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Fertilization and ooplasmic movements in the ascidian egg

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Summary

Using light microscopy techniques, we have studied the movements that follow fertilization in the denuded egg of the ascidian *Phallusia mammillata*. In particular, our observations show that, as a result of a series of movements described below, the mitochondria-rich subcortical myoplasm is split in two parts during the second phase of ooplasmic segregation. This offers a potential explanation for the origin of larval muscle cells from both posterior and anterior blastomeres.

The first visible event at fertilization is a bulging at the animal pole of the egg, which is immediately followed by a wave of contraction, travelling towards the vegetal pole with a surface velocity of 1·4 $\mu m\,s^{-1}$. This wave accompanies the first phase of ooplasmic segregation of the mitochondria-rich subcortical myoplasm. After this contraction wave has reached the vegetal pole after about 2 min, a transient cytoplasmic lobe remains there until 6 min after fertilization. Several new features of the morphogenetic movements were then observed: between the extrusion of the first and second polar body (at 5 and 24–29 min, respectively), a series of transient animal protrusions form at regular intervals. Each animal protrusion involves a flow of the centrally located cytoplasm in the animal direction. Shortly before the second polar body is extruded, a second transient vegetal lobe ('the vegetal button') forms, which, like the first, resembles a protostome polar lobe. Immediately after the second polar body is extruded, three events occur almost simultaneously: first, the sperm aster moves from the vegetal hemisphere to the equator. Second, the bulk of the vegetally located myoplasm moves with the sperm aster towards the future posterior pole, but interestingly about 20 % remains behind at the anterior side of the embryo. This second phase of myoplasmic movement shows two distinct subphases: a first, oscillatory subphase with an average velocity of about 6 $\mu m\,min^{-1}$, and a second steady subphase with a velocity of about 26 $\mu m\,min^{-1}$. The myoplasm reaches its final position as the male pronucleus with its surrounding aster moves towards the centre of the egg. Third, the female pronucleus moves towards the centre of the egg to meet with the male pronucleus. Like the myoplasm, the migrations of both the sperm aster and the female pronucleus shows two subphases with distinctly different velocities. Finally, the pronuclear membranes dissolve, a small mitotic spindle is formed with very large asters, and at about 60–65 min after fertilization, the egg cleaves.

Key words: ascidian development, fertilization, ooplasmic segregation, contraction waves, myoplasm, video microscopy.

Introduction

The unfertilized ascidian egg is usually arrested in the first meiotic division and the small meiotic spindle lies close to the surface at a point that defines the animal pole. Moreover, a mitochondria-rich layer is excluded from this meiotic region (Reverberi, 1956; Mancuso, 1963). Despite this visible polarity, any half of the unfertilized egg can develop into a normal larva (Reverberi & Ortolani, 1962; Bates & Jeffery, 1987). Thus, in the unfertilized ascidian egg, relatively little developmental pattern exists. However, after fertilization such a pattern is formed with remarkable speed. Well before first cleavage, the future embryonic axes of the ascidian embryo become apparent during a series of cytoplasmic movements known as ooplasmic segregation (Conklin, 1905; Jeffery, 1984). Three to five visible plasms become segregated into different regions of the egg cell, and are subsequently partitioned unequally during the early cleavages. As a result, these plasms become localized in different cells of the 64-cell-stage embryo. The most obvious of these plasms is the mitochondria-rich (and in some species pigment-rich) myoplasm localized in the cortex of the unfertilized egg.

Conklin's classical account describes two distinct phases of myoplasmic segregation: during the first phase, the myoplasm concentrates in the vegetal pole region and, during the second phase, it shifts from the

vegetal pole to the equatorial region of the zygote, where it marks the future posterior pole of the embryo (Conklin, 1905). The myoplasm is subsequently partitioned in the blastomeres that give rise to muscle cells. Since the components that specify muscle cell fate are still unknown, we conceive of the myoplasm as an entity that can be roughly identified as the mitochondria-rich mass. It is clear, however, that the mitochondria themselves are not the muscle cell determinants (Conklin, 1931).

In recent years, the mechanisms of ooplasmic segregation have been studied in several laboratories, and it has become clear that the first phase is driven by the contraction of a cortical actin network (Sawada & Osanai, 1981, 1984, 1985; Jeffery & Meier, 1983, 1984; Sawada, 1983; Jeffery, 1984). This cortical contraction is probably triggered by the wave of elevated free calcium that travels through the egg following fertilization (Speksnijder *et al.* 1986, 1988). The second phase of segregation seems to depend upon microtubule integrity (Sawada & Schatten, 1985, 1988).

We have investigated the movements that follow fertilization in the transparent denuded egg of *Phallusia mammillata* using epifluorescence microscopy and videomicroscopy. Besides establishing exact speeds and times for various movements, we have discovered new features that may have significant embryological implications. These include a series of animal protrusions in the period between the formation of the two polar bodies, the formation of a second cytoplasmic lobe reminiscent of a protostome polar lobe, the suggestion that there exists a physical connection between the myoplasm and the sperm aster, a splitting of the myoplasm into two parts which end up at opposite poles of the embryo and a division of the second phase of ooplasmic segregation into two subphases with distinctively different velocities. This work has been reported in part in abstract form (Sardet *et al.* 1986). A separate report on the calcium waves that we observed following fertilization and during meiosis in *Phallusia* eggs is in preparation (see also Speksnijder *et al.* 1986, 1988).

Materials and methods

Experimental animal and gametes

The European ascidian, *Phallusia mammillata*, was collected in France on the Meditteranean coast in Sète (Bassin de Thau), or on the Atlantic coast near Roscoff (Brittany). They were kept in aquaria in Villefranche-sur-mer or Woods Hole at 15–22 °C. Ripe gametes can be obtained from the gonoducts of these animals throughout the year. Concentrated sperm can be kept several days at 5 °C and diluted as needed. The eggs were washed in millipore-filtered seawater and dechorionated by incubation with trypsin (0·1 %) in seawater buffered to pH 8·0 with 10 mM-TAPS (Tris [hydroxymethyl] methylaminopropane sulphonic acid) (TAPS SW) for 2 h at 18 °C with gentle agitation (Zalokar, 1979). The eggs were extensively washed thereafter in TAPS SW and used within 6 h, although they can still be successfully fertilized 24 to 48 h later. To prevent adhesion and subsequent lysis of the dechorionated eggs, all surfaces were treated with gelatin as follows; all glassware and plastic dishes were immersed in a solution of 0·1 % gelatin and 0·1 % formaldehyde, drained, air dried, and subsequently rinsed extensively in running tap water. Provided they are prevented from adhering to each other, most trypsin-dechorionated eggs undergo normal development into swimming tadpoles (Zalokar, 1979; Zalokar & Sardet, 1984).

Light microscopic observation of fertilization

For the observation of fertilization, we perfused dechorionated eggs, which were slightly compressed between a slide and a coverslip, with a dilute suspension of sperm that had been preincubated for 30 min with 'chorionated' eggs, i.e. eggs from which the chorion had not been removed. This greatly improves the synchrony of fertilization of naked eggs. We also observed eggs using the microdrop method described by Lutz & Inoué (1986). Mitochondria were visualized by incubating the eggs for 30 min in the fluorescent carbocyanine dye $DiO(C_2)_3$ (Molecular Probes) at a concentration of 5 $\mu g\,ml^{-1}$, after which they were washed in TAPS SW, and observed under epi-illumination (excitation filter: 450–490 nm, emission filter: 520–560 nm, barrier filter: 510 nm). These vitally stained eggs develop normally after fertilization (Zalokar & Sardet, 1984).

Light microscopic observations were made on a Leitz Orthoplan, a Zeiss IM 35 and a Zeiss Axiophot (differential interference contrast and epifluorescence), or on a specially designed microscope (rectified differential interference contrast, polarization optics; see Inoué, 1986 figs III-21,22). DAGE/MTI Newicon and SIT cameras were used to record images on a time-lapse recorder (GYYR or Sony Umatic TV0 9000) or on an Optical Memory Disc Recorder (OMDR Panasonic 2021F). The observations reported in this paper were made over the last three years on many different eggs. Unless otherwise indicated, all experiments were done at 18–21 °C.

