**Abstract.** Several studies have indicated that the central pair of microtubules and their associated structures play a significant role in regulating flagellar motility. To begin a molecular analysis of these components we have generated central apparatus-defective mutants in *Chlamydomonas reinhardtii* using insertional mutagenesis. One paralyzed mutant recovered in our screen, D2, is an allele of a previously identified mutant, pf16. Mutant cells have paralyzed flagella, and the C1 microtubule of the central apparatus is missing in isolated axonemes. We have cloned the wild-type *PF16* gene and confirmed its identity by rescuing *pf16* mutants upon transformation. The rescued *pf16* cells were wild-type in motility and in axonemal ultrastructure. A full-length cDNA clone for *PF16* was obtained and sequenced. Database searches using the predicted 566 amino acid sequence of *PF16* indicate that the protein contains eight contiguous armadillo repeats. A number of proteins with diverse cellular functions also contain armadillo repeats including pendulin, Rch1, importin, SRP-1, and armadillo. An antibody was raised against a fusion protein expressed from the cloned cDNA. Immunofluorescence labeling of wild-type flagella indicates that the *PF16* protein is localized along the length of the flagella while immunogold labeling further localizes the *PF16* protein to a single microtubule of the central pair. Based on the localization results and the presence of the armadillo repeats in this protein, we suggest that the *PF16* gene product is involved in protein–protein interactions important for C1 central microtubule stability and flagellar motility.

Despite the diversity of eukaryotic cells using cilia and flagella for motility, the structural components of the flagellar axoneme are well conserved. These components include the inner and outer dynein arms, radial spokes, and a central apparatus comprised of two singlet microtubules and their associated structures. Although the mechano-chemical properties of the dynein ATPases and their contribution to microtubule sliding has been studied for some time (Gibbons and Rowe, 1965; reviewed in Witman, 1989), little is known about the role of other axonemal components in converting microtubule sliding into complex flagellar waveforms. Using a new gene disruption technique in the biflagellate alga *Chlamydomonas reinhardtii*, we have undertaken a molecular dissection of the central apparatus to examine its role in flagellar motility.

The central apparatus consists of two singlet microtubules and their associated projections. In cross-section the C1 microtubule has projections 18 nm in length whereas those of the C2 microtubule are 8 nm (Witman et al., 1978; Dutcher et al., 1984; Goodenough and Heuser, 1985). Combining ultrastructural and biochemical techniques to analyze several *Chlamydomonas* mutants with defects in the central apparatus, Adams et al. (1981) and Dutcher et al. (1984) determined that 23 polypeptides (in addition to α and β tubulin), comprise the central apparatus. Nine of these polypeptides are uniquely associated with the C1 microtubule, 13 are uniquely associated with the C2 microtubule and one is found associated with both microtubules. Therefore, each microtubule of the central pair has distinct associated proteins, perhaps indicating that the two microtubules are functionally specialized.

The role of the central apparatus has been suggested by studying motility in axonemes from wild-type and mutant cells. *Chlamydomonas* mutants in which the central apparatus is missing have paralyzed flagella (Warr et al., 1966), yet sliding between doublet microtubules can be induced in a sliding disintegration assay (Witman et al., 1978) albeit at a reduced velocity (Smith and Sale, 1994). Therefore, the motility defect in central apparatus mutants is not in generating force but in regulating force to produce flagellar bending. The central apparatus may serve to convert dynein-induced microtubule sliding into flagellar bending. How the central apparatus might accomplish this is unknown.

In structural studies of *Elliptio* gill cilia, Warner and Satir (1974) observed that the radial spokes maintain contacts with the central pair projections in bending regions of the axoneme, and they proposed that the radial spokes un-
undergo cyclic detachment and reattachment to the central pair projections. Studies of *Paramaecium* cilia by Omoto and Kung (1979, 1980) and *Chlamydomonas* axonemes by Kamiya (1982) and Hosokawa and Miki-Noumura (1987), demonstrate that the central pair rotates counterclockwise once per beat cycle, with a slight twist that has the same period as the propagating flagellar bend. Functional analyses using mutants lacking the radial spokes have suggested that the radial spokes regulate microtubule sliding along a posttranslational modification of dynein (Smith and Sale, 1992; Howard et al., 1994). One hypothesis to explain these observations is that the central pair projections periodically sweep the radial spoke heads, much like the distributor of a mechanical motor, inducing a series of events that ultimately regulate dynein. Additional support for this hypothesis comes from the analysis of extragenic suppressor mutations which partially restore flagellar motility when combined with paralyzed mutants lacking the central apparatus or radial spokes (Huang et al., 1982). These mutants restore partial flagellar motility without restoring the missing axonemal structures. Several of these suppressors are mutations in the recently identified dynein regulatory complex (Piperno et al., 1992) or in dynein components (Porter et al., 1994) indicating possible regulatory interactions between the central apparatus, radial spokes and dynein.

Interestingly, rotation of the central apparatus is not observed for all cilia and flagella (Tamm and Tamm, 1981). This observation has led to the hypothesis that central apparatus rotation represents a refinement of preexisting regulatory mechanisms, allowing flagella to beat with a variety of bending patterns. Support for this hypothesis comes from computer-assisted motion analysis of *Chlamydomonas* mutants (Brokaw et al., 1982; Brokaw and Luck, 1985). Suppressed central apparatus defective strains have restored motility, but their flagella produce only large amplitude, symmetric waveforms. Therefore, the central apparatus may be required to convert symmetric bends into the asymmetric ciliary waveforms required for efficient forward swimming in *Chlamydomonas*.

