Genetic and Biochemical Dissection of the Eucaryotic Flagellum

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ABSTRACT The axoneme is the basic functional unit of the eucaryotic flagellum. Periodic structures appended to its 9+2 microtubule core are responsible for generation of flagellar bending. An account of biochemical and genetic studies of flagellar-defective mutants of *Chlamydomonas reinhardtii* is presented. These studies provide insights into the complex molecular composition of the appended structures, their mode of assembly, and the way in which they interact to modulate flagellar function.

Microtubular structures appear in a variety of forms in dividing and nondividing eucaryotic cells. In each form the microtubular structure itself and its major component subunits α- and β-tubulin show close homology, not only among microtubular systems of a single organism but from organism to organism. It is likely that the particular form and function of a specific microtubular array depends mostly on molecules and structures appended to the basic tubulin framework.

Nowhere is this more obvious and more thoroughly studied than in the case of the eucaryotic flagellum or cilium. In these microtubular cell appendages, many of the functional accessory molecules are organized as periodic structures appended to the continuous microtubule outer doublets and central pair as is illustrated in Fig. 1.

On the basis of the work of Gibbons and his co-workers and studies of Satir and his colleagues (reviewed in reference 1), it has been established that the motive power for flagellar beating depends on dynein arms attached to the nine outer doublets. The arms, permanently attached to the A subfiber, make intermittent connections with the B subfiber, bringing about distal sliding of that doublet (2). It is clear that regulation and coordination is required for doublet sliding to lead to organized flagellar bending. Warner and Satir (3) proposed that some part of this function was provided by interactions between radial spokes projecting from the A subfiber of the outer doublet and projections from central-pair microtubules.

Possibility of Genetic Analysis

The unicellular green alga *Chlamydomonas reinhardtii* bears two flagella and is an ideal organism in which to use genetic analysis for studying assembly and function of flagellar structures. The possibility for this analysis was first demonstrated in a related organism more than 25 years ago by Ralph Lewin (4). Lewin also isolated 20 mutants in *C. reinhardtii*, (*pf*1–*pf*20), most of which have been mapped to independent loci (5). These mutants were first studied by Randall and Starling (6) and subsequently by other investigators (reviewed in reference 7). The usefulness of *Chlamydomonas* as a model has also been increased by extensive studies of flagellar morphology (8) and flagellar regeneration (9).

The major feature of the *Chlamydomonas* flagellum that makes it suitable for combined biochemical and genetic analysis is the ease with which flagella can be detached and recovered in highly purified fractions. In our experiments, pH shock is used for flagellar detachment, and the point of detachment is just distal to the basal body transition zone (7). Isolated flagella can be stripped of their membranes with nonionic detergent, and the microtubule structure is retained as an intact axonemal cylinder along with the accessory structures: dynein arms, radial spokes, and the central-pair projections (Fig. 1). Axonemal fractions isolated from *Chlamydomonas* by a somewhat different procedure may be reactivated by addition of ATP (10). These axonemes show different wave forms when the reactivation medium contains low or high Ca^{++} concentrations; one form at low Ca^{++} resembles the ciliary-type bending pattern of forward swimming cells, the other the flagellar-type bending pattern of backward swimming cells. The correlation between these motility properties of the isolated structure and those of the intact flagellum suggests that the axoneme represents the functional unit. At first approximation, the axoneme is structurally uniform with the morphological features illustrated in Fig. 1. Axonemal preparations contain a characterizeable number of component polypeptides. This number we presently estimate to range from 250 to 300. The effectiveness of

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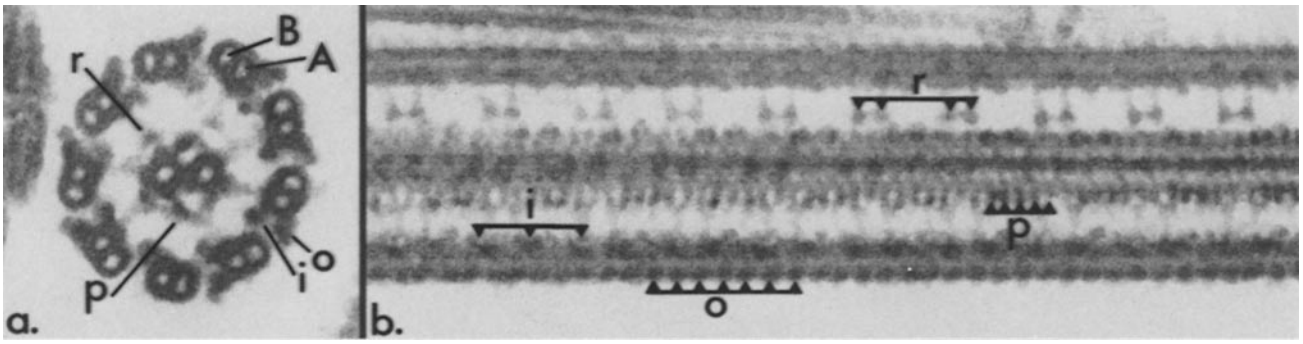

