*, the first phase of cytoplasmic reorganization (ooplasmic segregation) occurs immediately after fertilization and is completed within a few minutes. The second phase takes place just after meiosis is completed (25 minutes after fertilization) and takes about 12 minutes (Sardet et al., 1989; Speksnijder et al., 1993). The first phase of reorganization is triggered by a calcium wave that sweeps through the egg. This presumably begins at the point of sperm entry (Speksnijder et al., 1990a) and it is dependent on a cortical actomyosin basket with its opening in the animal pole region (Sawada and Osanai, 1981; Jeffery and Meiers, 1983; Jeffery and Meiers, 1984; Sawada and Osanai, 1985; Sawada, 1988; Jeffery and Bates, 1989; Sardet et al., 1992). Two transient surface protrusions form between fertilization and cytokinesis. The first, which we call the 'contraction pole', forms 3-5 minutes after fertilization, remains constricted for about 8-10 minutes and then relaxes. The

second, which we call the 'vegetal button', forms transiently in the lower part of the vegetal hemisphere about 20 minutes after the contraction pole (Sardet et al., 1989).

We have suggested previously that in *Phallusia mammillata* the sperm entry site and particularly the calcium wave help direct the first phase of cytoplasmic reorganization and play a role in defining the position of the contraction pole in the vegetal hemisphere and so participate in setting up the dorsoventral axis of the embryo (Speksnijder et al., 1990a).

We have re-examined this question in detail. First we analyzed the relationship between the point of sperm entry, the onset of the calcium wave, and the cortical contraction it triggers. We determined the location of the contraction pole with respect to the animal-vegetal axis and traced the developmental fate of the surface region corresponding to the contraction pole and the vegetal button - a later, but related, temporary protrusion.

Our findings indicate that the site of sperm entry and the direction of the calcium wave define the exact location of the contraction pole within a permissible region dependent on the organization of the egg established before fertilization.

MATERIALS AND METHODS

Biological material

The tunicate *Phallusia mammillata* (Ascidiae, Tunicata) was collected and handled as described previously (Sardet et al., 1989, 1992). Eggs were dechorionated with trypsin, fertilized and cultured as reported in these publications. Particular care was required in handling and culturing eggs and embryos; gelatin-formaldehyde coated pipettes, glass and plastic dishes were used and contacts between eggs and/or embryos avoided. Normal tadpoles developed under these conditions.

Vital labelling and marking of eggs and embryos

Covalent labelling of the surface proteins of the unfertilized denuded ascidian egg with FITC at basic pH was done exactly as described previously for sea urchin eggs (Peters et al., 1984). Eggs were handled very gently and fertilized immediately after labelling and washing. The meiotic chromosomes of the unfertilized egg and the entering sperm chromosomes were labelled with the DNA binding dye Hoechst 33342 (Molecular Probes) (Speksnijder et al., 1990a). In order to analyze the cortical contraction, we attached freshly and finely ground Nile Blue particles to the surface of dechorionated Hoechst-labelled eggs just prior to fertilization and selected eggs that had an adequate number and distribution of particles over their surface.

Culture and development of embryos

Eggs and embryos were compressed slightly in a wedge made of gelatin-formaldehyde coated glass (Speksnijder et al., 1990b). For long term observation of development they were mounted between a coated slide and coverslip in a sealed microdrop chamber (Lutz and Inoué, 1986).

Actin microfilament labelling

Actin microfilaments were labelled with phalloidin-TRITC (Sigma or Molecular Probes) on whole eggs essentially as described before (Sardet et al., 1992). Hoechst 33342 was used to reveal the position of male and female chromosomes and nuclei, and DiOC2(3) (Molecular Probes) was used to label mitochondria (Sardet et al., 1989). Whole mounts of labelled eggs were observed using epifluorescence or confocal microscopes as described below.

Micoinjection, intracellular calcium visualization and flash photolysis of $InsP_3$

Dechorionated eggs were coinjected using a high pressure injection

system (McDougall and Sardet, 1995) with either calcium green-1 dextran (10,000 M_r ; Molecular Probes) at a concentration of 2-10 mM in the pipette and Hoechst 33342 (2 mM in the pipette), or calcium green dextran and caged $InsP_3$ to give final concentrations of 10 μM calcium green dextran, 5 μM $InsP_3$, and 2 μM Hoechst 33342 in the egg. About 30 minutes after injection, the eggs were observed using an epifluorescence Zeiss Axiophot microscope (40 \times /n.a. 0.9 oil objective) as described below. To photolyse the caged $InsP_3$ we irradiated the eggs using a UV filter set (excitation 353-377, emission 397 and FD 395) and inserting a neutral density filter to reduce the intensity of excitation light falling on the egg (this probably released a small amount of $InsP_3$ since only a local cortical contraction was initiated). The eggs loaded with calcium green dextran and Hoechst 33342 were fertilized and observed in time lapse video microscopy using a SIT (Silicon Intensified Target) camera and an image processor as described below.

Video and confocal light microscopy

For video-enhanced microscopy we used a Zeiss Axiophot microscope equipped with SIT and Newicon cameras (Lhesa). The images (low light epifluorescence or differential interference contrast - DIC) were processed through a video processing unit (Matrox card installed in an IBM PC/AT compatible personal computer and Universal Imaging software - Image 1) and recorded on video tape or disc (Sony UMatic SP recorder or Panasonic OMDR).

Epifluorescence observations were made on a Zeiss Axiophot microscope equipped with the following filters: excitation 353-377, emission 397 and FD 395 for Hoechst 33342, excitation 450-490, emission 520-560 and FD 510 for FITC and DiOC2(3), and excitation 534-558, emission 590 and FD 580 for TRITC.

Confocal laser scanning microscopy (CLSM) observations were performed on a Leica confocal microscope equipped with an Argon/Krypton laser as described (McDougall and Sardet, 1995)

Electron microscopy

Scanning electron microscopy was performed by I. Erk (CGM, Gif-sur-Yvette) on eggs fixed with a mixture of glutaraldehyde (2%) and paraformaldehyde (3.6%) in seawater.

RESULTS

The cortical contraction is initiated near the site of sperm entry and carries the sperm nucleus vegetally

At fertilization, the egg of *Phallusia mammillata* undergoes a drastic change in shape which is due to a cortical contraction. This contraction is triggered by the calcium wave initiated by the fertilizing sperm. Unfortunately, the site of sperm entry is undetectable by simple microscopic inspection (Sardet et al., 1989; Speksnijder et al., 1990a). On the basis of experiments with cytochalasin, we have suggested that, as in many other invertebrate and vertebrate eggs, the calcium wave in ascidians begins at the site of sperm entry (Speksnijder et al., 1990a). About 30-40 seconds after the initial increase in free intracellular calcium, a cortical contraction is triggered, propagating through the egg in approximately 120 seconds (McDougall and Sardet, 1995; Sardet et al., 1989). The cortical contraction starts in the animal hemisphere region and often causes an asymmetric deformation of the egg (Speksnijder et al., 1990a).

