

Actomyosin contraction of the posterior hemisphere is required for inversion of the *Volvox* embryo

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SUMMARY

During inversion of a *Volvox* embryo, a series of cell shape changes causes the multicellular sheet to bend outward, and propagation of the bend from the anterior to the posterior pole eventually results in an inside-out spherical sheet of cells. We use fluorescent and electron microscopy to study the behavior of the cytoskeleton in cells undergoing shape changes. Microtubules are aligned parallel to the cell's long axis and become elongated in the bend. Myosin and actin filaments are arrayed perinuclearly before inversion. In inversion, actin and myosin are located in a subnuclear position throughout the uninverted region but this localization is gradually lost towards the bend. Actomyosin inhibitors cause enlargement of the embryo. The bend

propagation is inhibited halfway and, as a consequence, the posterior hemisphere remains uninverted. The arrested posterior hemisphere will resume and complete inversion even in the presence of an actomyosin inhibitor if the anterior hemisphere is removed microsurgically. We conclude that the principal role of actomyosin in inversion is to cause a compaction of the posterior hemisphere; unless the equatorial diameter of the embryo is reduced in this manner, it is too large to pass through the opening defined by the already-inverted anterior hemisphere.

Key words: Actin, Myosin, Microtubule, Multicellular deformation, *Volvox*

INTRODUCTION

Folding of a multicellular sheet is a widespread embryological phenomenon observed during gastrulation, neurulation and various other aspects of morphogenesis. Such processes have been analyzed extensively at the cellular level, where it has been repeatedly observed that the cells constituting a folding sheet undergo a predictable series of morphological changes in a spatiotemporally regulated fashion (Ettensohn, 1985; Fristrom, 1988). There have, however, been significantly fewer analyses of the subcellular events that underlie these coordinated changes in cell behavior during folding.

Inversion of *Volvox* embryo provides a useful model to study sheet folding (Kirk, 1998; Kirk et al., 1982). A *Volvox* embryo is a hollow spherical monolayer of about 3,000 cells. All cells in the embryo, including the 16 larger cells that are destined to become 'gonidia' (reproductive cells), are structurally connected by numerous cytoplasmic bridges. There is, however, a region at the anterior pole where the syncytium is interrupted by an opening called the phialopore (Green et al., 1981). As inversion starts, a portion of the sheet encircling the phialopore curls outward to produce a circular bend region. The bend then moves progressively toward the opposite pole (Kelland, 1977), eventually causing the spherical sheet to turn inside out. *Volvox* inversion bears some similarity to metazoan gastrulation, except that it progresses reversely and goes to completion.

During bend formation, two cellular events, i.e., specific shape changes and movements of the cytoplasmic bridges, have been reported to take place (Kelland, 1977; Viamontes and Kirk, 1977; Viamontes et al., 1979; Green et al., 1981). Prior to inversion, the cells are spindle shaped and linked by cytoplasmic bridges at their widest points. Cells in the bend form long, narrow projections at their outer ends and the cytoplasmic bridges move so that they become bundled at the tips of these projections. The bend is then propagated progressively from the phialopore toward the opposite pole where these two cellular events are taking place. Propagation of the bend obviously requires precise spatiotemporal regulation of cell shape and movement (Green et al., 1981).

Kirk and his colleagues have implicated microtubules and actin filaments in the two cellular events of inversion (Viamontes et al., 1979). An ultrastructural analysis revealed abundant microtubules aligned along the thin stalks of flask cells (Viamontes and Kirk, 1977), and colchicine or low-temperature treatments, which inhibited inversion, abolished elongated cells, otherwise present in the bend (Viamontes et al., 1979). Inversion was also blocked by inhibitors of actin filaments (Viamontes et al., 1979). Since the cellular location of actin filaments was not determined, the cytological basis for the cytochalasin effect was not fully explained.

In this study, we analyze the spatial distributions of actin filaments, myosin, microtubules and other structures in an inverting embryo by fluorescence and electron microscopy.

Combining such studies with the use of actomyosin inhibitors and microsurgery, we provide evidence that localized actomyosin contraction, which is generated so as to make the posterior hemisphere compact, appears to be essential for inversion. This constitutes a previously unrecognized aspect of the inversion process.

MATERIALS AND METHODS

Culture and preparation of embryos

Volvox carteri f. *nagariensis*, strain HK-10, was obtained from the University of Texas Culture Collection of Algae. The axenic culture was maintained in a synchronous and asexual growth with the standard *Volvox* medium (SVM; Kirk and Kirk, 1983) at 32°C under the light cycle previously reported (Tam and Kirk, 1991). *Volvox* spheroids 2–4 days after inoculation were harvested by filtration using 90 µm mesh (Cell Strainer; Nippon Becton Dickison Co. Ltd., Tokyo, Japan). They were then ruptured in SVM by five passages through a 25-gauge needle. The released embryos were retrieved on a 40 µm mesh after having been passed through a 90 µm mesh to remove spheroid debris. Embryos were then rinsed in excess SVM. All experiments were done at room temperature.

Drug treatments

Stock solutions of cytochalasin B, cytochalasin D and cytochalasin E (Sigma Chemical Co., St Louis, MO, USA) were prepared following the method described previously (Viamontes et al., 1979). They were dissolved at 10 mg/ml in DMSO (Infinity Pure, Wako Chemicals, Osaka, Japan) and diluted 100-fold in SVM before use. A myosin II inhibitor, 2, 3-butandione monoxime (BDM; Sigma) was diluted to 40 mM with SVM from a freshly prepared 0.5 M solution (Cramer and Mitchison, 1995). Sucrose at 50 mM was present in all drug solutions to avoid non-specific osmotic damages (Viamontes et al., 1979). The concentration of sucrose was lowered to 10 mM in the case of BDM to balance the osmolarity.

To treat embryos with the drugs, isolated embryos were placed on glass coverslips precoated with 0.01% polyethyleneimine (Kirk et al., 1993). Prior to drug application, embryos were incubated in 50 mM sucrose in SVM for about 60 minutes. An appropriate amount of the drug solution was placed onto the embryos and the solution was changed quickly two more times to facilitate solution exchange. Removal of drugs was done similarly with 50 mM sucrose in SVM.

Live recording

Embryos were observed by differential interference contrast (DIC) on an Olympus inverted microscope (IMT-2, Olympus, Tokyo, Japan) and the images were recorded with a cooled CCD video camera (Image Point; Photometrics, Ltd., Tucson, AZ, USA) onto S-VHS video tapes. Images were enhanced with an image-processor, Σ-III (Nippon Avionics Co., Tokyo, Japan) and captured on a Macintosh 840AV (Apple Computer, Cupertino, CA, USA) with NIH Image 1.59. Edited digital images were printed with a color printer (Pictography 3000; Fujifilm, Tokyo, Japan). For higher resolution, images were recorded with a high resolution cooled CCD camera (PXL-1400; Photometrics) mounted on a Nikon Optiphot II (Nikon, Tokyo, Japan) or on a Olympus AX-70.

Sectioning plastic-mounted specimens for optical microscopy

To acquire images with single-cell resolution, embryos were processed as follows. Embryos on a coverslip were fixed in 2% formaldehyde, 2.5% glutaraldehyde, 2.5% tannic acid in 0.02 M phosphate buffer at pH 8.0 for 12–16 hours and postfixed in 1% OsO₄ in the same buffer for 3 hours (Viamontes and Kirk, 1977). Specimens

were then dehydrated in a graded series of ethanol solutions and infused with Technovit 8100 (Kulzer, Wehrheim, Germany). To capture embryos in a Technovit block mountable on a microtome, a fresh Technovit solution contained in a gelatin capsule was placed upside-down on the coverslip and allowed to polymerize in vacuum for 1 hour. After polymerization, 2.5 µm serial sections were cut with a glass knife on an ultramicrotome (Reichert Ultracut E; Leica, Vienna, Austria). Sections were mounted on coverslips with Entellan (Merck, Darmstadt, Germany) and observed with DIC optics.

