Outer Doublet Heterogeneity Reveals Structural Polarity Related to Beat Direction in *Chlamydomonas* Flagella

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ABSTRACT Analysis of serial cross-sections of the *Chlamydomonas* flagellum reveals several structural asymmetries in the axoneme. One doublet lacks the outer dynein arm, has a beak-like projection in its B-tubule, and bears a two-part bridge that extends from the A-tubule of this doublet to the B-tubule of the adjacent doublet. The two doublets directly opposite the doublet lacking the arm have beak-like projections in their B-tubules. These asymmetries always occur in the same doublets from section to section, indicating that certain doublets have consistent morphological specializations. These unique doublets give the axoneme an inherent structural polarity. All three specializations are present in the proximal portion of the axoneme; based on their frequency in random cross-sections of isolated axonemes, the two-part bridge and the beak-like projections are present in the proximal one quarter and one half of the axoneme, respectively, and the outer arm is absent from the one doublet >90% of the axoneme's length. The outer arm-less doublet of each flagellum faces the other flagellum, indicating that each axoneme has the same rotational orientation relative to the direction of its effective stroke. This strongly suggests that the direction of the effective stroke is controlled by a structural component within the axoneme. The striated fibers are associated with specific triplets in a manner suggesting that they play a role in setting up or maintaining the 180° rotational symmetry of the two flagella.

In forward swimming *Chlamydomonas*, the two flagella beat with an effective stroke in approximately the same plane, but in opposite directions (17, 32, 34). Although the fine structure (6, 11, 14, 30, 32, 39, 40), biochemistry (10, 15, 21, 27, 28, 31, 40), and waveform (3, 5, 17, 32, 34) of the *Chlamydomonas* flagellum have been extensively investigated, little information is available on the mechanism by which the direction of the effective stroke is established. The isolated flagellar apparatuses of *Chlamydomonas* appear to retain the same beat pattern as in intact cells (16, 17), so the direction of beat must be controlled by a structural component within the flagellar apparatus. However, it is not known whether this component is internal or external to the axoneme. Structural differences have been observed in some of the outer doublets of *Chlamydomonas* (15, 38), but it has not been determined whether these asymmetries always occur in the same doublets along the axoneme, nor whether they are oriented in a way that is related to the plane of beat. The central pair of *Chlamydomonas* rotates during flagellar movement (19, 20), so it can not give polarity to the axoneme.

To determine whether the axonemes of *Chlamydomonas* have an intrinsic polarity related to the plane of beat, we made a detailed examination of the fine structure of the flagellar axoneme in serial thin sections having a known orientation relative to the two basal bodies. The results indicate that: (*a*) there are consistent morphological differences between several outer doublets in the axoneme; (*b*) as a consequence, the axonemes do indeed have an inherent structural polarity; and (*c*) this polarity is appropriately oriented to be of functional significance in establishing beat direction. We also found that the striated fibers connecting the two basal bodies are always associated with specific triplets, suggesting that the striated fibers play a role in determining the rotational orientation of the axonemes.

MATERIALS AND METHODS

Chlamydomonas reinhardtii wild-type strains 1132d− and NO+ were grown in 125-ml cultures as previously described (40). Conventional fixation procedures, including those using sodium cacodylate-buffered glutaraldehyde, often caused the flagellar matrix to appear dense, thus obscuring the delicate markers used in this study. For this reason, a double glutaraldehyde fixation was employed in which the cells were first fixed at room temperature with 1%

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glutaraldehyde in medium for 10–15 min and then with 1% glutaraldehyde in 100 mM sodium cacodylate, pH 7.2, for 50 min. After buffer washes, the sample was embedded in 1% agar, postfixed with 1% OsO_4 in 50 mM sodium cacodylate at room temperature, stained en bloc with 1% aqueous uranyl acetate, dehydrated, and embedded in Epon or Epon-Araldite. Demembranated axonemes were prepared as previously described (3), fixed with 1% glutaraldehyde in 100 mM sodium cacodylate, postfixed, en bloc stained, dehydrated, and embedded as described above. Serial sections were picked up on Formvar-coated or carbon-over-Formvar-coated grids, and viewed with a Philips 301 TEM. All micrographs are printed as if viewed from inside the cell.

