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Indicators of the dorsoventral axis in medaka (*Oryzias latipes*) zygotes

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Abstract Using time-lapse video microscopy and immunofluorescence, we found that the vector of saltatory motion of subcellular parcels in the vegetal pole region of medaka zygotes is parallel to (1) microtubules in this region of the zygote and (2) the future dorsoventral axis of the embryo.

Introduction

A transient array of parallel microtubules at the vegetal pole of amphibian zygotes plays in important role in establishing the dorsoventral axis (Gerhart *et al.*, 1989; Houliston and Elinson, 1992). These microtubules apparently serve as tracks with which kinesin (or kinesin-like) motors interact to cause the cortex to rotate relative to the inner mass of the zygote (Vincent *et al.*, 1987; Elinson and Rowning, 1988; Houliston and Elinson, 1991a; Houliston and Elinson, 1992; Houliston, 1994). The direction of this "cortical rotation" is normally determined by the sperm entry site (Manes and Barbieri, 1977), which has been proposed to bias the rotation initially (Elinson and Rowning, 1988; Gerhart *et al.*, 1989; Houliston and Elinson, 1991b). The rotation forms a gray crescent at a site in the animal hemisphere and 180° longitude from the sperm entry site; the gray crescent marks the dorsal surface of the embryo.

A cortical rotation has not been observed in teleosts, but a transient array of parallel microtubules does form at the vegetal pole of medaka zygotes (Abraham *et al.*, 1995). In order to determine whether the orientation of these microtubules corresponds to the dorsoventral axis in medaka embryos, we monitored the saltatory motion of subcellular parcels in the thin, peripheral layer of ooplasm in the vegetal pole region of medaka zygotes. The motion of such parcels is absent from medaka zygotes treated with microtubule poisons such as demecolcine (Abraham *et al.*, 1993; Webb *et al.*, 1995), and the motion can be regenerated in demecolcine-treated zygotes by irradiating them with UV light (360 nm), which photolyzes demecolcine and thus enables tubulin to polymerize into microtubules (Aronson and Inoué, 1970; Webb *et al.*, 1995). These results suggest that the movement of the parcels is mediated by microtubules. In the present study, we found that (1) the vector of saltatory motion of subcellular parcels at the vegetal pole of medaka zygotes is parallel to the axis of the microtubules in the vegetal array and (2) the vector of saltatory motion along microtubules at the vegetal pole points directly from the future ventral surface to the future dorsal surface of the embryo.

Materials and Methods

Methods for dissecting gonads from breeding medaka, preparing gametes, and fertilizing eggs *in vitro* have been described previously (Abraham *et al.*, 1993). Gonads, gametes, and zygotes were prepared in a balanced saline solution (BSS: 111 mM NaCl; 5.37 mM KCl; 1.0 mM $CaCl_2$; 0.6 mM $MgSO_4$; 5 mM HEPES, pH 7.3). For microscopic observation, the zygotes were transferred to a microscope slide on which a cover glass was supported by four pillars of petroleum jelly (Abraham *et al.*, 1993). The zygotes were oriented with their vegetal pole uppermost, placed on the stage of a phase-contrast microscope, and illuminated with light (filtered through KG5 and Wratten 58 filters) from a quartz-halogen lamp. The images were recorded via a Newvicon video camera and a time-lapse video cassette recorder. The saltatory motion of subcellular parcels in the thin, peripheral layer of ooplasm was analyzed during playback of the tape at a time-frame speed-up of 72-fold (Abraham *et al.*, 1993). In order to determine whether the vector of saltatory motion correlated with the orientation of microtubules at the vegetal pole, we recorded saltatory motion in zygotes until $T_n = 0.50$ (T_n, normalized time, in which the