We have now been able to observe sperm entry and displacement directly by injecting eggs with calcium green dextran to reveal intracellular calcium increases and the DNA binding dye Hoechst 33342 to label the egg and sperm chromosomes (Fig. 1A). Upon fertilization a local area of calcium increase was

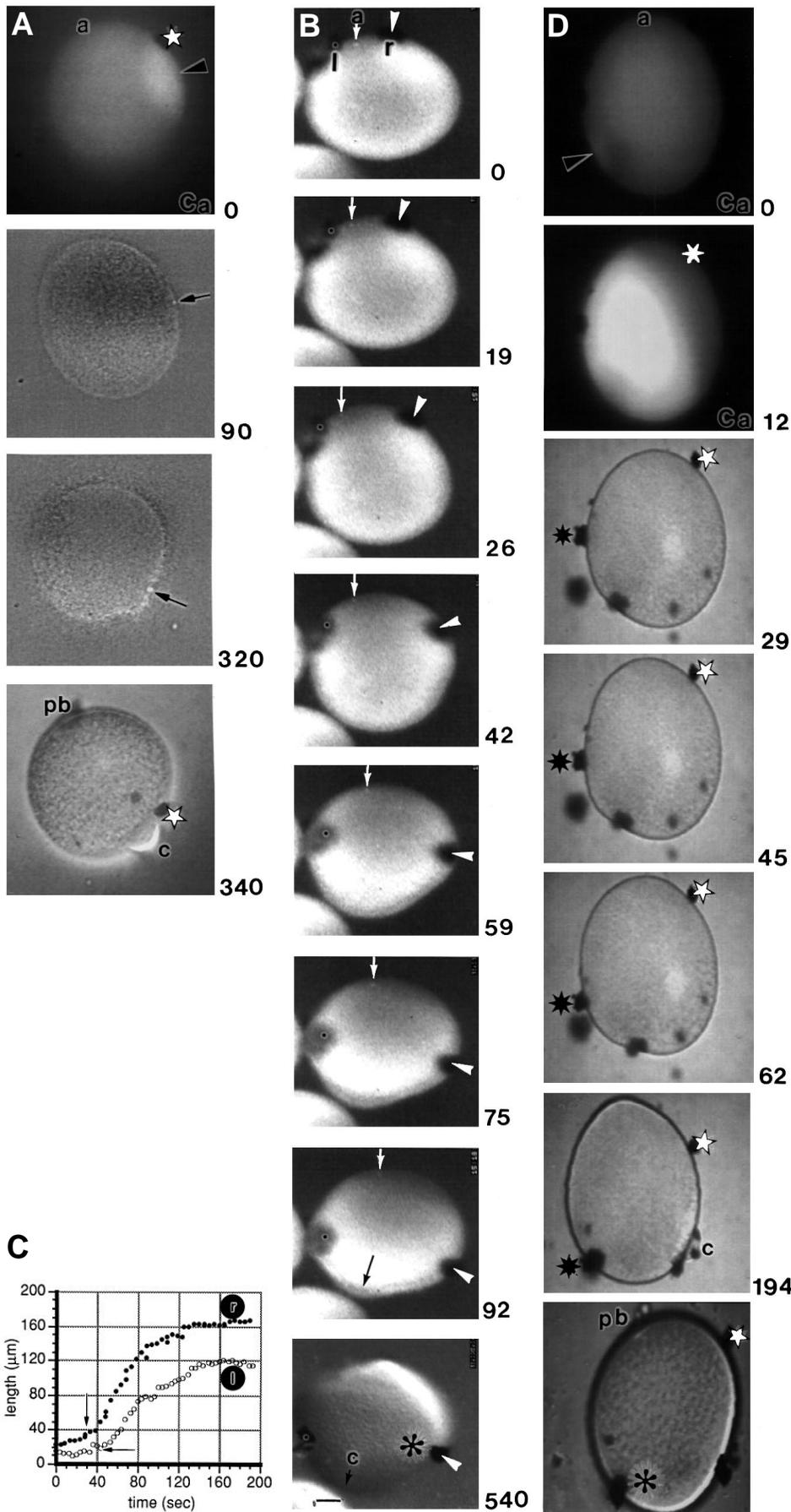


Fig. 1. Fertilization and the cortical contraction. (A) The fertilizing sperm moves vegetally with the contraction wave. An egg co-injected with calcium green dextran (10,000 M_r) and Hoechst 33342: the sequence shows four images extracted from a time lapse recording (1 image/10 seconds). Times in seconds are shown on the right. At time 0, the calcium wave (black arrowhead) is initiated at the egg equator (a: animal pole). A Nile Blue particle situated just above the fertilization site (white star) started moving 60 seconds after the onset of the calcium wave, moving vegetally toward the contraction pole (c) opposite the polar body (pb). The fertilizing sperm nucleus (black arrow) also moves vegetally. The sperm nucleus and the particle are not exactly in the same plane of focus. The middle two images were processed (image subtraction) to enhance the faint Hoechst signal from the sperm nucleus (white spot). (B,C) The asymmetry of the cortical contraction triggered by fertilization. (B) An egg is labelled with Hoechst 33342 marking the egg chromosomes at the animal pole (white arrow: a) and decorated with Nile Blue particles to follow the cortical contraction. At fertilization, the particle on the right (r: white arrowhead) although situated the furthest from the animal pole starts moving before the closer particle on the left (l: dot). The positions of the male aster on the side of sperm entry (asterisk) and of the contraction pole (c and black arrow) are shown (at 92 and 540 seconds). These images were selected from a low light level time lapse video recording (1 image/4-5 seconds). Bar, 20 μm . (C) Analysis of particle movements in the sequence shown in B. 'Length' is the linear distance between the animal pole and the right particle (black dots, ●) or the left particle (open circles ○). The particle (r) starts moving about 30 seconds after the start of the recording (vertical arrow) at an average speed of 2.0 $\mu\text{m}/\text{seconds}$ for about 50 seconds, slows down and stops at 120 seconds. The left particle (l) starts moving later (horizontal arrow), at an average speed of 1.7 $\mu\text{m}/\text{seconds}$ for about 50 seconds. It slows down and stops at about 130 seconds. (D) Asymmetric contraction and calcium wave. In this sequence the calcium wave (black arrowhead) begins below the equator (a: animal pole). A particle (black star) situated on the same side of the egg as the calcium wave starts moving at 45 seconds, whereas the particle on the opposite side (white star) is seen moving only after 62 seconds. The contraction pole (c) forms on the side opposite the site of the onset of the calcium wave. The location of the polar bodies (pb) and the sperm aster (asterisk) 26 minutes after fertilization are shown in the bottom image. Extracted from a time lapse video sequence (1 image/4 seconds).

observed within which was found the nucleus of the entering sperm labelled with the Hoechst dye. We then followed the migration of the male pronucleus and of particles affixed to the egg surface using low level light and time-lapse video microscopy. In three sequences where the sperm nucleus could be located between the start of the calcium wave and the onset of the cortical contraction the sperm nucleus moved toward the vegetal pole within 30-40 seconds of the local rise in calcium. Nile Blue particles on the surface of the egg close to the sperm nucleus moved with the same speed and direction. This demonstrates directly that the fertilizing sperm is carried towards the vegetal hemisphere by the cortical contraction.

