

Cellular asymmetry in *Chlamydomonas reinhardtii*

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Summary

Although largely bilaterally symmetric, the two sides of the unicellular alga *Chlamydomonas reinhardtii* can be distinguished by the location of the single eyespot. When viewed from the anterior end, the eyespot is always closer to one flagellum than the other, and located at an angle of approximately 45° clockwise of the flagellar plane. This location correlates with the position of one of four acetylated microtubule bundles connected to the flagellar apparatus. Each basal body is attached to two of these microtubule rootlets. The rootlet that positions the eyespot is always attached to the same basal body, which is the daughter of the parental/daughter basal body pair. At mitosis, the replicated basal body pairs segregate in a precise orientation that maintains the asymmetry of the cell and results in mitotic poles that have an invariant handedness. The fusion of gametic cells during mating is also

asymmetric. As a result of asymmetric, but different, locations of the plus and minus mating structures, mating preferentially results in quadriflagellate dikaryons with parallel flagellar pairs and both eyespots on the same side of the cell. This asymmetric fusion, as well as all the other asymmetries described, may be necessary for the proper phototactic behavior of these cells. The invariant handedness of the spindle pole, eyespot position, and mating structure position appears to be based on the inherent asymmetry of the basal body pair, providing an example of how an intracellular pattern can be determined and maintained.

Key words: *Chlamydomonas*, asymmetry, basal bodies, acetylated tubulin, eyespot, *uni* mutants.

Introduction

The number and position of organelles within a cell of *Chlamydomonas* is highly stereotyped. Most of the organelles are symmetrically positioned around the long axis of the cell (Fig. 1). The most striking exception to this rotational symmetry is the position of the eyespot, a red-pigmented structure that acts as an antenna for the photoreceptor in the plasma membrane above it (Foster and Smyth, 1980). Each cell normally contains a single eyespot, which is always located at the periphery of the cell, in a location closer to one flagellum than the other. The asymmetric position of the eyespot can be used to differentiate the cell into two halves. By convention, the half of the cell containing the eyespot is denoted *cis*, and the other half *trans* (Huang *et al.* 1982). An imaginary *cis/trans* plane that passes between the two basal bodies can be used to differentiate these two halves. To aid in description, we have added the terms *syn* and *anti* to describe the two halves of the cell differentiated by a second imaginary plane in which the two flagella beat. This is commonly referred to as the flagellar plane. The locations of these two planes of reference are shown diagrammatically in Fig. 2.

Using the eyespot as a marker, it has been shown that the flagellar apparatus has a physiological *cis/trans* asymmetry. Kamiya and Witman (1984) have shown that reactivated *cis* and *trans* axonemes have different and opposite responses to calcium concentration, and Huang *et al.* (1982) have described a number of mutations (the *uni* mutants) whose defect appears to be at the level of the basal body and whose distinguishing feature is that they fail to assemble the *cis* flagellum, but remain able to assemble the *trans* flagellum. Clearly the *cis* and *trans* basal bodies and axonemes are not equivalent.

The basal bodies of *Chlamydomonas* replicate and segregate in a manner similar to that of mammalian centrioles. During mitotic prophase in *Chlamydomonas* the two probasal bodies extend to full length and are segregated in a semiconservative manner, so that one parental and one daughter basal body go to each mitotic pole (Gaffel, 1988). The parental/daughter difference in the two basal bodies is postulated to be the source of the physiological difference between the two basal bodies and the basis of the *Uni*⁻ phenotype (Huang *et al.* 1982). *Chlamydomonas* is not unique in having two different flagella, and in fact no example of truly identical flagella on a biflagellate algal cell has yet been reported. In several

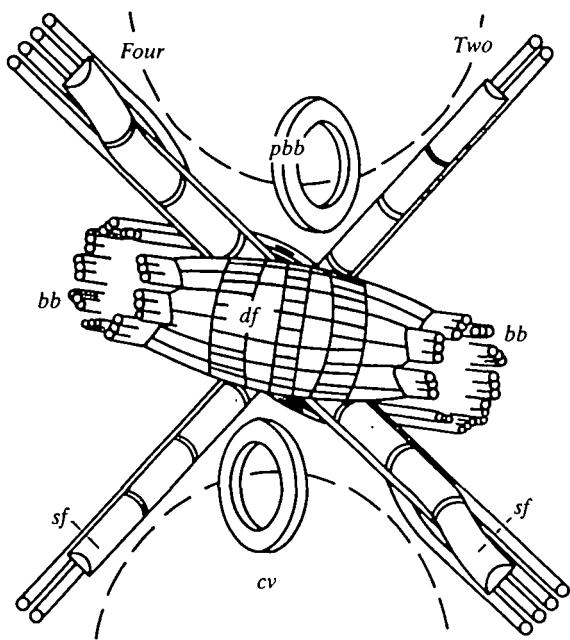


Fig. 1. Schematic diagram of the flagellar apparatus of *Chlamydomonas reinhardtii*. The view is from the anterior end of the cell and demonstrates the 180° rotational symmetry of the flagellar apparatus. *bb*, The two mature basal bodies. *ppb*, The probasal bodies that assemble the daughter basal bodies prior to mitosis. Their connections to the flagellar apparatus are omitted. *df*, The distal striated fiber that connects the basal bodies. Two smaller proximal striated fibers are mostly obscured in this diagram. *cv*, Contractile vacuoles whose location is represented by the broken circles. Two types of microtubule rootlets emanate from the flagellar apparatus, composed of two and four microtubules, respectively. *sf*, Striated fibers associated with both rootlet types. All four microtubule rootlets terminate in the region beneath the distal fiber, although the striated component of the two-membered rootlets is continuous.

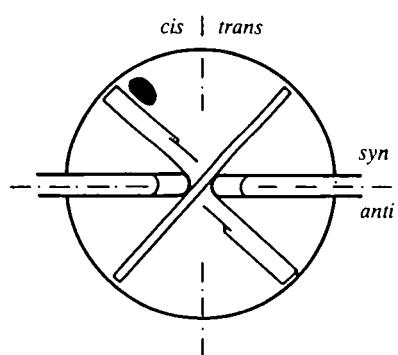


Fig. 2. Diagram of the eyespot position in an anterior view. Two imaginary planes bisecting the cell are used for reference. The *cis/trans* plane divides the cell between the two basal bodies. The *syn/anti* plane, or flagellar plane, contains the two basal bodies and the two flagella. The eyespot is always located at an angular position of 45° clockwise of the *syn/anti* plane, coincident with the meridian containing the four-membered microtubule rootlets.

algal species it has now been shown that the differences between the flagella correlate with the parental/daughter history of the two basal bodies (Beech *et al.* 1980; Melkonian *et al.* 1987; Wetherbee *et al.* 1988). Because of this we believe the correct positioning of the eyespot occurs with respect to an inherently asymmetric parental/daughter basal body pair.

The position of the eyespot is believed to be determined by a unique bundle of microtubules attached to the flagellar apparatus (Melkonian, 1984a; Moestrup, 1978). Each of the two basal bodies has two cytoplasmic microtubule roots or rootlets attached to it (Ringo, 1967). These rootlets differ from one another both in the number of microtubules they contain and in their angular position with respect to the flagellar plane. The four-membered microtubule rootlets extend at an angle of approximately 45° clockwise of the flagellar plane (all clockface distinctions are made from a point of reference outside the cell, facing the cell's anterior end) and the two-membered rootlets extend at an approximately 45° counterclockwise angle. The hypothesis that the eyespot is positioned by a rootlet is supported by the coincidence of the eyespot position and the angular position of the four-membered rootlet, as well as by the observation of a band of microtubules seen in association with the eyespot in electron micrographs (Gruber and Rosario, 1974). In other green algae, the eyespot is always found associated with the microtubule rootlet that corresponds, both in microtubule number and position, to the four-membered rootlet of *Chlamydomonas* (Moestrup, 1978).

Presumably the angular position of the rootlets is determined by a specific site of attachment for each rootlet type to each basal body. *Chlamydomonas* basal bodies are not rotationally symmetric and each microtubule triplet can be numbered around the basal body cylinder (Hoops and Witman, 1983). Examination of a *Chlamydomonas* mutant (*vfl-3*) in which the fibers connecting basal bodies are incomplete (Hoops *et al.* 1984; Wright *et al.* 1983) as well as observations of other green algae, which undergo a rotation of their basal bodies during development (Greuel and Floyd, 1985; Hoops, 1984; Taylor *et al.* 1985), support the idea that the rootlets and other components of the flagellar apparatus are physically connected to the basal bodies at unique sites.

