

OVERSIZED FLAGELLAR MEMBRANE PROTEIN IN PARALYZED MUTANTS OF *CHLAMYDOMONAS REINHARDTII*

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ABSTRACT

A mutant strain of *Chlamydomonas reinhardtii* is shown to possess an oversized flagellar membrane protein. The mutant has paralyzed flagella, is temperature sensitive for flagellar assembly, and has an abnormal axonemal protein composition. All phenotypes appear to derive from a single Mendelian mutation, and genetic analysis suggests that the mutation, which we call *ts222*, is in the gene *pf1*. Because *pf1* mutants are known to have radial-spoke defects (Piperno et al., 1977, *Proc. Natl. Acad. Sci. U. S. A.* **74**:1600–1604; and Witman et al., 1978, *J. Cell Biol.* **76**:729–797), a relation as yet undefined appears to exist between radial-spoke and flagellar membrane biogenesis.

The eukaryotic flagellum consists of a motile engine, the axoneme, bounded by a specialized membrane. Preparations of flagellar membrane from *Chlamydomonas reinhardtii* contain one predominant polypeptide, a glycoprotein of apparent mol wt >300,000 (1, 37). Other proteins are present, but in much smaller amounts. The *Chlamydomonas* flagellar membrane is not unique in possessing a single major protein of high molecular weight; the same is true for a number of other flagellar or ciliary membranes, such as those of molluscan and echinoderm sperm (39), of *Paramecium* (15), and of *Euglena* (5). The orientation of the membrane protein on or within the flagellar membrane is not known.

Ultrastructural investigations have revealed some distinct physical relationships between axoneme and membrane. All of the axonemal microtubules possess specialized structures at their distal ends, where they abut the membrane. The central-pair tubules seem to be directly embedded in the membrane by means of a "cap" structure, and the A-tubules of the outer doublets end in filamentous

projections into the membrane (8). In addition, there appear to be lateral attachments along the length of the flagellum between the membrane and the underlying microtubules (4, 8, 28, 31). The intimate structural relationship between membrane and axoneme suggests that there exists a functional relationship as well.

In addition to its known functions (1, 37), the membrane may conceivably play a role in flagellar assembly. Autoradiography of *Chlamydomonas* labeled during flagellar regeneration has shown that the bulk of flagellar assembly occurs at the organelle's distal tip (40), but how the components find their way to their assembly sites is unknown. If the flagellum were hollow, diffusion would certainly suffice but, in fact, the flagellum is quite densely filled. The identification of a motility system that acts at the flagellar surface (3, 4, 25) raises the possibility that the components travel between the axoneme and the membrane, with the membrane motility system being actively involved in their transit.

Although both the kinetics of flagellar assembly

(32) and the specific induction of protein synthesis induced by deflagellation (23) have been examined in some detail, next to nothing is known about flagellar assembly at the molecular and supramolecular levels. That is to say, the paths that the various proteins follow from their synthesis to their inclusion in the flagellum—paths that may include processing and subassembly steps—are unknown. Because of its size and abundance, the flagellar membrane protein can be tracked in different partitions of the cell, and so it may prove to be a valuable indicator of critical intracellular morphogenetic processes.

While screening the flagella of newly isolated motility mutants by SDS gel electrophoresis, we identified one mutant with an abnormal flagellar membrane protein. Here we describe some of the mutant's biochemical and genetic properties.

MATERIALS AND METHODS

Chlamydomonas Strains

Wild-type strains NO mating type plus (mt^+) and NO mating type minus (mt^-) were obtained from Dr. U. Goodenough, Washington University, St. Louis, Mo. Strains *pf1* (*pf1.31D mt^+*), *pf14* (*pf14.5D mt^+*), and *137c mt^+* were obtained from Dr. G. Piperno, The Rockefeller University, New York. Other strains were from our own collections. We isolated *ts222* in a NO mt^- background. The strain used for the genetic analysis was mt^- ; it had been twice backcrossed to NO mt^+ .

Cell Culture

Cells were grown on a 14-h light/10-h dark cycle with constant aeration in medium I of Sager and Granick (34) supplemented with $10^{-3}\%$ thiamine and $10^{-3}\%$ nicotinic acid. All experiments were begun at ~ hour 4 of the light segment of the cycle. Gametes were prepared by washing cells into nitrogen-free medium at hour 12 of the light segment of the cycle and placing them in continuous light, with aeration, for 18–20 h.

Chemicals and Isotope

Dibucaine was purchased from Ciba-Geigy Corp. (CIBA Pharmaceutical Co., Summit, N. J.). We purchased ^{35}S as $\text{H}_2^{35}\text{SO}_4$ (specific activity, 43 Ci/mg) from New England Nuclear, Boston, Mass. Cycloheximide was purchased from Sigma Chemical Co., St. Louis, Mo.

Flagellar Amputation

Two deflagellation methods were used. (a) Mechanical agitation: 10–20 ml of a cell suspension were treated for 30 s in a VirTis 45 homogenizer (VirTis Co., Gardiner, N. Y.) at setting 5 (32). (b) Dibucaine treatment: a cell suspension was made 2.5% sucrose and 2 mM in the tertiary amine anesthetic dibucaine (41).

Isolation of Flagella

Cells were grown in 150-ml volumes to a density of $\sim 2 \times 10^6$ cells/ml, pelleted by centrifugation (5 min at 1,500 rpm in an

IEC 253 rotor, International Equipment Company, Needham Heights, Mass.), resuspended at 1/100 volume in medium containing 2.5% sucrose, deflagellated by dibucaine treatment, and centrifuged at 3,000 g for 5 min (IEC 253 rotor at 3,200 rpm). The supernate containing the flagella was removed and centrifuged for 10 min at 8,000 rpm in a Sorvall SS-34 rotor (Du Pont Co., Sorvall Biomedical Div., Wilmington, Del.) to pellet the flagella. The flagellar pellet was resuspended in 100 μl of 0.0625 M Tris, pH 6.8. For detergent extraction, this flagellar preparation was made 0.04% in Nonidet P-40 (Shell Chemical Co., New York), and after 10 min it was centrifuged for 10 min at 15,000 rpm in a Sorvall SS-34 rotor to pellet the axonemes (40).

SDS Gel Electrophoresis

Samples in 0.0625 M Tris, pH 6.8, were mixed with equal volumes of $2 \times$ sample buffer (22), boiled for 1 min, and either frozen or immediately subjected to electrophoresis. The discontinuous buffer system of Laemmli was used (21), with 3% acrylamide in the stacking gel and a gradient of both urea (3–8 M) and acrylamide (4–16%) in the separation gel (6). Gels were poured at room temperature, and no SDS was included in the stacking or separation gels. Practical grade SDS (J. T. Baker Chemical Co., Phillipsburg, N. J.) was used in the running buffer. 40- μl Loads on gel slabs (21 cm \times 30 cm \times 0.15 cm) were run for ~12 h at 20 mA.

Gels were stained for protein with Coomassie blue by the method of Sloboda et al. (36), and for carbohydrate with periodic acid/Schiff's base (PAS) as by Fairbanks et al. (11). For autoradiography, gels were dried onto no. 1 filter paper (Whatman, Inc., Clifton, N. J.) in a Hoefer SE540 gel dryer (Hoefer Scientific Instruments, San Francisco, Calif.) and exposed to Kodak X-Omat IR-5 film for ~1 wk. Fluorography was done according to Laskey and Mills (22); prefogged X-Omat XR-5 film was exposed at -70°C for ~1 wk.

Two-dimensional isoelectric focusing/electrophoresis followed the method of O'Farrell (29) as modified by T. McKeithan (personal communication).

Preparation of ^{35}S -Labeled Flagella

LABELING DURING FLAGELLAR REGENERATION: Cells at 2×10^6 cells/ml were washed from complete to sulfur-deficient medium, 18–24 h before labeling (23). At the time of labeling, they were concentrated 5-fold and deflagellated by mechanical agitation. 0.1 mCi of [^{35}S]sulfate was added and flagellar assembly was allowed to proceed for 60 min. The regenerated flagella were amputated by dibucaine treatment and purified. The final 100- μl flagellar preparations contained ~0.5 μCi each.

LABELING DURING GAMETOGENESIS: 50-ml Cultures at 2×10^6 cells/ml were washed from complete to sulfur- and nitrogen-deficient medium, and 0.1 mCi of [^{35}S]sulfate was added (23). The cultures were maintained under constant illumination with aeration for 18–24 h, at which time they were concentrated 20-fold. Flagella were removed by dibucaine treatment and purified.

