Proc. Natl. Acad. Sci. USA
Vol. 74, No. 8, pp. 3456–3460, August 1977
Cell Biology

Flagellar mutants of *Chlamydomonas*: Studies of radial spoke-defective strains by dikaryon and revertant analysis

(cell motility/axonemal structure/protein assembly/mutant gene product)

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Communicated by Zanvil A. Cohn, June 6, 1977

ABSTRACT **The motility mutant of *Chlamydomonas reinhardtii pf14* lacks radial spoke structures in its flagellar axonemes, and 12 proteins present in wild type are missing from a two-dimensional map (isoelectrofocusing/sodium dodecyl sulfate electrophoresis) of its ^{35}S-labeled flagellar proteins. Six of these same proteins are missing in *pf1*, which lacks spokeheads. To determine whether any of the missing proteins represent the mutant gene product two experimental approaches have been applied. The first makes use of the fact that gametes of either mutant strain when fused with wild-type gametes to form quadriflagellate dikaryons undergo recovery of flagellar function. Recovery at the molecular level was monitored by prelabeling the mutant proteins with ^{35}S and allowing recovery to occur in the absence of protein synthesis. It is to be expected that the mutant gene product would not be restored as a radioactive protein and that recovery would depend on the assembly of the wild-type counterpart that is not labeled. The second technique makes use of revertants induced by UV irradiation. Dikaryon rescue in the case of *pf14* leads to restoration of 11 radioactive components; only protein 3 fails to appear as a radioactive spot. For *pf1* only two radioactive proteins are restored; proteins 4, 6, 9, and 10 were not radioactive. Analysis of revertants of *pf1* gave evidence (altered map positions) that protein 4 is the mutant gene product. In the case of *pf14*, analysis of 22 revertants has not provided similar positive evidence that protein 3 is the gene product.**

Previously we have analyzed flagellar proteins in paralyzed mutants of *Chlamydomonas reinhardtii* using a two-dimensional system of isoelectric focusing followed by electrophoresis in the presence of sodium dodecyl sulfate (1). In the case of the mutant *pf14*, a two-dimensional map of flagellar proteins was shown to lack 12 characteristic polypeptides that were regularly present in wild-type flagella. This deficiency could be correlated with the total absence of radial spokes and associated spokeheads in the axonemal structures of *pf14*. Another mutant, *pf1*, was shown to lack 6 of these 12 flagellar polypeptides, and analysis of axonemal structures revealed that the radial spokes were present but that spokeheads were absent.

Since *pf14* and *pf1* have been shown to be independent single-site mutations (2), the deficiency of all spoke structures in one case and of spokeheads in the other indicates that the mutant gene products play critical roles in the assembly of as many as 12 different proteins in the case of *pf14* and 6 in the case of *pf1*. Two complementary approaches have been undertaken to determine if the mutant gene products of *pf14* and/or *pf1* are structural components of the radial spoke.

The first type of experiments makes use of quadriflagellate dikaryon cells which are produced after gametic fusion in the *Chlamydomonas* mating cycle. It has been observed that in dikaryons of some paralyzed mutants of *C. moewusii* (3) or *C. reinhardtii* (4) with the counterpart wild-type strains, mutant flagella recover function within 1–2 hr after establishment of cytoplasmic mixing. In some cases restoration of function may require removal of preexisting flagella and flagellar regeneration (4). It has been suggested that in both cases recovery is brought about by the transfer of flagellar components from the wild-type cytoplasm to the mutant flagella. Since vegetative cells partially regenerate flagella in the presence of inhibitors of protein synthesis (5), it seemed possible that in dikaryon flagellar recovery the required protein molecules could be provided from pools of flagellar components already present in the gametic cells. Based on these observations, we proposed to carry out synchronous matings between mutant gametes, prelabeled by growth on $^{35}SO_4$-containing media, and unlabeled gametes of wild type in the presence of an inhibitor of protein synthesis. The inhibitor was included to prevent incorporation of radioactive amino acids into wild-type flagellar proteins. Assuming that adequate pools of flagellar components were present in the mutant, we anticipated that restoration of function of preexisting flagella (*in situ* rescue) or after regeneration (regeneration rescue) would be accompanied by the restoration in the flagellar protein map of many radioactive polypeptides. It would also be expected that those products whose assembly is directly affected by the mutation would not be restored and thus could be identified as missing radioactive components in the flagellar protein map. Of course, the recovery of flagellar activity would be dependent on incorporation of the corresponding wild-type component, which in this experiment would not be radioactive.

