Genetic Dissection of the Central Pair Microtubules of the Flagella of *Chlamydomonas reinhardtii*

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ABSTRACT   Mutations at two loci, which cause an altered mobility of the flagella, affected the central pair microtubule complex of *Chlamydomonas reinhardtii* flagella. The mutations at both loci primarily affected the C1 microtubule of the complex. Three alleles at the *PF16* locus affected the stability of the C1 microtubule in isolated axonemes. This phenotype has allowed us to determine that at least ten polypeptides of the central pair complex are unique to the C1 microtubule. The motility defect was correlated with the failure to assemble three of these ten polypeptides in vivo. The structural gene product of the *PF16* locus was a polypeptide with molecular weight 57,000 as shown by analysis of five intragenic revertants and by analysis of axonemes from dikaryon rescue experiments. Three alleles at the *PF6* locus affected the assembly of one of the two projections of the C1 microtubule and this projection was formed by at least three polypeptide components, which are a subset of polypeptides missing in isolated *pf16* axonemes. No structural gene product has been identified for the *PF6* locus. The gene product is probably not one of the identified projection constituents as shown by analysis of dikaryon rescue experiments. Chemical extraction of isolated wild-type axonemes suggests that at least seven polypeptide components are unique to the C2 microtubule.

The central pair microtubules of flagella more closely resemble the cytoplasmc microtubules than the outer microtubules of the flagella by virtue of their structure and their lability. They are singlet microtubules with 13 protofilaments unlike the nearby outer doublet microtubules that are composed of a composite set of microtubules with 13 and 11 protofilaments (1). The outer doublet microtubules, in contrast to the central pair microtubules, arise as a continuation of the microtubules of the basal bodies. The central pair complex originates distal to the transition zone of the basal body and terminates in specialized structures associated with the flagellar membrane at its tip (2). In *Chlamydomonas reinhardtii* the central pair microtubules are sensitive to depolymerization by low ionic strength dialysis and by exposure to detergents (3, 4), whereas the outer doublet microtubules retain their integrity under these conditions.

The two microtubules of the central pair complex also differ from one another (5–7). In squid sperm, Linck and co-workers (8) have shown that the morphology of the C1 microtubule differs from that of the C2 microtubule. In *Chlamydomonas* the C1 microtubule has two projections that are 18 nm long as measured by thin-section electron micros-

copy. The C2 microtubule has two shorter projections of ~8 nm each. Furthermore, the C2 microtubule is more labile than the C1 microtubule (4).

We have analyzed mutations at two unlinked loci in *Chlamydomonas* that affect the C1 microtubule specifically with the aim of understanding the assembly of microtubules in vivo and the differentiation of different types of microtubules.

MATERIALS AND METHODS

Mutant Strains: Three independent mutations at the *PF16* locus on linkage group IX have been identified, as well as six independent mutations at the *PF6* locus on linkage group X. The first allele of each gene was isolated by R. Lewin (10) following ultraviolet irradiation. Two additional alleles of *pf16* (*pf16A* and *pf16B*) and five additional alleles of *pf6* (*pf6A–E*) were isolated following mutagenesis of strain 137c by nitrosoguanidine (11) and by ICR 191 (12), respectively. Each allele segregated two mutant and two wild-type zygospores in backcrosses to wild-type cells (137c) as would be expected for a single gene mutation. Each allele was shown to be recessive to its wild-type allele in temporary dikaryons. Allelism was then determined by the lack of complementation between new alleles and strains carrying known *pf* mutations in temporary dikaryons (13). For both new alleles of *pf16* (*pf16A* and *pf16B*) and two new alleles of *pf6* (*pf6A* and *pf6B*), identification was confirmed by linkage to *sr1* and to *pf19*, respectively (14).

Motility: In contrast to other mutations affecting the central pair complex (*pf15*, *pf18*, *pf19*, and *pf20* [9, 6, 7]), the *pf6* and *pf16* mutants do not show paralyzed, rigid flagella. Rather *pf16* mutations lead to paralysis but the flagella show slight twitching at the flagellar tips and occasional wave forms. Flagella of *pf6* cells show limited jiggling movements, which occasionally may propel the cells forward. These motility phenotypes were unchanged at 17°, 25°, or 32°C. Further studies were performed with cells grown at 25°C.

Evidence for a Second Mutation in Two pf16 Strains: In the course of biochemical analysis of isolated *pf16* and *pf16B* axonemes, an alteration in the mobility in the pH gradient of two-dimensional gels was observed for a constellation of polypeptides (Fig. 3). The constellation consists of at least three polypeptides; all were shifted to a more basic location in these two strains. These polypeptides have an apparent molecular weight of 125,000. Because this electrophoretic change was observed in two of the three independently isolated alleles, we examined its linkage to the *PF16* locus. In eight tetrads from a cross of *pf16B* × 137c (and eight random spores from a cross of *pf16* × 137c), the alteration segregated as expected of a single gene and there was no detectable linkage between the alteration and the *PF16* locus (2:1:5; parental ditype:nonparental ditype:tetratype for *pf16B*). It was surprising that the same alteration was observed in two independently isolated alleles. The alteration when separated from the *pf16* or *pf16B* lesion did not produce any noticeable change in the motility phenotype of the mutants nor did the new single mutant have a motility phenotype by itself. One possible explanation is that second mutation has some selective advantage during storage of mutants.

Reversion Analysis: Revertants of *pf16B* and *pf6* were screened following mutagenesis by ultraviolet irradiation of gametic cells for 30 and 60 s as previously described (15). 47 and 87% killing was observed for *pf16B* cells, and 70 and 97% for *pf6* cells. Each revertant was crossed to wild-type strain 137c and analyzed by standard techniques (16).

