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Microtubule translocation and polymerisation during cortical rotation in *Xenopus* eggs

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SUMMARY

The development of dorsal axial structures in frogs depends on a process of cortical rotation in which the cortex of the fertilised egg becomes displaced with respect to the cytoplasm. An array of aligned microtubules that develops between the vegetal cortex and cytoplasm is implicated in generating movement. Rhodamine-tubulin was injected into eggs to allow patterns of microtubule movement and polymerisation in the vegetal array to be examined. Time-lapse video microscopy of living eggs showed that most of these microtubules move with the vegetal cytoplasm relative to the cortex, at the same speed as cytoplasmic pigment granules. This implies that movement is generated between the microtubules of the vegetal array and the cortex. A few microtubules were also detected that appeared immobile with respect to the cortex.

Rhodamine-tubulin became incorporated into vegetal microtubules when injected at any time during the period of cortical rotation. The newly formed microtubules connected the vegetal array and internal cytoplasm. This indicates that local outward-directed polymerisation continues in this region as the cortex translocates. Experiments with low doses of nocodazole showed that this continuing polymerisation does not contribute to the rotation. Concentrations of the drug that prevented tubulin polymerisation had no effect on the speed of rotation if applied after the vegetal array had formed. The same doses prevented movement if applied early enough to prevent the formation of the array. These observations support the idea that mechanochemical enzymes associated with the vegetal microtubules translocate the cortex along microtubules anchored in the subcortical cytoplasm.

Key words: cortical rotation, microtubule, rhodamine-tubulin, *Xenopus*, nocodazole, egg, axis

INTRODUCTION

The dorsal-ventral axis of many amphibians is specified during the second half of the first cell cycle as the result of a process of cortical rotation (see Gerhart et al., 1989). The entire outer cortex of the egg rotates relative to the bulk of the cytoplasm by 30° about a horizontal axis (Ancel and Vintemberger, 1948; Vincent et al., 1986; Fujisue et al., 1991), at speeds of 7-10 μm/minute in *Xenopus*. Close observation of the rotation and evidence from irradiation and bisection experiments indicate that the mechanism that generates this movement is located beneath the vegetal surface of the egg (Manes and Elinson, 1980; Scharf and Gerhart, 1983; Vincent et al., 1986; Vincent and Gerhart, 1987). An array of aligned microtubules appears in this region during the second half of the first cell cycle, the time of rotation. (Elinson and Rowning, 1988). It lies in the shear zone between the cortex and the yolky vegetal cytoplasm and arises, at least in part, from the outward growth of microtubules from the centre of the egg, which turn when they reach the cortex to run in the direction of cortical movement (Houliston and Elinson, 1991a; Schroeder and Gard, 1992; Elinson and Palacek, 1993). In the fertilised egg, the first microtubules to reach the vegetal cortex extend from the sperm aster (Houliston and Elinson, 1991a). These may be responsible for biasing the orientation of the array, usually directed away from the side of the egg where the sperm entered (Manes and Barbieri, 1977; Elinson and Rowning, 1988). The vast majority of the microtubules are aligned with their plus ends pointing in the direction of cortical movement (Houliston and Elinson, 1991b). Treatments that disrupt the vegetal microtubule array, including drugs that specifically depolymerise microtubules but not ones that disrupt actin filaments, block the cortical rotation and prevent the development of dorsal and anterior structures (Manes et al., 1978; Scharf and Gerhart, 1983; Elinson and Rowning, 1988; Vincent et al., 1987).

Various mechanisms have been proposed to account for the generation of directed force that translocates the cortex (Fig. 1; see also Houliston and Elinson, 1992). One idea is that mechano-chemical enzymes associated with microtubules in the vegetal array are responsible (‘microtubule motors’: see Vallee and Shpetner, 1990). As indicated in Fig. 1, there are three basic possibilities depending on where the microtubules are anchored and what type of motors are present. (1) The microtubules of the array could become attached to the cortex and components of the cytoplasm translocated relative to them by a minus end-directed microtubule motor. (2) Cortical elements could be translocated relative to microtubules anchored in the vegetal cytoplasm by a plus end-directed

motor. (3) Motors that cause microtubules to slide relative to one another could push apart microtubule populations attached to different elements in the subcortical region. A distinct hypothesis (4) is that directed microtubule polymerisation occurring in the subcortical region could provide part of the force that moves the vegetal cortex.

In order to discriminate between these possibilities, we need to know how microtubules behave in the shear zone between cortex and vegetal cytoplasm. Tubulin labelled with rhodamine was injected into eggs so that it incorporated into the microtubules of the vegetal array, allowing microtubule movement to be followed directly in live eggs by time-lapse video microscopy. Patterns of tubulin incorporation in fixed eggs following injections at different times were also examined, and the role of directed polymerisation in force generation assessed by treatment of eggs with low doses of the microtubule destabilising drug nocodazole.

MATERIALS AND METHODS

Induction of ovulation in *Xenopus laevis*, insemination and dejellying were as described in Zisckind and Elinson (1990). Variations in ambient temperature affect the duration of the first cell cycle (85-115 minutes), so a normalized time scale is used. Insemination is 0 NT and the time of first cleavage in untreated control eggs 1.0 NT. One cell cycle unit (c.c.u.) is defined as 1/100 of the time to first cleavage.