The second type of experiment made use of induced revertants of *pf1* and *pf14* with the expectation that in some cases alterations in polar amino acid content of the putative gene products might be identified by shifts in isoelectric point as detected in the first dimension of the mapping procedure.

The communication that follows gives an account of the application of these two experimental approaches.

MATERIALS AND METHODS

Reagents, Mutant Strains, and Preparative and Protein Mapping Methods. Unless otherwise indicated these were as previously described (1).

Culture and Radioactive Labeling of Gametes and Preparation of Zygotes. Cells were grown on petri dishes containing 25 ml of modified minimal 1 medium (6) with 1.5% agar which had been washed with distilled water. The sulfate content of modified minimal 1 medium was reduced by lowering the $MgSO_4$ concentration to $1/15$ of that normally used and $MgCl_2$ was added to restore the original Mg^{2+} concentration. For radioactive gametes, [^{35}S]sulfuric acid was added at 0.1 Ci/liter. Plates were inoculated with approximately 4×10^6 cells and

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Table 1. UV induction of reversions of *pf1* and *pf14*

Cell type	UV exposure time, sec*	% survival	Estimated reversion frequency†
pf1 mt$^+$			
Gametes	0	100	—
	30	7.9	2×10^{-7}
	60	0.5	7.3×10^{-6}
Vegetative cells	0	100	—
	30	47	3.2×10^{-8}
	60	7.6	1.9×10^{-7}
pf14 mt$^-$			
Gametes	0	100	2.6×10^{-8}
	30	2.2	1.9×10^{-6}
	60	0.006	4.1×10^{-5}
Vegetative cells	30	75	1.7×10^{-7}
	60	14.4	3.1×10^{-7}

* Cultures were stirred and exposed in an open dish at 20 cm from a Sylvania (Salem, MA) 15 watt germicidal lamp-G15T8 for the times indicated.

† The reversion frequency among surviving cells was computed according to the method described by Luria and Delbrück (7). In this representative experiment each data point is based on a sample size of 20 tubes. A dash indicates that no revertants were found.

grown at 25° for 4 days with constant illumination of about 20,000 lux. Cells were suspended in nitrogen-free medium at a density of approximately 4×10^7 cells per ml and allowed to differentiate into gametes for about 3 hr before they were mixed with gametes of the opposite mating type. Each plate yielded about 10 ml of gametes at 4×10^7 cells per ml. Dikaryon flagella preparations were obtained from 20 to 30 ml of mixed gametes.

Isolation and Testing of Revertants. Aliquots (10 ml, about 3×10^7 cells per ml) of *pf1* and *pf14* gametes or vegetative cells were exposed to UV light for varying times. After exposure, each aliquot was divided among 20 tubes containing 3 ml of modified minimal 1 medium plus acetate (6) and held in the dark overnight, before transfer to constant light. After 6–7 days of growth, each tube was examined for the presence of swimming cells, which were found in no more than 8 of 20 tubes. Aliquots from each tube containing swimming cells were plated, and a single swimmer clone from each tube was chosen for backcrossing to strain 137c. Table 1 gives typical results from this type of experiment.

RESULTS

Flagellar recovery in prezygotic dikaryons

As already pointed out in the introduction, the use of the dikaryon rescue approach requires that no new protein synthesis take place after fusion of radioactive mutant gametes with unlabeled wild-type gametes. To select the conditions for inhibition of protein synthesis we studied the effect of different concentrations of anisomycin on the incorporation of [^{3}H]arginine into tubulin of flagella from wild type by wild-type dikaryons during flagellar regeneration. For dikaryons exposed to 60 μM anisomycin, the incorporation of arginine into tubulin was inhibited by approximately 95%. Anisomycin at this concentration was used in subsequent experiments and it was added at the time gametes were mixed.