LABELING DURING VEGETATIVE GROWTH: Log-phase cells were washed into 50 ml of sulfur-deficient medium at a density of 5×10^5 cells/ml, and the cultures were bubbled with air on a light/dark cycle at 25°C . After 24 h, 0.1 mCi of carrier-free [^{35}S]sulfate was added. Growth was allowed to proceed an additional 24 h, at which time flagella were removed with dibucaine and purified.

Flagellar Length Determinations

Cells were fixed in 0.5% glutaraldehyde, and flagellar lengths were measured by phase-contrast microscopy at $\times 400$ with an ocular micrometer. For each sample, the flagella of ≥ 20 cells were measured, and their average length was computed.

Isolation of Mutants

Cells were spread on 1% agar plates and illuminated with UV light to a survival of $\sim 25\%$ (60 s at 25 cm from a Sylvania model G15T8 germicidal lamp). Plates were kept in the dark for 4 h (14) and then incubated, on a 14-h light/10-h dark cycle for 4 d at 25°C (until small colonies were visible), and then for an additional 4 d at 33°C to allow for the expression of any induced temperature-sensitive mutations. It was expected that cells that could not grow at one or the other temperature would give rise to small colonies at the end of the period, and that cells that were nonmotile at 33°C would give rise to characteristic "heaped" colonies (17, 25). These colonies were picked with sterile capillary tubes, each tube containing a small volume of liquid medium, and the tubes—each with a plug of agar at the bottom, a *Chlamydomonas* colony on top of the agar, and some liquid medium above that—were placed in beakers and illuminated from above at 33°C. Wild-type cells, when subjected to this regime, collect at the meniscus in the capillary as a result of their positive phototaxis, and, to the naked eye, they are clearly visible there as a green fringe. Capillaries in which the cells did not collect at the meniscus contained putative motility mutants. The cells from each were blown into tubes of growth medium and, after they had grown to a density of $\sim 5 \times 10^8$ cells/ml, they were diluted to three new tubes, which were incubated at 13°, 25°, and 33°C. When these cultures had grown up, the cells were examined by phase-contrast microscopy at $\times 400$ to determine their phenotypes.

Genetic Analysis

Crosses were performed following the methods of Levine and Ebersold (24). Diploids were constructed as described by Ebersold (10); the auxotrophic markers used for selection were *arg 1* (10) and a determinant of nicotinic acid auxotrophy isolated by the authors. Complementation in dikaryons followed Starling and Randall (25, 38).

RESULTS

ts222

Using the techniques described in Materials and Methods, we isolated the 153 new *Chlamydomonas* motility mutants whose phenotypes are summarized in Table I. By SDS gel electrophoresis/autoradiography of ^{35}S -labeled flagella, we found, among the first six mutants examined, one—called *ts222*—with a flagellar membrane protein of unusually large apparent size. (Even in wild type, the protein is immense, with an apparent mol wt of $>300,000$ (2, 37).) To confirm that this protein was in fact the major flagellar membrane protein, the flagella were treated with the nonionic detergent Nonidet P-40, and the extracted material was run

TABLE I
Phenotypes of New *Chlamydomonas* Mutants

Phenotype			Number of mutants
13°C	25°C	33°C	
Fla ⁻	Fla ⁻	Fla ⁻	99
Mot	Mot	Fla ⁻	24
Fla ⁻	Mot	Fla ⁻	2
Para	Para	Para	4
Para	Para	Fla ⁻	8
Fla ⁻	Para	Fla ⁻	2
Ab	Ab	Ab	9
Ab	Ab	Fla ⁻	2
Short	Short	Short	2
Long	Mot	Mot	1

Abbreviations are *Mot*, normal motility; *Fla⁻*, without flagella; *Para*, paralyzed flagella; *Ab*, aberrant swimming; *Short*, very short flagella; *Long*, very long flagella (21). Motility mutants were recovered at a frequency of $\sim 7.5 \times 10^{-3}$ (153 mutants from $\sim 20,000$ colonies screened). By contrast, auxotrophs were recovered in parallel experiments at a frequency of $\sim 5 \times 10^{-4}$ (8 auxotrophs from $\sim 15,000$ colonies).

on gels and stained both with periodic acid/Schiff's base, to indicate carbohydrate, and with Coomassie blue, to indicate protein. The gels are shown in Fig. 1. Clearly, the mutant protein is extractable, like the wild type, and contains carbohydrate. On the gel stained with Coomassie blue, a 1:1 mixture of the *ts222* and wild-type samples was also run. The pattern shows a simple additive combination of the *ts222* and wild-type bands, indicating that the difference in mobility is not an artifact produced during the electrophoresis. Even when wild-type and mutant cultures are mixed before flagellar isolation, this additive pattern is observed (data not shown). All preparations of *ts222* flagella show an apparently oversized membrane protein, and all preparations of wild-type flagella show predominantly the normal-sized species. However, we have observed variability of several types. (a) Sometimes wild-type vegetative-cell flagella show a small amount (no more than 10%) of oversized protein. (b) Some mutant preparations show significant amounts (up to 50%) of normal-sized protein. (c) Sometimes a mutant preparation shows more than one oversized protein species (as in Fig. 1). At present, we cannot explain this variability.

The most prominent difference between the SDS gel patterns of *ts222* and wild-type flagella was the membrane protein band, but a number of

additional differences were also apparent. To resolve the proteins more fully, we employed two-dimensional isoelectric focusing/electrophoresis. Fig. 2 shows the patterns obtained for *ts222* and wild-type axonemes. The differences in the patterns are numerous and consistent with those observed in one dimension. Not only is the *ts222* pattern missing a number of spots present in the wild-type pattern, but it includes many spots not found in wild type. The more prominent differences are marked in the figure by arrowheads.

Rapid Growth of Flagella in ts222 Shifted from 33° to 25°C, and Slow Loss of Flagella in ts222 Shifted from 25° to 33°C

The phenotype of *ts222* is temperature conditional. When grown at 13° or 25°C, the cells possess flagella that are paralyzed; when grown at 33°C, they lack flagella altogether. When *ts222* was grown at 33° and then shifted at 25°C, new flagella began to grow almost immediately, and this was true even when the experiment was performed in the presence of the protein synthesis inhibitor cycloheximide (Fig. 3). Conversely, when cells grown at 25° were shifted to 33°C, flagellar shortening and disappearance were gradual, so that after 24 h ~ half of the cells were still flagellated. Tables II and III detail this slow loss of flagella.

Assembly of ts222 Flagella at 25°C

Because *ts222* cells retained their flagella for a considerable period after shift to 33°C, their ability to assemble flagella at 33°C could be assessed by determining whether or not they could regenerate their flagella at that temperature. Cells were grown at 25°, placed at 33°C, deflagellated by mechanical agitation, and monitored thereafter for flagellar regeneration. Fig. 4 shows that *ts222* regenerates its flagella significantly in such an experiment; the flagella rapidly grow to approximately half-length and then gradually shorten, as they would if the cells were placed at 33°C without deflagellation.

ts222 Recessive to Wild Type

We tested *ts222* against wild type for complementation in both dikaryons and diploids. When *ts222*/wild-type dikaryons were constructed, two of their four flagella were initially paralyzed, but within 30 min the paralyzed flagella became mo-

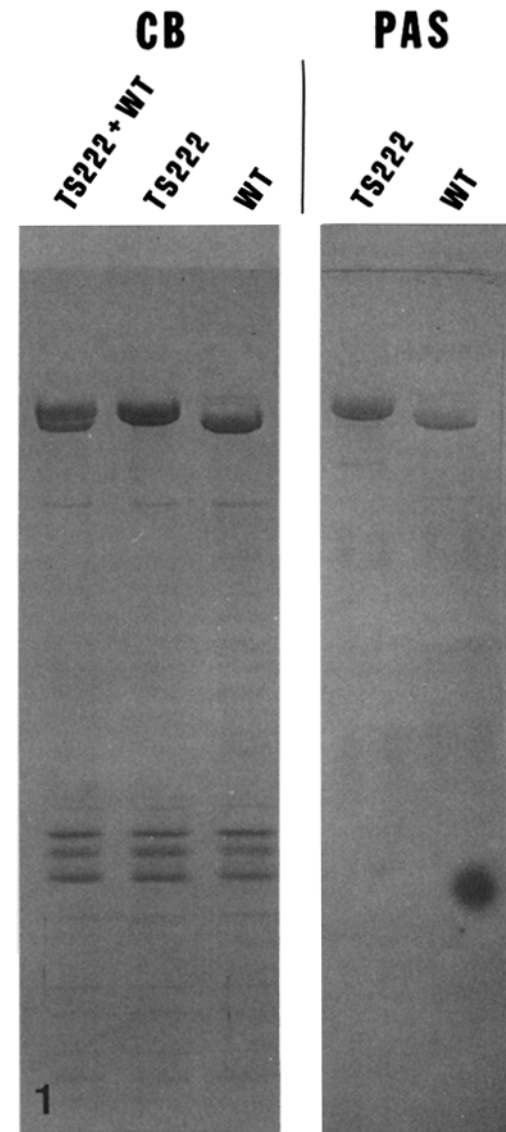


FIGURE 1 Nonidet P-40-extracted protein of *ts222* and wild-type flagella (NO *mt*⁻) stained with Coomassie blue (CB) and PAS. The leftmost lane is a mixture of equal parts *ts222* and wild-type samples.

