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Axis establishment and microtubule-mediated waves prior to first cleavage in *Beroe ovata*

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SUMMARY

The single axis (oral-aboral) and two planes of symmetry of the ctenophore *Beroe ovata* become established with respect to the position of zygote nucleus formation and the orientation of first cleavage. Bisection of *Beroe* eggs at different times revealed that differences in egg organisation are established in relation to the presumptive oral-aboral axis before first cleavage. Lateral fragments produced after but not before the time of first mitosis developed into larvae lacking comb-plates on one side. Time-lapse video demonstrated that waves of cytoplasmic reorganisation spread through the layer of peripheral cytoplasm (ectoplasm) of the egg during the 80 minute period between pronuclear fusion and first cleavage, along the future oral-aboral axis. These waves are manifest as the progressive displacement and dispersal of plaques of accumulated organelles around supernumerary sperm nuclei, and a series of surface movements. Their timing and direction of propagation suggest they may be involved in establishing cytoplasmic differences with respect to the embryonic axis.

Inhibitor experiments suggested that the observed cytoplasmic reorganisation involves microtubules. Nocodazole and taxol, which prevent microtubule turnover, blocked plaque dispersal and reduced surface movements. The microfilament-disrupting drug cytochalasin B did not prevent plaque dispersal but induced abnormal surface contractions. We examined changes in microtubule organisation using immunofluorescence on eggs fixed at different times and in live eggs following injection of rhodamine-tubulin. Giant microtubule asters become associated with each male pronucleus after the end of meiosis. Following pronuclear fusion they disappear successively, those nearest the zygote nucleus shrinking first, to establish gradients of aster size within single eggs. Regional differences in microtubule behaviour around the time of mitosis were revealed by brief taxol treatment, which induced the formation of small microtubule asters in the region of the nucleus or spindle during both first and second cell cycles. The observed wave of change may thus reflect the local appearance and spreading of mitotic activity as the zygote nucleus approaches mitosis.

Key words: cleavage, ctenophore, embryonic axis, microtubule, mitosis, aster

INTRODUCTION

Ctenophores have a single body axis (oral-aboral) with two perpendicular planes of symmetry dividing the embryo into four quadrants. Each quadrant derives from one blastomere at the 4-cell stage. These blastomeres undergo two unequal divisions to give rise to two types of macromere and two types of micromere at the 16-cell stage. These all have different fates and develop autonomously in classical mosaic fashion. Two of the eight characteristic rows of ciliated comb-plates of ctenophores derive from one micromere in each quadrant (see Reverberi, 1971; Freeman, 1979; Sardet et al., 1990 for reviews). The high degree of organisation in cleavage stage embryos becomes established entirely after fertilisation, the egg having no pre-determined developmental axis. The position of the oral pole coincides with the site of initiation of the first cleavage furrow, and not to the 'animal pole' where the polar bodies are emitted (Freeman, 1977). The site of first cleavage depends in turn on the position of sperm entry, which can be anywhere over the egg surface (Freeman, 1977; Carré and Sardet, 1984). The female pronucleus migrates to join a stationary male pronucleus, 'choosing' one in cases where polyspermy has occurred (Carré and Sardet, 1984). At this site, the zygote nucleus forms, first mitosis occurs and the first cleavage furrow starts, cleavage being unipolar. The planes of symmetry of the larvae can be predicted by the orientation of the first cleavage, and may be related to the path of migration of the female pronucleus (Carré et al., 1991). Pronuclear migration, mitosis and all other cytoplasmic events occur in a 5-50 μm thick peripheral layer of 'ectoplasm' that surrounds a central ball of yolky 'endoplasm'. This

layer changes thickness locally during the course of early developmental events. It contains naturally fluorescent granules which normally become segregated to the micromeres. When separated from the rest of the egg, the ectoplasm can support the development of most larval structures, including comb-plates (La Spina, 1963).

Experiments involving the removal of certain regions of ctenophore eggs or embryos have been performed since the end of the last century to try to define where and when developmental 'potentials' become localised. Particular attention has been paid to the comb-plates, which provide a convenient marker for embryonic organisation. Early studies described the morphology of larvae that developed (Driesch and Morgan, 1895b; Ziegler, 1898; Fischel, 1903; Yatsu, 1912). More recently, Freeman (1976, 1977) quantified the localisation of comb-plate forming potential toward the future aboral pole by counting the number of 4- or 8-cell-stage blastomeres from operated embryos that went on to develop comb-plates. We have summarised these various studies in Fig. 1. It is clear that developmentally significant inhomogeneities become established by the 2-cell stage. At this stage, differences are apparent both along the length of the axis and perpendicular to it. Isolated 2-cell stage blastomeres develop only four rows of comb-plates (Driesch and Morgan, 1895a) while eggs bisected before first cleavage can regulate their development to give eight. Freeman's experiments (1976, 1977) showed that factors required for the development of comb-plates are lost following removal of cytoplasm from the aboral pole (opposite the nuclei) of 2-cell blastomeres but not from the aboral pole of eggs (stippling in Fig. 1). Fischel (1903) found similarly that removing fragments from the aboral pole at the time of cleavage did not cause loss of larval structures. However, when he removed cytoplasm from near the site of cleavage initiation (future oral pole) at this time, larvae with reduced numbers of comb-plates, even lacking entire comb-plate rows, developed. Some cases with similar abnormalities were described in other studies performed on uncleaved eggs (Driesch and Morgan, 1895b; Yatsu, 1912). The exact time and orientation of the cuts that gave rise to the abnormal larvae in these studies is not known. Eggs bisected before second polar body formation always developed normally. These results suggest that inhomogeneity in the egg cytoplasm starts to develop during the first cell cycle, and that comb-plate-forming potential may be concentrated around the future oral pole (i.e. near the nuclei) at the time first cleavage starts, rather than in its final location at the opposite pole. This kind of localisation would be hard to detect using Freeman's approach since only fragments containing nuclei develop, and the assay quantifies loss but not concentration of comb-plate forming potential in fragments.

We have reexamined the question of when inhomogeneities relating to axial organisation are first established in ctenophores by bisecting *Beroe ovata* eggs at well defined times during the first cell cycle and examining the larvae that developed. We concentrated on the change in behaviour of lateral fragments from developing eight comb-plate rows to developing only four. We then examined the changes in organisation of cytoplasmic components that occur in the egg during the crucial period between pronuclear fusion and first cleavage, to see what might underlie the establishment of axial organisation. Inhibitor studies suggested that microtubules play a central role in the observed cytoplasmic reorganisation, so we went on to examine microtubule organisation over the first cell cycle using fixed and live eggs, and to examine how regional variation in microtubule organisation become established. Brief reports of some of the observations reported here have been presented previously (Carré et al., 1990; Johnston et al., 1991).

MATERIALS AND METHODS

Collection, handling and observation of eggs

Adult *Beroe ovata* were collected and maintained, and spawned eggs denuded and handled as described previously (Carré and Sardet, 1984; Carré et al., 1991). Image processing and time-lapse video recording of live specimens with epifluorescence illumination to reveal natural fluorescence and DNA stained with the vital dye Hoechst 33342 were made using the imaging station described previously (Carré et al., 1991). This was based around a Zeiss Axiophot microscope with filters for Hoechst (ex 353-377, em 395, FD 395) and FITC (ex 450-490, em 520-560, FD 510). Eggs were bisected on glass slides using razor blade fragments and cultured within their envelopes.

Immunofluorescence

Whole denuded *Beroe* eggs were rinsed in EMC, a divalent cation-free sea water (Detering et al., 1977), to remove extracellular material and fixed at −20°C in methanol containing 1% formaldehyde (from 37% solution) for 30 minutes or more. They were rehydrated to PBS/0.025% Triton X-100, pipetted gently to break the eggs and free internal yolk, extracted for 10-20 minutes with 0.5% Triton X-100/PBS, rinsed in PBS and then incubated in anti-tubulin rat monoclonal YL1/2 (Kilmartin et al., 1982) followed by texas-red-conjugated anti-rat IgG/IgM (Jackson) with 0.5 μg/ml Hoechst 33342 to stain nuclei.

