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Spatiotemporal pattern of microtubules in parthenogenetically activated *Oryzias latipes* (medaka) eggs

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Abstract The overall pattern of ooplasmic segregation, the movement of the female pronucleus, and the spatiotemporal pattern of microtubules were all very similar in parthenogenetically activated *vs.* fertilized medaka eggs. With respect to microtubules, a monaster formed at the animal pole by $T_n \approx 0.25$ (T_n, normalized time, in which the time between activation and the first cleavage of a fertilized eggs is represented by one unit), persisted until $T_n \approx 0.75$, and began to disintegrate at $T_n \approx 0.8$–0.9, when fertilized eggs entered mitosis. Interpolar ooplasm contained a network of microtubules, most of which showed no apparent preferred orientation. The density of this network decreased at $T_n \approx 0.75$–1.0, especially near the animal pole. At the vegetal pole of the egg, an array of parallel microtubules began to form at $T_n \approx 0.25$. This array was the dominant feature of the vegetal pole region at $T_n \approx 0.45$, but by $T_n \approx 0.75$ it had begun to disintegrate. These results show that the egg itself can assemble and disassemble complex networks of microtubules and organize the movements that constitute ooplasmic segregation in this species.

Introduction

During the first cell cycle of medaka zygotes, networks of microtubules appear in three domains of the egg (Abraham *et al.*, 1995). At the animal pole, a monaster first appears and then disappears, while at the vegetal pole, an array of parallel microtubules appears and then disappears. A network of criss-crossed microtubules, having no apparent preferred orientation, forms in interpolar ooplasm. Treatment of medaka eggs with microtubules poisons, (*e.g.*, demecolcine) blocks the appearance of these microtubules (Webb *et al.*, 1995), and slows the growth of the blastodisc, inhibits the movements of the male and female pronuclei, inhibits the saltatory motion of small ooplasmic inclusions, and inhibits the movement of one specific class of ooplasmic inclusions (oil droplets) toward to vegetal pole of the egg (Abraham *et al.*, 1993). The inhibitory effects of demecolcine can be reversed, even within small regions of the eggs, by UV-irradiation (Webb *et al.*, 1995). These results suggest that the networks of microtubules are required for normal ooplasmic segregation and pronuclear movement in medaka eggs.

In the present study, we compared the microtubule networks in parthenogenetically activated (pricked) eggs (referred to hereafter as activated eggs) *vs.* fertilized eggs in order to assess the possible role(s) of structural components contributed by sperm in the organization of the microtubule networks and in ooplasmic segregation.

A preliminary account of these findings has been published (Webb and Fluck, 1995).

Materials and Methods

The methods of removing gonads from breeding medaka and preparing eggs have been described (Yamamoto, 1967; Abraham *et al.*, 1993). Eggs were divided into two groups and placed in petri dishes containing BSS (111 mM NaCl; 5.36 mM KCl; 1 mM $CaCl_2$; 0.6 mM $MgSO_4$; 5 mM HEPES, pH 7.3; Abraham *et al.*, 1993). Eggs were activated either by fertilizing them or by pricking them once with a glass micropipette in the interpolar region of the egg. Both groups of eggs were incubated at room temperature (19.0–22.7°C) until they were fixed.

Using established procedures, the activated eggs were fixed at regular intervals from $T_n \approx 0.02$ to $T_n \approx 1.0$, dechorionated with watchmaker's forceps, and incubated with a mouse monoclonal antibody against alpha tubulin (DM1A) and a rhodamine-conjugated goat anti-mouse IgG (Gard, 1991; Abraham *et al.*, 1995; Webb *et al.*, 1995).

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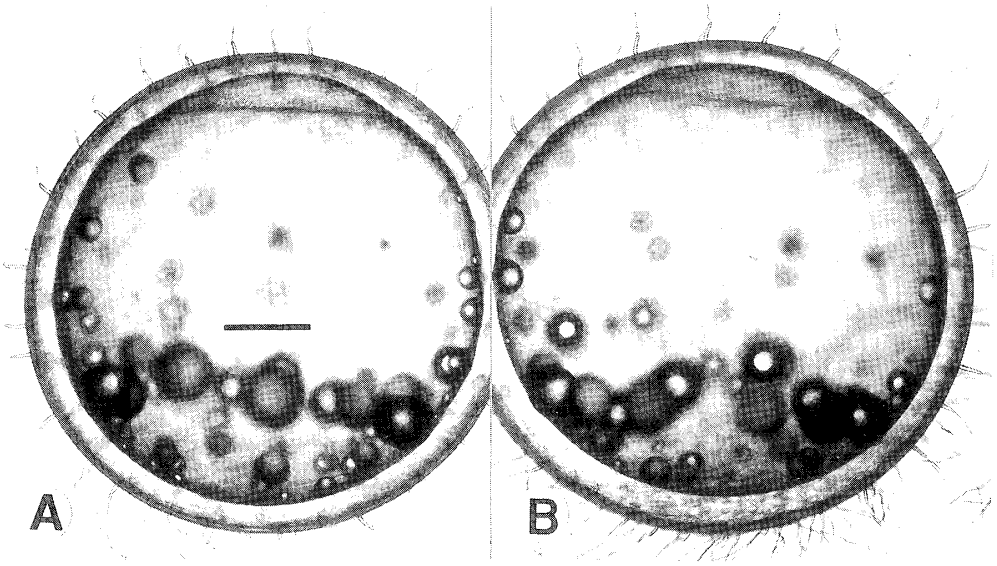

Fig. 1. Ooplasmic segregation in activated (A, $T_n \approx 0.97$) and fertilized (B, $T_n \approx 1.0$) eggs. In both eggs a blastodisc has formed at the animal pole (uppermost in these figures) and oil droplets have segregated toward the vegetal pole. Scale bar, 250 μm.