In order to examine the asymmetry of the contraction, we compared the movement of Nile Blue particles positioned evenly around the animal pole (Fig. 1B,C) or close to and far from the point of sperm entry as indicated by the starting position of the calcium wave (Fig. 1D). We recorded and analyzed 13 video sequences in which the position of the meiotic chromosomes, and thus of the animal pole, could be located unambiguously after vital labelling of the egg chromosomes with Hoechst 33342. Seven sequences showed clear asymmetric movement of particles situated on either side of the animal pole. In the other sequences the movements appeared symmetrical. In two of the sequences in which movements were asymmetric, the particle that moved first was close to the sperm entry site and ended up close to the final location of the sperm aster and away from the contraction pole, despite being the furthest from the animal pole at the onset of the contraction (see sequence in Fig. 1B and its analysis in Fig. 1C). In these cases it was particularly clear that the contraction started first on the side of sperm entry and that the final position of the contraction pole was on the side opposite the sperm entry site.

We also tracked particles attached to eggs which were previously injected with calcium green dextran. Fig. 1D is an example of such an experiment. In this sequence the sperm enters subequatorially (indicated by the onset of the calcium wave) and the particle situated slightly above the entering sperm clearly moves before the particle situated at the antipode of the sperm entry point. The resulting contraction

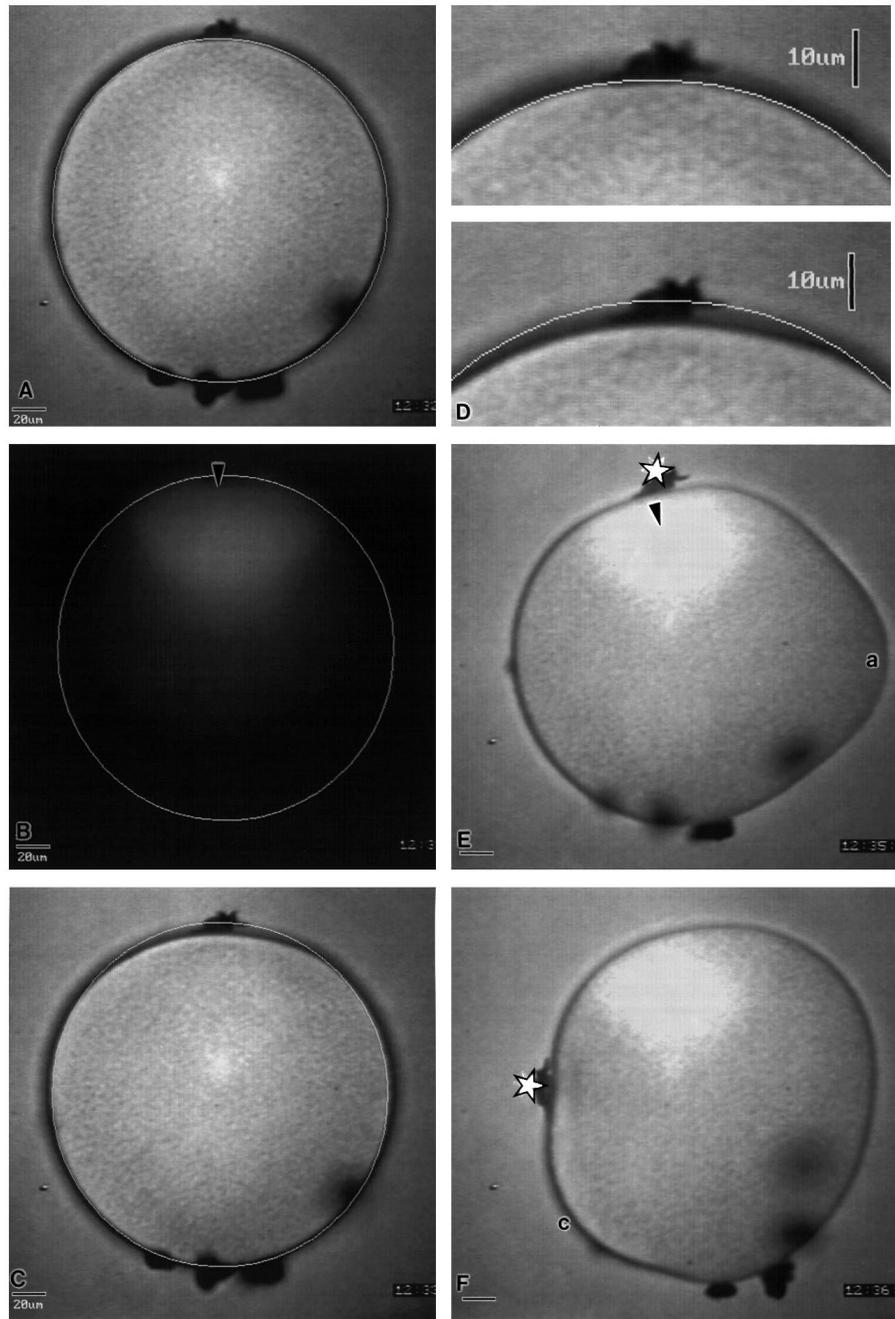


Fig. 2. A local release of caged InsP_3 causes a local contraction of the cortex. An unfertilized egg with Nile Blue particles fixed to its surface co-injected with calcium green dextran (10,000 M_r) and caged InsP_3 . The figures show five images from a time lapse recording (1 image/4 seconds). (A,B) The egg has particles on both sides. A short burst (1 second) of UV irradiation (black arrowhead) is delivered in the region of the top particle (irradiated area visible in B). (C) The egg is slightly deformed by the contraction in the region of irradiation. The particles situated on the opposite side of the egg remain stationary. (D) Close up view of the top egg region in A and C. The top particle is displaced approximately 3 μm in the vertical direction by the localized contraction triggered by the irradiation. (E,F) The same egg is given a much stronger exposure to UV light (90 seconds and neutral density filter removed: black arrowhead in E) and shown respectively 194 (E) and 250 (F) seconds later. The intense irradiation causes a propagated cortical contraction like that seen at fertilization. The egg first bulges at the animal pole region a in E, and is forming a contraction pole (c) in the vegetal hemisphere in F. Note that in F the particle (white star) now moves toward the contraction pole and away from the irradiation site where InsP_3 is generated and calcium released. Bar, 20 μm .

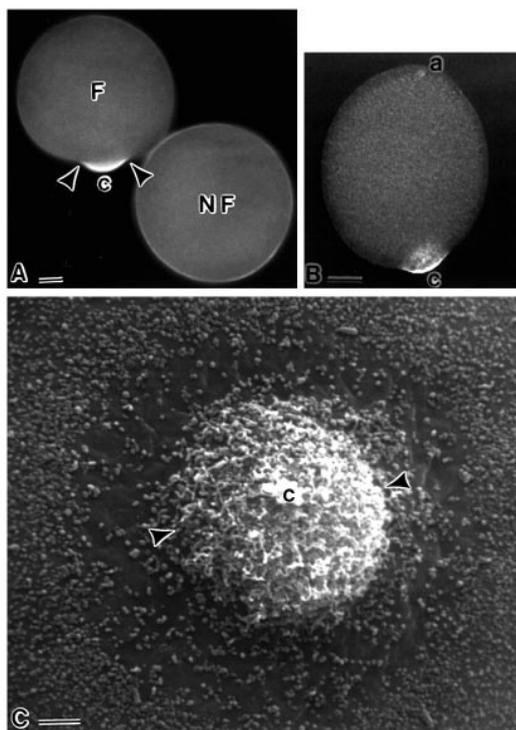


Fig. 3. The contraction pole. (A) A fertilized (F) and a non-fertilized (NF) egg in which amino groups of surface macromolecules have been covalently labelled with the impermeable fluorescent dye FITC at basic pH. The macromolecules labelled on the surface congregate on the constriction that forms within 5 minutes of fertilization at the contraction pole (c and arrowheads) after the egg is fertilized (F). The surface label is evenly distributed on the surface of the non fertilized egg (NF). Bar, 20 μ m. (B) Microfilament distribution about 5 minutes after fertilization visualized with confocal laser scanning microscopy after phalloidin-TRITC labelling. The constriction at the contraction pole (c) is highly enriched in microfilaments. a : animal pole. Bar, 20 μ m. (C) View of the constriction at the contraction pole seen by scanning electron microscopy 5 minutes after fertilization. Three concentric regions with different densities of microvilli can be distinguished (see also Fig. 5C). The contraction pole is in the center (c and black arrowheads). Bar, 5 μ m.