Preparation and microinjection of rhodamine-tubulin

Rhodamine tubulin was prepared from twice cycled pig or mouse brain tubulin as described previously (Houliston et al., 1993). It was injected beneath the vegetal surface of dejellied eggs inverted on glass slides in a minimum volume of 80% Steinberg's, using pulled glass capillaries (Clarke) and a Singer micromanipulator. Pressure was supplied by compressed air or a mouth pipette and the injection volume was about 20 nl, as determined by counting the number of injections from a known volume. Most injected eggs cleaved and developed normally.

Immunofluorescence and confocal microscopy of fixed eggs

Eggs were fixed at –20°C in methanol containing 1% formaldehyde (from 37% solution) for 2 hours or more, then rehydrated through a methanol/PBS series. Anti-tubulin immunofluorescence was performed as in Elinson and Rowning (1988) using a rat monoclonal anti-alpha tyrosinated tubulin YL1/2 (Kilmartin et al., 1982) or mouse monoclonal anti-alpha tubulin (DMIA: Sigma) followed by fluorescein anti-rat or anti-mouse Ig (Jackson Immunoresearch). Eggs were mounted in Citifluor (City University, London) and examined with a ×40 objective on a Leica confocal laser scanning microscope equipped with an argon/krypton laser. Images were averaged from 8 scans of the laser beam.

Observation of microtubules in live eggs

After removing the fertilisation envelope, injected eggs were embedded in 8% gelatin/80% Steinberg's to hold the egg cortex still (Black and Gerhart, 1985) and mounted between a glass slide and coverslip using a silicone rubber spacer. Time-lapse recordings were made using a SIT camera mounted on an inverted fluorescence microscope (Zeiss) with shutters controlled by computer using Image I software (Universal Imaging). Images were recorded at 20 second intervals on an Optical Disc Memory Recorder (OMDR, Panasonic) following averaging over four frames. Speeds of movement of microtubules and pigment granules were calculated by tracing their positions from the video screen onto acetate sheets. Parallax error was assumed to be negligible since the vegetal cortex was immobilised flat against the coverslip. To improve the clarity of printed still frames, images were treated with a non-linear (median) filter and the contrast enhanced.

Measurement of cortical rotation in nocodazole-treated eggs

Initial studies with nocodazole on eggs embedded as above were unsatisfactory because of variability in drug penetration. To avoid this problem, unembedded eggs with intact fertilisation membranes were transferred to 3.5% Ficoll 400 (Sigma) in 20% Steinberg's to collapse the perivitelline space. The characteristics of cytoplasmic movement under these conditions were equivalent to those measured by other methods, presumably because the egg cortex becomes stuck against the fertilisation envelope (as also observed in other studies: Savage and Danilchik, 1993). The fluorescent lipophilic dye $DiOC_6(3)$ (Molecular Probes) was used to visualise patches of subcortical germ plasm which move with the vegetal cytoplasm (Savage and Danilchik, 1993). Dejellied eggs were incubated for 3 minutes in a 2.5 μg/ml solution of $DiOC_6(3)$ in water, diluted from a 2.5 mg/ml stock in ethanol. Movements of the germ plasm were recorded with the system described above, and speeds calculated similarly.

Nocodazole was diluted from a 1.2 mM stock in DMSO into 20% Steinberg's/3.5% Ficoll immediately prior to use. Groups of 5 or 6 control and nocodazole-treated eggs from the same batches were fixed at various times during each experiment and stained by immunofluorescence with anti-tubulin antibodies.

RESULTS

Displacement of microtubules relative to the vegetal cortex

Microtubules in the vegetal array were labeled effectively following shallow vegetal injections of rhodamine-tubulin

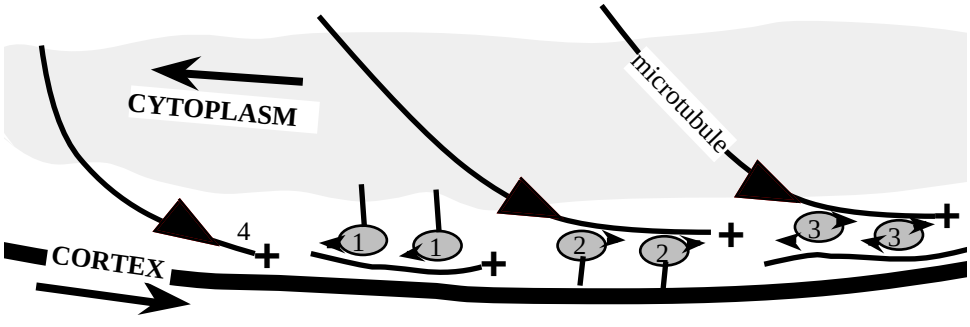

Fig. 1. Hypotheses for the mechanism of cortical rotation. A section of the vegetal region is represented in an egg where cytoplasmic microtubules have grown out towards the cortex from the left (sperm aster) side. The relative movement of the cytoplasm and cortex are shown by long arrows. Microtubule plus ends (+) mostly point in the direction of cortical movement. Ovals represent motor molecules moving along microtubules in the direction of the small arrowheads. These could carry the cytoplasm along microtubules attached to the cortex (1), the cortex along microtubules attached to the cytoplasm (2), or slide different microtubule populations relative to one another (3). Directed microtubule polymerisation (large arrowheads) could also provide force (4).

