Abstract. Strains of Chlamydomonas reinhardtii with a mutant allele at the BOP2 locus swim slowly and have an abnormal flagellar waveform similar to previously identified strains with defects in the inner arm region. Double mutant strains with the bop2-1 allele and any of 17 different mutations that affect the dynein arm region swim more slowly than either parent, which suggests that the bop2-1 mutation does not affect solely the outer dynein arms, the II or ida4 inner dynein arms, or the dynein regulatory complex. Flagellar axonemes isolated from bop2-1 cells are missing a phosphorylated polypeptide of 152 kD. Electron microscopic analysis shows that bop2-1 axonemes are missing density in the inner dynein arm region. Surprisingly, two populations of images were observed in longitudinal sections of axonemes from the bop2-1 strain. In the 10 longitudinal axonemes examined, a portion of the dynein regulatory complex and a newly identified structure, the projection, are affected. In five of these 10 longitudinal axonemes examined, two lobes of the ida4 inner arm are also missing. By examining the cross-sectional images of wild-type and bop2-1 axonemes at each outer doublet position around the axoneme, we have determined that the bop2-1 mutation affects the assembly of inner arm region components in a doublet specific manner. Doublets 5, 6, and 8 have the most severe deficiency, doublet 9 has an intermediate phenotype, and doublets 2, 3, 4, and 7 have the least severe phenotype. The bop2-1 mutation provides the first evidence of radial asymmetry in the inner dynein arm region.

In the unicellular green alga Chlamydomonas reinhardtii, two flagella generate force to move the cell. The beat stroke is highly asymmetric and consists of two stages. In the effective stage, a power stroke, which travels almost entirely in two dimensions, is initiated by a single bend that forms near the base of the flagellum while the remainder of the flagellum is straight. In the recovery stage, a three-dimensional stroke is initiated near the base of the flagellum and propagates distally, leaving the flagellum in the starting position (Brokaw et al., 1982). An asymmetric beat stroke suggests that the flagella are structurally asymmetric. The focus of much research in recent years has been the regulation of dynein activity, but the relationship between asymmetry of the beat stroke and dynein regulation is not well understood.

Three types of asymmetry have been observed in the flagella of C. reinhardtii. Asymmetries have been reported among specific outer doublets of the flagella (radial asymmetry), along the length of the flagellum (proximal/distal asymmetry), and between the two flagella (cis/trans asymmetry). For example, outer doublets 1, 5, and 6 are different from the other outer doublets. Doublets 1, 5, and 6 contain a B-tubule projection, which extends partially across the lumen of the microtubule (Hoops and Witman, 1983). These three doublets are located in the plane of the power stroke; doublets in these positions are predicted to exhibit the least microtubule sliding relative to their neighbors during the power stroke (Hoops and Witman, 1983). Several mutant strains (mbo1,2,3), which are missing the B-tubule projections from doublets 5 and 6, are able to swim only backwards and the flagella utilize a sinusoidal waveform that is similar to sperm flagella waveforms (Segal et al., 1984). A two-membered cross-bridge that extends from doublet 1 towards doublet 2 and the absence of an outer dynein arm from over 90% of the length of doublet 1 are two additional radial asymmetries (Hoops and Witman, 1983).

The second flagellar asymmetry exists between regions that are proximal to the basal bodies and regions that are distal. Several of the high molecular weight (HMW), inner arm dynein chains are hypothesized to be localized to specific regions of the flagella. Electron microscopic and biochemical examinations of partially extracted axonemes suggest that HMW polypeptide 3 is present in proximal but not in distal portions and the HMW polypeptide 2 is localized predominantly to proximal regions of the axoneme.
A second example of proximal/distal asymmetry is the sensitivity of flagella to the addition of colchicine. When colchicine is added to the medium, the distal 25–50% of the flagella is disassembled. The biochemical basis for this sensitivity is not known (Dentler and Adams, 1992), but it is possible that some factors that affect flagellar length may be asymmetrically positioned along the length of the axoneme. A third example of proximal/distal asymmetry is the localization of the B-tubule projections and two-membered cross-bridge described above. The B-tubule projections are present only in the proximal 40–57% of the flagella and the two-membered cross-bridge is present only in the proximal one quarter of the axoneme (Hoops and Witman, 1983).

The observed asymmetry is based on functional differences between the two flagella. The two flagella are termed cis and trans based on their position relative to an eyespot; the cis flagellum is closer to the eyespot (Huang et al., 1982a). The trans flagellum is always initiated from the parental basal body while the cis flagellum is initiated from the daughter basal body (Holmes and Dutcher, 1989). Cis/trans asymmetries may play a role in phototactic behavior. Supporting this hypothesis, different responses between the two flagella to light (Rüffer and Nultsch, 1991) and Ca²⁺ (Kamiya and Witman, 1984) stimuli have been observed. The beat patterns of the two flagella are altered coordinately to changes in light stimuli. The beat amplitude of one flagellum is increased while the beat amplitude of the other flagellum is decreased in response to changes in light intensity in vivo (Rüffer and Nultsch, 1991). Preferential beating of the two axonemes is observed in response to different Ca²⁺ concentrations in detergent-extracted cell models. The cis axoneme is more active at 10⁻⁷ M Ca²⁺ whereas the trans axoneme is more active at 10⁻⁴ M Ca²⁺ (Kamiya and Witman, 1984). No biochemical or morphological difference between the two flagella has been reported.