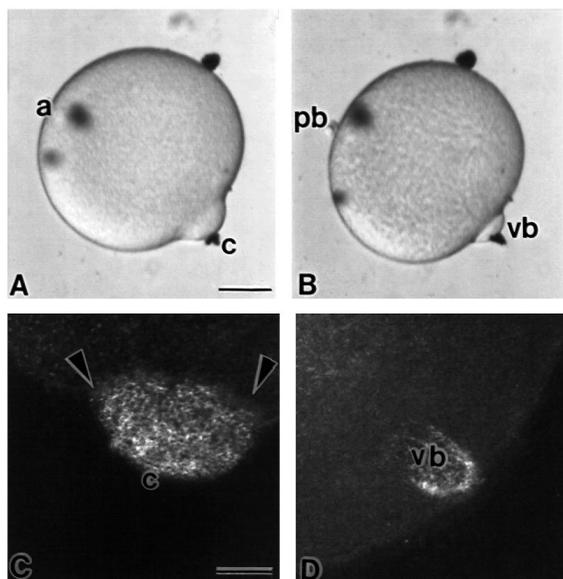


Fig. 4. Contraction pole and vegetal button. (A) An egg 3 minutes after fertilization. The contraction pole (c) marked with a Nile Blue particle is not situated exactly opposite the animal pole (a). Bar, 40 μ m. (B) The same egg 25 minutes after fertilization. The Nile Blue particle is now on a smaller transient protrusion: the vegetal button (vb). Polar bodies (pb). (C) Lower part of the vegetal hemisphere region of a fertilized egg (fixed 3 minutes after fertilization and labelled with phalloidin-TRITC to reveal microfilaments). Note the high density of actin microfilaments in the contraction pole (c) and the lower density of actin microfilaments and microvilli (white dots) in a ring surrounding it (arrowheads show the zone of transition). (D) Vegetal hemisphere region of an egg 25 minutes after fertilization (labelled with phalloidin-TRITC). There is a high density of microfilaments in the vegetal button. Bar, 10 μ m in C and D.

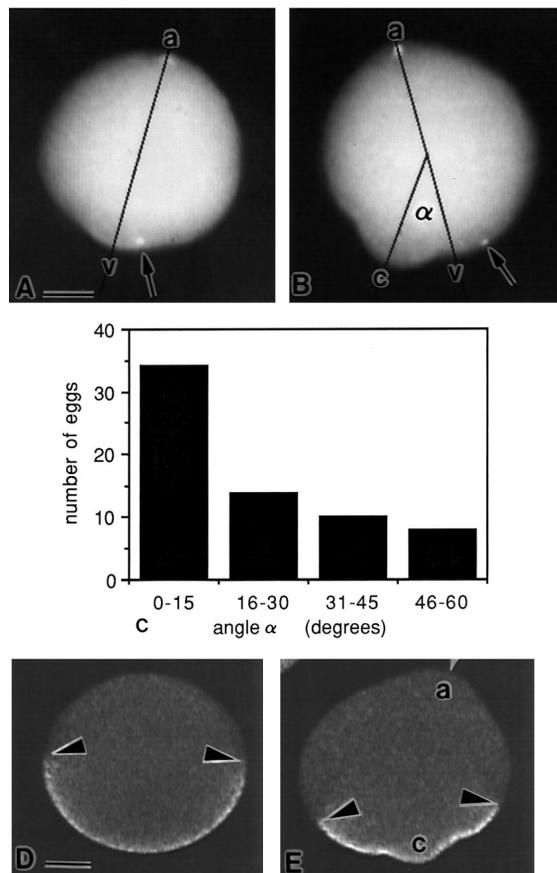


Fig. 5. Position of the contraction pole and myoplasmic cap relative to the animal-vegetal axis. (A,B) Fertilized eggs were fixed and labelled with Hoechst 33342. We analysed the angle (α) that formed between the contraction pole (c) and the vegetal pole (v). To be consistent, we measured α in eggs that were oriented in the same way (that is, with their paternal (arrow) and maternal chromosomes (a) in the same focal plane. The center of gravity of the egg and the position of the animal-vegetal (a-v) axis have been determined as described in methods. The angle α provides a measure of the position of the contraction pole with respect to the animal-vegetal axis. In A the angle is 0°, in B it is about 40°. Bar, 20 μ m. (C) Distribution of the angles (as defined above). (D,E) An unfertilized egg (D) and fertilized egg 3 minutes after fertilization (E) were recorded in an equatorial confocal section after labelling the mitochondria-rich myoplasm with DiOC2(3). The myoplasmic cup (between edges shown by black arrowheads) concentrates in the vegetal hemisphere and is centered around the contraction pole (c) excentered with respect to the animal-vegetal axis (animal pole: a).

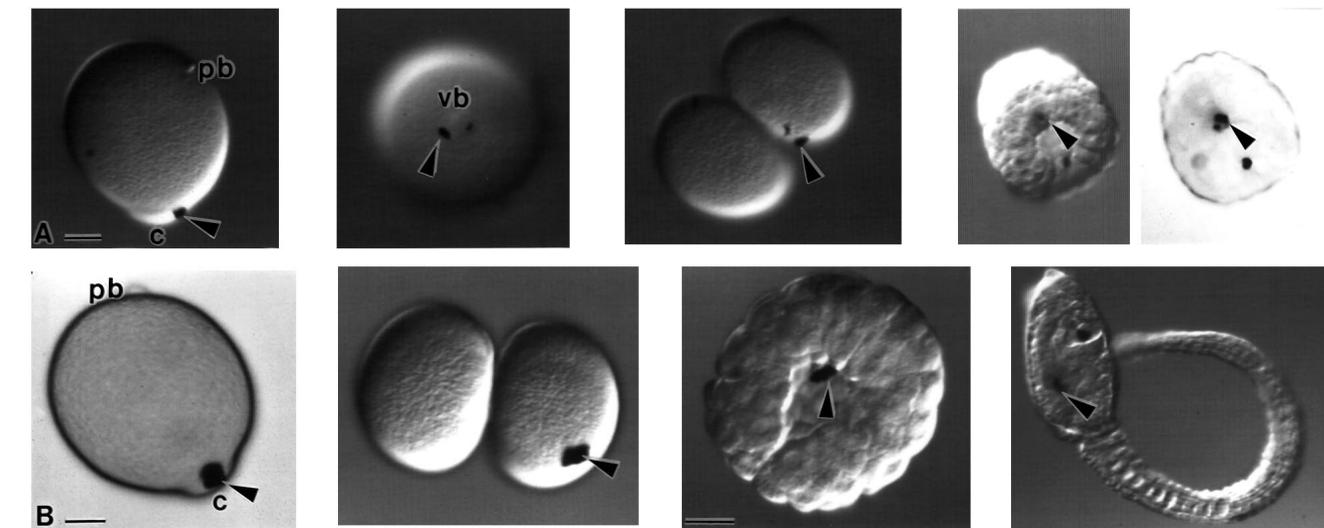
pole lies off the animal-vegetal axis and is again on the side opposite the sperm entry site.