Preparation and microinjection of rhodamine-tubulin

Rhodamine-tubulin was prepared by modifying the methods of Leslie et al., 1984 and Hyman et al., 1991. Twice cycled porcine brain tubulin was repolymerised, pelleted at 100 000 ***g*** and the microtubules resuspended to about 4 mg/ml in Mes buffer (100 mM Mes, 2 mM EGTA, 1 mM EDTA, 0.5 mM $MgCl_2$ pH 6.9) with 4 M glycerol and 1 mM GTP at 37°C. NHSR (5 (and 6) carboxy tetramethylrhodamine succinimidyl ester, Molecular Probes) was mixed in gradually from a freshly made 25 mg/ml stock in DMSO to give a molar dye:tubulin ratio of 40:1 and incubated for a total of 10 minutes at 37˚C. Labelled microtubules were pelleted through a cushion of 5% DMSO, 40% glycerol in GB(1M sodium glutamate pH 6.6, 1 mM EGTA, 0.1 mM GTP), depolymerised in GB at 4°C and clarified at 40 000 ***g***. The preparation was cleaned of unreacted dye and assembly-incompetent tubulin by one or two further cycles of polymerisation /depolymerisation in GB with final resuspension in injection buffer (50 mM Hepes, 0.1 mM $MgCl_2$, pH 6.9) to give 2-4 mg/ml protein. This rhodamine-tubulin was virtually MAP free, as determined by electrophoresis using 7.5% SDS polyacrylamide gels and Coomassie staining.

Eggs, in sea water, were held in position with forceps and microinjection was performed using pulled glass capillaries (Clarke) and a Singer micromanipulator. Air pressure was sup-

plied by a mouth pipette and the injection volume was about 20 nl, as determined by counting the number of injections from a known volume. Injected eggs underwent mitoses and cleaved normally in most cases.

Confocal microscopy

A Leica confocal laser scanning microscope equipped with an argon/krypton laser was used to examine immunofluorescence samples and rhodamine-tubulin injected eggs. 40× and 63× objectives were used and images averaged from 4 or 8 scans of the laser beam. Live eggs were mounted in chambers made from gelatin-coated slides and coverslips (Zalokar and Sardet, 1984) sealed with silicone grease, and images acquired every 5-20 minutes. Occasionally pronuclear fusion or first mitosis was inhibited in eggs under observation. This effect varied between batches of eggs and occurred more frequently in eggs incubated in Hoechst 33342. Data from blocked eggs was discarded.

Drugs

Nocodazole (Sigma), Taxol (Molecular Probes) and Cytochalasin B (Sigma) were diluted rapidly into sea water from stocks of 1-10 mM in DMSO, and then used within two hours. Drug-treated eggs were rinsed briefly in EMC before fixation for immunofluorescence.

RESULTS

Establishment of axial organisation before first cleavage

We followed the development of fragments of eggs bisected at different times during the first cell cycle to see whether differences arise with respect to the oral-aboral axis before first cleavage. Fragments from eggs bisected perpendicular to this axis (equatorial cuts) at the time of first cleavage develop into normal larvae with eight rows of comb-plates (see Fig. 1: Ziegler, 1898; Fischel, 1903; Freeman and Reynolds, 1973; 5 out of 5 operations performed by us). We therefore decided to bisect eggs parallel to this axis (axial cuts) to assess the establishment of symmetry across, rather than localisation along it. In fragments that continued to cleave, the number of micromeres and then the number of comb-plate rows that developed was counted. The time of bisection was assessed in relation to the normal sequence of events in fertilised eggs described previously (Carré and Sardet, 1984; Carré et al., 1991). The behaviour of pronuclei and polar bodies, the appearance and disappearance of plaques around male pronuclei and the behaviour of the zygote nucleus preceding mitosis can all be observed with a dissecting microscope.

The results of our experiments are given in Table 1 and examples of fragments shown in Fig. 2. Prior to pronuclear fusion, eggs were bisected with random orientation since the position of the zygote nucleus and thus of the oral-aboral axis is not decided at this stage. Fragments need to contain the female pronucleus plus at least one male pronucleus in order to develop. Almost all fragments that cleaved developed eight well-organised rows of comb-plates (Table 1, groups 1-3; Fig. 2A). In the exceptions, comb-plates were present but disorganised. Bisection performed with random orientation just before first mitosis (Table 1, group 4a) gave similar results, although comb-plate rows were often less well organised. Most fragments from axial cuts at this stage (Table 1, group 4b) also developed eight comb-plate rows, however, these were truncated on one side of the larva in some cases (as in Fig. 2D), and one case developed with only 4 rows.

When axial cuts were made at the time the cleavage furrow was first visible, the progression of cleavage was affected in one of three ways. Either one fragment cleaved immediately and the other never did (Table 1, group 5a; Fig. 2B), or one fragment cleaved immediately into a uncleaving and a cleaving part, while the other did not cleave until the second division (Table 1, groups 5c and 5b respectively; Fig. 2E, F) or neither fragment cleaved until the time of second cleavage in controls (Table 1, group 5d; Fig. 2I,J). We interpret this as reflecting the precise position of the cuts (see Fig. 2L) such that in group 5a both daughter nuclei and the cleavage furrow fell on one side of the cut, in groups 5b and c the cut separated one daughter nucleus from the furrow and in group 5d, the first cleavage

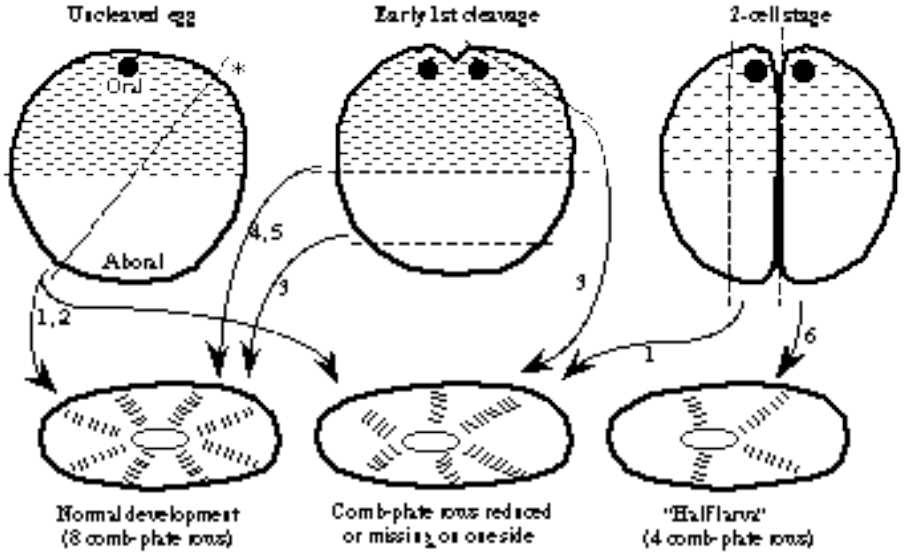

Fig. 1. Diagram summarising previous experiments in which regions of ctenophore eggs or 2-cell blastomeres were removed. Embryos are shown from the side with the presumptive oral pole at the top. Stippling represents the amount of comb-plate-forming potential retained in oral fragments following equatorial cuts (Freeman 1976, 1977). Dotted lines represent the position of cuts by: 1. Driesch and Morgan (1895b); 2. Yatsu (1912); 3. Fischel (1903); 4. Ziegler (1898); 5. Freeman and Reynolds (1973); 6. Driesch and Morgan (1895a). * In uncleaved eggs, cuts were made at random with respect to the future oral pole (eventual position of zygote nucleus formation).