tile, indicating a donation by the wild-type parent of functions essential for *ts222* motility. A *ts222*/wild-type diploid strain was also constructed. The strain was motile, whether grown at 25° or 33°C, and contained a normal-sized flagellar membrane protein. The presence of the *ts222* allele in the diploid was confirmed by backcrossing it to NO *mt*⁺ and recovering paralyzed progeny. Meiotic-

WT

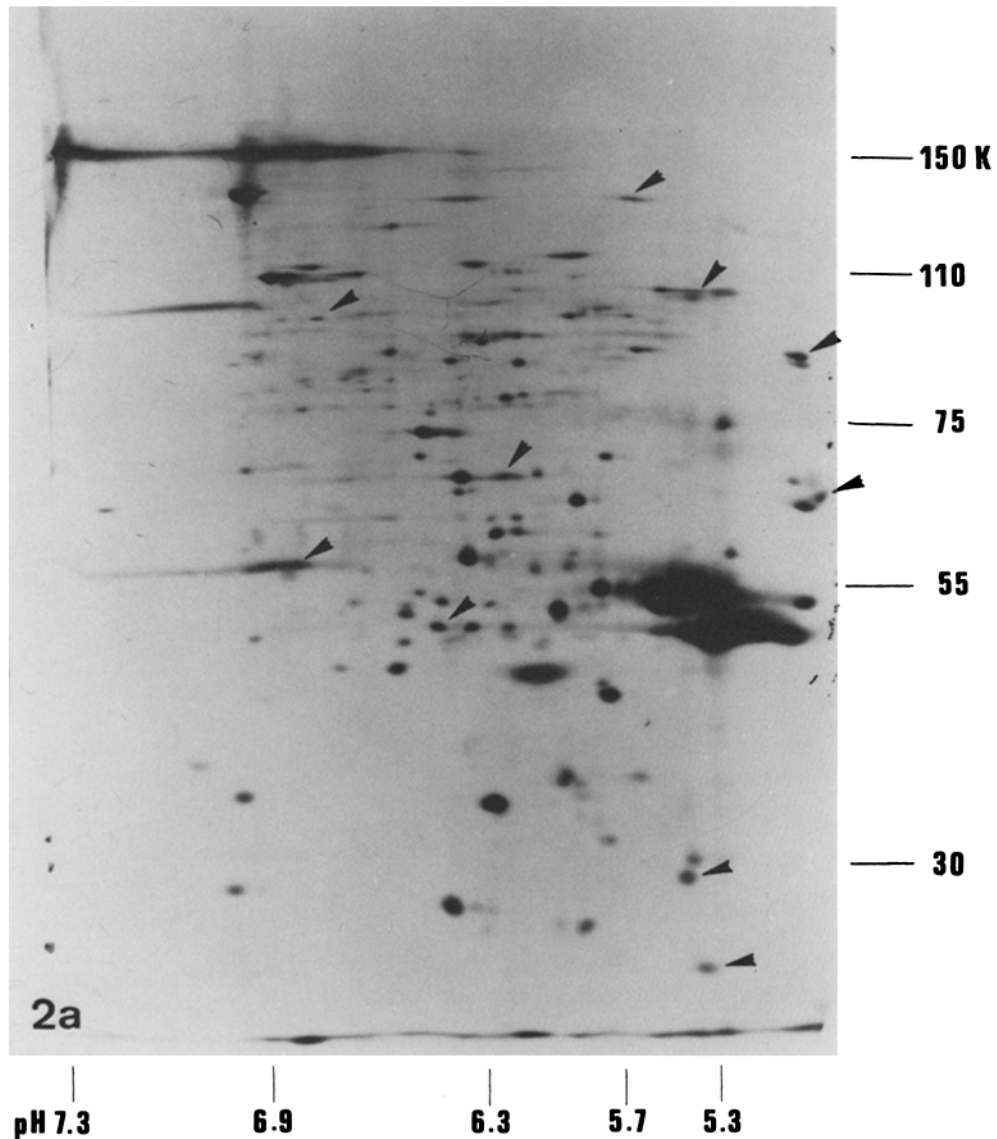


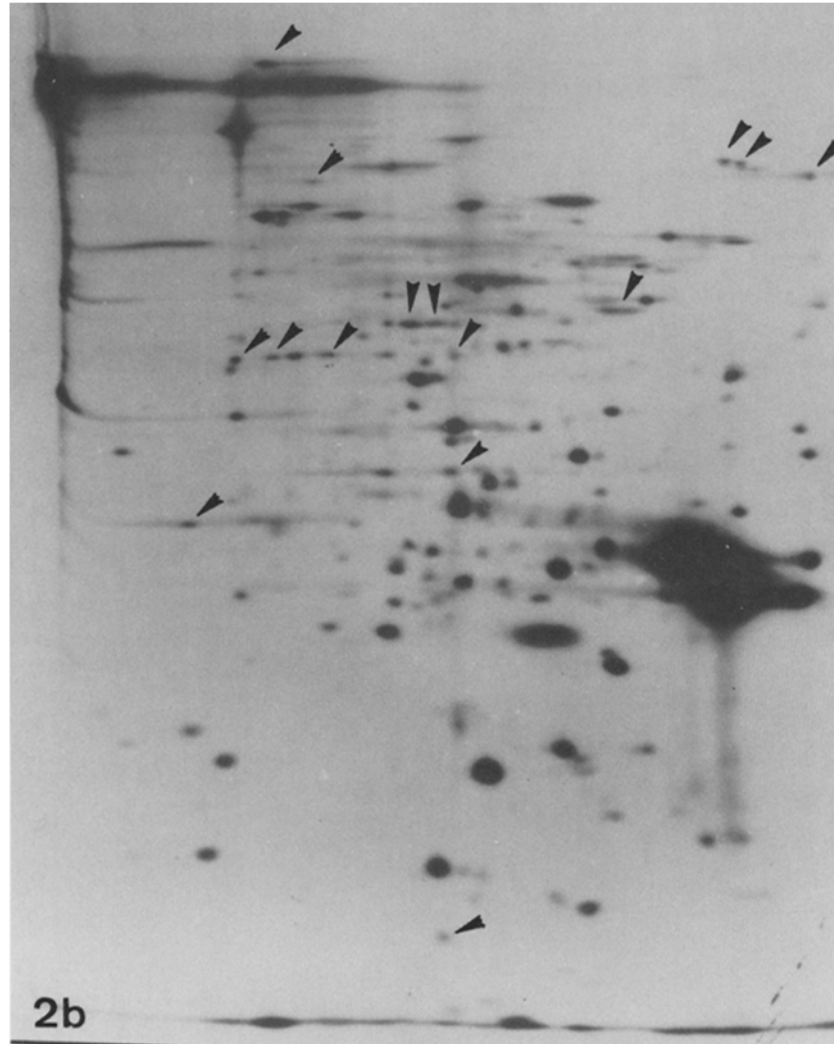
FIGURE 2 Two-dimensional gel patterns for (a) wild-type and (b) *ts222* flagella. ^{35}S -labeled *ts222* and NO *mt*⁻ gametic flagellar proteins were separated by isoelectric focusing/electrophoresis and fluorographed. Ampholines (pH 5–7 and pH 3.5–10) were from Bio-Rad Laboratories, Richmond, Calif. The pH gradient is basic on the left, acidic on the right. Electrophoresis was done through 10% acrylamide. Arrows point to protein spots present in one pattern but not the other.

product survival was partial as expected (10); of six tetrads dissected, only one was complete, and one paralyzed product was found among the 10 products tested. We conclude that the *ts222* phenotypes are recessive.