Fluorescence microscopy

Embryos attached to PEI-coated coverslips were fixed for 15 minutes in 3.7% formaldehyde, 1 mM DTT, 0.1% Triton X-100, 2 mM MgCl₂, 5 mM EGTA, 150 mM KCl, 10 mM sodium glycerophosphate and 10 mM Hepes at pH 7.1, a solution modified from a fixative originally designed for preservation of labile actin filaments of leukocytes (Redmond and Zigmund, 1993). The coverslips were then washed (with gentle agitation) in a Coplin jar containing 1% NP-40, 1 mM DTT, 1% BSA, 2 mM MgCl₂, 5 mM EGTA, 150 mM KCl and 10 mM sodium glycerophosphate for 1 hour to extract chlorophyll and minimize autofluorescence. After washing three times in TPBS (0.1% Tween 20) supplemented with 0.1% BSA, embryos were stained for 4 hours with 4 µM fluorescein-phalloidin (Molecular Probes, Inc., Eugene, USA) in the same buffer and again washed three times in TPBS. Samples were mounted in 50% glycerol, 1 mg/ml phenylenediamine (an anti-fading agent) in PBS.

For immunostaining, embryos were similarly fixed and incubated for 12 hours either with 10–20 µg/ml anti-tubulin monoclonal antibody (Amersham, Buckinghamshire, England), or 10 µg/ml antibodies against *Physarum* myosin II (Ogihara et al., 1983). Samples were then washed and stained with an FITC-conjugated secondary antibody (Amersham) at 1:200 dilution for 4 hours. To visualize the nucleus, DAPI (4 µg/ml) was included in the secondary antibody solution. The secondary antibodies were preabsorbed following the protocol described by Harlow and Lane (1988). The anti-myosin II from *Physarum* plasmodium used in this study was affinity purified and is monospecific for the heavy chain. Staining without the primary antibody as a control gives no specific patterns (data not shown).

Embryos were imaged on a cooled CCD camera. Serial images in z-axis were precisely incorporated by the use of a computer-assisted focusing device (Micro Z-mover; Photometrics). Digitally deconvoluted images were obtained using IPLab and Confocal extension (Signal Analytics Corporation, Vienna, VA, USA), following the method by Agard et al. (1989).

Electron microscopy

Specimens were prepared for electron microscopy as described by Viamontes et al. (1979). Embryos were fixed at 20°C for 15 seconds in 2 ml of freshly prepared 2.5% glutaraldehyde in SVM (pH 7.2), and then 0.5 ml of 2% OsO₄ in H₂O was added. Fixation was continued in the resulting mixture for 10–15 minutes. After washing with SVM, the specimens were transferred to 2.5% tannic acid in H₂O for 15 minutes, washed again in SVM and postfixed in 2% OsO₄ in H₂O for 15 minutes. After another SVM wash, specimens were transferred to 2.5% uranyl acetate in H₂O for 15 minutes, washed in H₂O, dehydrated, embedded and sectioned.

Microsurgery

Prior to dissection, the 'gonidial vesicle' surrounding an embryo was removed following the previously described method (Ransick, 1991) with modifications as follows. Gonidia were isolated by the method described above for embryos and then were incubated in SVM containing 0.1 mg/ml trypsin (Sigma) for 15–30 seconds and washed with an excess volume of SVM containing 1% BSA. The softened vesicles were then removed from the embryos with micromanipulation using a fine tungsten needle. Vesicle-free embryos were transferred to

SVM contained in a plastic ELISA plate and allowed to develop for about 8 hours. After the final cleavage, vesicle-free embryos were transferred to a ~200 μ l drop of SVM placed on a silicon-coated coverslip. Most of the anterior half was then removed from the embryo by pressing a fine glass needle along the equatorial plane.

Morphometry

To measure the width of the posterior hemisphere of inverting embryos with high geometrical accuracy, the digitized images were rotated so that the anteroposterior axis became the y-axis of the analysis coordinate. The posterior hemisphere diameter was then obtained as the maximal surface-to-surface dimension parallel to the x-axis. Quantification and calculation were done using NIH Image 1.59.

RESULTS

Microtubule rearrangements accompany cell shape changes that occur during inversion

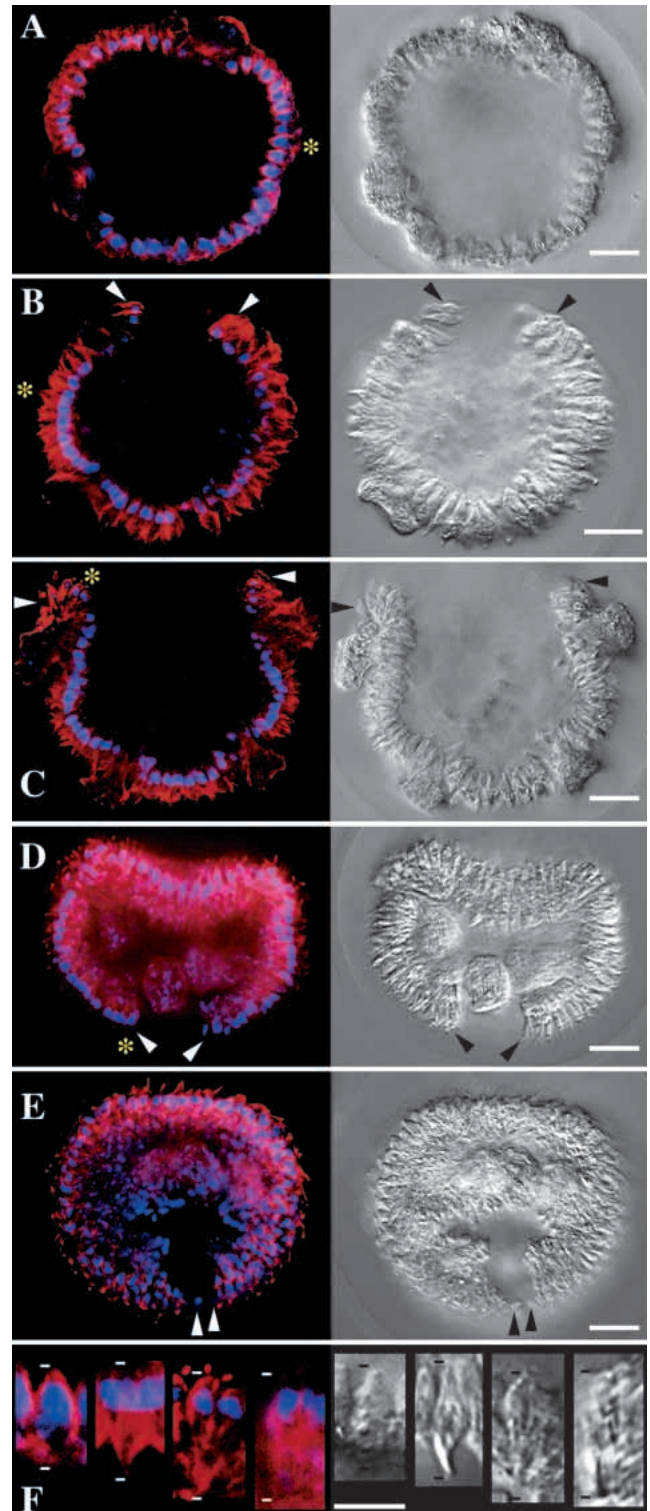
Cells changed shape conspicuously during inversion, as seen in the DIC images (Fig. 1). The sequential changes of the cell shape are consistent with the previous report by Viamontes *et al.* (1979), who named the characteristic shapes, pear, spindle, flask and column, according to the progression of inversion. The pear-shaped preinversion cells (Fig. 1A,F) were nearly round with slightly conical anterior ends, the apices of which were directed toward the interior of the embryo. The nucleus was also conical, with its apex aligned with that of the cell. The phialopore opened, as cells assumed an elongated spindle-shape with a short protuberance pointing outward (Fig. 1B,F). In the characteristic bend region near the phialopore, there were flask-shaped cells with the long stalks (Fig. 1C,F). Cells remained elongated throughout inversion (Fig. 1C,D,F), but then assumed a less-elongate column shape again after inversion had been completed (Fig. 1E,F). Concomitant with these cell shape changes, the distribution of microtubules changed dynamically. In preinversion embryos, the microtubule signal outlined each cell in fairly uniform manner (Fig. 1A,F) but, once cells became elongated, they exhibited an much more intense microtubule signal in regions distal to the nuclei, at the chloroplast outer ends of the cells (Fig. 1B,C,F). Although individual microtubules were not resolved highly, the microtubules appeared to be aligned parallel to the long axis of each cell. Nuclei were located close to the inner