We have characterized a mutation in C. reinhardtii that is involved in the control of flagellar waveform (Dutcher et al., 1988). The bop2-1 strain was isolated as an extragenic suppressor of the motility phenotype observed in a pflO strain (bop = bypass of paralysis). The bop2-1 strain exhibits a swimming phenotype that is similar to those of inner dynein arm region mutant strains. Flagellar axonemes from the bop2-1 strain are missing a 152-kD phosphoprotein that was not localized previously to a particular axonomere structural component. Electron micrograph analyses of bop2-1 axonemes demonstrate that this mutation affects the assembly of inner arm region components in a doublet specific manner. The bop2-1 mutation provides the first evidence of radial asymmetry in the inner dynein arm region of the flagella.

**Materials and Methods**

**Microscopic Techniques**

Slides were prepared and phase microscopy was performed as described previously (Inwood, 1985). The swimming patterns of cells and flagellar movements were observed and recorded on a Zeiss Universal microscope using darkfield optics with a 16 x 40 x Plan objective and an oil darkfield condenser (NA = 1.2–1.4). Samples were illuminated by a xenon lamp powered by a Chadwich Helmuth model 136 power supply. The power supply, kindly supplied by E. D. Salmon (University of North Carolina, Chapel Hill, NC), was pulsed by a Grass Instruments pulse generator for stroboscopic effects. Images were recorded with a 35 mm Nikon camera on Tri-X film.

**Electron Microscopy, Digitization, and Averaging Techniques**

Axonemes were prepared for electron microscopy as described in Porter et al. (1992). Sections of 60-nm nominal thickness were used for the cross-sectional analysis; 40-nm sections were used for the longitudinal analysis. All microscopy was performed on a Philips CM10 electron microscope (Phillips Electronic Instruments Co., Mahwah, NJ) operating at 80 kV. The methods for selecting, digitizing, averaging, normalizing, and comparing cross-sectional outer doublets were as described previously (Mastronarde et al., 1992). Sample averages were obtained from 50 to 100 outer doublets.

**Gel Electrophoresis**

Two-dimensional SDS-PAGE was performed as previously described (Piperno et al., 1981). In the 2D analysis of wild-type and bop2-1 axonemes, cells were harvested from low-phosphate medium plates into nitrogen-free, phosphate-free medium and then incubated for 10 min with [32P]orthophosphoric acid at a ratio of 1 mCi/10⁶ cells (Segal et al., 1984). Over 90% of the radiolabeled was taken up by the cells. One-dimensional Neville gels with a 4–8% acrylamide gradient were prepared as in Adams et al. (1981). Autoradiography was performed as described by Segal et al. (1984). Gels were silver stained by the method of Blum et al. (1987) or the method of Merrill et al. (1981) with the following modifications. Gels were washed three times for 30 s each after potassium dichromate treatment; they were left for 45 min in bright light in the silver nitrate solution; and a wash with sodium carbonate preceded the wash with sodium carbonate and formaldehyde. After staining, these gels were photographed in a 2% glycerol solution with a Watten No. 8 filter and then dried onto a dialysis membrane. Molecular weights were calculated from the MW-SDS-200 standards from Sigma Chemical Co. (St. Louis, MO).

**Dikaryon Rescue Experiments**

Temporary dikaryons were constructed as described in Dutcher et al. (1984). For the biochemical analysis of the dikaryons, a wild-type parent was grown on solid medium with a reduced concentration of sulfate and the second parent (bos2 pflO) was grown on similar medium containing 25 mCi of [35S]sulfuric acid/liter for 3 d at 25°C in constant light (25 µE/m²/s) and then for 2 d at 21°C in subdued light (6 µE/m²/s). Cells of each strain were harvested into nitrogen-free medium at a density of ~1 x 10⁶ cells/ml and allowed to differentiate for 5 to 10 h. The gametes of opposite mating types were mixed. After 10 min, anisomycin was added to 60 µM to inhibit protein synthesis. (Anisomycin was a gift of Pfizer, Inc., Groton, CT). After 1 h, flagellar axonemes were isolated as described previously (Porter et al., 1992). Because only 30% of the cells mated in the experiment, overexposures of the autoradiographs were examined.

**Reversion Analysis**

A bop2-1 oda9 enh2 parental strain was mutagenized by ultraviolet irradiation to 80% survival (Dutcher et al., 1988). oda9 is a mutation that affects the assembly of the entire outer dynein arm (Kamiya, 1988) and enh2 is a mutation found in our stock collection that enhances the phenotype of the bop2-1 oda9 double mutant strain. bop2-1 oda9 strains swim slower than either parental strain and enh2 has no phenotype alone or with bop2-1 or oda9, singly. Enrichment screens for swimming cells were performed by transferring 10% of the medium near the nemoxic to new medium. Once enriched, the cells were allowed to grow to ~1 x 10⁶ cells/ml and the procedure was repeated. After four rounds of enrichment, cells were plated for single colonies and the swimming phenotypes were analyzed. To identify intragenic reversion events at one of the three loci, the strains were crossed by a wild-type strain. Between 6 and 10 tetrads were analyzed for each strain with an average of 137 tetrads. Intragenic revertants or extragenic suppressors that are closely linked to bop2-1, oda9, or enh2 should produce tetrads with four meiotic progeny that swim and no meiotic progeny that fail to swim. The revertants that produced all swimming progeny were crossed to three additional strains (bop2-1, oda9, and oda9 enh2) and the meiotic progeny were analyzed to determine the locus where reversion may have occurred. Intragenic revertants at the BOP2 locus (or closely linked extragenic suppressors) would produce meiotic progeny that cannot swim only when crossed to the bop2-1 tester, which could generate bop2-1 oda9 enh2 progeny. Events at or near the ODA9 locus would give rise to nonswimmers when crossed to either the oda9 or oda9 enh2 tester, and events at or near the ENH2 locus would give rise to nonswimmers only when crossed to the oda9 enh2 tester.
selected from each preparation. To control for preparation variability, averages from at least five different preparations of the same strain were normalized on the optical density of the outer dynein arms and combined to obtain a grand average (Mastronarde et al., 1992).