Just before viewing the eggs, we stained the nuclei with Hoechst 33258 (10 μg ml^{-1}; Abraham *et al.*, 1993). The stained eggs were transferred to a microscope slide on which a cover glass was supported by four pillars of petroleum jelly. The cover glass was pressed gently against the egg to facilitate optical studies and to enable us to roll the egg in order to view specific regions of the egg (Abraham *et al.*, 1993). We examined the eggs with conventional epifluorescence microscopy via a Nikon Optiphot microscope coupled to a Dage-MTI SIT camera, a Dage-MTI DSP-2000 image processor and a video monitor. Photographs were taken from the monitor through Ronchi grating (Rolyn Optics Co., Covina, California; Inoué, 1981).

The results summarized herein represent 13 replicate experiments with fertilized eggs and 15 replicate experiments with parthenogenetically activated eggs. A total of 160 eggs (6 unfertilized eggs, 75 fertilized eggs, and 79 activated eggs) from 24 females were examined.

Chemicals

Formaldehyde and glutaraldehyde were obtained from Electron Microscopy Sciences (Fort Washington, Pennsylvania); anti-alpha-tubulin antibody from ICN (Costa Mesa, California); the rhodamine-conjugated goat anti-mouse IgG from Organon Teknika (Malvern, Pennsylvania); and other chemicals from Sigma (St. Louis, Missouri).

Results

(a) Ooplasmic segregation in activated and fertilized eggs.

The formation of a blastodisc at the animal pole and the movement of oil droplets toward the vegetal pole were very similar in activated and fertilized eggs (Fig. 1).

(b) Microtubules in unfertilized eggs.

The predominant pattern of fluorescence in the ooplasm of unfertilized eggs was punctate (Fig. 2A). However, a low to very low density of microtubules was present as well (Fig. 2B).

(c) Microtubules in activated eggs and fertilized eggs.

The remainder of the results will be presented in terms of the changes that occurred in three regions of the egg: (1) the animal pole region, located within ≈30° (≈300 μm) arc of the animal pole; (2) an interpoler region, located within ≈60° (≈600 μm) arc on both sides of the equator; and (3) the vegetal pole region, located within ≈30° arc (≈300 μm) arc of the vegetal pole (Abraham *et al.*, 1995).

Animal pole region. In unfertilized eggs and in eggs fixed at $T_n \approx 0.02$, the predominant pattern of fluorescence throughout this region in activated eggs was punctate (Fig. 3A) except for microtubules in and near the meiotic apparatus (not shown). Similar results were obtained with fertilized eggs.

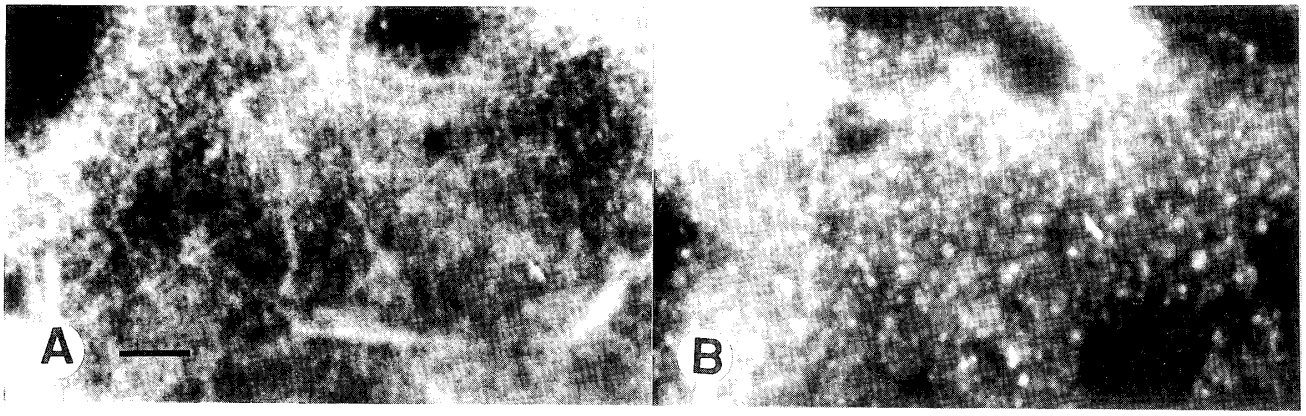

Fig. 2. Microtubules in unfertilized eggs. A) A few microtubules are surrounded by much punctate fluorescence in this interpolar region of the egg. Such microtubules were scattered unevenly throughout all regions of unfertilized eggs. B) This region near the vegetal pole contains only punctate fluorescence, which was the predominant pattern in unfertilized eggs. Scale bar, 5 μm.

At $T_n \approx 0.17$, the second meiotic division was in late anaphase. The predominant pattern of fluorescence in activated eggs was still punctate throughout the region (Figs. 3B and 4A), but a few microtubules were present near the meiotic apparatus in one of four eggs. Such microtubules were present in all four of the fertilized eggs examined.