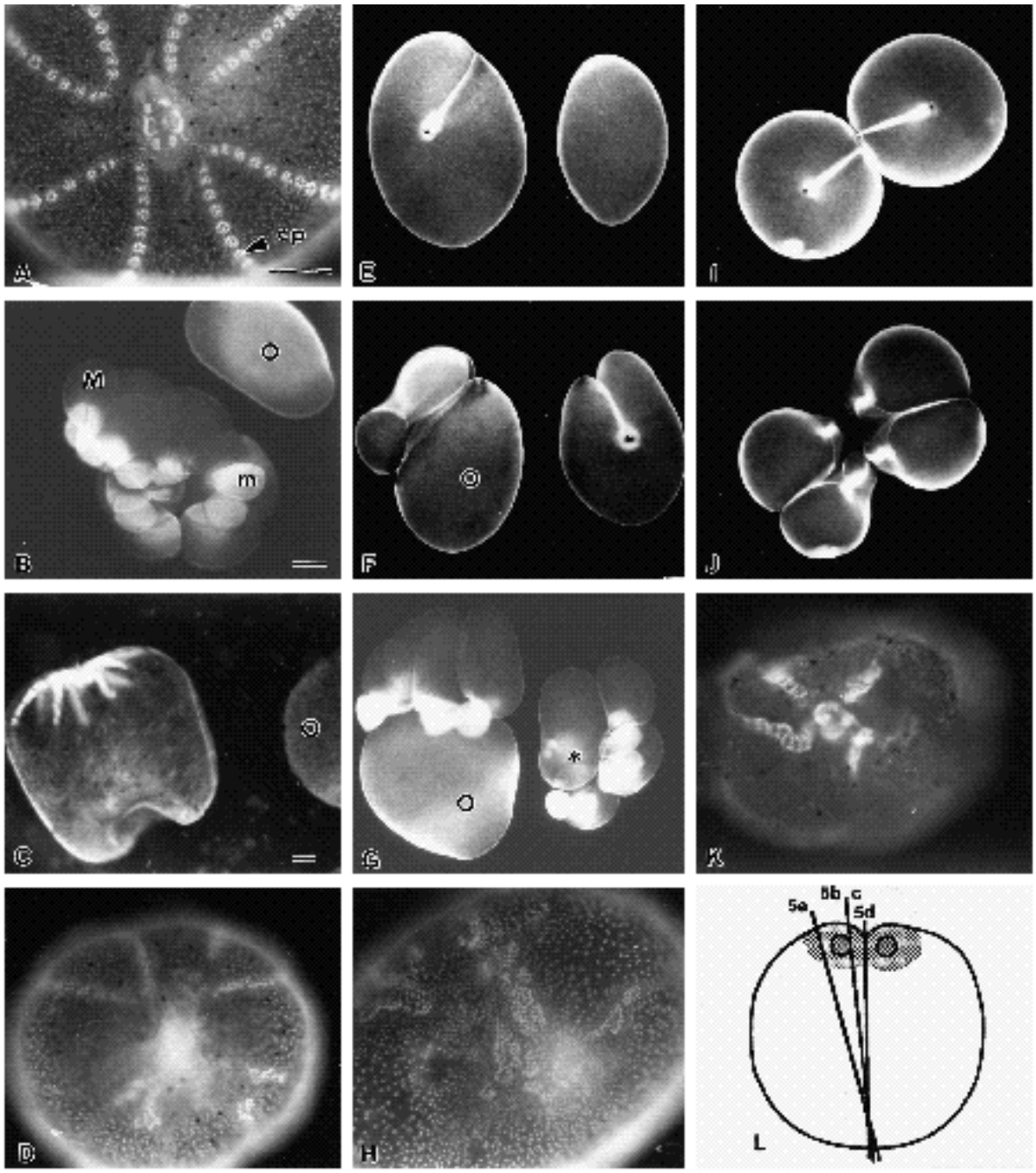

Fig. 2. Development of fragments from bisected eggs. Larvae in A, C, D, H and K are stained with Hoechst 33342 to visualise the clusters of larger nuclei beneath each comb-plate (cp). Other images are of unstained blastomeres with naturally fluorescent granules concentrated in the cleavage furrows and then in micromeres (m). (A) View from the aboral pole of a larva derived from an egg bisected during meiosis (group 1 in Table 1): 8 well-organised comb-plate rows have formed. (B) Fragment from egg bisected at the beginning of cleavage (group 5a) that developed 8 micromeres (m) and 8 macromeres (M) at the 16-cell stage while the sister fragment remained uncleaved (o). (C) Larva derived from same fragment viewed from the side, showing 8 comb-plate rows. (D) Another larva from group 5a viewed from the aboral pole showing 4 normal comb-plate rows on one side and four truncated ones on the other side. (E) Fragments from another egg bisected at the beginning of cleavage, photographed a few minutes later. The left fragment cleaved immediately (group 5c) and the right fragment (group 5b) did not. (F) At the next cleavage division, the fragments shown in E both divided, leaving one undividing portion created by the first cleavage (O). (G) At the 16-cell stage each of these fragments gave four micromeres (one is still in the process of forming (*)). (H) An embryo derived from a class 5b fragment seen from the aboral pole with 3 or 4 comb-plate rows present but poorly organised. (I) Fragments of an egg bisected at the beginning of cleavage in which neither divided until the time of the second division in controls (group 5d). (J) The same fragments dividing to give 4 macromeres at the next cleavage. (K) A larva from a group 5d fragment with 4 comb-plate rows, seen from the aboral pole. (L) Diagram showing the probable position of cuts performed at the beginning of first cleavage relative to the cleavage furrow and daughter nuclei. Shading represents the proposed location of comb-plate-specifying potential. Bars, 100 μm (D-K same magnification as B).

Table 1. Development of comb-plate rows in fragments of *Beroe* eggs bisected at different times

Group†	Stage of operation*	Number of fragments	Number with comb-plates organised in: 0-2 rows	3-4 rows	5-6 rows	7-8 rows	no order
1	Meiotic	21	0	0	0	21 (100%)	0
2	Early pronuclear migration	17	0	0	0	13 (76%)	4
3	End of pronuclear migration	6	0	0	0	6 (100%)	0
4a	Just before first mitosis	8	0	0	0	6 (75%)	2
4b		8	0	1	0	7 (88%)	0
5a	Cleavage beginning	8	0	1	3	4 (50%)	0
5b		17	2	10 (59%)	1	1	3
5c		18	6	10 (56%)	0	0	2
5d		8	0	8 (100%)	0	0	0

Only fragments that cleaved and developed to give larvae after 3 days at 20°C are included.

†Groups 1, 2, 3 and 4a were from randomly oriented bisection. Only one fragment of each pair cleaved. Groups 4b and 5a-d were from bisection parallel to the future oral-aboral axis (see diagram in Fig. 1), close to it in all cases except for 3 fragments in group 4b. In group 5a only one fragment of each pair cleaved. The fragments in groups 5b and c were from operations where the first cleavage continued in one fragment (5c) giving an uncleaved part while the other fragment (5b) cleaved only at the second division. Group 5d are from operations where the first cleavage was suppressed completely and both fragments cleaved at the second division. See Fig. 2 for examples.

*Stages were defined as follows: 1, before second polar body emission; 2, polar bodies emitted, plaques not yet formed around male pronuclei; 3, plaques present across egg; 4, plaques dispersed, zygote nucleus forming bulge; 5, furrow just visible.

division was replaced by the manual bisection. Some of the fragments in all these groups developed with entire rows of comb-plates lacking. Half of the group 5a fragments did develop 8 micromeres and 8 comb-plate rows (Fig. 2B,C) but these were often truncated on one side of the embryo (Fig. 2D). The other group 5a fragments developed 4 or 6 comb-plate rows. In cases where the two daughter nuclei were separated (groups 5b, c and d), most fragments developed 4 micromeres and then 3 or 4 comb-plate rows (Fig. 2E-K). The organisation of the comb-plate rows was sometimes poor (eg Fig. 2H).

These bisection experiments demonstrate that the symmetry of the embryo with respect to the oral-aboral axis, manifest by the appearance and arrangement of the 8 comb-plate rows, is established prior to first cleavage. The change in location of cytoplasmic components responsible for this symmetrical plan and/or their ability to redistribute following removal of one region seems to start at around the time of mitosis. The process of unipolar cleavage is not required for this organisation to become established, since fragments containing a single daughter nucleus developed like isolated 2-cell blastomeres, even when the first cleavage furrow was replaced by mechanical bisection. Our results support the idea that factors responsible for directing the development of comb-plate rows become arranged close to the future oral pole prior to first cleavage, i.e. around the first mitotic spindle and daughter nuclei (grey region in Fig. 2L).

