

# Radial Spokes of *Chlamydomonas* Flagella: Genetic Analysis of Assembly and Function

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**ABSTRACT** In addition to the previously studied *pf-14* and *pf-1* loci in *Chlamydomonas reinhardtii*, mutations for another five genes (*pf-17*, *pf-24*, *pf-25*, *pf-26*, and *pf-27*) have been identified and characterized as specifically affecting the assembly and function of the flagellar radial spokes. Mutants for each of the newly identified loci show selective alterations for one or more of the 17 polypeptides in the molecular weight range of 20,000–130,000 which form the radial spoke structure. In specific instances the molecular defect has been correlated with altered radial spoke morphology. Biochemical analysis of in vivo complementation in mutant X wild-type dikaryons has provided indirect evidence that mutations for four of the five new loci (*pf-17*, *pf-24*, *pf-25*, and *pf-26*) reside in structural genes for spoke components. In the case of *pf-24*, the identity of the mutant gene product was supported by analysis of induced intragenic revertants. In contrast to the other radial spoke mutants thus far investigated, evidence suggests that the gene product in *pf-27* is extrinsic to the radial spokes and is required for the specific in vivo phosphorylation of spoke polypeptides.

In the preceding communication (1) analysis of axonemal polypeptides from the radial spokeless mutant, *pf-14*, led to the identification of 17 polypeptides as likely components of the radial spokes in flagella of *Chlamydomonas reinhardtii*. The localization of a subset of five of these polypeptides to the distal portion of the radial spoke, the spokehead, was obtained by analysis of the spokeheadless mutant, *pf-1*, and confirmed in experiments of chemical dissociation of wild-type axonemes.

Given the evidence that as many as 17 different polypeptides form the radial spoke structure, we sought to identify mutations for additional loci specifying the assembly and function of the spokes. Because of the precise and reproducible identification of each of the 17 spoke components in two-dimensional axonemal maps, the group taken together served as a signature for screening other flagellar-defective mutants. With this approach, mutations for five additional genes affecting the radial spokes were identified and characterized.

In the studies reported here the genetics, axonemal ultrastructure, and axonemal polypeptide composition of these mutants are described. By use of methods that previously led to the identification of gene products in *pf-14* and *pf-1* (2), evidence was obtained that the mutations for four of the five new loci reside in structural genes for spoke polypeptides. In the remaining case in which the mutant gene product appears to be extrinsic to the radial spokes, the mutant shows a specific defect for in vivo phosphorylation of spoke polypeptides. This

mutant gives the first indication of the existence of regulatory genes specific for the assembly and/or function of the radial spokes.

## MATERIALS AND METHODS

The methods for the following were as previously described: culture of cells (2), labeling with [<sup>35</sup>S]sulfuric acid (3) and [<sup>32</sup>P]phosphoric acid (1), isolation of flagella and axonemes (3), electron microscopy (3), formation of prezygotic dikaryons (2), and two-dimensional and one-dimensional electrophoresis (1).

## Mutant Strains

A culture of *pf-17*, a mutant previously isolated by R. A. Lewin, was kindly provided by Dr. N. Chua (The Rockefeller University). The strain was backcrossed to wild-type 137c and its map location on the right arm of linkage group VII was confirmed in a cross to *ac-5*. The newly isolated mutants were obtained after chemical mutagenesis of 137c mt<sup>+</sup>. The methods used for mutagenesis with nitrosoguanidine or methylmethanesulfate were as previously described (4). The protocol used for mutagenesis with ICR-191 (Terochem Laboratories, Ltd., Edmonton, Canada) was as follows: aliquots (25 ml, ~2–4 × 10<sup>6</sup> cells/ml) of vegetative 137c mt<sup>+</sup> cells in M-media (modified as in reference 4) were exposed to 20 μg/ml ICR-191 in the dark at 25°C for 90 min with constant stirring. The cells were pelleted, washed twice, and finally resuspended in 20 ml of media. 1-ml aliquots were placed in tubes containing 4 ml of media and held in the dark overnight before transfer to constant light. The mutagenic efficiency with this protocol was tested for by determining the rate of induction of streptomycin-resistant clones (at 50 μg/ml) in wild type as previously described (4). The frequency of resistant clones was 48/10<sup>6</sup> survivors as compared to a spontaneous frequency of 2/10<sup>6</sup> survivors. Nonconditional and thermosensitive flagellar mutants were selected for as previously described (4).

## Genetic Analysis

Standard techniques of crossing and tetrad analysis were used to determine segregation patterns and recombination frequencies (5). The newly isolated mutants were crossed to single and multiple marker stocks to determine the map locations of the mutations. The centromere distances were calculated from tetraploid frequencies observed in crosses to *ac-17* by the method of Gowans (6).

### Isolation and Genetic Analysis of Revertants

The revertants described in this paper were induced by exposing the *pf* radial spoke mutants to UV irradiation and were selected for as previously described (2). Each revertant was analyzed genetically to determine if restoration of motility was caused by a back mutation in the original gene or by a second-site extragenic mutation. This analysis was performed by crossing each revertant to wild-type strain 137c and analyzing at least 10 complete tetrads from each cross. Those revertants in which no segregation of the original *pf* phenotype was observed were classified as likely to represent intragenic reversions.

## RESULTS

### Identification and Genetic Analysis of Radial Spoke Mutants

The axonemal molecular phenotype of the radial spokeless mutant *pf-14* (1) provided the basis for the identification of other mutations affecting the radial spokes. A large number of nonconditional and thermosensitive motility mutants isolated in our laboratory or previously isolated by R. A. Lewin, were screened at the level of a two-dimensional analysis of isolated <sup>35</sup>S-labeled axonemal polypeptides. Among these mutants, mutations for five new loci were found to be correlated with a selective and specific alteration for one or more of the 17 polypeptides missing in *p-14* (Table I). One of the newly identified radial spoke mutants, *pf-17*, was previously isolated by Lewin with UV irradiation and mapped by Ebersold et al. (7) to linkage group VII. The other radial spoke mutants were isolated in our laboratory after mutagenesis with nitrosoguanidine, methylmethanesulfate, or ICR-191. Each of the mu-

TABLE I  
Origin and Genetic Map Location of Radial Spoke Mutants

Mutant	Mutagenic agent	Map location (linkage group)
<i>pf-1*</i>	UV irradiation	V
<i>pf-14*</i>	UV irradiation	VI
<i>pf-14A</i>	Nitrosoguanidine	VI
<i>pf-17*</i>	UV irradiation	VII
<i>pf-24</i>	Nitrosoguanidine	X
<i>pf-25</i>	Nitrosoguanidine	
<i>pf-25A</i>	Methylmethanesulfate	
<i>pf-25B</i>	ICR-191	
<i>pf-25C</i>	ICR-191	X
<i>pf-25D</i>	ICR-191	
<i>pf-25E</i>	ICR-191	
<i>pf-25F</i>	Nitrosoguanidine	
<i>pf-26</i>	ICR-191	V
<i>pf-27</i>	Methylmethanesulfate	XII

\* Mutants previously isolated by R. A. Lewin, and mapped by Ebersold et al. (7). The other radial spoke mutants described in this paper were isolated in our laboratory.

tants was back-crossed to wild-type strain 137c, and the flagellar motility phenotype was found to segregate 2:2 as expected for single-site mutations.