By $T_n \approx 0.25$ in both activated and fertilized eggs, the second meiotic division had been completed. The second polar body, which could be seen by phase-contrast microscopy at the surface of the egg, was stained with Hoechst 33258 and also reacted with the anti-tubulin antibody and the rhodamine-labeled secondary antibody (not shown). A radial array of microtubules surrounded a central region that contained only punctate fluorescence (Figs. 3C and 4B), suggesting the presence of a microtubule-organizing center (MTOC) near the center of the animal pole region. Both the polar body and the radial array of microtubules could be seen in the same optical section with a 40x objective lens (N.A. $\approx$ 1.3), suggesting that the radial array of microtubules is near the surface of the egg. The diameters of the punctate region (about 50 μm) and the radial array (about 275 μm) were similar in activated eggs and fertilized eggs.

Eggs (both activated and fertilized) fixed at $T_n \approx 0.30$, 0.45, 0.49, and 0.75 were similar to eggs fixed at $T_n \approx 0.25$ (Figs. 3D, 3E, 4C, and 4D). At these later stages, however, we could clearly see that the female pronucleus was closer to the MTOC than the second polar body was. The female pronucleus was typically in the region that contained punctate sources of fluorescence, while the polar body was over the region that contained radially oriented microtubules (Figs. 3D and 4C). By $T_n \approx 0.75$, the diameter of the central region of punctate fluorescence and the diameter of the radial array of microtubules had increased to 178 $\pm$ 84 μm (n = 2) and 952 $\pm$ 126 μm (n = 2). The diameter of the radial array at $T_n \approx 0.75$ was larger in fertilized eggs (1303 $\pm$ 125 μm, n = 4) than in activated eggs, but the difference was not statistically significant.

By $T_n \approx 0.8$ and 0.9, the nucleus of the zyogote was in metaphase and anaphase, respectively, and the mitotic spindle was clearly visible (not shown). The cytoplasm immediately adjacent to the spindle contained only punctate fluorescence, but the periphery of the animal pole region contained either radially oriented microtubules or microtubules having no apparent preferred orientation. Activated eggs were similar, except that there was no mitotic apparatus in the center of the region.

By $T_n \approx 1.0$, two well defined MTOCs were present in zygotes, which had begun to undergo cytokinesis. Other microtubules were oriented perpendicular to the cleavage furrow (not shown). Throughout the remainder of the animal pole region of fertilized eggs and throughout the entire animal pole region of activated eggs we saw only punctate fluorescence (Fig. 3F).

Interpolar region. Only punctate fluorescence was present in this region until $T_n \approx 0.17$, when a network of microtubules having no apparent preferred orientation was inter-mixed with punctate fluorescence throughout this region (Figs. 3B and 5A). The density of microtubules appeared to be slightly higher in the vegetal hemisphere than the animal hemisphere.

At $T_n \approx 0.25$, 0.30, and 0.45, the density of microtubules had increased and the amount of punctate fluorescence had decreased in both