The position of the eyespot not only makes cells of *Chlamydomonas* asymmetric, but enantiomeric as well. That is, they exist in only one mirror-image configuration. When viewed from the anterior end, the eyespot is always located at an angle of approximately 45° from the plane determined by the two flagella (Fig. 2). Only the enantiomorph in which the eyespot is clockwise of the flagellar plane exists in nature (Ruffer and Nultsch, 1985); the counterclockwise enantiomorph has not been observed. This handedness of the cell is distinct from *cis/trans* asymmetry, since it is an asymmetry within the *cis* half of the cell itself.

Like the cells of *Chlamydomonas*, basal bodies exist in only one enantiomeric configuration. When viewed from the anterior end, the microtubule triplets overlap in a clockwise direction (Fulton, 1971; Melkonian, 1984a).

Basal bodies or centrioles of the opposite handedness, where the overlap is in a counterclockwise direction, have not been observed in any organism to our knowledge. The enantiomeric position of the eyespot in the cell is thus believed to be a reflection of the fact that each basal body has only one attachment site for the four-membered rootlet and basal bodies exist in only one enantiomeric form. In summary, *Chlamydomonas* exhibits two types of cellular asymmetry. First, a *cis/trans* asymmetry differentiates the two halves of the cell and is apparently based on the parental/daughter relationship of the basal bodies. Second, within each half-cell an invariant enantiomeric asymmetry exists that reflects the inherent asymmetry around each basal body.

To understand better the origin and maintenance of asymmetry in *Chlamydomonas*, we have examined several stages in the *Chlamydomonas* life cycle with an eye toward their handedness, as determined by the position of the eyespot. Both mating and mitosis proved to be asymmetric. The mating asymmetry demonstrates that the *cis/trans* polarity of the flagellar apparatus extends to types of cellular behavior other than motility. The enantiomeric configuration of the spindle pole probably reflects how the asymmetric pattern of the *Chlamydomonas* cells is transmitted from generation to generation and suggests that the centrosome may itself be asymmetric.

Materials and methods

Strains and culture conditions

The 137c strain of *C. reinhardtii* was used as the wild-type strain for this study. Unless otherwise noted, all cultures were grown under constant illumination on agar plates at 21°C. General growth medium was Medium I of Sager and Granick (1953) supplemented with 8 mM-sodium acetate and with the K₂HPO₄ concentration raised to 1 mM. To obtain synchronous cultures for observation of mitosis and cytokinesis, cells were plated at a low density on minimal medium plates (Medium I without any added acetate) and grown at 21°C on a 12-h light/dark cycle for at least 3 days. For microscopic observation, cells were resuspended in liquid minimal medium and approximately 8 µl were placed under a coverslip and sealed with lanolin. Division occurred within the first hour into the dark cycle. For mating experiments, gametic cells were produced either by growth on low-sulphate plates, starvation on acetate-containing plates, or synchronous gametogenesis in liquid (Martin and Goodenough, 1975). The artificial activation of mating-type plus gametes was achieved with 10 mM-dibutyryl-cyclic AMP and 1 mM-isobutyrylmethylxanthine (IBMX) according to the method of Pasquale and Goodenough (1987).

Immunofluorescence

Three monoclonal antibody preparations were used in this study. Antibodies from the E7 hybridoma are specific for most forms of β-tubulin (Chu and Klymkowsky, 1987). Monoclonal 3A5 is specific for all forms of α-tubulin so far tested and gave staining patterns in *Chlamydomonas* identical to those of E7. The 6-11B-1 monoclonal antibody is specific for acetylated α-tubulin (Piperno and Fuller, 1987). E7 was a gift from M. Klymkowsky (University of Colorado), 3A5 and 6-11B-1 were gifts from M. Fuller (University of Colorado). All antibodies were used as culture supernatants. E7 and 3A5 were diluted

1:100; 6-11B-1 was diluted 1:10. *Chlamydomonas* cells were grown in liquid to a density of approximately 10⁶–10⁷ cells ml⁻¹ and pelleted in a clinical centrifuge. The cells were resuspended in an equivalent volume of a buffer containing 30 mM-Pipes, 25 mM-KCl, 5 mM-MgSO₄, 5 mM-EGTA, and 12% hexylene glycol (pH 6.8) for about 1 min and then repelleted. They were then lysed in 0.5% saponin (Sigma) in the same buffer for 1–2 min, pelleted, and fixed by the addition of 100–200 µl of a solution containing 4% freshly depolymerized paraformaldehyde and 0.1% glutaraldehyde in the Pipes buffer to the wet pellet. Resuspended cells were immediately pipetted onto polylysine-coated coverslips (1 mg ml⁻¹ in water) and allowed to settle and fix for 20 min. Coverslips were then rinsed in PBS (0.137 M-NaCl, 2.7 mM-KCl, 1.5 mM-potassium phosphate, 8 mM-sodium phosphate, pH 7.3) containing 0.1% Triton X-100 for 10 min. Coverslips were then incubated with the antibody dilutions for 40–60 min, rinsed five times for 30 s with the PBS plus Triton solution and then incubated with fluorescein-labeled goat anti-mouse IgG (Cappel Laboratories), diluted 1:100, for 20–40 min. Coverslips were rinsed in PBS, briefly in water, and mounted in 2% *n*-propyl gallate, 90% glycerol, 0.1 M-NaHCO₃ (pH 8.2). Epi-fluorescent observations were made with a Zeiss IM35 with a 63× objective.

The hexylene glycol in the lysis buffer appeared both to stabilize the microtubules and to improve the permeability of the cells. It did not appear to force polymerization of tubulin, since cells that were fixed in its absence and then lysed had approximately the same number and length of microtubules when stained. However, cells prepared in this manner had more lightly stained microtubules and had significantly higher levels of background fluorescence from the chloroplast (data not shown). This method does not rely on the use of mating autolysin and so is not restricted to use on non-dividing gametes. However, there were significant differences in the degree of cell wall permeabilization for genetically identical cultures of cells.

Microscopy

A Zeiss Axiophot equipped with a 100× plan-neofluor objective was used for bright-field and differential interference contrast (DIC) observation. Observation of living cells were made under lanolin-sealed coverslips using light filtered through a green filter (Zeiss 467806). Light intensities were kept to a minimum. In order to observe cytoplasmic fusion during mating it was necessary to siliconize the slide and coverslip (Sigmacote); without this treatment, few cells shed their walls and most did not proceed beyond flagellar agglutination. Synchronized cells were used for observing eyespot position following cytokinesis. Cells carrying the *unil* mutation remain aflagellate for a longer period of time following cytokinesis and thus develop more prominent eyespots before the zoospores are released from the mother cell wall. For this reason they were preferable for photography, but similar results were observed in the 137c strain when they formed visible eyespots prior to zoospore release. Photomicrographs of eyespots were taken with a green filter in order to enhance the contrast of the red eyespot. Glutaraldehyde at 1% in phosphate buffer was used to fix cells for microscopy.