FIGURE 1 Electron micrographs of transverse (*a*) and longitudinal (*b*) sections of a wild-type *Chlamydomonas reinhardtii* axoneme. A and B subfibers of the outer microtubule doublets are indicated. Periodic appended structures are shown: *r*, radial spoke; *p*, projections from central pair microtubules; *o*, outer dynein arms; and *i*, inner dynein arms. For methods of preparation see reference 13. (*a*) × 162,000. (*b*) × 142,000.

our studies has greatly depended on the possibility of identifying a large number of these components.

Use of Mutants to Identify Polypeptide Components of Accessory Structures

Mutations have been identified that result in deletions of three of the major structures appended to the doublet A subfiber, i.e., radial spoke (11, 12), outer dynein arm, and inner dynein arm (13). In addition, there are mutants lacking both central tubules (14), one central tubule (15), or a single type of projection from one of the central tubules (15). We have used these examples of structural deficiency to compare proteins from mutant axonemes with wild-type. The comparison reveals a specific loss of polypeptides for each deleted structure, i.e., a characteristic signature which may define the polypeptide composition of the structure in question. The primary method of analysis of axonemal proteins is a modification of the two-dimensional electrophoretic method devised by O'Farrell (16). A typical analysis from wild type is illustrated in Fig. 2. The first dimension is electrophoresis in an ampholine-generated pH gradient (the acidic side is on the right) and the second dimension is electrophoresis in SDS (for detailed methods see references 17 and 18). For any polypeptide its position on the x-axis reflects its isoelectric point and its y-axis position its size. Maps are made by autoradiography of axonemal proteins made radioactive by prolonged cell growth on $H_2{}^{35}SO_4$. As expected from axonemal morphology, ~70% of the axonemal mass is accounted for by α- and β-tubulin. Tubulin subunits in Fig. 2 are overloaded to reveal the more than 200 other polypeptides constituting the remaining 30% of the axonemal mass. In Fig. 2, most of the wild-type axonemal proteins are revealed, but as will be shown later, important axonemal polypeptides with molecular weights >240,000 cannot be resolved in this type of gel and require different electrophoretic methods for study.

In Fig. 2 the notations identify sets of polypeptides regularly present in wild-type axonemes but absent or markedly deficient in mutants bearing deletions of radial spoke, central pair, or outer or inner dynein arms. For each type of structural defect the signature of deficient polypeptides is unique.

Significance of Multiple Polypeptide Defects in pf Mutants

It was surprising that in so many cases, single mutations resulted in loss of entire axonemal substructures and in multiple axonemal polypeptide deficiencies. The radial spoke was a typical case. The phenotype of the mutant *pf*14 is loss of all radial spokes and absence of 17 polypeptides from the axonemal map (12). Two other mutations *pf*1 and *pf*17 (12) result in the loss of a subset of this group (polypeptides r 1, 4, 6, 9, and 10),[1] and this loss is correlated with absence only of the radial spokehead. It seemed possible that in each of these cases the mutation altered only a single polypeptide and that the deficiency of a total of five or 17 polypeptides revealed some interdependent assembly of components of radial spokes, i.e., loss or structural deficiency of a single polypeptide interrupts the subsequent stable assembly of radial spoke or spokehead components.