RESULTS

Structural Polarity of the Axoneme

Cross-sections of the *Chlamydomonas* axoneme frequently reveal several structures that depart from the overall rotational symmetry of the axoneme (Fig. 1). Three outer doublet microtubules contain "beak-like projections" (38) that extend partway across the lumen of their B-tubules. Two of the doublets containing the projections are adjacent; the third is on the opposite side of the axoneme. The latter doublet lacks an outer dynein arm, although the inner arm appears to be present. A previously undescribed two-part bridge extends from the A-tubule of this doublet (n) to the B-tubule of the adjacent doublet (n + 1). This bridge consists of two roughly parallel, wedge-shaped components, each of which spans the gap between the doublets. The inner component is ~14.5 nm long and tapers toward the A-tubule; the outer one is ~12.5 nm long and tapers toward the B-tubule. These components appear to be ~4.0–5.0 nm wide at their centers, with the inner one usually slightly wider than the outer.

In every case of isolated and in situ axonemes examined, the beak-like projections and the two-part bridge are associated with the same doublets from section to section; similarly, the outer dynein arm is always missing from the same doublet (Figs. 2 and 3). These observations show that some of the outer doublet microtubules are morphologically distinct from the others. Consequently, the axoneme has an *inherent structural polarity* defined by the positioning of these unique doublets.

All flagella cross-sections from within the flagellar collar, and out to at least 0.7 μm (10 sections) distal to the flagellar collar, have the beak-like projections and the two-part bridge, and lack the above-mentioned outer arm. However, the projections and the bridge do not occur over the entire length of the flagellum. In isolated axonemes, the bridge was observed in 17 of 75 randomly chosen cross-sections and always occurred in association with the three beak-like projections. All three beak-like structures were present in 30 cross-sections; one or two were present in an additional 13. A single outer arm was lacking in 69 of the cross-sections, including all of those having one or more beak-like projections. In all cross-sections, the relative positions of these structural asymmetries around the axoneme was consistent with the arrangement described above. These observations indicate that the asymmetries have unique and regular distributions along the axoneme: the two-part bridge and the beak-like projections are present in the proximal quarter and half of the axoneme, respectively, and the outer arm is missing from one of the doublets over at least 90% of its length.

Rotational Orientation of the Axoneme Relative to the Cell Body

Because specific doublets of the *Chlamydomonas* axoneme have unique structural features, these features can be used as polarity markers to ascertain the rotational orientation of the flagella in situ. To determine the rotational orientation of one flagellum of a cell relative to the other, we examined serial sections that showed the origin of both flagella in the cell body (Figs. 2 and 3). In every such cell examined, the outer arm-less doublet of one flagellum faces the other flagellum—i.e., the two-part bridge is located on the medial side of the axoneme. No exceptions to this pattern were observed. Therefore, the two axonemes must have a consistent rotational orientation relative to one another and the cell body.

Because the two flagella of *Chlamydomonas* exit from the cell at about a 90° angle from one another, cross-sectional information can usually be obtained from only one flagellum of a cell. However, occasionally one flagellum is abnormally bent, or both flagella have reverse bends at their bases, so that good cross-sections are obtained of both flagella (Fig. 3). In these cases, both axonemes have all three polarity markers. In each flagellum the doublet bearing the two-part bridge and