Results

Eyespot–rootlet association

Because of the evidence for the involvement of cytoplasmic microtubules in positioning the eyespot (Moestrup, 1978), we developed a method for visualizing the micro-

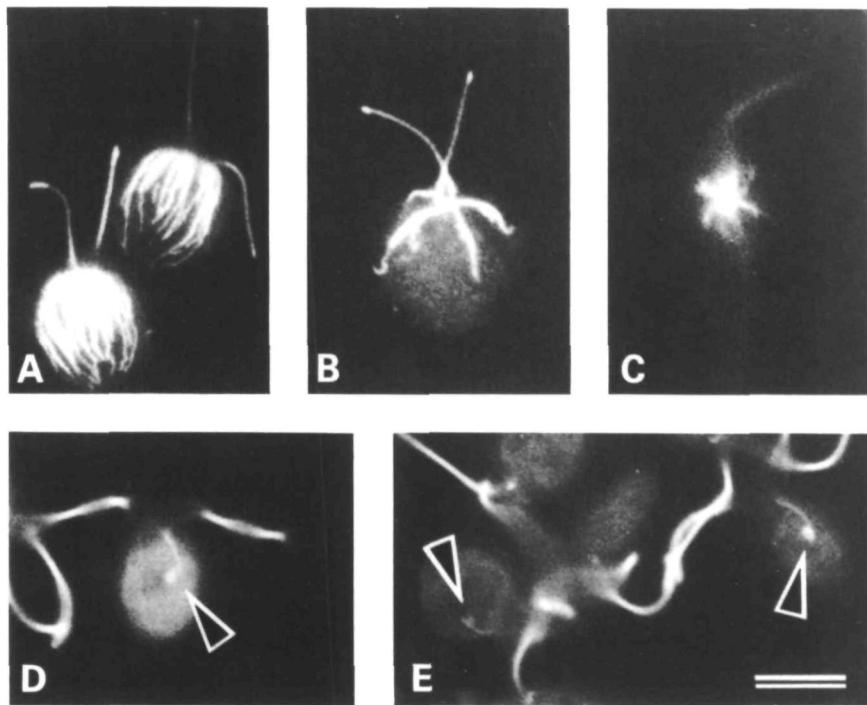


Fig. 3. A. The immunofluorescent staining of a *Chlamydomonas* cell labeled with the 3A5 monoclonal antibody, which binds to all α -tubulins tested. B. A cell from the same preparation as that used in A, only stained with the monoclonal antibody 6-11B-1, which is specific for acetylated α -tubulin. Only four of the radiating microtubule bundles stain with this antibody. C. The microtubule staining pattern obtained when cells are washed in PBS after lysis, but before fixation. This treatment results in the depolymerization of most cytoplasmic microtubules, but the remaining stable ones are arranged in a cruciate pattern reminiscent of the acetylated α -tubulin pattern. The antibody is E7, which stains all forms of β -tubulin. D,E. Cells were prepared by the described method with the exception that hexylene glycol was omitted from the lysis mixture, which leads to the depolymerization of the non-rootlet cytoplasmic microtubules. In this preparation some of the eyespots remained intact and can be identified by their reflection of the epi-illumination (arrowheads). They are all found just clockwise of a rootlet. The anti-tubulin antibody is 3A5. Bar, 5 μ m.

tubules of the cell by indirect immunofluorescence microscopy in order to confirm the eyespot–rootlet association. The microtubule cytoskeleton of *Chlamydomonas* consists of a number of cortical microtubules, in addition to the four rootlets, that emanate from the flagellar apparatus and run as far as the base of the cell (LeDizet and Piperno, 1986; and Fig. 3A). To distinguish the rootlets from the other cytoplasmic microtubules, we made use of two unique properties of the rootlets: their relative stability and their high proportion of acetylated α -tubulin. Using immunofluorescence, LeDizet and Piperno (1986) identified a subpopulation of cytoplasmic microtubules in *Chlamydomonas* that were stable to depolymerization and contained acetylated α -tubulin. Using a slightly different protocol we have been able to repeat these results with the same antibody, but with improved resolution. The acetylated α -tubulin is present primarily in four bundles that radiate from the flagellar apparatus in a cruciate pattern (Fig. 3B), which is identical to the known pattern of the rootlets. A few segments of other microtubules stain occasionally, but never in any identifiable pattern. A cruciate pattern of microtubule bundles can also be obtained when cells are subjected to slightly depolymerizing conditions before fixation (Doonan and Grief, 1987). We have achieved this either by omitting the hexylene glycol from the lysis

mixture or by briefly rinsing the lysed cells in PBS prior to fixation and staining with anti-tubulin antibody, either 3A5 or E7 (Fig. 3C). From their number and position, we assume that these microtubules correspond to the rootlets.

This method is not ideally suited for examining the rootlet/eyespot association because of the predominantly lipid nature of the eyespot. Hence, most methods for permeabilizing the cells also solubilize the eyespot. However, on occasion both structures can be identified: the rootlets by their immunofluorescence and the eyespots by their general reflection of the epi-illumination (Fig. 3D,E). In every case we have examined, the eyespot was positioned next to one of the stable microtubule bundles, which could be identified as the four-membered rootlet from its position with respect to the flagellar pair. Electron microscopy has previously demonstrated that the eyespot is always positioned just to one side of a microtubule rootlet (Gruber and Rosario, 1974). What had not been previously recognized is that the eyespot is also always on the same side of the rootlet. When looking from the anterior end of the cell the eyespot is always just clockwise of the rootlet that is associated with it. This was true for all 11 cells for which both rootlet and eyespot position could be unequivocally determined. We have subsequently confirmed this clock-

wise position of the eyespot from serial section electron micrographs of a *Chlamydomonas* cell provided by K. McDonald (University of Colorado, Boulder).

Cytokinesis and eyespot position

When cells of *Chlamydomonas* divide, the flagella are resorbed, from one to four rounds of consecutive mitosis and cytokinesis take place within the mother cell wall, and then each daughter cell assembles a new pair of flagella and the daughter cells are released. All the daughters have the stereotypical cytoplasmic organization described. To determine how the eyespot asymmetries are generated, we examined this cellular morphogenesis by light microscopy. Just before a cell of *Chlamydomonas* enters mitosis, the eyespot begins to lose its red pigment and birefringence, and so it cannot be followed by light microscopy. Following cell division, however, eyespots often reappear in each daughter cell before the daughters are released from the mother cell wall. To determine the position of the eyespot with respect to the cleavage plane, cells containing the *unil* mutation were used because they have a longer lag time between cytokinesis and flagellar assembly, and as a result have more prominent eyespots before daughter cells are released. The eyespot is invariably found opposite the site of the cleavage plane (Fig. 4A) ($N=200$). This was true even of pairs of daughter cells that had failed to

complete cytokinesis (Fig. 4B), thus ruling out rotation of the cells following division as a mechanism for achieving this position. We have found the same to be true of wild-type cells (137c) on the occasions when their eyespots were apparent. Within the clumps of four or eight cells generated by multiple rounds of division, relationships of sister cells may be distorted, but it appeared that eyespots were always located opposite the last cleavage furrow whether cells divided once, twice or three times (Fig. 4C). We have not at any stage in the life-cycle of *Chlamydomonas* seen an exception to the rule that eyespots are positioned opposite the cleavage plane.

In synchronized cultures of unfixed *Chlamydomonas* cells undergoing division, the eyespot does not always disappear during early prophase, and occasionally persists all the way through cytokinesis. These persistent eyespots were never seen to end up directly opposite the cleavage plane following division. Instead, the first cleavage furrow, which originates from the flagellar apparatus, invariably passed close to the location of the old eyespot. These old eyespots were never seen to move following cytokinesis and occasionally began to lose their pigmentation. At the same time, the new eyespots were occasionally observed forming opposite the furrow (Fig. 5L-O) as the new flagella began to extrude from the daughter cells. From these data we conclude that the old eyespot is lost, either prior to or following division, and that a new eyespot forms *de novo* in the correct position in each of the daughter cells.

Prior to division, the *Chlamydomonas* protoplast undergoes a 90° rotation within the cell wall so that the anterior/posterior axes of the two daughter cells end up perpendicular to that of the former mother cell (Johnson and Porter, 1968). We were interested in what role, if any, the 90° rotation of the cell played in generating or maintaining the cellular asymmetry. The rotation proved to be one of the most light-sensitive aspects of division, so that it did not always proceed a full 90° when being followed under the microscope, and in some cases did not occur at all. The rotation almost always occurs after the flagella have resorbed, but before separation of the basal body pairs signals the beginning of mitotic prophase. Regardless of how far the rotation proceeded, from 0° to 90° , mitosis and cytokinesis were normal and the resulting daughter cells had the stereotypical cytoplasmic organization. It thus appears that the rotation is not required for generating cellular asymmetry.

Asymmetry of the spindle pole

Because of the invariant position of the new eyespots with respect to the cleavage furrow, we formed the hypothesis that the basal bodies must have an invariant position as well. By the time the protoplast rotation has been completed the two probasal bodies have grown to full length and the four basal bodies can be visualized as four small dots arranged in a diamond shape (Fig. 6A). This configuration of the replicated basal bodies has been well documented by electron microscopy (Cavalier-Smith, 1974; Gaffel, 1988; Johnson and Porter, 1968).