during the first half of the cell cycle (between 0.30 and 0.45 NT). The rhodamine-tubulin usually incorporated into the developing microtubule array in a diffuse patch around the injection site. Time-lapse video recordings were made towards the edge of this patch, where background fluorescence from unpolymerised rhodamine-tubulin was lowest. The microtubule fibres observed were probably segments of longer microtubules or microtubule bundles, the rest of which lay out of the plane of focus or were not well enough labelled to detect. Single microtubules may not be detected by this system. Injected eggs were removed from their fertilization envelopes and embedded in gelatin to immobilise the egg surface flat against a coverslip. Under these conditions, the rotation is manifest as movement of the internal cytoplasm relative to the stationary cortex (Vincent et al., 1986).

Most of the pigment granules in the subcortical region, i.e. in the same plane of focus as the microtubules, moved at speeds of 3-6 μm/minute, while those close to the vegetal surface showed little or no net movement, confirming that the cortex was immobile. The speed of movement of subcortical pigment granules and of yolk platelets corresponds to that of nile blue-stained yolk measured in *Xenopus* eggs with their fertilisation envelopes similarly removed (Satoh and Shinagawa, 1990). In eggs with intact fertilisation envelopes, which are less flattened, nile blue spots move slightly faster (Gerhart et al., 1989).

In all sequences analysed, the vast majority of detectable microtubule fibres appeared to move with the subcortical pigment granules relative to the stationary cortex (Fig. 2). This was most obvious in recordings where microtubule fibres were bent or crossed, forming characteristic patterns in relationship to one another (Fig. 2A). When microtubule fibres were