To obtain cross-sectional average images for any particular doublet, we identified and grouped corresponding microtubule doublets from the nine positions around the axoneme. Doublet 1 was identified by the absence of an outer dynein arm. In images where we could see B-tubule projections, the doublet identified as doublet 1 by the absence of the outer dynein arm always correlated with doublet 1 as identified by the location of the B-tubule projections. A computer program written by Dr. David Mastronarde (Boulder Laboratory for 3-D Fine Structure, Boulder, CO) was then used to identify and average doublets from the other eight positions. Individual outer doublet averages and group outer doublet averages, which are comprised of any subset of individual outer doublet averages, were then compared in an analysis of variance as described below. Images of doublet 1 could not be normalized on the density of their outer dynein arms since this structure is missing. Images of doublet 1 were therefore normalized on the basis of individual microtubule protofilaments (Mastronarde et al., 1992). 12% of axonemal cross-sections did not have a clear doublet 1 and these images were obtained from a pixel-by-pixel nested analysis of variance (Mastronarde et al., 1992). We selected images of axonemes with clear outer arms, radial spokes, and five or more of the 96-nm inner arm region repeating units. The selected repeating units from each longitudinal image were aligned and averaged to provide an individual axoneme average. Either a subset or all of the individual axoneme averages from a particular strain were then aligned and averaged together to give a longitudinal group or grand average, respectively.

Quantitative comparisons between wild-type and bop2-1 averages were performed for both cross-sectional and longitudinal images. Difference images were obtained from a pixel-by-pixel nested analysis of variance (Mastronarde et al., 1992). For the cross-sectional difference images, differences not significant at the 0.05 level were set to zero. For the longitudinal difference images, differences not significant at the 0.0025 level were set to zero. During the course of our work, it came to our attention that the longitudinal model published in Mastronarde et al. (1992) was a vertical mirror image of the correct orientation of inner arm region structures. The dynein arm structures in the figures of Mastronarde et al. (1992) should be interpreted as protruding through the back of the microtubule instead of extending toward the viewer.

The techniques used for digitizing and averaging the longitudinal images and the methods for comparing images were as described in Mastronarde et al. (1992). We selected images of axonemes with clear outer arms, radial spokes, and five or more of the 96-nm inner arm region repeating units. The selected repeating units from each longitudinal image were aligned and averaged to provide an individual axoneme average. Either a subset or all of the individual axoneme averages from a particular strain were then aligned and averaged together to give a longitudinal group or grand average, respectively.

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Other Methods

The media for culturing cells were as described previously (Holmes and Dutcher, 1989). Many of the mutant strains were kindly provided by Dr. Elizabeth Harris of the Chlamydomonas Genetics Center (Duke University, Durham, NC). The ida4 and ida4 mutant strains were provided by Dr. Ritsu Kamiya (University of Tokyo, Tokyo, Japan). The wild-type strain used throughout this work was strain 137c (CC-125). Genetic analysis was performed essentially as described by Levine and Ebersold (1960) and Harris (1989).

Results

Phenotypic Analysis

The bop2-1 mutation was isolated as an extragenic suppressor that restored partial motility to the pf10 mutant strain (Dutcher et al., 1988). pf10 mutant cells have an altered flagellar waveform; the beat stroke is nearly symmetric and the cells are unable to move forward (Inwood, 1985). In the pf10 bop2-1 double mutant strain, the cells swim with a velocity of $88 \pm 15 \mu m/s$ ($n = 50$ cells) compared to a velocity of $176 \pm 7 \mu m/s$ ($n = 50$ cells) for wild-type cells. bop2-1 cells swim with a velocity of $102 \pm 10 \mu m/s$ ($n = 50$ cells) and exhibit a flagellar waveform that resembles the pattern observed in previously characterized inner arm region mutant strains (Brokaw and Kamiya, 1987; Kamiya, 1988; Porter et al., 1992). The BOP2 locus maps to a previously unidentified locus on linkage group IV (Dutcher et al., 1988).

Genetic Interactions of the bop2-1 Mutation

We performed epistasis tests with bop2-1 and other flagellar mutations. Since the bop2-1 strain had a motility phenotype similar to other dynein region mutant strains, double mutant strains were constructed with four classes of dynein region mutant strains. We examined representative alleles at each of 10 loci that fail to assemble outer dynein arms, alleles at three loci that fail to assemble a complete dynein regulatory complex (drc), alleles at one locus that fail to assemble the II inner dynein arms, and one allele that fails to assemble the inner dynein arms affected by the ida4 mutation. In all cases, the double mutant strains have a new phenotype. The double mutant strains have a reduced velocity compared with either parental strain (Table I). Therefore the bop2-1 mutation is unlikely to affect solely the II or ida4 inner dynein arms, the outer dynein arms, or the drc. Many of the double mutant strains showed increased percentages of aflagellate cells in the population (data not shown). A similar afagellate phenotype has been observed previously in many double mutant strains and is not specific to combinations of mutations in the dynein arms (Piperno et al., 1990; Porter et al., 1992).