Attempts to solubilize and purify central apparatus components biochemically have had only limited success (Witman et al., 1972; Piperno and Luck, 1979). To identify components that may be involved in regulating motility, we have begun a molecular dissection of the central apparatus using a new gene disruption technique in *Chlamydomonas reinhardtii* (Tam and Lefebvre, 1993). New central apparatus mutants were generated by transforming cells with a selectable marker gene that integrates into the host's genome. The advantage of this approach is that the selectable marker serves as a molecular tag to identify and ultimately clone the gene responsible for the mutant phenotype.

In our first screen of transformants, several central apparatus defective mutants were identified. One mutant, D2, is an allele of the previously identified mutant, pf16. Ultrastructural analysis indicated that two central microtubules were present in intact flagella from pf16 cells but upon demembranation to produce axonemes, the C1 microtubule was lost (Dutcher et al., 1984). Using the integrated plasmid as a tag, a fragment of DNA flanking the site of insertion was cloned, and full-length genomic and cDNA clones were subsequently obtained. The PF16 gene encodes a protein of ~60 kD, containing eight contiguous 42-amino acid armadillo repeats (see Peifer et al., 1994). Several proteins that participate in a variety of cellular processes also contain armadillo repeats. Although these proteins have diverse cellular functions, the repeat domains in each have been implicated in protein–protein interactions necessary for their function. Therefore, the PF16 gene product may be involved in protein–protein interactions necessary for central microtubule stability and flagellar motility.

### Materials and Methods

#### Cell Strains and Media

Strain A54-e18 (nit1-1, acl17, sl1, mut+) was the host strain for transformation experiments to obtain insertion mutants. Strains L5 (nit1, apm1-19, mt-) and L8 (nit1, apm1-19, mut-) were provided by W. W. Tam (University of Minnesota, St. Paul, MN) and were used as parents in back crosses with Nit+. A54-e18 transformants to determine cosegregation of the Nit+ phenotype with the motility defect. The central apparatus-defective mutants pf15 (CC807+), and pf20 (CC22- and CC1030+) were provided by the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). The arg7 strain (Lux and Dutcher, 1991) used to construct double mutants for cotransformation experiments and the central pair mutants pf6, pf16, pf18, and pf19 were provided by Mary Porter (University of Minnesota).

All cells were grown in constant light in SGII, SGII-N, or SGII-NO3. Cells were provided by L. W. Tam (University of Minnesota). All cells were grown in constant light in SGII, SGII-NO3 (see Sager and Granick, 1953; Kindle, 1990; Tam and Lefebvre, 1993), or TAP media (Gorman and Levine, 1985).

#### Transformation

High-efficiency transformation was achieved using the glass bead procedure of Kindle (1990). For transformation using the plasmid pMN56 (containing the full-length nitrate reductase gene; Fernandez et al., 1989; Kindle et al., 1989; Nelson et al., 1984), nitl mutant cells were grown in SGII liquid culture, concentrated by centrifugation to 4 x 10^6/ml, and stirred under bright light for ~4 h (Nelson et al., 1994). After the 4-h incubation 0.5 ml of cells, 1 mg of pMN56 plasmid linearized by digestion with EcoRI, 5% PEG-8000 (Sigma Chem. Co., St. Louis, MO) and 0.3 g of acid-washed glass beads (0.7-1.2 mm diam; Sigma) were vortexed together at top speed on a Vortex Genie II mixer in 15-ml conical plastic tubes for 45 s. The cells were suspended in 10 ml of SGII-NO3 media, transferred to a fresh tube, pelleted by centrifugation, and then plated onto SGII-NO3 agar plates. For transformation of arg7 cells, cells were grown in arginine-supplemented (0.005%) TAP media and concentrated and treated with autolysin (Harris, 1985) for 1 h to dissolve cell walls. Cells were transformed by the glass bead method as described, using 1 mg of plasmid pARG7.8 (containing the wild-type *Chlamydomonas* arginino-succinate lyase gene; Debuchy et al., 1989) linearized by digesting with BamHI. The transformants were plated on TAP agar plates without arginine supplement. For transformation using a dominant selectable marker for emetine resistance, cells were transformed as described using the plasmid pPN4 that carries a mutant *Chlamydomonas* gene for the ribosomal protein S14 that confers resistance to emetine upon transformation (Nelson et al., 1994). Following vortexing, cells were resuspended in 10 ml of SGII media and shaken under light for 3.5 h. The cells were then pelleted and resuspended in 100 ml of SGII-N in a 250-ml flask and bubbled for 4 d in constant light. After 4 d the cells were pelleted and resuspended in SGII and shaken under light for 8 h. The cells were then pelleted and plated onto SGII plates containing 80 mM emetine dihydrochloride (Sigma). To test genomic lambda clones for rescue of mutant phenotypes, 1-3 µg of each genomic clone was cotransformed with plasmid DNA carrying a selectable marker gene.