Genetic mapping data have established that these new mutants represent mutations for four previously unmarked loci which we have designated *pf-24*, *pf-25*, *pf-26*, and *pf-27* (Table II). Except for the *pf-25* locus, for which seven independently isolated alleles have been found, only single isolates for each of the new loci were identified.

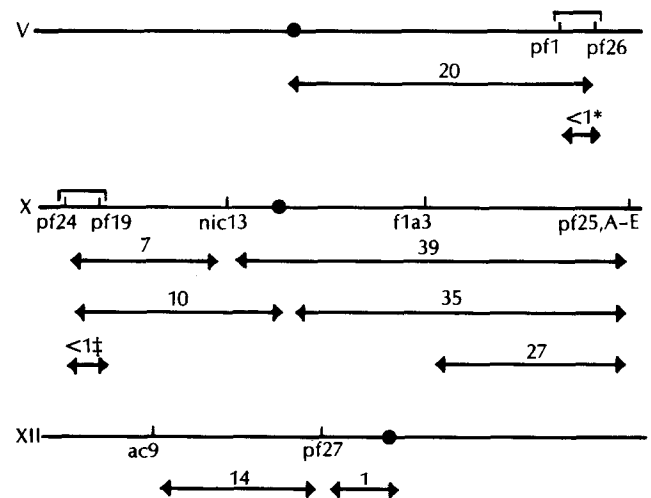
Of the seven genes identified as specifying radial spoke structure and function one case of close linkage was detected, that of the *pf-1* and *pf-26* loci on linkage group V. Although we have observed no recombination between *pf-26<sub>ts</sub>* and *pf-1* mutants in an analysis of 990 zygote clones or dissected tetrads, mutations for the two sites were found to be complementing in a stable diploid strain, and unique gene products were identified for the two loci. These data are included in our subsequent description of *pf-26<sub>ts</sub>*.

### Gene Product Analysis by Dikaryon Rescue and Revertant Analysis

In our analysis of the newly identified radial spoke mutants we have applied the two techniques used successfully in earlier studies on *pf-14* and *pf-1* mutants to identify mutant gene products (2). These methods are described briefly here using recent results obtained from *pf-14* for illustration.

The first technique, dikaryon rescue, takes advantage of a feature of the normal mating cycle of *Chlamydomonas*. Biflagellate plus and minus gametes fuse efficiently to give a population of quadriflagellate, temporary dikaryons with fused cytoplasm. Lewin (8) observed that in some cases when one of the gametic pair was a paralyzed flagellar mutant and the other wild type, flagellar function was restored to the preexisting mutant flagella during the lifespan of the dikaryon. To analyze rescue at the molecular level, we carried out the mating reaction with mutant gametes, prelabeled by growth on <sup>35</sup>SO<sub>4</sub>-containing media, and unlabeled wild-type gametes in the presence of an inhibitor of protein synthesis. Under these conditions it

TABLE II  
Genetic Data on Map Locations of *pf-24*, *pf-25*, *pf-26*, and *pf-27*



\* *pf-26* and *pf-1* are closely linked, but complementing mutations based on diploid analysis. The observed recombination frequency was <1 in 990.

‡ In crosses of *pf-24* × *pf-19*, the observed frequency of recombination was 1 in 362.

could be expected that in the course of restoration of flagellar function to radial spoke-defective mutants, most molecular components would be drawn from the combined pool of  $^{35}\text{S}$ -labeled mutant proteins and unlabeled wild-type proteins. However, if the mutant could not make one of the components of the radial spoke, or made it in a form which could not be assembled, this component could be supplied only from the wild-type pool and would be unlabeled. It would be absent in the two-dimensional autoradiogram of axonemes from dikaryons because it would not be radioactive, while all other normal components present in the mutant would contain radioactivity and would be present in the flagella. It has been observed (8, 9) that dikaryon rescue of flagellar function may occur with the flagella preexisting in gametes, *in situ* rescue, or may require deflagellation and flagellar regrowth, "regeneration rescue." We observed that for *pf-1*, *pf-14*, and all nonconditional mutants to be described subsequently, flagellar function was restored within 1 h of *in situ* rescue.

In Fig. 1 an example of the results of an *in situ* dikaryon rescue experiment is given for the radial spokeless mutant, *pf-14*. As is shown in the two-dimensional map of the quadriflagellate axonemal preparation, 16 of the 17 components originally missing from the mutant were restored in a radioactive form (for comparison, see map of *pf-14* gametic axonemes in Fig. 1B of the preceding paper).<sup>1</sup> Component 3, which is not present as a radioactive protein, is likely to represent the gene product of *pf-14*. The same results were obtained when the analysis was carried out by regeneration rescue.

We have recently obtained direct morphological evidence that *in situ* restoration of function and rescue of molecules in *pf-14* × wild-type dikaryons is associated with the assembly of radial spokes onto the preformed mutant axonemes. These data were obtained in dikaryon rescue experiments in which a recombinant strain carrying the *pf-14* mutation and a mutation for the *pf-18* locus was crossed to wild type. As previously described (10), *pf-18* is a mutant defective for the assembly of the central pair microtubules, and axonemes isolated from the mutant are characteristically missing the central tubules. In addition, as first shown by Starling and Randall (9) and confirmed by us, unlike *pf-14*, when *pf-18* is crossed to wild type, function of the paralyzed flagella is not restored to the preexisting flagella and occurs only after flagellar regeneration in the dikaryon.

The morphology of axonemes isolated from a *pf-14 pf-18* recombinant is illustrated in Fig. 2A. As seen in the cross section and longitudinal sections, the axonemes of the recombinant lacked both central pair microtubules and radial spokes. 3 h after mating of *pf-14 pf-18* gametes × wild type, the axonemes from the dikaryons were isolated and fixed for electron microscope examination. In such preparations axonemes derived from the mutant flagella were readily differentiated from those of wild type because they lacked central pair microtubules. In most images of central pairless axonemes, radial spokes with normal morphology could be identified attached to the outer doublets. In some longitudinal sections, as illustrated in Fig. 2B, rows of paired radial spokes were seen along extended regions of individual doublets.