We also asked if the bop2-1 mutation was a bypass suppressor of several paralyzed flagellar mutations. Some classes of mutant strains are able to suppress the paralysis that results from mutations that disrupt radial spoke and/or central pair assembly (Huang et al., 1982b; Piperno et al., 1992; Porter et al., 1992). 10 mutations that affect structures or processes that are required for normal flagellar beatin

| Table I. Genetic Interactions between bop2-1 and Other Dynein Arm Mutations |
| Mutant strain | Structure/polypeptide affected | Single mutant phenotype | Double mutant phenotype |
|----------------|-------------------------------|-------------------------|------------------------|
| bop2-1         |                               | Slow swimming           | Slower swimming         |
| oda1           | ODA                           | Slow swimming           | Slower swimming         |
| oda2           | ODA                           | Slow swimming           | Slower swimming         |
| oda3           | ODA                           | Slow swimming           | Slower swimming         |
| oda4           | ODA                           | Slow swimming           | Slower swimming         |
| oda5           | ODA                           | Slow swimming           | Slower swimming         |
| oda6           | ODA                           | Slow swimming           | Slower swimming         |
| oda7           | ODA                           | Slow swimming           | Slower swimming         |
| oda8           | ODA                           | Slow swimming           | Slower swimming         |
| oda9           | ODA                           | Slow swimming           | Slower swimming         |
| oda10          | ODA                           | Slow swimming           | Slower swimming         |
| supfl          | ODA                           | Slow swimming           | Slower swimming         |
| pf2            | DRC                           | Slow swimming           | Slower swimming         |
| pf3            | DRC                           | Slow swimming           | Slower swimming         |
| sup3           | DRC                           | Wild-type motility      | Slower swimming         |
| pfl-1, pf9-2   | ADA                           | Slow swimming           | Slower swimming         |
| ida1           | ADA                           | Slow swimming           | Slower swimming         |
| ida4           | ADA                           | Slow swimming           | Slower swimming         |

Structure/polypeptide affected compiled with data from McVittie, 1972, Huang et al., 1982a, Kamiya, 1988, Kamiya et al., 1991, and Porter et al., 1992. ODA, outer dynein arms; DRC, dynein regulatory complex; ADA, inner dynein arms.
were tested (Table II). In all cases, the double mutant strains were paralyzed, so the bop2-I mutation does not act as a bypass suppressor of the mutations tested.

**Biochemical Phenotype of bop2-I Axonemes**

We examined bop2-I axonemes by two-dimensional SDS-PAGE to determine if a biochemical phenotype existed. Both wild-type and bop2-I cells were labeled with [35S]sulfuric acid and flagellar axonemes were isolated. A single prominent polypeptide present in wild-type axonemes is missing from bop2-I axonemes (Fig. 1). This polypeptide has a molecular weight of 152 kD and an isoelectric point of 6.2 and is not missing in any previously characterized mutant strain.

We examined axonemes from the progeny of tetrads from a bop2-I × wild-type cross that had been incubated with [32P]orthophosphoric acid to ask if the 152-kD polypeptide is present in axonemes in a phosphorylated form. The axonemes were analyzed by one-dimensional PAGE. A 152-kD polypeptide was present in wild-type segregants and absent in bop2-I segregants (Fig. 2). It is likely that the 152-kD polypeptide is present only in a phosphorylated form in the axoneme since only one spot is observed by 35S two-dimensional SDS-PAGE of wild-type axonemal polypeptides in this region (Fig. 1). Therefore, the bop2-I mutation results in the loss of a single 152-kD phosphoprotein from the axoneme.

Cosegregation of the biochemical and flagellar phenotypes was assayed in meiotic progeny from a bop2-I pf10 × wild-type cross. When analyzed by one-dimensional PAGE, the 152-kD polypeptide was missing from eighteen independent meiotic progeny that exhibited the bop2-I motility phenotype and each of these strains was able to suppress the pf10 mutation in an additional cross (data not shown). Therefore, the motility, suppression, and biochemical phenotypes cosegregate with the bop2-I mutation and are at most 5.3 centimorgans apart.

**Biochemical Phenotype of Double Mutant Strains**

We examined the biochemical phenotype of several double...
Figure 3. Silver stained one-dimensional gel electrophoretograms of a meiotic tetrad from a cross of p9-2 BOP2 × PF9 bop2-1 parents. Molecular weight standards of 205, 116, 97, and 66 kD are shown on the left. Arrows indicate the missing polypeptides with apparent molecular weights of 152,000, 140,000, and the additional polypeptides with apparent molecular weights of 100,000, and 97,000. (a) Axonemal polypeptides from bop2-1 PF9 cells. A polypeptide of 152 kD is missing. (b) Axonemal polypeptides from BOP2 PF9 cells. A polypeptide of 140 kD is missing. This gel system does not resolve the 110-kD polypeptide missing from p9-2 axonemes. (c) Axonemal polypeptides from bop2-1 pf9-2 cells. The 152- and 140-kD polypeptides are missing as in lanes a and b. In addition, axonemes from the double mutant strain contain two new additional polypeptides with apparent molecular weights of 100,000 and 97,000. (d) Axonemal polypeptides from wild-type (BOP2 PF9) cells.