Fig. 1. Localization of microtubules in *Volvox carteri* embryos during (A) the preinversion stage, (B) phialopore opening, (C) early inversion, (D) late inversion and (E) the postinversion stage. (F) 4 pairs of magnified images of the cells characteristic to embryos in the specific stages of inversion are shown; 'pear', 'spindle', 'flask' and 'column'-shaped cell (Viamontes *et al.*, 1979), from left to right. The approximate locations of the corresponding cells in the low magnification images are indicated with asterisks in A-D. The left image in each pair shows the location of tubulin (red) and the nucleus (blue), and the right one shows the same embryo by DIC. Cells changed shape conspicuously during inversion (F). Microtubules outlined the cell shape initially in the pear-shaped cells (A), and then became much more abundant in the posterior (outer) ends as the cells elongated in the spindle- and flask-shaped cells (B,C). Enhanced tubulin signals on the nuclear ends of the cells (D,E) reflects the beginning of the flagellar elongation following inversion in the columnar cells. Arrowheads in A-D indicate the opening of the phialopore. Bar, 10 μ m (A-E); 5 μ m (F).

surface in uninverted portions of the embryo. Flagella became visible on the newly exposed outer surface, in close proximity to the nucleus, in embryos approaching the end of inversion (Fig. 1D-F).

Actin filaments also undergo dynamic changes during inversion

We next examined the cellular localization of actin filaments



by use of fluorescein-phalloidin. When non-fluorescent-phalloidin was added in varying proportions to the fluorescein-phalloidin staining solution, it caused a substantial, concentration-dependent decrease in the fluorescent signal (Fig. 2I-K) leading us to conclude that the fluorescent signal obtained with fluorescein-phalloidin is phalloidin-specific and, hence, presumably actin-specific.

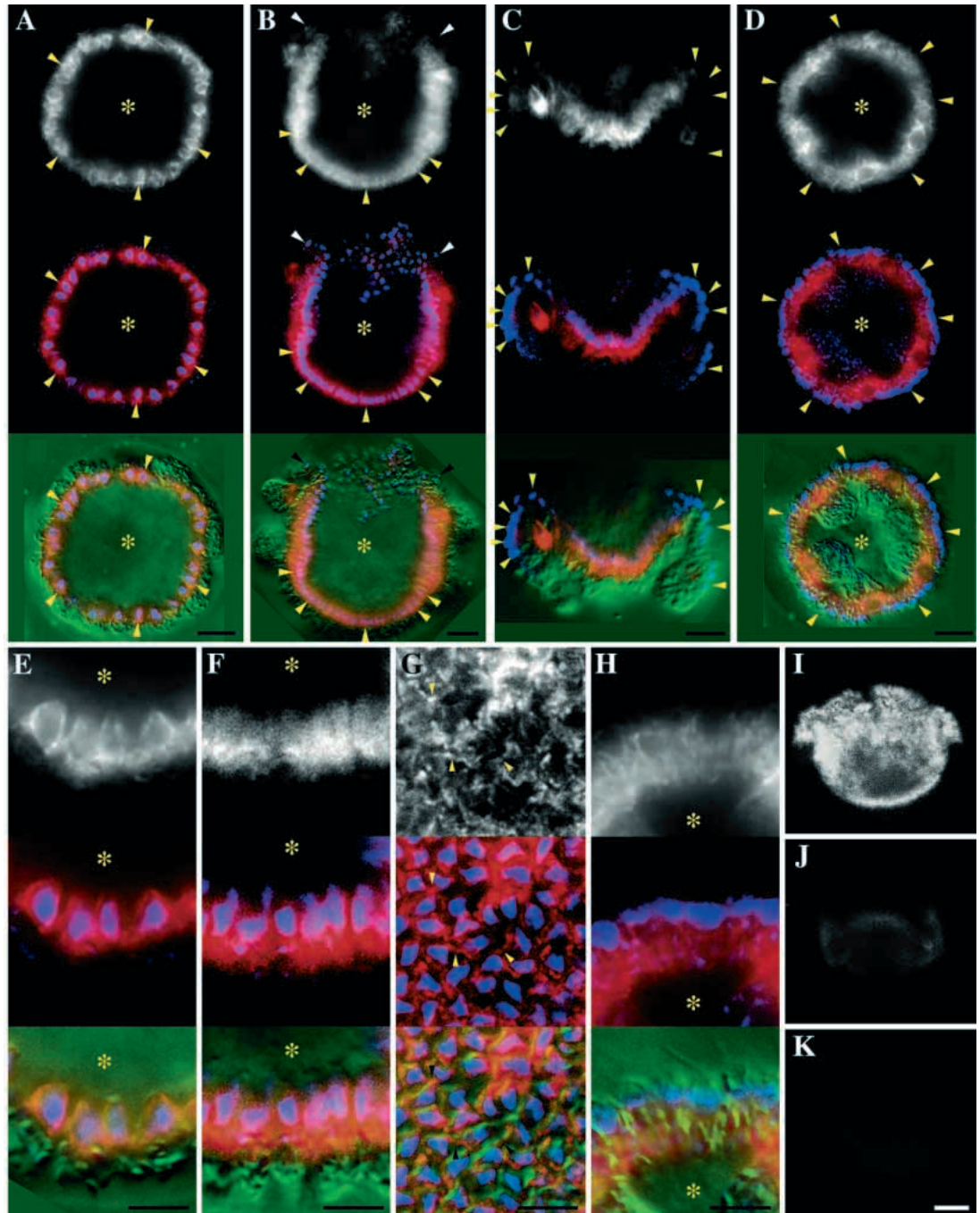
In preinversion embryos, most actin filaments were

restricted to the perinuclear cytoplasm; none were detected in the chloroplast region (Fig. 2A,E). As inversion began, a dramatic change in the organization of actin filaments occurred; the intense perinuclear signals were replaced by a more-or-less continuous, subnuclear actin filament array that traversed the entire embryo from one pole to the other (Fig. 2B,F). Indeed, this filament array appeared to be continuous through the cytoplasmic bridges known to be linking adjacent

Fig. 2. Localization of actin filaments in embryos during inversion. Each inversion stage (A-H) is represented by a set of images, in which the top one shows only the localization of actin, the second one adds the location of the nucleus (blue) to actin (red) and the bottom one adds the DIC image (green). Arrowheads are placed to indicate the outer ends of the nucleus so that the cellular locations of actin filaments relative to the nucleus and the chloroplasts may be recognized easily among the sets of images. Asterisks indicate the embryo coeloms. (A,E) At the preinversion stage, actin filaments were localized in the

perinuclear region and absent in the chloroplast region (arrowheads). (B,F) As the phialopore opens (black and white arrowheads), actin filaments moved toward the chloroplast (outer) ends of cells throughout the embryo (B, yellow arrowheads; F). (C,G) As inversion proceeds actin filaments disappeared from cells entering the bend region and remained absent until inversion had completed. (D,H) Then, once inversion had been completed a diffuse actin filament signal returned to the chloroplast (now inner) ends (*) of all cells (D, arrowheads; H). As evident in the magnified images, the perinuclear

signal (E) moved to regions between the nucleus and the chloroplast (F) to form the characteristic continuous belt-like zone. This intense signal was found not only in the bodies of the cells but also in the bridge-like structures linking adjacent cells (arrowheads in G). (I-K) The specificity of the phalloidin staining was demonstrated. Embryos were stained with fluorescein-labeled phalloidin only (I), an equimolar mixture of labeled and unlabeled phalloidin (J) and a 10-fold excess of unlabeled phalloidin (K). Bar, 10 μ m (A-E,J); 5 μ m (F-I).



cells at this stage (Fig. 2G). This shift in actin-filament location appears to be much more extreme in the anterior (phialopore-proximal) hemisphere than in the posterior hemisphere (Fig. 2B). As inversion proceeded (Fig. 2C), the actin filaments were displaced somewhat toward the chloroplast ends of the cells of the uninverted cells, where they formed an intensely staining band that was continuous throughout the posterior portion of the embryo. In marked contrast, actin filaments virtually disappeared from cells that had entered or passed through the bend region of the embryo (Fig. 2C, arrowheads). After inversion was complete, diffuse actin signals returned to somatic cells (Fig. 2D) and more intense signals were observed in the gonidial cells.