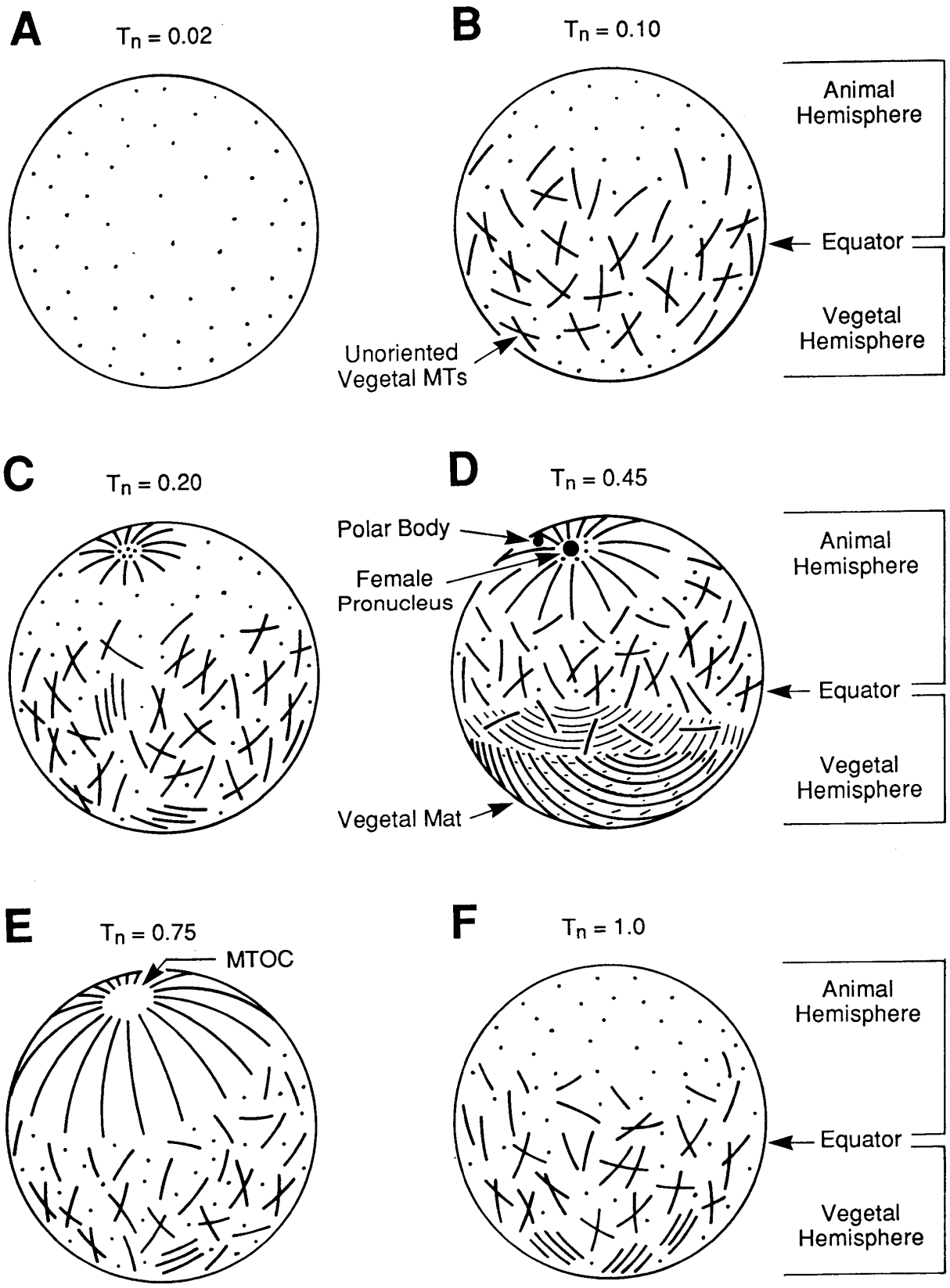

Fig. 3. Diagrammatic representation of microtubules in activated eggs. To simplify the drawings, oil droplets are not shown in the diagrams. The animal pole is uppermost in all the diagrams. The diameter of the egg is approximately 1 mm. A) $T_n \approx 0.02$. Punctate fluorescence is present throughout the ooplasm. B) $T_n \approx 0.17$. At the animal pole, the fluorescence is punctate. Interpolar ooplasm contains a mixture of punctate fluorescence and microtubules; the density of microtubules is slightly higher near the vegetal pole. A few microtubules are oriented along the animal-vegetal axis of the egg. At the vegetal pole, a central area containing punctate fluorescence is surrounded by a region containing criss-crossed microtubules. C) $T_n \approx 0.25$. At the animal pole, microtubules radiate from a central region that contains punctate fluorescence. Interpolar ooplasm contains punctate fluorescence and a network of microtubules, most of which have no apparent preferred orientation. Near the vegetal pole, punctate fluorescence is mixed with a dense network of microtubules, most of which have no apparent preferred orientation. D) $T_n \approx 0.45$. The animal pole region is similar to that at $T_n \approx 0.25$, except that in this diagram we have shown the approximate positions of the second polar body and the female pronucleus. In interpolar ooplasm, the amount of punctate fluorescence has decreased, and the density of microtubules has increased. The vegetal pole region contains an array of parallel microtubules. E) $T_n \approx 0.75$. Interpolar ooplasm contains a gradient of microtubule density, increasing toward the vegetal pole. Near the vegetal pole, a central region containing parallel microtubules is surrounded by a region containing a mixture of punctate fluorescence and criss-crossed microtubules. F) $T_n \approx 1.0$. Only punctate fluorescence is present near the animal pole. At the vegetal pole, small patches of parallel microtubules are mixed with punctate fluorescence and criss-crossed microtubules.