The second technique for gene product analysis makes use

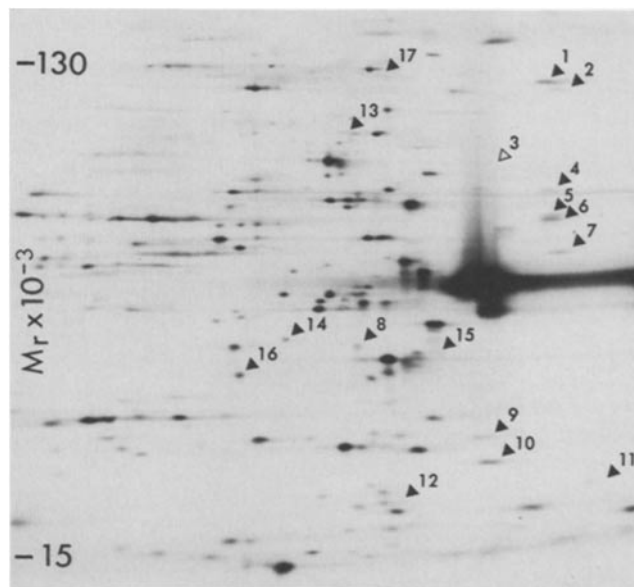


FIGURE 1 Autoradiogram of axonemal polypeptides, as resolved by two-dimensional electrophoresis, from dikaryons of *pf-14* mt<sup>+</sup> × 137c mt<sup>-</sup> 2.5 h after gametic mixing. Only *pf-14* gametes were radioactively labeled by growth on  $^{35}\text{S}$ -containing medium. A portion of the original map is shown. The proteins were applied at the anode and run under conditions of nonequilibrium pH gradient electrophoresis as described in the preceding communication (1). The gel is oriented with basic polypeptides on the left. The solid arrowheads indicate positions of radioactive proteins characteristically missing in the mutant but restored in the dikaryon. The open arrowhead indicates the position of polypeptide 3 which was not rescued in the dikaryon as a radioactive component.

of induced, intragenic revertants of the various mutants. The expectation is that in some cases alterations in the content of polar amino acids of putative gene products will lead to shifts in the isoelectric point, which will be recognizable in the two-dimensional maps. In Fig. 3A an example of a revertant obtained from *pf-14A* is shown in an axonemal mixing experiment with wild type. Protein 3 of the revertant is shown to have a higher isoelectric point than the wild type counterpart. The isoelectric focusing step in this experiment was carried to equilibrium. Other intragenic revertants of *pf-14A* showed shifts in isoelectric points to the acidic side of wild-type, and in some cases the acidic polypeptides showed a marked increase in mobility in the second dimension of the mapping procedure. The results confirm that polypeptide 3 is the *pf-14* gene product.

### Characterization of the New Radial Spoke Mutants

The newly identified radial spoke mutants fall into 2 groups based on their axonemal molecular phenotypes.<sup>2</sup> The first group includes nonconditional and thermosensitive mutations affecting a subset of 5 of the 17 polypeptides missing in *pf-14*, and identified through our analysis of *pf-1* as localized to the radial spokehead (1). The second group includes nonconditional mutations affecting one or more of the remaining 12

<sup>1</sup> Several of the components observed to be rescued in autoradiograms of *pf-14* × wild-type dikaryon maps were detected at very low intensities (for example, components 11 and 12). This may reflect differences in the pools of these components in *pf-14*.

<sup>2</sup> It should be noted that the molecular phenotypes given for each of the new radial spoke mutants were derived from an analysis of two-dimensional maps obtained from many different preparations. Only those reproducible differences between the mutants and wild type are described.

radial spoke polypeptides likely to be found in the proximal stalk.

In our analyses of the new mutants differences outside of the group of 17 components found to be absent in *pf-14* were not observed. In addition, in an analysis of high molecular weight components present in axonemal and flagellar preparations each of the mutants, like *pf-14* and *pf-1* (1), was observed to be wild-type-like.

A summary of the results of our analysis of the new radial spoke mutants, and of *pf-14* and *pf-1* appears in Table III.

### Mutations Affecting Radial Spokehead Polypeptides

#### NONCONDITIONAL MUTANTS

*pf-17*: *pf-17* is a paralyzed mutant which shows the same selective molecular deficiency for radial spoke components 1, 4, 6, 9, 10 as observed for *pf-1* (Fig. 4). At the ultrastructural level *pf-17* is also indistinguishable from *pf-1*. In transverse and longitudinal images of isolated axonemes from *pf-17* (Fig. 5 B), radial spoke stalks are seen to extend from A tubules with wild-type periodicities. However, in contrast to wild-type axonemes (see Fig. 5 A) the prominent distal spokehead is missing. As previously described for *pf-1* and *pf-14* (11, 12), the central pair microtubule complex in *pf-17* is often seen to be displaced toward one side of the axoneme.

In dikaryon rescue experiments components 1, 4, 6, and 10 are restored as radioactive components; only component 9 is not rescued. This indirect evidence that component 9 may be the defective gene product has yet to be confirmed by revertant analysis. 10 independent intragenic revertants of *pf-17* were isolated and examined by two-dimensional mapping procedures. In each instance, component 9 and components 1, 4, 6, and 10 were observed to be present at their wild-type positions.

*pf-24*: *pf-24* is a paralyzed mutant, which like *pf-1* and *pf-17*, shows a deficiency for the spokehead components 1, 4, 6, 9, and 10. However, *pf-24* differs from *pf-1* and *pf-17* at the molecular level in two important aspects. In contrast to *pf-1* and *pf-17*, where the spokehead components are completely absent from <sup>35</sup>S-labeled axonemal maps, in *pf-24* these components are present in reduced amounts. *pf-24* also differs from the previously described spokehead mutants in that the putative stalk components 2 and 16 are also found to be present in diminished intensities. In fact, in *pf-24* only a trace of radioactivity is detected at the position where wild-type component 2 is normally resolved. At a qualitative level similar deficiencies for these seven polypeptides are evident in maps of intact flagella isolated from the mutant. This observation indicates that the presence of the seven components in reduced amounts in axonemal maps of *pf-24* is not caused by a selective loss of these components during the preparation of axonemes but is likely to reflect a selective defect for the assembly of these components.

At the ultrastructural level spokeheads are often observed to be missing from *pf-24* axonemes. While spoke stalks can be identified in axonemal longitudinal sections they sometimes appear truncated or are completely absent.