Because the images obtained with whole embryos made it appear that the actin array in the uninverted portions of an embryo was continuous from cell to cell (Fig. 2C,F), we next examined optically sectioned embryos from a different angle, in an attempt to obtain higher resolution and to determine if this actually was the case. Fig. 2G shows a cross section of the posterior hemisphere of an inverting embryo in which some cells appear to have protrusions linking them to adjacent cells (which presumably is a reflection of the cytoplasmic bridge system known to link cells at this level at this time; Green et al., 1981). Actin staining is present in such protrusions.

Myosin II is co-localized with actin filaments in the uninverted portion of the embryo

Antibodies against myosin II of *Physarum* plasmodia (Ogihara et al., 1983) cross-reacted with *Volvox* myosin. Before inversion, myosin II was localized in the perinuclear region in a pattern similar to that of the actin filaments (Fig. 3A). In

addition, however, a discrete spot of intense myosin staining was observed at the inner end of each cell, in a region known to be occupied by the basal bodies and contractile vacuoles (Fig. 3D).

During inversion, myosin II, like actin, became localized to a subnuclear cytoplasmic region, formed arrays that appeared to be continuous from cell to cell in the posterior portion of the embryo, but became diffusely distributed in cells within and beyond the bend region (Fig. 3B). Concomitant with these changes, the myosin signal in the vicinity of the basal body disappeared (Fig. 3E). Postinversion embryos had very faint myosin signals in the cytoplasm; but a bright spot near the basal bodies reappeared (Fig. 3C,F).

In short, the dynamic changes that occur in myosin II localization during inversion are parallel to those observed for actin filaments and are as tightly coupled to the morphogenetic events of inversion.

Inhibition of actomyosin function causes stage-specific inversion arrest

To determine what role the observed changes in co-localized actin and myosin might be playing in the inversion process, we examined the effects of four actomyosin inhibitors: cytochalasin B, cytochalasin D, cytochalasin E and BDM (Cramer and Mitchison, 1995). Even when added 2 hours prior to the beginning of inversion, all three kinds of cytochalasin inhibited inversion partially (Fig. 4F-J), despite the fact that such treatments had led to almost total disappearance of actin filaments in the subnuclear region within the first an hour (Fig. 4P,Q). BDM had the same effect (Fig. 4K-O). In all four cases, the phialopore opened (Fig. 4F,K) and, subsequently, the lips

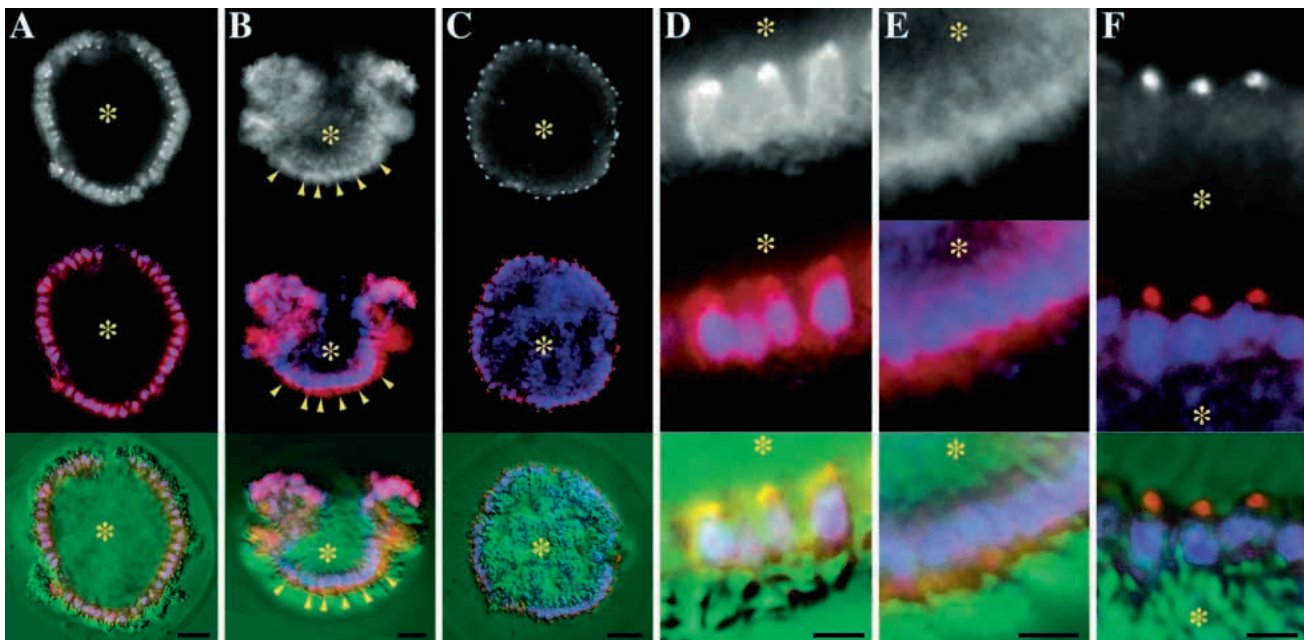


Fig. 3. Localization of myosin II in various stages of inversion. Each stage is represented by a set of images as in Fig. 2. Asterisks indicate the embryo coeloms. (A,D) In a preinversion embryo, myosin was observed in the perinuclear region and as an intense-labeled spot near each basal body, contractile-vacuole region clearly seen in the magnified image. (B,E) During inversion, myosin first became localized in the central, subnuclear regions of the cells and, as in the case of actin filaments, the signal appeared to be continuous from cell to cell (arrowheads), but then as the cells entered and passed the bend region myosin distribution became much more diffuse. (C,F) Following inversion, intense myosin signals reappeared in the basal-body, contractile-vacuole regions of all cells. Virtually no myosin signal was found at the basal body during inversion (B,E). Bar, 10 μ m (A-C); 5 μ m (D-F).