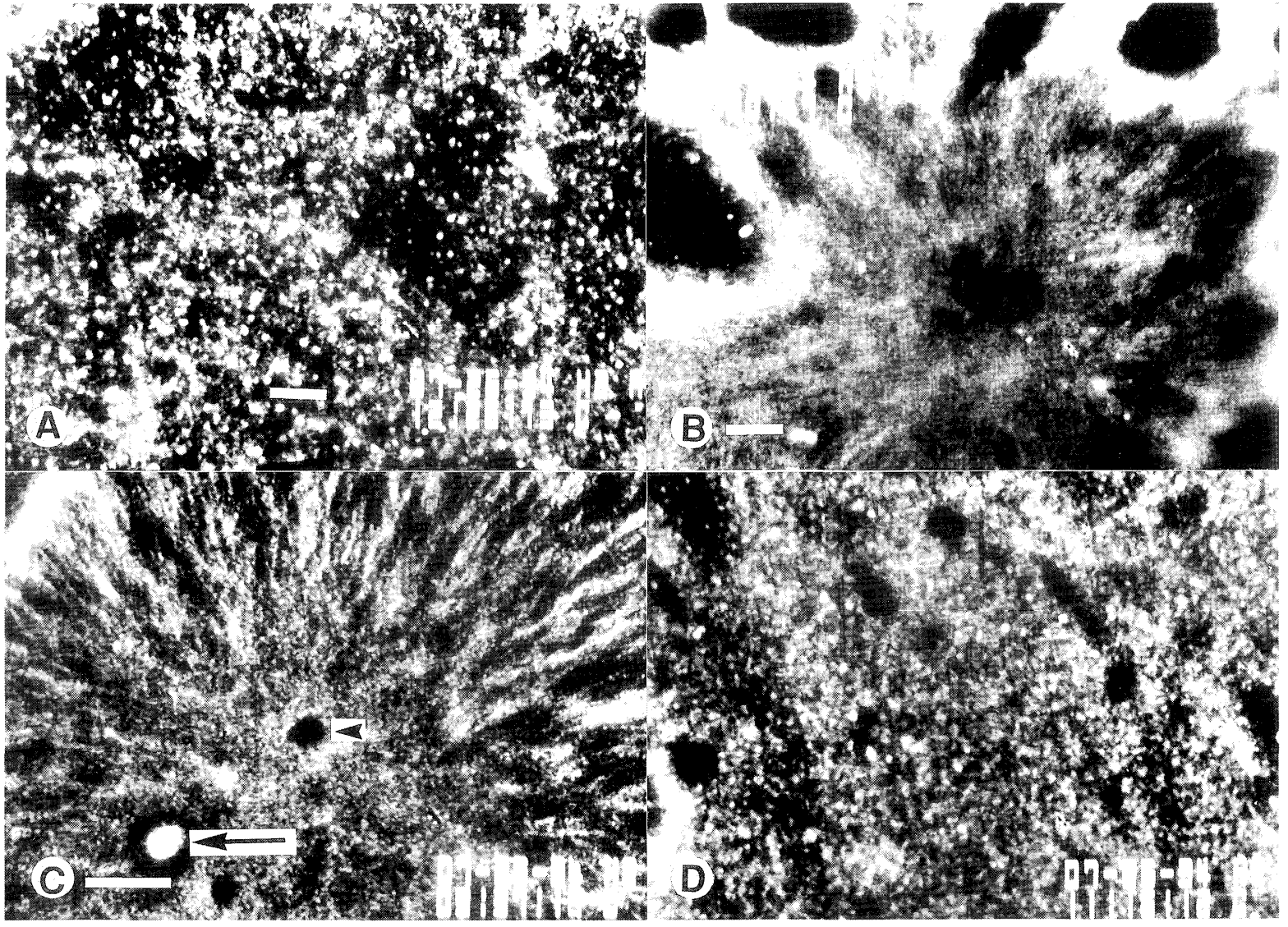

Fig. 4. Microtubules in the animal pole region of activated eggs. Scale bars: A, 5 μm, B, 25 μm; C, 20 μm. A and D were printed at the same magnification. A) $T_n \approx 0.17$. Punctate fluorescence is present throughout the region. B) $T_n \approx 0.25$. A central region containing punctate fluorescence is surrounded by an array of radially oriented microtubules. C) $T_n \approx 0.45$. The female pronucleus (arrowhead) is near the central region that contains punctate fluorescence, and the second polar body (arrow) is over the radially oriented microtubules. D) $T_n \approx 1.0$. Punctate fluorescence is present throughout the region.

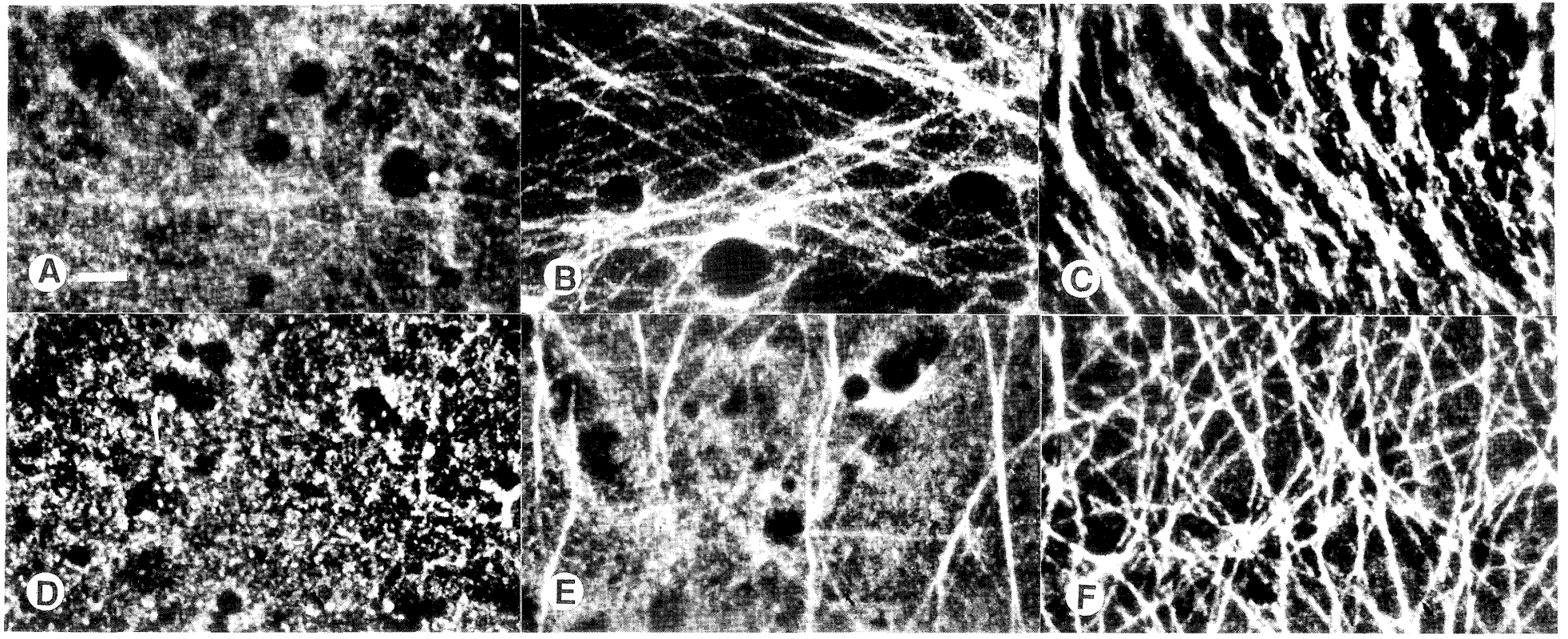

Fig. 5. Microtubules in interpolar ooplasm of activated and fertilized eggs. Scale bar, 5 μm. A) $T_n \approx 0.17$. A sparse network of microtubules is mixed with punctate fluorescence. The animal pole is off the right edge of the photograph. B) $T_n \approx 0.45$. The density of microtubules is higher, and there is little punctate fluorescence. The animal pole is off the right edge of the photograph. C) $T_n \approx 0.25$. Small regions of interpolar ooplasm contain microtubules oriented along the animal-vegetal axis (which runs from upper left to the lower right of this figure) of this fertilized egg. D–F) $T_n \approx 1.0$. The density of microtubules is low near the animal pole (D), higher near the equator (E), and even higher near the vegetal pole (F). The animal pole is off the left edge of these photographs.