Although basal bodies can be followed by light microscopy, it is difficult because of their small size.

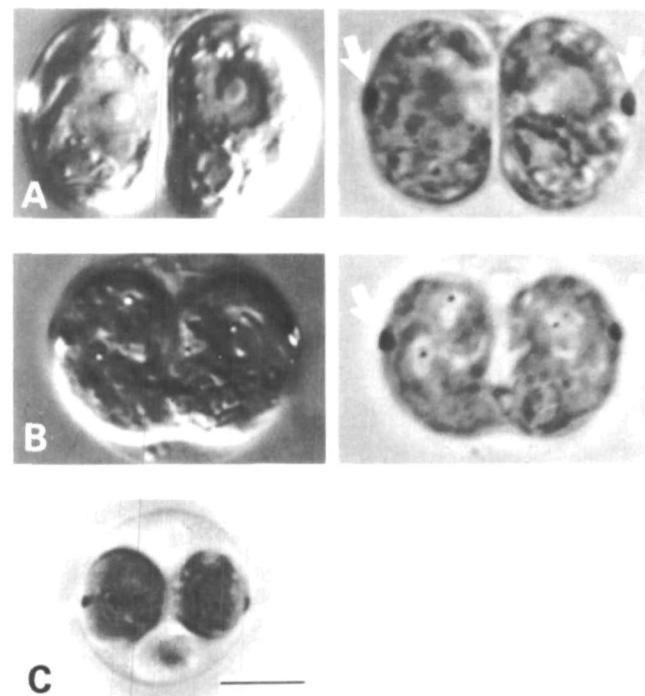


Fig. 4. Eyespot position following cytokinesis. A. A typical pair of sister cells following cytokinesis in DIC optics on the left and in brightfield on the right. The eyespots (arrowheads) are always located opposite the site of cleavage furrow formation. B. A similar pair of cells in which cytokinesis failed and the sisters remain connected by a cytoplasmic bridge. C. A brightfield image demonstrating this relationship for sister cells from the second division. Bar, 5 μ m.

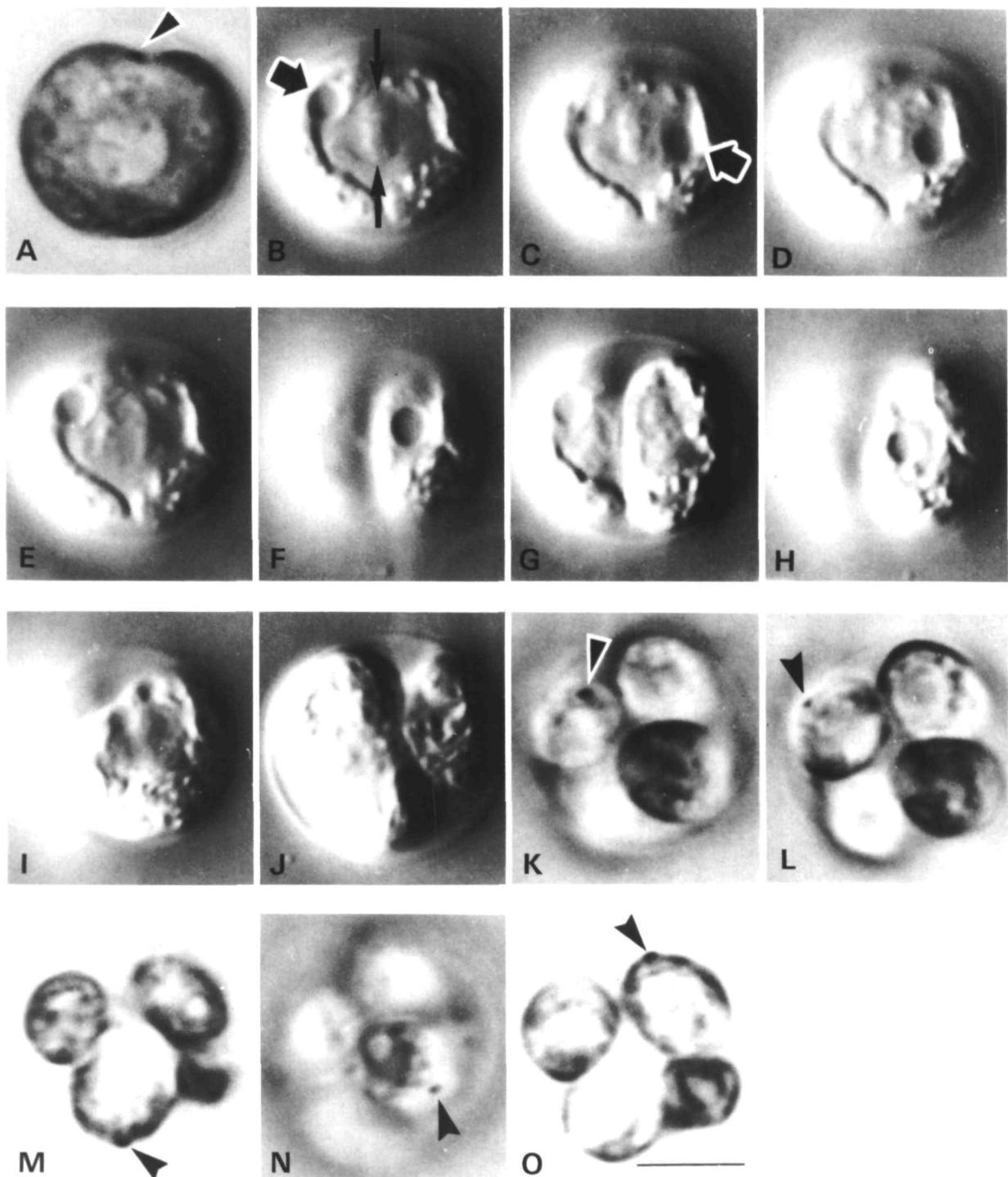


Fig. 5. Division of one cell into four. A. The black and white arrowhead points to the position of the eyespot adjacent to the incipient cleavage furrow. B-E. Metaphase and anaphase. The black arrows point to the chromosomes at the metaphase plate. The two contractile vacuoles (black-and-white arrows) do not lie on the spindle axis, but are located just clockwise of it, when viewed from the anterior end of the cell. F,G. First cytokinesis. H. Each sister cell develops a second contractile vacuole. I,J. Each cell rotates with respect to its sister so that the second spindles are at right angles to one another. K. Second cytokinesis. The black-and-white arrowhead points to the persistent old eyespot. L. The new forming eyespot at a different focal plane from that of the old eyespot (black arrowhead). M,N,O. New eyespots in the other three sister cells. From A to O represents about 90 min. From B to G took 5 min. Bar, 5 μ m.

However, the contractile vacuoles are useful markers for their location. The contractile vacuoles remain unrepli-cated until after cytokinesis is complete. One goes to each

spindle pole, along with one basal body pair, and a new one forms in each sister cell following cytokinesis. The position of the old contractile vacuole at each spindle pole

is asymmetric with respect to the half spindle; it does not lie along the spindle axis. When looking from the metaphase plate towards the pole, with the anterior end of the cell up, the contractile vacuole is always just to the right of the spindle pole (Fig. 5B-E). This was true for 100 poles examined from 50 spindles of both the first and second division.

In six cases, all four basal bodies could be visualized and followed as they separated into two pairs and migrated to the future spindle poles. The four basal bodies were always arranged in a diamond, and that diamond always bore the same relationship to the two contractile vacuoles, as illustrated in Fig. 6B. When the basal bodies separate, the same two basal bodies always go to the same pole and maintain a constant orientation to one another (Fig. 6). When looking down on the spindle from outside the cell, the basal body pair is always 45° counterclockwise of the spindle axis. From the position of

the contractile vacuoles, this was true for the 100 spindle poles examined.

If the daughter cells are going to divide a second time, they each rotate again prior to their second mitotic divisions. Following these second divisions, the four cells are generally arranged in a tetrahedral configuration within the mother cell wall. The tetrahedral configuration is usually achieved by rotation of each sister cell in the opposite direction by about 45° (Fig. 5I,J) rather than 90°. As the cells rotate they elongate along what will be the axis of their future spindles. Except for the extent of rotation, every other aspect of the second division is identical to what is seen in the first, including the segregation of basal bodies and the handedness of the spindle pole.