Extraction of the C2 Microtubule: Approximately 300 μg of axonemal protein was collected and resuspended in 50 mM NaCl, 4 mM $MgCl_2$, 0.25 mM HEPES, 0.005 mM $SrCl_2$, 0.025 mM dithiothreitol. The suspension is made 500 mM NaCl, 4 mM $MgCl_2$, 10 mM HEPES, 0.0001 mM $SrCl_2$, 4 μM dithiothreitol, and 4 μM ATP (pH 7.2) in a total volume of 600 μl and placed at room temperature for 10 min. The suspension was dialyzed at 4°C for 4 h against 10 mM 2(N-morpholino) ethanesulfonic acid (MES), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (pH 6.4). It was centrifuged at 110,000 g for 30 min (17). Supernatant and the residual pellet were collected. Both fractions were examined by gel electrophoresis and the residual pellet was examined by thin-section electron microscopy.

Other Methods: Methods for culturing cells and formation of dikaryons (15), radiolabeling with [^{35}S]sulfuric acid, and electron microscopy (11) have been previously described. The isolation protocol for flagella and axonemes was modified (11) in the final step by adding 0.1% NP-40 rather than 0.5% for preparation of axonemes and by collecting the flagella or axonemes by centrifugation at 35,000 g for 1 h. With these modifications we were able to obtain axonemes from wild-type 137c cells in which 87% of the axonemal cross sections had both central pair microtubules as compared with 52% for the previous method. Two-dimensional gel electrophoresis was performed as described by Piperno et al. (18), with the modification that the pH gradient electrophoresis was run for 16 h at 1.5 mA. One-dimensional gel electrophoresis on 4–8% gradient gels was performed as described by Adams et al. (7).

RESULTS

Ultrastructural Analysis

Morphology of the central pair complex was studied in flagella attached to the cells, in isolated flagella, and in isolated axonemes. With intact flagella, there is poor penetration of tannic acid stain, and thin-section electron microscopy shows little detail of the central pair complex (7). In these flagellar cross sections, we simply were able to determine whether both central pair microtubules were present or absent. No differences in central pair morphology were detected between mutant and wild-type preparations in attached flagella. Both tubules were present in 97% of wild-type (137c mt^+) cross-sectional images ($n = 407$), in 98% of *pf16B* images ($n = 59$), and in 97% of *pf6* images ($n = 73$). Similar observations were made for isolated flagellar fractions from these strains (data not shown).

In isolated axonemal preparations, staining was effective and additional features of the central pair complex were

TABLE I
Number of Microtubules in the Central Complex

No. of micro-tubules	Genotype			Chemically extracted 137c*
	137c	pf16B	pf6	
0	4 (0.01)†	139 (0.32)	8 (0.02)	19 (0.07)
1	34 (0.11)	256 (0.60)	43 (0.09)	250 (0.90)
2	264 (0.87)	35 (0.08)	409 (0.89)	9 (0.03)
Total	303	430	460	278

* See Materials and Methods.
† Numbers in parentheses indicate the frequency of each class.

visible. When we determined the frequency of cross-sectional axonemal images that had two central microtubules, *pf16B* axonemes were clearly different from both *pf6* and 137c mt^+ preparations (Table I). For 137c mt^+, 87% of axonemal cross sections ($n = 303$) showed two central microtubules and for *pf6* the value was 89% ($n = 460$). In the case of *pf16B* preparations, only in 8% of the images ($n = 430$) were both microtubules present. In the remainder of the *pf16B* cross-sectional images, 60% showed only one tubule, and 32% showed no central tubules.

In addition, we examined wild-type axonemes that had been subjected to chemical extraction. Axonemes were treated with high salt concentrations (17) and then dialyzed against low ionic strength buffer for 4 h. The suspension was subjected to centrifugation and the residual axonemal pellet and the extracted material in the supernatant were collected. By thin-section electron microscopy, 90% of the images of the residual axonemes in the pellet fraction ($n = 278$) had a single microtubule, 3% had two microtubules, and 7% had no central microtubules.

In axonemal images the C1 central microtubule can be seen to have projections of ~18 nm, which are longer than the projections of the C2 microtubule (5). We used this criterion to identify the persistent tubule among 110 *pf16B* axonemal cross sections showing a single central microtubule. In 106 cases (95%), the C2 microtubule persisted. Similar observations were made for *pf16* axonemes (96%; $n = 71$). In direct contrast, it had been observed previously that in wild-type axonemal preparations the C2 microtubule is more likely to be labile (4, 7). In our preparations most cross sections with only a single microtubule from *pf6* axonemes (96%; $n = 43$), from 137c axonemes (93% $n = 34$), or from extracted 137c axonemes (97%; $n = 250$) contained the C1 microtubule. Thus, it appeared that *pf16* lesions specifically altered the stability of the C1 microtubule in the majority of isolated axonemes. Since in *pf16B* axonemes, 32% of the cross sections (compared with 1.3% in 137c and 1.7% in *pf6* axonemes) showed the absence of both microtubules, it is likely that the mutation secondarily affects the stability of the C2 microtubule.

As already noted isolated axonemes from *pf6* cells showed no significant instability of the central microtubules. A mutant defect became apparent when samples of C1 and C2 microtubules from axonemal cross sections of *pf6* and 137c were analyzed for the number of projections that each carried. As shown in Table II, there was in *pf6* preparations a specific paucity of C1 images that showed two projections and a marked excess of C1 images that showed only a single projection. No such difference was found in the analysis of C2 images from *pf6* and 137c. These observations suggest that lesions at the *PF6* locus specifically affect formation of one

Table II

Number of Cross-sectional Images from 137c and pf6 Gametic Cells

No. of projec-tions	C1 microtubule		C2 microtubule	
	137	pf6	137	pf6
0	3 (0.04)*	8 (0.13)	8 (0.10)	4 (0.06)
1	25 (0.33)	52 (0.84)	34 (0.45)	24 (0.39)
2	47 (0.63)	2 (0.03)	34 (0.45)	34 (0.55)
Total	75	62	76	62

* Numbers in parentheses indicate the frequency of each class.