Knowledge of the mutant gene product is required to test the possibility of stepwise assembly, and two approaches have been applied to obtain this information. The first is based on an assumption that in *pf* mutants only the gene product would be missing from the cellular precursor pool of axonemal proteins known to exist (9). To identify pool components, we took advantage of a feature of the regular mating cycle of *Chlamydomonas*. In the mating reaction, plus and minus biflagellate gametes fuse efficiently to give a population of quadriflagellate, temporary dikaryons. Lewin (4) observed that in some dikaryons derived from matings of *pf* and wild-type gametes, the paralyzed flagella recovered function after fusion. He interpreted the rescue of function as in situ complementation of the defective mutant pool by a wild-type component or components. To adapt this method to gene product diagnosis, we labeled the mutant cells by growth on $^{35}SO_4$ and carried out fusion with nonradioactive wild-type cells in the presence of anisomycin to inhibit protein synthesis (12, 19). The expectation was that in restoration of function to the *pf* flagella, mutant (^{35}S-labeled) and wild-type polypeptides would have an equal chance of being incorporated into the newly assembled radial spoke structure, except in the case of the mutant gene product where only polypeptides from the wild-type pool (unlabeled) could be incorporated. The expectation for axonemes derived from *pf*14 wild-type dikaryons was that 16 polypeptides of the group of 17 missing in *pf*14 axonemes would be restored in a radioactive form when flagellar function was restored. The 17th component, representing the gene product, would be drawn only from the wild-type pool and would therefore not be radioactive. When the

[1] For clarity, sets of axonemal components found to be missing in mutants associated with deletion of an appended structure are identified by a letter preceding the number, i.e., r, radial spoke, i, inner arm, o, outer arm.