#### Mutant Selection

Colonies of successful transformants were picked into 96-well microtiter dishes containing SGII-NO3 liquid media and screened for motility defects using a stereomicroscope (Zeiss DR-C) at 80X magnification. For further analysis of flagellar defects, cells were examined using phase optics at 400X magnification. Axonemes from mutants of interest were prepared
for ultrastructural analysis by electron microscopy. Flagella were severed from cell bodies using dibucaine (King et al., 1987; Watanabe, 1988) isolated by differential centrifugation in HMDedNA (10 mM Hepes, 5 mM MgSO4, 1 mM DTT, 0.5 mM EDTA, 30 mM NaCl, pH 7.4). Axonemes were isolated using 0.5% NP-40 in HMDedNA to remove flagellar membranes. For thin section electron microscopy, specimens were fixed with 1% glutaraldehyde and 1% tannic acid in 0.1 M sodium cacodylate, post-fixed in osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in epoxy resin (EM Sciences, Fort Washington, PA). Uniform silver-gray sections were mounted on formvar-coated, carbon stabilized copper grids, stained with uranyl acetate and Reynolds lead citrate, and then examined at 80 kv in the transmission electron microscope (either a model 100XC or 1200CX [JEOL U.S.A., Inc., Peabody, MA] or Hitachi H-600 [Hitachi Sci. Instrs., Mountain View, CA]).

Genetic Analysis

Techniques for mating and tetrad analysis are described by James et al. (1988). Meiotic progeny were scored for Nit+ or Nit- phenotypes by replica plating on SGII-NO3 and SGII agar plates. The motility phenotype was scored by resuspending meiotic progeny in liquid media. Pairs of mutants were tested for complementation by mating strains containing complementing auxotrophic markers (D2 tarts were tested for complementation by mating strains containing complementing auxotrophic markers (D2

DNA-Blot Analysis

Genomic DNA was isolated as described previously (Schnell and Lefebvre, 1993). Size fractionation of genomic DNA and transfer to nylon filters, colony lifts, plaque lifts, and hybridizations were performed using Magna NT nylon membranes (Micron Separations Inc., Westborough, MA) following protocols supplied by the manufacturer. For all hybridizations, probes were isolated from agarose gels using the GeneClean II kit (BIO 101 Inc., La Jolla, CA). Probes for genomic Southern were prepared by random primer labeling using either the Genius 2 nonradioactive DNA labeling kit (Boehringer Mannheim Biochemicals, Houston, TX) or using [32P]dCTP (Feinberg and Vogelstein, 1983). For plaque and colony lift hybridizations, 3P-labeled probes were used.

Isolation of Genomic Sequence Flanking the Integrated Plasmid

Genomic DNA was isolated flanking the site of insertion of the integrating plasmid in the mutant D2. A 4-kb fragment was identified by hybridization that consisted of the 3' end of the N1T1 gene (from pMN56) along with genomic DNA flanking the insertion site. This fragment was cloned by constructing and screening a partial genomic DNA library. Genomic DNA (30 µg) was digested with BamHI and SphI and fractionated on a 1% Sea Plaque agarose gel. After electrophoresis, 1-mm slices containing the 4-kb size fraction were excised from the gel; DNA was isolated from the gel slices by melting at 65°C followed by sequential extractions using phenol, phenol/chloroform (1:1), and chloroform. To identify which DNA size fraction contained the junction fragment, one-tenth volume of each fraction was electrophoresed on an agarose gel, transferred to Magna NT filters, and then hybridized with a labeled probe recognizing the 3' end of the N1T1 gene. The DNA fraction containing the junction fragment was ligated into the pBluescript vector (0.1 µg) digested with BamHI1 and SphI. The ligation mixture was transformed into MC1061 cells by electroporation using the BTX transfection 100 (Biotechnologies and Experimental Research, Inc., San Diego, CA) following protocols in the Bio-Rad GenePulsar Application Guide. The transformed cells were then plated on LB agar plates containing 100 µg/ml ampicillin to select for resistant colonies. Ampicillin-resistant colonies were transferred to Magna NT nylon membranes (Sambrook et al., 1989) and hybridized with a 32P-labeled probe from the 3' end of the N1T1 gene to identify clones containing the junction fragment.

Cloning PF16: Library Screening and Mutant Rescue

A library constructed with DNA from the wild-type strain 21gr m+ (Schnell and Lefebvre, 1993) in the lambda phage vector XfixII (Stratagene, La Jolla, CA) was screened using a fragment of cloned sequence flanking the insertion site in the mutant D2 as a probe. Approxi-
nent PF16 protein that had been electropho­ted onto PVDF membrane (Millipore Corp., Bedford, MA). Binding and elution of the antibody was performed as described by Talia­n et al. (1983). For immunoblots, flagella were isolated as described above and 75 μg of protein loaded into each lane of a 10% polyacrylamide minigel. The proteins were transferred elec­trophoretically to a PVDF membrane. Incubation with the purified PF16 antibody and alkaline phosphatase conjugated secondary antibodies, and antibody detection was performed according to the Western Light Chemi­luminescent Detection System kit (Tropix Inc., Bedford, MA).