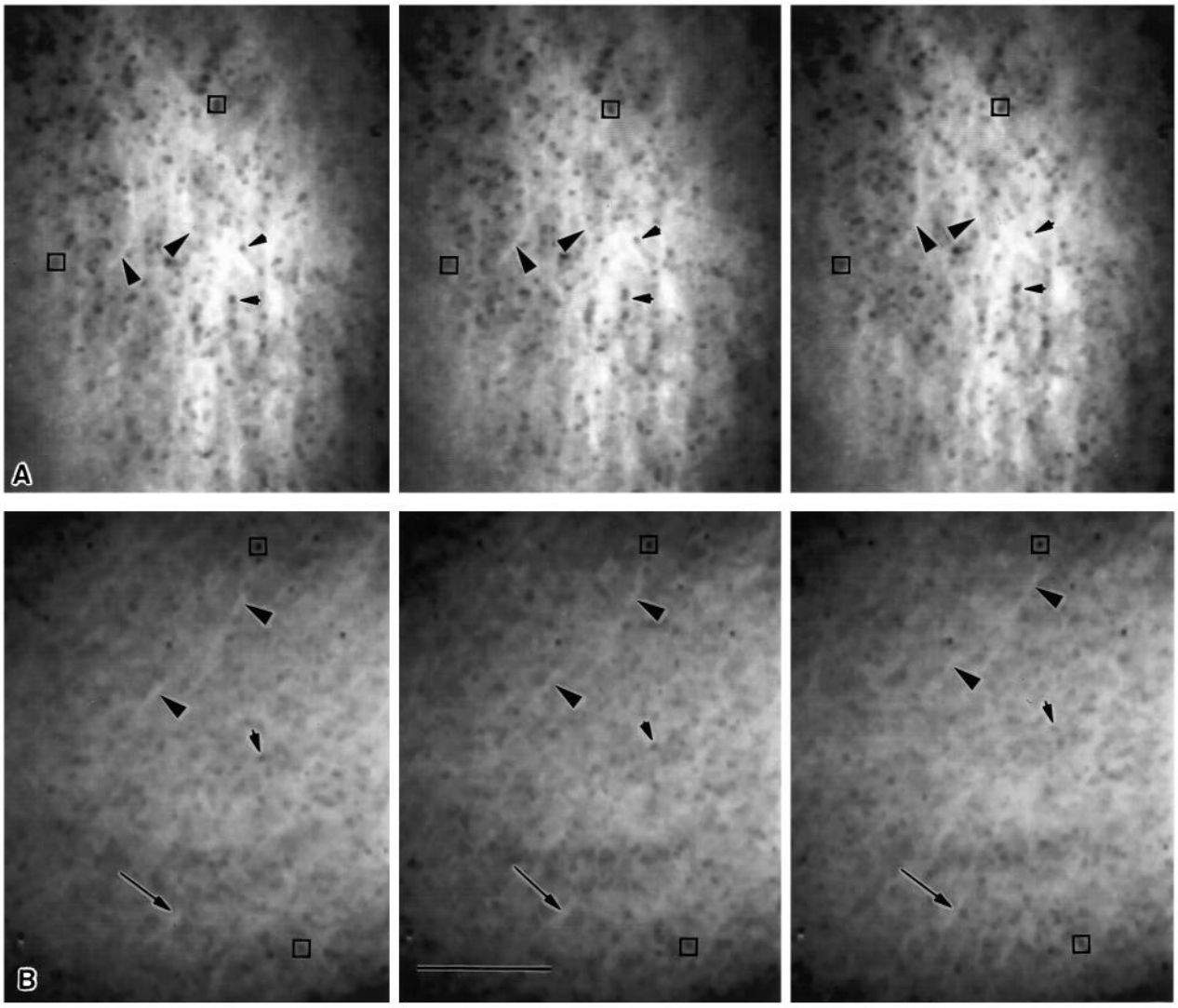

Fig. 2. Displacement of microtubules relative to the vegetal cortex. Rhodamine-tubulin was injected during the first half of the cell cycle and recordings made during the period of rotation, with the cortex immobilised in gelatin. (A) Video frames taken at 20 seconds intervals. Pigment granules in the cortex (e.g. in black boxes) remain stationary while those deeper in the cytoplasm (e.g. at small arrowheads) and the bulk of labeled microtubule fibres (e.g. at large arrowheads) moved up the screen. (B) Another egg, frames taken at 40 seconds intervals. Symbols as in A. In this recording most microtubule fibres moved with the cytoplasmic pigment granules towards the top right of the screen, however some appeared to remain stationary with respect to the cortex (e.g. at arrow). Bar 25 μm.

straight and parallel it was much harder to be sure whether they were moving. Microtubule fibres that did not appear to move were present in three of the clearest seven recordings (e.g. at arrow in Fig. 2B). Additional 'stationary' microtubules may have been present but not detected.

Quantification of the speeds of movement of microtubule fibres was imprecise because of the difficulty in defining single points on them. Measurements were made where segments could be followed over periods of 2-3 minutes. They moved at 3-7 μm/minute, with the average speed of 26 segments taken from 6 sequences being 4.8 μm/minute (s.d. 1.1 μm/minute). The average speed of 26 subcortical pigment granules from the same six sequences, 4.5 μm/minute (s.d. 0.8 μm/minute), was not statistically different from that of the microtubules (*t*-test).

The angle between the direction of movement and the orientation of the microtubules was always less than 25°, such that the cortical rotation was directed along the aligned microtubules, as inferred previously from studies on fixed eggs (Elinson and Rowning, 1988; Ziskind and Elinson, 1990). This is additional evidence that the movement observed was not artifactual shifting of the whole egg or its contents.

Microtubule polymerisation during cortical rotation

During the course of these experiments, I noted that rhodamine-tubulin incorporation into microtubules in the vegetal array occurred even when injections were performed during the second half of the cell cycle, after the array had formed. This suggested that polymerisation continues in the vegetal region during the period of the rotation. To follow up this observation, rhodamine-tubulin was injected beneath the vegetal surface of eggs at different times during the first cell cycle, and its incorporation into microtubules assessed after fixation about 10 c.c.u. (10-15 minutes) later (Fig. 3). Rhodamine tubulin injected any time between 0.45 NT and 0.75 NT became incorporated into microtubules in the vicinity of the injection site (Fig. 3A-C), indicating that polymerisation indeed continues