A wave of cytoplasmic reorganisation occurs prior to first cleavage

Having established that the establishment of the future oral-aboral axis has begun by the time of first cleavage, and knowing that this axis is specified with respect to the site of pronuclear fusion, we examined the cytoplasmic events during the period between the meeting of the pronuclei and cleavage furrow formation. First, the distribution of naturally fluorescent granules was followed using time-lapse video microscopy. These granules are a good marker of the peripheral layer of yolk-poor 'ectoplasm' which lies around a core of yolky 'endoplasm' (see Reverberi, 1971). The fluorescence comes through the FITC filters strongly (Fig. 3B) or Hoechst filters more weakly (Fig. 3A, C). We observed previously that the fluorescent granules accumulate around male pronuclei following fertilisation, forming characteristic plaques (see Fig. 3A) also containing an accumulation of mitochondria and other organelles (previously termed sperm pronuclear zones or SPZs). These plaques develop progressively between 40 and 60 minutes after sperm entry and disperse following pronuclear fusion (Carré and Sardet, 1984, Carré et at, 1991). The video recordings revealed that the plaques did not disperse synchronously. The one around the zygote nucleus started to disperse first, followed sequentially by those further and further away (Fig. 3A). By the time of first cleavage all the plaques had disappeared, except in eggs where supernumerary sperm had entered at the opposite pole to the zygote nucleus, where plaques remained. The dispersal of the plaques was accompanied by waves of surface movement that propagated from the region of the newly formed zygote nucleus to the opposite pole. Early movements were manifest by the displacement of plaques, relative to the underlying male pronuclei, toward the zygote nucleus (Fig. 3A). Subsequent movements resembling peristaltic contractions were evident as the zygote nucleus rose at the egg surface just before mitosis (Carré et al., 1991) and prior to first cleavage.

To summarise, a wave of transformation of cytoplasmic organisation with various components starts from the zygote nucleus and spreads through the ectoplasm layer. It takes the wave of plaque dispersal the entire period between pronuclear fusion and first cleavage (around 80 minutes) to reach the opposite pole of the egg. Its speed is thus in the range of about 10-20 μm per minute. It slows down as it reaches the opposite pole of the egg. The direction of propagation corresponds to the future oral-aboral axis of the animal.

Cytoplasmic reorganisation during the wave requires dynamic microtubules

We treated eggs with drugs that disrupt the cytoskeleton to see whether the observed changes were dependent on

microtubules or microfilaments. The drugs were diluted in sea water and introduced into chambers containing eggs under observation at around the time of pronuclear fusion. Treatment with 10 μM nocodazole to depolymerise microtubules (checked by immunofluorescence) prevented the redistribution of the naturally fluorescent granules (Fig. 3B). Treatment with 2 μm taxol to stabilise microtubules also blocked the wave of cytoplasmic redistribution (Fig. 3C). Taxol treatment at this time froze the existing pattern of microtubule organisation (see below for details). We conclude that the reorganisation of fluorescent granules requires dynamic behaviour of microtubules. Waves of surface movement were still observed in the presence of these drugs. These were difficult to quantify but they appeared less extensive than those seen in control eggs. In particular, the early movements resulting in the displacement of the plaques with respect to supernumerary male pronuclei were prevented. Both nocodazole and taxol prevented first cleavage, presumably because mitosis was blocked.

Cytochalasin B, which disrupts actin filaments, was used at doses of 1-4 μM. The effectiveness of the drug at blocking cleavage was somewhat variable, but in cases where cleavage was prevented, the redistribution of fluorescent granules and dispersal of plaques was still observed. Cytochalasin-treated eggs often underwent abnormal and erratic bulging at the egg surface, making it difficult to

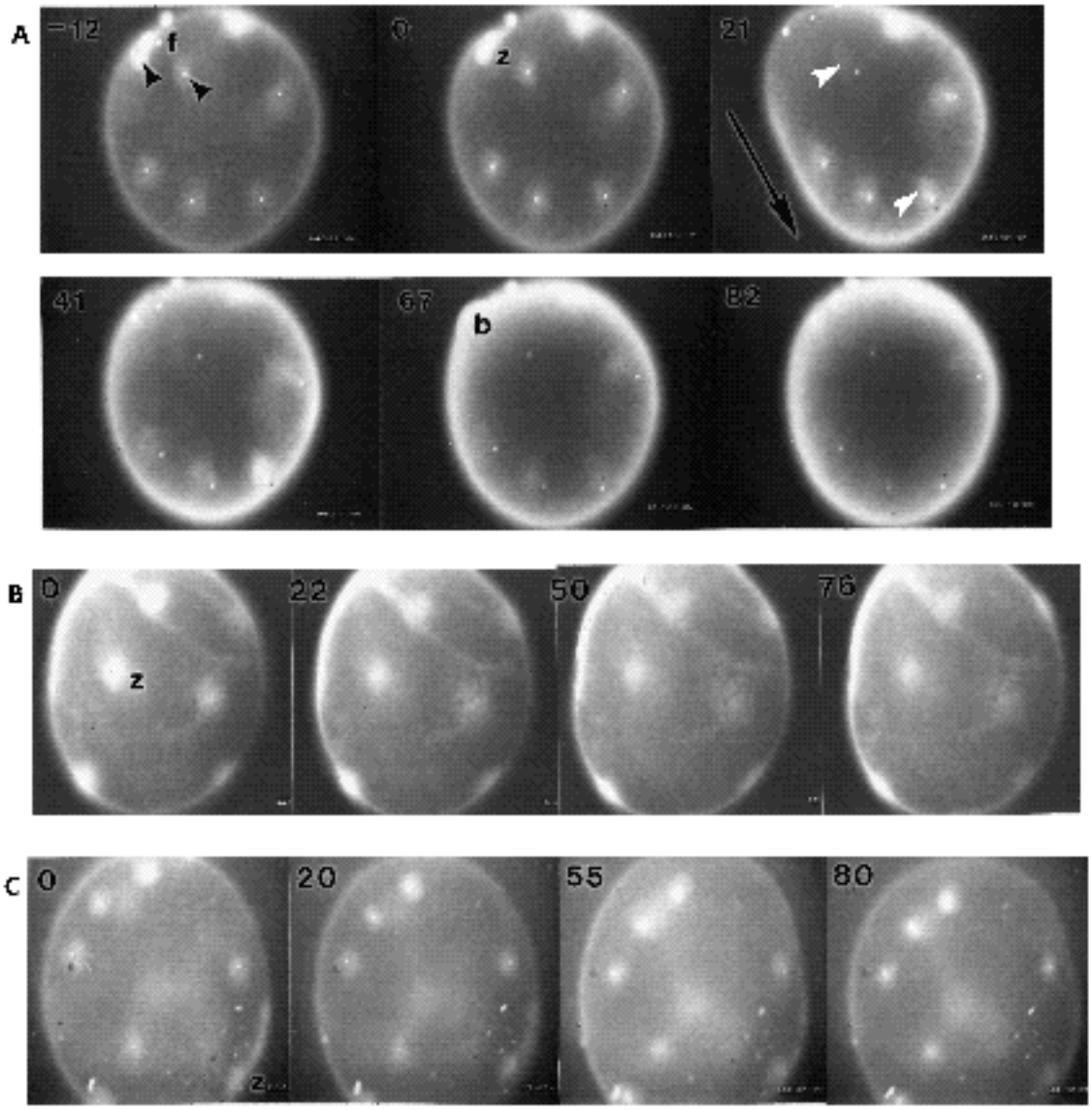

Fig. 3. Images from video sequences of untreated (A), nocodazole treated (B) and taxol treated (C) eggs. Small white spots in A and C are nuclei stained with Hoechst 33342. Time in minutes is indicated in the top left corner, with pronuclear fusion occurring at around time 0 in each case. f, female pronucleus ending migration. Note the plaque of accumulated fluorescent granules around each male pronucleus before pronuclear fusion (e.g., at black arrowheads). These subsequently became displaced towards the zygote nucleus concomitant with surface movements (white arrowheads) and then dispersed. The changes progressed in a wave from the zygote nucleus (z) in the direction of the arrow. Further waves of surface movement were associated with the formation of a bulge at the surface (b) containing the zygote nucleus at around prophase. Both nocodazole and taxol prevent the dispersal of the plaques and reduce the degree of surface movement (B and C). All egg diameters about 1 mm.