Results

Fertilization of Phallusia mammillata *eggs*

The eggs of ascidians are surrounded by an inner layer of test cells and an acellular chorion with adhering follicle cells. These extracellular structures greatly impair visualization of the events that follow fertilization in the living egg. Therefore, their removal is required for the detailed observation of these fertilization events. Usually, this involves dechorionation with microneedles, which is necessarily limited to a few eggs. An additional problem is that fertilization of naked ascidian eggs is not always reliable. We found, however, that large numbers of chemically dechorionated *Phallusia* eggs could be reliably and synchronously fertilized by sperm that had been previously exposed to chorionated eggs (data not shown). Using this method, we have been able to observe that sperm does not penetrate preferentially in the vegetal pole area as previously thought (Conklin, 1905). By fixing eggs soon after insemination and observing the relative position of female and male pronuclei, we have found that in fact sperm show a tendency to fuse with the egg in the animal hemisphere (Speksnijder *et al.* 1987; J. E. Speksnijder, C. Sardet & L. F. Jaffe, submitted). Unfortunately, there are no obvious surface modifications, such as fertilization cones, associated with

sperm entry that allow us to determine readily the point of sperm entry in living eggs, although we were able to get a glimpse of it on one exceptional occasion (arrowhead in Fig. 1A).

Polarity of the Phallusia *egg*

The egg of *Phallusia* shows a distinct animal–vegetal polarity in its organization, which can be readily visualized. The chromosomes, which are in metaphase of the first meiotic division, are located right underneath the egg surface at a point that defines the animal pole. The meiotic spindle is clearly visible in polarized optics (arrow in Fig. 1A). A clear zone surrounding the meiotic spindle can be detected with differential interference contrast optics (not shown). Finally, the subcortical mitochondria-rich mass is located underneath the entire egg surface except for the most animal part, and is thus organized as a basket with its opening at the animal pole. This mass can be visualized by staining with the vital dye $DiO(C_2)_3$ (Fig. 2, see also Zalokar & Sardet, 1984), or by its autofluorescence under u.v. epi-illumination (Deno, 1987).

The contraction wave and first phase of ooplasmic segregation

The first visible sign of fertilization in *Phallusia*, as in other ascidians (Conklin, 1905; Ortolani, 1955; Reverberi, 1971; Sawada & Osanai, 1981; Villa & Patricolo, 1987), is the formation of a bulge at the animal pole (Fig. 1A,B). Simultaneously, a constriction appears near the animal pole, which travels towards the vegetal pole in 2·0 min (S.E.M. = 0·04; $n = 4$). The velocity of this contraction wave ranges from 1·2–1·6 $\mu m\,s^{-1}$, with a mean of 1·4 $\mu m\,s^{-1}$ (S.E.M. = 0·1; $n = 5$). After the contraction wave has reached the vegetal pole area, a transient cytoplasmic lobe remains present from 2 to 6 min after fertilization (Fig. 1F–H).

The contraction wave accompanies the segregation of the subcortical mitochondria-rich myoplasm towards the vegetal pole of the egg. At the end of the contraction wave, this mass is entirely located in the lower third of the vegetal hemisphere (Fig. 2). Upon focusing through the mass in eggs stained with $DiO(C_2)_3$, one can observe that it shows characteristic folds in its most vegetal part, and is located further away from the plasma membrane in this vegetal area than in its most equatorial parts.

It is possible to follow the movement of the mitochondria-rich mass simultaneously with the movement of small Nile blue particles attached to the egg surface (Fig. 2A–D). These two elements, located on the internal and the external sides of the egg plasma membrane, move simultaneously and in a coordinated fashion with an average velocity of 1·4 $\mu m\,s^{-1}$ (S.E.M. = 0·1; $n = 4$) which is a value comparable to the one we calculated for the speed of the contraction wave.

In accordance with previous observations (Zalokar, 1974; Reverberi, 1975; Sawada & Osanai, 1981), we observed that the contraction wave and the segregation of the myoplasm are both blocked by cytochalasins (preincubation for one hour and insemination in 10 $\mu g\,ml^{-1}$ cytochalasin B, or 2 $\mu g\,ml^{-1}$ cytochalasin D). The movement of particles attached to the egg surface is also inhibited by these drugs (data not shown).

Meiosis

At 18 °C, the first polar body forms at about 5 min after fertilization (mean = 5·1; S.E.M. = 0·1; $n = 7$), and the second polar body at 24–29 min (mean = 26; S.E.M. = 1; $n = 7$). During either the entire period, or part of it, we observed from 6 to 15 pulsating movements consisting of alternating protrusions and retractions at the animal pole every 60–90 s (see Fig. 1I–N). These pulsating movements accompany a flow of the central cytoplasm towards the animal pole and back, which is clearly visible in time lapse. Furthermore, shortly after first polar body extrusion, we observed the formation of a small, transient, polar body-like protrusion at the animal pole (7–9 min after fertilization, see arrowhead in Fig. 1J). By this time, a sperm aster starts forming in the vegetal hemisphere (arrowhead in Fig. 1K). Its position relative to the vegetal pole is variable and probably depends on the point of sperm entry (Speksnijder *et al.* 1987); the more animally the sperm enters, the closer to the equator it is likely to end up after the first segregation phase. The aster progressively grows (Fig. 1K–N), and the male pronucleus forms by coalescence of karyomeres at the time of second polar body formation (arrowhead in Fig. 1O). Meanwhile, the finely granular cytoplasm has progressively changed to a coarser texture (Fig. 1I–M). The periodic oscillations at the animal egg surface and the internal cytoplasm cease just before the extrusion of the second polar body (Fig. 1N). In differential interference contrast optics, the mitochondria-rich mass extends 5–10 μm below the surface in the vegetal hemisphere (Fig. 1K–N). By focusing through the mass, one can see that it is folded and seems detached from the surface near the vegetal pole, which confirms our observations in $DiO(C_2)_3$-labelled eggs. In its more equatorial position, the mass tapers off and is more closely associated with the surface. The mitochondria-rich mass can also be readily visualized in polarized optics (arrowhead in Fig. 1W).

Just before second polar body formation, the eggs start to flatten at the vegetal pole (Figs 1O and 3A), and subsequently form a second transient cytoplasmic lobe in this area (arrowhead in Fig. 3B–D). This small polar lobe-like structure lasts 7–8 min, and varies in size depending on the time of the year and the batch of eggs. It does not seem to contain granules or mitochondria (Figs 3B–D and 4B–D). We have called this structure 'the vegetal button'. This structure has not been described before, probably because it is difficult to observe in chorionated eggs. This vegetal lobe is very different from the one described in lectin-activated *Phallusia mammillata* eggs which in fact corresponds to the transient vegetal lobe normally formed 2–6 min after fertilization but is delayed by about 15 min during lectin activation (Zalokar, 1979; Sardet & Speksnijder, unpublished observations).