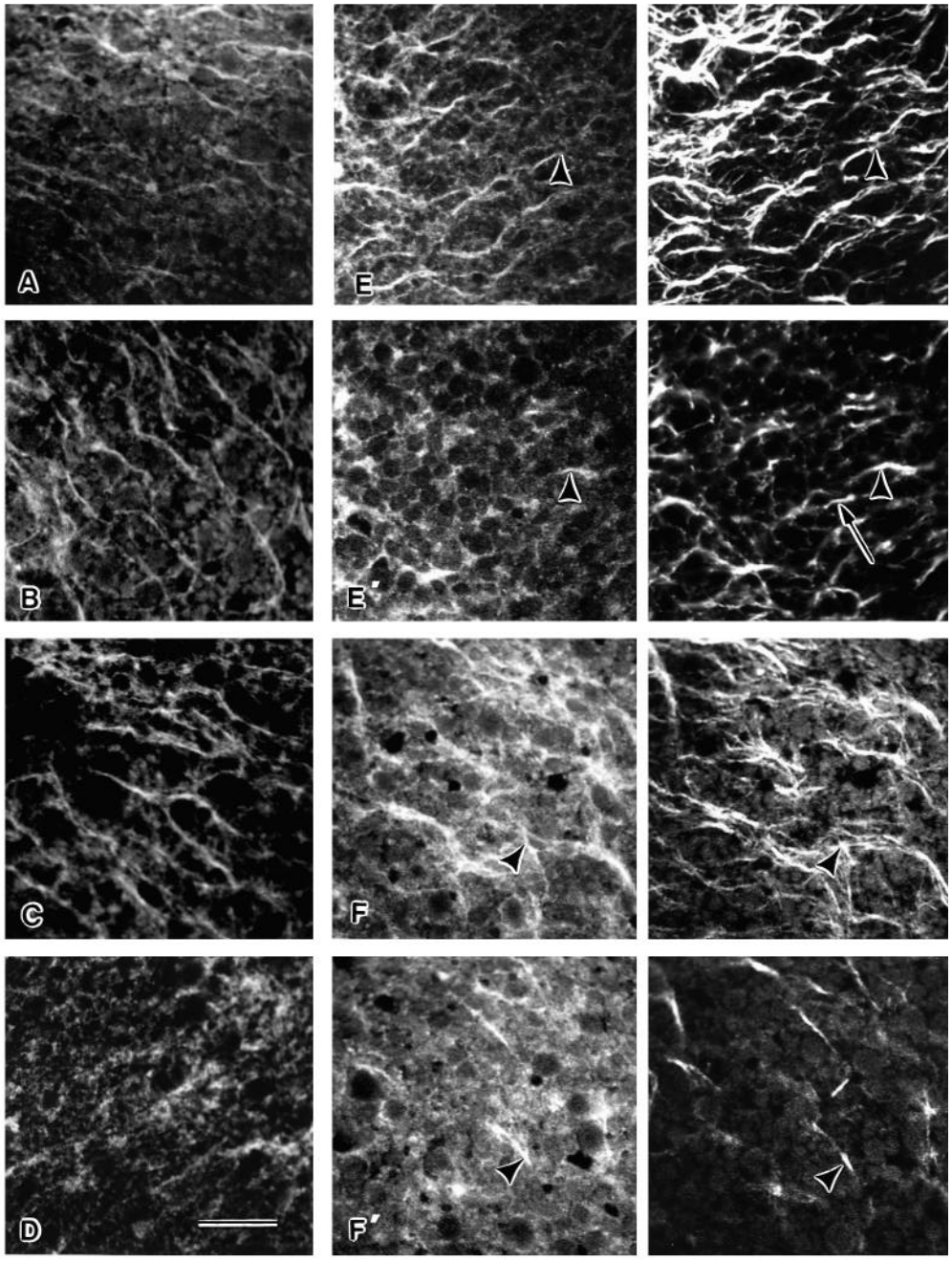

Fig. 3. Incorporation of rhodamine-tubulin into microtubules during the period of cortical rotation. Confocal images taken from the vegetal surface of fixed eggs. Bar 25 μm. (A-D) Incorporation over short periods (less than 10 c.c.u.). (A) Injection at 0. 48 NT, fixation at 0.57 NT. (B) Injection at 0. 56 NT, fixation at 0.64 NT. (C) Injection at 0.77 NT, fixation at 0.84 NT. (D) Injection at 0.82, fixation at 0.94 NT (no incoroporation). (E-F) Incorporation into outward-growing microtubules revealed following longer incubation times and counterstaining with anti-tubulin (right-hand panels); (E) egg injected at 0.56 NT and fixed at 0.69 NT; (F) egg injected at 0.67 NT and fixed at 0.88 NT. E′ and F′ are optical sections 3 μm below the level of aligned microtubules. Deep and surface microtubule segments are continuous (e.g. at arrowheads). Most surface segments, but not all corrresponding deeper segments have incorporated rhodamine-tubulin (arrow).

throughout the period of rotation. No incorporation into microtubules below the vegetal cortex could be detected from similar injections outside this period, that is, before the array forms at 0.45 NT or as it is starting to thin after 0.8 NT (Fig. 3D). The lack of incorporation in these cases argues against the possibility that introduction of excess tubulin induced local polymerisation.

Many of the microtubules in the vegetal array appear to arise from the outward polymerisation of sperm aster and other radially extending cytoplasmic microtubules, which turn at the cortex to run in the direction of rotation (Houliston and Elinson, 1991a; Schroeder and Gard, 1992, Elinson and Palacek, 1993). To determine whether the polymerisation in the vegetal hemisphere during the second half of cell cycle continued to be directed outward, eggs were injected with rhodamine-tubulin as above and allowed 10 to 20 c.c.u. for incorporation. They were then counter-stained by immunofluorescence with anti-tubulin antibodies and examined by confocal microscopy. After injections early or late during the rotation, microtubules containing rhodamine-tubulin were found connecting the array to internal microtubules, some having incorporated rhodamine-tubulin preferentially at the surface (Fig. 3E,F). Assuming that polymerisation occurred predominantly on microtubule plus ends, which lie in the direction of rotation (Houliston and Elinson, 1991b), this indicates that outward directed polymerisation continues during the rotation period.

Cortical rotation in nocodazole-treated eggs

To test the hypothesis that polymerisation contributes force to the cortical rotation, eggs were treated with low doses of nocodazole after the vegetal array had formed. Preliminary trials showed that nocodazole at concentrations of 0.2-0.5 μM did not destroy the vegetal microtubule array. Eggs fixed at the end of the nocodazole incubation period showed arrays equally dense or sparser than those fixed at the beginning, while control eggs showed much denser arrays. The first differences between nocodazole and control eggs were apparent after about 5 c.c.u., suggesting that this time is required for the drug to penetrate to the subcortical region and bind to tubulin. No incorporation of rhodamine-tubulin into vegetal microtubules was detected in the presence of 0.3 μM of nocodazole (not shown). It is impossible to prove that polymerisation has been blocked completely since low levels may go undetected.