In the 16 experiments where the start of the calcium wave and the final position of the contraction pole were recorded 8 showed the contraction pole formed on the side opposite the site of sperm entry and only one showed a contraction pole on the same side as the entering sperm. The remaining 7 contraction poles were located close to (within 6°) the vegetal pole (see below).

InsP₃ and calcium trigger a contraction of the egg surface

InsP₃, a second messenger molecule that has been shown pre-

viously to activate ascidian eggs (Dale, 1988; McDougall and Sardet, 1995). To examine the identity of the trigger for the cortical contraction, we injected caged InsP₃ into unfertilized eggs and photolysed it using UV illumination. In uninjected controls, UV illumination did not cause a cortical contraction. To visualize the contraction of the cortex in injected eggs, we attached Nile Blue particles to the egg surface and administered a UV flash near some of those particles (Fig. 2A,B). A brief and localized UV flash (1 second) provoked a localized contraction of the cortex in the illuminated region in about 40-50 seconds (Fig. 2A-D). This timing corresponds approximately with the events at fertilization, since the contraction of the cortex occurs 30-40 seconds after the onset of the calcium wave triggered by



C

end of first phase of ooplasmic segregation cortical contraction (5 min PF)	beginning of second phase of ooplasmic segregation (25 min PF)	gastrula (3 h PF)	tadpole (8-12h PF)
<p>contraction pole on axis: 45</p>	<p>vegetal button on:16 off:0 not recorded:29</p>	<p>archenteron in:39 out:6</p>	<p>head in:25 out:2 not recorded:18</p>
<p>contraction pole off axis: 19</p>	<p>vegetal button on:7 off:0 not recorded:12</p>	<p>archenteron in:17 out:2</p>	<p>head in:11 out:1 not recorded:7</p>

Fig. 6. Developmental fate of the contraction pole and vegetal button surface regions. (A,B) Two sequences of developing eggs which have contraction poles (c) excentered with respect to the preexisting animal-vegetal axis. In A, the big (black arrowhead) and little (not seen in first image) Nile Blue particle straddle the contraction pole (c). The particles were found 20 minutes later straddling the vegetal button (vb), and 40 minutes later close to the cleavage furrow. After 4 hours the particles are in the center of the invaginating archenteron (2 images in DIC and bright field optics). Bar, 20 μm). (B) A similar sequence is shown, but in this case the particle was found far from the cleavage furrow. It was later found in the center of the archenteron and in the anterior region of the tadpole larva after 14 hours. pb: polar bodies. Bar, 20 μm. (C) This table summarizes observations of 64 embryos marked on their contraction poles and/or vegetal buttons with Nile Blue particles as shown in A and B and followed by time lapse video microscopy. ‘On axis’ means that the angle, α, between the animal-vegetal axis and the contraction pole (see Fig. 4) is less than 10°. ‘Off axis’ means that α is between 10° and 55°. Embryos were scored

for the presence of the particle in the archenteron of the gastrula, and later for its presence in the head endoderm. If particles located on the contraction pole or vegetal button were not found in the archenteron and/or head endoderm they were counted as out.

the fertilizing sperm. Particles located opposite the UV flash did not move after photolysis of a small amount of caged InsP_3 (Fig. 2C,D). However, the same egg could be subjected to a more intense flash of UV illumination (by removal of a neutral density filter), generating a larger release of caged InsP_3 and a large global calcium signal (not shown here). In this case, the whole egg cortex contracted in a manner similar to that seen at fertilization (Fig. 2E,F). This contraction lead to the formation of a contraction pole in the vegetal hemisphere, and to the migration of particles and the subcortical myoplasm towards a contraction pole area (labelled c in Fig. 2F). These experiments suggest that in the unfertilized egg, the cortical actomyosin basket can respond locally to a small non-propagating calcium increase and only contracts as a whole in the wake of a calcium wave or a large global rise in calcium.

The contraction pole and the vegetal button: two microfilament rich constrictions

The contraction pole is a readily identified constriction (between about 20 μm and 40 μm in diameter) situated in the lower part of the vegetal hemisphere. It results from an actomyosin-driven cortical contraction (Jeffery and Bates, 1989; Sawada, 1988). It has been known for a long time that microvilli concentrate there, and that sperm and lectin binding sites also congregate in this region (Sawada and Osanai, 1981). We wondered if surface and/or transmembrane proteins present on dechorionated eggs also accumulated in this region, so we covalently labelled exposed amino groups at the surface of the dechorionated unfertilized eggs with FITC at pH 10 (at this pH the dye does not penetrate into eggs; Peters et al., 1984). In unfertilized eggs, the labelling is uniform over the egg surface, whereas five minutes after fertilization most of the label is concentrated in the contraction pole region (Fig. 3A). As observed previously in other species (Jeffery and Meier, 1984; Sawada and Osanai, 1985), in *Phallusia mammillata*, actin microfilaments are also concentrated in the contraction pole region (Fig. 3B). In addition, higher magnification views of confocal images and scanning electron microscopy reveal that there is also a microfilament- and microvilli-poor ring surrounding the contraction pole (see Figs 3C and 4C). The 3 zones of actin microfilament labelling detected correspond to the 3 different zones of density of surface microvilli seen in scanning electron micrographs (Fig. 3C). Therefore, the contraction pole is a transient surface constriction containing a very high density of microvilli, surface proteins and microfilaments. Ten minutes after fertilization there remains no obvious trace of the tight constriction of the contraction pole, yet that region keeps a characteristic shape and appearance in DIC and fluorescence microscopy due to the presence of a myoplasmic cup centered around the contraction pole (Fig. 5D,E). During the next 15 minutes, as meiosis is completed, the egg undergoes periodic phases of contraction and relaxation due to the propagation of periodic calcium waves originating from the ER-rich contraction pole region (McDougall and Sardet, 1995; Speksnijder et al., 1990b).

Soon after the completion of meiosis, as the newly formed female and male pronuclei start to migrate, a small constriction (10–30 μm depending on egg batches), which we call the vegetal button, appears transiently in the vegetal hemisphere

(Sardet et al., 1989). Following eggs where Nile Blue particles selectively marked the contraction pole, we found that the vegetal button always appeared exactly where the contraction pole was located previously (Fig. 4A,B). This did not depend on the position of the contraction pole with respect to the animal-vegetal axis. Labelling microfilaments with phalloidin-TRITC indicated that the vegetal button, like the contraction pole, is a microfilament-rich region of the egg surface (Fig. 4C,D). Also like the contraction pole, the vegetal button disappears after a few minutes. It is precisely at this time that a second phase of cytoplasmic relocation (or ooplasmic segregation) begins (see Sardet et al., 1989).