We observed that in the presence of anisomycin, dikaryons of *pf14* or *pf1* with wild type showed restoration of beating of the paralyzed flagella. Because it was difficult to be certain of the timing of this process when making observations against the background of the actively beating wild-type flagellar pair in each dikaryon, we repeated the observations using, instead of wild type, the paralyzed mutant *pf18*. Flagella of this mutant lack central pair microtubules and do not become active in dikaryons unless deflagellation and flagellar regeneration take place (4). In dikaryons of *pf14* or *pf1* with *pf18*, the transition from four paralyzed flagella to two paralyzed and two actively beating flagella is striking and in each case could be observed to take place within 1 hr of gamete mixing. Therefore, it was to be expected that molecular rescue could be observed *in situ* without a requirement for flagellar regeneration in these mutants.

Gametes of *pf14* and *pf1* were prepared on solid media containing ^{35}S (see *Materials and Methods*) and mixed in the presence of anisomycin with equal numbers of unlabeled wild-type gametes. Gametic fusion was extensive so that in typical experiments 70–80% of the cell population was quadriflagellate within 30 min. The dikaryons were held an additional 2 hr at 25° under very low light, at which time flagellar axonemes were prepared and the protein components analyzed by two-dimensional mapping. The results of typical experiments are shown in Fig. 1. When the radioautogram from the dikaryon *pf14* by wild type (Fig. 1*B*) is examined with respect to the 12 polypeptides that are characteristically absent from flagellar maps of *pf14*, a striking change is apparent for 11 of the 12 proteins; radioactive spots at each of the characteristic positions are visible. Only for protein number 3 is a radioactive spot missing. In all cases (for example, see Fig. 1 *A* and *C*), the protein designated 3 consists of two closely apposed spots. In all observations so far the behavior of these two components is coordinate.

A paralyzed mutant *pf14A*, which is allelic to *pf14*, was isolated in our laboratory after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (6). When *pf14A* is studied by the dikaryon rescue technique, the results are indistinguishable from those seen in Fig. 1*B*.

The rescue experiment for *pf1* dikaryons is shown in Fig. 1*C*. In this case, examination of the positions for the six components typically missing in *pf1* reveals that only two radioactive components are restored (proteins 1 and 2) and that four components (proteins 4, 6, 9, and 10) are not restored as radioactive spots.

Electron microscopic studies in which we made use of *pf18*, which lacks central microtubules, as a morphological marker gave clear evidence that the recovery of flagellar function in dikaryons of *pf14* or *pf1* is accompanied by restoration of spoke and spokehead structures. Therefore, we assumed that during dikaryon rescue, wherever a flagellar protein failed to reappear as a radioactive spot, it had been supplied from a pool of unlabeled wild-type components. For *pf14* and *14A* this was true for only one component, protein 3. For *pf1*, four components, proteins 4, 6, 9, and 10, showed this behavior. To interpret these results in terms of mutant gene products we turned to the analysis of revertants.

Analysis of revertants

Revertants of *pf14* and *pf1* were induced by exposure to UV light and further characterized by backcrossing to wild type as outlined in *Materials and Methods*. Using the two-dimensional mapping procedure we analyzed flagellar axonemal proteins from 22 independently isolated revertants of *pf14* and 19 revertants of *pf1*. For *pf14* revertants we have so far not detected any deviation from wild type in the behavior of protein number 3 or of any other of the 12 proteins that are char-