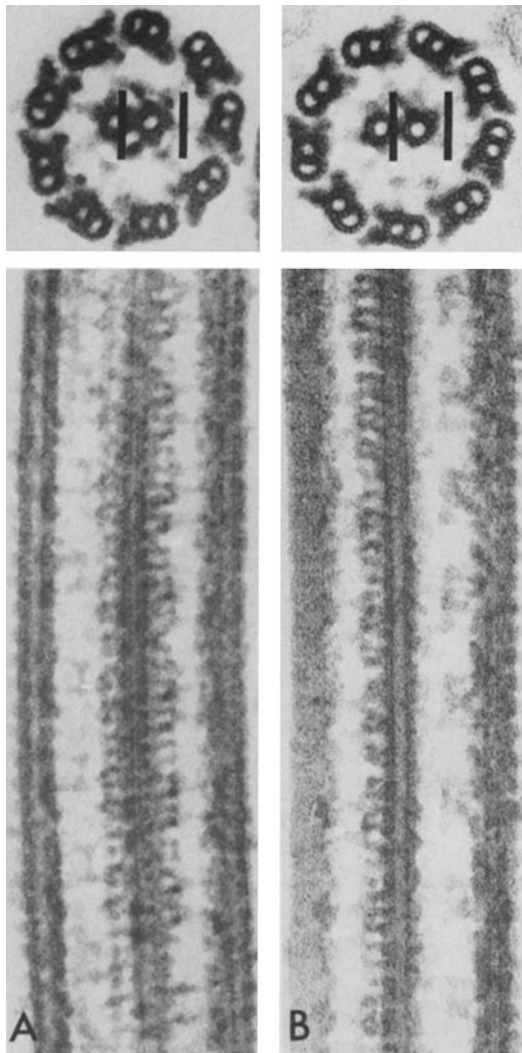Figure 1   Thin-section electron micrograph illustrating the morphology of isolated axonemes from wild-type (A) and pf6 (B) cells. (A, top) a transverse section of a wild-type axoneme in which the central tubules are clearly seen to be differentiated by the presence of two long projections (C1 microtubule on the right) and two short projections (C2 microtubule on the left); (bottom) a longitudinal section that passes through a wild-type axoneme in the plane indicated by slashes at top. The long projections on the C1 microtubule extend from the microtubule in two rows with a longitudinal periodicity of ~17 nm. (B, top) a transverse section of a pf6 axoneme in which one of the two long projections on the C1 microtubule is missing; (bottom) a longitudinal section that demonstrates that pf6 cells are defective for the assembly of one specific row of long projections normally associated with the C1 microtubule. × 128,000.

of the long projections of the C1 microtubule. The favorable longitudinal section of a pf6 axoneme shown in Fig. 1 further suggests that with respect to lateral location on the C1 microtubule, a specific population of long projections is missing from the axoneme. This specificity was confirmed by an analysis of 54 pf6 axonemal cross sections oriented as shown in Fig. 1B with the C1 microtubule on the right and the dynein arms directed clockwise. In every case the single C1 projection pointed upwards.

Biochemical Analysis

Previous work had shown that the central pair complex in axonemes isolated from pf18 mutant cells (6, 7) was unstable and was lost during the preparation of axonemes. In comparing radiolabeled polypeptides from isolated pf18 and 137c axonemes, a set of 18 polypeptides was missing or reduced consistently in this mutant (7). However, in these preparations only 52% of the axonemes from wild-type cells had a complete central pair complex by electron microscopic analysis. Using minor modifications of the isolation protocol (see Materials and Methods) we were able to obtain wild-type preparations in which 87% of the axonemal cross sections had a complete central pair complex. In comparing pf18 and 137c axonemes using this protocol, 23 polypeptides were missing from the pf18 preparations compared with wild-type preparations. Two different electrophoretic gel systems were used for this analysis. The first method resolves polypeptides with molecular weights from 360,000 to 160,000 by one-dimensional SDS acrylamide gradient gels. Four polypeptides in this molecular weight range (CP1-CP4) are missing (6, 7) and are indicated in Fig. 2. The second method resolves polypeptides from 160,000 to 15,000 by two-dimensional gel electrophoresis. 19 polypeptides in this molecular weight range (CP5-CP23) are missing and are indicated in Fig. 3. It should be noted that CP19-CP23 had not been identified as consistently deficient in the earlier analyses of pf18 axonemes (6, 7).

Axonemal polypeptides from pf16 and pf6 cells were ana-

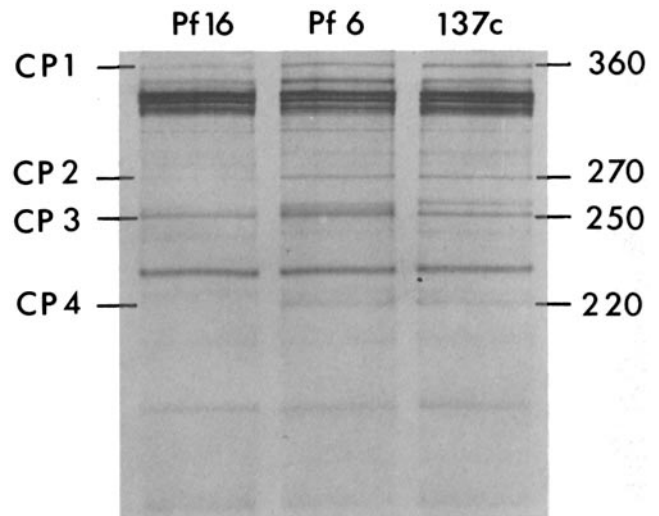|	Pf 16	Pf 6	137c	
CP1				360
CP2				270
CP3				250
CP4				220