**Immunolocalization**

For immunofluorescence experiments flagella were isolated and resus­pended in HMDEKNa as described above. Fixation, permeabilization, and antibody incubation steps were carried out as described in Sanders and Salisbury (1995) using purified PF16 antibody as the primary antibody, and goat anti–rabbit, FITC-conjugated secondary antibodies (Cappel, Or­ganon Technika, Durham, NC) diluted 1:500. For immunogold labeling flagella were isolated from A54-e18 cells, resuspended in HMDEK (10 mM Hepes, 5 mM MgSO4, 1 mM DTT, 0.5 mM EGTA, and 25 mM KCl), and allowed to settle on formvar-coated, carbon-stabilized nickel grids. All subsequent antibody incubation steps were carried out as described in Bernstein et al. (1994) using purified PF16 antibody as the primary antibody, and 12 nm gold-conjugated secondary antibodies (Jackson Immuno­logicals, Westgrove, PA) diluted 1:40. Control experiments in which the primary antibody was omitted were also performed.

**Results**

**Transformation and Mutant Screen**

Mutants were generated by transforming nit1 (nitrate reductase deficient) cells with the plasmid pMN56 containing the cloned NIT1 gene (Fernandez et al., 1989). To identify mutants with motility defects, colonies were picked into liquid media in 96-well microtiter dishes and ana­lyzed by phase microscopy to identify paralyzed mutants with rigid flagella (Randall et al., 1964, 1967). Immotile cells were easily identified by eye since they sink to the bottom of the dish. Putative mutants were further characterized by phase microscopy to identify paralyzed mutants with rigid flagella. Axonemes were isolated from all cells with para­lyzed flagella of wild-type length and analyzed by electron microscopy. Of 1,700 transformants screened, 17 mutants were selected for electron microscopic analysis and of these, 6 showed clear defects in the central apparatus (see Table 1).

An analysis of axonemal cross sections revealed that the majority of axonemes from the mutants A10 and A7 completely lacked central microtubules, a phenotype previously described for the mutants pf15, pf18, pf19, and pf20 (Randall et al., 1964, 1967; Warr et al., 1966). Axonemes from the mutants D2 and C11 had either one central microtubule or no central apparatus present, similar to axonemes from the mutant pf16 (Dutcher et al., 1984) that contain only the C2 microtubule of the central pair. In cross sections of axonemes from the mutant F9, either one or two central microtubules were present, a phenotype not previously described. Axonemes from the mutant H2 had two central pair microtubules but further analysis revealed that a subset of projections was absent from the C2 central microtubule.

**Genetic Analysis**

Each of the mutants was backcrossed to a nit1/ parent to determine whether the central apparatus defect cosegregate with the Nit+ phenotype. If a mutant phenotype was produced by gene disruption due to plasmid integration into the genome, then the Nit+ phenotype should coseg­egrate with the flagellar motility defect. Tetrad analysis revealed that in four of six of the central apparatus mutants, the Nit+ phenotype cosegregated with flagellar paralysis in a minimum of 24 complete tetrads in each of two back­crosses. For two of the mutants (C11 and A7), Nit-, para­lyzed progeny were obtained, indicating that the paralyzed phenotype was not linked to the insertion of the nitrate re­ductase gene; these mutants were not analyzed further.

The insertional mutants were tested for allelism with previously identified mutants with central apparatus de­fects. For example, axonemes from the mutant H2 and from the previously described mutant pf6 are both missing a subset of central pair projections. However, crosses of pf6 with H2 indicated that H2 is not closely linked to or al­lelic with pf6. Both nonparental ditype (2 paralyzed cells: 2 motile cells) and tetrateype (3 paralyzed cells: 1 motile cell) tetrads were obtained indicating that recombination be­tween these two loci had occurred. Of the mutants exam­ined genetically, only A10 and D2 appeared to be allelic with previously identified mutants. In crosses of A10 with pf20 no recombinants were obtained in 24 complete tetrads. Allelism was established by constructing stable dip­loids. Heterozygous diploids of A10 and pf20 have para­lyzed flagella, indicating that these mutations are allelic. In crosses of D2 with pf16 no recombinants were obtained in 24 complete tetrads and no complementation was ob­served in heterozygous diploids. D2 was chosen for a de­tailed molecular analysis.

**Cloning the PF16 Gene**

Because the Nit+ and paralyzed flagella phenotypes of D2 cosegregated in backcrosses with Nit− cells, the lesion in D2 was most likely caused by the integration of the transform­ing plasmid DNA. To begin cloning the PF16 gene, a fragment of DNA flanking the site of plasmid integration was cloned from D2 genomic DNA. We determined that the mutant contained a single copy of the plasmid by prob­ing Southern blots of genomic DNA using the pUC119 vector as a hybridization probe. Unfortunately, the vector

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**Table 1. Central Apparatus Mutants Obtained by Insertional Mutagenesis**

| Mutant | Number of central tubules present | Motility | Cosegregation of Nit+ with pf |
|--------|----------------------------------|----------|-----------------------------|
|        | 0 | 1 | 2                        |
| Wild-type | 6% | 13 | 81 | Normal | NA |
| A10    | 73 | 27 | 0 | Paralyzed/rigid | Yes |
| C11    | 53 | 45 | 2 | Paralyzed/twitch | No |
| F9     | 4  | 58 | 38 | Twitch/bend at base | Yes |
| D2     | 55 | 45 | 0 | Paralyzed | Yes |
| A7     | 87 | 13 | 0 | Paralyzed | No |
| H2     | 0  | 19 | 81 | Uncordinated, missing C2 projections | Yes |