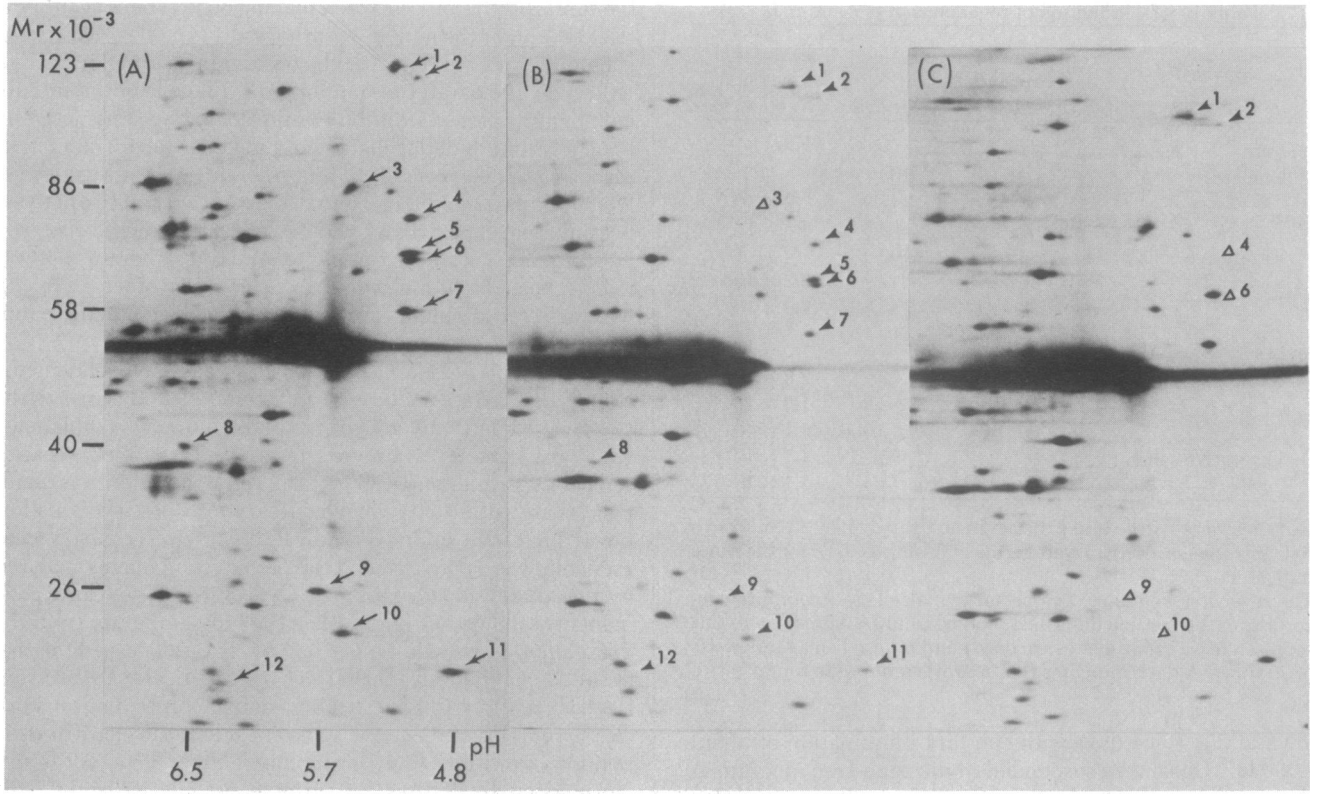

FIG. 1. Autoradiograms of the polyacrylamide gel slab used for the two-dimensional separation of axonemal polypeptides from *Chlamydomonas*. Only a portion of the original map is shown comprising the molecular weight (M_r) range 125,000–15,000 and the pH range 7–4.5. (*A*) Axonemes from gametes of the 137c wild-type strain. Arrows indicate 12 polypeptides that are to be studied in detail. (*B*) Axonemes from dikaryons of *pf14 mt*$^+$ by 137c *mt*$^-$ 2.5 hr after gametic mixing. Only *pf14* gametes were radioactively labeled by growth on ^{35}S-containing medium. (*C*) Axonemes from dikaryons of *pf1 mt*$^+$ by 137c *mt*$^-$ 2.5 hr after gametic mixing. Only *pf1* gametes were radioactively labeled. In (*B*) and (*C*) solid arrowheads indicate positions of radioactive proteins characteristically missing in the mutant but restored in the dikaryon; open arrowheads indicate positions where radioactive proteins were not restored.

acteristically missing in the parental strain (see *Note Added in Proof*).

The situation is different for revertants of *pf1*, where we have detected striking alterations in the map position of protein number 4 in 4 of the 19 revertants so far analyzed. In all four cases the altered behavior of spot 4 was apparent immediately, and was confirmed later by mixing experiments in which wild-type axonemal proteins or axonemal proteins from other revertants served as markers. No differences have been detected in the position for the other 5 proteins that, together with protein 4, are characteristically missing in *pf1*.