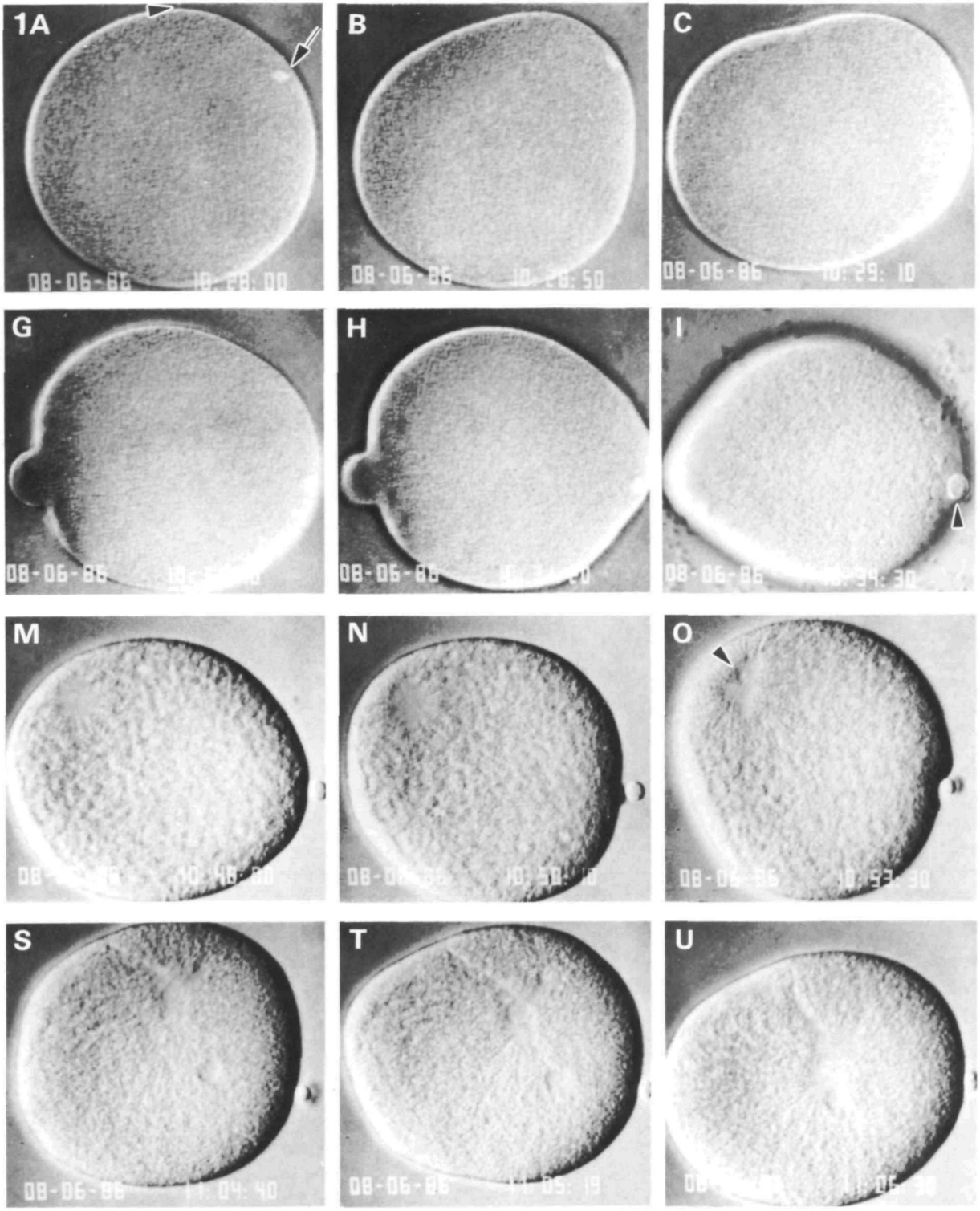
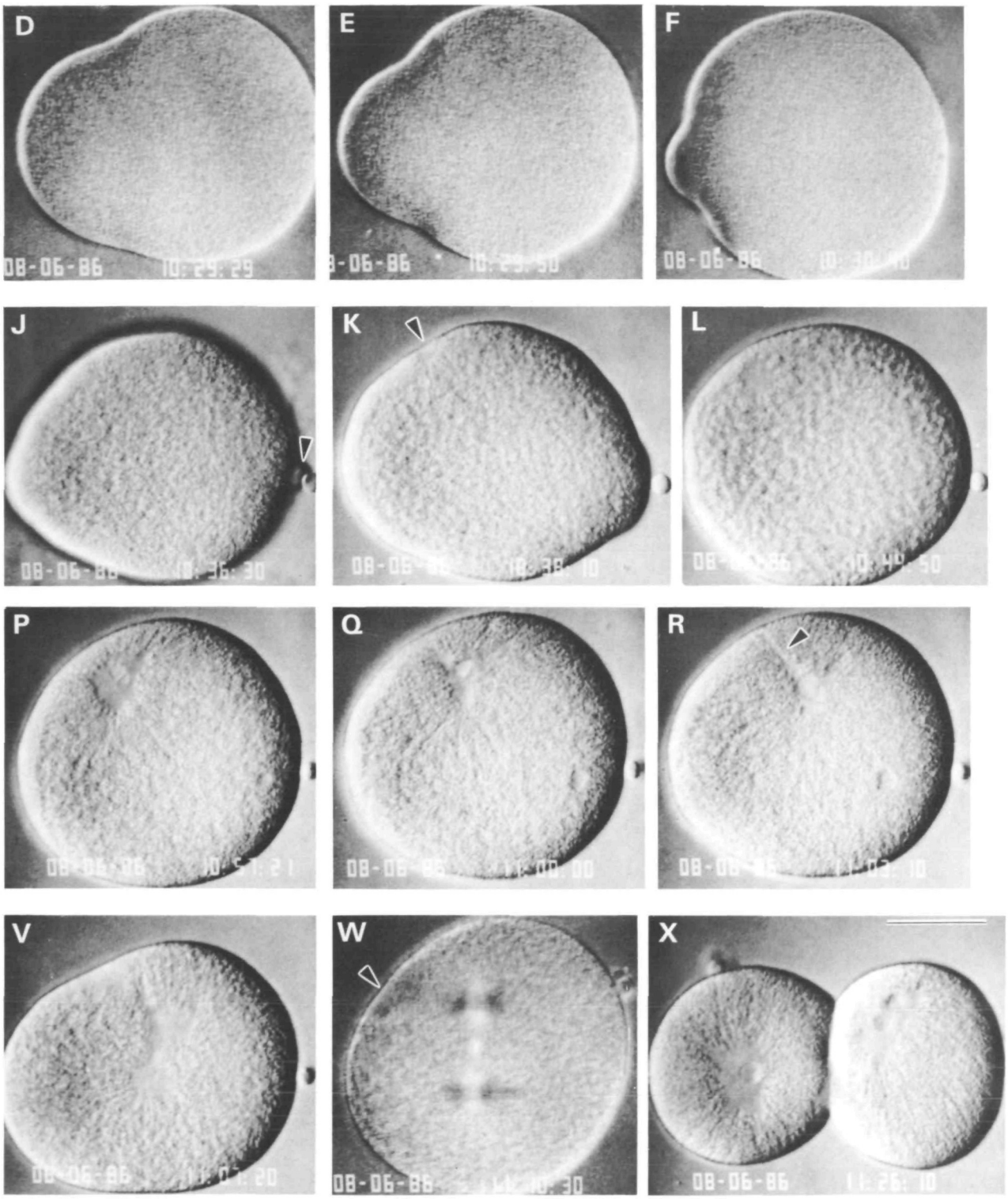


Fig. 1. Fertilization and segregation in *Phallusia mammillata* (recorded from an OMDR time-lapse sequence). (A–H) The contraction wave. In this sequence, the sperm enters near the arrowhead (A). Note that the birefringence of the meiotic spindle at the animal pole becomes weaker (A–C), and that the contraction wave progresses toward the vegetal pole (B–H). Rectified polarized optics. (I–O) Polar body formation and meiotic oscillations. The first polar body forms (arrowhead in I). A small, transient polar body-like protrusion is formed at the animal pole (arrowhead in J). The sperm aster appears in the vegetal hemisphere (arrowhead in K). The cytoplasm becomes more granular (I–N) and oscillations are observed at the animal pole (protrusions at K, M; retraction at L, N). Membranes reform around the paternal chromosomes (arrowhead in O). The second polar body is extruded 23 min after fertilization (N, O). Differential interference contrast.
(P–V) Movements of pronuclei and myoplasm. The sperm aster enlarges (P). The pronuclei move toward the centre of the



egg, where their membranes dissolve (P–V). The myoplasm located in the vegetal pole area undergoes oscillatory motions towards the equator (see deformations of the vegetal pole in O–Q) and a final smooth motion to its final position (see deformations of the vegetal pole area in T–V), as if dragged by the male aster (see structural connection between the two indicated by arrowhead in R). Differential interference contrast. (W–X) Mitosis. Regions of additive and subtractive positive birefringence (mitotic asters and spindle respectively) are shown with rectified polarized optics. Note the position of the myoplasm (arrowhead in W). The egg divides; in this case the cleavage plane is slightly tilted with respect to the animal–vegetal axis, probably due to the compression of the egg (X; differential interference contrast). Bar, 50 μm. (This sequence was recorded at a slightly higher temperature, at 23–25 °C rather than the usual 18–21 °C, which causes a slight decrease in developmental times).