In dikaryon rescue experiments all of the *pf-24*-deficient polypeptides, except for component 2, are increased in intensity. This evidence that polypeptide 2 is the mutant gene product in *pf-24* has been supported by revertant analysis. Seven intragenic revertants were isolated and examined by two-dimensional electrophoresis in which the isoelectric focus-

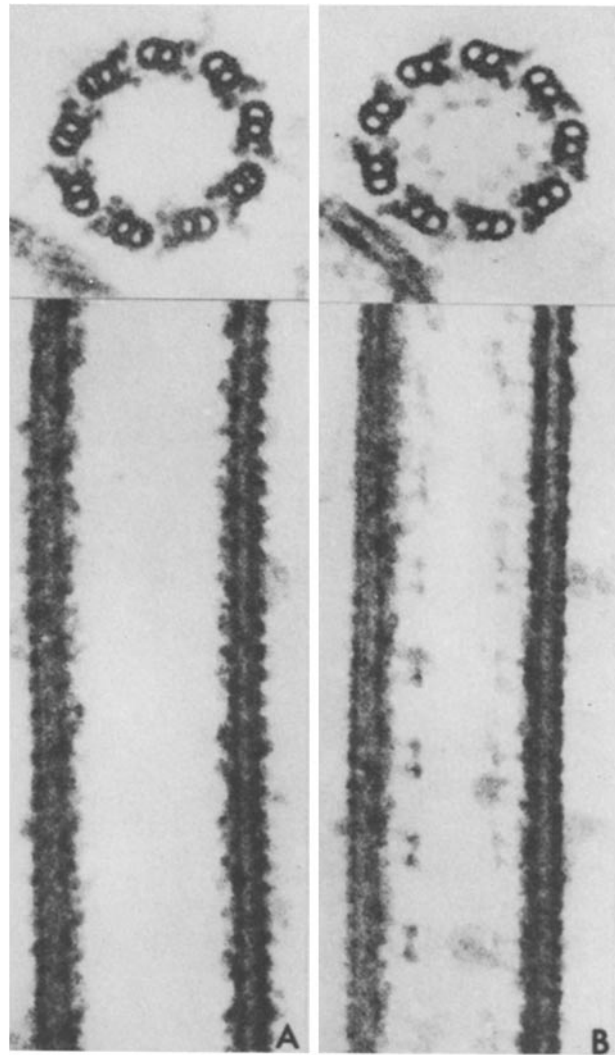


FIGURE 2 Thin-section electron micrographs.  $\times 125,000$  (A) Representative transverse and longitudinal sections of isolated axonemes from a *pf-14 pf-18* recombinant strain. In the double mutant, radial spokes and the central pair microtubules are absent (for a comparison with wild type, see Fig. 5 A). (B) Selected transverse and longitudinal sections of axonemes isolated from dikaryons of *pf-14 pf-18 mt<sup>+</sup>  $\times$  137c mt<sup>-</sup> 3.0 h* after gametic mixing. These micrographs illustrate the morphology of axonemes in which the central microtubules were missing, but radial spokes with wild-type morphology and longitudinal periodicities were present.

ing step was carried to equilibrium. In two of these revertants a component 2 was resolved which was more acidic and had a slower mobility than its wild-type counterpart. The shift in component 2 observed in these revertants is illustrated in Fig. 3 B in an axonemal mixing experiment of one of the revertants with wild-type.

#### THERMOSENSITIVE MUTANTS

*pf-1-R-84<sub>ts</sub>*: *pf-1-R-84<sub>ts</sub>* was isolated as an induced intragenic revertant of *pf-1* with an altered polypeptide 4 which is lower in apparent molecular weight and slightly more acidic than its wild-type counterpart (compare Fig. 6 A and B). This revertant of *pf-1* has been examined in detail because it was found to be thermosensitive for flagellar motility. At the permissive temperature (20°C), *pf-1-R-84<sub>ts</sub>* displays active swimming, but within 10 min of shift to 32°C the preassembled flagella become paralyzed.

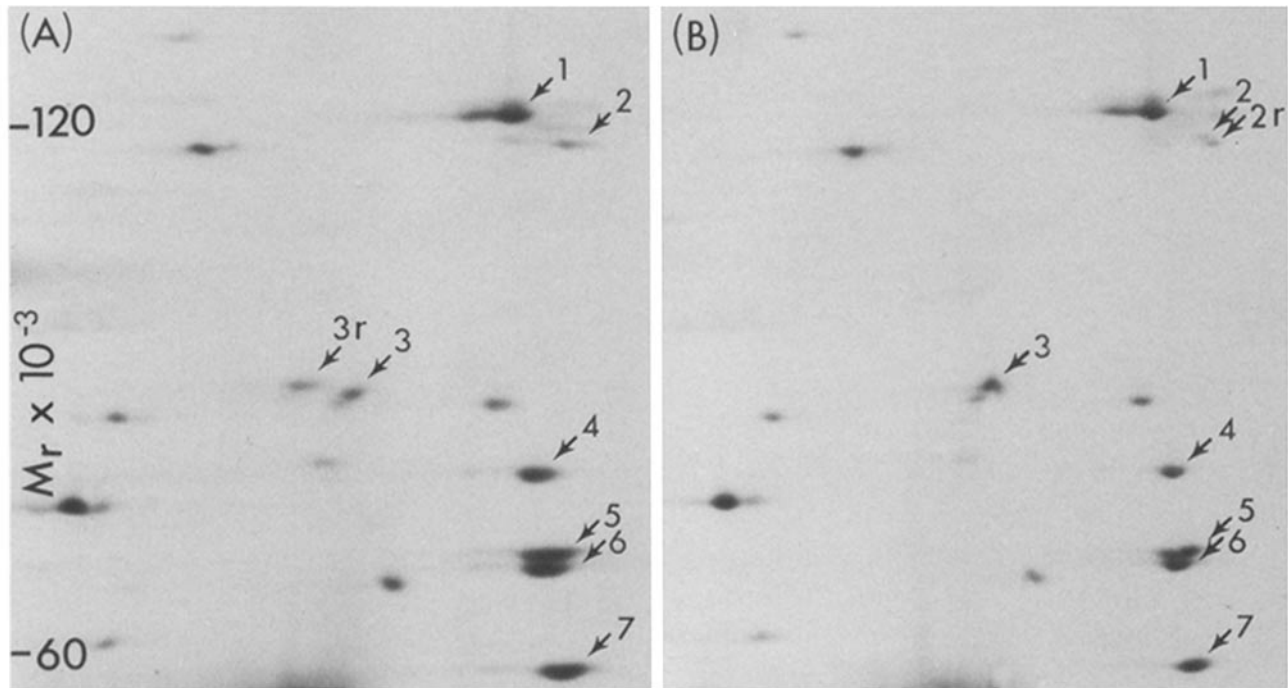


FIGURE 3 Portions of autoradiograms of polyacrylamide gel slabs oriented as in Fig. 1. Proteins were applied at the cathode and run under conditions of equilibrium pH gradient electrophoresis as previously described (1). Arrows point to radial spoke components 1-7 which are present in this region of the map.  $^{35}\text{S}$ -labeled axonemal polypeptides from 137c and from the revertants were mixed in a 1:1 ratio before electrophoresis. Polypeptides in the revertants with altered isoelectric points are designated r. (A) *pf*-14A-R-11 and 137 c axonemal polypeptides. (B) *pf*-24-R-1 and 137c axonemal polypeptides.