Asymmetry of mating fusion

The fusion of gametes in *Chlamydomonas* proved to be

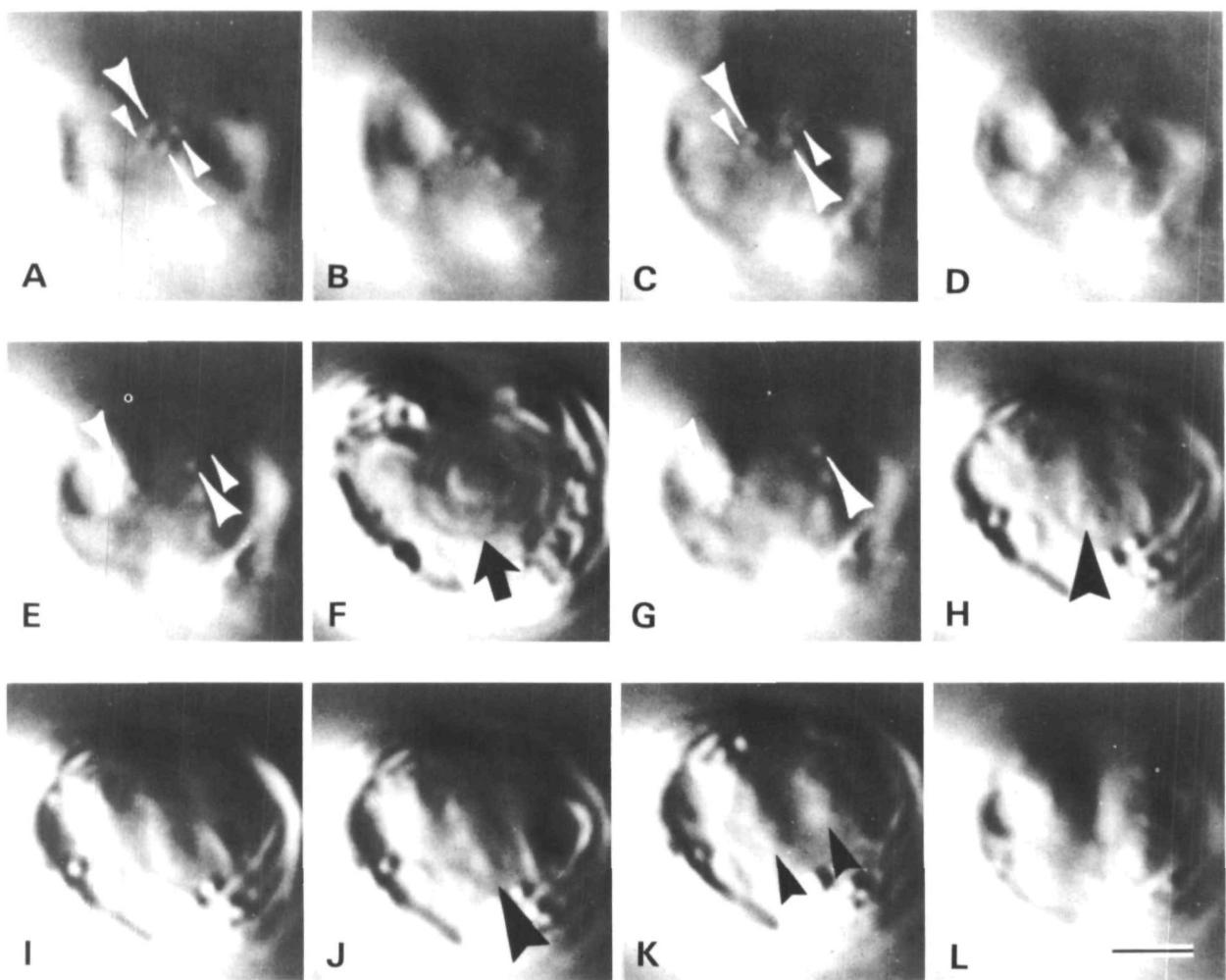


Fig. 6. Migration of basal bodies at mitosis. A,B. The four basal bodies are arranged in the diamond configuration. Large white arrowheads point to the parental basal bodies (based on ultrastructural data; Gaffel, 1988) and the smaller arrowheads to the daughter basal bodies. C,D,E. The two pairs separate and move to the future spindle poles without rotating with respect to one another. F. Chromosome condensation begins within the nucleus (arrow). H. The chromosomes move to the metaphase plate (black arrowhead) of the intranuclear spindle. J. The spindle poles are at the approximate location of the basal body pairs and the asymmetry of the spindle pole with respect to the contractile vacuole is apparent. K. At anaphase sister chromatids (smaller black arrowheads) move to the poles. L. The beginning of cytokinesis. Bar, 2 μ m.

asymmetric as well. When gametes of the plus and minus mating-types are mixed together they initially adhere along the length of their flagella so that the cells approach one another in a head-to-head configuration. Then the mating-type plus cell extends a mating structure, a thin actin fiber-filled tube, from an area adjacent to the flagellar bases. This tube fuses with a smaller mound erected on the mating-type minus cell, which is the mating-type minus structure (Friedmann *et al.* 1968; Goodenough and Weiss, 1975). Cellular fusion then rapidly proceeds along the length of the two cells so that they 'jackknife' into a dikaryon with the four flagella at one end. The dikaryon remains binucleate and quadriflagellate for 1–2 h before retracting its flagella and beginning its differentiation into a zygote. During this time we observed that the quadriflagellate dikaryons were fully motile and displayed strong phototactic behavior.

Our microscopic observations of dikaryons indicate that the fusion of gametes nearly always produces dikaryons in which one of the two eyespots is located adjacent to the region of fusion, while the other eyespot is opposite the region of fusion (Fig. 7). In other words, one gamete has fused along its *syn* side while the other has fused along its *anti* side (Fig. 2). This pattern is not invariant, but was observed 93% of the time in 300 dikaryons, formed between 137c strains.

One explanation for this result is that gametes are only able to adhere to one another with their flagella in a *cis*-to-*cis*, *trans*-to-*trans* orientation and that fusion is then constrained to one side or the other of their common flagellar plane. The differences already described between the *cis* and *trans* flagella make this plausible. However, microscopy of mating pairs of cells did not support this idea. Plus and minus gametes adhere to one another with their flagella in both a *cis*-to-*cis* and a *cis*-to-*trans* orientation in roughly equal numbers, and in both cases fuse to form dikaryons with one eyespot adjacent to the region of fusion and the other opposite it. Because the adhesion between the flagella was broken as soon as the mating structures fused, the flagella played no role in restricting the direction of fusion.

Another explanation of the observed fusion asymmetry is that the mating-type of the gamete restricts the site or orientation of fusion. To test whether the mating-type phenotype correlated with the side of the gamete that fused, we 'marked' one of the parents in a cross with the *unil* mutation so that we could determine whether that parent had fused its *syn* or *anti* side. Reciprocal crosses between wild-type (137c) and *Uni*⁺ (*unil*) cells were performed and the resulting dikaryons fixed and examined with the light microscope to determine whether the unpaired flagellum of the triflagellate dikaryon was associated with the gamete that had fused its *syn* or *anti* side. When the *unil* parent was mating-type plus, the single flagellum was associated with the gamete that had fused along its *anti* side. When the *unil* parent was mating-type minus, fusion occurred along the *syn* side (Fig. 8). It thus appeared that the mating-type of a gamete restricts its orientation of fusion.