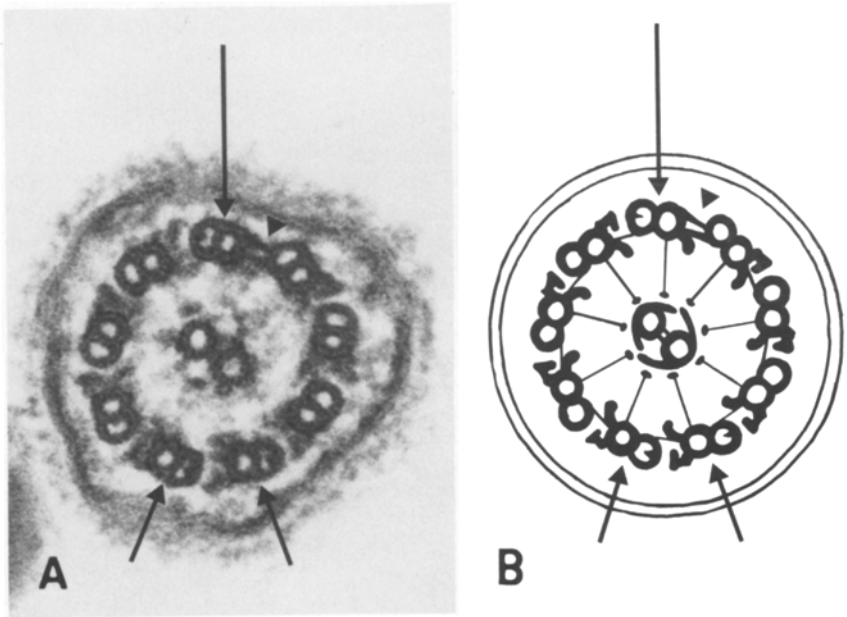


FIGURE 1 (*A*) Cross-section of the *Chlamydomonas* flagellum. Three doublets have beak-like projections in their B-tubules (arrows). One of these doublets (long arrow) lacks the outer arm, and a two-part bridge (arrowhead) extends from the A-tubule of this doublet to the B-tubule of the adjacent doublet. × 218,000. (*B*) Diagramatic representation of *A*.