TABLE III  
Summary of the Radial Spoke Mutants

Mutants	Motility phenotype	Radial spoke morphology	$^{35}\text{S}$ -Labeled polypeptides missing	$^{35}\text{S}$ -Labeled polypeptides present in reduced amounts	Gene product*
<i>pf</i> -14	Paralyzed	-Radial spokes	1-17	—	3‡
<i>pf</i> -1	Paralyzed	-Spokeheads	1, 4, 6, 9, 10	—	4‡
<i>pf</i> -17	Paralyzed	-Spokeheads	1, 4, 6, 9, 10	—	9
<i>pf</i> -24	Paralyzed	<Spokeheads and stalks	2§	1, 4, 6, 9, 10, 16	2‡
<i>pf</i> -25	Abnormal motility	Wild-type-like	11	8	11
<i>pf</i> -26 <sub>ts</sub>	Paralyzed at the restrictive temperature	Wild-type-like	6	—	6
<i>pf</i> -27	Paralyzed	<Spokeheads and stalks	—	2, 3, 13	?

\* As suggested by dikaryon rescue experiments.

‡ Confirmed by revertant analysis.

§ A trace of radioactivity at the wild-type location of this polypeptide is seen in this mutant.

In two-dimensional axonemal maps of *pf*-1-R-84<sub>ts</sub> grown at 20°C, altered component 4\* and the other spokehead components 1, 6, 9, and 10 are present in reduced amounts (Fig. 6B). When axonemes were prepared from cells grown at 32°C, all five spokehead components were absent from the autoradiograms. These data correlate well with ultrastructural observations that spokeheads are present in *pf*-1-R-84<sub>ts</sub> axonemes isolated from cells grown at the permissive temperature, but are missing from cells grown at the restricted temperature.

*pf*-26<sub>ts</sub>: At the permissive temperature of 20°C *pf*-26<sub>ts</sub> shows wild-type-like motility. The preformed flagella of *pf*-26<sub>ts</sub> retain their motility when the cells are shifted from 20° to 32°C. However, when the flagella are assembled at the restrictive temperature they are paralyzed. This occurs whether the flagella are formed at 32°C during the normal division cycle or after experimental deflagellation.

Axonemal maps from *pf*-26<sub>ts</sub> grown at the permissive tem-

perature show a complete absence of wild-type spokehead component 6, with all other spoke polypeptides present at wild-type positions and intensities (see Fig. 6A). The absence of wild-type component 6 in *pf*-26<sub>ts</sub> is correlated with the appearance of a new axonemal polypeptide. In comparison to wild-type component 6, which has an apparent molecular weight of 67,000 and pI of 5.1, this new polypeptide (labeled 6\* in Fig. 6A) migrates slightly faster and has a pI of 5.7. Axonemal maps obtained from *pf*-26<sub>ts</sub> grown at the restrictive temperature are similar to those obtained from the mutant grown at the permissive temperature. In some preparations polypeptide 6\*, as well as the other spokehead components 1, 4, 9, and 10, are present in reduced amounts. Morphologically, *pf*-26<sub>ts</sub> axonemes isolated from cells grown at 20° or 32°C appear wild-type-like for the structure of the radial spokehead and stalks.

To determine if polypeptide 6\* is altered in its primary structure or is differentially modified by an unknown mecha-

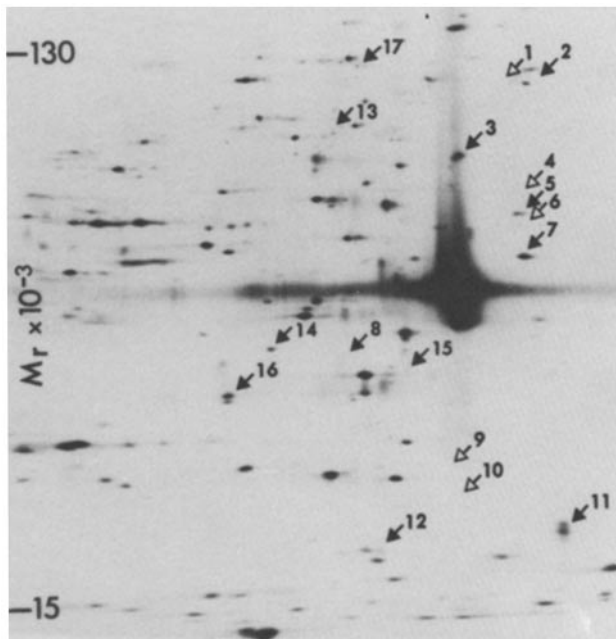


FIGURE 4 Autoradiogram of axonemal polypeptides from *pf-17* run under the same conditions as Fig. 1. The arrows and numbers indicate the positions of the 17 polypeptides missing in *pf-14*. Those polypeptides that are absent in *pf-17* are indicated by open arrowheads.

nism, we tested for coexpression of polypeptides 6 and 6\* in dikaryons of wild-type and *pf-26<sub>ts</sub>*. For this experiment mutant and wild-type gametes both prelabeled with <sup>35</sup>S-SO<sub>4</sub> were mated in the absence of protein synthesis inhibition. After gametic fusion, the dikaryons were deflagellated and flagellar regeneration was allowed to occur at 32°C. The regenerated flagella were isolated and axonemal preparations examined in two-dimensional maps. In the dikaryon maps both wild-type component 6 and mutant component 6\* were found assembled into the axonemes. Their coexpression in the dikaryon gives indication that polypeptide 6\* is altered in primary sequence and therefore is likely to be the gene product in *pf-26<sub>ts</sub>*.

As we have previously noted, in crosses between *pf-26<sub>ts</sub>* and *pf-1* mutants no recombination was observed above the 0.1% level. However, in a cross between *pf-26<sub>ts</sub>* and *pf-1-R-84<sub>ts</sub>*, a spontaneous presumptive diploid strain was recovered under nonselective conditions. The presence of both the *pf-26<sub>ts</sub>* and *pf-1-R-84<sub>ts</sub>* alleles in the diploid was confirmed by back-crossing the strain to 137c *mt<sup>+</sup>* and recovering temperature-sensitive paralyzed progeny with axonemal molecular phenotypes of *pf-26<sub>ts</sub>* and *pf-1-R-84<sub>ts</sub>*.<sup>3</sup>

The diploid is motile whether grown at 20° or 32°C. In axonemal maps obtained from the diploid grown at 20°C, radial spoke polypeptides 4 and 6 are clearly present. *pf-1-R-84<sub>ts</sub>* polypeptide 4\* and *pf-26<sub>ts</sub>* polypeptide 6\* are also found in the diploid maps, but at very low intensities.