Eggs were transferred to nocodazole at times before, during and after the formation of the vegetal microtubule array, and the cytoplasmic movement relative to the cortex measured (Fig. 4). Cytoplasmic movement was followed by time-lapse video microscopy by virtue of staining germ plasm in the subcortical cytoplasm with a fluorescent vital dye (Savage and Danilchik, 1993). Examples of the effects of various treatments are shown in Fig. 4. Groups of 5 or 6 eggs treated in parallel were fixed in order to examine the organisation of the vegetal microtubules by immunofluorescence (Fig. 5). When eggs were transferred to 0.5 μM nocodazole after the array had started to form, movement continued in line with that of controls (five experiments using eggs from four frogs; Fig. 4A, B). During such treatments the vegetal array thinned out in the presence of nocodazole, while it became denser in control eggs (Fig. 5A-C). The same dose of nocodazole completely blocked movement if applied during the first half of the cell cycle (three

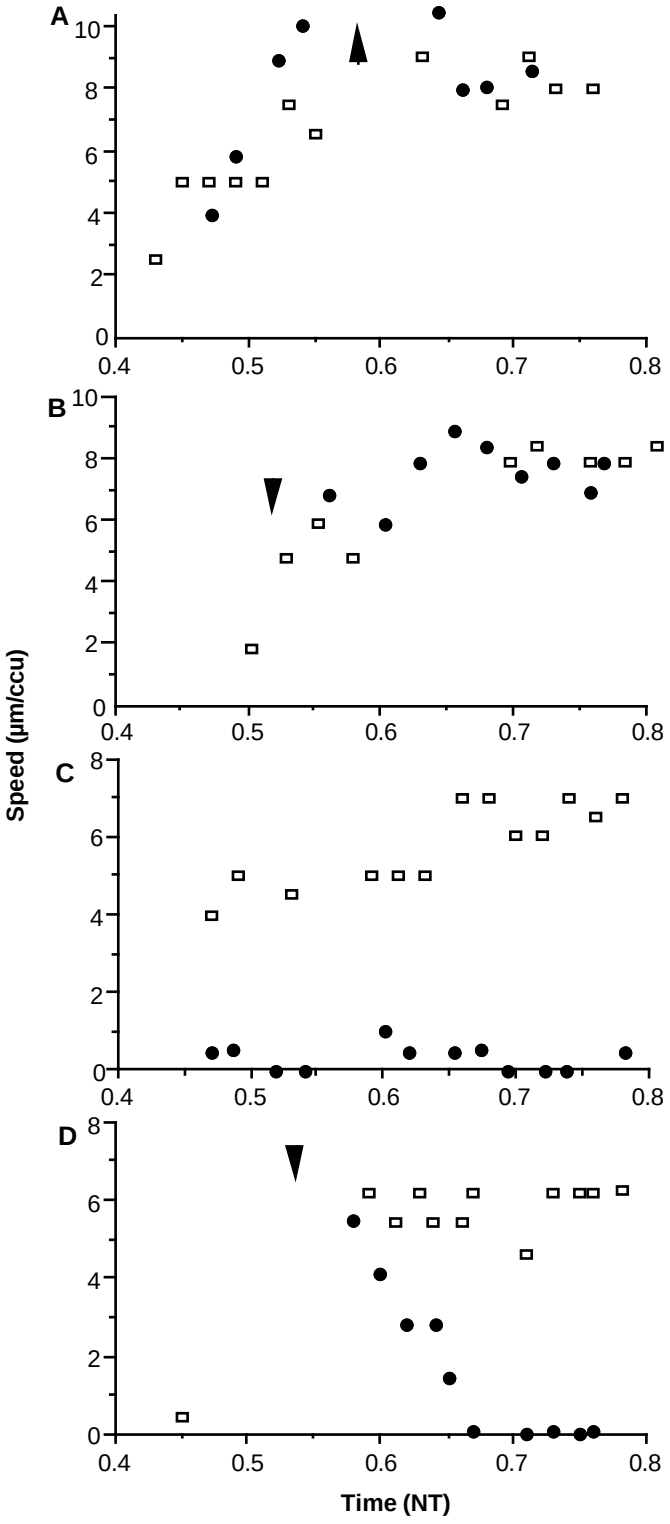

Fig. 4. Effects of nocodazole on the speed of cortical rotation. Movement of germ plasm aggregates in the vegetal cytoplasm were measured relative to the stationary cortex. (A) Treatment with 0.5 μM nocodazole from 0.59 NT. (B) Treatment with 0.5 μM nocodazole from 0.50 NT. (C) Treatment with 0.5 μM nocodazole from 0.35 NT. (D) Treatment with 1.0 μM nocodazole from 0.54 NT. Treated eggs (black circles) are compared to a control eggs from the same female (open squares). Arrows mark the time of nocodazole addition.

experiments using eggs from three frogs; Fig. 4C), thus preventing the array from forming (Fig. 5D-F).

Treatment with 1.0 μM nocodazole had a somewhat variable effect on the microtubule array between experiments. Out of six experiments using eggs from three frogs, the array was essentially destroyed in three cases, residual arrays of sparse aligned microtubules were found in two cases and denser arrays were present in one. The effect on the rotation correlated with the extent of the microtubule array. In eggs from batches where residual microtubules were found, the movement was reduced but not completely blocked. Where no or very few microtubules were found the movement stopped rapidly and completely (Figs 4D, 5G-I). 2.0 μM nocodazole also rapidly destroyed the array and arrested the rotation (two experiments).