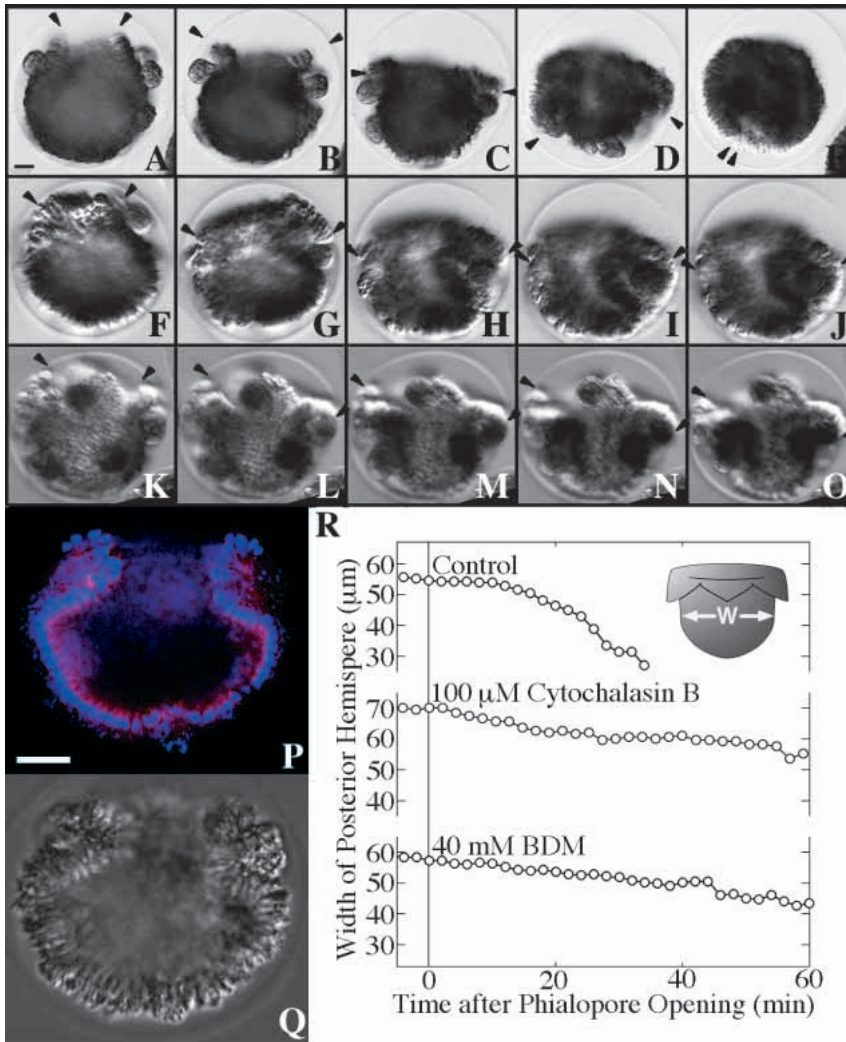


Fig. 4. Cytochalasin B and BDM cause an arrest of inversion. Preinversion embryos were treated with control buffer only (A-E), 100 $\mu\text{g}/\text{ml}$ cytochalasin B (F-J) and 40 mM BDM (K-O) for 2 hours prior to the onset of phialopore opening. Images were captured at 10 minutes intervals after phialopore opening. In the first 20 minutes, the shape of embryos, whether treated or untreated, were similar and the phialopore opened normally. However, after that time, propagation of the bend was arrested. With cytochalasin treatment (1 hour), (P, actin filaments in red and the nuclei in blue; Q, DIC image) actin filaments were not very abundant and were restricted to the basal body ends of the cells, whereas, in control embryos, at this stage actin filaments are very abundant (Fig. 2B,C,F). (R) The diameter of the posterior hemisphere was measured. Inversion arrest was found to be accompanied by a failure with shortening of the posterior hemisphere to decrease in diameter. Bar, 10 μm .

of the phialopore began to turn outward as in a control embryo (Fig. 4G,L). The propagation of the bend, however, stopped in ~ 20 minutes, leaving the anterior hemisphere inverted incompletely (Fig. 4H-J,M-O). The posterior hemisphere was never inverted.

In embryos that underwent such an inversion arrest, we noticed that the posterior hemisphere appeared wider than in control embryos. To quantify this, we measured the diameter of the posterior hemisphere of the treated and untreated embryos, with the results shown in Fig. 4R. In the first 15

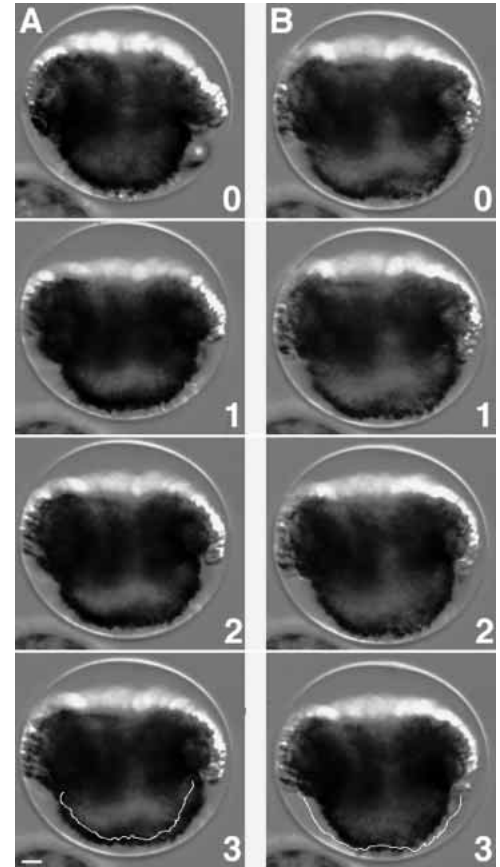


Fig. 5. BDM relaxes the embryo quickly. (A) Time course of the BDM effect. The phialopore of an embryo had opened and the bend region was being propagated, at the time when 40 mM BDM was added (top) at time zero the posterior hemisphere became swollen within the next 3 minutes (bottom). The solid line in the bottom panel represents a tracing of the posterior edge of embryo in the top panel added to assist in visualizing the difference that occurred in 3 minutes. (B) Time course of recovery from BDM. After the embryo shown in A had been exposed to BDM for a total of 5 minutes, it was washed with drug-free medium at the new time zero (top). The embryo quickly regained the compact posterior hemisphere. The solid line in the bottom panel represents a tracing of the posterior edge of the embryo at the time the drug was washed out, added to assist in visualizing the extent of the change that occurred in 3 minutes. Bar, 10 μm .

minutes after the onset of phialopore opening, the diameter of the posterior hemisphere decreased slowly in both treated and control embryos. But then, whereas the posterior hemisphere of the control embryos decreased in diameter by nearly half during the next 20 minutes, no such rapid decrease in diameter occurred in the treated embryos; they simply continued the initial slow contraction. The time at which abrupt contraction occurred in the control embryos corresponded to the time at which the posterior hemisphere began to pass through the phialopore.

Actomyosin inhibition becomes irreversible beyond a certain time point

We discovered that BDM acts very rapidly on inverting *Volvox* embryos, and took advantage of this to study the time course of onset and reversal of inhibition more closely (Fig. 5). When BDM was supplied to an embryo in which the phialopore had already opened, the posterior hemisphere including the equator expanded significantly within 3 minutes (Fig. 5A). Our measurements (not shown) indicated that the diameter of the embryo at the equator increased by 10–15%. As expected, removal of BDM resulted in a quick contraction (Fig. 5B). In this quick expansion and contraction, we could not detect any specific cells or regions that expanded and contracted more than the rest of the posterior hemisphere; the posterior hemisphere appeared to contract almost uniformly. The embryos contracted about 10 μm in diameter, which corresponds to a 15 μm change in the circumference. Since an optical section of the posterior hemisphere (Figs 4, 5) contained approximately 30 cells, individual cells are expected to contract much less than 1 μm . The action of BDM could be fully reversed, even in embryos exposed to the drug for more than 3 hours before phialopore opening, if – but only if – the BDM was washed out before inversion had been underway for 20 minutes. When the drug was washed out after this time, the treated embryos never completed inversion. This critical time point corresponded roughly with the time at which the abrupt contraction of the posterior hemisphere began in control embryos, as shown in Fig. 4.

In contrast, the effects of cytochalasins were irreversible in all cases (data not shown). Removal of cytochalasins either before or after the critical time point was unsuccessful. This was probably because of the slow permeation of these chemicals into and out of the embryos.

Cell shape changes are propagated to the posterior pole in arrested embryos

Results presented in the previous section indicated that a critical point for drug action is passed about 20 minutes after the beginning of inversion, just about the time when the posterior hemisphere of control embryos abruptly becomes smaller and begins to pass through the phialopore opening (Fig. 4). We speculated that beyond this time BDM and cytochalasins deprive the embryos of the ability to undergo the changes of individual cell shapes that are required to complete the inversion process. However, this speculation was not supported by further examination of cytochalasin B-arrested embryos (Fig. 6).