The flagellar motility and axonemal molecular phenotype of the diploid strain give evidence that although the *pf-1* and *pf-26* loci are closely linked, mutations for the two sites can be

<sup>3</sup> The meiotic product survival was partial as expected (13); of ~300 zygotes dissected (which yielded 100% germination to four zoospores), only 13 were complete. Among the viable products showing thermosensitivity for flagellar motility, two products were found to contain the *pf-26<sub>ts</sub>* allele, and one product the *pf-1-R-84<sub>ts</sub>* allele.

complementing. Although wild-type components 4 and 6 were found to be preferentially assembled into the diploid flagella, the observation that the *pf-1-R-84<sub>ts</sub>* and *pf-26<sub>ts</sub>* variant polypeptides are partially expressed provides further evidence that they are likely to be altered in primary structure.

### Mutations Affecting Radial Spoke Stalk Polypeptides

*pf-25*: In contrast to the other radial spoke mutants described in this study, which are either nonconditionally or conditionally

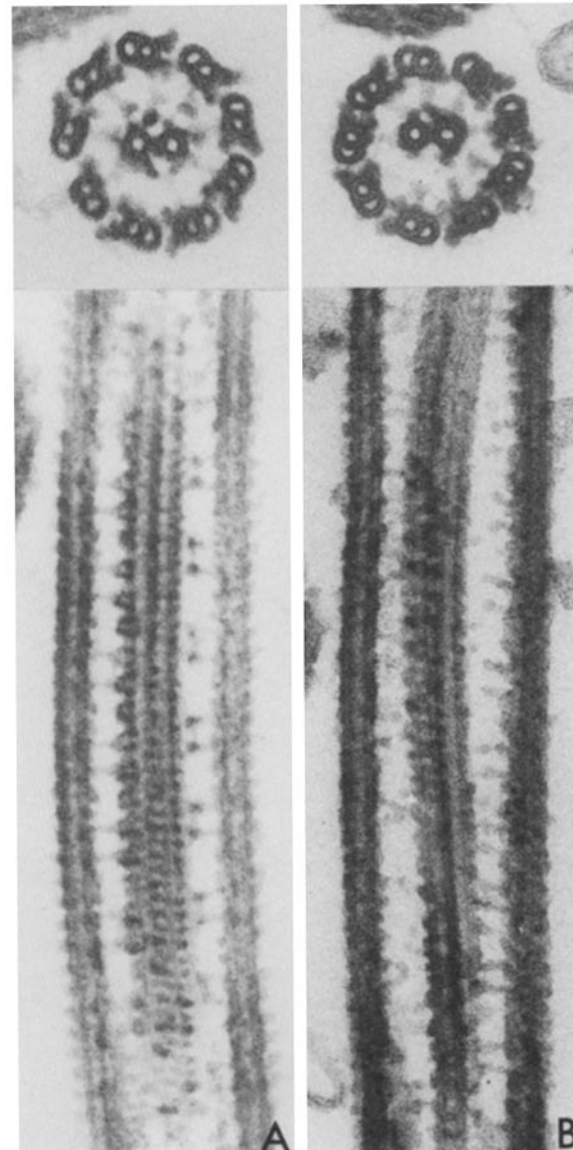


FIGURE 5 Electron micrographs of transverse and longitudinal sections of wild-type and *pf-17* axonemes.  $\times 125,000$ . (A) Wild-type 137c axonemes. In the top figure radial spokes are seen to extend from subfiber A of the peripheral doublets toward the central pair microtubules. In the bottom figure the radial spokes are seen to project in pairs along the length of two outer doublets. Each spoke is differentiated into two segments, a proximal stalk, and distal spokehead. (B) *pf-17* axonemes. Radial spoke stalks are seen to project from subfiber A of the peripheral doublets with wild-type periodicity. The stalks are often found to extend at abnormal angles from the peripheral doublets and to display flexibility along their length. Absent from the distal end of the stalks are the spokeheads prominent in wild-type axonemes.