The number of central tubules present was determined for isolated axonemes and is expressed as a percentage (n > 100 cross sections in each case).
portion of this construct was not intact, eliminating the use of a plasmid rescue technique as a potential cloning strategy. Therefore a partial genomic library was constructed from D2 DNA to clone sequence flanking the insertion site. Genomic DNA from D2 was digested with BamHI, which cuts at the 3' end of the NIT1 gene, and SphI, which cuts in the genome (Fig. 1), and the appropriately sized fragments were ligated into the pUC119 vector. Bacteria containing the plasmid DNA of interest were identified using the 3' end of the NIT1 gene as a probe. A restriction map of PstI sites in the plasmid was prepared, and a 1.3-kb PstI fragment that did not contain the NIT1 gene or vector was used as a hybridization probe on Southern blots and in screening a wild-type lambda library.

**Figure 1.** Cloning a DNA fragment flanking the insertion site of the pMN56 plasmid. Solid wide lines represent genomic DNA, thin lines represent NIT1 gene sequence, and the box represents the pUC119 vector. The broken lines of the pUC119 box indicate that the vector was not intact. Flanking sequence was cloned into pBluescript using the enzymes BamHI and SphI. A 1.3-kb PstI restriction fragment that did not contain the NIT1 gene or vector was used as a hybridization probe on Southern blots and in screening a wild-type lambda library.

**Figure 2.** Restriction map of overlapping lambda clones. Probe 1 is the 1.3-kb PstI fragment from Fig. 1 used in the first screen of the library to obtain clones 6, 8a, and 9a. Probe 2 is the 3-kb NotI fragment used to screen the library a second time to obtain additional clones. Clones 5, 7, 8b, and 9b were obtained in the second screen and rescued the paralyzed flagella phenotype when transformed into D2 mutent cells. The plasmid pB6D2 was constructed by digesting clone 9b with XbaI and ligating the indicated fragment into the pBluescript vector. Probe 3 is a NotI/XbaI fragment of pB6D2 used to screen a cDNA library and as a probe on RNA blots. The extent of deleted DNA in the mutant was determined by using each of the NotI fragments as probes on Southern blots of wild-type and D2 DNA digested with NotI. The deletion may extend beyond the left side of clone 8b.

Different sizes of DNA from D2 and wild-type cells confirming that this probe represented a piece of DNA flanking the site of plasmid insertion in the mutant.

The 1.3-kb PstI probe (Fig. 2, Probe 1) was then used to screen a bacteriophage lambda library constructed from wild-type genomic DNA (Schnell and Lefebvre, 1993). In the first screen several overlapping clones were identified but none of these rescued the motility defect in D2 upon transformation. One possible explanation for failed rescue is that the complete wild-type copy of the gene was not contained in the clones tested. Insertion of exogenous DNA into *Chlamydomonas* frequently results in rearrangement and deletion of large portions of DNA (Tam and Lefebvre, 1993). Thus, if a sizable deletion occurred at the integration site, DNA clones flanking that site might be far from the PF16 gene. Restriction maps of our genomic lambda clones were prepared using the enzyme NotI, and the labeled restriction fragments were used as probes on Southern blots of genomic DNA from D2. A sizable deletion was detected that extended beyond the lambda clones we had obtained. A 3.5-kb NotI fragment at the end of clone 8a extended into the region deleted in the mutant, and this fragment was used as a probe to rescreen the library (Fig. 2, Probe 2). A new set of overlapping clones was obtained that rescued the motility and structural defects in D2 and pf16 upon transformation.

**Rescue of D2 and pf16**

To determine which of the lambda clones obtained in our screen contained a functional PF16 gene, the lambda clones were transformed into D2 and pf16 cells and the transformants were screened for rescue of flagellar paralysis. In these experiments the cells were transformed with both a lambda clone being tested and a selectable marker gene. For D2 rescue, a D2, arg7 double mutant was constructed and transformed with pARG7.8 (arginino-succinate lyase; Debuchy et al., 1989) and putative genomic clones for PF16. Transformants were selected as colonies.
Table II. Rescue of D2 and pf16 by Transformation

| Mutant           | 0   | 1   | 2   |
|------------------|-----|-----|-----|
|                  | %   |     |     |
| Wild-type        | 6   | 12  | 81  |
| D2               | 55  | 45  | 0   |
| D2 Transformant  | 0   | 1   | 99  |
| pf16*            | 32  | 60  | 8   |
| pf16 Transformant| 9   | 14  | 75  |

Percentile values represent at least 100 cross-sections for each strain.

*Dutcher et al., 1984.

Figure 3. Thin section electron microscopy of axonemes from the mutant D2 (a) and from rescued mutants D2 (b) and pf16 (c) transformed with the cloned PF16 gene. Panels are representative axonemal cross sections. Bar, 200 nm.