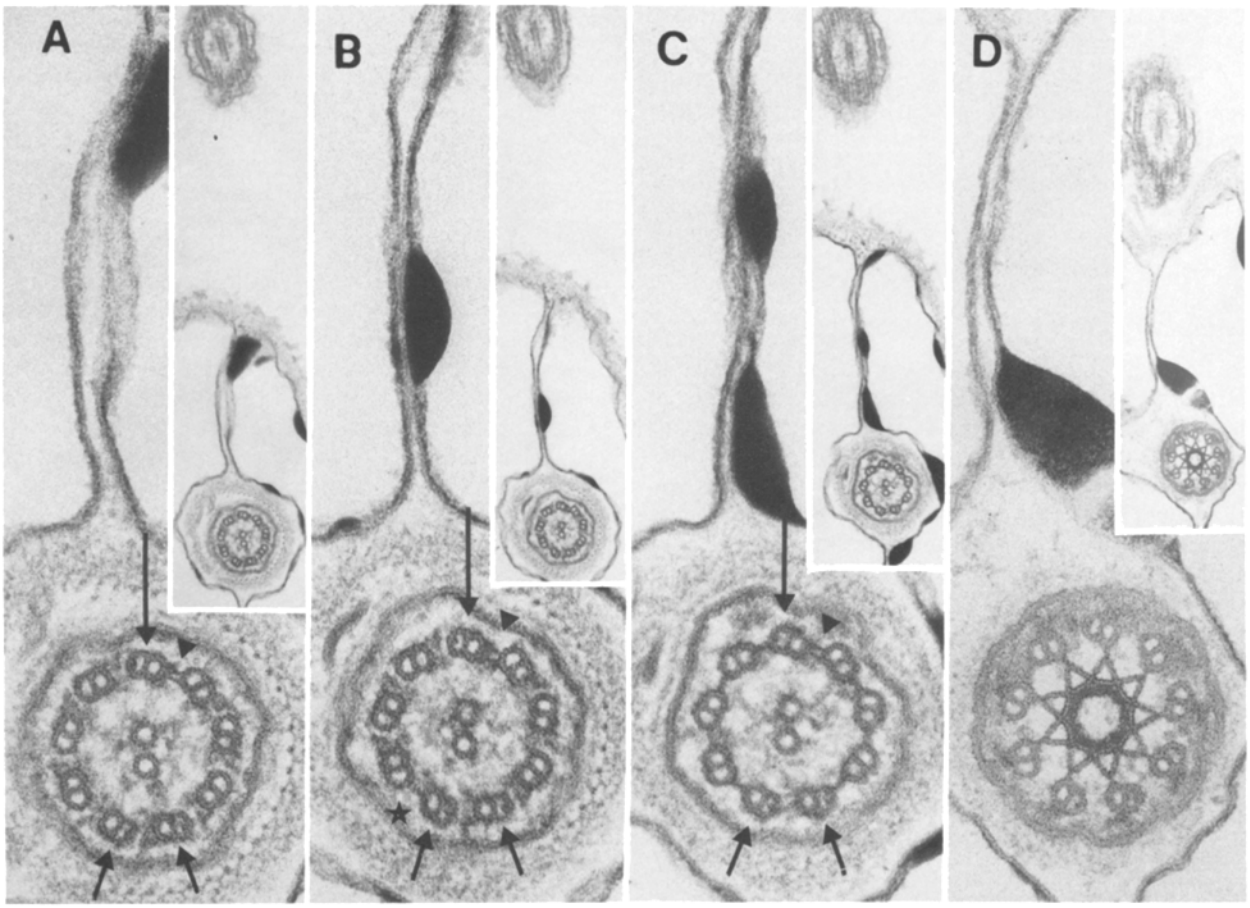


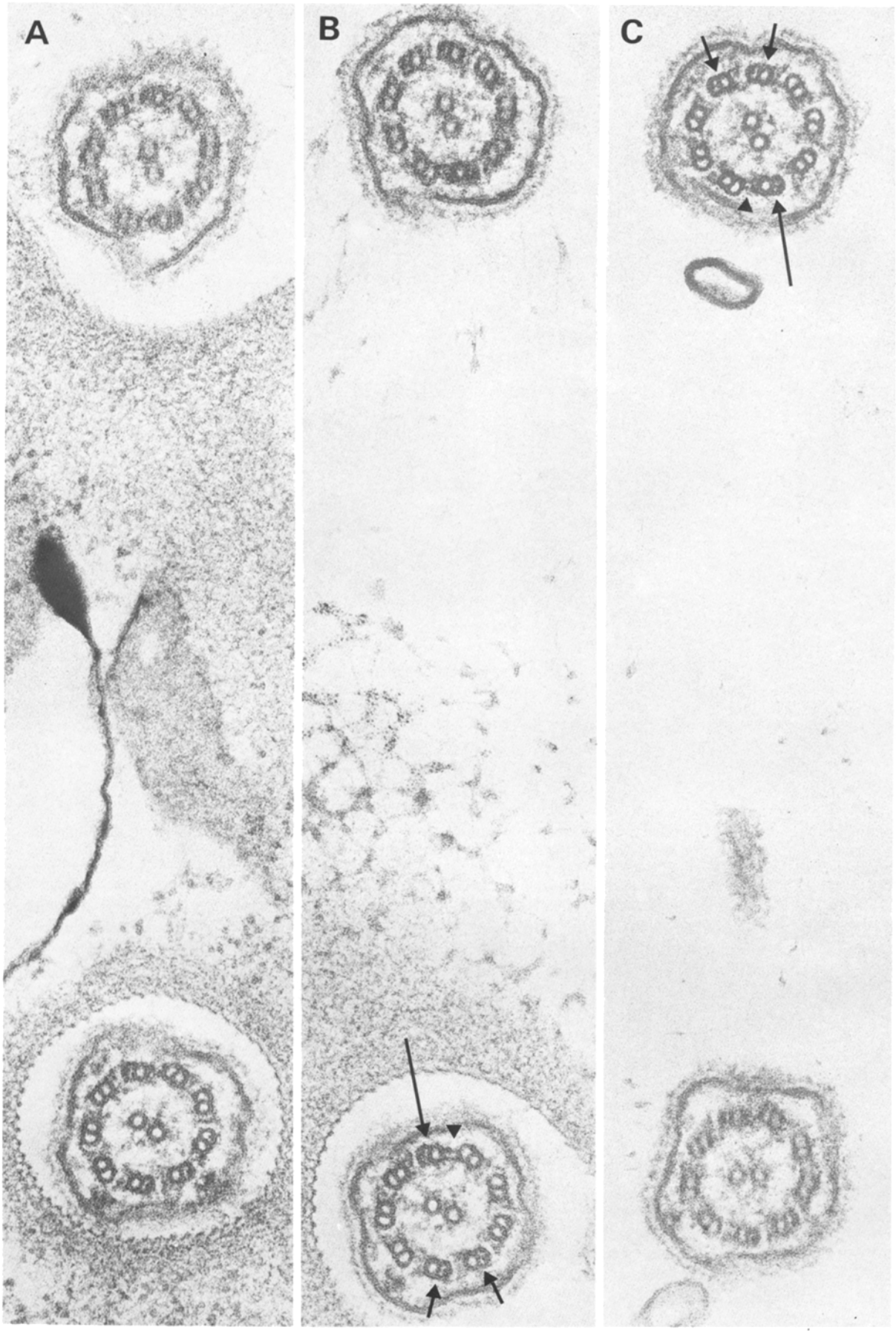
FIGURE 2 Sections 1, 2, 5, and 9 from a series. Although the figures are arranged from distal to proximal to correspond with the text, each micrograph is printed in the conventional manner (i.e., as if viewed from inside the cell). Insets show the relative positions of the two flagella in each section; the lower flagellum is the one shown at higher magnification. (*A*) In the distal portion of the flagellar collar, three doublets (arrows) contain beak-like projections, and the two-part bridge (arrowhead) is present. The doublet with the bridge attached to its A-tubule faces the other flagellum (*inset*). Both inner and outer arms are associated with all but one doublet (long arrow), which lacks the outer arm. (*B*) The next most proximal section. The polarity markers (arrows and arrowhead) are associated with the same doublets as in the previous section. Slightly more proximally the arms are absent; even at this level an outer arm is lacking from one doublet (star) that normally has both arms. (*C*) Further proximally, all arms are absent from the doublets. Both the beak-like projections (arrows) and the two part bridge (arrowhead) are present. Peripheral links and inner links (see text) also occur at this level. Occasionally, as at the bottom of this micrograph, these two links together can resemble the two-part bridge. That such apparent "bridges" actually represent a peripheral and an inner link can be ascertained by examination of adjacent serial sections (not shown). (*D*) In the region of the stellate structure, the two-part bridge is no longer present. Although some doublets may contain structures similar to the beak-like projections, these structures are not necessarily arranged in the "two-opposite-one" pattern seen more distally. The upper flagellum has just exited from its flagellar collar (*inset*). × 135,000; *insets*, × 38,000.