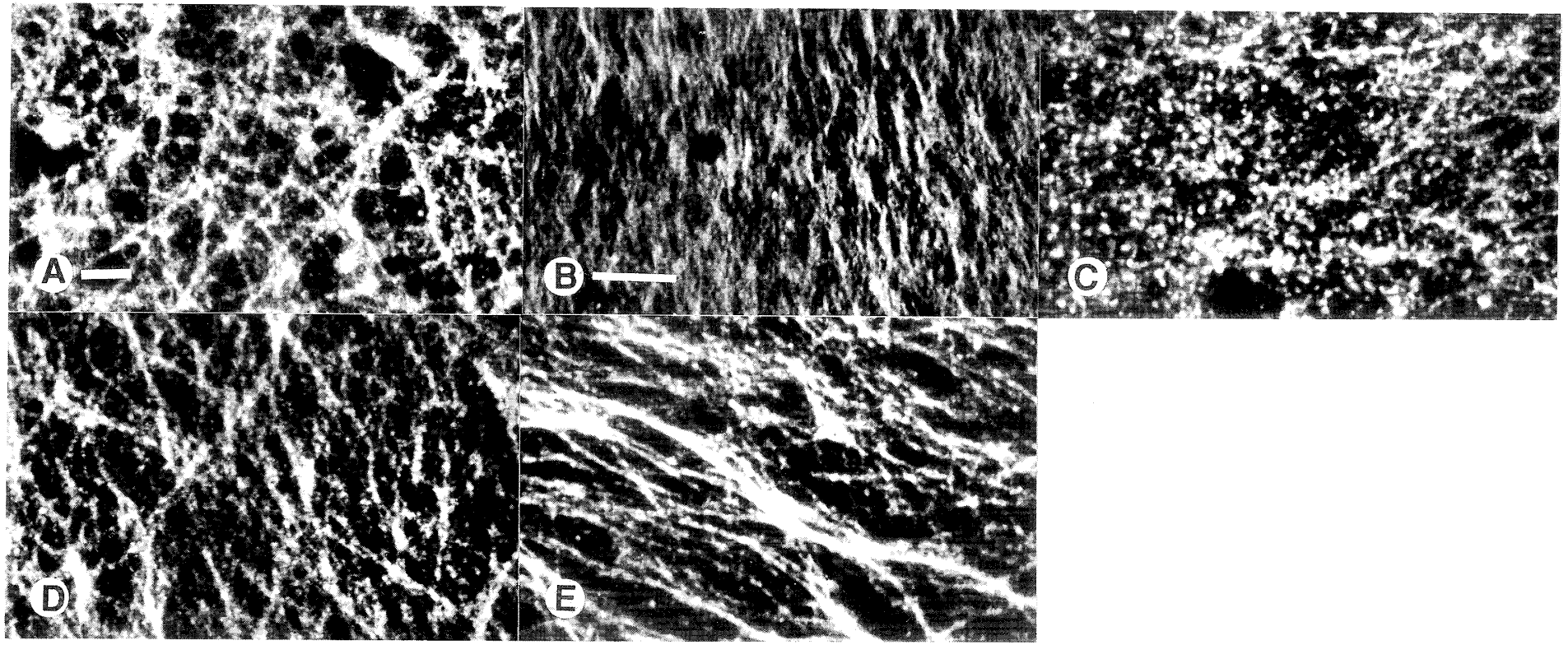

Fig. 6. Microtubules in the vegetal pole region of activated eggs. Scale bars: A, 5 μm; B, 20 μm. A and C-E were printed at the same magnification. A) $T_n \approx 0.17$. Punctate fluorescence is mixed with a criss-crossed network of microtubules. B) $T_n \approx 0.45$. An array of parallel microtubules is present throughout the region. C–E) $T_n \approx 1.0$. Some areas contain punctate fluorescence (C), others a network of criss-crossed microtubules (D), and others arrays of parallel microtubules (E).

activated and fertilized eggs (Figs. 3C, 3D, and 5B). Most of the microtubules had no apparent preferred orientation, but in a small proportion of eggs fixed at $T_n \approx 0.25$ (one of six activated eggs and two of six fertilized eggs) we found areas in which the microtubules were aligned approximately along the animal-vegetal axis of the egg (Figs. 3C and 5C). Similarly aligned microtubules were also seen in activated (but not fertilized eggs) fixed at $T_n \approx 0.30$ (one of five eggs) and $T_n \approx 0.45$ (four of six eggs).

At $T_n \approx 0.75$–1.0, the density of microtubules in this region decreased, especially near the animal pole, and a pronounced gradient of microtubule density was seen along the animal-vegetal axis of both activated (Figs. 3E, 3F, and 5D-F) and fertilized eggs.