DISCUSSION

Data presented here show that most of the aligned microtubules of the subcortical vegetal array in fertilised *Xenopus* eggs move with the cytoplasm relative to the cortex during the rotation period of the first cell cycle. Some of these microtubules maintain direct connections with ones deeper in the egg throughout the rotation period (Houliston and Elinson, 1991a,b; Schroeder and Gard, 1992; this study). Thus the

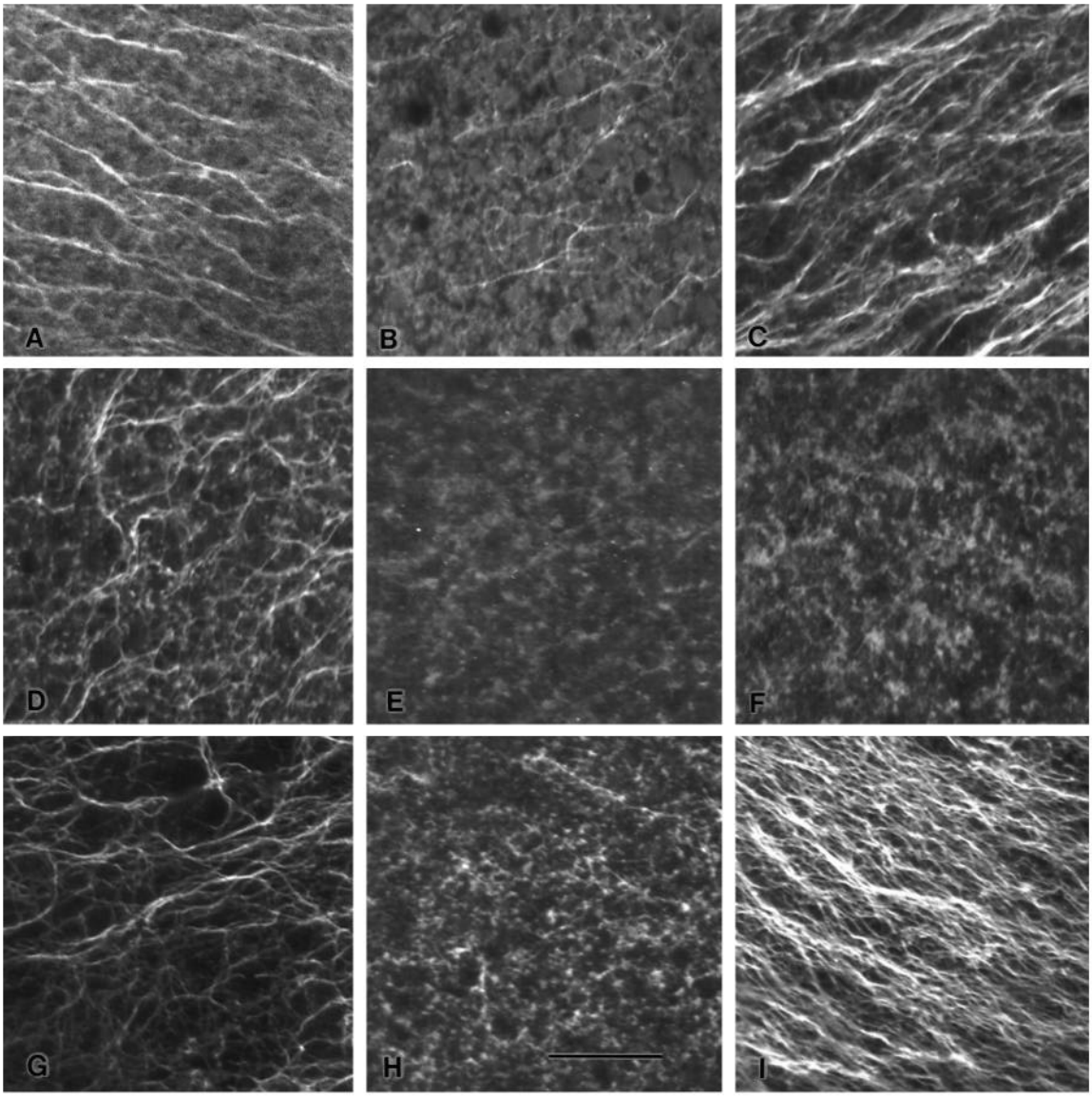

Fig. 5. Vegetal microtubules in nocodazole-treated eggs. Groups of 5-6 eggs from batches used to test the effect of nocodazole treatments on cortical rotation (see Fig. 4) were fixed at various points during the experiment and the microtubules stained by immonofluorescence. Representative confocal images are shown. Bar 25 μm. (A-C) Treatment with 0.5 μM nocodazole after array formation. (A) Egg fixed at time of nocodazole addition (0.53 NT) ; (B) nocodazole-treated egg fixed at 0.74 NT; (C) control egg fixed at 0.74 NT. (D-F) Treatment with 0.5 μM nocodazole before array formation (0.35 NT). (D) Control egg fixed at 0.51 NT; (E) nocodazole-treated egg fixed at 0.51 NT; (F) nocodazole-treated fixed at 0.74 NT. (G-I) Treatment with 1.0 μM nocodazole after array formation. (G) Egg fixed at time of nocodazole addition (0.54 NT); (H) nocodazole-treated egg fixed at 0.82 NT; (I) control egg fixed at 0.82 NT.

vegetal cytoplasm with attached aligned microtubules in the shear zone can be considered to move as a block, with movement being generated between these microtubules and the overlying cortex, rather than between the microtubules and the vegetal cytoplasm.