Growing on media without arginine supplement. These colonies were picked into 96-well microtiter plates containing liquid media. Successful transformants in which the putative genomic clones rescued the mutant phenotype were identified as colonies with swimming cells. Similar experiments involving pf16 used emetine resistance as a dominant selectable marker. The cells were transformed with both a lambda clone being tested and the plasmid pJN4, containing a mutant copy of the gene for ribosomal protein S14 that confers emetine resistance upon transformation (Nelson et al., 1994). Successful transformants were selected as colonies growing on plates containing emetine. Emetine-resistant colonies were transferred to liquid media; rescued transformants were identified as swimming cells. For both D2 and pf16 rescue of the mutant phenotype by the transforming DNA was confirmed by isolating genomic DNA from several transformed strains and identifying the DNA from the appropriate lambda clone by Southern analysis using Probe 2 (Fig. 2). In all cases the rescued cells contained the transforming lambda clone, indicating that rescue was most likely due to the function of the transgene.

Ultrastructural analysis by electron microscopy of axonemes isolated from rescued cells revealed that for both D2 and pf16 the central apparatus was restored (Fig. 3). Table II summarizes these results quantitatively. In the rescued mutants, the number of central microtubules observed in axonemal cross-sections was comparable to the number of central microtubules observed in cross-sections of axonemes from wild-type cells.

Northern Analysis and cDNA Sequence

To characterize the transcript of the PF16 gene and to obtain the amino acid sequence of its predicted gene product, cDNA clones were isolated and sequenced. First, the PF16 gene was more precisely defined by subcloning lambda clone 9b into smaller portions and testing each subclone for the ability to rescue flagellar paralysis. The smallest subclone that rescued pf16 was a 4.5-kb XbaI fragment subcloned into pBluescript (Fig. 2, pB6D2). A NotI/XbaI fragment from this clone (Fig. 2, Probe 3) was used as a probe on RNA blots. Total RNA was prepared from D2, pf16, and wild-type cells before and 45 min after deflagellation. Hybridization of Probe 3 (Fig. 2) to blots of size-fractionated RNA revealed a 2.2-kb transcript that was induced following deflagellation in wild-type cells (Fig. 4, lanes 1 and 2). Deflagellation in Chlamydomonas has been shown to cause an accumulation of transcripts from genes encoding flagellar proteins (reviewed in Lefebvre and Rosenbaum, 1986). As expected, no detectable transcript was observed in RNA from the mutant D2 (Fig. 4, lanes 3 and 4) since the PF16 gene is deleted in the mutant. A transcript of the expected size was present in RNA from pf16 cells, although in reduced amounts. A Chlamydomonas cDNA library was screened with Probe 3 and resulted in the isolation of two clones, one ~1 kb in length and one ~2 kb, which were sequenced (Fig. 5). The 1-kb sequence was incomplete, representing only the 3' end of the transcript. The sequence of the 2-kb clone appeared to be full length. A single large open reading frame extending 566 amino acids, was present in the sequence. The putative initiation codon was preceded by an upstream in-frame termination codon (Fig. 5, double underline). The two cDNA sequences overlapped at the 3' end of the gene and were
and 6 contain pfl6 RNA prepared pre- and postdeflagellation. The blot was probed with Probe 3, (Fig. 2). The S14 gene (encoding the ribosomal 14 protein) is constitutively expressed and was used as a control for equal loading. Lanes 1 and 2 contain RNA from wild-type cells pre- and postdeflagellation, lanes 3 and 4 contain D2 RNA prepared pre- and postdeflagellation, and lanes 5 and 6 contain pfl6 RNA prepared pre- and postdeflagellation. The band of 1 kb represents the transcript of the S14 gene.

identical in the region of overlap, although the 1-kb cDNA used a different putative polyadenylation site (Fig. 5).

Sequence Analysis

The predicted protein of 566 amino acids has an isoelectric point of 7.7 and mass of 60.2 kD. The PF16 gene product contains eight contiguous 42-amino acid repeats in the amino-terminal half of the protein (Fig. 5, underlined). The seventh and eighth repeats are separated by a single amino acid whereas repeats one through seven are organized in perfect tandem array. These repeats were first identified in the Drosophila segment polarity gene product armadillo (Riggleman et al., 1989; see review in Peifer et al., 1994). These so-called armadillo repeats have been found in a variety of different proteins from other eukaryotic systems including pendulin (mouse and Drosophila, Kussel and Frasch, 1995; Torok et al., 1995), Rch1 (human, Cuomo et al., 1994), importin (Xenopus, Gorlich et al., 1994), and SRPI (yeast, Yano et al., 1992). PF16 is only homologous to these proteins in the repeat region and it is therefore not the Chlamydomonas homologue of any of these proteins. The repeats within PF16 are more similar to each other than are the repeats in the other proteins of this class (Fig. 6; see Peifer et al., 1994). For example, in pairwise comparisons of the PF16 repeats, the identity between any two repeats ranged from 14-43%. In addition, for the other proteins containing armadillo repeats, gaps are often introduced to maximize alignment. In the case of the PF16 protein, the eight repeats are contiguous and perfectly aligned with the exception of a single amino acid between repeats seven and eight. Database searches using only the amino acid sequence from the carboxy third of the protein found no sequence similarities with other proteins. However, the carboxy terminus is glycine rich having 26% glycine residues over a stretch of 72 amino acids. The presence of a glycine-rich region at the carboxy terminus is similar to the armadillo protein, that contains 32% glycines over a stretch of 70 amino acids.

To determine the gene structure and confirm the sequence of the PF16 coding region we sequenced the 4.5-kb insert of the smallest genomic clone that rescued the PF16 defect, pB6D2. There are six introns in the coding region (Fig. 7), all found within the repeat region of the protein (Fig. 5, see carets). None of the introns separates two repeats precisely. The genomic clone contained ~0.97 kb of sequence upstream of the 5' end of the cDNA clone and 1.0 kb downstream of the polyadenylation site.