Figure 2   Portion of autoradiograph from one-dimensional gradient gel electrophoretogram resolving polypeptides from 360,000 to 220,000. Axonemes from pf16B, pf6, and 137c radiolabeled cells are shown. Approximately 0.5 μg of protein (specific activity = 100,000 cpm/μg) were loaded per lane. The band above CP3 is variable in its presence and probably represents contamination by flagellar matrix proteins. Values at right represent molecular weight × 10⁻³.
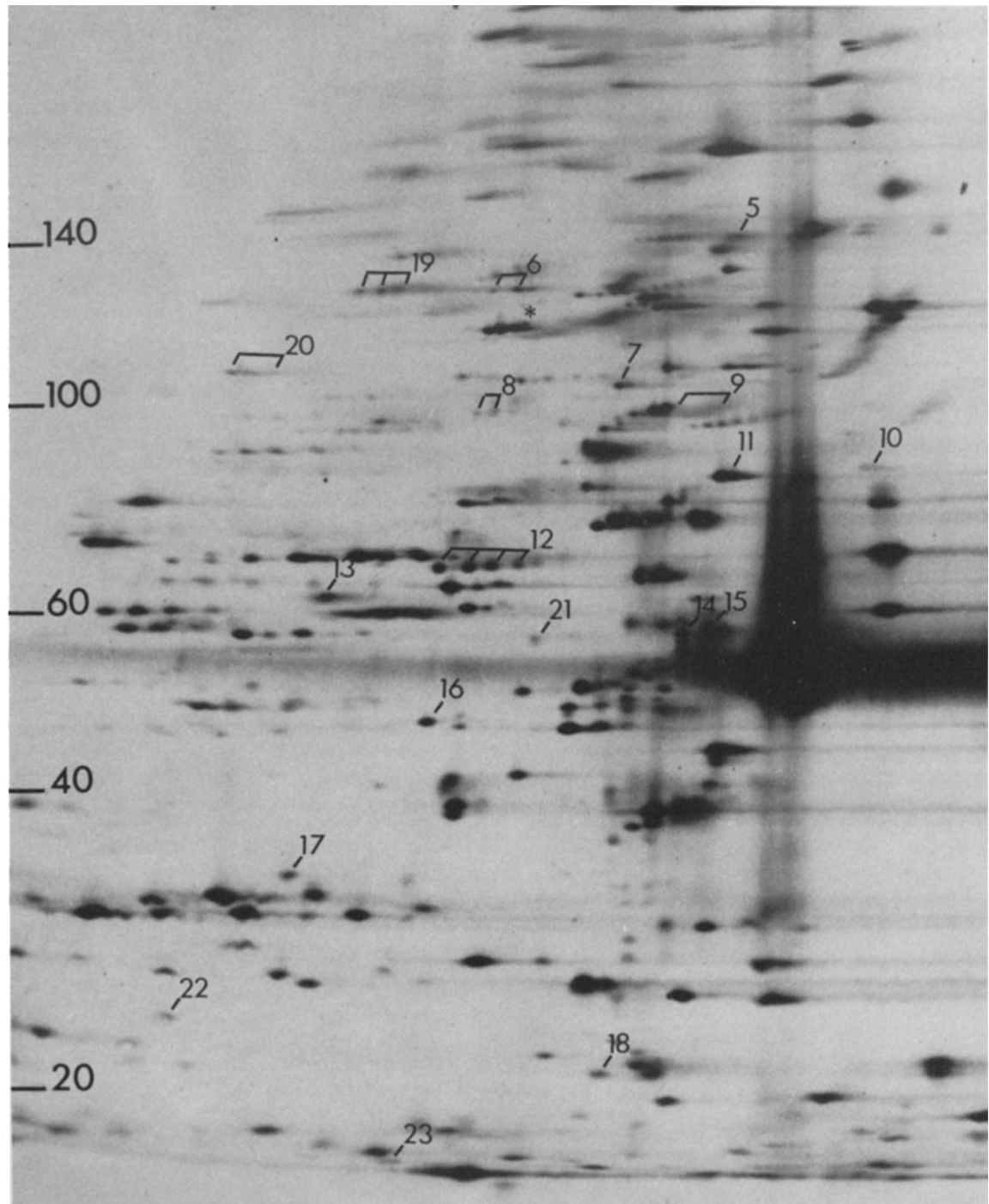
FIGURE 3 Autoradiograph of [^{35}S]sulfuric acid radiolabeled polypeptides from isolated 137c axonemes resolved by two-dimensional electrophoresis. The first dimension is nonequilibrium isoelectric focusing in a pH gradient and the second dimension is 4–11% acrylamide gradient gel electrophoresis in the presence of SDS. Nineteen polypeptides missing or diminished in the mutant *pf18* are indicated. This mutant completely lacks a central pair microtubule complex in isolated axonemal preparations (6, 7). The constellation of polypeptides indicated by the asterisk is the polymorphism described in Materials and Methods.

lyzed and compared with wild-type axonemes. In both cases, the sets of polypeptides consistently found to be deficient in these mutants comprised a subset of the group of polypeptides deficient in the isolated axonemes from *pf18* cells. The subset of 10 polypeptides missing from *pf16B* axonemes is listed in Table III. In three independent alleles (*pf16*, *pf16A*, *pf16B*) the same complement of polypeptides was defective. However in *pf16A* axonemes, CP12 and CP18 were reduced but were

not missing. The presence of these two prominent polypeptides may indicate the severity of the defect associated with this allele.