Dikaryon Rescue Analysis

Dikaryon rescue experiments in Chlamydomonas have provided evidence about gene–gene product relationships (Luck et al., 1977; Dutcher et al., 1984). An analysis of temporary dikaryons was used to examine the relationship between the suppression of the pfl0 motility phenotype and the 152-kD polypeptide. Dikaryons were examined from three different matings, BOP2 pfl0 × BOP2 PF10, bop2-1 pfl0 × BOP2 PF10, and bop2-1 pfl0 × BOP2 pfl0 (Table III). Examination of the flagellar beat strokes in quadriflagellate zygotic cells from the BOP2 pfl0 × BOP2 PF10 mating showed that pfl0 and wild-type flagella maintain different beat strokes in temporary dikaryons, as previously observed (Dutcher, 1986). However, in bop2-1 pfl0 × BOP2 PF10 and bop2-1 pfl0 × BOP2 pfl0 dikaryons, the suppression of the pfl0 phenotype by the bop2-1 mutation is lost within one hour in two of the four flagella. Presumably, the wild-type BOP2 gene product is present in the cytoplasm of the BOP2 parent, and incorporation into the flagella results in the loss of suppression. Therefore the bop2-1 allele is recessive to the wild-type allele in dikaryons as well as in heterozygous diploid strains (Dutcher et al., 1988).

To determine if the 152-kD polypeptide is present in the bop2-1 pfl0 cytoplasmic pool of axonal proteins, we mated 35S-labeled bop2-1 pfl0 cells with unlabeled BOP2 PF10 cells and isolated the axonemes from the heterozygous dikaryons one hour after mating. The recovery of the pfl0 phenotype in dikaryons is not accompanied by the appearance of a labeled 152-kD protein on one-dimensional gels (data not shown). This result implies that the 152-kD phosphoprotein is not available from the cellular pool of labeled polypeptides contributed by the bop2-1 pfl0 cells.

Reversion Analysis

We performed a reversion analysis of the bop2-1 allele with the goal of identifying the gene product of this locus. We hoped to find pseudorevertants that would have an altered biochemical phenotype compared to both the wild-type and original mutant strains (Luck et al., 1977). Since revertants of the bop2-1 slow swimming phenotype would be difficult to identify without a laborious microscopic screen, we mutagenized a mutant strain that had a synthetic phenotype. The triple mutant strain that contains bop2-1, the outer dynein arm mutation od69, and a third mutation enh2, has very few motile cells in the supernatant when grown in the presence of 25 μE/m2/s of white light and forms a pellet that contains primarily aflagellate cells. We screened for new mutational events that restored motility to this triple mutant strain as as-

Table III. Heterozygous Dikaryon Phenotypes

| Parental genotypes                  | Motility phenotype of dikaryon flagella directly after mating | Motility phenotype of dikaryon flagella 1 h after mating |
|-------------------------------------|-------------------------------------------------------------|----------------------------------------------------------|
| BOP2 pfl0 × BOP2 PF10              | 2 wild-type                                                 | 2 wild-type                                              |
|                                     | 2 pfl0                                                      | 2 pfl0                                                   |
| bop2-1 pfl0 × BOP2 PF10            | 2 wild-type                                                 | 2 wild-type                                              |
|                                     | 2 suppressed                                                | 2 pfl0                                                   |
| bop2-1 pfl0 × BOP2 pfl0            | 2 pfl0                                                      | 4 pfl0                                                   |
|                                     | 2 suppressed                                                |                                                          |
sayed by the presence of cells in the supernatant of liquid medium. 53 independent revertants were isolated and analyzed as described in Materials and Methods. 38 of the 53 revertants contained extragenic suppressors. One strain appeared to be an intragenic revertant at the ENH2 locus. The remainder (n = 14) appeared to be intragenic revertants at the ODA9 locus. Since no bop2-1 revertants were found, the reversion analysis was unable to provide evidence about the gene product of the BOP2 locus.

**Structural Phenotype of the bop2-1 Strain**

Axonemes from bop2-1 cells were examined by electron microscopy to see if they displayed a structural phenotype. Because the motility phenotype of the bop2-1 strain is similar to that of previously characterized inner arm region mutant strains, we focused our analysis on this region. Axonemes from wild-type and bop2-1 strains were examined as described in Materials and Methods. The grand averages of cross-sectional images from these strains are shown in Fig. 4 (a and b). From a comparison of the contour lines in the inner arm region in Fig. 4 (a and b), a loss of density is apparent in the bop2-1 grand average compared to the wild-type grand average. A difference image between the wild-type and bop2-1 grand averages shows that two lobes of density are missing in bop2-1 axonemes compared to wild-type axonemes (Fig. 4 c). The more severe loss falls in the inner domain of the inner arm region and an additional loss is located near the base of the inner arm region. The bop2-1 structural phenotype is statistically different from the phenotypes of axonemes from the pf23, pf9, ida4, or pf2 mutant strains, which also have defects in the inner arm region (data not shown; Mastronarde et al., 1992). Therefore, the bop2-1 allele represents a new class of mutation that affects the structure of the inner arm region.

We performed a longitudinal analysis of axonemes from the bop2-1 strain to ask which of the major lobes of density within each 96-nm repeating unit of the inner arm region were affected. The grand averages from wild-type and bop2-1 longitudinal images are shown in Fig. 5 (a and b), respectively. A different image between the wild-type and bop2-1 grand averages shows that the bop2-1 axonemes are deficient in four structures (Fig. 5 c). Structure 1 (hereafter called the projection) extends distally between lobe 4 (described below) and the outer domain of the inner arm region, is lightly stained, and was not identified previously (Mastronarde et al., 1992). Structure 2 is a portion of the drc and was missing in pf2 axonemes. Structure 3 is a portion of the most distally located ida4 structure in the 96-nm repeat (Mastronarde et al., 1992). Structure 4 is located under the proximal radial spoke; it was missing in ida4 axonemes and was termed lobe 4 (Mastronarde et al., 1992). As might be expected from the cross-sectional phenotype, the longitudinal phenotype of the bop2-1 strain is significantly different from the phenotypes of the pf23, pf9, ida4, or pf2 mutant strains (data not shown; Mastronarde et al., 1992). Thus, the bop2-1 mutation affects the assembly of one newly identified structure and three previously characterized structures.