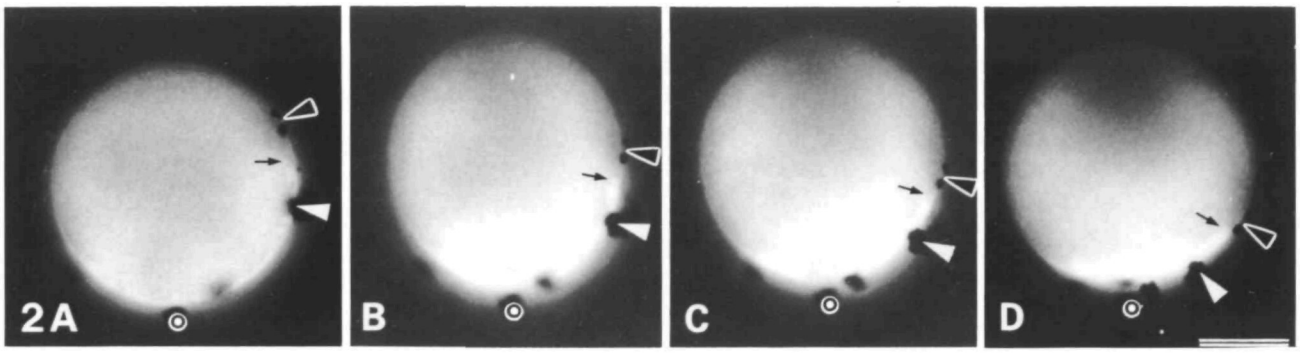


Fig. 2. The contraction wave and the first segregation phase of the mitochondria-rich myoplasm. The mitochondria-rich myoplasm is revealed in epifluorescence after staining with the vital dye $DiO(C_2)_3$. Nile blue particles (arrowheads) are attached to the surface and move toward the vegetal pole (dot marker) with the edge of the subcortical myoplasm (thin arrow). A–D were taken at approximately 20 s intervals, with A being close to the onset of the contraction wave. Bar, 50 μm.

The second phase of ooplasmic segregation

After the second polar body has been extruded, a series of complex events occur, which involve the movement of at least three different entities, i.e. the mitochondria-rich myoplasm, the sperm aster and the female pronucleus. We have observed these various events in many eggs, on the basis of which we describe the movements in a more general way. In addition, we were able to analyse the movements in more detail in two cases, in which the orientation of the egg, which obviously is very critical at this stage, was just right for the observation of the entire process.

Within a minute after the second polar body has been extruded, the vegetally localized myoplasm starts moving towards the equator of the egg. This movement occurs in two distinct subphases: the first one is an oscillatory movement over a period of about 15 min (see Fig. 1N–S), with an average speed of the anterior edge of the myoplasm of 6 μm min^{-1} ($6{\cdot}1 \pm 0{\cdot}3$ and $5{\cdot}7 \pm 0{\cdot}8$ μm min^{-1}, respectively, in the two cases analysed; see Table 1). The second subphase of this movement is a smoother and faster motion of the mass towards its final position at the equator (Fig. 1S–U), which occurs over a period of about 5 min with an average speed of its anterior edge of 26 μm min^{-1} ($25{\cdot}9 \pm 4{\cdot}5$ and $27{\cdot}1 \pm 9{\cdot}0$ μm min^{-1}, respectively; see Table 1). The direction and the velocity of the final movement of the mass are clearly related to the movement of the male pronucleus and surrounding aster towards the female pronucleus and the centre of the egg (observe the deformation of the egg surface in Fig. 1T–U). Photographic and video records strongly suggest that there is a cable-like structural connection between the myoplasm and the male pronuclear region (see arrowhead in Fig. 1R, and shape of the fluorescent mass in Fig. 4C–F).

Interestingly, we found that not all the mitochondria-rich myoplasm moves towards the equator and future posterior pole of the egg. About 20% of the mass, which is located at the most anterior side of the egg, gets separated from the remainder of the mass and remains located in the anterior vegetal region as the bulk of the myoplasm moves posteriorly (see arrowhead in Fig. 4C,F). This finding may have major implications for the lineage of the muscle cell line, as will be discussed later.

In contrast to the first segregation phase, which is accompanied by the movement of surface components (Fig. 2), there are no detectable surface movements associated with this second segregation phase of the mitochondria-rich mass. As the mass moves towards the future posterior pole of the embryo, particles attached on the surface remain still (Figs 5 and 6).

The movements of the pronuclei

Several minutes after the formation of the second polar body, the female pronucleus becomes visible at the animal pole of the egg, starts to migrate towards the centre of the egg and then curves to meet with the male pronucleus (Fig. 1P–V; and arrows in Fig. 3C–E). Similar to the movement of the myoplasm, this movement also seems to occur in two subphases, a slow and a fast phase (Table 1). During the first 7 to 8 min, it moves with an average speed of 4 μm min^{-1} ($3{\cdot}8 \pm 0{\cdot}4$ and $4{\cdot}8 \pm 1{\cdot}1$ μm min^{-1}, respectively, in the two cases analysed), whereas during the final movement towards the male pronucleus, which takes several minutes, it travels at an average velocity of about 12 μm min^{-1} ($9{\cdot}3 \pm 2{\cdot}2$ and $15{\cdot}1 \pm 1{\cdot}1$ μm min^{-1}).

The very large and asymmetric sperm aster, which is still closely associated with the egg cortex in the vegetal hemisphere at the time of the polar body formation, starts moving up together with the mitochondria-rich mass towards the equator of the egg (Fig. 1O–Q). The exact time at which the sperm aster starts moving up seems somewhat variable (being either simultaneous with, or a few minutes later than, the movement of the myoplasm) and may well depend on its position relative to the vegetal pole after the first segregation phase (and thus ultimately on the point of sperm entry; see previous section on Meiosis). The movement of the sperm pronucleus and surrounding aster again shows two distinct subphases (Table 1); a first subphase directed towards the equator with an average velocity

of 3 μm min^{-1} (4·0 ± 0·2 and 2·5 ± 0·1 μm min^{-1}, respectively) and a second subphase towards the centre of the egg (Fig. 1R–U) with an average velocity of 11 μm min^{-1} (10·9 ± 1·0 and 10·4 ± 2·5 μm min^{-1}, respectively). The second subphase of both the female and the male pronucleus starts at about the same time, as illustrated in Fig. 7, in which the distance between the two pronuclei is plotted as a function of time. During the first subphase, the pronuclear distance decreases at a rate of about 5 μm min^{-1} (Table 1) and, as both pronuclei speed up and curve towards the centre of the egg, their distance decreases at about 13 μm min^{-1} (Fig. 7 and Table 1). At the final stage of these pronuclear movements, both pronuclei meet in the centre of the egg, after which their membranes break down at approximately 40 min after fertilization (at 23–25 °C) (Fig. 1U–V).

Mitosis and cleavage

The mitotic apparatus is characterized by very large asters that radiate to the cortex and a small mitotic apparatus (Fig. 1W–X). Cytokinesis starts at the animal pole at about 62 min at 18–21 °C (*versus* about 50 min at 23–25 °C). The newly formed blastomeres possess large and prominent asters (Fig. 1X). The mitochondria-rich myoplasm is divided equally between the two daughter cells (Fig. 5D).

Discussion

In the present study, we have investigated the various cytoplasmic and surface movements that accompany fertilization in ascidians. Using microscopy techniques on the extremely clear and naked eggs of *Phallusia mammillata*, we were able to observe several hitherto undescribed aspects of fertilization and ooplasmic segregation in the ascidian egg, which will be discussed in the following sections. A schematic representation of the various events is shown in Fig. 8.