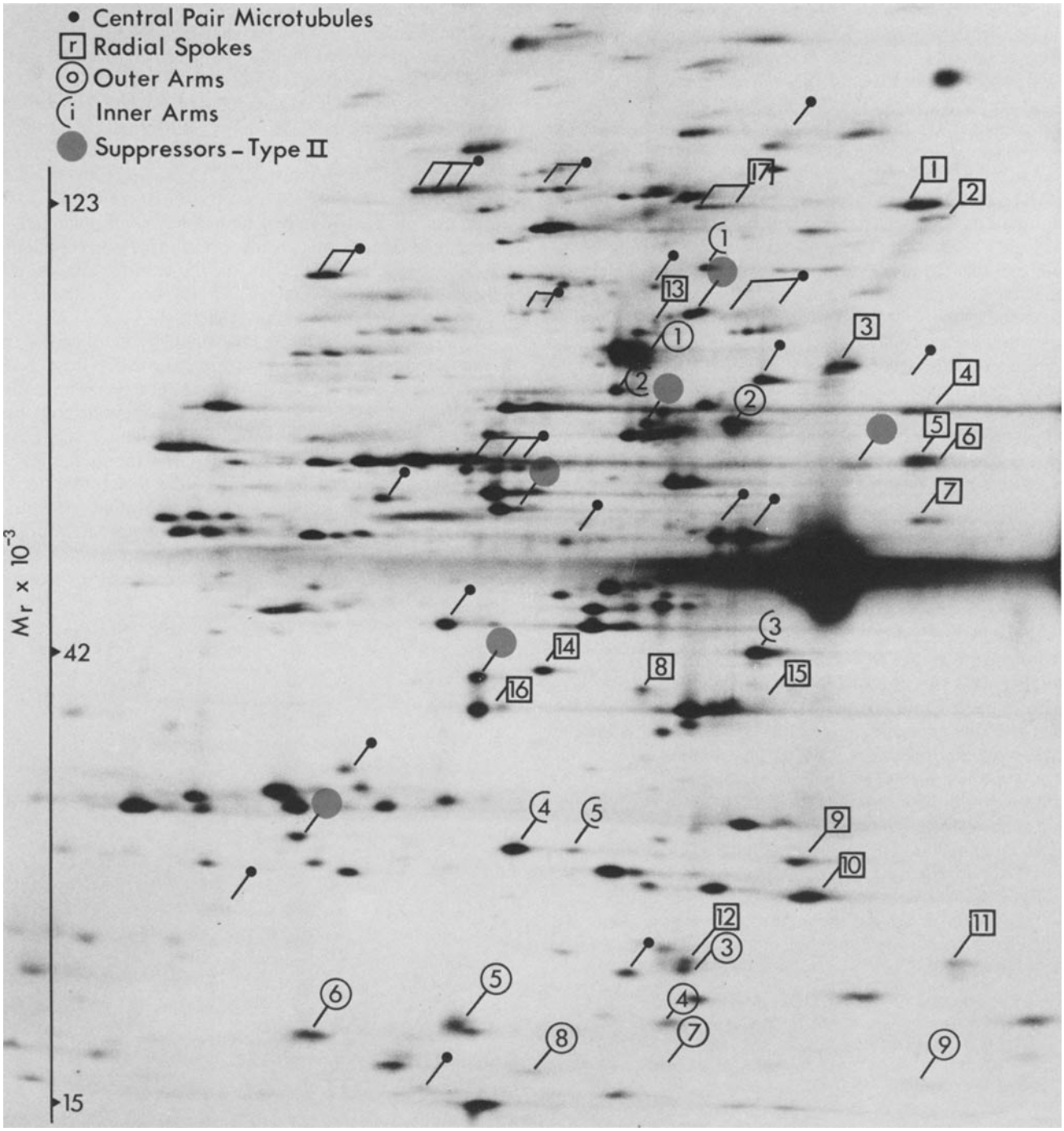

FIGURE 2 An autoradiogram of the polyacrylamide slab gel used for the two-dimensional separation of wild-type axonemal polypeptides. Only a portion of the original map is shown comprising the M_r range 130,000–15,000. Acidic polypeptides are located on the right side of the map. Polypeptides indicated by the appropriate symbol are regularly present in wild-type but are absent or extensively reduced in mutant axonemes deficient for the structure shown in the key. The heavily exposed components at M_r 55,000 shown with associated streaking are α- and β-tubulin subunits overloaded here to reveal the numerous minor axonemal polypeptides. For methods of preparing gels see references 17 and 18.

experiment was carried out, paralyzed $^{35}SO_4$-labeled *pf*14 and unlabeled wild-type gametes mated efficiently and nearly synchronously (12). Within 90 min flagellar beating was observed in all four flagella of the dikaryons, and isolated axonemes analyzed by two-dimensional electrophoresis showed restoration in radioactive form of 16 polypeptides of the 17 originally missing in *pf*14. The 17th polypeptide, component r 3 was never found in a radioactive form and was considered to be the putative gene product.

This conclusion could be verified by application of a second technique, namely the production by mutagenesis of intragenic revertants of *pf*14. The expectation was that we would find among the revertants cases where changes in polar amino acid composition of the gene product had occurred. The altered gene products would be easily identified because of their different behavior in isoelectric focusing. Several revertants of *pf*14B showed easily detectable changes in the position of component r 3 in two-dimensional gels (12, 19). Within

the limits of recombinational analysis carried out by crossing *pf*14B revertants to wild-type, the revertant strains with shifted components r 3 appear to be intragenic. Therefore, data obtained by application of the two different methods are congruent and indicate that for the *pf*14 locus, radial spoke component r 3 is the gene product. The two methods have been used extensively in our studies and have always given congruent results.

The radial spoke has been extensively analyzed making use of mutations representing six independent chromosomal loci (12). Fig. 3 identifies the gene products for these loci and indicates that the products fall into two classes. In some cases defective products result in assembly defects leading to failure to assemble the spokehead or the entire structure; in other cases the structure is assembled in the absence of the mutant component although function is impaired. It is interesting that in both types of mutants the repair process observed in dikaryons occurs on the pre-existing flagella, apparently without a requirement for extensive axonemal reorganization. This is also true for structure-deleting mutants affecting the outer and inner dynein arms and for some mutants affecting the central pair (13, 15). It is likely that each of these cases also reveals the interdependence of components in assembly of an appended structure.

Dynein Arms

As already mentioned, outer and inner dynein arms appear to provide the motive power for axonemes. Gibbons (reviewed in reference 1) first showed that dynein arms could be selectively extracted from axonemal doublets with high salt-containing buffers and he identified a Ca^{++}- or Mg^{++}-activated ATPase of the isolated molecules. The ATPase activity was highly specific for ATP. Dyneins were shown to be large molecules with high sedimentation values, complex in structure, and containing polypeptide subunits with molecular weights >300,000. The ATPase activity of isolated dynein complexes appeared to be the in vitro counterpart of in vivo ATP-driven, arm-dependent, sliding of adjacent doublets.