Polypeptides from isolated *pf16B* and *pf16* flagella were also analyzed. They were missing only three of the ten polypeptides absent in axonemes. The missing components were CP14, CP17, and CP19 (Table III). In summary, there are 10 unique polypeptides associated with the C1 microtubule. The

TABLE III

Summary of Axonemal and Flagellar Polypeptide Deficiencies

CP	Mol wt × 10⁻³	pf16B		pf6		137c extracted[+] axonemes	
		axonemes	flagella	axonemes	flagella	extracted	residual
1	360*	−/+	+	+	+	−/+	+
2	270*	−	+	+	+	−	+
3	250*	+	+	+	+	+	−
4	220*	−	+	+	+	−	+
5	142*	+	+	+	+	+	−
6	128*	+	+	+	+	+	−
7	106*	+	+	+	+	+	−
8	97*	+	+	+	+	+	+
9	97*	−	+	−	−	−	+
10	84*	+	+	+	+	+	−
11	84*	+	+	+	+	+	−
12	66*	−	+	−	−	−	+
13	62*	+	+	+	+	+	+
14	57*	−	−	+	+	−	+
15	56*	+	+	+	+	+	−
16	45*	+	+	+	+	+	+
17	32*	−	−	+	+	−	+
18	20*	−	+	−	−	−	+
19	128	−	−	+	+	−	+
20	110	−	+	+	+	−	+
21	56	+	+	+	+	+	+
22	25	+	+	+	+	+	+
23	14	+	+	+	+	+	+

* Polypeptides identified by Adams et al. (7).
[+] Column entitled extracted refers to polypeptides removed from axonemes by salt treatment and dialysis and recovered in the supernatant after centrifugation at 110,000 g for 30 min. Column entitled residual refers to polypeptides remaining bound to the axonemes and recovered in the pellets.

absence of three of these polypeptides results in paralyzed flagella (as shown by the analysis of the isolated flagella) and their absence destabilizes the normally stable C1 microtubule in isolated axonemes.

Polypeptides from isolated pf6 axonemes were examined and it was found that the isolated axonemes were missing a subset of the pf16 axonemal signature. They were lacking CP9, CP12, and CP18 (Table III). Two additional alleles of pf6 (pf6A and pf6B) showed the same molecular phenotype in axonemes. Isolated flagella from pf6 were also missing CP9, CP12, and CP18. The lesions of PF6 appeared to affect one of the two projections of the C1 microtubule. By electron microscopic analysis, a single lateral array of projections was deficient and by biochemical analysis, it appeared to comprise a unique set of three polypeptides. Furthermore, this projection did not appear to play a role in stabilizing the C1 microtubule.

We also analyzed supernatant and pellet fractions of chemically extracted 137c axonemes. Based on the electron microscopic phenotype (Table I) of the residual axonemal pellet, we expected that central pair polypeptides representing components of the C2 microtubule would be enriched in the supernatant fraction. The supernatant fraction may also include labile components of the C1 microtubule. We found that 14 of the 23 central pair signature polypeptides were extracted into the supernatant by the chemical treatment. Comparing supernate and pellet, seven polypeptides were found predominantly in the supernatant fraction (Table III; CP3, 5, 6, 7, 10, 11, and 15). These polypeptides are likely to represent unique components of the C2 microtubule. The other seven polypeptides were extracted partially; they are CP1, 8, 13, 16, 21, 22, and 23. Except for CP1 in the second set, the central pair polypeptides that were solubilized by the

chemical treatment did not include the 10 polypeptides found missing from pf16 axonemes. The polypeptides that are solubilized partially might be localized in both the C1 and C2 microtubules.

Gene Product Analysis of pf16B

To ask about the identity of the gene product of the PF16 locus and to determine its role in the flagellum, we attempted to isolate intragenic revertant mutations of the motility defect following ultraviolet-irradiation of gametic cells. Seven potential intragenic revertants of pf16B were isolated. Each was an independent isolate and was found to be linked to the original pf16B mutation as assessed by the absence of recombination between each of the new mutations and the pf16B mutation in 12 tetrads in backcrosses to 137c (<4.0 centiMorgans [cM][1]). One isolate (R3) was examined further and no recombination was observed between the pf16B mutation and the second mutation in 138 tetrads from crosses to wild-type cells (137c mt⁻). Although these secondary mutations may define a closely linked locus (ranging from <4 cM to <0.34 cM), we will assume that these secondary mutations are intragenic revertants.

Two of the revertants (R6 and R10) restored wild-type motility to the cells, whereas five of the revertants (R1, R3, R5, R7, and R8) showed some departure from wild-type motility at 25°C. Biochemical analyses of isolated axonemes from R6 and R10 cells showed the return of the 10 polypeptides missing in axonemes of the parent pf16B cells to their wild-type position. The second group of revertants (R1, R3,