The location of the contraction pole and the site of gastrulation

We have shown previously that the contraction pole need not

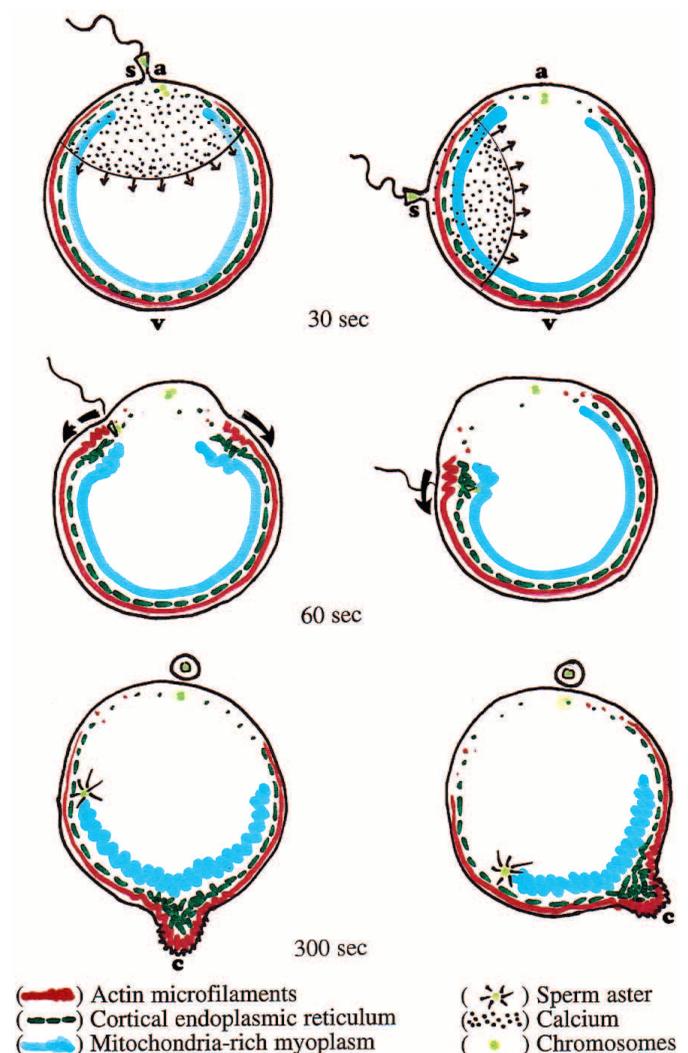


Fig. 7. A schematic representation of fertilization and the first phase of ooplasmic segregation in the ascidian egg. (Left column) The sperm (s) fertilizes the egg near the animal pole (a) triggering a calcium wave and a symmetrical contraction. The contraction pole (c) is close to the vegetal pole (v). (Right column) The sperm (s) fertilizes the egg near the equator and the cortical contraction is asymmetric. The contraction pole (c) and cytoplasmic domains are excentered with respect to the animal-vegetal axis.

be situated exactly at the vegetal pole, but can be up to 50° away from the animal-vegetal axis (Speksnijder et al., 1990a). To analyze this phenomenon quantitatively, we fixed batches of eggs 5 minutes after fertilization and labelled them with Hoechst 33342 to locate the male and female chromosomes. Under the microscope we selected eggs that had the maternal meiotic chromosomes, male chromosomes, and contraction pole in the same equatorial plane of focus to analyze their relative position (Fig. 5A,B). The positions of the maternal meiotic chromosomes and the center of the egg ('center of gravity' determined by image analysis) were used to define the animal-vegetal axis. The position of the contraction pole was then measured with respect to this animal-vegetal axis. The number of contraction poles found in each of four quadrants (0-15°, 16-30°, 31-45°, and 46-60°) is shown in Fig. 5C. In 51% of the eggs the contraction pole formed in the quadrant nearest to the vegetal pole. In the other cases, the contraction pole was situated further than 15° from the vegetal pole and could be as far as 54° from it (Fig. 5C). There was no systematic relationship between the position of the sperm nucleus after the contraction and the location of the contraction pole. The cup of mitochondria-rich myoplasm was repeatedly found centered around the contraction pole irrespective of its position (with respect to the vegetal pole). This was particularly obvious in confocal sections of live eggs labelled with the mitochondrial dye DiOC2(3) (Fig. 5D,E).

The rearrangements of cytoplasmic domains in the ascidian egg appear to be involved in setting up the embryonic axes and in directing the fate of specific blastomeres during the early cleavage stages. For example, the B4.1 blastomeres which inherit a large percentage of the mitochondria-rich myoplasm are fated to form primary muscle cells (reviewed in Jeffery and Swalla, 1990; Nishida, 1992; Satoh, 1994). To determine whether the location of the contraction pole and vegetal button predict the location of the future site of gastrulation of the embryo, we followed the fate of particles situated on the contraction pole and/or on the vegetal button. As previously reported, these particles did not follow the movements of the subcortical myoplasm during the second phase of ooplasmic segregation (Bates and Jeffery, 1987; Sardet et al., 1989). Particles attached to the contraction pole or the vegetal button were observed and recorded through successive cleavages, gastrulation and the formation of the tadpole larvae (Fig. 6A,B). The Nile Blue-labelled plasma membrane covering the contraction pole (or later vegetal button) was found to be situated later on the large cells in the center of the invaginating archenteron in about 90% of the embryos studied (summarized in Fig. 6C). This was not dependent on the position of the contraction pole relative to the animal-vegetal axis (see Fig. 6C). We had expected to see a fixed relationship between the position of the particle labelling the contraction pole, and later the vegetal button, with the plane of the cleavage furrow. However, irrespective of the positioning of the contraction pole, there was no systematic relationship between the two (see Fig. 6A,B) and in half the cases ($n=56$) the particles situated on the contraction pole or vegetal button were not found in the cleavage plane. Similarly, the polar bodies situated at the animal pole were not found systematically in the first plane of cleavage. Understanding the rules governing the exact positioning of the first cleavage plane will clearly require a detailed analysis of the movement of pronuclei and of the localization and orien-

tation of the small mitotic spindle that forms in the center of the egg about 35 minutes after fertilization.

Since the particles placed on the contraction pole label the first cells to invaginate at gastrulation, we expected to find the particle on the endoderm cells, which are localized in the head region at the larval stage. Indeed, the label was found to be in the anterior region in 36 out of 39 tadpole larvae observed (Fig. 6).

DISCUSSION

The sequence of events from fertilization through the first phase of ooplasmic segregation in the ascidian

Based on our present study on *Phallusia mammillata* together with previous reports on this and other species, we can now provide a spatial and temporal description of the events triggered by sperm in ascidians that are involved in cortical and cytoplasmic reorganizations (the first phase of ooplasmic segregation). This is depicted schematically in Fig. 7.

The egg is arrested in metaphase of the first meiotic cycle at the time of fertilization. As in many other deuterostome eggs, a calcium wave due to intracellular calcium release (probably from the ER) is initiated at the site of sperm entry (Brownlee and Dale, 1990; McDougall and Sardet, 1995; Speksnijder et al., 1990a; and this report).