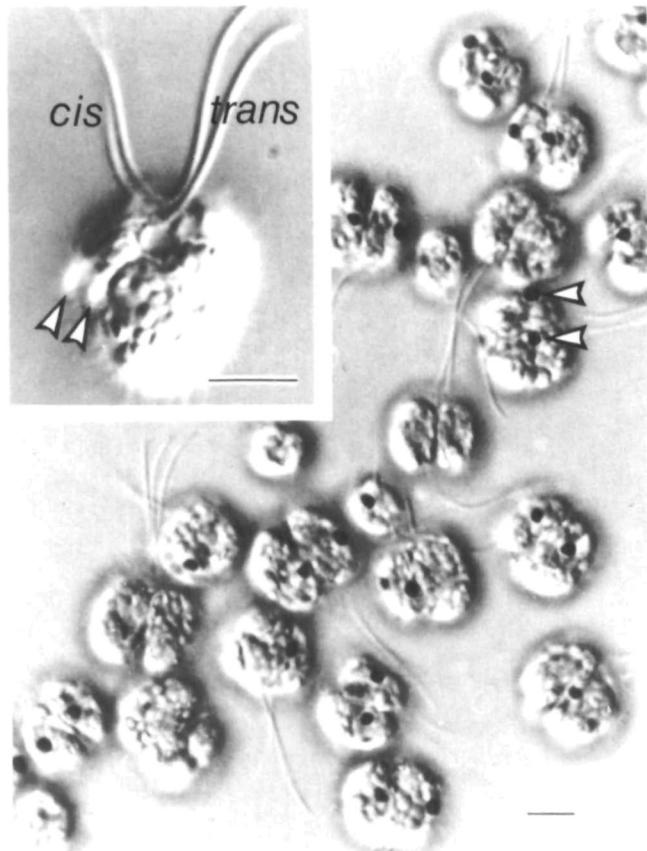


Fig. 7. A field of 15-min-old dikaryons formed by the mating of wild-type mating-type plus and minus cells. From the position of the eyespot (arrowheads) it can be seen that each dikaryon has formed from the fusion along the *syn* side of one gamete and the *anti* of the other. The inset is a view perpendicular to the flagellar planes of a dikaryon illustrating how the flagella are oriented so that the two *cis* and two *trans* flagella could beat coordinately. The eyespots are birefringent when viewed from the side because of their stacked nature. They are nearly always positioned on the same side of the dikaryon (denoted *cis*). Bars, 5 µm.

Asymmetry of the mating structure

Mating in *Chlamydomonas* is mediated by the plus and minus mating structures. The mating structures form near the flagellar apparatus, but slightly to one side of the two flagellar bases, between the two-membered rootlet of one basal body and the four-membered rootlet of the other (Goodenough and Weiss, 1978; Melkonian, 1984b). The mating structure thus has an asymmetric location with respect to the flagellar apparatus, and its location can be determined with respect to the eyespot. The incipient mating structures are present immediately following the gametogenic division (Martin and Goodenough, 1975), but can only be seen at this stage with the electron microscope. However, when the mating-type plus cell is activated by the events following adhesion with a mating-type minus cell, it extrudes a thin membrane tubule that can be visualized with DIC optics (Pasquale and Goodenough, 1987). The position of this tubule with respect to the eyespot was assayed by mixing plus and minus gametes together for a short time (2–5 min) during which

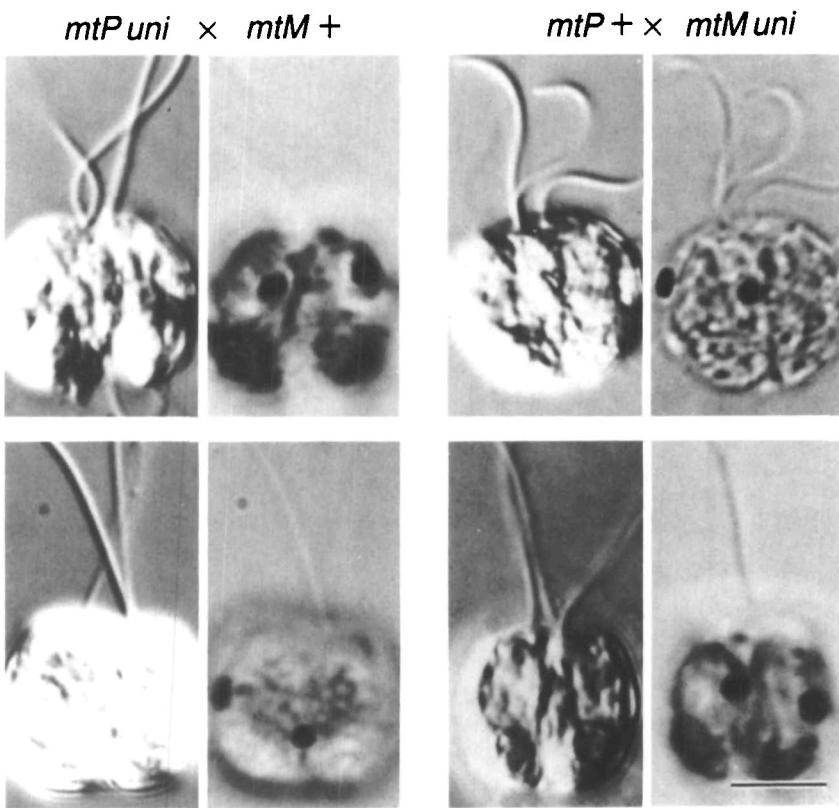


Fig. 8. Reciprocal crosses of *uni* and wild-type strains. When the *uni* strain is mating-type plus (left panel) the unpaired flagellum of the dikaryon is associated with the gamete that fused along its *trans* side. When the *uni* strain is mating-type minus (right panel), it fuses along its *cis* side. Each pair of micrographs is a DIC (left) and bright-field (right) image of a 1-h-old dikaryon. Bar, 5 μ m.

many of the plus gametes had elongated their mating structure, but not all had yet fused. They were then fixed in glutaraldehyde, which does not preserve the flagellar adhesion, and examined under the microscope. In those cases where it could be clearly defined, the mating-type plus mating structure always formed on the side of the flagella opposite the eyespot, the *anti* side. In order to show that the position of the mating-type plus structure was not induced by the mating-type minus gametes, mating structure activation was induced artificially by the method of Pasquale and Goodenough (1987). Dibutyryl-cyclic AMP and IBMX were used to stimulate the mating response in a pure culture of mating-type plus gametes, and the mating structure of these cells was also found to be on the *anti* side of the flagella.

The mating-type minus mating structure does not protrude enough to be visible in the light microscope but its position can be inferred by locating where the plus mating structure fuses. Fixed pairs of cells in which mating structures had fused revealed that the thin tube always ran from the *syn* side of one cell to the *anti* side of the other, regardless of whether the flagella had adhered in a *cis*-to-*cis* or *cis*-to-*trans* orientation. Knowing that the plus mating structure forms on the *anti*-side, we conclude that the minus mating structure forms on the *syn* side. Thus the mating structure of the gamete forms on the side that is going to fuse during dikaryon formation. The geometry of mating fusion is shown diagrammatically in Fig. 9.

Discussion

Chlamydomonas is not the first cell with right/left

asymmetry to be examined. The symmetry properties of ciliates have been well described (Frankel, 1984) and provide some interesting parallels to what we have observed in *Chlamydomonas*. The cells of ciliates, even

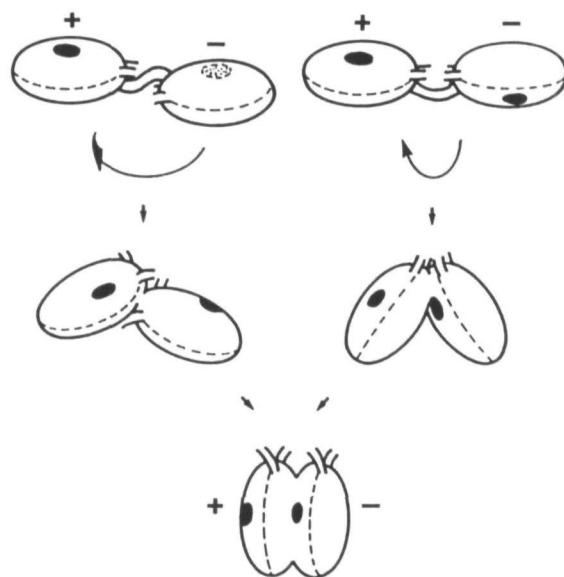


Fig. 9. Diagram of mating fusion in gametes that have adhered to one another with their flagella in either a *cis*-to-*trans* orientation, on the left, or a *cis*-to-*cis* orientation, on the right. In both cases the gamete on the left has its mating structure on the *anti* side while the gamete on the right has its on the *syn* side. From the position of the mating structure we can infer that the left gamete is mating-type plus and the right gamete is mating-type minus. In either case they fuse to produce identical dikaryons.

simple holotrichous ones like *Tetrahymena*, have an overall asymmetry based on the position of the contractile vacuole pore, cytoproct and other organelles (Nelsen and Frankel, 1989). Only one enantiomorph appears to exist in nature, but cells of the opposite handedness can be generated by either surgical manipulation or treatments that either arrest cells in late cleavage or prevent conjugating cells from separating. These mirror-image cells are unstable, but can be propagated indefinitely with selection. They have two striking properties. One is that they can be generated in the absence of any genetic change, and thus represent true examples of a non-nucleic-acid-based inheritance. The second is that the mirror-image reversal is not complete. It does not extend to the basal bodies, their microtubule rootlets or their striated fibers. Because the position of the ciliary rows in the oral apparatus is reversed while the cilia themselves are not, the reversed individuals are poor feeders, which necessitates their periodic selection from revertants of the normal handedness. On the basis of examples like these, Frankel (1984) has differentiated the surface patterning of ciliates into 'global' patterning, which can be reversed, and 'local' patterning at the level of the basal body, which is not reversible.