A conspicuous feature of the individual longitudinal averages obtained from bop2-1 axonemes was the heterogeneity of the phenotypes. Of the 10 bop2-1 individual axoneme averages, five were missing the lobe 4 density and five contained the lobe 4 density. The lobe 4 density was present in all nine wild-type individual axoneme averages. In the bop2-1 axonemes whose individual averages were missing lobe 4 density, the density was missing in all of the repeats. In the axonemes whose individual averages contained lobe 4 density, the density was present in all of the 96-nm repeats (data not shown). One individual axoneme average from bop2-1 axonemes that is deficient for lobe 4 is shown in Fig. 5 d and two individual axoneme averages that contain lobe 4 are shown in Fig. 5 e and f.

Because of the heterogeneity in individual axoneme averages, we averaged the bop2-1 axonemes that contained or lacked the lobe 4 density to define the fine structure of both classes of axonemes. The group average of the five axonemes that contained the lobe 4 density and a difference image compared to wild-type are shown in Fig. 5 g and h. There was no significant difference from wild-type structure in either lobe 4 or the distally located ida4 density. The group average of the five axonemes that lack lobe 4 density and a difference image compared to wild-type are shown in Fig. 5 i and j. In this subset, in addition to lobe 4, the more distally located ida4 structure is also deficient. Both sets of bop2-1 axonemes
Figure 5. Longitudinal phenotypes of bop2-1 axonemes. Outer dynein arms are at the top; radial spokes are at the bottom; the proximal end of the 96-nm repeating unit is at the left. Inner arm region structure is located between the radial spokes and outer dynein arms. (a) The grand average of wild-type from nine axonemes and 62 repeating units. (b) The grand average of bop2-1 from 10 axonemes and 69 repeating units. (c) A difference image between wild-type and bop2-1 grand averages. The structures labeled 1 through 4 are described in the text. (d) The individual axoneme average of a bop2-1 axoneme that is missing the lobe 4 density (n = 6 repeating units). The arrow shows the expected position of lobe 4. (e and f) The individual axoneme averages of two bop2-1 axonemes that contain the lobe 4 density (n = 6 and 7 repeating units, respectively). The arrowheads show the position of lobe 4. (g) The group average of five bop2-1 axonemes that contain lobe 4 density (n = 32 repeating units). (h) A difference image between the wild-type grand average and the group average in g. (i) The group average of five bop2-1 axonemes that are missing lobe 4 density (n = 37 repeating units). (j) A difference image between the wild-type grand average and the group average in i. In c, h, and j, differences not significant at the 0.0025 level of significance were set to zero. Bar, 25 nm.
are deficient for a portion of the drc and for the projection. Thus, the longitudinal grand average was a composite of axonemes missing a portion of the drc and the projection and axonemes missing those structures and two ida4 inner arm structures.

**Inner Arm Region Asymmetry Revealed by the bop2-1 Mutation**

Two alternative models could explain the heterogeneity in the longitudinal images of bop2-1 axonemes. First, the loss of structure in bop2-1 axonemes could be a result of random loss of inner arm region structures. In this scenario, the deficiencies in the axonemes would not correlate to known flagellar asymmetries. In the second model, the loss of structure in bop2-1 axonemes could be revealing one of the three inherent asymmetries in the axoneme; these are the radial, proximal/distal, and cis/trans asymmetries.

The longitudinal images do not contain sufficient positional information to determine if the heterogeneity correlates with one of the known asymmetries. However, cross-sectional images contain morphological markers for both radial and proximal/distal asymmetries. Therefore we reanalyzed the cross-sectional images to determine if a correlation existed between an axonemal asymmetry and the two classes of bop2-1 axonemal defects. The relative position of each outer doublet in the data sets was determined based on its position relative to doublet 1, which lacks an outer dynein arm and contains a two-membered cross-bridge and a B-tubule projection (Hoops and Witman, 1983). Images from each specific outer doublet were pooled, aligned, averaged, and normalized to provide grand averages for each outer doublet position. A comparison of the integrated intensities of the whole inner arm region of doublets 2–9, individually and combined, from both bop2-1 and wild-type axonemes is shown in Fig. 6. Our analysis shows that bop2-1 outer doublets 5, 6, 8, and 9 contain significantly less inner arm region intensity than the corresponding wild-type doublets. bop2-1 outer doublets 2, 3, 4, and 7 do not have significantly less intensity than the corresponding wild-type doublets even though the average intensity of these bop2-1 outer doublets is less than the wild-type average (see below). Thus, it appears that the bop2-1 mutation affects the assembly of inner arm region structures in a doublet specific manner. The bop2-1 mutation provides the first evidence of radial asymmetry in inner arm region structures.