In Fig. 2 are shown typical maps for three of these four revertants. Fig. 2*A* shows that in revertant 65, protein 4 has a strikingly altered isoelectric point shifted to the acidic side of the wild-type marker. Mixing experiments of the type shown in Fig. 2*A* revealed another feature of *pf1*-R-65 protein 4, namely, a slightly increased mobility in the second dimension, sodium dodecyl sulfate electrophoresis. This alteration is shown more clearly in Fig. 2*B*, where the second dimension map was carried out in a gel of lower acrylamide content to accentuate differences in mobility. The R-65 protein 4 (labeled r) runs ahead of wild-type marker. Included also is protein 4 from another revertant, R-108 (labeled r′), which is seen to have an isoelectric point shifted to the basic side of wild type and to have a mobility only slightly faster than the marker. In Fig. 2*C* a reconstruction experiment of the type shown in Fig. 2*B* illustrates the situation in revertant R-85. Here, R-85 proteins replace the wild type of Fig. 2*B* and the R-85 protein 4 (labeled r″) is seen to have a mobility faster than R-65 protein 4 (labeled r) but no detectable alteration in isoelectric point. In a fourth revertant, R-105, protein 4 is shifted to the basic side in a position intermediate between R-108 and wild type (the map is not shown).

In summary, axonemes of four revertants of *pf1* show protein 4 with altered coordinates in the two-dimensional mapping procedure: in all cases the mobility in the second dimension is somewhat increased. In one case there is no shift in isoelectric point (R-85); in one case the isoelectric point is shifted to the acidic side (R-65); and in two cases (R-108 and R-105) it is shifted to the basic side.

Each of these revertants has been backcrossed to wild type and analyzed by tetrad analysis. Segregation of the *pf* phenotype has not been observed in 120 tetrads for R-65, 20 for R-85, 30 for R-105, and 40 for R-108.

Since each of these strains appears to represent a secondary mutation within the same gene, it is unlikely that the several types of chemical behavior of protein 4 can be explained on the basis of post-transcriptional protein modifications. To explore further this possibility we have tested for coexpression of protein 4 in dikaryons of wild-type and *pf1*-R-65. For these experiments gametic fusion and flagellar regeneration were allowed to take place without inhibition of protein synthesis since we wished to observe the behavior of protein 4 from its time of synthesis. Fig. 3*A* shows an experiment in which ^{35}S-labeled gametes of wild type and *pf1*-R-65 were allowed to form dikaryons and to regenerate flagella after deflagellation by pH shock. The axonemal protein map for spot 4 shows unbiased coexpression of the two forms of the protein. In Fig. 3*B* a similar