The asymmetry of eyespot placement in *Chlamydomonas* is probably an example of local patterning. The position of the eyespot correlates with the position of the four-membered rootlet, whose angular position with respect to the flagellar plane is probably determined by its unique site of attachment on the basal body. We have been unable to find, in several different screens, any mutations in *Chlamydomonas* that reverse the handedness of the eyespot position, and all known green algae with eyespots have this same handedness (Melkonian, 1984a). The cruciate arrangement of rootlets is common in green algae, and in all of them the eyespot is associated with the rootlet analogous to the four-membered rootlet of *Chlamydomonas* (Moestrup, 1978). The observation made here that the eyespot is always associated with the clockwise side of the four-membered rootlet underscores the specificity of the rootlet–eyespot association. In addition, it suggests that the two sides of the rootlet are different. This is supported by the previous observation (Weiss, 1984) that a striated fiber that runs along the top of the four-membered rootlet (Fig. 1) is displaced in a clockwise direction. An eyespot invariably located clockwise of its rootlet has been found in at least one other alga (Watson, 1975), and it would not be surprising if this turns out to be a universal feature of this highly conserved structure.

It has previously been shown that the mating structures of *Chlamydomonas* are attached to the two-membered rootlets (Goodenough and Weiss, 1978). Thus, the plus mating structure must be attached to the *cis* two-membered rootlet, while the minus mating structure is attached to the *trans* two-membered rootlet. The difference in position of the plus and minus mating structures thus appears to be based on the *cis/trans* asymmetry rather than the inherent rotational asymmetry of the basal body. This could be due to a difference between the *cis* and *trans* two-membered rootlets.

One possible reason that *Chlamydomonas* goes to the trouble of fusing gametes in only one orientation and tightly regulates the angular position of the eyespot with respect to the two flagella is that phototactic tracking in *Chlamydomonas* is based on the cell's ability to sense the direction of a light beam (Foster and Smyth, 1980). As the cells swim they rotate along their long axis and the photoreceptor–eyespot complex scans the light intensity around the cell. If the light intensity is greater on one side of the cell's swimming path, the beating of the two flagella is altered to turn the cell either towards or away from the light source, depending on whether the phototaxis is positive or negative. The turning mechanism appears to be inherent to the *cis* and *trans* flagella (Kamiya and Witman, 1984), so the signaling device, the photoreceptor–eyespot, has to be properly positioned in order that the signal produces the correct turn response at the proper point during the cell's rotation.

It is likely that the light signal from the photoreceptor–eyespot is transduced into a diffusible signal, like Ca^{2+} (Kamiya and Witman, 1984), so that in the dikaryon the signals from each photoreceptor–eyespot would be added and the sum interpreted by each flagellar apparatus in the same way. In the dikaryon, the two equivalent *cis* axonemes beat in the same direction, and the two equivalent *trans* axonemes in the other (Fig. 7). We therefore believe that *Chlamydomonas* has evolved asymmetrically positioned mating structures to ensure *syn-to-anti* fusion, which leads to dikaryons with parallel flagellar pairs and both eyespots on the same side of the dikaryon. Other configurations of gametic fusion should produce dikaryons that are not phototactic.

There have been numerous studies of mitosis and cytokinesis in *Chlamydomonas*. Our observations of living cells provide a temporal basis for understanding how these high-resolution studies fit together, as well as providing data on the more global aspects of division that are difficult to obtain from thin-section electron microscopy. Fig. 10 describes the events of mitosis and cytokinesis, with the inferred position of the rootlets, that lead to the replication of the asymmetric *Chlamydomonas* cytoskeleton.

Prior to division, the flagella of *Chlamydomonas* are usually resorbed (Johnson and Porter, 1968). One of the first visible indications of a commitment to division is a rounding up of the cell within the ovoid cell wall and the formation of an incipient furrow that bisects the flagellar apparatus and extends approximately half way round the cell. Johnson and Porter (1968) observed a band of four microtubules present just to one side of the forming furrow, but were unable to confirm that it actually derived from a four-membered rootlet. Our observation that the furrow forms along a meridian coincident with that of the two four-membered microtubule rootlets and passes close to the position of the old eyespot supports this suspicion.

The rotation of the protoplast that occurs during division has been known for some time but its significance has remained unknown. One explanation for this rotation is that a dividing cell elongates along its spindle axis, perhaps as a result of constriction at the forming