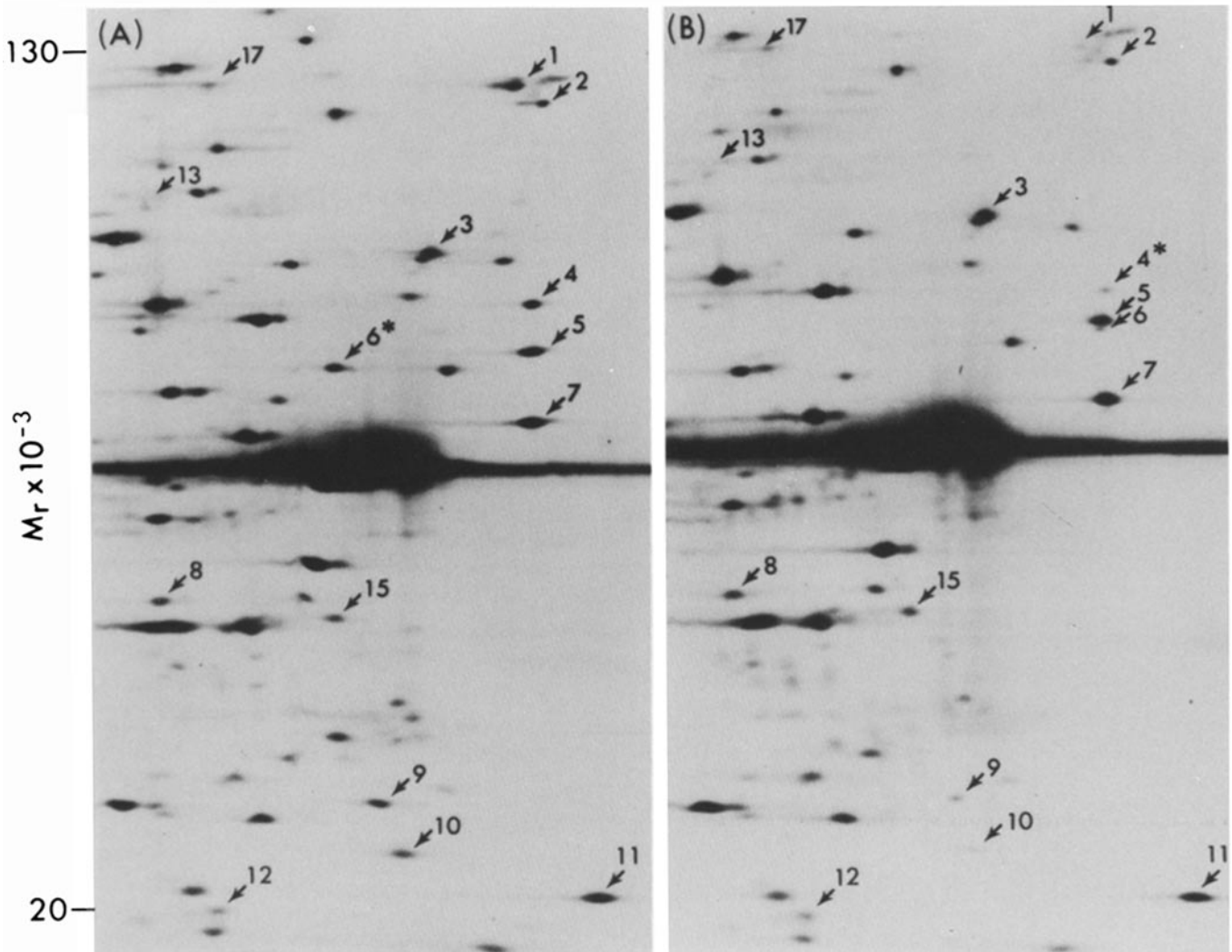


FIGURE 6 Portions of autoradiograms of polypeptide gel slabs run under the same conditions as in Fig. 3. Axonemal polypeptides isolated from thermosensitive mutants grown at the permissive temperature (20°C). All radial spoke polypeptides that are present in the illustrated portion of the maps are indicated by arrows and numbers. The maps of the two mutants are complementary in that the polypeptide with altered isoelectric point (indicated with asterisks) in one is present in the other at its wild-type position. (A) *pf-26<sub>ts</sub>* axonemal polypeptides. As compared to wild-type 6, 6\* migrates slightly faster and has a more basic isoelectric point. (B) *pf-1-R-84<sub>ts</sub>* axonemal polypeptides. As compared to wild-type 4, 4\* shows a slightly more acidic isoelectric point and increased motility. Polypeptide 4\* and spokehead components 1, 6, 9, and 10 are present at diminished intensities.

paralyzed flagellar mutants, *pf-25* swims actively, but in an abnormal fashion. At the molecular level, axonemes from *pf-25* and six independently isolated alleles (*pf-25-A-F*) are characterized by deficiencies for two putative radial spoke stalk polypeptides; component 11 is missing and component 8 is present at a diminished intensity. All other radial spoke polypeptides appear to be present at wild-type positions and intensity. At the ultrastructural level we have been unable to associate the molecular deficiencies for components 11 and 8 in *pf-25* with any detectable alteration in the structure of the radial spokes.

In dikaryon rescue experiments performed in the *in situ* and flagellar regeneration modes, component 8 is increased in intensity, but component 11 is not rescued. Several attempts have been made to isolate intragenic revertants of *pf-25*. Thus far we have not obtained such revertants for this mutant.

*pf-27*: *pf-27* is a paralyzed mutant in which spoke stalk components 2, 3, and 13 are present in reduced amounts in both <sup>35</sup>S-labeled axonemal and flagellar maps (not illustrated).

The level of deficiency for these components varied quantitatively with different preparations. Morphologically, axonemes of *pf-27* resemble those of *pf-24* in which a deficiency for spokeheads and sometimes entire spokes is seen.

*pf-27* is rescued *in situ*, and in autoradiograms of polypeptide maps obtained from dikaryon experiments, components 2, 3, and 13 are all restored to wild-type levels. These observations suggest that, unlike the previously described mutants, the mutant gene product in *pf-27* is likely to be extrinsic to the radial spokes.

In the preceding communication, 5 of the 17 radial spoke components (2, 3, 5, 13, and 17) were shown to be phosphorylated *in vivo* in wild type in a short 10- to 20-min pulse with [<sup>32</sup>P]phosphoric acid (1). A striking observation was made when <sup>32</sup>P-labeled axonemal maps from *pf-27* were compared to wild type. In *pf-27* (see Fig. 7) each of the five radial spoke polypeptides observed to be phosphorylated in wild type are either absent or only a trace of radioactivity is seen. It should be noted that the apparent reduction in mass for three of the

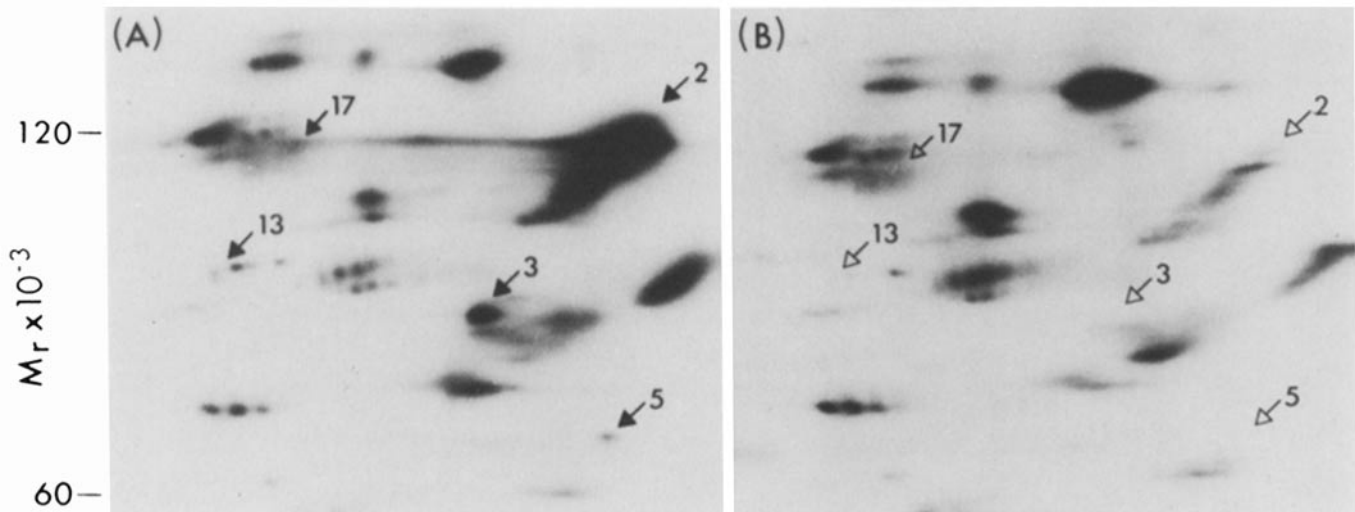


FIGURE 7 Portions of autoradiograms of polypeptide gel slabs run under the same conditions as Fig. 1. Labeling was obtained with an in vivo pulse (10–20 min) of [ $^{32}\text{P}$ ]phosphoric acid. (A) 137c axonemal polypeptides. Arrows point to  $^{32}\text{P}$  label associated with five radial spoke polypeptides. (B) *pf-27* axonemal polypeptides. Arrows with empty arrowheads indicate the positions of the five  $^{32}\text{P}$ -labeled radial spoke polypeptides that are missing or found at greatly diminished intensity in the mutant.

phosphorylated spoke polypeptides detected in  $^{35}\text{S}$ -labeled maps of *pf-27* does not correspond to the extreme deficiencies for  $^{32}\text{P}$  labeling of these components. In *pf-27* all other  $^{32}\text{P}$ -labeled axonemal components are present at wild-type intensities.