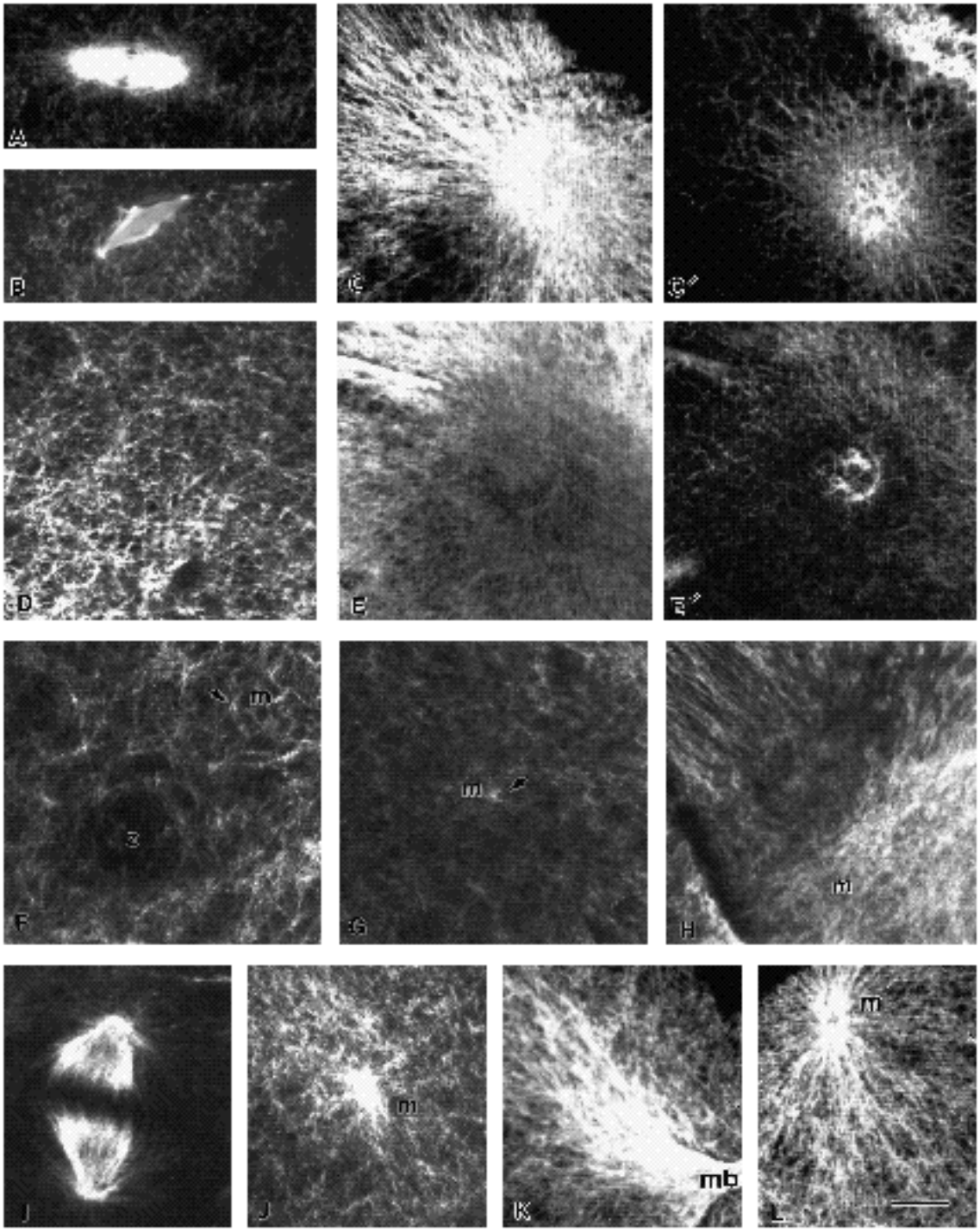

Fig. 4. Anti-tubulin immunofluorescence (confocal microscope images). (A, B) Eggs fixed at the time of second meiosis with spindles in metaphase and anaphase respectively and sparse randomly oriented microtubules elsewhere. (C, C′) Surface and deeper planes of focus, 4.6 μm apart, through a giant aster fixed at the time of pronuclear migration, showing surface microtubules emanating from the region of the sperm centrosome. (D) Dense network of randomly oriented microtubules in an unfertilised egg fixed about 1 hour after the end of meiosis. (E, E′) Surface and deeper planes of focus, 1.4 μm apart, through a giant aster fixed shortly after pronuclear fusion. Astral microtubules are still abundant at the egg surface. (F) Region around the zygote nucleus of an egg fixed between pronuclear fusion and first mitosis. No asters can be discerned associated with the zygote nucleus (z) and only a tiny remnant (arrow) associated with a nearby accessory male pronucleus (m). (G) Another region of the same egg about half way to the opposite pole, where a small aster (arrow) is associated with a supernumerary male nucleus (m). (H) Giant aster at the opposite pole of the same egg with microtubules radiating (top left and right) from a region containing a supernumerary male nucleus (m). (I) Spindle in a mitotic egg. (J) A small aster opposite this spindle. (K) Microtubules emanating from a midbody (mb) at the end of first cleavage. (L) Elsewhere in newly formed 2-cell blastomeres, microtubules are distributed fairly evenly. This aster, associated with supernumerary male pronuclei (m), is much smaller than during the first cell cycle. Bar (for all parts), 20 μm.