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[1] Abbreviations used in this paper: cM, centiMorgans; MAP, microtubule-associated protein.
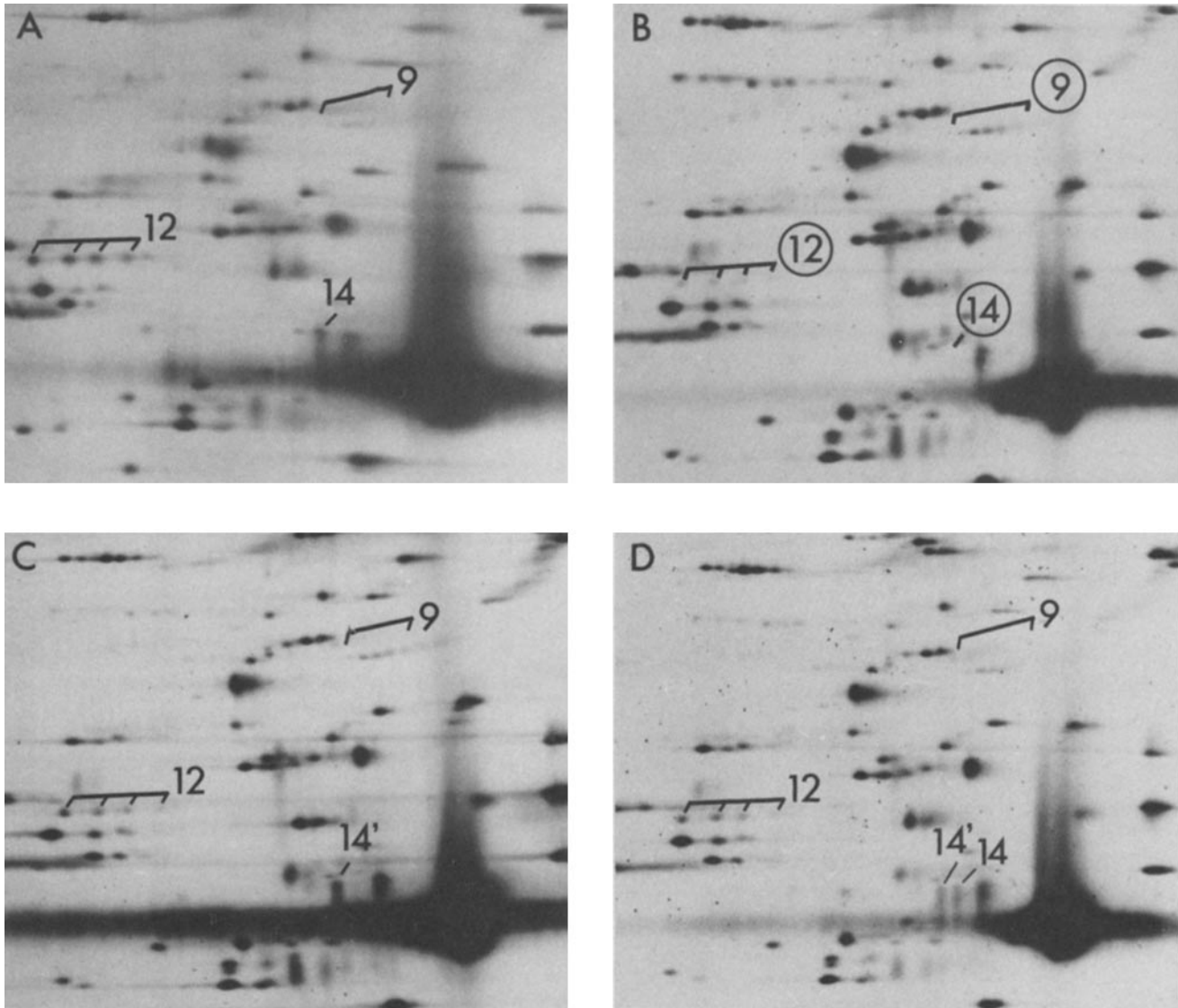
FIGURE 4   Portions of autoradiographs from two-dimensional electrophoresis. (A) 137c axonemes; (B) *pf16B* axonemes; (C) *pf16B-R3* axonemes; (D) *pf16B-R3* and 137c axonemes mixed in vitro. Polypeptides CP9, CP12, and CP14 are indicated. Numbers encircled designate absence or reduction of the polypeptides indicated. *CP14'* is the putative altered gene product of the *PF16* gene.

R5, R7, and R8) showed the return of nine of the ten polypeptides to their wild-type positions and the tenth polypeptide, CP14, was replaced by a polypeptide with a more basic isoelectric point (*CP14'*) (Fig. 4). From in vitro mixing experiments of isolated axonemes from R1 or R3 cells with wild-type axonemes, both polypeptides (*CP14* and *CP14'*) were observed (Fig. 4). When R1 and R3 axonemes were mixed together in vitro, a single polypeptide (CP14') was observed (data not shown).

The observed change in the mobility of CP14 in some of the revertants suggests that CP14' may be an altered structural product of the *PF16* locus; however, it is not proof. Further evidence that CP14 is the structual gene product was provided by dikaryon analysis (15). Temporary dikaryons were constructed between *pf16B* and 137c cells, in which the *pf16B* parent was radiolabeled with [^{35}S]sulfuric acid but the wild-type parent (137c) was not radiolabeled. When in situ rescue of the motility defect of the *pf16B* flagella occurs in the dikaryon in the presence of a protein synthesis inhibitor,

anisomycin, any polypeptide that must come exclusively from the pool of wild-type flagellar polypeptides rather than the combined mutant and wild-type pools will return unlabeled. It was observed that all the polypeptides except CP14 contained radiolabel when the axonemes from *pf16B* × 137c dikaryons were isolated and gel electrophoretograms were analyzed by autoradiography. Thus, CP14 could only be supplied by the pool of polypeptides from the unlabeled wild-type parent. Both methods suggest that CP14 is the gene product of the *PF16* locus.

The revertants were also analyzed in dikaryons to assess dominance relationships. In dikaryons of *pf16B* × *pf16B-R3*, in which both parents were radiolabeled, the paralyzed flagella regained motility. The altered CP14' polypeptide and not the normal CP14 was observed in axonemes isolated from these dikaryons (data not shown). This datum shows that the revertant is dominant to the *pf16B* allele. Additionally, the altered gene product of two revertants, *pf16B-R1* and *pf16B-R3*, were each found to be co-dominant with the wild-type

PF16 gene product as shown by the presence of both CP14 and CP14' in axonemes isolated from wild-type by revertant dikaryons in which the flagella had been amputated and regenerated in the mixed cytoplasm (data not shown). These results show that the *pf16* flagella in the presence of wild-type or revertant cytoplasm regained motility and that the two microtubules were again stable in isolated axonemes, which again suggests that CP14 is the *PF16* gene product.