## DISCUSSION

Flagellar mutants representing seven independent loci in *Chlamydomonas reinhardtii* have been identified as affecting the assembly and function of the radial spokes. In the majority of these mutants, the single-site mutations result in the defective assembly of more than a single polypeptide into the axoneme. However, the mutations appear to be specific for the radial spokes. In each mutant the polypeptide deficiencies were restricted to subsets of the group of 17 components found to be missing in the spokeless mutant, *pf-14*.

To differentiate between mutations affecting structural components of the radial spokes and possible regulatory gene products, mutants for each of the seven loci were studied by dikaryon rescue techniques. These studies gave indication that in six of the mutants the gene product represented a component of the radial spoke structure. In the case of *pf-14A*, *pf-1*, and *pf-24*, the identification of the gene products was supported by reversion analysis. Intragenic revertants were isolated with gene products showing variant isoelectric points.<sup>4</sup>

With knowledge of the gene product, the mutant phenotypes may be interpreted in terms of the assembly hierarchy of the radial spoke. When polypeptide 3, a component of the proximal stalk, is missing from flagella, the entire radial spoke fails to assemble. Deficiencies for either spokehead polypeptides 4 or 9 result in the failure of assembly of the radial spokehead complex. These defects in assembly are complete and stand in

<sup>4</sup> Because components 2 and 3, the putative gene products in *pf-24* and *pf-14*, respectively, are phosphorylated, it is possible that the altered map positions of the proteins observed in revertants are caused by quantitative differences in phosphorylation. This possibility must be seriously considered, particularly in the case of *pf-24* in which the two revertants showed component 2 with similar small shifts in isoelectric point.

contrast to the situation in *pf-24* and in *pf-25*. In *pf-24* the gene product, stalk component 2, is virtually absent from axonemes and flagella, but there is only a partial deficiency of components 1, 4, 6, 9, 10, and 16. Apparently, component 2 may be required for stability of the spoke structure but its role in assembly is not absolute. The smallest effect on spoke assembly is seen in *pf-25* where the apparent gene product, polypeptide 11, a stalk component, is absent. Aside from a diminished quantity of stalk component 8 there appear to be no assembly defects. It should be noted that unlike all other mutants examined here which are paralyzed, *pf-25* flagella show some motility.

The results obtained in the dikaryon rescue experiments have demonstrated that either portions of the radial spokes or the complete structures themselves can be assembled into preexisting axonemes. The experiments indicate further that in the mutants, many, if not all, of the unassembled polypeptides are present in a precursor pool of flagellar proteins. There is evidence that radial spoke polypeptides with isoelectric points and molecular sizes identical to those of the axonemal components are present in cell bodies. It is likely that these polypeptides provide the pool from which radial spokes are assembled in mutant  $\times$  wild-type dikaryons. In recent experiments (G. M. Adams, unpublished observations) using protocols similar to those described by Lefebvre et al. (14) in their analysis of induction of flagellar protein synthesis in wild-type *Chlamydomonas*, several radial spoke components were identified in the cell bodies of deflagellated gametic *pf-14* and *pf-1* mutants. The identification was made on the basis of coincidence in cell body and axonemal two-dimensional maps. In the case of *pf-1*, all of the spokehead components except for polypeptide 4 were detected in the cell bodies.

In contrast to these nonconditional mutants in which the gene product is missing from axonemes and flagella, two temperature-sensitive mutants have been identified in which the altered gene products can be assembled into structure. In the case of *pf-26<sub>ts</sub>*, the gene product, altered component 6, is assembled at both permissive and nonpermissive temperatures without consequence for the assembly of the other radial spoke components. However, when assembly occurs at the nonper-



missive temperature, the flagella are paralyzed. On the other hand, radial spokes assembled at the permissive temperature remain functional after the cells are shifted up. Component 6 in *pf-26<sub>ts</sub>* is modified in such a way as to dissect its role in spoke assembly from its role in spoke function. In contrast, altered component 4 in the temperature-sensitive revertant *pf-1-R-84<sub>ts</sub>*, is modified in such a way that its function in assembly of the radial spokehead is lost at high temperature even in radial spoke structures assembled at permissive temperatures.

We have observed that five of the six genes likely to code for structural proteins of the radial spoke are unlinked or distantly linked to one another, suggesting that relevant genes for spoke assembly are not extensively clustered. However, it is of interest to note that in the one example of close linkage of genes specifying spoke polypeptides (*pf-1* and *pf-26*), the gene products are components of the spokehead complex. At the present time we have no evidence that this close linkage is functionally significant, but the possibility exists that expression of these two genes is under coordinate control.

In one mutant, *pf-27*, dikaryon rescue analysis gave evidence that the mutant gene product is extrinsic to the radial spokes. The <sup>32</sup>P-labeling experiments indicate that it is required for the specific phosphorylation of the spoke polypeptides 2, 3, 5, 13, and 17. Because we have been unable to detect in *pf-27* any alteration for axonemal polypeptides falling outside the spoke system, the mutant gene product is likely to be extrinsic to the axoneme itself.

As shown in the preceding communication (1), >80 axonemal components in wild type are phosphorylated *in vivo* with a short 10- to 20-min pulse with [<sup>32</sup>P]phosphoric acid. The observation that *pf-27* shows a selective defect only for phosphorylation of the five radial spoke polypeptides demonstrates the specificity with which post-translational modification of these components may occur.

Because *pf-27* flagella are paralyzed, it appears that phosphorylation of the five radial spoke components is not only specific in nature, but is required for normal flagellar function. The observation that these polypeptides are rapidly modified *in vivo* in wild type suggests that phosphorylation of the spoke components may regulate radial spoke function in flagellar motility. However, because *pf-27* shows a deficiency in <sup>35</sup>S-labeled maps of axonemes and flagella for components 2, 3, and 13, it is not excluded that phosphorylation is in some way involved in radial spoke assembly.

In addition to the radial spoke mutants described in this paper, we have recently identified two alleles for another locus,

*pf-5*, which show molecular deficiencies for radial spoke polypeptides. However, in contrast to the mutants already described, the two *pf-5* alleles are also missing axonemal polypeptides outside the spoke system. The missing polypeptides in *pf-5* and *pf-5A* include radial spoke components 13 and 15, component 1 of the inner dynein arms (3), and a low molecular weight axonemal polypeptide (mol wt <15,000) whose structural localization is yet unknown. The observation that single-site mutations for the *pf-5* locus can affect the assembly of polypeptides into different substructures of the axoneme suggests that in addition to specific regulatory genes, such as *pf-27*, there are gene products that may influence at an integrative level the assembly and function of the axoneme.

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