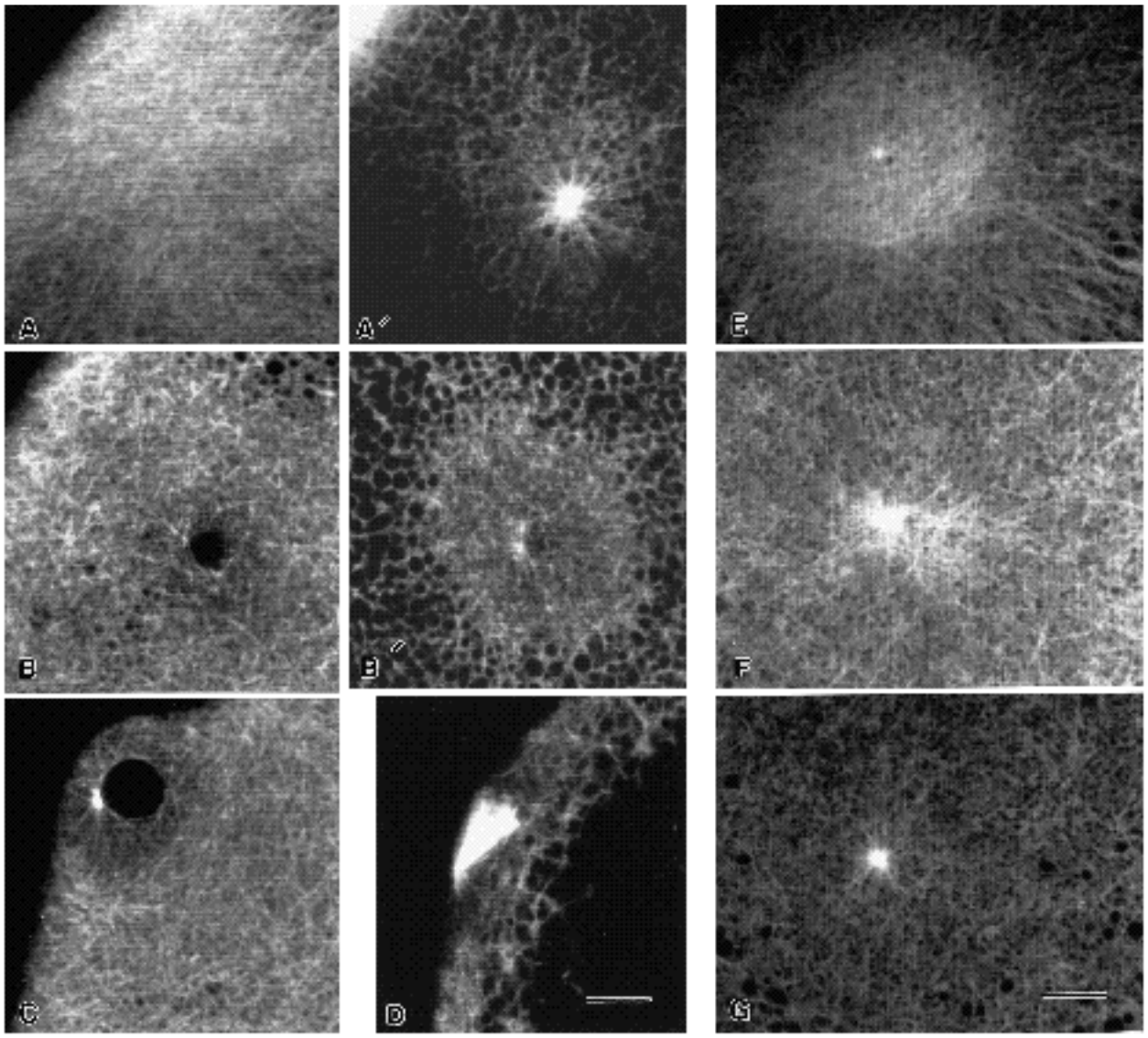

Fig. 5. Successive confocal microscope images from two live eggs injected with rhodamine-tubulin. (A, A′) Optical sections at the surface and centrosome (20 μm deep) of a giant aster 10 minutes before arrival of female pronucleus. (B, B′) Same region 40 minutes after pronuclear fusion. The aster has dispersed. (C) At 55 minutes the nucleus has risen in a bulge with an even microtubule network at the surface with a prophase aster developing. The egg has turned slightly. (D) At 76 minutes a mitotic spindle formed at the same site. (E-G) Images at the level of the centrosome of a giant supernumerary aster in another egg during a 90 minute period, from before pronuclear fusion (E) to first cleavage (G). F is 30 minutes before G. Bars, 20 μm.

assess the normal function of actin filaments in the egg at this time.

Microtubule organisation changes progressively across the egg

We examined microtubule organisation in eggs fixed at different times up until the end of first cleavage by whole-mount immunofluorescence using anti-tubulin antibodies. In freshly spawned eggs (undergoing meiosis) microtubules were seen evenly distributed throughout the ectoplasm and in the meiotic spindles (Fig. 4A, B). Following fertilisation and the completion of meiosis, giant microtubule asters developed beside each male pronucleus, presumably on the associated centrosome (Fig. 4C). These flattened asters spread to fill the ectoplasm, with only a few microtubules penetrating the central yolky mass. In cases where only one or two sperm had entered the egg, the microtubules in a single aster could extend further than 1 mm. In unfertilised eggs, a dense even network of randomly oriented microtubules developed (Fig. 4D).

Giant asters around male pronuclei were found to persist in eggs fixed shortly after pronuclear fusion, i.e. they were present in eggs containing partly condensed male chromatin in the zygote nucleus (Fig. 4E). At later times the giant asters diminished in size, those nearest the zygote nucleus shrinking first. Thus a gradient of aster sizes could be found within single eggs (Fig. 4F-H). At the time of mitosis, asters were found associated with male pronuclei opposite the mitotic spindle in some eggs (Fig. 4I, J), whereas the asters on nearer sperm centrosomes had shrunk almost completely.

In cleaving eggs many microtubules were found emanating from the advancing cleavage furrow and ultimately became associated with the midbody (Fig. 4K). A random network dominated in the rest of the egg, with some nucleation by supernumerary sperm centrosomes evident (Fig. 4L).

Since batches of *Beroe* eggs do not undergo first cell cycle events synchronously, the time taken for pronuclear migration being variable (Carré and Sardet, 1984; Carré et al., 1991), the sequence described above was deduced by examining Hoechst-stained chromatin in fixed eggs. We were able to follow the progression of events directly in different regions of live eggs following injection of rhodamine-tubulin. These observations in live eggs confirmed the immunofluorescence results. Giant microtubule asters around the zygote nucleus shrank and were replaced by a random microtubule network by the time a bulge at the egg surface indicated immanent nuclear membrane breakdown (Fig. 5A-C). Microtubules then became sparse and short in this region as the mitotic spindle formed (Fig. 5D). Giant asters by supernumerary male pronuclei also shrank and were replaced by a random microtubule network (Fig. 5E-G). The changing organisation of microtubules over this period deduced from these observations on fixed and live eggs is summarised in Fig. 6 (left column).

Regional differences can be revealed by taxol treatment

In the course of the drug experiments we noticed that taxol treatment resulted in the appearance of multiple small plaques of natural fluorescence if the treatment was carried out during meiosis or late in the cell cycle. Taxol is known to affect microtubule organisation in a cell-cycle dependent

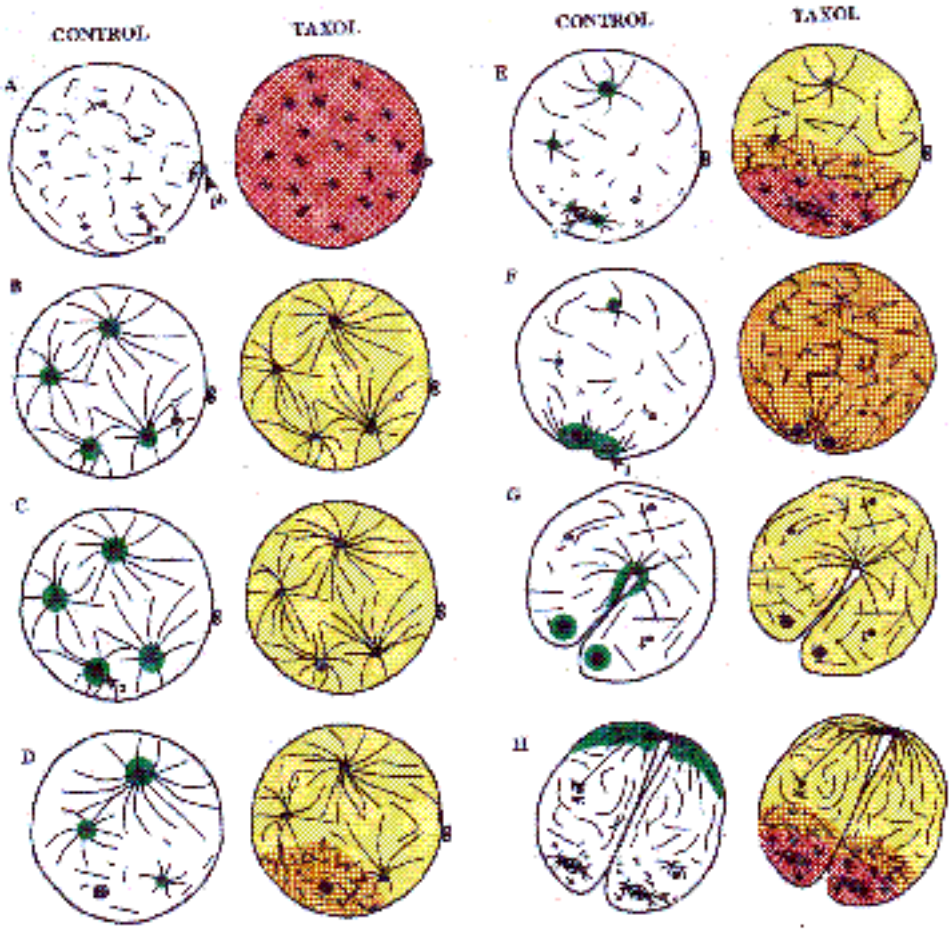

Fig. 6. Diagram summarising microtubule organisation during early events in *Beroe* development in control (left column) and taxol-treated (right column) eggs. Black lines represent microtubules and green indicates concentrations of naturally fluorescent granules. (A) During meiosis microtubules are distributed evenly throughout the peripheral cytoplasm and form the meiotic spindles. (B) At the time of pronuclear migration giant microtubule asters form around each male centrosome and fluorescent granules and mitochondria accumulate in plaques at their centres. (C) These are still present immediately after pronuclear fusion. (D) The asters then shrink as the associated plaques disperse in a wave starting from the zygote nucleus. (E) By the time of first mitosis microtubules are sparse around the spindle but asters are still present at the other pole of the egg. (F) As cleavage starts the plaques have mostly dispersed. (G) During cleavage many microtubules spread from the furrow but elsewhere the network is even. Fluorescent granules concentrate at the leading edge of the furrow and in the region of the daughter nuclei. (H) At the time of second mitosis microtubules again become sparse around the spindles while remaining denser elsewhere, especially around the midbody. Following 10 minute pulses of 2.5 μm taxol, small asters are induced throughout meiotic eggs and around mitotic spindles (red shaded areas) while long microtubules are stabilised at other times (yellow), with bundling of microtubules in intermediate zones (orange). pb, polar body; m, male pronucleus; f, female pronucleus; z, zygote nucleus; s, mitotic spindle; d, daughter nucleus.