lacking the outer arm faces the opposite flagellum, and the two adjacent doublets with the beak-like projections face away from it. These results confirm that the position of the markers—and hence, the orientation of the axoneme—is the same in each flagellum relative to the direction of its effective stroke.

Basal Body Orientation and the Attachment of the Striated Fibers

The dynein arms are present part way into the flagellar collar (Fig. 2, *a* and *b*). At this level, the outer arm is missing from the one doublet, and both the beak-like projections and the two-part bridge are associated with their respective doublets. More proximally, arms are no longer present, but the bridge and the beak-like projections are still observed (Fig. 2*c*). Rod-like "peripheral links" (38) connect adjacent doublets in this region. An additional, previously undescribed, linking structure is present to the inside of each peripheral link; this inner link is bowed towards the center of the axoneme and is less opaque and well defined than the periph-

FIGURE 3 Sections 1, 4, and 9 from a series showing cross-sections of both flagella of a single cell. (*A*) The upper flagellum is sectioned just distal to the flagellar collar and the lower flagellum is sectioned within the collar. (*B* and *C*) Further distally, the presence of beak-like projections in three doublets (arrows), the two-part bridge (arrowhead), and the lack of an outer arm on one doublet (long arrow) can be seen in both flagella. Note that the doublet lacking the outer arm and bearing the two-part bridge faces the opposite flagellum. × 130,000.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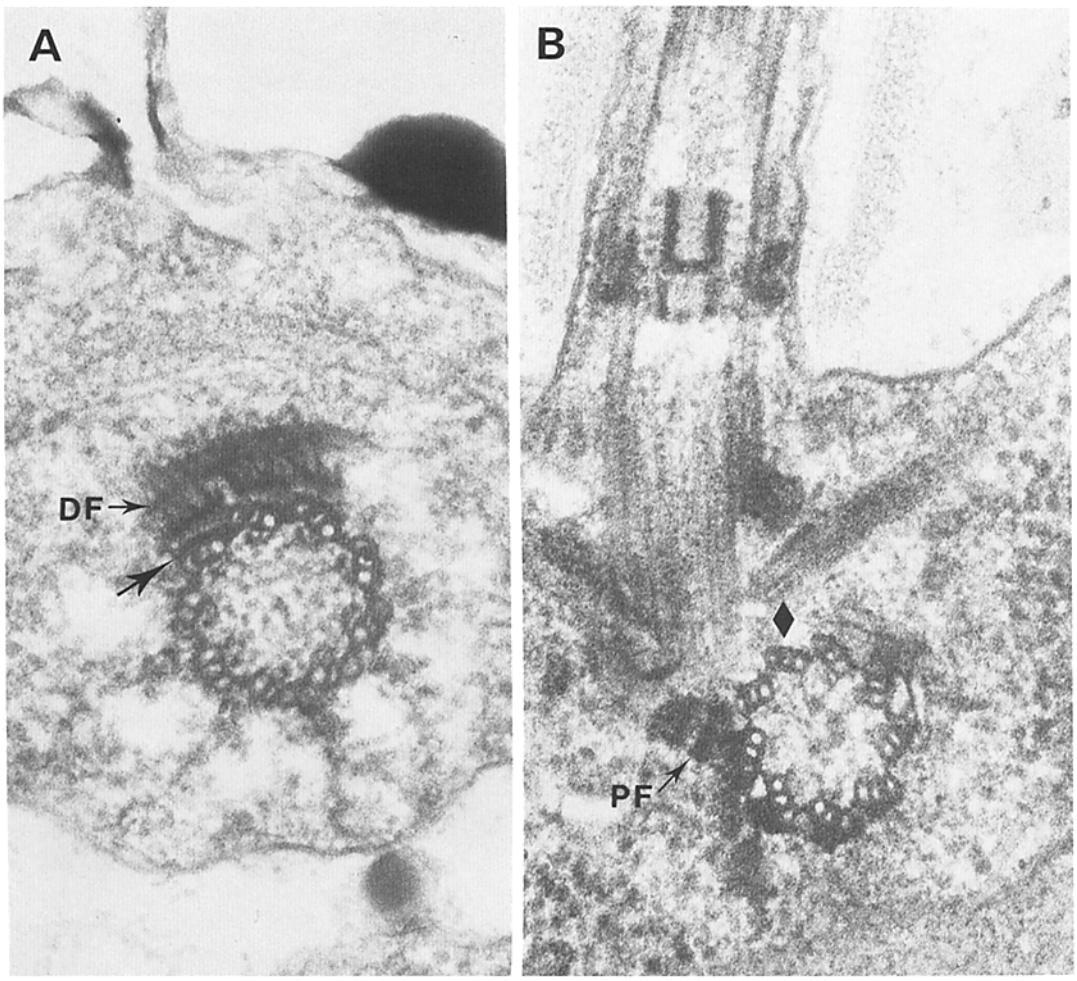

FIGURE 4 Sections through the *Chlamydomonas* basal body. (*A*) The distal striated fiber (*DF*) attaches to an electron dense covering (arrow) on three triplets; the middle of these triplets is continuous with the doublet lacking the outer arm. × 155,000. (*B*) The proximal striated fiber (*PF*) likewise attaches to specific triplets. The triplet continuous with the doublet lacking the outer arm is marked (diamond). Note that the two basal bodies are not exactly in the same plane, but are offset by about one half a basal body diameter. × 115,000.

eral link (Fig. 2*c*). The two-part bridge ends just distal to the stellate pattern (Fig. 2*d*). Here and more proximally, the lumens of the B-tubules of some doublets or triplets often contain material that is morphologically similar to the beak-like projections, but the material does not necessarily occur in the same regular pattern as in the axoneme. Therefore, none of the polarity markers described above can be used to identify individual doublets or triplets in these regions. However, specific microtubules can be traced in serial sections from the axoneme down into the basal body, so that the structural relationship of specific triplets to the striated fibers and other basal structures of the flagellar apparatus can be determined.

The two basal bodies are connected by the distal striated fiber (Fig. 4*a*). The filaments of this fiber insert into electron-dense structures that cover three adjacent triplets of each basal body (Fig. 4*a*). The middle of these triplets is continuous with the doublet that has the two-part bridge attached to its A-tubule. The proximal striated fibers also attach to specific doublets (Fig. 4*b*), but their attachment is more complex and will not be described here.

Tangential longitudinal sections of the basal body and proximal portion of the *Chlamydomonas* flagellum indicate that the triplets or doublets are not strongly twisted in this region. However, the two flagella are not in exactly the same plane (Fig. 4*b*), but are offset by one-half to one basal body diameter. This offset is in the clockwise direction as viewed from above (Fig. 5). As a result, the triplet that is continuous with the doublet having the two-part bridge attached to its A-tubule is no longer exactly opposite the corresponding triplet of the other basal body.