All ts222 Phenotypes the Result of a Single Lesion in a Mendelian Gene

Two lines of evidence indicate that the four basic *ts222* phenotypes—flagellar paralysis, tem-

TS222



perature-sensitive flagellar assembly, oversized membrane protein, and abnormal gel pattern—all result from a single mutation in a Mendelian gene. (a) Tetrad analysis: *ts222* was crossed to wild type and the resulting meiotic products were examined. All (41/41) of the tetrads tested showed 2:2 segregation of motile and paralyzed products. In addition, temperature-sensitive flagellar assembly and the presence of an oversized membrane protein cosegregated with flagellar paralysis in each of 12 tetrads examined. Fig. 5 shows the flagellar proteins from a single tetrad; 2:2 segregation of the

oversized membrane protein determinant is apparent. (b) Reversion analysis: on four occasions, spontaneous motile revertants appeared in liquid cultures of *ts222*. From the rarity of their appearance, the spontaneous reversion frequency of *ts222* can be calculated to be $<10^{-8}$ per generation. The phenotypes of these revertants are given in Table IV; significantly, two of them, RE2 and RF3, show fully wild phenotypes. Fig. 6 shows the gel patterns of the revertants' flagellar proteins: the pattern for each is identical, or nearly identical, to that of wild type. Because all of the *ts222* phenotypes can co-

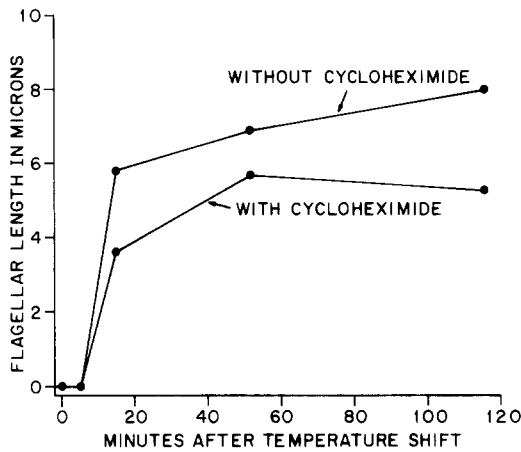


FIGURE 3 Growth of *ts222* flagella after shift from 33° to 25°C. Cells were grown at 25°C to a density of 10^6 cells/ml, diluted 1:20 into fresh medium, and placed at 33°C for 3 d. Two samples, one to which 15 μ g/ml cycloheximide was added, were shifted to 25°C, and flagellar lengths were determined at intervals.

TABLE II
Percentage of Flagellated Cells in Cultures Shifted from 25° to 33°C

Time after temperature shift	Flagellated cells	
	<i>ts222</i>	Wild type
<i>h</i>	%	%
0	90	98
24	42	98
48	6	95
72	0	95

Cell titers were constant over the first 24 h, and they doubled over the next 24. We generally observe such a lag in growth for cells shifted to 33°C.

revert in a (presumed) single step, we find it most probable that they are all a result of a single, pleiotropic, genetic lesion.

ts222 Apparently Allelic to *pf1*

(a) *ts222* and *pf1* have non-complementing defects. We noted that the two-dimensional pattern of *ts222* axonemes (Fig. 2) was missing a number of acidic proteins that appeared to be the same as those missing in the published gel patterns of radial-spoke defective *pf1* and *pf14* mutants (26). We therefore investigated the possibility that *ts222* was an allele of one of the genes already known to be involved in radial-spoke assembly.

ts222 was tested for its ability to complement

pf1, *pf14*, and *pf17* in quadriflagellate dikaryons. Three matings—*ts222 mt⁻ × pf1 mt⁺*, *ts222 mt⁻ × pf14 mt⁺*, and *ts222 mt⁻ × pf17 mt⁺*—were performed and the resulting quadriflagellates were observed at intervals for the appearance of motility in the four originally paralyzed flagella. Both *ts222/pf14* and *ts222/pf17* showed complementation. In the case of *ts222/pf14*, many of the quadriflagellates showed active swimming, using all four flagella, at 30 min after mating. The *ts222/pf17* complementation was less dramatic, but by 30 min most quadriflagellates showed rapid movements at the tips of all four flagella, and at 2 h many were swimming. In contrast, the *ts222/pf1* flagella never acquired motility. Even when the *ts222/pf1* dikaryons were deflagellated at 1 h and allowed to regenerate, the four regenerated flagella were fully paralyzed, indicating that flagella assembled from a mixed *ts222* and *pf1* cytoplasm are defective. We conclude from this lack of complementation that *ts222* and *pf1* strains share the same functional defect.

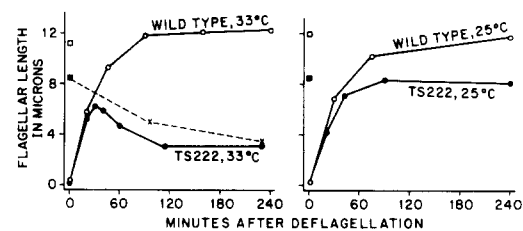


FIGURE 4 Flagellar regeneration by *ts222* at 25° and 33°C. Right panel: cells were grown at 25°C to a density of 2×10^6 /ml, deflagellated by mechanical agitation, and monitored for flagellar regeneration. Left panel: cells were grown at 25°C to a density 2×10^6 /ml, shifted to 33°C for 10 min, deflagellated by mechanical agitation, monitored for flagellar regeneration at 33°C. The dashed line shows flagellar length for *ts222* shifted to 33°C without deflagellation. Opened and filled squares show the initial lengths of wild type and *ts222*, respectively.

TABLE III
Flagellar Lengths in Cells Shifted from 24° to 33°C

Time after temperature shift	Average flagellar length	
	<i>ts222</i>	Wild type
<i>h</i>	μ m	μ m
0	8.3	11.3
24	5.9	10.5
48	3.0	10.5
72	0	10.2

Cells without flagella were not included.

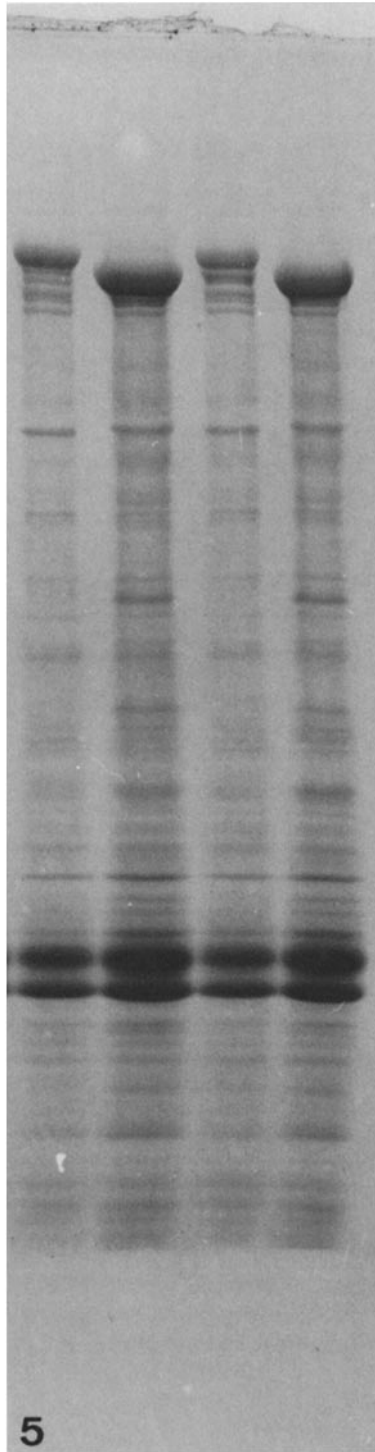


FIGURE 5 Flagellar protein from the four products of a *ts222*/wild-type meiosis. Gel was stained with Coomassie blue.

TABLE IV
Phenotypes of *ts222* Revertants

Strain	25°C	33°C
Wild type	Mot	Mot
<i>ts222</i>	Para	Fla ⁻
RE2A	Mot	Mot
RE4A	Mot	Fla ⁻
RF1A	Mot/Ab	Mot/Ab
RF3A	Mot	Mot

Abbreviations as in Table I.