Gene Product Analysis of pf6

We attempted to isolate intragenic revertants of the motility defect of *pf6* cells. Only a single potential intragenic revertant (*pf6-R4*) was isolated. No recombination was observed between *pf6* and the second mutation in 43 tetrads (<1.1 cM). These cells showed wild-type motility patterns. Biochemical analysis of isolated axonemes from *pf6-R4* cells showed the return of the three polypeptides missing in axonemes of *pf6* cells to their wild-type position. No evidence for a structural gene product of the *PF6* gene was obtained using dikaryon rescue analysis. When radiolabeled *pf6* cells were mated to unlabeled 137c cells, all three polypeptides that were missing returned with label in the isolated axonemes. Two alternative explanations exist. One is that the gene is a structural component of the axoneme but that we have not resolved it in either of our gel systems. Alternatively, the gene product may be extrinsic to the axoneme.

DISCUSSION

Mutations at the *pf16* locus result in the singular loss from axonemal preparations of the C1 microtubule of the central pair complex. When compared with axonemes from wild-type cells, the mutants showed loss or striking diminution of ten axonemal polypeptides regularly present in the wild-type preparations. The missing polypeptides, CP1, 2, 4, 9, 12, 14, 17, 18, 19, and 20, range in relative molecular weight from 360,000 to 20,000 and constitute a subset of 23 polypeptides missing from isolated axonemes of mutants at the *pf18* locus. Axonemes from *pf18* cells lack both the C1 and C2 central microtubules (5).

When wild-type axonemes were treated with high salt concentrations and dialyzed against low ionic strength buffer, the C2 central pair microtubule is selectively solubilized. Examination of the distribution between supernatant and pellet of central pair axonemal polypeptides, as defined by the *pf18* axonemal deficiencies, showed that a subset of seven polypeptides CP3, 5, 6, 7, 10, 11, and 15 are exclusively present in the supernatant fraction. These data together with the *pf16* results, suggest that each subset of central pair components is unique respectively to the C1 or the C2 microtubule.

At present there is no evidence to indicate that tubulin subunits, the major components of the central pair, show any structural variation between the C1 and C2 tubules. An analysis of α and β tubulin in two-dimensional gels showed no differences between wild-type and central pair defective (*pf18*) axonemes (7). Since only two α tubulin and two β tubulin genomic sequences have been identified in *C. reinhardtii* (20, 21), any explanation of the specific properties of the variety of flagellar and cellular microtubules based exclusively on tubulin subunits would require that the diversity be based on transcriptional processing or post-translational modification. An explanation based on the association of other polypeptides with the tubulin subunit lattice seems equally plausible.

A variety of proteins have been found associated with preparations of cytoplasmic microtubules, including microtubule-associated protein (MAP) 1 ($M_r = 350,000$) and MAP 2 ($M_r = 270,000$) (22, 23), the tau (τ) MAPs ($M_r = 55,000$–62,000) (24), and lower molecular weight MAPs ($M_r = \sim 30,000$) (25). It has been shown that MAP 1 or MAP 2 proteins reassembled onto microtubules appear as filamentous projections or arms at regularly spaced intervals (26–28). The high molecular weight MAPs and the tau MAPs (29) have been found to influence the stability of microtubules reassembled from brain tubulins.

It is likely too, in the case of *Chlamydomonas* flagellar central microtubules, that the specific and different properties of the C1 and C2 microtubules are based on the unique subset of nontubulin polypeptides associated with each microtubule. The case seems clear for one class of projections of the C1 microtubule. The absence from *pf6* flagella and axonemes of polypeptides CP9, 12, and 18, a subset of the unique C1 polypeptides, is correlated with the absence of one class of projections. In this case the mutant defect appeared not to alter the stability of the C1 microtubule in axonemal preparations.

In the case of *pf16* mutants, three polypeptides were missing from flagella (CP14, 17, and 19) but it has not been possible to determine whether loss of the polypeptides is associated with a structural alteration. When mutant flagella are converted to axonemes, there was very extensive loss of the C1 microtubule, the more stable of the central pair microtubules in wild-type axonemes, and partial loss of the C2 microtubule. The altered stability of the C1 central microtubule suggests that one of the three polypeptides missing from *pf16* flagella may interact with the tubulin lattice. The likely candiate for such direct or indirect interaction is CP14, the polypeptide ($M_r = 57,000$) that appears to be the *PF16* gene product.

It is interesting that *pf16B-R3*, a strain selected from reversion experiments and likely to represent an intragenic revertant (<0.34 cM) showed a heat-sensitive phenotype. At temperatures below 21°C, motility was wild-type and the complement of axonemal polypeptides was complete (see Fig. 4). The cells become progressively more paralyzed with increasing temperature and the C1-specific polypeptides were absent from axonemes maintained and isolated at 32°C. Preliminary results suggest that it will be possible to use this mutant to isolate additional suppressors at the restrictive temperature with the aim of identifying other genes involved in the same morphogenetic process (30, 31).

Analysis of mutants at the *PF6* and *PF16* loci provides additional evidence that specialized properties of cellular microtubules may depend on nontubulin polypeptides associated with the tubulin lattice. The *pf16* mutants appeared to affect stability of the tubulin lattice, and genetic analysis using a temperature-sensitive variant may permit extensive dissection of the interaction of α and β tubulins with associated proteins.