To confirm that the cloned wild-type gene corresponded to pfl6, the genetic lesion in a pfl6 mutant (pfl6B) was de-
aligned by cloning and sequencing a copy of the gene containing the pfl6 mutation. Within the first 2.1 kb sequenced, a mutation was found at the 3' splice acceptor site of the second intron. For all Chlamydomonas introns identified so far, the splice acceptor consensus sequence is AG. In the case of pfl6, the AG is mutated to AA. If this intron is not removed by splicing, the resulting transcript would be 90 bp larger than the wild-type transcript, a change not detectable on RNA blots. Two potential splice acceptor sites were found downstream at nucleotides 501 and 503. These cryptic splice sites might be used in the mutant. However, both the unspliced mRNA and the mRNA that would be produced by splicing at positions 501 or 503 would contain a frameshift introducing a stop codon. In other systems, mRNAs containing an in-frame nonsense codon are preferentially degraded (e.g., Peltz and Jacobson, 1993), which could account for the observation that the PF16 transcript from pf16 cells was wild-type in size but reduced in amount. The same sequence alteration (AG to AA at a splice acceptor site) was recently identified for another gene in Chlamydomonas. For the mutant ida4-3, whose wild-type gene product encodes an intermediate chain of inner arm dynein (LeDizet and Piperno, 1995a), an AG to AA mutation was found at intron 4 (LeDizet and Piperno, 1995b). In this case, the intron was not spliced and a larger than wild-type transcript was detected on RNA blots.

**Fusion Protein Expression and Purification, Antibody Production, and Localization**

To localize the PF16 gene product in axonemes, antibodies were generated to a fusion protein expressed in Escherichia coli cells. A large portion of the cDNA was cloned into the pRSET B vector using the enzyme NcoI. The resulting construct expressed a PF16 protein lacking 39 amino acids from the amino terminus and 23 amino acids from the carboxy terminus for a total of 503 amino acids. The expressed protein also contained six additional histidine residues at its amino terminus, making it possible to purify the expressed fusion protein. The pRSET-PF16 construct was transformed into BL21(DE3) cells carrying the pLysS plasmid, and protein expression was induced by the addition of IPTG to the growth media. Cells were lysed by freezing and thawing and the bacterial lysate was purified by binding to a nickel column (QIAGEN NTA Ni²⁺ resin). As assessed by SDS PAGE, large quantities of pure fusion protein of the expected size (~50 kD) were found in the eluate. Polyclonal antisera were prepared in rabbits using the purified protein as antigen.

The resulting sera was purified against the fusion protein and analyzed on western blots of flagella prepared from three cell strains: wild-type (A54-e18), pfl6 and pfl6 cells in which the mutant phenotype had been rescued by transformation with pB6D2. The purified sera recognized a protein of the expected size in flagella from wild-type and rescued pf16 cells (Fig. 8, lanes 1 and 3) but no band was observed in flagella from the mutant pf16 (Fig. 8). These results indicate that the antibody was specific for the PF16 protein.

For immunolocalization, the same purified sera was used in whole mount preparations of flagella from wild-type and mutant cells. In immunofluorescence experiments staining was observed along the length of wild-type flagella whereas no staining was observed in flagella from pfl6 cells (not shown). To more precisely localize the PF16 gene product, immunogold electron microscopy was performed. Whole mounts of wild-type flagella were prepared for immunogold labeling and negatively stained for electron microscopy. In samples in which the axonemal doublets splayed and the two microtubules of the central apparatus separated, gold particles were observed to be bound along the length of only one microtubule of the central apparatus (Fig. 9). The central microtubule recognized by the antibody is presumably C1 because axonemes...
Immunoblot of flagellar protein. Approximately 75 µg of isolated flagella were loaded into each lane. The purified PF16 antibody recognizes a protein of the expected size present in flagella of wild-type (lane 1) and rescued pf16 cells (lane 3). No band is seen in flagella of the mutant pf16 (lane 2).

Discussion

The central apparatus in Chlamydomonas flagella is composed of two singlet microtubules and at least 23 associated polypeptides. Despite the variety of mutants that exist, little is known about these proteins and their functions. Using new cloning strategies developed for Chlamydomonas reinhardtii, we have begun a molecular dissection of the central apparatus with the hope of eventually understanding how it regulates dynein-driven microtubule sliding during flagellar beating. In addition, these central apparatus polypeptides may be unique microtubule-associated proteins having microtubule stabilizing or nucleating functions.

Several studies of central apparatus components have already identified polypeptides of general interest. For example, Miller et al. (1990) identified capping structures at the tips of the central pair microtubules that are recognized by an antibody to a kinetochore antigen. This tip protein, by analogy, may serve to capture and stabilize the plus ends of the microtubules. The plus ends of central pair microtubules are distal to the cell body (Euteneuer and McIntosh, 1981), consistent with a role for this antigen in microtubule capture. Also, recent studies of Bernstein et al. (1994), Fox et al. (1994), and Johnson et al. (1994) have identified members of the kinesin family of proteins that localize to the central apparatus. So far, the role these proteins play in flagellar motility or assembly has not been determined.