While the analysis above shows that inner arm region structure in outer doublets 5, 6, 8, and 9 is affected in bop2-1 axonemes, we wanted to know if the affected doublets carried equivalent deficiencies. First, we compared the structure of wild-type doublets 5, 6, 8, and 9 to wild-type doublets 2, 3, 4, and 7. Except for the presence of the B-tubule projection (present in doublets 5 and 6), no differences were present between these two groups (data not shown). We then generated individual doublet averages for bop2-1 doublets 5, 6, 8, and 9 and compared them to the overall grand average from all the wild-type outer doublets. Difference images are shown in Fig. 7 (a–d). bop2-1 outer doublets 5, 6, and 8 are missing intensity in both lobes whereas bop2-1 doublet 9 is missing density only from the lobe in the inner domain (compare Figs. 4 c and 7 e). Therefore, the bop2-1 outer doublets with significantly less intensity in the inner arm region (Fig. 6) can be put into two classes with doublets 5, 6, and 8 missing more inner arm region density than doublet 9.

Even though the entire inner arm region intensities of doublets 2, 3, 4, and 7 from bop2-1 axonemes are not significantly less than the intensities in the corresponding wild-type doublets (Fig. 6), we examined the structure of these doublets as a group. The inner arm region structure of bop2-1 doublets 2, 3, 4, and 7 was compared to the wild-type grand average and a difference image is shown in Fig. 7 f. This difference image shows that doublets 2, 3, 4, and 7 exhibit a less severe deficiency in the inner arm region. It is likely that this less severe deficiency did not show up in the analysis shown in Fig. 6 because our first analysis compared the entire inner arm region intensity between bop2-1 and wild-type axonemes and the second analysis compared the inner arm region structure pixel-by-pixel.

We also examined the structure of doublet 1 from wild-type and bop2-1 strains to determine if the bop2-1 mutation affects the morphology of doublet 1. The morphology of doublet 1 is clearly different from the structure of the other eight doublets (Mastronarde et al., 1992). Further complexity may exist in the biochemical composition of doublet 1 since
two classes of doublet 1 morphology are present along the length of the doublet. We did not include the doublet 1 images that contained the two-membered cross-bridge in our analysis because of the large variability this would introduce. Cross-sectional images from wild-type and bop2-1 axonemes were normalized on the microtubule doublet, rather than the outer dynein arm, and then compared as described previously. We constructed a difference image between wild-type doublet 1 (n = 22 images) and bop2-1 doublet 1 (n = 29 images) averages. No differences were present between the two images (p = 0.214). Therefore, the bop2-1 mutation does not affect the structure of the inner arm region of doublet 1.

Our analyses have identified three classes of bop2-1 doublets. The class with the most severe phenotype is comprised of doublets 5, 6, and 8. Doublet 9 has an intermediate phenotype and doublets 2, 3, 4, and 7 have the least severe phenotype. Therefore, not only does the bop2-1 mutation affect the assembly of inner arm region structures differently in specific doublets, but there are several different degrees of severity.

We examined the structural phenotype of bop2-1 axonemes from proximal and distal/medial regions of the axoneme. 38% of the bop2-1 images were from proximal regions of the axoneme as defined by the presence of either B-tubule projections or the two-membered cross-bridge. The proximal and the distal/medial regions of the axoneme contain equal percentages of affected outer doublets, and a comparison between the images of bop2-1 doublets from these regions shows no differences (p = 0.120). Therefore, the structures affected in the bop2-1 strain are not correlated to a known proximal/distal asymmetry.

**Discussion**

**The Structural Phenotype of bop2-1 Axonemes**

The bop2-1 mutation affects the assembly of several structures in the inner dynein arm region and reveals a new asymmetry in the inner arm region. In cross-sectional images, doublets 5, 6, and 8 have the most severe phenotype, doublet 9 is intermediate, and doublets 2, 3, 4, and 7 have the least severe phenotype (Fig. 7). A subset of the density missing in doublets 5, 6, and 8 is missing in all the other doublets. We have attempted to correlate the radial asymmetry that we observe in cross-sectional images with the heterogeneity that we characterized in the individual longitudinal images (Fig. 8). Four inner arm region densities are missing in the most defective longitudinal images; these include two of the densities missing in ida4 axonemes, a portion of the drc, and the projection (Fig. 5 j). These images are likely to be of doublets 5, 6, and 8, which have the most severe defect in the cross-sectional analysis (Fig. 8 b). The remainder of the longitudinal images are missing a portion of the drc and the projection (Fig. 5 h). We propose that these images are of doublets 2, 3, 4, and 7, which have a less severe defect (Fig. 8 c). Doublet 9 has an intermediate phenotype in the cross-sectional images and we do not see an intermediate phenotype among the longitudinal images. It is possible that we have not sampled doublet 9 among the 10 longitudinal im-
morphic analyses have shown that five of these structure missing in a doublet 9 may not be different enough from the images of doublets 2, 3, 4, and 7 to be detected in the longitudinal analysis of an individual axoneme. Our analyses are quite convincing that radial asymmetry alone accounts for the view of the axoneme (Fig. 8a). Thus the projection could be a part of the three uncharacterized dynein species (Kagami and Kamiya, 1992). We have attempted to ask if the 152-kD polypeptide is the gene product of the BOP2 locus using both dikaryon rescue analysis and reversion analysis. We failed to find any intragenic reversion events at the BOP2 locus from the reversion analysis. The results of the dikaryon analysis are consistent with the 152-kD polypeptide being the gene product of the BOP2 locus. However, an alternative explanation for the dikaryon analysis results is that the 152-kD polypeptide is the gene product but is assembled into a complex in the cytoplasm that requires the Bop2 gene product for stability. Results from Luck and Piperno (1989) and Porter et al. (1992) on other dynein mutations support this second model. The identification of the gene product of the BOP2 locus must await further genetic and molecular analyses.