Fertilization of Phallusia mammillata *eggs*

As in other ascidian eggs (Conklin, 1905; Mancuso, 1963; Sawada & Osanai, 1981, 1985), the unfertilized egg of *Phallusia mammillata* is arrested in metaphase of the first meiotic division and displays a distinct animal–vegetal polarity. After staining with the vital dye $DiO(C_2)_3$, a subcortical layer rich in mitochondria can be observed under the entire egg surface, except for the animal pole area where only granules and endoplasmic reticulum are found in the cortex (Reverberi, 1956; Sardet & Gualtieri, in preparation).

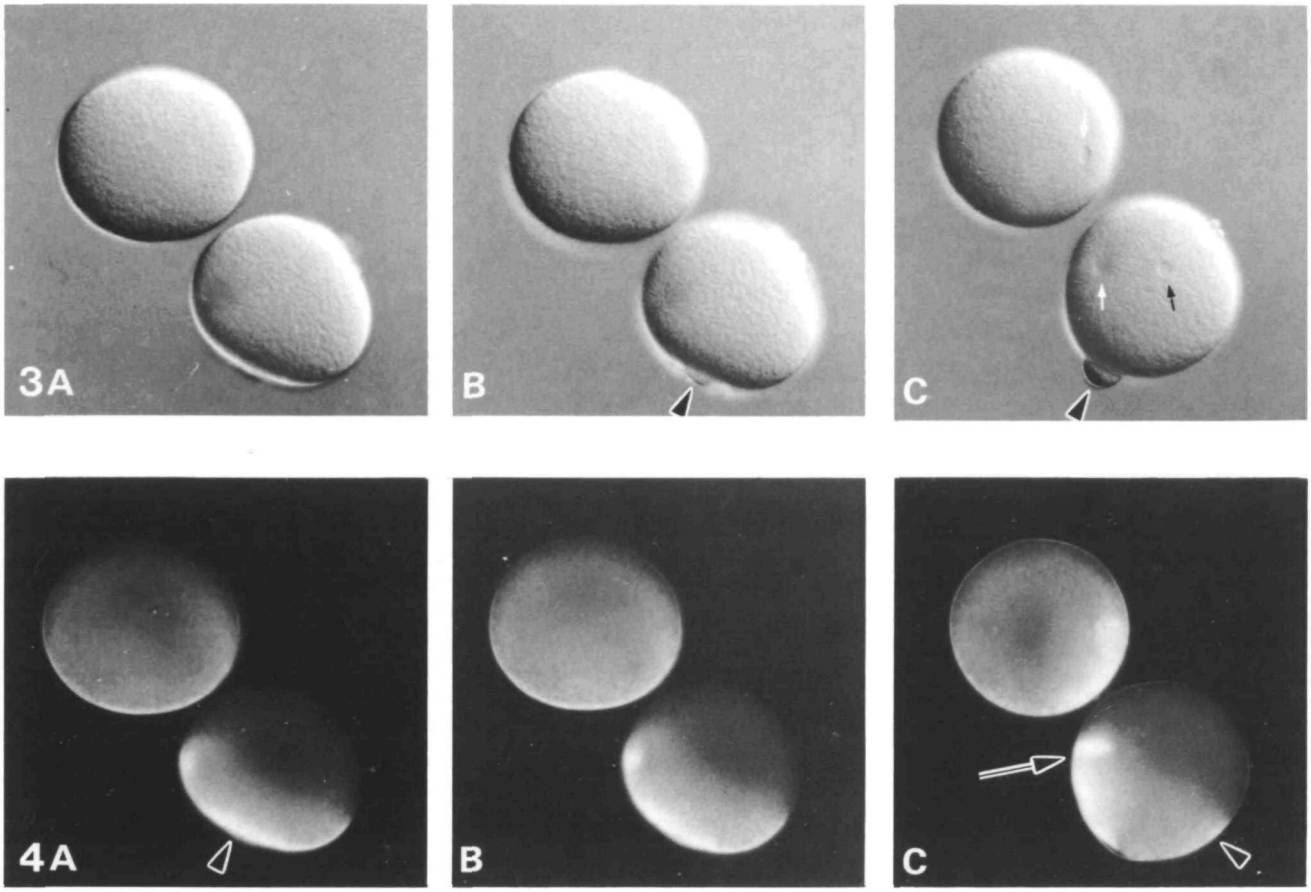
The eggs of *Phallusia mammillata* can be obtained throughout the year and can be easily dechorinated in large batches without affecting early development. However, in order to ensure rapid, reliable and synchronous fertilization, we found that it was necessary to preincubate the sperm with eggs that had kept their chorions. We presume that some activation of sperm occurs upon contact with the chorion, which facilitates fusion of the sperm with the egg (Honegger, 1986). Sperm do not seem to attach lastingly to the naked egg surface, and we could not detect any modifications of the egg surface at the site of sperm fusion. By using such preactivated sperm, we have previously shown that, in contrast to what was generally believed, the sperm does not enter at the vegetal pole of the ascidian egg, but rather tends to fuse with the egg in the animal hemisphere (Speksnijder *et al.* 1987). Subsequently, it is dragged towards the vegetal pole during the cortical contraction wave that accompanies the first phase of ooplasmic segregation (see Fig. 8).

The first phase of ooplasmic segregation

In *Phallusia mammillata*, as in other ascidians, whether chorionated or dechorionated, the first manifestation of fertilization is a flow of cytoplasm towards the animal pole and a constriction in the animal hemisphere, resulting in a bulging of the animal pole region (Sawada, 1983; Jeffery, 1984; Sawada & Schatten, 1988). It has been convincingly demonstrated that a network of actin microfilaments is present in the cortex of ascidian eggs (Jeffery & Meier, 1983; Sawada & Osanai, 1985). In *Ciona*, this actin network is organized as a basket with its opening towards the animal pole. The width of the animal actin-free area corresponds with the diameter of the constriction at the earliest stage of the contraction wave (Sawada & Osanai, 1985; Sawada, 1986). As the contraction wave travels towards the vegetal pole, the actin network regresses with the constriction into the vegetal hemisphere. This finding, and the observation that cytochalasins block the contraction wave (Zalokar, 1974; Reverberi, 1975; Sawada & Osanai, 1981; this paper), suggest that it is indeed a contraction of the cortical actin network that provides the force and direction for the contraction wave (Sawada, 1983; Jeffery, 1984). It is possible that, as in *Xenopus* eggs (Christensen *et al.* 1984), an actomyosin contractile mechanism is involved in this cortical contraction, but so far myosin has not been demonstrated in the cortex of ascidian eggs. The wave of elevated free calcium that we have observed at fertilization in both *Ciona* and *Phallusia* eggs (Speksnijder *et al.* 1986, 1988) is a likely candidate for the trigger of the contraction wave.

In many aspects, the wave of contraction in ascidian eggs resembles the mechanisms involved in the locomotion of cells like neutrophils and amoebae. Recently, Bray & White (1988) have proposed an interesting model for 'cortical flow' in animal cells based on a cortically located motor which drives processes such as cell locomotion, growth cone migration and cytokinesis. The contraction wave in ascidian eggs seems to be another example of such a process.