In *Phallusia*, 30 to 40 seconds after the calcium wave (i.e., about 50 to 60 seconds after fertilization) a dramatic cortical contraction, triggered in the animal hemisphere on the side of sperm entry (this report), crosses the 120 µm egg in about 90 seconds. The cortical contraction is an actomyosin driven contraction of a microfilament network situated under the plasma membrane (Jeffery and Bates, 1989; Sawada, 1988). The cortical contraction acts as a wave that carries, in its wake, elements located on the surface of the egg (attached sperm or particles, surface and transmembrane macromolecules) as well as components of the egg interior (sperm nucleus, cortical ER and subcortical organelles). The contraction is directed towards the vegetal hemisphere, where a contraction pole forms. The contraction pole is a 20-40 µm surface protrusion rich in microvilli and microfilaments. The contraction pole remains constricted for about 10 minutes and then relaxes, leaving this region of the egg surface curved in a characteristic way.

As a consequence of the cortical contraction, the contents of the egg become reorganized. In particular, the mitochondria-rich myoplasm layer translocates to the vegetal hemisphere, where it forms a subcortical cup centered around the contraction pole. The exact location of the contraction pole and thus of the myoplasm depends on the site of sperm entry and on the direction of the calcium and contraction waves (as shown in Fig. 7 and discussed in the following sections). The cortical contraction also results in the formation of a new cytoplasmic domain consisting of accumulated ER sheets and tubes within the contraction pole (Gualtieri and Sardet, 1989; Speksnijder et al., 1993). This ER accumulation acts as the cortical pacemaker that initiates periodic cortical calcium waves during meiosis (McDougall and Sardet, 1995; Speksnijder et al., 1989a, 1990b).

The vegetal button, another temporary microfilament-rich protrusion smaller than the contraction pole, appears exactly

where the contraction pole formed previously. The vegetal button is vaguely reminiscent of the small polar lobe-like structure observed in some annelids and molluscs (reviewed by Conrad et al., 1990). The vegetal button forms about 25 minutes after fertilization, once meiosis is completed. By this time, the sperm aster has reached its maximum size and the newly formed egg pronucleus is starting to migrate towards the sperm aster (Sardet et al., 1989; Sawada and Schatten, 1989). The vegetal button varies in size and persists for about 3-6 minutes depending on egg batches. Unlike the formation of the contraction pole, the formation of the vegetal button is probably independent of calcium signals (repetitive calcium signals initiated in that area cease just before the second polar body is extruded). The role of the vegetal button is unclear at present but, we suggest that it may be at the origin of the changes that signal the second phase of ooplasmic segregation during which the bulk of the myoplasm and accumulated ER are relocated to the future posterior pole of the embryo (Sardet et al., 1989, 1991; Sawada and Schatten, 1989; Speksnijder et al., 1993).

Calcium and the cortical contraction

The unfertilized ascidian egg cortex is characterized by an abundant network of actin microfilaments which are arranged as a basket with its opening (a region apparently depleted of microfilaments) around the animal pole (Sawada, 1988; Sawada and Osanai, 1985). Observations on isolated cortices further suggest that the microfilaments, which are directly apposed to the inner face of the plasma membrane, are distributed in a gradient of increasing density toward the vegetal pole (Sardet et al., 1992). We do not yet know if the cortical contraction results from the reorganization of these preexisting actin microfilaments or involves the polymerization of actin into additional microfilaments. The considerable delay (30-40 seconds) between the local elevation of intracellular calcium and the onset of the contraction at that site suggests that polymerization of microfilaments may occur before the contraction is initiated.

We have presented direct evidence that local release of InsP_3 and calcium (probably from the ER) are sufficient to trigger a cortical contraction. Clearly there must exist a threshold of calcium concentration in the cortex at which the cortical contraction can propagate across the entire egg. At present the respective roles that calcium and the members of the phosphatidylinositol cycle play in the regulation of microfilament assembly and contraction is unclear (see review by Janmey, 1994). It is likely that calmodulin activated kinases and/or myosin light chain kinase are intermediates between calcium and the actomyosin-driven contraction. The cortical contraction in the ascidian egg may be a good model (being an amplified cortical contraction triggered by a large calcium signal) for the study of the mechanisms involved in cytokinesis, since there is good evidence that localized small calcium signals trigger cleavage initiation (Fluck et al., 1991; Snow and Nuccitelli, 1993).

We have observed that, depending on the site of sperm entry, the cortical contraction can be more or less asymmetric with respect to the animal-vegetal axis. We propose that the cortical actomyosin basket responds locally to calcium increase by contracting. If the sperm enters close to the animal pole the opening of the actomyosin basket will contract evenly and the

contraction will be symmetrical (Fig. 7, left column). If the sperm enters equatorially, the calcium wave it triggers will first reach one side of the actomyosin basket and then reach the other side only 15-20 seconds later, inducing a delayed contraction there (Fig. 7, right column). This time delay is close to that observed for setting in motion particles respectively close or far from the site of fertilization (see Fig. 1B).

The site of fertilization and the the site of gastrulation in the ascidian embryo

Our studies confirm our earlier observations that the contraction pole does not always form exactly at the vegetal pole (i.e., exactly opposite the meiotic site) but that it can be as far as 50-55° away from it (Speksnijder et al., 1990a; Fig. 5 in this report). There is a good correlation between the site of sperm entry and the position of the contraction pole. We have previously observed that 43% of sperm enter within 45° of the animal pole (Speksnijder et al., 1989b, a) and, correspondingly, the contraction pole has the greatest probability of forming within 15° of the vegetal pole (half the cases examined in the present report; see Fig. 5). This is the situation one would expect when a calcium wave and subsequent contraction wave are initiated near the animal pole and are both propagated symmetrically along the animal-vegetal axis (Fig. 7, situation shown in left column). In the remaining half of the cases examined, the contraction pole was situated between 15° and 55° away from the vegetal pole proper (Fig. 5). Correspondingly, we have previously observed that in 57% of the cases, sperm penetrates more than 45° away from the animal pole (Speksnijder et al., 1989b). In such cases, the direction in which the calcium wave propagates does not coincide with the animal-vegetal axis, and the cortical contraction is thus asymmetric (Fig. 7, situation shown in right column). The site of initiation of the contraction is on the side of sperm entry and the contraction pole forms opposite the side of sperm entry with respect to the animal-vegetal axis.

Thus we conclude that because of an animal-vegetal asymmetry already established in the egg cortical cytoskeleton before fertilization, the contraction pole is fated to be located in the vegetal pole region within a 110° cone centered on the vegetal pole. Its precise location within this cone depends on the site of sperm entry and the calcium wave initiation site. It is as if a rough tuning mechanism (preexisting before fertilization) and fine tuning mechanism (established after fertilization) were superimposed to reorganize the cytoplasmic domains along an axis that can be shifted with respect to the original animal-vegetal axis.

Our experiments confirm and document the fact that particles placed on, or very near, the vegetal pole (when the contraction pole is situated there) are found on the endoderm cells that are first to invaginate during gastrulation and later found in the head region of the tadpole (Bates and Jeffery, 1987). Particles placed on a contraction pole or vegetal button situated far (40-50°) from the vegetal pole have the same final destination, suggesting that the future site of gastrulation of the embryo corresponds to the location of the contraction pole whether it is coincident or not with the vegetal pole itself. In this context, it is interesting to note that Bates and Jeffery (1987) by ablating an area (5-15%) of the myoplasmic cup region or irradiating the entire vegetal hemisphere with UV in *Styela clava* zygotes between the 2 phases of ooplasmic seg-

regation, inhibited gastrulation. Our attempts to perturb gastrulation by UV irradiation of the small zone where the contraction pole or the vegetal button are located, have not been successful for the moment. Further experimentation along these lines may define precisely when and where such a gastrulation center forms.