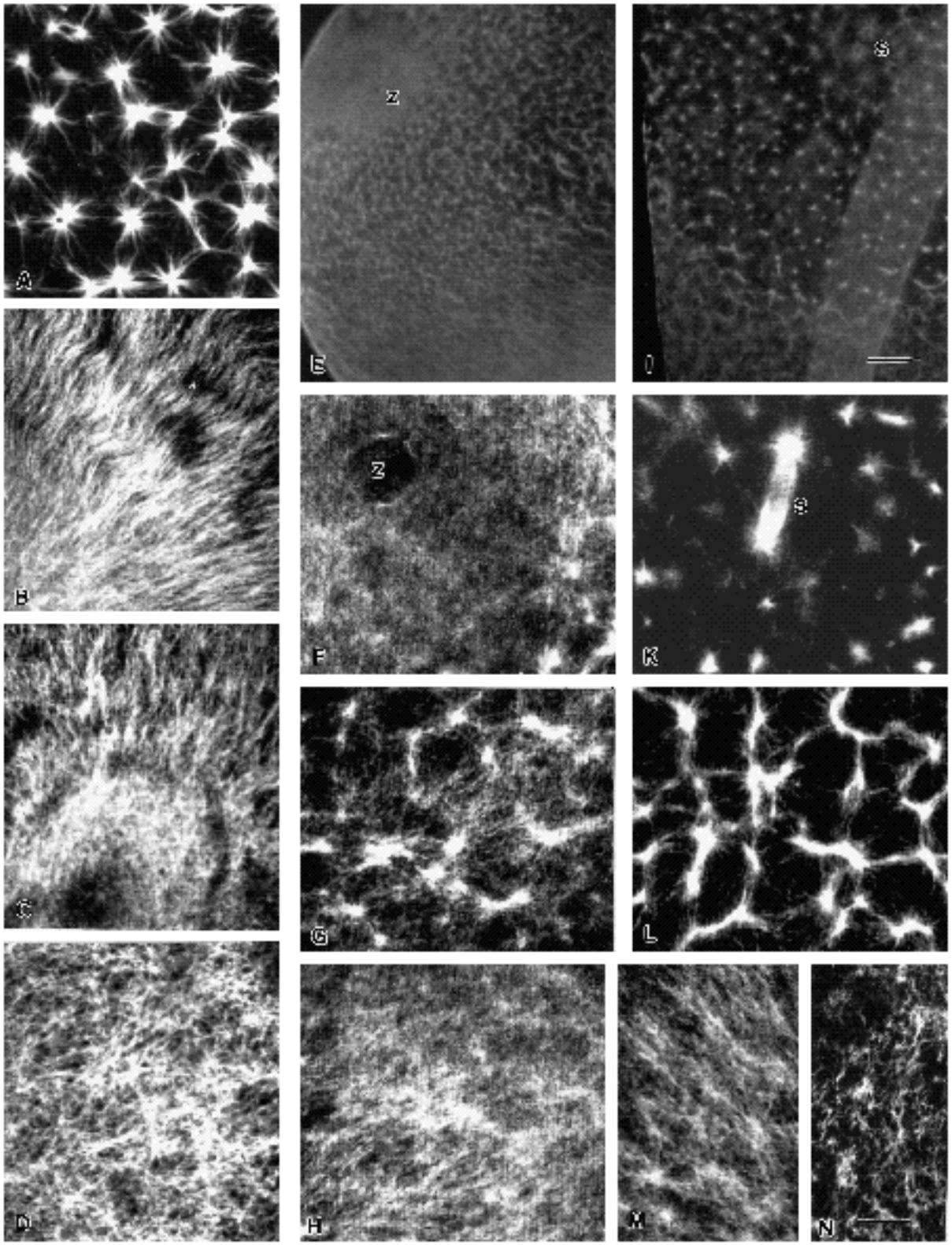

Fig. 7. Anti-tubulin immunofluorescence of taxol treated eggs. (A) Small asters induced in a meiotic egg. (B) Long microtubules preserved in giant asters in egg treated before pronuclear fusion. (C) Similar effect of taxol applied just after pronuclear fusion. (D) Dense microtubule network in egg treated during cleavage. (E-H) Taxol treatment shortly before mitosis induced bundling of short microtubules in the hemisphere containing the zygote (z) nucleus (F, G) but not at the opposite pole (H). (I-N) During mitosis small asters were induced around the spindle (s), while microtubules form interconnected bundles further away (L) and retain a more even network at the opposite pole (M,N). All images from confocal microscopy except E and I. Bar (E and I), 50 μm; for all other parts, 20 μm.

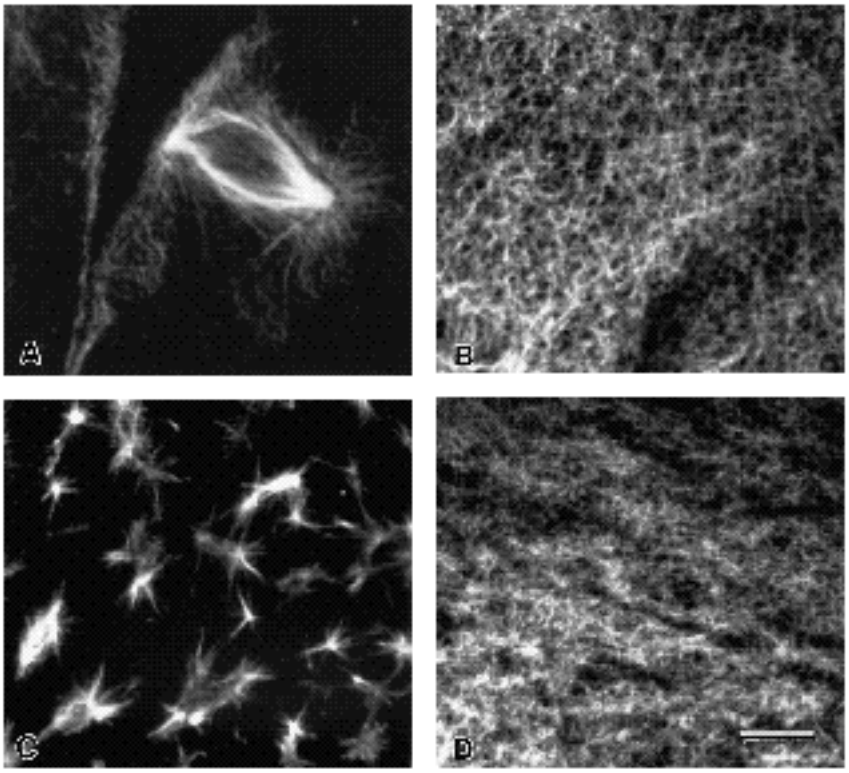

Fig. 8. Anti-tubulin immunofluorescence (confocal images) of control (A, B) and taxol treated (C, D) blastomeres at the time of second mitosis. (A, C) Region of spindle. (B, D) Region in centre of blastomere. Bar (for all parts), 20 μm.

manner. During interphase it stabilises long microtubules, whereas in mitotic cytoplasm it induces small microtubule asters (De Brabander et al., 1981). The mitotic asters are thought to form as the result of the interaction of short taxol-stabilised microtubules with material able to cause sliding of the microtubules against each other (Verde et al., 1991). Factors promoting the sliding activity and ones accelerating microtubule turnover are activated during mitosis.

We treated eggs with 2.5 μM taxol for 10 minutes at different times and then fixed and stained them by anti-tubulin immunofluorescence. Eggs treated during meiosis had tiny asters distributed evenly throughout the cytoplasm (Fig. 7A). Eggs treated once plaques had formed showed no such tiny asters, indeed a pattern of giant sperm asters very similar to that seen in control eggs, fixed at the same time, was seen (Fig. 7B, C). Similarly, the pattern of microtubules during cleavage was not altered by taxol treatment (Fig. 7D). Many of the eggs treated with taxol between pronuclear fusion and cleavage showed regional variation in their response to taxol. This was clearest in eggs treated at the time of mitosis (Fig. 7I-N), when small asters were induced in a region around the spindle (Fig. 7K), while at the opposite pole of the egg microtubules appeared essentially undisturbed (Fig. 7M, N). In between these two regions was a transition zone in which short microtubules and longer microtubules were arranged in connected bundles (Fig. 7L). Aggregation of short microtubules was also seen in a region around the zygote nucleus in eggs treated before nuclear membrane breakdown (Fig. 7E-H), and more extensive bundling in large regions of eggs treated at the beginning of cleavage. Longer microtubules were found in the region of the daughter nuclei and cleavage furrow at this time. A similar regional induction of small asters around the spindle was obtained following taxol treatment at the 2-cell stage, the taxol amplifying differences perceptible as variation in the density of the microtubule network in controls (Fig. 8).