(b) Recombination between *ts222* and *pf1.31D* was not detected. We cross *ts222 mt⁻* to *pf1.31D* and analyzed 89 complete tetrads. All 89 yielded four paralyzed progeny, indicating no recombination between the respective mutations in 356 meiotic products. In addition, young *ts222* × *pf1* zygote-derived colonies were collected en masse from germination plates and ~30 zygote colonies per tube were distributed to each of 25 tubes containing 2 ml of medium. After a 4-wk incubation at 25°C without shaking, the cultures were examined for the presence of motile cells in the culture medium. All cells visible to the naked eye were at the bottom of the tubes, and, when the medium above those cells was examined in the microscope, very few cells, all paralyzed, were observed. We conclude that there is no recombination between *ts222* and *pf1* above the 0.1% level. (Reconstruction experiments in which mixtures of 10⁸ mutant and 10¹ wild-type cells were incubated showed that wild-type recombinants would have been detected in these experiments.) Our failure to detect recombination in these experiments is consistent with the mutations being in the same gene. Of course, the *ts222* and *pf1* mutations need not be identical. One or both could be a complex mutation such as a deletion or inversion, or they could be in adjacent genes.

(c) *ts222* and *pf1* display similar phenotypes. Having obtained evidence that *ts222* and *pf1* are allelic, we asked whether the multiple phenotypes characteristic of *ts222* were also displayed by *pf1.31D*. We found that the *pf1* mutant—already known to show flagellar paralysis and to be missing a number of acidic proteins—is also temperature sensitive for flagellar assembly, and that it too carries an oversized membrane protein. Fig. 7 shows an autoradiograph of *ts222*, *pf1*, and NO *mt⁺* flagellar protein. The mutant patterns look much alike, sharing a number of differences with respect to the wild type. They are not identical,

however, but some or all of the differences between them may be a result of differences in their respective genetic backgrounds. Evidence for this is presented in Fig. 8, which shows that the wild-type strains NO *mt*⁻ (the parent of *ts222*) and 137c *mt*⁺ (which is related to the *pf1* strain) themselves differ in flagellar protein composition.

Oversized Membrane Protein Not Accumulated in Cell Body of Wild-type Vegetative Cells, But Observed in Gametes

Having observed an oversized membrane protein in *ts222*, we considered the possibility that it might represent an unprocessed precursor to the normal protein. If such processing were coupled to flagellar assembly, then membrane protein in the intracellular pool (unassembled protein) of wild type would be oversized, whereas protein in the flagellar membrane (assembled protein) would be normal sized. Wild-type vegetative cells were labeled with ³⁵S during flagellar regeneration and deflagellated, and the proteins of cell bodies and flagella were compared by SDS gel electrophoresis/autoradiography (Fig. 9). The synthesis of the membrane protein, indicated by the arrows, was stimulated by deflagellation (23), but whether from cell body or flagellum, it had the same electrophoretic mobility. We conclude that processing of the membrane protein, if it occurs, is not coupled to the flagellar assembly process.

Although we typically observe only a single species of flagellar membrane protein in wild-type vegetative cells (occasionally we do observe a minor, more slowly migrating component), a protein migrating behind the flagellar membrane protein has been observed in similar experiments done with gametic cells (23). Gametic cells are made by starving vegetative cells for nitrogen; they have smaller cell bodies and a low basal rate of protein synthesis, but they turn on flagellar protein synthesis after deflagellation, like vegetative cells (23). When we compared gametic and vegetative flagella, we saw the patterns shown in Fig. 10. Two membrane protein species are seen in the wild-type gametic cells, and the more slowly migrating of the two comigrates with the oversized *pf1* protein. These results show that wild type has the capacity to synthesize an oversized membrane protein—a species that we believe to be the same as that made by *ts222* and *pf1*. It should be noted that, for reasons we do not understand, not all gamete preparations show both species. If, indeed,

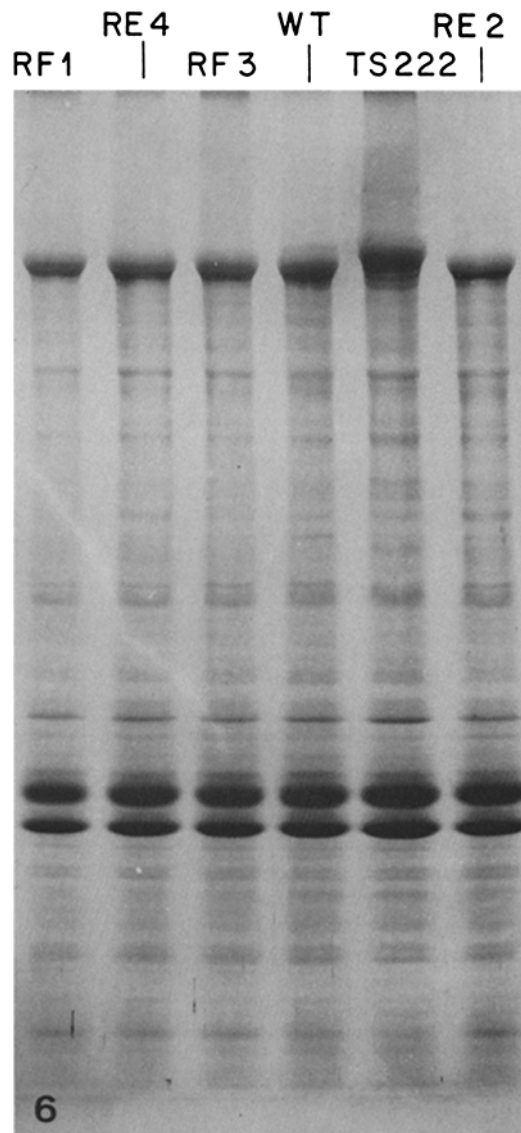
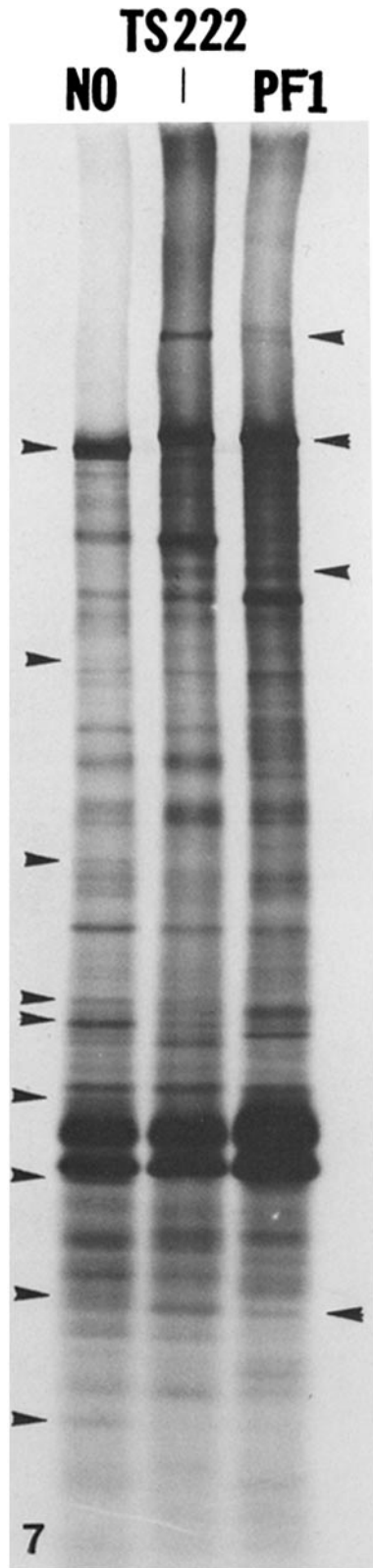


FIGURE 6 Flagellar proteins of *ts222*, wild type (NO *mt*⁻), and four *ts222* revertants. Gel was stained with Coomassie blue.

the smaller protein is derived from the larger by processing, then perhaps the processing is more efficient in some cultures than in others.

Membrane-Protein Differences Not Confined to pf1 and ts222

We have examined the flagellar protein patterns of a number of additional paralyzed mutants and are in the process of testing more. Fig. 11 shows



the flagellar proteins of *pf14*, *pf1*, *ts222*, and *NO mt⁻*, the wild-type parent of *ts222*. *pf1* and *pf14* have been shown to be missing radial-spoke components (30); each shows the abnormal membrane protein pattern. Fig. 12 shows that a paralyzed mutant lacking central-pair microtubules, *pf20*, has normal membrane protein. Two common *Chlamydomonas* wild-type strains, 21GR and 137c, also show the normal pattern (see Fig. 8 for 137c). Although it is tempting to ascribe the membrane-protein phenotypes of *pf1* and *pf14* to their paralysis mutations, we do so with caution in the absence of genetic data such as we have obtained for *ts222*. Nonetheless, it is clear that membrane protein differences are not confined to *ts222* and *pf1* alone.