We have shown that the first phase of ooplasmic segregation causes mitochondria, ER, surface proteins and microvilli to become centered around the contraction pole that can be anywhere (within a 110° cone) in the vegetal hemisphere. There was no fixed relationship between the contraction pole and the plane of first cleavage, nor was the first cleavage plane related to the position of the animal pole. Since the first cleavage plane coincided with the future plane of bilateral symmetry of the tadpole, our data suggest that the body axes of the embryo are not specified until after the first phase of ooplasmic segregation. It is known that the final position of the myoplasm is related to the first cleavage plane, the myoplasmic crescent always being bisected. We thus conclude that exact axis specification in ascidians occurs during the second phase of ooplasmic segregation. The first phase probably serves to collect the myoplasm prior to its final relocalization during the second phase of cytoplasmic reorganization that depends on the location and movements of the sperm aster.

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REFERENCES

- Bates, W. R. and Jeffery, W. R. (1987). Localization of axial determinants in the vegetal pole region of ascidian eggs. *Dev. Biol.* **124**, 65-67.
- Brownlee, C. and Dale, B. (1990). Temporal and spatial correlation of fertilization current, calcium waves and cytoplasmic contraction in eggs of *Ciona intestinalis*. *Proc. R. Soc. Lond.* **239**, 321-328.
- Conklin, E. G. (1905). The organization and cell lineage of the ascidian egg. *J. Acad. Natl. Sci. Phil.* **13**, 1-119.
- Conrad, G. W., Schantz, A. R. and Patron, R. R. (1990). Mechanisms of polar lobe formation in fertilized eggs of molluscs. *Ann. New York Acad. Sci.* **582**, 273-294.
- Dale, B. (1988). Primary and secondary messengers in the activation of ascidian eggs. *Exp. Cell Res.* **177**, 205-211.
- Dale, B. (1990). *Mechanism of Fertilization: Plants to Humans*. Berlin: Springer Verlag.
- Elinson, R. P. (1990). Cytoskeleton and embryonic polarity. *Curr. Opin. Cell Biol.* **2**, 75-79.
- Fluck, R. A., Miller, A. L. and Jaffe, L. F. (1991). Slow calcium waves accompany cytokinesis in medaka fish eggs. *J. Cell Biol.* **115**, 1259-1265.
- Gualtieri, R. and Sardet, C. (1989). Endoplasmic reticulum network in the ascidian egg: localization and calcium content. *Biol. Cell* **65**, 301-304.
- Janmey, P. A. (1994). Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Ann. Rev. Physiol.* **26**, 169-191.
- Jeffery, W. R. and Bates, W. R. (1989). Ooplasmic segregation in the ascidian *Styela*. In *The Molecular Biology of Fertilization*, vol. (ed. H. Schatten and G. Schatten), pp. 341-367, San Diego: Academic Press.
- Jeffery, W. R. and Meier, S. (1984). Ooplasmic segregation of the myoplasmic actin network in stratified ascidian eggs. *Wilhelm Roux's Arch. Dev. Biol.* **193**, 257-262.
- Jeffery, W. R. and Meiers, S. (1983). A yellow crescent cytoskeletal domain in ascidian eggs and its role in early development. *Dev. Biol.* **96**, 125-143.
- Jeffery, W. R. and Swalla, B. J. (1990). The myoplasm of ascidian eggs: a localized cytoskeletal domain with multiple roles in embryonic development. *Sem. Cell Biol.* **1**, 253-261.
- Lutz, D. A. and Inoué, S. (1986). Techniques for observing living gametes and embryos. *Meth. Cell Biol.* **27**, 89-110.
- McDougall, A. and Sardet, C. (1995). Function and characteristics of repetitive calcium waves associated with meiosis. *Curr. Biol.* **5**, 318-328.
- Nishida, H. (1992). Regionality of egg cytoplasm that promotes muscle differentiation in embryo of the ascidian, *Halocynthia roretzi*. *Development* **116**, 521-529.
- Peters, R., Sardet, C. and Richter, H. P. (1984). Mobility of membrane lipids and proteins in the animal and vegetal poles of the sea urchin egg. *Dev. Growth Diff.* **26**, 105-110.
- Sardet, C., McDougall, A. and Houlston, E. (1994). Cytoplasmic domains in eggs. *Trends Cell Biol.* **4**, 166-172.
- Sardet, C., Rouvière, C., Flannery, B. and Davoust, J. (1991). Time lapse confocal microscopy of mitochondrial movements in ascidian embryos. *Amer. Inst. Physics* **226**, 77-82.
- Sardet, C., Speksnijder, J., Inoue, S. and Jaffe, L. (1989). Fertilization and ooplasmic movements in the ascidian egg. *Development* **105**, 237-249.
- Sardet, C., Speksnijder, J., Terasaki, M. and Chang, P. (1992). Polarity of the ascidian egg cortex before fertilization. *Development* **115**, 221-237.
- Satoh, N. (1994). *Developmental Biology of Ascidians*. Cambridge: Cambridge University Press.
- Sawada, T. (1988). The mechanism of ooplasmic segregation in the ascidian egg. *Zool. Sci.* **5**, 667-675.
- Sawada, T. and Osanai, K. (1981). The cortical contraction related to the ooplasmic segregation in *Ciona intestinalis* eggs. *Wilhelm Roux's Arch. Dev. Biol.* **190**, 208-214.
- Sawada, T. and Osanai, K. (1985). Distribution of actin filaments in fertilized egg of the ascidian *Ciona intestinalis*. *Dev. Biol.* **111**, 260-265.
- Sawada, T. and Schatten, G. (1989). Effects of cytoskeletal inhibitors on ooplasmic segregation and microtubule organization during fertilization and development of the ascidian *Molgula occidentalis*. *Dev. Biol.* **132**, 331-342.
- Schatten, H. and Schatten, G. (1989a). *The Cell Biology of Fertilization*. San Diego: Academic Press.
- Schatten, H. and Schatten, G. (1989b). *The Molecular Biology of Fertilization*. San Diego: Academic Press.
- Snow, P. and Nuccitelli, R. (1993). Calcium buffer injections delay cleavage in *Xenopus laevis* blastomeres. *J. Cell Biol.* **122**, 387-394.
- Speksnijder, J. E., Corson, D. W., Sardet, C. and Jaffe, L. (1989a). Free calcium pulses following fertilization in the ascidian egg. *Dev. Biol.* **135**, 182-190.
- Speksnijder, J. E., Jaffe, L. and Sardet, C. (1989b). Polarity of sperm entry in the ascidian egg. *Dev. Biol.* **133**, 180-184.
- Speksnijder, J. E., Sardet, C. and Jaffe, L. (1990b). Periodic calcium waves cross ascidian eggs after fertilization. *Dev. Biol.* **142**, 246-249.
- Speksnijder, J. E., Sardet, C. and Jaffe, L. F. (1990a). The activation wave of calcium in the ascidian egg and its role in ooplasmic segregation. *J. Cell Biol.* **110**, 1589-1598.
- Speksnijder, J. E., Terasaki, M., Hage, W. J., Jaffe, L. F. and Sardet, C. (1993). Polarity and dynamics of the endoplasmic reticulum during fertilization and ooplasmic segregation in the ascidian egg. *J. Cell Biol.* **120**, 1337-1346.

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