REFERENCES

1. Tilney, L. G., J. Bryan, D. J. Bush, K. Fujiwara, M. S. Mooseker, D. B. Murphy, and D. H. Snyder. 1973. Microtubules: evidence for 13 protofilaments. *J. Cell Biol.* 59:267–275.
2. Dentler, W. L., and J. L. Rosenbaum. 1977. Flagellar elongation and shortening in *Chlamydomonas*. III. Structures attached to the tips of flagellar microtubules and their relationship to the directionality of flagellar microtubule assembly. *J. Cell Biol.* 74:747–759.
3. Jacobs, M., J. M. Hopkins, and J. Randall. 1969. Biochemistry of *Chlamydomonas* flagella. *Proc. R. Soc. Lond. B Biol.* 173:61–62.
4. Witman, G. B., K. Carlson, J. Berliner, and J. L. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *J. Cell Biol.* 54:507–539.
5. Hopkins, J. M. 1970. Subsidiary components of the flagella of *Chlamydomonas reinhardtii. J. Cell Sci.* 7:823–839.
6. Witman, G. B., J. Plummer, and G. Sander. 1978. *Chlamydomonas* flagellar mutants lacking spokes and central tubules. *J. Cell Biol.* 76:729–747.
7. Adams, G. M. W., B. Huang, G. Piperno, and D. J. L. Luck. 1981. Central- pair microtubular complex of *Chlamydomonas* flagella: polypeptide composition as revealed by analysis of mutants. *J. Cell Biol.* 91:69–76.
8. Linck, R. W., G. E. Olson, and G. L. Langevin. 1981. Arrangement of tubulin subunits and microtubule-associated proteins in the central- pair microtubule apparatus of squid (*Loligo pealei*) sperm flagella. *J. Cell Biol.* 89:309–322.
9. Chasey, D. 1974. The three-dimensional arrangement of radial spokes in the flagella of *Chlamydomonas reinhardtii. Exp. Cell Res.* 84:374–380.
10. Lewin, R. A. 1954. Mutants of *C. moewusii* with impaired motility. *J. Gen. Microbiol.* 11:358–363.
11. Huang, B., G. Piperno, and D. J. L. Luck. 1979. Paralysed flagella mutants of *Chlamydomonas reinhardtii. J. Biol. Chem.* 254:3091–3099.
12. Huang, B., Z. Ramanis, S. K. Dutcher, and D. J. L. Luck. 1982. Uniflagellar mutants of *Chlamydomonas*: evidence for the role of basal bodies in transmission of positional information. *Cell.* 29:745–753.
13. Starling, D., and J. Randall. 1971. The flagella of temporary dikaryons of *Chlamydomonas reinhardtii. Genet. Res.* 18:107–113.
14. Harris, E. H. 1982. Nuclear gene loci of *Chlamydomonas reinhardtii. In* Genetic Maps. S. J. O'Brien, editor. National Cancer Institute, National Institutes of Health, Bethesda, Maryland. 142–146.
15. Luck, D. J. L., G. Piperno, Z. Ramanis, and B. Huang. 1977. Flagellar mutants of *Chlamydomonas*: studies of radial spoke-defective strains by dikaryon and revertant analysis. *Proc. Natl. Acad. Sci. USA.* 74:3456–3460.
16. Levine, R. P., and W. T. Ebersold. 1960. The genetics and cytology of *Chlamydomonas. Annu. Rev. Microbiol.* 14:197–216.
17. Piperno, G., and D. J. L. Luck. 1979. Axonemal adenosine triphosphatases from flagella of *Chlamydomonas reinhardtii. J. Biol. Chem.* 254:3084–3090.
18. Piperno, G., B. Huang, Z. Ramanis, and D. J. L. Luck. 1981. Radial spokes of *Chlamydomonas* flagella: polypeptide composition and phosphorylation of stalk components. *J. Cell Biol.* 88:73–79.
19. Warr, J. R., A. McVittie, J. T. Randall, and J. M. Hopkins. 1966. Genetic control of flagellar structure in *Chlamydomonas reinhardtii. Genet. Res.* 7:335–351.
20. Silflow, C. D., and J. L. Rosenbaum. 1981. Multiple α- and β-tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after deflagellation. *Cell.* 24:81–88.
21. Brunke, K. J., E. E. Young, B. U. Buchbinder, and D. P. Weeks. 1982. Coordinated regulation of the four tubulin genes of *Chlamydomonas reinhardtii. Nuc. Acid Res.* 10:1295–1310.
22. Sloboda, R. D., S. A. Rudolph, J. Rosenbaum, and P. Greengard. 1975. Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci.* 72:177–181.
23. Murphy, D. B., R. B. Vallee, and G. G. Borisy. 1977. Identity and polymerization—stimulatory activity of the nontubulin proteins associated with microtubules. *Biochemistry.* 16:2598–2605.
24. Weingarten, M. D., A. Lockwood, S.-Y. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA.* 72:1858–1862.
25. Berkowitz, S. A., J. Katagiri, H. K. Binder, and R. C. Williams, Jr. 1977. Separation and characterization of microtubule proteins from calf brain. *Biochemistry.* 16:5610–5617.
26. Herzog, W., and K. Weber. 1978. Fractionation of brain microtubule-associated proteins. Isolation of two different proteins which stimulate tubulin polymerization *in vitro. Eur. J. Biochem.* 92:1–8.
27. Kim, H., L. I. Binder, and J. L. Rosenbaum. 1979. The periodic association of MAP2 with brain microtubules *in vitro. J. Cell Biol.* 80:266–276.
28. Vallee, R. B., and S. E. Davis. 1983. Low molecular weight microtubule-associated protein are light chains of microtubule-associated protein (MAP 1). *Proc. Natl. Acad. Sci. USA.* 80:1342–1346.
29. Cleveland, D. W., S.-Y. Hwo, and M. W. Kirschner. 1977. The physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. *J. Mol. Biol.* 116:227–247.
30. Gutherie, C., H. Nashimoto, and M. Nomura. 1969. Structure and function of *E. coli* ribosomes. VIII. Cold-sensitive mutants defective in ribosome assembly. *Proc. Natl. Acad. Sci. USA.* 63:384–391.
31. Jarvick, J., and D. Botstein. 1975. Conditional-lethal mutations that suppress genetic defects in morphogenesis by altering structural proteins. *Proc. Natl. Acad. Sci. USA.* 72:2738–2742.