In this study new central apparatus defective mutants were generated in which the mutated gene could be cloned using the transforming DNA as a molecular tag. A variety of central apparatus mutants were obtained, some of which represent alleles of previously identified mutants. Among 1,700 transformed strains examined, six had clear ultrastructural defects in the central apparatus. The first central pair gene cloned from our mutant collection is an allele of pf16; axonemes of this mutant are missing the C1 microtubule of the central apparatus. Using two-dimensional gel analysis, Dutcher et al. (1984) determined that...
pf16 flagella contain two intact central pair microtubules but are missing three polypeptides. Upon demembranation, however, the C1 microtubule is destabilized, and the resulting axonemes contain only the C2 microtubule. Most likely, the three polypeptides missing from pf16 flagella are critical for flagellar motility and presumably contribute to the stability of the C1 microtubule. Using a combination of dikaryon rescue experiments, reversion analysis, and 2-D SDS PAGE, Dutcher et al. (1984) proposed that the gene product of the Pf16 locus is one of the three polypeptides missing in mutant flagella, a 57-kD protein designated CP14.

Using the insertional mutant, D2, to identify a fragment of genomic DNA flanking the insertion site, a wild-type genomic clone and a full-length cDNA clone containing the Pf16 gene were isolated and sequenced. The genomic clone rescued the motility and structural defects in both D2 and the original pf16 mutant upon transformation. The Pf16 gene encodes a predicted 566-amino acid protein with a molecular mass of ~60 kD, in close agreement with the findings of Dutcher et al. (1984). However, the isoelectric point predicted from the amino acid sequence (7.7) is very different from that observed by Dutcher et al. (~5.5; see Piperno et al., 1981; Dutcher, 1995). The difference in isoelectric point seems too large to be due to posttranslational modification (such as phosphorylation) altering the primary gene product. Thus, the relationship between CP14 and the Pf16 gene product is not yet clear.

Database searches using the predicted amino acid sequence of Pf16 placed the protein in a family of proteins that contain repeat domains first identified in the Drosophila segment polarity gene armadillo (Riggleman et al., 1989). Eight contiguous 42-amino acid repeats comprise most of the amino-terminal two-thirds of the protein. The deduced universal consensus for armadillo repeats (7.7) have been mutated or deleted and assessing for restoration of motility and C1 stability.

Further analysis of the polypeptides associated with the central pair of flagellar microtubules should reveal important clues as to the regulation of dynein-driven doublet sliding in flagellar motility, and should also provide clues as to the assembly of the central apparatus. The central pair of microtubules assemble with their plus ends distal (Euteneuer and McIntosh, 1981), the same polarity observed for the nine doublet microtubules (Allen and Borisy, 1974; Binder et al., 1975); but unlike the nine doublet microtubules, they are not nucleated from the basal bodies. The central pair microtubules are more similar to cytoplasmic microtubules than doublet microtubules for example, in their cold lability and colchicine sensitivity (Behnke and Forer, 1967; Shelanski and Taylor, 1967). Although no difference in tubulin composition or protofilament organization for the two tubules has been observed, different polypeptides are assembled specifically to one microtubule or the other. The Pf16 protein is attached preferentially to the C1 microtubule (this study) whereas this family have very different cellular functions, genetic analyses as well as functional knockout experiments have indicated that the repeat domains mediate specific protein–protein interactions required for function. Truncated armadillo protein, for example, retains some function but the degree of function is strictly correlated with the length of the truncated protein (Peifer and Weishaus, 1990). The authors suggest that the repeats form discrete structures that are independent and additive in function. Because the flagellar axoneme is one of the most highly ordered cellular organelles, the finding of an axonemal protein containing such highly conserved and precisely ordered repeats may not be surprising. In the case of the Pf16 gene product, given that the mutant flagella are paralyzed and the C1 microtubule is destabilized when flagella are demembranated, one possibility is that the repeat region is involved in protein–protein interactions important for C1 microtubule stability and flagellar motility. This hypothesis can be tested by transforming pf16 cells with modified copies of the Pf16 gene in which some or all of the repeats have been mutated or deleted and assessing for restoration of motility and C1 stability.

Polyclonal antibodies were generated using the expressed Pf16 cDNA as an antigen. Western blots of flagella isolated from wild-type cells and pf16 cells that had been rescued by transformation with the Pf16 gene, showed immunoreactivity with a single band of the expected size. Using the affinity-purified sera for immunofluorescence and immunogold labeling of wild-type flagella, the Pf16 gene product was localized to a single microtubule of the central apparatus. Because axonemes from pf16 cells are missing the C1 microtubule, the Pf16 gene product is most likely a component of the C1 microtubule complex, perhaps comprising part of the C1 projections. In the future these antibodies will be invaluable in determining which polypeptides interact with the Pf16 gene product. For example, one or more of the polypeptides identified by Dutcher et al. (1984) as missing in axonemes from pf16 cells may interact with the Pf16 gene product. Using the antibody in cross-linking and immunoprecipitation experiments, we can identify these interacting components and begin to elucidate the composition of the C1 microtubule complex.
KLP-1 is preferentially attached to the C2 microtubule (Bernstein et al., 1994). Further characterization of the polypeptides associated with the central pair of microtubules should provide new insights into the binding specificity of other microtubule associated proteins.

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