One possible mechanism for the cortical rotation was suggested by the observation that directed microtubule polymerisation continued beneath the vegetal cortex during the second half of the cell cycle. It seemed possible that such directed polymerisation might provide a concerted force to push the cortex in one direction, usually away from the growing sperm aster (Fig. 1 (4)). Equivalent mechanisms of force generation by microtubule polymerisation pushing against the cortex have been proposed to explain pronuclear migration (eg. Manes and Barbieri, 1977) and the positioning of astral mitotic spindles (Bjerknes, 1971). The role of tubulin polymerisation was tested by applying nocodazole to eggs at concentrations sufficient to block microtubule elongation without destroying existing microtubules. The ability of microtubules to persist during the rotation period in the presence of nocodazole suggests that microtubule turnover in this region is relatively slow, although the array microtubules are not sufficiently stable to become acetylated by enzymes present in the egg (Chu and Klymkowsky, 1989). Cortical rotation was not affected by nocodazole applied after the array of aligned microtubules had begun to form, despite the reduced density of the microtubules compared to control eggs. Thus directed polymerisation does not contribute significantly to the cortical rotation, nor does the number of microtubules in the array appear to limit the speed of cortical movement. The observation that the extent of rotation is not exaggerated when D_2O is used to force extra polymerisation during the rotation period (Vincent et al., 1987) supports this conclusion.

Biochemical measurements show a burst of microtubule polymerisation half way through the first cell cycle as the rotation starts (Elinson, 1985). The level of polymerised tubulin in the egg then falls until around 0.8 NT, the time of mitosis. The observed incorporation of rhodamine-tubulin into vegetal array microtubules throughout the period of cortical rotation was thus surprising. It suggests that microtubule polymerisation is not controlled in a uniform manner through different regions of the egg. Microtubule growth continues locally in the vegetal cortical region in spite of depolymerisation elsewhere.

After first mitosis (around 0.8 NT) the situation reverses. The vegetal array thins progressively until first cleavage (Elinson and Rowning, 1988), while the global level of microtubules in the egg rises (Elinson, 1985). Despite the time lag between mitosis and the loss of vegetal microtubules, the breakdown of the array has been shown to depend on biochemical events accompanying mitosis (Schroeder and Gard, 1992). The delay may reflect the progressive activation across the egg of mitotic factors that promote microtubule severing and changes in microtubule dynamics in mitotic cytoplasm (Vale, 1991; Verde et al., 1990; Belmont et al., 1990; Gotoh et al., 1991). Thus, microtubules in the animal hemisphere could be depolymerising and fragmenting at the time of mitosis, while vegetal microtubules continue to behave as in interphase. Mitotic changes in microtubule behaviour originating in the animal half may eventually spread to cause the loss of microtubules and reduction of microtubule motor activity (Allan and Vale, 1990) beneath the vegetal cortex, explaining why cortical rotation and the microtubule array in isolated vegetal halves persist longer than in controls (Vincent et al., 1987; Elinson and Palacek, 1993).

Given that directed microtubule polymerisation appears not to be responsible for moving the cortex, the most likely hypothesis is that a microtubule motor enzyme is involved (see Fig. 1). Since most of the array microtubules are oriented with their plus ends pointing in the direction of cortical rotation (Houliston and Elinson, 1991b) and the cortex moves with respect to these microtubules (this study), a plus end-directed microtubule motor molecule attached to the cortex (Fig. 1 (2)) rather than a minus end motor anchored in the cytoplasm (Fig. 1 (1)) is implicated. It remains possible that relative sliding between microtubules (Fig. 1 (3)) occurs. The occasional 'stationary' microtubule segments seen in the video recordings may have become attached to a cortical structure and be sliding against other microtubules. Alternatively they may simply have become detached from the array, perhaps because force is not generated evenly across the vegetal cortex. To resolve these points, a more sensitive technique, perhaps labeling of microtubules in a restricted area using photoactivatable fluorescent tubulin, would be needed.

One candidate plus end-directed microtubule motor molecule for the cortical rotation is frog egg kinesin, known to be associated with the microtubules of the vegetal array (Houliston and Elinson, 1991b). This kinesin co-distributes with ER, so could transport cortically anchored ER and hence the cortex in the direction of rotation (possibility 2 in Fig. 1). Another candidate is Eg5, a plus end directed motor in the kinesin family, particularly abundant in the *Xenopus* egg (Le Guellec et al., 1991; Sawin et al., 1992a). In addition, there are at least another four proteins in this family in the *Xenopus* egg (Sawin et al., 1992b; Vernos et al., 1993). The involvement of these molecules in the rotation now needs to be tested.

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