In control embryos, as previously reported (Viamontes et al., 1977), a small bundle of extremely elongated ‘flask’ cells (usually about seven cells in a cross section) is present in each bend region (Fig. 6A). This continues to be true as the bend is propagated toward the posterior pole (Fig. 6B,C). In cytochalasin B-treated embryos, the bend is not propagated beyond the equator to the posterior pole (Fig. 6D,E). However, a cluster of elongated cells is not only present in the bend region at the time that inversion is arrested, but formation of such clusters of elongated cells is propagated beyond the bend within the next 10 minutes (Fig. 6D), eventually reaching the vicinity of the posterior pole about 30 minutes after inversion had been arrested (Fig. 6E). As in a normal bend region, the thin ends of such cells were bundled together. In the uninverted

portion of the inhibited embryo (as in the inverted portion), microtubule staining revealed presence of flagella pointing toward inside the embryo sphere (Fig. 6F).

Migration of cytoplasmic bridges between cells is not inhibited in the arrested embryos

The presence in the posterior hemisphere of arrested embryos

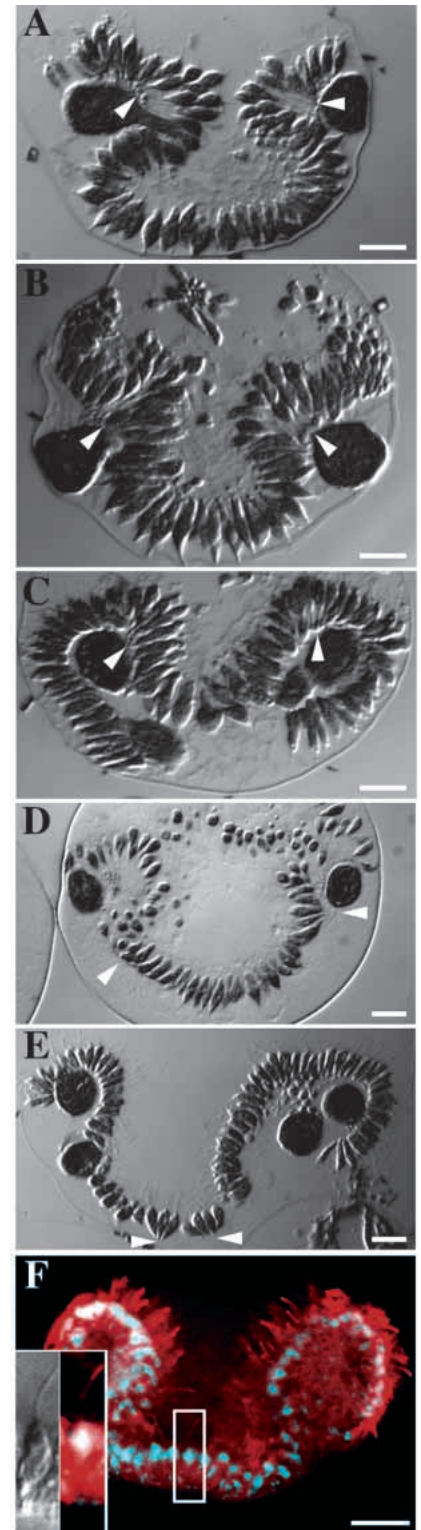


Fig. 6. Propagation of cell shape changes to the posterior pole in CCB-treated embryos. In control embryos, the elongated cells that constitute the bend region were bundled together at the thin ends of the cell (A) and, as the bend moved down, this bundling was propagated from the phialopore toward the posterior pole (B,C), as indicated by the arrows. A somewhat similar propagation of cell shape change (arrowheads) occurred in CCB-treated embryos (D,E). Cells were slightly rounder, the number of cells constituting the bundle was smaller than those of untreated embryos and, in some cases, the embryo appeared to be fragmented between regions where cells had undergone such apparent bundling. Cytochalasin B-treated embryo had flagella inside the uninverted embryo stained with antibodies to β -tubulin (red). The nuclei were stained with DAPI (blue). Times after the phialopore opening were 20 (A), 30 (B), 40 (C), 30 (D) and 50 minutes (E). Bar, 10 μm .

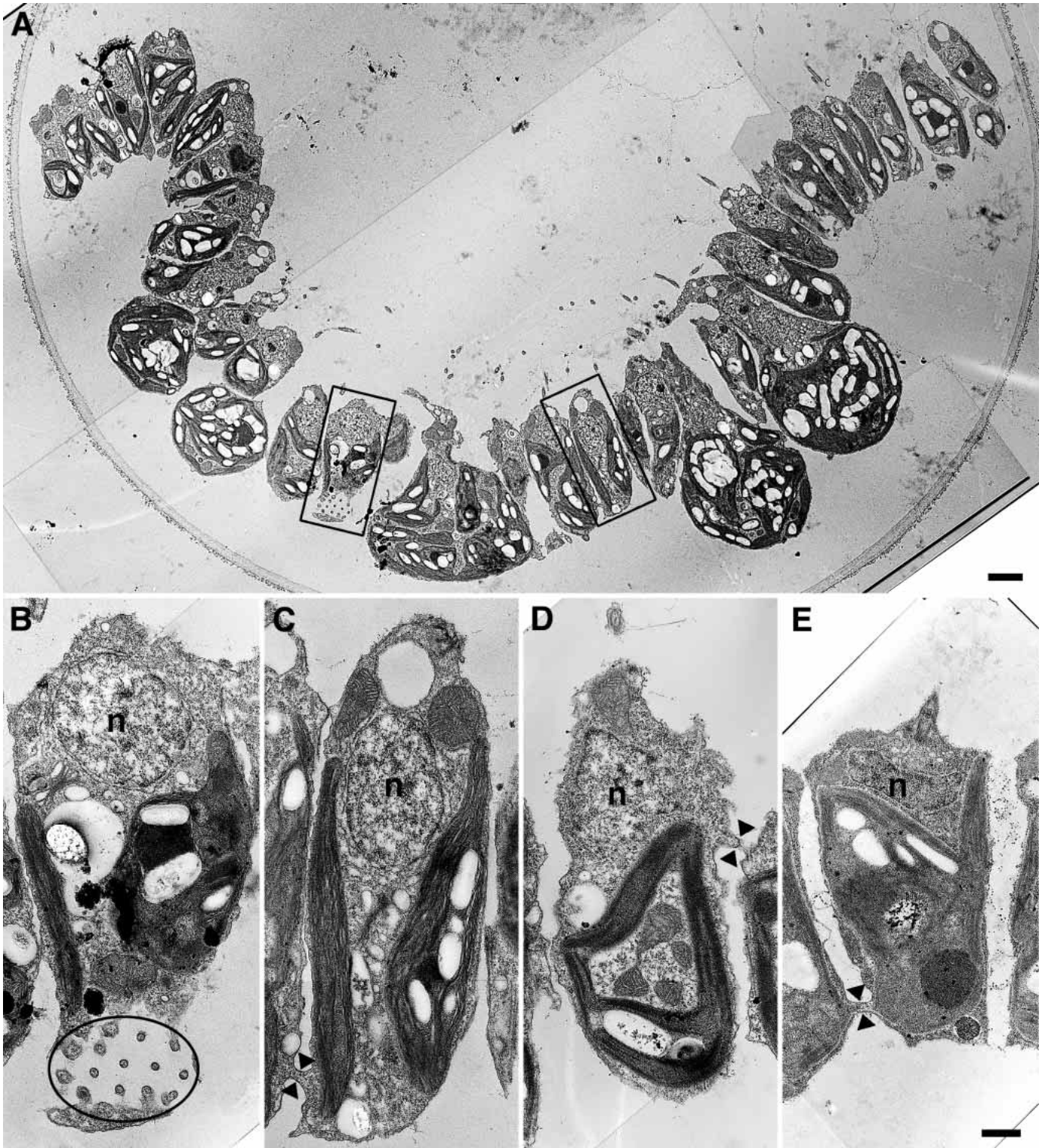


Fig. 7. Cytoplasmic bridges migrated to the chloroplast ends of cells in BDM-treated embryos. An arrested embryo was examined ultrastructurally in sagittal section (A) to locate the cytoplasmic bridges. The embryos had been treated with 40 mM BDM for 120 minutes prior to fixation. Cytoplasmic bridges of cells near the posterior pole shown in rectangles in A are shown at higher magnification in an en-face section (B; see bridge profiles inside the ellipse) and in a transverse section (C). These bridges are clearly located at the chloroplast ends of cells, far from the nuclear region. An untreated, early-inversion embryo was chosen for comparison that had an overall morphology similar to that of an arrested embryo. In this untreated embryo, cells in locations corresponding to those of B or C had cytoplasmic bridges at the nuclear level (D), and bridges were found close to the chloroplast end only in cells that had entered or passed through the bend region (E). Such repositioning of cytoplasmic bridges in normal inversion was previously analyzed in detail (Green et al., 1981) N, nucleus. Bar, 2 μ m (A); 500 nm (B-E).