DISCUSSION

ts222 Defective in Flagellar Assembly at 33°C

We believe that *ts222* is defective for flagellar assembly at 33°C because flagellar outgrowth occurs after a shift from 33° to 25°C even in the absence of new protein synthesis (Fig. 3). This means that all proteins required for flagellar assembly are present in the cell at 33°C in a potentially assembly-competent state. The mutant, therefore, is not defective in the synthesis of an essential protein at high temperature, nor are essential proteins tied up irreversibly in aberrantly assembled structures (27). At least with regard to its protein, then, *ts222* has a defect in the assembly of its components, not in their synthesis.

It appears that the temperature-sensitive component (most likely a protein) in *ts222* is not rapidly inactivated by a shift to 33°C. This is indicated by the ability of *ts222* cells grown at 25° to regenerate flagella at 33°C (Fig. 4). The mutant protein could be insensitive to heat inactivation because of interaction with other proteins (7), or by virtue of a stable conformation assumed at 25°C (19, 33).

To explain the paralysis of *ts222* and *pf1* at low temperature and their lack of flagella at high, we

FIGURE 7 Flagellar protein patterns of *pf1* and *ts222* are similar. Figure shows an autoradiograph of flagella labeled during vegetative growth. Arrowheads at the left point to proteins that are present in wild type (*NO*, *NO mt⁻*) but absent in both *pf1* and *ts222* flagella. Arrowheads at the right point to protein species present in the mutant flagella but absent from wild type.

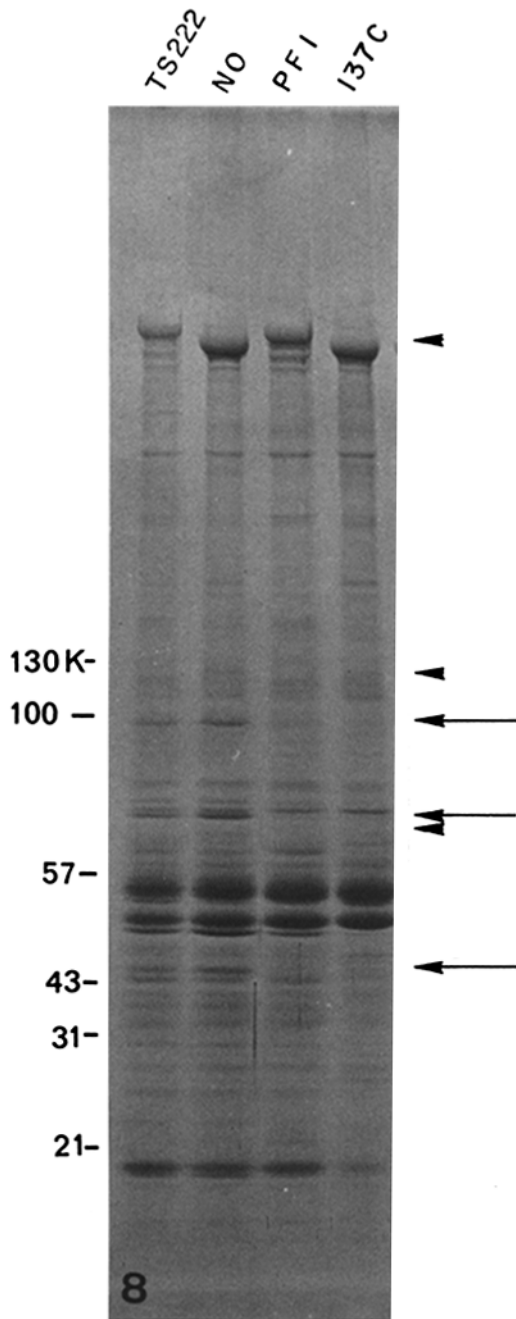


FIGURE 8 Some (or all) of the differences between *ts222* and *pf1* gel patterns result from different genetic backgrounds. Arrows point to differences between the wild-type strains NO *mt*⁻ and I37c. Arrowheads point to differences with respect to both wild-type patterns shared by the mutants. The positions of the molecular weight standards β -galactosidase, phosphorylase a, py-

propose two possibilities. Proteins in both mutants may be affected so that they have altered or incomplete function at low temperature and no function at all at high temperature (12). The mutations arose independently; thus, by this hypothesis, that both show the same phenotype would be ascribed to coincidence. (Note that the possibility that the mutations are at the same site has not been ruled out.) Alternatively, a temperature-sensitive phenotype could be a general property of null mutations in the *pf1* gene—i.e., the gene product might be required for flagellar motility at low temperatures, but it might, in addition, be required for flagellar assembly at high temperatures.

pf1 Mutants Possibly Defective in Protein Processing

At present, we do not know why *pf1* mutants make a membrane glycoprotein that migrates abnormally slowly in SDS gels. We suspect that the migration results from an overlong polypeptide chain, but this may not be so; the protein could, for example, carry extra carbohydrate (18). Perhaps oversized protein is synthesized in wild type and is rapidly processed to normal size. Such processing is known for many proteins, including those associated with, or transported through, membranes (2, 16). *pf1* Mutants, by this hypothesis, would be defective in a protein-processing activity (possibly residing in protein 4), and their pleiotropic phenotype, which includes the presence of proteins not seen in wild type, could be the result of a failure to process a number of different protein species.

We have looked for processing of the *ts222* membrane protein *in situ* by constructing quadri-flagellate dikaryons between ³⁵S-labeled *ts222* and unlabeled wild-type gametes, and then looking for the appearance of normal-sized labeled membrane protein in the dikaryon flagella. No processing was observed (experiment not shown). However, because the processing (if it exists) would normally have to occur before insertion of the protein into the membrane (Fig. 9), such *in situ* processing may not be possible.

Lefebvre et al. (23) observed a protein of somewhat greater apparent size than the membrane protein in gametic cells that were pulse labeled

ruvate kinase, ovalbumin, and soybean trypsin inhibitor are shown at the left (top to bottom).

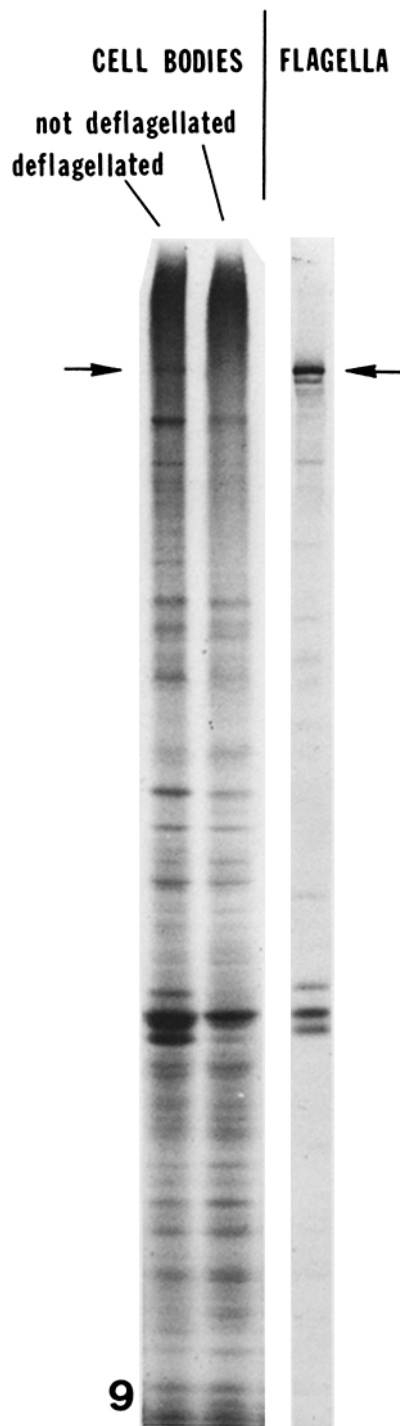


FIGURE 9 Comparison of ^{35}S -labeled flagellar and cell-body protein. Wild type (NO mr^+) was grown to a density of 2×10^8 cells/ml, and an aliquot was deflagel-

during flagellar regeneration. A comparison of the mobility of this protein with the *pf1* oversized protein suggests that they are the same (Fig. 10).