Discovery of *Chlamydomonas* mutants showing selective deficiency of either the outer dynein arm or the inner dynein

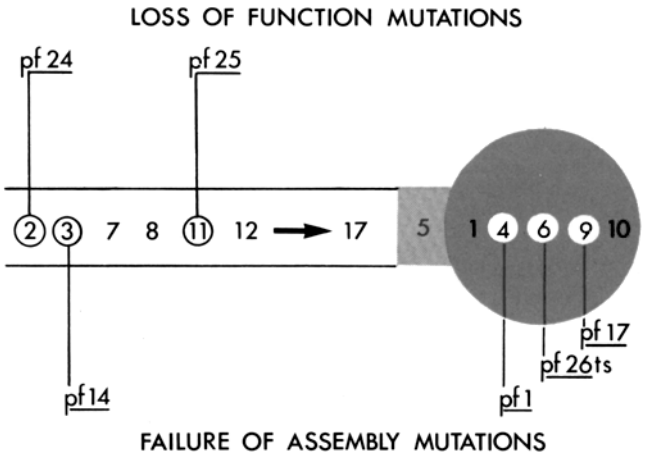

FIGURE 3 A diagram of the putative arrangement of polypeptides in the axonemal radial spoke stalk and the radial spokehead. The spatial assignments are based on analysis of mutants (12) and results of chemical extraction procedures on wild-type axonemes (17). As indicated, mutant gene products have been identified and the consequence of their deficiencies has been shown (12).

arm (13) offered new opportunities to analyze these structures. Two-dimensional methods for analyzing axonemal proteins were supplemented with a one-dimensional electrophoretic procedure that resolved 10 polypeptides in the M_r range 330,000–300,000 as illustrated in Fig. 4. When both methods were applied to the comparison of wild-type axonemes with those from a typical outer arm–deficient mutant *pf*13A and from an inner arm mutant *pf*23, it became clear that each structure was different (13). As illustrated in Figs. 2 and 4, outer arm mutants were deficient in high molecular weight polypeptides I, II, V, and X along with nine polypeptides in the M_r range 86,000–15,000; the inner arm mutant was deficient in polypeptides III, IV, VI, VII, and VIII and in five polypeptides M_r range 110,000–28,000.

The relationship between polypeptides identified by the mutants and polypeptide subunits of dyneins has been made clear by the isolation from wild-type axonemes of four different dynein ATPases (18, 20, 21). The polypeptide compositions of these ATPases are shown in Table I and account for all of the polypeptides missing in the outer arm mutant *pf*13A and for most of those deficient in the inner arm mutant *pf*23. The data suggest that each outer arm is a complex consisting of two dynein ATPases, 12S and 18S. It is less certain how 10–11S dynein and 12.5S dynein are organized within inner arms. An especially interesting finding concerning the inner

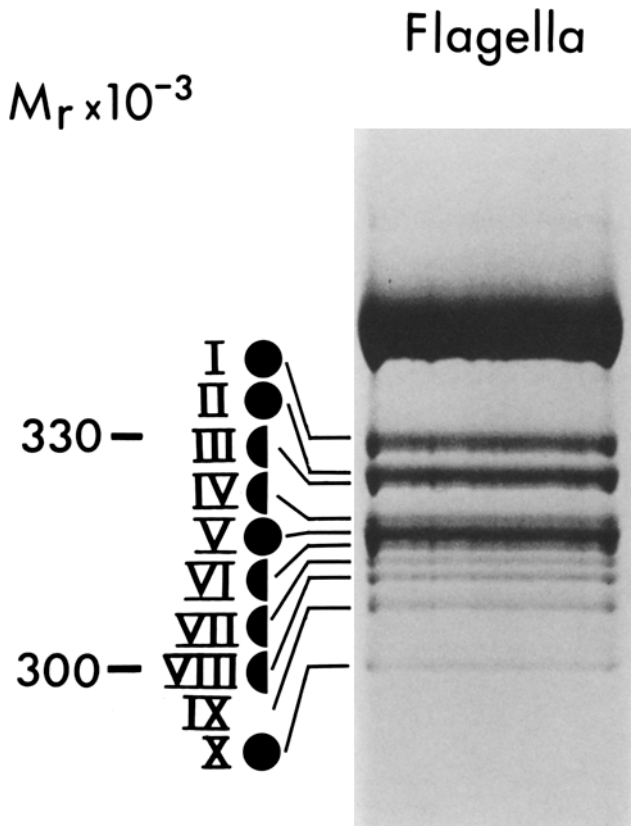

FIGURE 4 Radioautogram of the one-dimensional gel electrophoresis used to analyze axonemal polypeptides of M_r 400,000–290,000. Illustrated here are the polypeptide components of wild-type flagella. Polypeptides absent or extensively reduced in outer arm–deficient mutants are indicated ●, and in an inner arm–deficient mutant ◖. The very dense component shown above polypeptide I is reduced to negligible amounts in axoneme preparations and appears to be a flagellar membrane component. For methods see reference 18.