of clustered cells that are bundled at their thin, chloroplast ends implied that cytoplasmic bridges might be capable of becoming relocated to the chloroplast ends of the cells even in the presence of actomyosin inhibitors. To determine whether this might be the case, the location of cytoplasmic bridges was examined ultrastructurally (Fig. 7). In BDM-treated embryos (Fig. 7A), cells in the posterior hemisphere clearly had cytoplasmic bridges at their chloroplast ends (Fig. 7B,C). This embryo had been fixed at a time when inversion would have been completed in the absence of BDM. For comparison, we chose a control embryo with curvature of the posterior hemisphere approximately the same as that of the BDM-arrested embryo, meaning that cells in the posterior hemisphere had not yet experienced inversion at the time of fixation. As previously reported, cytoplasmic bridges were found at the nuclear level (Fig. 7D), indicating that bridges in these cells had not yet migrated (Green et al., 1981). In contrast, the cytoplasmic bridges in postinversion regions (Fig. 7E) were located at the chloroplast ends of the cells, like those near the uninverted posterior pole of the BDM-inhibited embryo. Cytoplasmic bridges migrated similarly in cytochalasin B-treated embryos (data not shown).

Inversion arrest can be reversed by isolation of embryonic fragments

If actomyosin inhibitors do not block the propagation of cell shape changes or the migration of cytoplasmic bridges into the posterior hemisphere, why do such inhibitors block inversion of the posterior hemisphere? We postulated that this might be because, *in the absence of actomyosin-mediated contraction*, the diameter of the embryo in the region of the equator is larger than (and hence unable to pass through) the opening that is present in the already-inverted anterior hemisphere.

To test this hypothesis, we performed two kinds of experiment. First, we added BDM to the embryo just after the equatorial region had passed through the bend region. Our hypothesis predicted that, at this stage, BDM would have no effect on inversion at all, because the remaining uninverted portion of the posterior hemisphere would be smaller than the equator. This turned out to be the case (data not shown). Second, we used microsurgery to isolate subhemispherical fragments of BDM-inhibited embryos (Fig. 8A) in which the cut edge formed an opening as wide as the fragment itself (Fig. 8). Again, our hypothesis predicted that BDM should have no inhibitory effect on inversion under these conditions. Fig. 8 shows that this was the case.

Fig. 8. Inversion of a posterior fragment can be achieved in the presence of BDM. A preinversion embryo was treated with 40 mM BDM at time zero. With a fine glass needle, it was cut below the equator, closer to the posterior than to the anterior pole (A). In a side view, three gonidial cells are seen outside the fragments initially (A-C). Between A and H, the dissected posterior fragment rotated about 90° (the initial side view in A became the top view in D) and underwent inversion, so that cells that were initially on the outside (A-C) ended up on the inside (F-H). Flagella (arrowheads) were present on the inverted embryo surface. Time in minutes:seconds after BDM treatment: 12:20 (A), 14:51 (B), 19:50 (C), 22:20 (D), 27:20 (E), 32:20 (F), 37:34 (G) and 37:59 (H). Bars, 10 μ m.

DISCUSSION

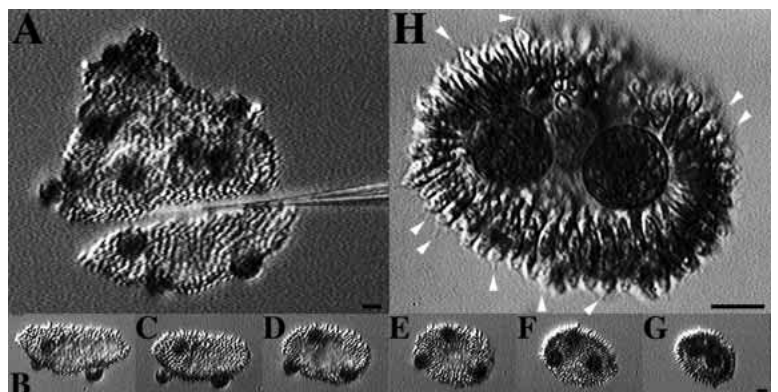
The immunofluorescence studies performed here indicate that microtubules are present abundantly during the entire course of *Volvox* inversion and that changes in their organization correlate with changes in cell shape and embryo morphology. As the clearest example, the long stalks of the flask-shaped cells that are characteristic of the bend region are shown to be richly supplied with aligned microtubules. These observations support the conclusion, drawn from ultrastructural observations made by others, that microtubule-mediated changes in cell shape play an important role in the inversion process (Green et al., 1981; Viamontes et al., 1979; Viamontes and Kirk, 1977).

The present study also extends the analysis of *Volvox* inversion in a new direction by examining for the first time the distribution of myosin and actin filaments in inverting embryos and by reexamining the effects of actomyosin inhibitors on the inversion process. These studies have uncovered an important aspect of the inversion mechanism that had not previously been detected.

Distribution of actin and myosin in inverting embryos

As inversion begins, actin and myosin move in concert from the perinuclear region to the subnuclear region of each cell and then, as cells approach the bend region, their actomyosin filaments move progressively further from the nuclei, toward the chloroplast ends of the cells. This is reminiscent of the progressive change that has been shown to occur in the locations of the cytoplasmic bridges during the inversion process (Green et al., 1981, figure 10). Moreover, some actin filaments appear to be continuous from cell to cell (Fig. 2G). This leads to the obvious suggestion that actomyosin filaments may actually traverse the cytoplasmic bridges that link neighboring cells (Green et al., 1981). This possibility should be examined at the ultrastructural level but, unfortunately, we have not yet succeeded in preserving the actin filaments of *Volvox* embryos in specimens that are suitable for EM examination (unpublished observation). A similar lack of success has been reported by others (Green et al., 1981; D. Kirk, personal communication).

It is particularly noteworthy that the actin-filament network disappears, and myosin becomes diffusely distributed, as soon cells enter the bend region of the inverting embryo (Figs 2C, 3B). This clearly indicates that, whatever role(s) the actomyosin complex plays in inversion, that role must be



completed before the cells participate in formation of a bend region. This assumption was reinforced by our observation on the effects of actomyosin inhibitors, to be discussed next.

Effects of actomyosin inhibitors on various aspects of the inversion process

All four of the actomyosin inhibitors used here (cytochalasin B, cytochalasin D and cytochalasin E, plus BDM) had a similar effect when applied to *Volvox* embryos continuously from 2 hours before the beginning of inversion through the time at which control embryos had completed inversion. They permitted inversion to be initiated relatively normally and to proceed to the halfway of the anterior hemisphere, but they blocked inversion of the posterior hemisphere irreversibly (Fig. 4).