Genetic Analysis of the bop2-1 Mutation
The bop2-1 allele was identified as a suppressor of the non-swimming phenotype of the pf10 mutation (Dutcher et al., 1988). Only one bop2 allele was found among 70 independent extragenic suppressors of pf10 and this allele was one of only 13 suppressor mutations that was able to suppress in the absence of light (Dutcher et al., 1988). We have screened a new collection of suppressors of the pf10 nonswimming phenotype that act in the absence of light (Dutcher et al., 1988). Among 73 newly isolated suppressed strains, we have failed to find any bop2 alleles (Shirley, R. L., S. J. King and S. K. Dutcher, work in progress). This preliminary result suggests that the bop2-1 allele is a highly unusual allele and that different alleles at this locus may have dramatically different phenotypes. Thus, it remains an important question to determine if the bop2-1 allele makes a product with partial or altered activity or is a null allele and makes no product.

Role of Radial Asymmetry in Flagellar Motility
To use the asymmetric waveform it is necessary to activate

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and inactivate the dynein arms in a specific pattern radially around the axoneme (Omoto and Kung, 1979; Fox and Sale, 1987). It is unlikely that this asymmetrical activation/inactivation is achieved simply through the outer dynein arms. Structural, biochemical and genetic analyses have suggested that the outer dynein arms are structurally equivalent and the waveform of oda- mutant strains is not altered from that of wild-type axonemes (Piperno et al., 1981; Mitchell and Rosenbaum, 1985; Brokaw and Kamiya, 1987; Kamiya, 1988). However, the motile flagellar mutant strains with defects in the assembly of inner arm dyneins or in the dynein regulatory complex have waveforms that are more sinusoidal in appearance than the asymmetric waveforms of wild-type axonemes (Brokaw et al., 1982; Brokaw and Kamiya, 1987).

A complete complement of structures in the inner arm region may be necessary for asymmetric waveforms. In wild-type axonemes this asymmetrical activation is likely to be imposed on the inner dynein arms at least in part by the interactions of the central pair microtubules and radial spokes. This statement is supported by three observations. First, in vitro reactivated axonemes with extruded central pair microtubules produce a symmetric waveform (Fukokawa and Miki-Noumura, 1987). Second, when paralyzed mutant strains that lack radial spokes are suppressed by mutations in the drc, they show a symmetric waveform (Brokaw et al., 1982; Brokaw and Kamiya, 1987). Finally, Smith and Sale (1992) have shown that the radial spokes modify the inner dynein arms; the in vitro activity of isolated inner dynein arms as monitored by rates of microtubule sliding is slower when inner arms are isolated from axonemes with mutant radial spokes compared to wild-type axonemes. Therefore, morphological and biochemical asymmetries in the inner dynein arms may provide one way to generate the characteristic asymmetric waveform. Given the biochemical complexity of the dyneins in the inner arms (Kagami and Kamiya, 1992), they are clearly a reasonable site for this type of control.

Four radial asymmetries have now been identified. These are the B-tubule projections, the two-membered cross-bridge, the absence of outer dynein arms, and the inner arm region structures described in this work. The two-membered cross-bridge and the B-tubule projections, because of their localization to the proximal portions of outer doublets and their location in the beat plane of the flagella, are ideal candidates for structures that are involved in the control of the planar power stroke with the correct spatial orientation. The absence of outer dynein arms from doublet 1, along with the two-membered cross-bridge, may act to prevent effective microtubule sliding between doublets 1 and 2 along the length of the axoneme. One possible role for radial asymmetry in the inner dynein arm region may be to allow certain subsets of arms to function as units in the generation of force (Sale, 1986; Lindemann et al., 1992).

Identification of radial asymmetries in both sea urchin and rat sperm flagella supports the hypothesis that different groups of outer doublets may function as a unit in the generation of force. Sea urchin and rat sperm flagella use the sinusoidal beat pattern exclusively, whereas this pattern is observed rarely in Chlamydomonas flagella. Two functional outer doublet groups have been identified in sea urchin sperm flagella; one group contains doublets 8, 9, 1, 2, 3, and the central pair whereas the other group contains doublets 4, 5, 6, and 7 (Sale, 1986). Three functional outer doublet groups have been identified in rat sperm flagella; the first group contains doublets 9, 1, and 2, a second group contains doublets 4, 5, 6, and 7, and the final group contains doublet 8, the central pair complex, and doublet 3 (Lindemann et al., 1992). In each of the sperm flagella, the organization of the functional groups is thought to promote microtubule sliding only in the plane of the sinusoidal beat. A second difference between the sperm flagella and Chlamydomonas flagella is that both sea urchin and rat sperm flagella contain central pair complexes that do not rotate. In contrast, Chlamydomonas flagella contain a central pair complex that rotates and is thought to regulate specific doublet dyneins in a spatial and temporal manner (Kamiya, 1982; Kamiya et al., 1982). A complex pattern of dynein activation and inactivation would be required to generate both a planar power stroke and a three-dimensional recovery stroke. Therefore, while the three classes of affected bop2-1 doublets do not fit into a pattern as simple as those seen in the sperm flagella, outer doublets 5, 6, 8, and 9 may also be members of one or more functional outer doublet groups. The effect of the bop2-1 mutation may be to disrupt the structure of a subset of outer doublet functional groups that are required to work in coordination in the complex Chlamydomonas flagellar beat pattern.

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Note Added in Proof. With the characterization of the ida5 and ida6 strains, only two of the eight biochemically defined HMW dyneins remain unidenti-fied by mutational analyses.

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