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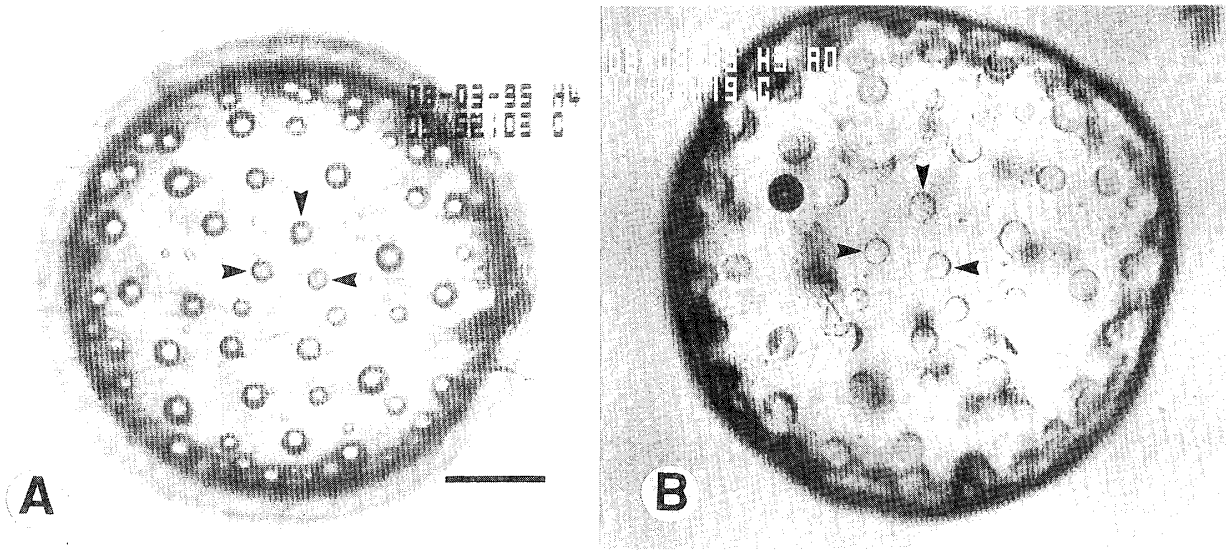

Fig. 1. Cytoplasmic oil droplets near the vegetal pole of living and fixed zygotes. The pattern of cytoplasmic oil droplets in an image made just before fixing the zygote (A) was compared with that of a fixed zygote (B) in order to orient the zygote on the stage of the microscope before viewing the microtubules in the vegetal array. For example, the same three oil droplets in the two images of a zygote (zygote #2 in Fig. 3) are marked by arrowheads. Scale bar, 250 μm.

elapsed time between fertilization and the beginning of cytokinesis is 1.0 unit) and then fixed the zygotes and prepared them for immunolocalization of alpha-tubulin (Gard, 1991; Abraham *et al.*, 1995). Just before fixing each zygote, we made a video print of its vegetal pole region, which contains numerous oil droplets. This image subsequently enabled us to orient the fixed zygote on the stage of the microscope just before viewing microtubules (Fig. 1A, B). Photographs and video prints of at least six microscopic fields of microtubules were made, and the orientation of microtubules in each was measured (Fig. 2).

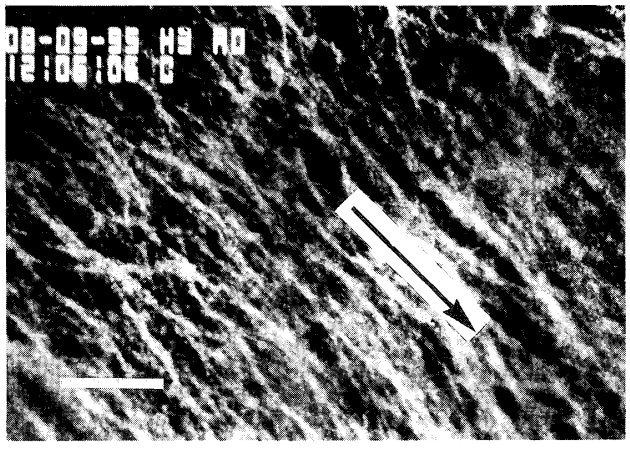

Fig. 2. Vector of saltatory motion and the parallel array of microtubules. The vector (arrow) of saltatory motion has been superimposed upon an image of the parallel microtubules in the vegetal array of a zygote (zygote #2 in Fig. 3). Scale bar, 10 μm.

In order to determine whether the vector of saltatory motion correlated with the dorsoventral axis of the embryo, we recorded saltatory motion until T_n = 10, then replaced the BSS on the microscope slide with 3% Instant Ocean (Aquarium Systems, Mentor, Ohio), and grew the embryos overnight at 20°. The next day, we observed the embryos with a stereomicroscope and marked the dorsal midline (the midline of the embryonic shield).

We recorded saltatory motion at the vegetal pole of 30 zygotes, compared the vector of saltatory motion with the orientation of parallel microtubules in the vegetal pole region of 15 zygotes, and compared the vector of saltatory motion with the position of the dorsal midline in eight zygotes.