Primary *pf1* Defect Not in Flagellar Membrane Protein Gene

The major flagellar membrane protein is abnormal in *pf1* and *ts222*, but there exists multiple evidence that the mutants are not defective in the structural gene for this protein. Luck et al. (26) obtained strong evidence that an acidic 76,000-mol-wt axonemal protein, which they named protein 4, is the *pf1* gene product. (In our two-dimensional gels, the presumptive protein 4 spot runs somewhat faster, with an apparent molecular weight of $\sim 65,000$; it is the spot in Fig. 2 a (marked by an arrowhead) above and to the right of the prominent tubulin proteins.) They showed that some motile revertants of *pf1* mutants carry axonemal protein 4 species with isoelectric points or electrophoretic mobilities that differ slightly from those of wild type. That these alterations can differ in various independent revertants is evidence that the gene for protein 4 is mutant, with the various species presumed to carry different revertant amino-acid replacements. In addition, our complementation results strongly suggest that *pf1* is not the gene for the membrane protein, and they argue against the hypothesis, not ruled out by the experiments of Luck et al., that protein 4 is a cleavage product of the oversized protein, with *pf1* or *ts222* mutations in the gene rendering its product uncleavable. We know that the oversized protein is incorporated "normally" into flagella; in other words, *ts222* flagella, even when newly regenerated, carry approximately normal amounts of the protein. If the membrane protein gene were mutant (if, for example, it contained an insertion of genetic material, or if it contained a point mutation that rendered its RNA or polypeptide product insensitive to processing), then we would expect a phenotype of codominance to wild type; i.e., the *ts222*/wild-type diploid should carry both oversized and normal-sized protein in its flagellar

lated by mechanical agitation. The deflagellated and the untreated cells were labeled with ^{35}S for 60 min and deflagellated with dibucaine. The cell bodies and the regenerated flagella were purified, electrophoresed, and autoradiographed. The arrows point to the flagellar membrane protein. Electrophoresis was done through a 6-16% acrylamide gradient.

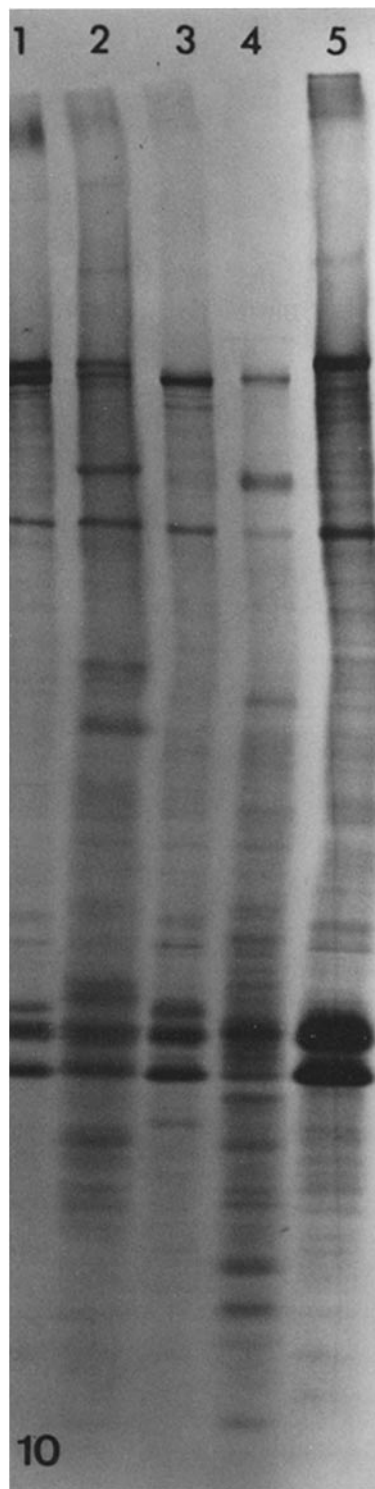


FIGURE 10 Gametic cells produce oversized membrane protein. Figure is an autoradiograph. All samples

membrane. This is not the case; *ts222* expression is recessive. Almost surely, then, *ts222* is not in the gene for the major flagellar membrane protein.

Relation of the Various *pf1* Phenotypes

Luck et al. (26) and Piperno et al. (30) have provided evidence that protein 4 is the product of the *pf1* gene. They also demonstrated that *pf1* mutants are missing a distinct morphological component of the axoneme—the head of the radial spoke—and they assigned protein 4 to this spokehead. (The assignment, as the authors were fully aware, was speculative.) The spokeheads, it should be remembered, are located near the center of the flagellum, $\sim 1,000$ Å from the membrane. Our finding that the flagellar membrane protein is altered in *pf1* mutants indicates an unsuspected relation between these distant flagellar components. When the functions and physical locations of the membrane protein and of protein 4 are better known, the nature of this relation may become clear. Perhaps the radial spoke, which is defective in *pf1* and *pf14* mutants, is required to produce a normal, rather than oversized, membrane protein. If so, then this function must normally occur intracellularly and not in the flagellum, because wild-type *Chlamydomonas* produces normal-sized membrane protein in its cell body (Fig. 9).

The presence, in the *ts222* flagellum, of proteins not found in wild-type flagella needs explanation. Perhaps, as discussed above, these proteins represent the unprocessed precursors of other species missing from the mutant flagella. Alternatively, they may be species that, in the course of normal flagellar morphogenesis, are digested or expelled from the structure, but that, in the mutant, remain trapped in assembly intermediates (16, 21).

As a final speculation, we mention the possibility that the spoke defects in *pf1* mutants may be a consequence of their membrane defect. This speculation is based on the recent discovery that flagella possess a motile system whereby particles attached to the surface of the organelle can be transported up and down its length (3, 4). If, as is thought, this surface motility reflects a motile system within or below the membrane, then this

were labeled during flagellar regeneration except *pf1*, which was labeled during vegetative growth. Wild type was NO *mi*⁺. Lane 1, wild-type gametic flagella; lane 2, wild-type gametic cell bodies; lane 3, wild-type vegetative flagella; lane 4, wild-type vegetative cell bodies; lane 5, *pf1* flagella.

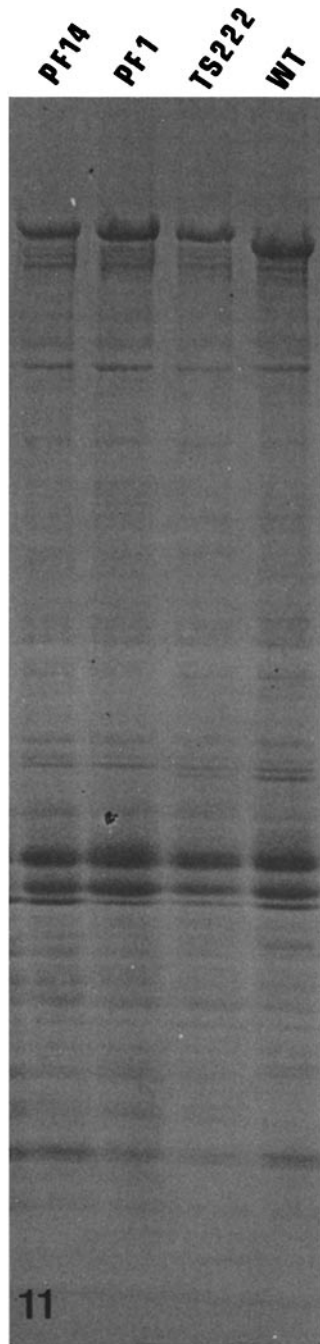


FIGURE 11 *pf14* Also shows an oversized membrane protein. Wild-type strain was NO *mt*⁻. Gel was stained with Coomassie blue.

motile system may play an essential role in transporting axonemal components to their sites of assembly during flagellar morphogenesis. *ts222*

has been shown to possess flagellar surface motility (R. Bloodgood, personal communication); however, if the major membrane protein is involved in carrying proteins up the inside of the flagellum, *ts222* may be defective in this carrier function, and this may be the source of its pleiotropy.