The contraction wave travels over most of the eggs at an average velocity of 1·4 μm s^{-1}. From our results, it is clear that particles attached to the egg surface as well as the subcortical mitochondria-rich mass travel in a coordinated fashion at the same speed during the first phase of ooplasmic segregation. We presume that the sperm that enter in the animal hemisphere also travel at the same speed towards the vegetal pole (Speksnijder *et*



Figs 3 and 4. The second phase of ooplasmic segregation. Two eggs are followed in differential interference contrast (Fig. 3) and epifluorescence after staining of the mitochondria with $DiO(C_2)_3$ (Fig. 4). Times after fertilization of Figs 3 and 4, respectively, are indicated in the legend. (A) The mitochondria-rich myoplasm (arrowhead in Fig. 4A) is situated beneath the surface in the vegetal pole region (16 and 17 min). (B) The second polar body has just been extruded and a cytoplasmic lobe ('the vegetal button') appears at the vegetal pole (arrowhead in Fig. 3B). The bulk of the myoplasm moves with the male pronuclear aster towards the equator (20 and 21 min). (C,D) Male (thin white arrows) and female (thin black arrows) pronuclei are moving closer to each other, the 'vegetal button' reaches its maximum size (arrowhead in Fig. 3C), and then regresses quickly (Fig. 3D). Note that mitochondria are excluded from the button (Fig. 4C). The bulk of the myoplasm has moved with the sperm aster towards the equator (arrow in Fig. 4C). A small fraction remains in the future anterior side of the embryo (arrowhead in Fig. 4C). Note the fluorescent area in the most animal part of the myoplasm which seems to correspond with the structural connection observed in Fig. 1R (25 and 26, and 27 and 28 min). (E,F) The pronuclei have met in the centre of the egg, and their pronuclear membranes have broken down. The myoplasm has split in two parts; the bulk of it is now situated at the future posterior pole and a small part is left behind at the opposite pole (arrowhead in Fig. 4F) (30 and 31 min, 38 and 39 min). Bar, 50 μm.

al. 1987). It must be noted, however, that toward the end of the contraction wave, the subcortical myoplasm lags behind the surface particles (see Fig. 2), confirming the notion that the subcortical components are progressively dragged down during the contraction of the egg cortex (Sawada, 1983).

It is interesting to note that, in the eggs of the Oligochaete *Tubifex*, a similar mechanism for ooplasmic segregation has been described (Shimizu, 1986). As in ascidians, the cortical actin network is believed to generate the motive force as well as determine the polarity of ooplasmic segregation and, in addition, a cosegregation of mitochondria and surface components with the cortical actin network has been demonstrated.

Meiosis

After the contraction wave has reached the vegetal pole of the ascidian egg, a cytoplasmic lobe remains present until shortly after the formation of the first polar body at about 5 min after fertilization. Then, at the time of second polar body formation, a second cytoplasmic lobe is formed at the vegetal pole. Both in their appearance and timing, these cytoplasmic lobes resemble the meiotic polar lobes formed in protostomes such as the gastropod *Ilyanassa obsoleta* (Morgan, 1933; Cather, 1963). The function of these meiotic polar lobes is unknown, but, like other modifications of the vegetal pole during meiosis in molluscan eggs, they may well be part of the general phenomenon of cytoplasmic localiz-

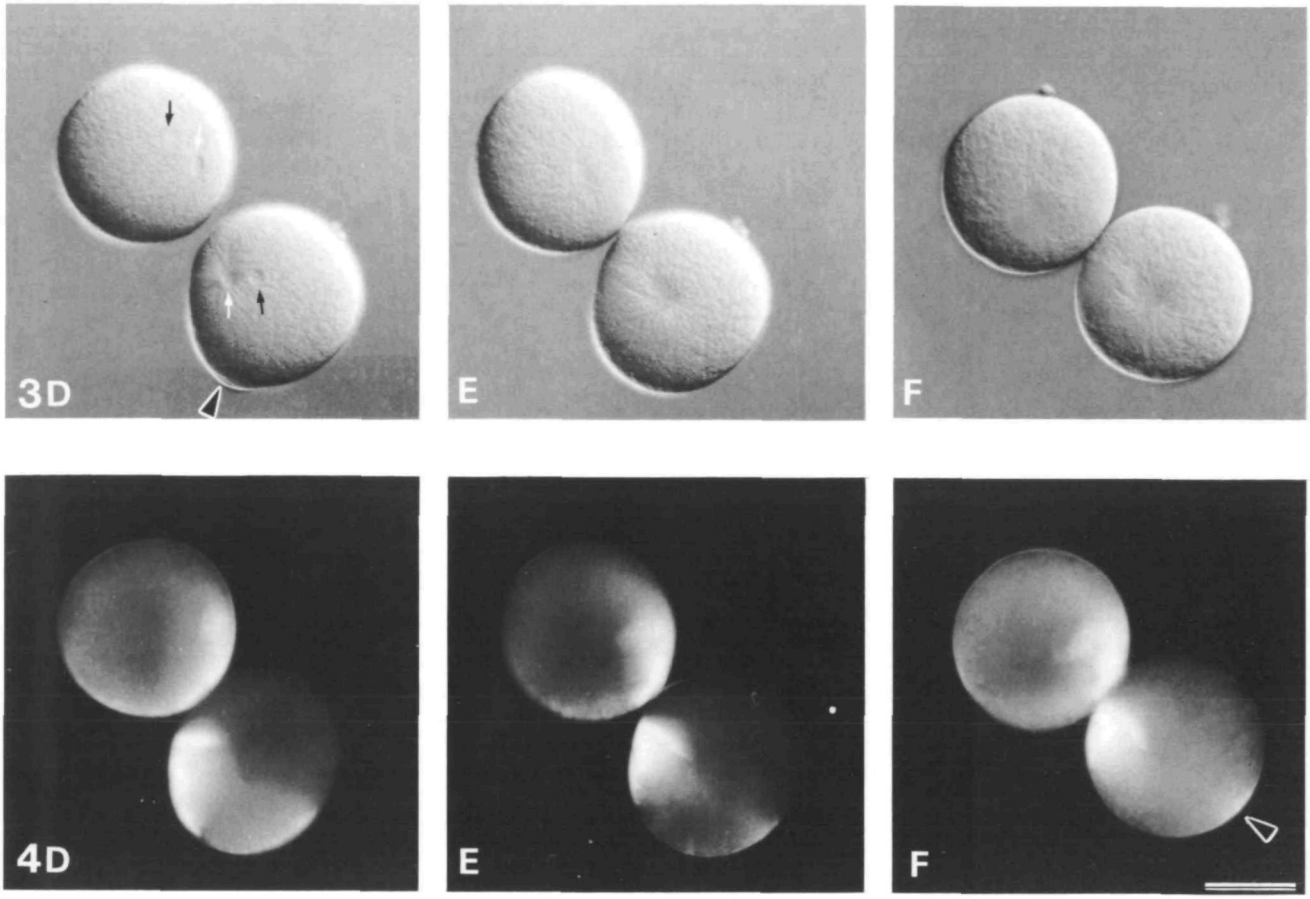


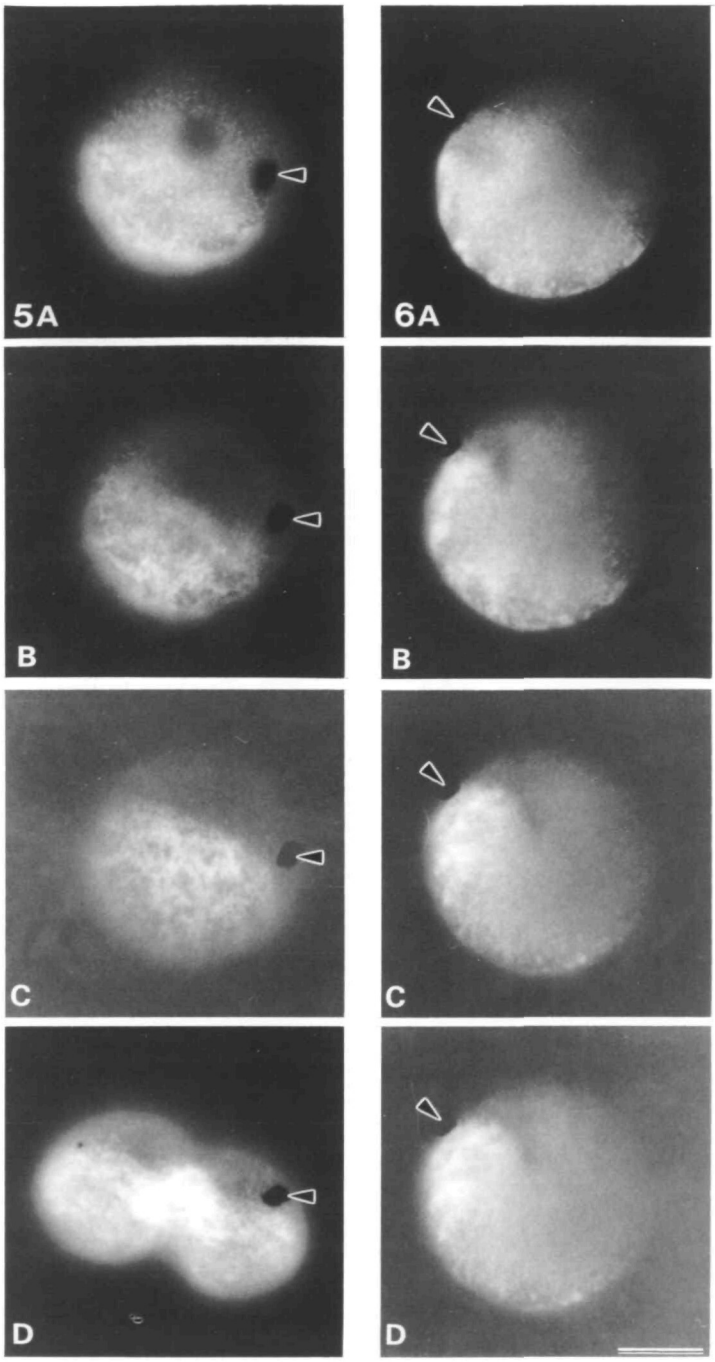
Table 1. *Mean velocity (in μm min^{-1}) of the motions of the myoplasm, female pronucleus and sperm aster; and the decrease in distance between the female pronucleus and the centre of the sperm aster (in μm min^{-1}) during the second phase of ooplasmic segregation in* Phallusia *eggs*