Vegetal pole region. In all nine activated eggs and 11 of 13 fertilized eggs, we saw only punctate fluorescence in eggs fixed at $T_n \approx 0.02$, but in two fertilized eggs, we saw a few microtubules in this region of the egg. By $T_n \approx 0.17$, microtubules were present in both activated (Figs. 3B and 6A) and fertilized eggs. In most eggs, microtubules were present near the periphery of the vegetal pole region, while only punctate fluorescence was present near the center of the region. At $T_n \approx 0.25$ and 0.30, punctate fluorescence was inter-mixed with a dense network of microtubules throughout this region (Fig. 3C). Although this network had no apparent preferred orientation in most eggs, small patches of parallel arrays of microtubules were present in some activated eggs (3 of 17) and fertilized eggs (4 of 15). These organized microtubules were more prevalent near the center of the region.

In both activated and fertilized eggs at $T_n \approx 0.45$, this region was dominated by a large array of parallel microtubules that extended ≈ 250 μm in all directions from the center of the region (Figs. 3D and 6B). At $T_n \approx 0.75$–0.9, this array was still present near the center of the region, but punctate fluorescence and a disorganized network of microtubules had appeared near the periphery of the region (Fig. 3E). At $T_n \approx 1.0$, the number of disorganized microtubules and the amount of punctate fluorescence had increased, but small patches of parallel microtubules could be found throughout the region (Figs. 3F and 6C-E).

(d) Movement of the female pronucleus in activated and fertilized eggs.

By $T_n \approx 0.45$, the lateral distance between the female pronucleus and the polar body was 65.6 ± 19.3 μm ($\bar{X} \pm$ SD, n = 11) and 78.9 ± 16.4 μm (n = 3) in activated and fertilized eggs, respectively (Fig. 7); the difference was not statistically significant. By $T_n \approx 0.75$, the depth of the female pronucleus below the polar body was 33.3 ± 11.5 μm (n = 3) and 38.4 ± 6.5 μm (n = 5) in activated and fertilized eggs (Fig. 7), respectively; the difference was not statistically significant.

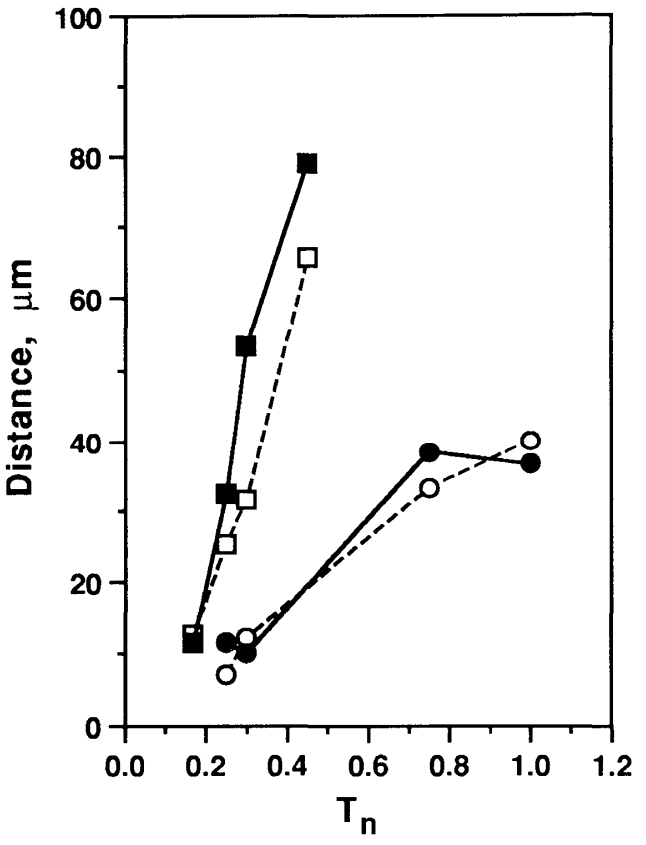

Fig. 7. Distance between the second polar body and the female pronucleus in activated and fertilized eggs. Lateral distance between the second polar body and the female pronucleus in activated (□---□) and fertilized (■—■) eggs. Vertical distance between the second polar body and the female pronucleus in activated (○---○) and fertilized (●—●) eggs.

Discussion

Two steps are required to fully activate an egg: A treatment that prompts the egg to do almost everything that it would do if it were fertilized and another that enables it to bipolarize (Mazia, 1978). Prick-activated medaka eggs clearly take the first step. Their ooplasm segregates normally, they assemble and disassemble a complex network of microtubules, and the female pronucleus moves away from the second polar body. It is also clear that they do not take the second step. The results of the present study thus enable us to assess the contributions of sperm to these two steps in the activation of medaka eggs.

The spatial pattern of ooplasmic segregation occurred normally in activated medaka eggs, with a blastodisc forming at the animal pole and oil droplets segregating toward the vegetal pole, even though these eggs were activated at an interpolar site and not at the animal pole. This result is consistent with others that have described the strong inherent polarity of teleost eggs (Huver, 1964; reviewed in Hart and Fluck, 1996). However, our crude measures of ooplasmic segregation—the bulk flow of ooplasm and the movement of one class of ooplasmic inclusions (oil droplets)—could have overlooked subtler movements of specific components of the ooplasm such as those reported in *Xenopus laevis* in which the presence of a sperm and an associated microtubule array is essential for normal cytoplasmic segregation (Ubbels *et al.*, 1983). The temporal pattern of segregation was also normal in activated medaka eggs, suggesting that the egg itself has the information to insure not only the proper assembly of the molecular machinery that mediates ooplasmic segregation but also the timing of this assembly.