Results

The saltatory motion of parcels at the vegetal pole of zygotes had no apparent preferred orientation until T_n = 0.32 ± 0.04 ($\bar{X}$ ± S.D., N = 21 zygotes), when the "tracks" of the parcels became parallel to each other. We found a close agreement between the axis of this motion and the orientation of microtubules in the vegetal pole region (Fig. 2 and 3), with an average difference of only 15.4° between the two ($z = -5.2$, $p < 0.000001$). If there were no relationship between the two parameters, the error would be randomly (uniformly) distributed between 0° and 90° with a mean of 45 degrees.

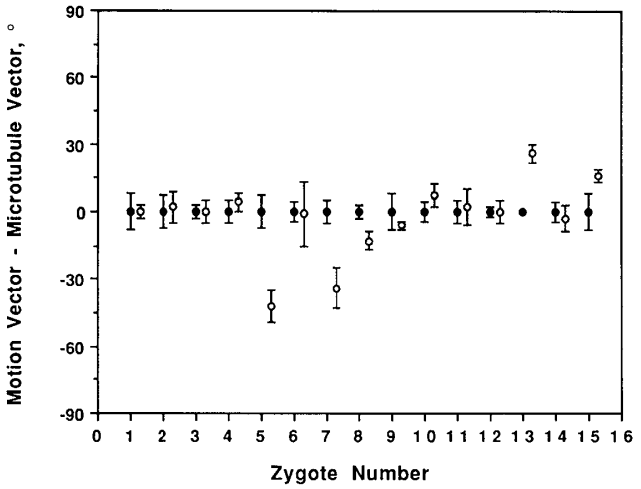

Fig. 3. Coincidence of the vector of saltatory motion and the orientation of parallel microtubules in the vegetal array. The vector of saltatory motion in each zygote (●) was normalized to 0°, and the difference (○) between this vector and the orientation of microtubules in the vegetal array was plotted. Note the generally small differences between the two angles and, also, the generally small error bars, which show one standard deviation on each side of the mean. The small error bars reflect the observations that the parcels moved parallel to each other and that the microtubules were oriented parallel to each other.

The vector of saltatory motion also pointed to the dorsoventral axis of the embryo (Fig. 4 and 5). This result is highly significant ($p = 0.00000518$) against the null hypothesis that the errors were randomly distributed between −180° and +180°.

Discussion

Several lines of evidence suggest that the saltatory motion we observed in the present study involves microtubules. First, the motion is absent from medaka zygotes treated with microtubule poisons such as demecolcine (Abraham *et al.*, 1993; Webb *et al.*, 1995). Second, the motion can be regenerated in demecolcine-treated zygotes by irradiating them with UV light (360 nm), which photolyzes demecolcine and thus enables tubulin to polymerize into microtubules (Aronson and Inoué, 1970; Webb *et al.*, 1995). Third, we have shown in the present study that the axis of saltatory motion in the vegetal pole region of the zygote coincides with the axis of the parallel microtubules there and that the motion of the parcels in this region becomes parallel at about the same time ($T_n = 0.32 \pm 0.04$) that the parallel array of microtubules forms ($T_n \approx 0.25$– 0.30, Abraham *et al.*, 1995).

We also found in the present study that both the orientation of microtubules at the vegetal pole of the zygote and the axis of saltatory motion correlated with the dorsoventral axis of the embryo. This correlation suggests that the mechanism that establishes the dorsoventral axis in teleosts may be similar to the one in amphibians. Although efforts to describe a cortical rotation in teleosts like the one in amphibians have been unsuccessful (Ho, 1992), primitive fish do form a crescent at the margin on one side of the animal region, giving the zygote a bilaterally symmetrical structure (Clavert, 1962; Ginsburg and Dettlaff, 1991; Bolker, 1993). However, even if teleosts do not undergo a cortical rotation, developmentally sig-