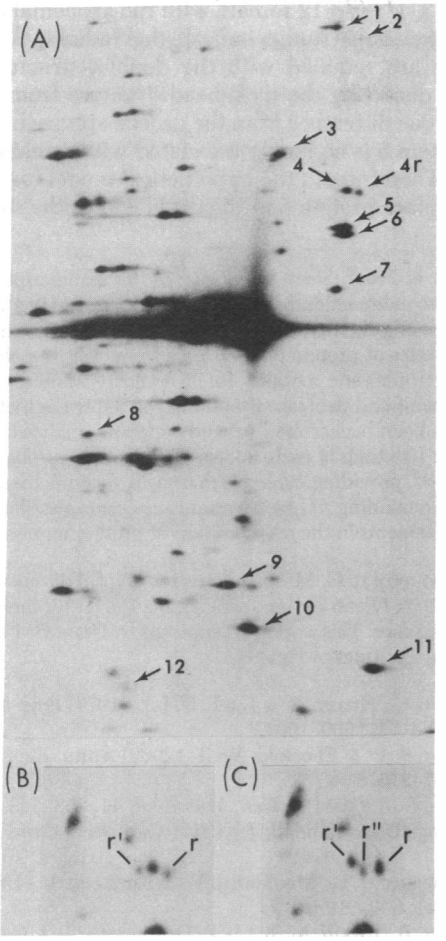

FIG. 2. Autoradiograms as in Fig. 1. (*A*) Axonemes from gametes of 137c and gametes of revertant *pf1*-R-65 mixed in about 1:1 ratio. In (*B*) and (*C*) small regions are illustrated of two-dimensional maps in which a 5% acrylamide gel slab replaces the usual gradient gel. (*B*) Axonemal mixture from gametes of 137c, revertant *pf1*-R-108 and *pf1*-R-65. The region shown cuts through protein 5. r′ indicates protein 4 of *pf1*-R-108; r, protein 4 of *pf1*-R-65; and the undesignated spot in between, protein 4 of wild type. (*C*) Same mixture as in (*B*) except that axonemes from revertant *pf1*-R-85 replace wild type. Protein 4 from *pf1*-R-85 is designated r″.

experiment is illustrated. However, in this case only the wild-type gametes were radioactively labeled. Under these conditions it was to be expected that any labeled *pf1*-R-65 protein 4 would be the result of new synthesis from pools of ^{35}S-labeled amino acids introduced by the wild-type gametes. After one cycle of flagellar regeneration (not illustrated), the radioactivity at the expected position for protein 4 of *pf1*-R-65 was faint; after a second cycle of deflagellation and regeneration, as shown in Fig. 3*B*, the radioactivity at this position is very clearly visible (labeled 4r).

A dikaryon experiment of the type shown in Fig. 3*A* was also carried out with revertants R-65 and R-108 (results not illustrated); in this case too, unbiased coexpression of the two types of protein 4 was observed.

These results would be expected if each of the revertants produced a protein 4 with altered primary structure. For the findings to be consistent with a model of protein modification it is necessary to postulate a tight association between the modification enzyme and the site of protein synthesis which persists through two cycles of flagellar regeneration in the presence of wild-type products. Furthermore, each mutational event would have had to alter the properties of a single modification enzyme in such a way as to produce protein 4 with four different types of chemical modification.

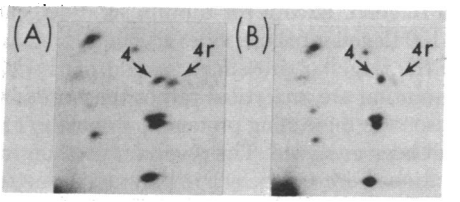

FIG. 3. Autoradiograms as in Fig. 1 of the map regions containing axonemal components 3, 4, 5, 6, and 7. (*A*) Axonemes from dikaryons 137c *mt*$^+$ by *pf1*-R-65 *mt*$^-$ after deflagellation and regrowth of flagella for 75 min at 25°. Both gametic cultures were labeled by growth on ^{35}S-labeled medium. (*B*) Axonemes from dikaryons as in (*A*). Only 137c *mt*$^+$ gametes were radioactively labeled. Axonemes were isolated after two cycles of deflagellation and flagellar regeneration.

DISCUSSION

We have described an application of dikaryon rescue (3, 4) in which the restoration of flagellar function in prezygotic dikaryons occurs by utilization of pools of flagellar proteins already present in gametes and in which the mutant proteins are exclusively labeled. Assuming that there is no special deficiency of the pool of a particular component in the mutant strains, the method may have general usefulness as a diagnostic tool to help identify defective mutant gene products. When applied to mutants lacking flagellar radial spokes and spokeheads (*pf14* and *pf14A*) the method gives a clearcut indication that one of the proteins (number 3) that is characteristically among those absent in the mutant state could be the gene product. So far this identification has not been confirmed by the technique of revertant analysis, so that any conclusion about the mutant product must be considered tentative (see *Note Added in Proof*). It is possible that the true mutant product is a modification enzyme for protein 3 or is another protein that forms an association with protein 3 and is required for its assembly. The first of these possibilities must be seriously considered because protein 3 always has two components and because preliminary studies indicate that both components may be phosphorylated. The second possibility is also real because of our uncertainty concerning the completeness of the two-dimensional mapping system. So far this method identifies about 130 flagellar components; its limitations have been discussed in an earlier publication (1).

In *pf1*, a mutant lacking radial spokeheads, the results of the rescue experiments are difficult to interpret because four proteins, numbers 4, 6, 9, and 10, of *pf1* origin are not restored to the dikaryon flagella. In this case, however, revertant analysis provides strong independent evidence that one of these four components, protein 4, is the mutant gene product.