The spatiotemporal pattern of microtubules in activated eggs was essentially identical to that in fertilized eggs, suggesting that the egg also contains information to ensure the proper assembly and disassembly of microtubules. We saw no evidence that microtubules formed earlier (Elinson, 1983; Elinson, 1985; Ubbels and Vermeulen, 1986; Houliston and Elinson, 1991) or persisted longer (Elinson and Rowning, 1988) in fertilized *vs.* activated medaka eggs as they do in *X. laevis*. This maternal capability to organize microtubules was especially remarkable in the animal pole region of the medaka egg, where a microtubule-organizing center (MTOC) is present near the two pronuclei in fertilized eggs (Abraham *et al.*, 1995). In the present study, we found that an MTOC was also present in activated eggs, apparently centered on the female pronucleus. This suggests that the female pronucleus itself or a closely associated structure can organize the assembly of microtubules. An association between the female pronucleus and microtubule-organizing activity in parthenogenetically activated eggs has been previously reported in *Lytechinus pictus* (Mar, 1980), and *X. laevis* (Houliston and Elinson, 1991). Whether the activity in activated medaka eggs is specifically associated with a maternal centrosome (Ubbels *et al.*, 1983; Schroeder and Gard, 1992; Sluder *et al.*, 1993) is an interesting question for the future.

The movements of oil droplets in medaka eggs (Abraham *et al.*, 1993; Catalone and Fluck, 1994; Webb *et al.*, 1995) and the female pronucleus in a number of species (Zalokar, 1974; Hiramoto *et al.*, 1984; Hamaguchi and Hiramoto, 1986; Sawada and Schattern, 1989; Abraham *et al.*, 1993) likely involve microtubules. Thus, because the movements of both oil droplets and the female pronucleus were normal in activated medaka eggs, one would predict that such eggs would have microtubule arrays essentially like those in fertilized eggs. The results of the present study are consistent with this prediction. Movement of the female pronucleus has been reported to occur in parthenogenetically activated eggs of other species (*X. laevis*, Manes and Barbieri, 1977; *L. pictus*, Mar, 1980; *Rana nigromaculata*, Sambuichi, 1981), but in general it moves less than it does in fertilized eggs. In contrast, its movements appeared to be normal in activated medaka eggs.

The similarity in the spatiotemporal pattern of microtubules outside the animal pole region of activated eggs and fertilized eggs is consistent with the suggestion that the microtubule arrays in the vegetal pole and interpolar regions of medaka eggs are independent of the sperm aster (Webb *et al.*, 1995) as they are in *X. laevis* (Houliston and Elinson, 1991; Elinson and Palaček, 1993). The polymerization of microtubules outside the animal pole region of activated eggs (and fertilized eggs as well) could be spontaneous (Karsenti *et al.*, 1984). Alternatively, it could be organized by a centrosome with a shape much different from the one that organizes the sperm aster (Mazia, 1984; McNiven and Porter, 1988; Tucker *et al.*, 1995) or multiple centrosomes. If either of the latter alternatives is correct, one would predict that centrosomal antigens, in particular gamma-tubulin, would be present throughout the interpolar and vegetal pole regions of the medaka egg (Palacios *et al.*, 1993; Stearns *et al.*, 1991; Gard, 1994)

The only developmental stage at which we saw a clear difference in the spatiotermporal pattern of microtubules in activated *vs.* fertilized eggs was at $T_n \approx 1.0$, when fertilized eggs were undergoing mitosis and cytokinesis. In fertilized eggs, we saw microtubules not only in the mitotic apparatus but also near the cleavage furrow, whereas the animal pole region of activated eggs had only punctate fluorescence. These results suggest that prick-activated medaka eggs cannot take the second step required to fully activate, that is to bipolarize (Mazia, 1978; Mazia, 1987). This limitation is

likely due to the absence of a functional centrosome (Iwamatsu and Ohta, 1974; Heidemann and Kirschner, 1975; Maller *et al.*, 1976; Mitchison and Kirschner, 1981). Perhaps in medaka, as in starfish (Sluder *et al.*, 1993), a maternal centrosome can organize microtubules in activated eggs but then loses its reproductive capacity during the first cell cycle. Such a cell would be unable to bipolarize.

The two predominant patterns of fluorescence in medaka eggs that had been processed for the immunocytochemical localization of alpha-tubulin —punctate and linear (microtubules)—appear to represent two interconvertible forms of tubulin. When the density of microtubules was low—for example, throughout eggs at $T_n \approx 0.02$, at the vegetal pole of eggs at $T_n \approx 1.0$, and in the animal pole region of activated eggs at $T_n \approx 1.0$—the amount of punctate fluorescence was high; and when the density of microtubules was high, for example, throughout eggs at $T_n \approx 0.45$, the amount of punctate fluorescence was low. Moreover, because the egg, whether activated or fertilized, can control the timing of the interconversion of the two forms, it may have a cytoplasmic clock like the one in *X. laevis* eggs (Kirschner *et al.*, 1985). Whether the mechanism(s) that controls the polymerization and depolymerization of tubulin involves calcium ion (Fluck *et al.*, 1994) and microtubule-severing proteins (Vale, 1991) and whether this mechanism operates as a wave (Harris, *et al.*, 1980) are questions for future study.

Acknowledgments

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