		Sequence no. 1*	Sequence no. 2*
Myoplasm	slow subphase	6·1 ± 0·3 (11)	5·7 ± 0·8 (5)
	fast subphase	25·9 ± 4·5 (2)	27·1 ± 9·0 (3)
Female pronucleus	slow subphase	3·8 ± 0·4 (7)	4·8 ± 1·1 (2)
	fast subphase	9·3 ± 2·2 (4)	15·1 ± 1·1 (4)
Sperm aster	slow subphase	4·0 ± 0·2 (3)	2·5 ± 0·1 (2)
	fast subphase	10·9 ± 1·0 (4)	10·4 ± 2·5 (3)
Decrease distance between female pronucleus and centre of the sperm aster	slow subphase	3·6 ± 0·6 (7)	5·8 ± 0·3 (2)
	fast subphase	13·2 ± 1·7 (4)	13·2 ± 0·4 (3)

*These data were obtained from two separate sequences – recorded with an Optical Memory Disk Recorder (OMDR) – in which the orientation of the egg was optimal for the analysis of these motions. In each egg, the position of the anterior edge of the myoplasm, the female pronucleus and the centre of the sperm aster was marked at 1- to 3-min intervals, and the average velocity of each of the motions as well as the decrease in distance between the female pronucleus and the centre of the sperm aster over that time interval was calculated. From these values, the mean velocity was determined. Indicated are the mean ± S.E.M. (*n*); *n* is the number of time intervals over which the average velocities were calculated.

ation during meiotic maturation (Longo, 1983). As in many polar lobe-forming species (Dohmen, 1983; Speksnijder & Dohmen, 1983; Speksnijder *et al.* 1985*a*,*b*), ascidian eggs display distinct surface differentiations at their vegetal pole after fertilization, such as a higher density of microvilli and lectin-binding sites (Sawada & Osanai, 1981; Zalokar, 1980; O'Dell *et al.* 1973). In addition, recent experiments by Bates & Jeffery (1987) have demonstrated the transient localization of specific (axial) determinants at the vegetal pole of the ascidian *Styela* during meiosis. The similarity in both appearance and timing of these two types of lobes in two groups of animals (ascidians *versus* molluscs and annelids) that are evolutionarily so far apart, is intriguing.

Another interesting aspect of meiosis is the occurrence of oscillatory cytoplasmic and surface movements during the period between the formation of the first and



Figs 5 and 6. Coordinated motion of the myoplasm and surface particles during the second phase of ooplasmic segregation. Two complementary views of this process are shown under epifluorescent illumination (Fig. 5: face-on view; Fig. 6: side view). Mitochondria are visualized after accumulation of the vital dye $DiO(C_2)_3$; arrowheads indicate Nile blue particles attached to the egg surface. Times after fertilization for Fig. 5 are 32, 42, 47 and 55 min; and for Fig. 6: 34, 43, 48 and 53 min. Bar, 50 μm.

second polar body. Preliminary experiments suggest that these periodic oscillations are coupled to oscillations in the concentration of intracellular free calcium (Speksnijder *et al.* 1986, 1988). At present, we do not know what their function is, but it is noteworthy that

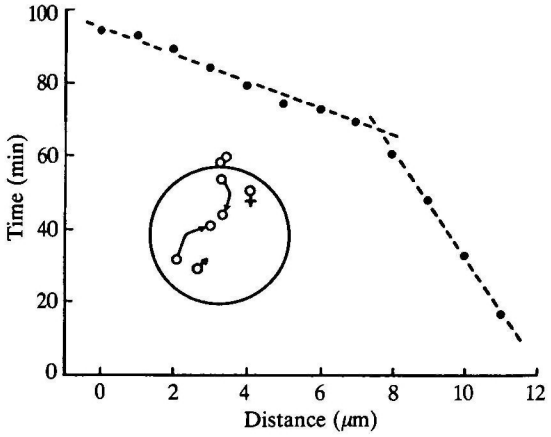


Fig. 7. Distance between the female pronucleus and the centre of the sperm aster as a function of time. The inset shows the path of each of the pronuclei. At $t = 0$, the female pronucleus is first visible. At about 7–8 min, both pronuclei speed up and curve towards the centre of the egg to meet at about 12 min. These data were obtained from the video sequence shown in Fig. 1 (sequence no. 1 in Table 1).

periodic oscillations in free calcium concentration have also been observed in mammalian eggs after fertilization and in hepatocytes after hormonal stimulation (Cuthbertson & Cobbold, 1985; Miyazaki *et al.* 1986; Woods *et al.* 1986). However, no associated cytoplasmic or surface motions have yet been reported in mammalian cells.

The second phase of ooplasmic segregation

The mitochondria-rich mass constitutes a distinct cytoplasmic domain which extends up to the growing sperm aster. Our observations suggest they may be physically connected (see Fig. 1R–U and Fig. 3C,4C). After second polar body formation, the bulk of the myoplasm slowly migrates with the sperm aster towards the equator in a succession of oscillatory motions. A faster steady motion then carries the bulk of the myoplasm towards its final position at the future posterior pole of the egg. This motion seems tied to the movement of the male and female pronuclei to the centre of the egg. It is not accompanied by surface motions. It has recently been reported that this second phase of ooplasmic segregation is sensitive to the microtubule inhibitor colcemid (Sawada & Schatten, 1985, 1988). Therefore, it seems likely that the microtubules of the sperm aster are involved in localizing the myoplasm in its posterior position. However, the exact mechanism of this microtubule-controlled movement is not yet clear. Sawada & Schatten (1988) have shown that microtubules of the sperm aster form a three-dimensional array extending through and beyond the myoplasm, and that the majority of these microtubules run parallel to the egg surface. If such microtubules were localized in the cortex in a parallel array, they could serve as tracks for shearing forces to drive the subcortical myoplasm, similar to the mechanism suggested for cortical rotation in fertilized *Xenopus* eggs (Elinson & Rowning, 1988). Alternatively, the myoplasmic domain might be pulled