DISCUSSION

Both the presence of the beak-like projections in the B-tubules (38) and the lack of an outer dynein arm (15) have been previously reported in the axoneme of *Chlamydomonas*. In the present study, we found that each of these structural asymmetries, as well as the newly discovered two-part bridge, is associated with a specific axonemal doublet or doublets, and that these morphologically differentiated doublets occur in specific positions with respect to one another. These observations indicate that the axonemes of *Chlamydomonas* have an inherent structural polarity. The morphological differences between doublets also indicate that the doublets almost certainly differ in some of their polypeptide components. Con-

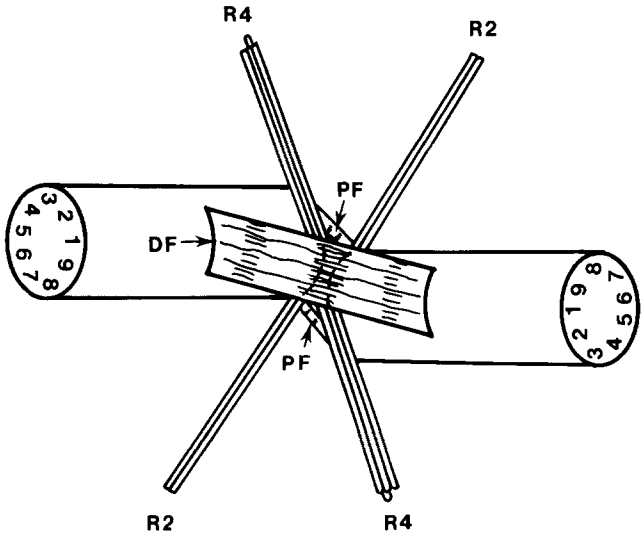

FIGURE 5 Diagrammatic representation of the flagellar apparatus of *Chlamydomonas* as seen from above. This diagram is in the correct absolute orientation. Note that the distal striated fiber (*DF*) and the proximal striated fibers (*PF*) are in a position to determine the rotational orientation of each axoneme. The relative positions of the doublets are indicated by numbers. *R2, R4:* two- and four-membered rootlets, respectively.

sequently, compositional heterogeneity among the doublets must now be considered in biochemical studies of the *Chlamydomonas* axoneme.

In discussing specific doublets, it is convenient to have a system of nomenclature for referring to individual doublets around the axoneme. The original basis for indexing doublets depended on the plane of the central pair of microtubules (1, 4). However, the central pair apparently rotates in *Chlamydomonas* (19, 20), as in certain other cilia and flagella (18, 24, 25, 26, 35), and thus cannot be used for this purpose. This is confirmed by the observation that the central pair of *Chlamydomonas* has a variable orientation at its proximal end (Hoops and Witman, unpublished results). In those organisms in which the doublets have been numbered relative to the plane of the central pair, the number 5 and 6 doublets are frequently connected by a bridge (1, 9, 33). These same two doublets are located on the side of the axoneme that is at the leading edge during the normal effective stroke (9, 33, 36), indicating that either the bridge *or* the direction of beat could be used as alternative devices for numbering the doublets. In *Chlamydomonas*, the situation is complicated by the fact that the two-part bridge is on the side opposite the direction of the effective stroke. We choose to use the latter as a basis for numbering the doublets of *Chlamydomonas*, because (*a*) the two-part bridge of *Chlamydomonas* may not be analogous with the 5–6 bridge of other organisms (see below), and (*b*) an indexing system based on beat pattern has the advantage that equivalently numbered doublets are apt to have similar roles in different organisms. In forward swimming *Chlamydomonas*, the flagella beat away from one another during the effective stroke (17, 32, 34). Therefore, if the doublets are indexed relative to the direction of the effective stroke in a manner consistent with that in other organisms, the two adjacent doublets with the beak-like structures are the number 5 and 6 doublets. The opposite doublet, also having a beak-like projection and lacking the outer arm, is the number 1 doublet. The doublets are numbered clockwise in the direction that the dynein arms point (Fig. 5).