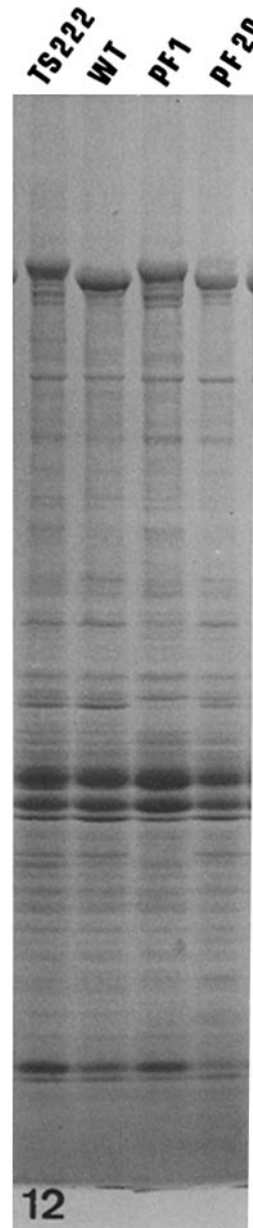


FIGURE 12 *pf20* Flagella have normal-sized membrane protein. Wild type was NO *mt*⁻. Gel was stained with Coomassie blue.

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REFERENCES

1. BERGMAN, K., U. GOODENOUGH, D. GOODENOUGH, J. JAWITZ, and H. MARTIN. 1975. Gametic differentiation in *Chlamydomonas reinhardtii*. II. Flagellar membranes and the agglutination reaction. *J. Cell Biol.* **67**:606-622.
2. BLOBEL, G., and B. DOBBERSTEIN. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* **67**:835-851.
3. BLOODGOOD, R. 1977. Motility occurring in association with the surface of the *Chlamydomonas* flagellum. *J. Cell Biol.* **75**:983-989.
4. BLOODGOOD, R., E. LEFFLER, and A. BOJCZUK. 1979. Reversible inhibition of *Chlamydomonas* flagellar surface motility. *J. Cell Biol.* **82**:664-674.
5. BOUCK, G. V., A. ROGALSKI, and A. VALAITIS. 1978. Surface organization and composition of *Euglena*. II. Flagellar mastigonemes. *J. Cell Biol.* **77**:805-826.
6. CASTILLO, C. J., C.-L. HSIAO, P. COON, and L. W. BLACK. 1977. Identification and properties of bacteriophage T4 capsid-formation gene products. *J. Mol. Biol.* **110**:585-601.
7. DAWES, J., and E. B. GOLDBERG. 1973. Functions of baseplate components in bacteriophage T4 infection. II. Products of genes 5, 6, 7, 8, and 10. *Virology*. **55**:391-396.
8. DENTLER, W., 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.
9. EBERSOLD, W. R. 1956. Crossing-over in *Chlamydomonas reinhardtii*. *Am. J. Bot.* **43**:408-410.
10. EBERSOLD, W. R. 1967. *Chlamydomonas reinhardtii*: heterozygous diploid strains. *Science (Wash. D. C.)*. **157**:447-449.
11. FAIRBANKS, G., T. STECK, and D. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. **10**:2606-2616.
12. GEORGIOPOULOS, C., and I. HERSKOWITZ. 1971. *Escherichia coli* mutants blocked in Lambda DNA synthesis. In *The Bacteriophage Lambda*. A. D. Hershey, editor. Cold Spring Harbor Laboratory, New York.
13. GIBBONS, B. H., and I. GIBBONS. 1972. Flagellar movement and adenosine triphosphatase activity in sea urchin sperm extracted with Triton X-100. *J. Cell Biol.* **54**:75-97.
14. GILLHAM, N., and R. P. LEVINE. 1962. Pure mutant clones induced by ultraviolet light in the green alga *Chlamydomonas reinhardtii*. *Nature (Lond.)*. **194**:1165-1166.
15. HANSMAN, H. 1975. The immobilization antigen of *Paramecium aurelia* is a single polypeptide chain. *J. Protozool.* **22**:257-259.
16. HERSHKO, A., and M. FRY. 1975. Post-translational cleavage of polypeptide chains: role in assembly. *Annu. Rev. Biochem.* **44**:775-797.
17. HUANG, B., M. RIFKIN, and D. LUCK. 1977. Temperature-sensitive mutations affecting flagellar assembly and function in *Chlamydomonas reinhardtii*. *J. Cell Biol.* **72**:67-85.
18. HUNT, L. A., J. ETCHISON, and D. F. SUMMERS. 1978. Oligosaccharide chains are trimmed during the synthesis of the envelope glycoprotein of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U. S. A.* **75**:754-758.
19. JARVIK, J., and D. BOTSTEIN. 1973. A genetic method for determining the order of events in a biological pathway. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2046-2050.
20. JARVIK, J., P. LEFEBVRE, and J. ROSENBAUM. 1976. A cold-sensitive mutant of *Chlamydomonas reinhardtii* with aberrant control of flagellar length. *J. Cell Biol.* **70**(No. 2, Pt. 2):149a (Abstr.).
21. KING, J., E. LENK, and D. BOTSTEIN. 1973. Mechanism of head assembly and DNA encapsulation in Phage P22. II. Morphogenetic pathway. *J. Mol. Biol.* **80**:697-731.
22. LASKEY, R., and A. MILLS. 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**:335-341.
23. LEFEBVRE, P., S. NORDSTROM, J. MOULDER, and J. ROSENBAUM. 1978. Flagellar elongation and shortening in *Chlamydomonas*. IV. The effects of flagellar detachment, regeneration, and resorption on the induction of flagellar protein synthesis. *J. Cell Biol.* **78**:8-27.
24. LEVINE, R. P., and W. T. EBERSOLD. 1960. The genetics and cytology of *Chlamydomonas*. *Annu. Rev. Microbiol.* **14**:197-216.
25. LEWIN, R. 1954. Mutants of *Chlamydomonas moewusii* with impaired motility. *J. Gen. Microbiol.* **11**:358-363.
26. LUCK, D., 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. U. S. A.* **74**:3456-3460.
27. LUFTIG, R., and L. LUNDH. 1973. Bacteriophage T4 head morphogenesis. Isolation, partial characterization, and fate of gene 21-defective tau-particles. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1636-1640.
28. LYNN, D. H. 1976. Comparative ultrastructure and systematics of the Colpoda (ciliphora): structural differentiation in the cortex of *Colpoda simulans*. *Trans. Am. Microsc. Soc.* **95**:581-599.
29. O'FARRELL, P. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.
30. PIPERNO, G., B. HUANG, and D. LUCK. 1977. Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U. S. A.* **74**:1600-1604.
31. RINGO, D. L. 1967. Flagellar motion and fine structure of the flagellar apparatus in *Chlamydomonas*. *J. Cell Biol.* **33**:543-571.
32. ROSENBAUM, J., J. MOULDER, and D. RINGO. 1969. Flagellar elongation and shortening in *Chlamydomonas*. I. The use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. *J. Cell Biol.* **41**:600-619.
33. SADLER, J. R., and A. NOVICK. 1965. The properties of repressor and the kinetics of its action. *J. Mol. Biol.* **12**:305-327.
34. SAGER, R., and S. GRANICK. 1953. Nutritional studies with *Chlamydomonas reinhardtii*. *Ann. N. Y. Acad. Sci.* **56**:831-838.
35. SATTLER, C., and L. STAEHELIN. 1974. Ciliary membrane differentiations in *Tetrahymena pyriformis*. *Tetrahymena* has four types of cilia. *J. Cell Biol.* **62**:473-490.
36. SLOBODA, R., W. DENTLER, R. BLOODGOOD, B. TELZER, S. GRANETT, and J. ROSENBAUM. 1976. Microtubule-associated proteins (MAPS) and the assembly of microtubules *in vitro*. In *Cell Motility*, R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, New York, 1171-1212.
37. SNELL, W. 1976. Mating in *Chlamydomonas*: a system for the study of specific cell adhesion. I. Ultrastructural and electrophoretic analysis of flagellar surface components involved in adhesion. *J. Cell Biol.* **68**:48-69.
38. STARLING, D., and J. RANDALL. 1971. The flagella of temporary dikaryons of *Chlamydomonas reinhardtii*. *Genet. Res.* **18**:107-113.
39. STEPHENS, R. E. 1977. Major membrane protein differences in cilia and flagella: evidence for a membrane-associated tubulin. *Biochemistry*. **16**:2047-2058.
40. WITMAN, G., K. CARLSON, J. BERLINER, and J. ROSENBAUM. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *J. Cell Biol.* **54**:507-539.
41. WITMAN, G., J. PLUMMER, and G. SANDERS. 1978. *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules. *J. Cell Biol.* **76**:729-747.