TABLE I

Comparison of Axonemal Polypeptides Present in Isolated Dynein ATPases with Those Missing in an Outer Arm–defective Mutant (pf13A) and an Inner Arm–defective Mutant (pf23)

	Outer arm defective		Inner arm defective	
Missing polypeptides	I, II, V, X* o 1–9		III, IV, VI, VII, VIII i 1–5	
	Dynein ATPases			
	12S	18S	10–11S	12.5S
Polypeptides	V o 3	I, II, X o 1, 2, 4, 5–9 [10]‡ [11]‡	VI i 3–5	VII i 3–5 [6]§

* Polypeptide components I–X are illustrated in Fig. 4, and components o 1–9 and i 1–5 are illustrated in Fig. 2.

‡ Components [10][11] were detected in 18S dynein ATPase but were not found to be missing from *pf*13A axonemes (18).

§ Component [6] was detected in 12.5S dynein ATPase but not found to be missing from *pf*23 axonemes (20).

arms is that one of their components i 3 closely resembles actin (22).

The complexity of dynein arms and the fact that a single arm consists of different dynein ATPases has now been demonstrated in other organisms. Considerable progress has been made in trying to relate this biochemical complexity to the ultrastructure of the dynein arm (23–26).

Function of Radial Spoke-Central Pair Interaction

It seemed unlikely that analysis of mutants would be helpful in analyzing the function of interactions of radial spokeheads with projections from the central microtubules. Strains defective in these structures were paralyzed, a finding that was unexpected because the appearance, the molecular composition, and apparent function of dynein arms were not different from those of wild type (12, 14, 27). Isolation of a new series of mutants (28) makes it clear that the paralysis of central pair/radial spoke–deficient mutants can be released. Because flagellar function is restored by these secondary mutations without alteration of the original central pair or radial spoke defect, we refer to them as suppressors. They might equally be termed by-pass mutants. The sup_{pf} mutants are independent of the radial spoke or central pair mutants from which they were isolated and several have been mapped to new chromosomal loci. They fall into two groups: group I restores motility to all central pair- and all radial spoke–defective strains, while group II restores motility only to radial spoke–defective strains. It is interesting that several of the sup_{pf} mutants have already been shown to have characteristic defects in axonemal proteins.

$sup_{pf}1$, a group I type suppressor, shows an altered electrophoretic mobility of the high molecular weight polypeptide II (Fig. 4) (28). As already mentioned, this polypeptide is a component of outer arm 18S dynein ATPase. The use of $sup_{pf}3$ and $sup_{pf}4$, type II suppressor mutants, and of motility mutants *pf*2 and *pf*3, later shown also to be type II suppressors, has made possible the identification of a new series of six interrelated polypeptides (Fig. 2). Deficiency of some of these polypeptides is correlated with suppressor activity (28).

Discovery of the sup_{pf} mutants suggests that flagellar paralysis observed in radial spoke- or central pair–defective mutants may represent the operation of some inhibitory mechanism for dynein arm-driven sliding. The origin of the inhibition could arise from altered interactions of the radial spokeheads with projections from the central pair. Transmission of the inhibitory stimulus might involve polypeptides of the sort identified by the Type II, radial spoke specific, sup_{pf} mutants. The terminus of the inhibitory mechanism could be the dynein arms themselves, as is suggested by the molecular defect in $sup_{pf}1$. The observation that in radial spoke- or central pair–defective mutants the inhibition of dynein arms is complete probably reflects the fact that in these mutants every central pair or every radial spoke structure is altered. It can be imagined that in the counterpart of this mechanism in wild-type flagella, localized alterations in the interaction of radial spokes with central pair projections may result in localized inhibition of dynein arms.