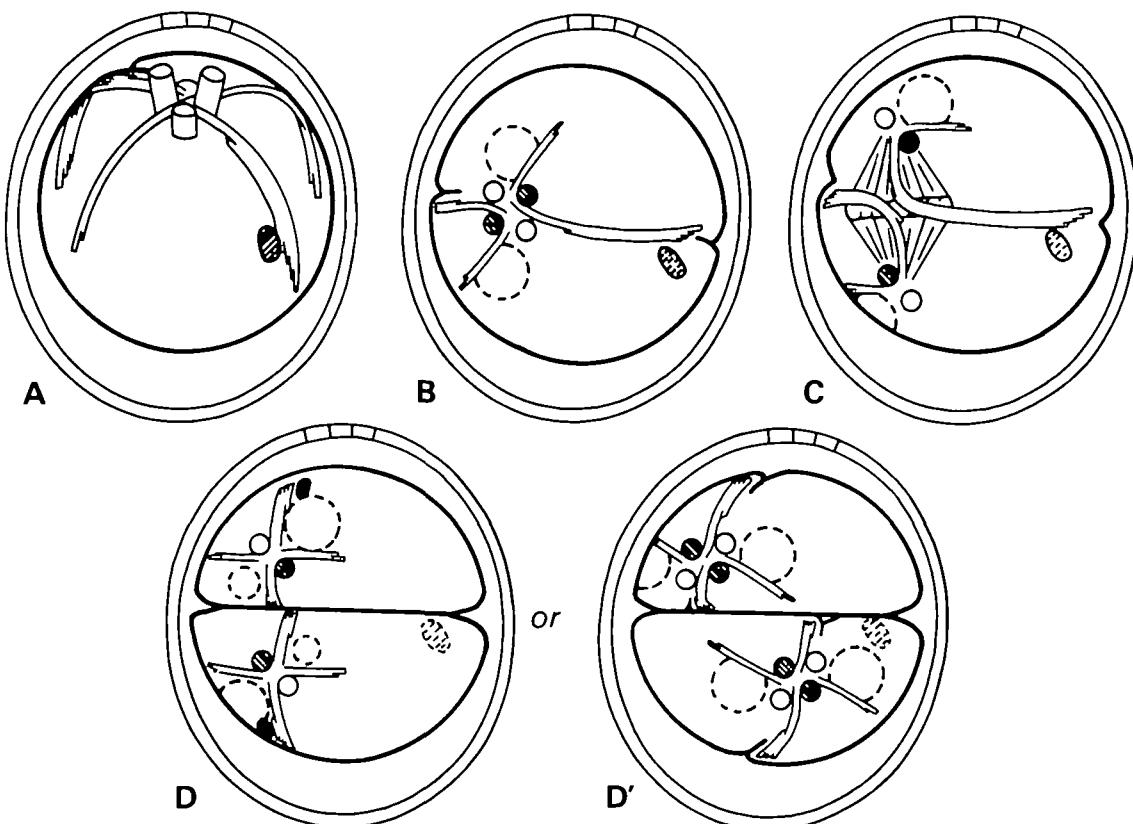


Fig. 10. A model for the orientations of basal bodies, rootlets and contractile vacuoles throughout division. A. Position of basal bodies and rootlets immediately following resorption of the flagella. The incipient cleavage furrow begins to form in the plane of the four-membered rootlets. The old eyespot begins to disappear. B. The 90° rotation of the protoplast within the cell wall takes place. The rotation is in approximately in the plane of the two-membered rootlets, but can be in either the *cis* or *trans* direction. The parental basal bodies are shaded and the daughters left open. The broken circles represent the position of the contractile vacuoles. C. The basal body pairs separate and the mitotic spindle is assembled. The parental/daughter asymmetry of the basal body pair at each pole generates the invariant asymmetry of the contractile vacuole position at the pole. D. If this is the final cell division, the mature daughter basal body grows its own rootlets, a new contractile vacuole is formed in the proper location, and a new eyespot assembles in association with the four-membered rootlet from the daughter basal body. D'. If the products of the first division are going to divide again then another round of basal body replication takes place. Each parental basal body rotates clockwise towards its respective daughter, which establishes the connections between the new pairs. This rotation rotates the attached rootlets as well, and results in the clockwise rotation of the contractile vacuole plane. Each sister cell then rotates within the mother cell wall so that their axes are 90° to one another prior to the second round of division.

furrow, and the force of elongation rotates the cell within the confines of the ovoid cell wall. This seems the simplest way to account for our observation of a 90° rotation prior to the first division, but opposite 45° rotations in the sister cells prior to the second division. The fact that mitosis and cytokinesis appear normal even when rotation does not occur would seem to exclude it from playing any role in setting up the proper orientation of division. In the colonial Volvocales (*Volvox*, *Gonium*, *Platydorina*) the direction and extent of rotation during the vegetative divisions could be crucial for determining the orientation of the *Chlamydomonas*-like cells to one another within the colony, but in *C. reinhardtii* it appears to play no role other than perhaps to aid in the efficient packing of the zoospores within the mother cell wall.

The replication and segregation of basal bodies occurs in a precisely oriented fashion. The extension of the probasal bodies to full length has been well documented to occur in early pre-prophase (Gaffel, 1988; Gould,

1975). At some time between the maturation of the daughter basal bodies and the separation of the new pairs to the poles, the connections between the parental basal bodies must be dissolved and new ones established between the parental and the daughter. If the microtubule triplets of the basal body are truly non-equivalent, this process would be expected to involve a rotation of the parental basal body so that the microtubule triplet that formerly faced the other parental basal body now faces its daughter basal body. On the basis of data about the behavior of probasal bodies in the colonial Volvocales (Greuel and Floyd, 1985; Hoops, 1984; Taylor *et al.* 1985), we believe that the probasal body across the two-membered rootlet from the mature basal body is its daughter. If this is true, the rotation of the parental basal body towards its daughter would be in a clockwise direction (Fig. 10). We have observed a clockwise rotation of the contractile vacuole pair prior to the second division (unpublished data) at a time when the new

connections between parental and daughter basal bodies are being established. Just such a basal body rotation has been previously postulated to be required for maintaining the absolute orientation of the basal bodies to one another in the daughter cells of other algae (O'Kelly and Floyd, 1984).

Prior to the separation of the new basal body pairs, they are arranged in a diamond configuration (Fig. 6A). From electron microscopy, it is known that only the two basal bodies across the short diagonal of the diamond have attached rootlets, and are therefore the presumed parental basal bodies (Gaffel, 1988). When the new basal body pairs separate from one another and move to the site of the future spindle poles, they do not rotate, but maintain their original relationship to one another (Fig. 10). As a result, the parental basal body of each pair always ends up as the one closer to the cleavage furrow. The eyespot always forms on the side of the cell opposite the cleavage furrow. So, as predicted, the *cis/trans* asymmetry of the cell corresponds to a parental/daughter asymmetry of the basal body pair; the *trans* basal body is the parental and the *cis* basal body is the daughter. We therefore conclude that the *uni* mutations affect the daughter basal body's ability to assemble a flagellum more strongly than they do the parental basal body.

From the position of the parental and daughter basal body at the spindle pole, the parental four-membered rootlet should be pointing back towards the metaphase plate (Fig. 10). Doonan and Grief (1987), in a study of division in *Chlamydomonas* using indirect immunofluorescence of tubulin, reported the observation of two microtubule bundles extending over the top of the mitotic spindle from each pole toward the metaphase plate, where they bend and run perpendicular to the spindle along the forming cleavage furrow. The position of these microtubule bands is exactly where we predict the four-membered rootlets should be. The bend at the metaphase plate is a result of the rotation of the parental basal body when the new parental/daughter connections were established and of the separation of the new pairs at right angles to the incipient furrow. One result of having the two parental four-membered rootlets oriented along the axis of the spindle is that the new four-membered rootlets, attached to the daughter basal bodies, will extend directly away from the spindle. This is probably the reason why the new eyespots, whose formation correlates with the new four-membered rootlets, are always directly opposite one another, and opposite the cleavage furrow.

It appears that part of what maintains the asymmetry of the *Chlamydomonas* cell is the non-random position of the basal bodies at the spindle poles. It is possible that the orientation of the basal bodies at the poles is maintained by the parental four-membered rootlets, which keep the basal body pairs from rotating. In this case, the centrosome that nucleates the microtubules of the spindle could be a radially symmetric structure whose asymmetry is imposed on it by the differential stability of microtubules incorporated into the spindle (Kirschner and Mitchison, 1986). The centrosome need only have a single specific attachment site to the basal body pair. Another possibility

is that the orientation of the basal bodies is maintained by the centrosome itself (Mazia, 1984). In this model the centrosome can attach to the spindle microtubules and the basal bodies in only one orientation; or in other words, the handedness of the spindle pole results from the handedness of the centrosome.

Parental/daughter asymmetry of basal bodies is not unique to algae. A parental/daughter asymmetry of centrioles in mammalian tissue culture cells is well established (Rieder and Borisy, 1982; Vorobjev and Chentsov, 1982). The two centrioles of a pair can be distinguished by their ultrastructure, their capacity to nucleate the assembly of cytoplasmic microtubules and their ability to assemble a primary cilium. The centriole pairs of mammalian cells also appear to have non-random orientations to the microtubule-nucleating centrosome. This is particularly evident at mitotic spindle poles, where the parental centriole of each pair is oriented perpendicular to the spindle axis in tissue culture cells (Vorobjev and Chentsov, 1982). In the embryonic divisions of some organisms the centriole pairs at either pole of a spindle are always oriented at right angles to one another (Costello, 1961). The orthogonal relationship of the centrioles at the poles predicts the direction of centrosome separation at the next division. It is likely that the centrioles in these situations are oriented in response to the orientation of the centrosome (Mazia, 1984), rather than vice versa. The centriole pairs of *Chlamydomonas* are not orthogonal, but parallel to one another at the spindle poles. The orthogonal orientation of cleavage planes in *Chlamydomonas* is instead a result of the 90° rotation of the cells prior to mitosis.

While right/left asymmetry is not a prominent feature of eukaryotic cells, it is quite apparent in *Chlamydomonas*. We confirm here that the *cis/trans* asymmetry of *Chlamydomonas* correlates with the parental/daughter asymmetry of the basal body pair, and suggest that this inherent asymmetry of the basal body pair is the source of the cellular asymmetry. Given the invariant position of basal bodies at the spindle poles, we cannot tell whether the *cis/trans* positioning of the eyespot and mating structure is a result of a difference between the *cis* and *trans* rootlets, or of some independent mechanism that senses the orientation of the mitotic apparatus and/or the cleavage furrow. However, it seems clear that the rootlets are responsible for maintaining the position of the eyespot and mating structure and that their position is determined by the orientation of the basal body to which they are attached. The basal body pair of *Chlamydomonas* is probably not significantly different from any centriole pair, but in *Chlamydomonas* the inherent asymmetries of the pair have been exploited in the elaboration of the cytoskeleton, providing an example of how a cellular form can be specified.

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