At present our data do not explain why four rather than only one of the six radioactive proteins missing in flagella of *pf1* are not incorporated during dikaryon rescue. It could be argued that there is in this mutant a specific pool deficiency. In that case the situation would be different from that with *pf14*, where dikaryon results indicate that pools of these proteins are present. An explanation consistent with our data is that the precursor pool consists of a complex of the four proteins which is formed at the time of their synthesis. It is also possible that the four components are synthesized as a single high molecular weight precursor.

It is obvious that the evidence obtained by both dikaryon rescue and two-dimensional mapping of revertants is indirect. However, direct approaches to characterizing mutant gene products by extensive structural analysis of proteins will be

difficult in flagella. Except for tubulin subunits, each of the more than 100 flagellar polypeptides accounts for a microscopic fraction of the flagellar protein mass, and methods for polypeptide resolution are analytical rather than preparative (1). For this reason the interesting proteins 4 of some *pf1* revertants have not yet been analyzed. The revertant proteins in question show alterations both in isoelectric point and electrophoretic mobility. Both charge and mobility differences could be consequences of single amino acid substitutions which result in substantial alterations of sodium dodecyl sulfate binding. It is also possible that the revertant polypeptide is truly smaller in size than the wild-type counterpart; the molecular weight change could be as large as approximately 1000 in the case of *pf1*-R-85. Data on the primary structure in these cases would provide a basis for understanding the original mutation of *pf1* and the nature of the second site alterations in its revertants.

It is possible that revertants of this type may have future application to the study of the function of radial spokes. Spoke structures are required for flagellar function (8), and it has been suggested that spokeheads interact with the central sheath to convert doublet sliding into axonemal bending (9). We observed that one of the four *pf1* revertants with easily detectable alterations in protein 4 (*pf1*-R-108) showed defective swimming behavior. Although flagellar function in this or other revertants has not been analyzed, the motility defect could indicate that the structural characteristics required for assembly of protein 4 may be different from those required for function.

The major application of the results presented here is to increase our knowledge of the flagellar radial spoke structure and the process of its assembly. On the basis of previous experiments (1) we could assign some or all of proteins 3, 5, 7, 8, 11, and 12 to the radial spoke stalk structure and 1, 2, 4, 6, 9, and 10 to the radial spokehead. It is now clear that radial spokes can be assembled onto preexisting axonemal doublets. It seems likely that in the assembly process protein 3 is assembled first or is required to form a stable structure; protein 4 plays a similar role in the assembly of the spokehead onto the stalk.

This picture of the organization of the spoke structure has been confirmed by another approach now under study in our laboratory. When axonemes are dialyzed against low ionic strength buffers according to the chemical dissection procedure of Gibbons (10), proteins 1, 2, 4, 5, 6, 9, and 10 are solubilized while 3, 7, 8, 11, and 12 remain with the axonemal doublets. Electron microscopic studies indicate that radial spokes but not spokeheads are retained with the doublet structures. This method of dissecting the spokehead structure from the stalk shows only one difference from the genetic approach described above; protein 5 is no longer associated with remaining stalk structure. Therefore, in the hypothetical model for assembly we would place protein 5 at the distal end of the stalk structure.

Note Added in Proof. Since submission of this manuscript, axonemal proteins of 13 independently isolated revertants of *pf14A* have been analyzed. Among the revertants, three showed clearcut shifts in the map coordinates of protein 3: a more basic isoelectric point and increased electrophoretic mobility for R-4 and R-5 and a more basic isoelectric point and decreased mobility for R-11. Each of these revertants has been backcrossed to wild type and analyzed by tetrad analysis. For 10 tetrads of each, no segregation of the *pf* phenotype has been observed, providing evidence that the reversions are intragenic. Among the remaining *pf14A* revertants are examples showing very large displacements in the map position of protein number 3.

We are grateful to G. M. W. Adams for helpful discussions and to Beth Weinstein, Diann Lewis, and Virginia Kozler for their excellent technical assistance. This work was supported by Grant GM 17132 from the National Institutes of Health.

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