Whatever may be their mechanisms, the sup_{pf} mutants made it possible to analyze flagellar function in mutants lacking central pair or radial spoke structures. The analysis was simplified by using a *Chlamydomonas* mutant, *uni*1, lacking one of the flagellar pair (29). Cells of this mutant rotate with little precession and, in favorable cases, elements of the flagellar beat cycle can be recorded by stroboscopic darkfield microphotography on rapidly moving film. Charles Brokaw has carried out extensive analyses of these cells and devised methods for quantitatively recording their bend patterns and for combining data from groups of cells (e.g., reference 30). Fig. 5 illustrates summary data in the form of computer-constructed forward motion bending patterns for wild-type, for $sup_{pf}1$ and $sup_{pf}3$, and for recombinants of each of the sup_{pf} mutants with a radial spokehead–deficient mutant (*pf*17). While no significant alteration from the wild-type bending pattern is seen for the sup_{pf} mutants by themselves, the recombinants with *pf*17 show a remarkable alteration during the recovery stroke of the mutant bend cycle. Propagation of the principal bend toward the flagellar tip is associated with formation of a striking reverse bend with much greater curvature and bend angle than is seen in wild-type flagella. It is interesting that a very similar bend pattern is seen in recombinants of central pair–deficient mutants with $sup_{pf}1$ (Brokaw and Luck, unpublished data). Therefore it appears that in the absence of radial spoke/central pair–

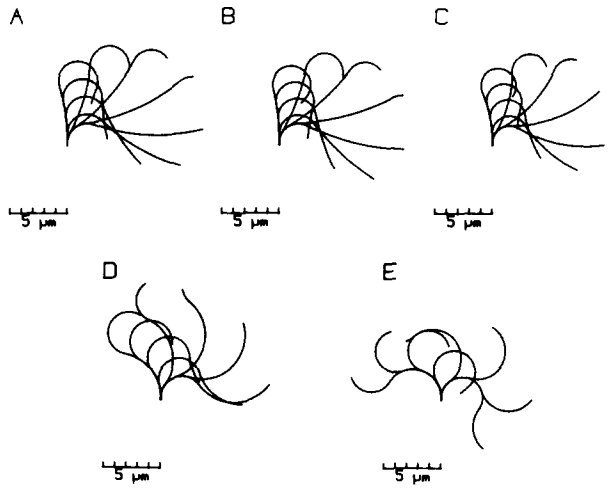

FIGURE 5 Artificial bending patterns constructed from computer-based analysis of recordings of flagellar bending for 9–15 individual cells. (*A*) *uni*1 cells; (*B*) *uni*1, $sup_{pf}3$ recombinant cells; (C) *uni*1, $sup_{pf}1$ recombinant cells; (*D*) *uni*1, $sup_{pf}3$, *pf*17 recombinant cells; (*E*) *uni*1, $sup_{pf}1$, *pf*17 recombinant cells. For analytic methods see reference 30.

projection interactions, a regular bend cycle can be initiated. The bend pattern is simpler than in wild type and is nearly symmetric. The function of spoke-projection interactions appears to be modification of a simple symmetric bend pattern to the highly asymmetric type required for effective motility in *Chlamydomonas*. The modification may be accomplished by inhibition of reverse bends.

Concluding Remarks

Analysis of axonemes using combined biochemical and genetic methods has revealed some of the enormous complexity of assembly and function of this organelle. Many of the 250 or more different axonemal polypeptides have been accounted for as components of complex appended structures or as regulatory components. Yet the summary presented here far underestimates even our present knowledge of the complexity of this system. We have not considered components, likely to be matrix components, present in flagella but not in axonemes. The view of axonemal structure discussed in relationship to Fig. 1 does not take into account structural variations among individual outer doublets and variation of structures along the length of flagella (31). The potential complexity of axonemal polypeptides is increased because many of them are phosphorylated proteins (17). Evidence from mutants already suggests that cycles of phosphorylation dephosphorylation may be important for the function of radial spokes (12) or for the Ca^{++}-dependent conversion of the asymmetric ciliary-type bending that characterizes forward movement of cells to the symmetric flagellar-type bending of reverse swimming (Segal, R. A., B. Huang, Z. Ramanis, and D. J. L. Luck, manuscript submitted for publication). The features of flagellar function that remain to be analyzed are legion, yet it is clear that genetical methods and a collection of well characterized mutants will be powerful assets for future discovery.

I am grateful to Bessie Huang for providing me with Fig. 1. Fig. 5 is reprinted from Brokaw, Luck, and Huang (30) with the permission of the publisher. My collaborators have been G. M. W. Adams (Louisiana State University), Susan K. Dutcher (University of Colorado, Boulder), Bessie Huang (Baylor College of Medicine), Gianni Piperno, Zenta Ramanis, and Rosalind Segal, whose first rate contributions are made clear in the reference citations.

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