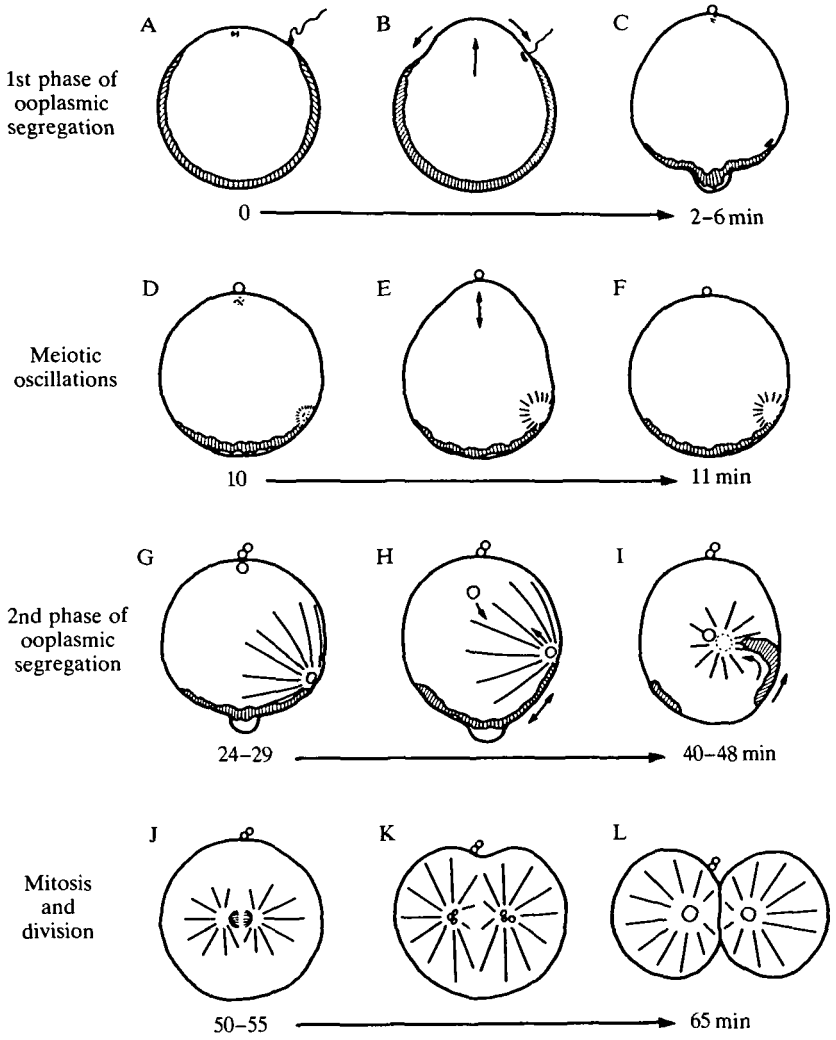


Fig. 8. Fertilization and morphogenetic movements in the ascidian egg. (A–C) The first phase of ooplasmic segregation. (A) The meiotic spindle defines the animal pole. A subcortical layer rich in mitochondria ('the myoplasm') is excluded from the animal pole region. Sperm have a tendency to enter in the animal hemisphere. (B) A wave of cortical contraction propagates from the animal to the vegetal pole regions at an average velocity of $1{\cdot}4\,\mu m\,s^{-1}$, dragging the sperm nucleus and the myoplasm down and pushing the central cytoplasm up towards the animal pole. (C) The first phase of ooplasmic segregation is completed 2 min after fertilization. The first polar body appears at about 5 min and a cytoplasmic lobe remains present at the vegetal pole from 2 to 6 min after fertilization. (D–F) Meiotic oscillations. Between first and second polar body formation (at 5 and 24–29 min, respectively), the animal pole region undergoes periodic cycles of protrusion and retraction movements, simultaneous with movements of the centrally located cytoplasm towards the animal pole and back, with a period of 60–90 s (an example is given in the sequence D,E,F). The sperm aster, located in the vegetal hemisphere, enlarges. (G–I). The second phase of ooplasmic segregation. (G) The second polar body is extruded at 24–29 min after fertilization, a second vegetal cytoplasmic lobe is formed and the female pronucleus migrates towards the centre of the egg to meet the male pronucleus. (H) Oscillating motions move the myoplasm towards the equator. (I) A smooth steady motion carries the bulk of the myoplasm towards the future posterior pole of the embryo. A small part of the myoplasm remains in a subcortical anterior position. Pronuclei meet in the centre of the egg, and their membranes break down. (J–L) Mitosis and division. (J) A mitotic spindle with extremely large asters is formed. (K). Cleavage starts at the animal pole. While karyomeres are fusing, the astral rays can be seen to extend all the way to the egg cortex. (L) Division. Nuclear membrane reforms. The myoplasm is divided equally between the daughter cells (not shown).

by microtubules attached to the male pronucleus or by growing astral microtubules.

An intriguing fact is that the myoplasm – with its much larger mass – seems to move about 2–3 times faster than the sperm aster and the female pronucleus (see Table 1). This suggests an independent motile mechanism such as a shearing force along microtubular tracks. In addition, all three entities (myoplasm, sperm aster and female pronucleus) show two distinct phases in their movement; a first relatively slow subphase (average velocity of 6, 4 and $3\,\mu m\,min^{-1}$, respectively) and a second, faster subphase (average velocity is 26, 12 and $11\,\mu m\,min^{-1}$, respectively). The velocities during the slow subphase correspond to rates of growth and

shrinkage of microtubules observed *in vivo* (see e.g. Schulze & Kirshner, 1986; Cassimeris *et al.* 1987). In addition, the velocities measured for the fast subphase of the pronuclear movements correspond with values reported for the movement of beads, organelles and female pronuclei along astral rays in sand dollar and sea urchin eggs (Schatten, 1982; Hamaguchi *et al.* 1986; Wadsworth, 1987). Therefore, all evidence points in the direction of microtubules as being the driving force for the various motions during the second phase of ooplasmic segregation (Sawada & Schatten, 1985, 1988; this paper), but clearly our results suggest a complex mechanism for these microtubule-based motile events in the ascidian egg.

The myoplasm and the origin of larval muscle cells

From a developmental point of view, our most interesting observation is that the mitochondria-rich mass becomes separated in two portions during the second phase of ooplasmic segregation. As the bulk of the mass moves towards the future posterior pole of the embryo, a small portion which is located most anteriorly, remains behind, and thus will be inherited by blastomeres of the A4.1 cell lineage. A similar observation has been made on the mitochondria-rich mass in *Ciona* (Deno, 1987). This finding may have important embryological implications. It was thought until recently that muscle cell determinants (situated in the so-called 'myoplasm' or 'yellow crescent' region) were only localized in the descendants of the posterior B4.1 blastomeres (Reverberi, 1971; Whittaker, 1979; Jeffery *et al.* 1984). However, work by Nishida & Satoh (Nishida & Satoh, 1983, 1985; Nishida, 1987), ourselves (Zalokar & Sardet, 1984), and recent careful reexaminations of traditional cell lineages (Meedel *et al.* 1987), have shown that descendants other than those of the B4.1 blastomere are able to differentiate into muscle cells in the embryo or even in isolation. Our observation that some of the myoplasm (an entity that roughly corresponds to the mitochondria-rich mass) also moves into the vegetal anterior part of the embryo would explain why descendants of the A4.1 blastomeres can differentiate into muscle cells. However, additional muscle cells are apparently also formed from the descendants of the b4.2 (animal posterior) blastomere. As a possible explanation, we suggest that, in cases where the sperm aster forms toward the equator rather than close to the vegetal pole, the myoplasm will end up at, and also slightly above, the equator. As a result, some of the myoplasm will be included in the b4.2 cells after the first equatorial division (i.e. third cleavage). This explanation seems plausible since the position of the sperm aster with respect to the equator will depend on the point of sperm entry which we have shown to be preferentially in the animal hemisphere (Speksnijder *et al.* 1987).

It remains to be defined what structures, organelles or molecules constitute the particular components that determine cell fate as they are segregated into specific areas of the egg and embryo during successive cleavages or by experimental manipulations (see Whittaker, 1980, 1982, 1987; Deno *et al.* 1984; Crowther & Whittaker, 1986; Jeffery *et al.* 1986; Nishikata *et al.* 1987). Our observations of the morphogenetic movements in the ascidian egg open the way for new studies of this unresolved problem.

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