The 5–6 bridge present in many animal cilia and flagella is diamond-shaped in appearance (1, 9, 33) and thus has a different morphology than the 1–2 bridge of *Chlamydomonas*. This, plus the fact that the two types of bridges are on opposite sides of the axoneme relative to the direction of the effective stroke, suggests that they have different origins. Nevertheless, it has been proposed that the 5–6 bridge prevents sliding between the doublets to which it is connected (37), and it seems likely that the 1–2 bridge has a similar function. To generate the approximately planar beat of *Chlamydomonas*, there must be either relatively little sliding or no sliding between doublets 1 and 2, depending on the exact plane of beat relative to a plane through the 1–2 bridge and the center of the axoneme. The 1–2 bridge may thus serve to restrain or prevent sliding between those two doublets in the region where it occurs. The absence of the outer arm on doublet 1 is consistent with there being little or no sliding between doublets 1 and 2.

Our analysis of the rotational orientation of the flagella showed that each axoneme is oriented in the same way relative to its direction of beat. This strongly suggests that an internal, asymmetrically distributed axonemal component determines the direction of the effective stroke. This is supported by the observation that isolated, reactivated axonemes of *Chlamydomonas* attached to a glass surface do not change the direction of their effective stroke over many beat cycles (3).

Because the dynein arms point clockwise when the axonemes are viewed from base-to-tip, and the number 1 doublets of each axoneme face each other, the two flagella of a cell have 180° rotational symmetry. Previously, it was suggested that the arrangement of striated fibers and microtubular rootlets of chlorophycean unicellular motile cells, including *Chlamydomonas*, display 180° rotational symmetry when viewed along the cell axis (7). The arrangement of these components in *Chlamydomonas* is diagrammed in Fig. 5 (unpublished data). The fact that both the flagella and the flagella-associated structures have the same type of symmetry suggests that the flagella-associated structures should interact with unique portions of the basal bodies. Indeed, our study revealed that the distal striated fiber always attaches to triplets 9, 1, and 2 of both basal bodies. Likewise, the striated proximal fibers attach to specific regions of each basal body. The information necessary for morphogenesis of the flagellar apparatus may therefore be present in the basal bodies themselves. Attachment of the striated fibers to specific sites on the basal bodies probably results in the axonemes being set up or maintained in the correct rotational orientation to give the observed beat pattern. This is supported by the observation that a *Chlamydomonas* mutant with missing or abnormal striated fibers also has abnormal rotational orientation of its axonemes (13).

Because the flagellar apparatus has 180° rotational symmetry, the beat envelopes of the two flagella of a swimming cell should also display 180° rotational symmetry. Isolated, reactivated axonemes of *Chlamydomonas* appear to have a slight three-dimensional component in both the asymmetrical (corresponding to forward swimming) and symmetrical (corresponding to reverse swimming) beating modes (3). The structural symmetry of the flagellar apparatus suggests that if aplanarity is also present in the beat envelopes of flagella in situ, the swimming cell should roll around its longitudinal axis. Indeed, under our conditions of observation, the cells do clearly roll as they swim through the media (R. Kamiya

and G. B. Witman, manuscript submitted for publication). This is in contradiction to previously published reports (29, 32), but is predicted by current theories of how *Chlamydomonas* phototaxes (8).

In many other green algae, particularly those belonging to the Ulvaphyceae, there are septations that extend all the way across the lumen of the B-tubule (12). Like the beak-like projections in *Chlamydomonas*, these are located in two adjacent doublets and in the doublet on the opposite side of the axoneme (12). Serial sections of one of these algae (*Ulvaria*; unpublished) indicate that these doublets are in the same position relative to the effective stroke as in *Chlamydomonas*. In addition, the flagella of *Chlorosarcinopsis* lack an outer dynein arm on one doublet (23). The markers used in the present study therefore appear to be widespread among green algae.

Doublets number 1, 5, and 6 also appear to be morphologically specialized in the cilia and flagella of many different animal species. In certain animal sperm, the lumens of one or more A-tubules may contain septa or other electron-dense material. In such cases, wherever three or more doublets contain electron-dense material, these doublets include numbers 1, 5, and 6 with only a single exception (2, 22). Although the functions of these doublet specializations are not yet known, the fact that they occur in the same or in closely related arrangements in a large number of phylogenetically diverse organisms suggests that they play an important role in flagellar axoneme morphogenesis or function.

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