It was previously reported that cytochalasin D acted on the *Volvox* embryo quickly and reversibly to block both the initial generation of, and the propagation of, the bend regions (Viamontes et al., 1979). In contrast, under our conditions of observation, all three cytochalasins acted much more slowly, and irreversibly, and did not prevent either the initial generation of bend regions or their progression to the halfway point of the treated embryos (Fig. 4G,P,Q). These slow effects of cytochalasins were taken into consideration in the design of our experiments. We observed with immunofluorescence that no effects on the actin filament network could be observed in the first 5 minutes of cytochalasin treatment (data not shown) and it took 30-60 minutes to fully depolymerize actin filaments in the subnuclear region (Fig. 4P). Thus, cytochalasin treatment for 2 hours before inversion, which we performed here, is thought to be long enough to induce the drug effects in the embryos, and hence the incomplete progression of the bend was never due to insufficient permeation of the drugs. It is unclear whether the different results obtained in this study and the earlier one (Viamontes et al., 1979), are due to subtle difference in strains, culture conditions, purity of the drugs, method of drug administration or some other unknown factor. In any case, the present study and the previous one indicate that the sequence of cell-shape transformations that accompany inversion are largely independent of actomyosin activity and, although they are undoubtedly necessary, they are not sufficient to allow inversion to be completed.

Role of the force generated by actomyosin in inversion

A clue to the mechanism by which actomyosin inhibitors prevent inversion from going to completion came with the observation that, in the presence of such inhibitors, the posterior hemisphere of the embryo appeared to be wider than it was in control embryos at the same stage. Measurements confirmed this impression: by 15-20 minutes after the beginning of inversion, the posterior hemisphere of control embryos began an accelerated contraction that reduced its diameter appreciably, but treated embryos underwent no such rapid contraction. The rapidity and irreversibility with which the myosin II inhibitor, BDM (Cramer and Mitchison, 1995) acted on the *Volvox* embryos permitted us to study this phase of the inversion process more closely and show that an actomyosin inhibitor exerts its inhibitory influence on the inversion process during this period. If BDM was present at the 20-minute time point, inversion of the posterior hemisphere was blocked. In contrast, even in embryos that had been

exposed to BDM for 3 hours, if the drug was washed out before the 20-minute time point, inversion was completed normally.

This led us to the hypothesis that actomyosin inhibitors block the last half of inversion by blocking a contraction of the equatorial region of the embryo that is necessary to make it small enough to pass through the opening that is formed by already inverted anterior hemisphere. Two additional pieces of evidence supported this hypothesis. The first was the observation that, if BDM was added after the equatorial region had passed through this opening, no inhibition of the rest of the inversion process was observed. The second was the observation that if a subposterior hemispherical fragment was isolated by microdissection, BDM failed to prevent it from inverting (Fig. 8). As shown in Figs 6 and 7, cell shape changes and migration of cytoplasmic bridges occurred normally even in the arrested

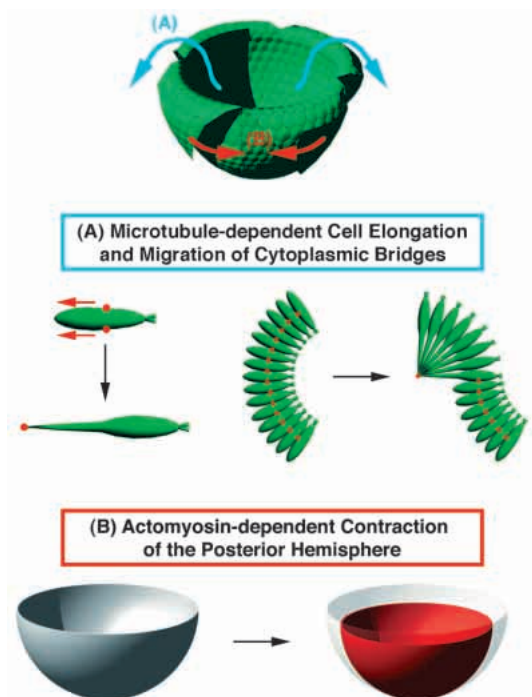


Fig. 9. Two cytoskeletal components cooperate in inversion of *Volvox* embryo. Inversion consists of two distinct motions of the multicellular sheet. One starts from the lip opening at the phialopore and the bend propagates toward the opposite pole, making the entire sheet inside-out (A). The other is the contraction of the posterior hemisphere (B). Microtubules are responsible for the former process and actomyosin for the latter. (A) The bend is formed by two different, but both microtubule-dependent changes in the individual cells; the shape changes from spindle to flask cells and there is migration of cytoplasmic bridges. As cytoplasmic bridges (red dots) move away from the basal body, the cell elongates, thus reversing the sheet curvature in the bend. Characteristics of the 'cytoplasmic bridge system' in *Volvox* was studied in detail by Kirk and his colleagues (Green et al., 1981). (B) Actomyosin-dependent contraction of the posterior hemisphere makes it small enough to pass through the opening that is formed by already-inverted anterior hemisphere. The 'snap-throw' behavior of the posterior hemisphere, a nomenclature used because of the posterior hemisphere inverts faster than the anterior hemisphere, reflects the elastic nature of the multicellular sheet of *Volvox* embryos (Viamontes et al., 1979). We suggest that the molecular mechanism for the 'snap-throw' is likely to be based on the active contraction of actomyosin.

embryos. Therefore, removal of the anterior hemisphere, which would otherwise have imposed a physical constraint on the swollen posterior hemisphere, permitted inversion of the arrested posterior hemisphere. In Fig. 9, we schematize how these mechanics cooperate in inversion with special reference to actomyosin and microtubules. Our hypothesis does not require any cellular differentiation between cells in the anterior and the posterior hemispheres to explain why anti-actomyosin drugs arrest inversion at approximately the equator.

The role of actomyosin in *Volvox* inversion appears similar to its role in reducing the dimensions of a multicellular epithelial sheet during animal morphogenesis (Owaribe and Masuda, 1982). It is noteworthy, however, that the function of actomyosin contraction may be different in these two cases. Whereas the effect of actomyosin inhibitors in the animal system is attributed to inhibition of the contraction that occurs at one end of each cell and causes them to assume 'flask' or 'bottle' shape, formation of such cellular shapes in *Volvox* is not prevented by actomyosin inhibitors and appears to be driven by microtubules (Fig.6; Viamontes et al., 1979). Nevertheless the role proposed here for actomyosin contraction (namely, to relieve the folding cellular sheet from the physical constraints) may be applicable in many other examples of epithelial folding, especially when these processes include the passage of a part of the multicellular sheet through a small opening, such as in gastrulation, evagination of imaginal disk (Fristrom, 1988) etc. Mathematical models of epithelial folding (for example, Odell et al., 1981) might also need to pay more careful attention to resolving such spatial constraints.

We speculate that the disappearance of actin filaments from cells in the already inverted anterior hemisphere (Figs 2C, 3B) may play an essential positive role in inversion. If the cells were to remain in a contracted state as they entered and passed through the bend regions, this would almost certainly counteract the effect of contractions in the posterior hemisphere, resulting in a decrease in the diameter of the opening through which the posterior hemisphere must pass. Thus loss of actomyosin filaments and relaxation of cells that have traversed the bend region may well be as indispensable for the completion of inversion as is the actomyosin contraction in the posterior hemisphere.

In conclusion, we have shown that actomyosin exerts a contractile force that causes the posterior hemisphere to become compact and thus facilitates the propagation of the bending region, which is generated largely by microtubules. It is necessary for these activities of actomyosin to be coordinated in space and time with the activities of microtubules during inversion. Genetic analysis of these dynamic phenomena may be feasible. A variety of *Volvox* mutants deficient in various phases of inversion has been isolated (Sessoms and Huskey, 1973; Kirk et al., 1982) and techniques suitable for analyzing the molecular basis of such defects have recently been developed (Miller et al., 1993; Schiedlmeier et al., 1994; Hallmann et al., 1997). We are now attempting to isolate transposon-tagged mutants with phenotypes that resemble the drug-arrested embryos of the molecular-genetic program involved in coordinating cytoskeletal activities in space and time to bring about this interesting morphogenetic transformation.

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