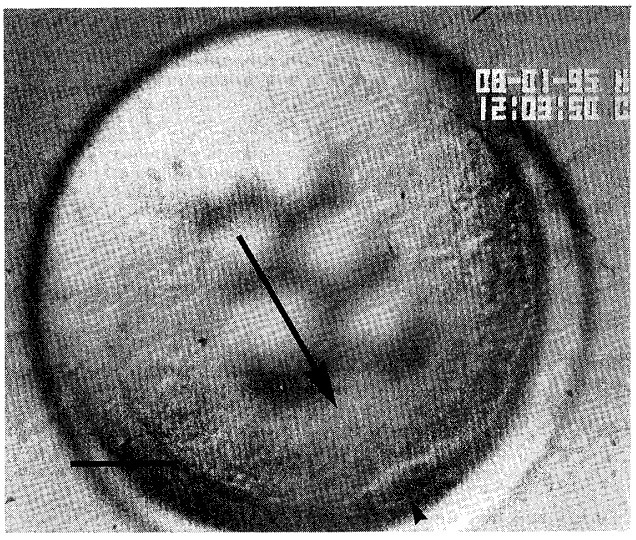

Fig. 4. Vector of saltatory motion points to the dorsal midline. The vector (arrow) of saltatory motion in the zygote (zygote #3 in Fig. 5) has been superimposed upon an image of the embryo after the embryonic shield had formed. The dorsal midline is marked by an arrowhead. Scale bar, 250 μm.

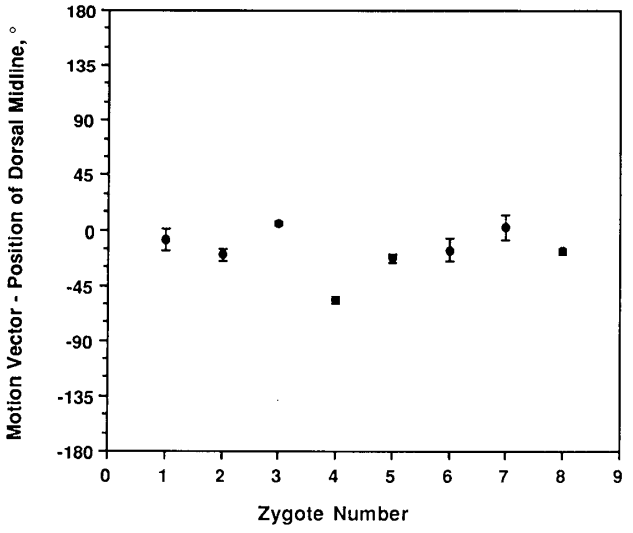

Fig. 5. Coincidence of the vector of saltatory motion and the dorsal midline. The difference between the vector of saltatory motion and the angular position of the dorsal midline for each of eight zygotes is shown. Note the small error bars for the vector of saltatory motion, which represent one standard deviation on each side of the mean, and the generally small differences between the two vectors.

nificant information could move along the parallel microtubules at the vegetal pole, which are also present at the vegetal pole of zebrafish zygotes (Strähle and Jesuthasan, 1993). Whether the parcels we observed in the present study contain such information (Ainger *et al.*, 1993) and whether molecular distinctions can be made between the future dorsal *vs.* the future ventral surface of the embryo remain to be determined (Elinson *et al.*, 1993; Wilhelm and Vale, 1993; Johnston, 1995).

The relationship between the orientation of the array of parallel microtubules and the dorsoventral axis may only be correlational and not cause-and-effect, but it should be possible to distinguish between these two alternatives experimentally. Possible approaches include selectively disrupting the vegetal array of microtubules with short-wave (254 nm) UV irradiation (Scharf and Gerhart, 1983) or using the Colcemid-UV method selectively to regenerate microtubules at the animal pole of the medaka zygote while preventing their regeneration at the vegetal pole (Aronson and Inoué, 1970; Webb *et al.*, 1995).

The orientation of the parallel microtubules at the vegetal pole in amphibian zygotes is normally determined by the sperm entry point (Gerhart *et al.*, 1989; Elinson and Rowning, 1988). However, in teleosts, sperm enters through a micropyle at the animal pole, and thus the sperm entry point would not seem to be a factor in determining the orientation of the array.

In the present study, we determined the orientation of the microtubules only after fixing the zygotes, but it should be possible to monitor these structures in living zygotes after microinjecting rhodamine-labeled tubulin into them (Houliston, 1994). Although the presence of cytoplasmic oil droplets in medaka zygotes made it easy to compare the orientation of saltatory motion in living eggs with the orientation of microtubules in fixed eggs, it should be possible to mark the zygotes of other species (Elinson and Rowning, 1988), for example the zebrafish, that lack such endogenous markers.

Acknowledgments

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Note added in proof — Using confocal microscopy, Rowning *et al.* [(1995) *Mol. Biol. Cell, Suppl.*, **6**: 345a) have monitored the movement of DiOC6(3)-labeled organelles near the vegetal pole of *X. laevis* zygotes and found that they move toward the future dorsal side of the zygote.

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