The distribution of microtubules following taxol treatment at different times is summarised in Fig. 6. The location of mitotic activity, as revealed by taxol treatment has been indicated in red. The bundling effect, indicated in orange may represent areas in which mitotic factors are not fully activated or where microtubules were longer at the start of treatment and so did not have time to form distinct asters. We favour the former interpretation since longer treatment did not appear to induce asters over a larger area (not shown).

DISCUSSION

In many species, inhomogeneities present in the unfertilised egg cytoplasm become elaborated progressively to give rise to the characteristic body-plan of the adult. Rearrangements of the cytoplasm directed by external cues may introduce new axes. In frogs and ascidians, sperm entry triggers the displacement of cytoplasm organised radially about a single animal-vegetal axis in the egg to complete the specification of the dorsal-ventral and anterior-posterior axes of the embryo (see Houliston and Elinson, 1992; Speksnijder et al., 1990). Eggs of ctenophores, like mammalian eggs, appear to have no cytoplasmic organisation relating to future axial organisation prior to fertilisation. In ctenophores, the position of the single body axis becomes specified at random, depending on where sperm enter and

which male pronucleus the female pronucleus fuses with (Freeman, 1977; Carré and Sardet, 1984). We have examined when and how inhomogeneities in egg organisation relating to future axial organisation first arise.

Establishing axial organisation during ctenophore development

Freeman (1976, 1977) analysed the segregation of comb-plate-forming potential towards the future aboral pole using a number of ctenophore species. He concluded that this process begins at the time of first cleavage and may involve cytoplasmic movements accompanying cytokinesis. Earlier studies, in apparent contradiction, found defects in the organisation of comb-plates in larvae that developed from eggs bisected at the beginning of cleavage, notably if cytoplasm was lost from around the future oral pole (Fischel, 1903; see Fig. 1). These findings can be reconciled by the proposal that comb-plate forming potential gathers around the zygote nucleus at the future oral pole before being relocated during cleavage.

Almost all 4-cell-stage blastomeres isolated from fragments from axial cuts at the beginning of cleavage develop comb-plates (Freeman, 1977). This could arise either from an even distribution of comb-plate-forming potential or a symmetrical arrangement at the future oral pole. We cultured intact equivalent fragments of *Beroe ovata* eggs to assess the organisation of the comb-plates. Most developed half the normal number of micromeres and comb-plate rows. Yatsu (1912) had observed similar development following axial bisection during cleavage. When we bisected eggs at earlier stages, perfect larvae developed in most cases. The transition in behaviour occurred at around the time of mitosis, suggesting that certain components become organised in a symmetrical arrangement with respect to the oral-aboral axis at this time. The results of older studies (Driesch and Morgan, 1985b; Fischel, 1903; Yatsu, 1912) are in general agreement with these conclusions. All these workers described larvae lacking entire or parts of comb-plate rows, in particular from eggs cut shortly before cleavage.

Microtubules and cytoplasmic reorganisation in *Beroe* eggs

We observed a wave of cytoplasmic reorganisation spreading from the zygotic nucleus following pronuclear fusion. It involves the displacement and dispersal of perinuclear organelles and movements of the surface relative to the underlying layer of cytoplasm. Microtubules appear to play a role in this wave of change. Drugs that depolymerise or stablilise microtubules prevented the cytoplasmic reorganisation. A correlation was observed between the reorganisation of microtubules and that of the ectoplasm, as revealed by the changing distribution of fluorescent granules (Fig. 6). Both are first distributed evenly in the peripheral cytoplasmic layer. Then giant microtubule asters form around each male pronucleus and plaques of accumulated organelles (fluorescent granules and mitochondria) form at the centre of each one. These organelles may thus be associated, directly or indirectly, with a minus end-directed microtubule motor. At the time of the wave, the plaques disperse and the asters shrink in parallel, starting with those closest to the zygote nucleus. Ectoplasm then reaccumulates around the telophase spindle and at the tip of the cleavage furrow, from where many microtubules emanate.

Our current hypothesis for the mechanism of propagation of the wave is that it reflects remodeling of the microtubule network as the zygote nucleus approaches and enters mitosis, with mitotic activity being initially concentrated in one place and then spreading across the egg. Localised mitotic activity was demonstrated by regional induction of characteristic small microtubule asters using taxol. Transitions between microtubule organisation in interphase and mitotic cells usually occur fairly synchronously throughout the cytoplasm, with microtubules being shorter and turning over more rapidly in mitosis (see Cassimeris et al., 1987). In large cells, however, cell cycle progression need not be uniform. Masui (1972) demonstrated that in maturing frog oocytes, about the same size as *Beroe* eggs, mitotic activity (assayed as maturation promoting factor) spreads from the animal to the vegetal cytoplasm. Precleavage waves have also been described, progressing in the same direction (e.g. Yoneda et al., 1982). In unfertilised (meiotic metaphase II) frog eggs, only the region around the spindle has sparse short ‘mitotic’ microtubules, many longer microtubules being present elsewhere in the cytoplasm (Houliston and Elinson, 1991).

Cytochalasin B, at concentrations sufficient to block cleavage, did not block the wave of plaque dispersal, suggesting that dynamic microfilaments are not required for this process. Abnormal surface movements were induced by the drug and may have disturbed cytoplasmic organisation. The progression of the unipolar cleavage, which is microfilament-dependent, is associated with extensive cytoplasmic and surface movements. Ectoplasm is taken with the leading edge of the first cleavage furrow to the future aboral pole. Comb-plate-forming potential first becomes translocated in this direction at this time (Freeman, 1976), however, first cleavage can be suppressed without preventing the later accumulation of ectoplasm in micromeres at the aboral pole and subsequent normal comb-plate development (Fig. 2K). The next two cleavages also carry ectoplasm to the aboral pole and may contribute to its final segregation into micromeres.

A role for the microtubule-mediated waves?

Cytoplasmic reorganisation during the period between pronuclear fusion and first cleavage may be important in the regulation of polyspermy in *Beroe*. Relocation away from supernumerary male pronuclei of components involved in chromosome decondensation or entry into mitosis, may prevent extra mitotic spindles and cleavage furrows from forming. Since the waves of cytoplasmic reorganisation occur at the time axial organisation first becomes detectable in *Beroe ovata* eggs, and propagate from the future oral pole, they may also play a part in establishing the oral-aboral axis. This could be tested, perhaps by inhibiting the waves using reversible drug treatment or by inducing secondary waves. An alternative candidate for setting up the axis is the spindle itself, which could cause the redistribution of particular cytoplasmic components into a symmetrical arrangement as a result of its inherent bipolar structure.

Ectoplasm accumulates around the furrow initiation site, where the bisection experiments suggest comb-plate forming potential is located (see above). After cleavage, it takes up an asymmetric distribution, being concentrated on the external face of two-cell blastomeres (Spek, 1926), an organisation that is maintained independent of cell contact (Freeman, 1976). It is tempting to relate this new stabilised distribution of ectoplasm during interphase to the inability of the blastomeres to regulate their development after this stage. Fischel (1903) and Spek (1926) proposed that in order for regulation to occur in egg fragments, there had to be sufficient time for the ectoplasm to redistribute before first cleavage. Redistribution of ectoplasm after first mitosis may be prevented following stabilisation of the microtubule network or other changes in the cytoskeleton. Such a mechanism has been proposed to fix dorsalising activity to one side of the frog egg at the end of the first cell cycle (Elinson, 1983). Fischel (1903) noted that the distribution of ectoplasm 'cycles' between a dispersed organisation and concentration in the advancing furrow, prompting the thought that processes of microtubule-dependent cytoplasmic reorganisation orchestrated by localised mitotic activity may occur at each of the early determinative cleavages in *Beroe*.

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