Mutations at Twelve Independent Loci Result in Absence of Outer Dynein Arms in *Chylamydomonas reinhardtii*

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Abstract. 35 strains of *Chlamydomonas* mutant missing the entire outer dynein arm were isolated by screening slow-swimming phenotypes. They comprised 10 independent genetic loci (*oda*1-10) including those of previously isolated mutants *oda*38 and *pf*28. The 10 loci were distinct from *pf*13 and *pf*22, loci for nonmotile mutants missing the outer arm. These results indicate that at least 12 genes are responsible for the assembly of the outer dynein arms. There were no mutants lacking partial structures of the outer arm, suggesting that lack of a single component results in failure of assembly of entire outer arms. Temporary dikaryons derived from mating of two different *oda* strains often, but not always, recovered the wild-type motility within 2 h of mating. Hence, outer arms can be transported and attached to the outer doublets independently of flagellar growth.

CILIA and flagella of eukaryotic cells beat through regulated sliding of the nine outer-doublet microtubules. Essential to this movement are the two types of dynein arms, inner and outer, which are attached at regular intervals to the doublet tubules and produce force by interacting with adjacent tubules in the presence of $MgATP^{2-}$ (see Gibbons, 1981). Both the inner and outer arms are complex assemblies of ~10 proteins including several heavy chains with ATPase activities (Piperno and Luck, 1979, 1981; Huang et al., 1979; Tang et al., 1982; Bell and Gibbons, 1982; Pfister and Witman, 1984). Recent studies have shown that the two types of arms are completely different in protein composition (Huang et al., 1979; Piperno and Luck, 1981) and in the spacing at which they are arranged on the doublet microtubule (Huang et al., 1979; Goodenough and Heuser, 1985).

Our present understanding of dynein arm structure has benefited greatly from the discovery of *Chlamydomonas* mutants devoid of the inner or outer dynein arm; Huang et al. (1979) found mutants at two different loci (*pf*13 and *pf*22) that resulted in failure of assembly of outer arms. One mutant, *pf*23, lacked inner arms. Analysis of axonemal proteins deficient in these mutant strains provided information about the composition of outer and inner dynein arms (Huang et al., 1979). The polypeptide composition of dyneins deduced from these analyses has been confirmed by studies of dynein ATPase subunits extracted from wild-type axonemes and purified by a variety of techniques (Piperno and Luck, 1979, 1981; Pfister et al., 1982; Goodenough et al., 1987; Piperno, 1988). These data indicate that each outer dynein arm is comprised of three heavy chains, α, β, and γ, of mol wt >400,000 (King and Witman, 1987) and at least nine polypeptides ranging from mol wt 86,000 to 15,000.

Outer and inner arm mutants have been found among nonmotile strains and identified by their characteristic short flagella. Because axonemes of sea urchin sperm depleted of outer arms by extraction retained motility (Gibbons and Gibbons, 1973), the finding of total paralysis in these mutants was unexpected. Recently, Kamiya and Okamoto (1985) and Mitchell and Rosenbaum (1985) isolated mutants that lacked the outer arm but retained motility. These strains had a reduced flagellar beat frequency and swam more slowly; their motility has been analyzed in detail (Brokaw and Kamiya, 1987).

In this paper, I report the isolation and characterization of additional mutant strains lacking the entire outer arm but retaining motility. The 35 isolates were classified into 10 complementation groups, which were studied by recombination analyses in crosses between isolates and to *pf*13a and *pf*22. 10 independent loci were defined. Axonemes of representatives of the new mutant loci were found to be grossly deficient in three high mol wt proteins that are missing in *pf*13a.

Materials and Methods

Mutant Strains

The following mutants of *Chlamydomonas reinhardtii* used in this study have been described previously: *pf*13a and *pf*22 (Huang et al., 1979), *oda*1 (*oda*38; Kamiya and Okamoto, 1985), and *pf*28 (Mitchell and Rosenbaum, 1985). *pf*13a, *pf*22, and strains used for genetic analyses (*pf*3 and *pf*27) were gifts from Dr. David Luck (Rockefeller University, New York) and *pf*28 from Dr. David Mitchell (State University of New York, Syracuse, NY). Other mutants were isolated based on the procedure developed by Lewin (1954) as described elsewhere (Kamiya and Okamoto, 1985). Briefly, a wild-type 137c strain (*wt*) (mating type + or −) was mutagenized by UV light and mutants with impaired motility were concentrated by collecting cells that tended to grow at the bottoms of the test tubes. These cells were

streaked on 1.5% agar plates and single colonies were transferred to 96-well culture plates, each well containing 0.2 ml of liquid medium. Early in this study, I selected flattened colonies similar to those of wild-type cells (Kamiya and Okamoto, 1985). Recently, however, I have been collecting as many types of colonies as possible (ranging from big, flattened ones to small, heaped ones) because the absence of outer dynein arms was not always correlated with the cell growth pattern on agar plates. In a typical experiment, 48 colonies were saved from an initial test tube culture and their motility was observed with an inverted microscope. Clones that were judged to be slow-swimming mutants were further analyzed by SDS-PAGE for the composition of the high mol wt peptides (HMWs)[1] in their axonemes. When more than one clone from an initial test tube shared a common phenotype, only one clone was saved. Medium I of Sager and Granick (1953) was used throughout these steps.

Genetic Analysis

Mutants that were missing the HMWs of outer dynein arm were crossed with the wild type to obtain strains of both mating types. Complementation in temporary dikaryons between different strains was tested according to Starling and Randall (1971). Changes in flagellar beat frequency in temporary dikaryons were detected by analyzing the frequency of bodily vibration of zygotes with a fast Fourier transform (FFT) analyzer (Kamiya and Hasegawa, 1987). If dikaryons between two different strains displayed a clear increase in flagellar beat frequency after 2 h of mating, the two strains were judged to be nonallelic. If a given strain displayed the frequency increase with all of the representative strains of known complementation groups, this strain was regarded as a new allele and crossed with other strains for genetic analysis. Their segregation patterns were examined in dissected tetrads using standard techniques of tetrad analysis (Levine and Ebersold, 1960); for scoring mutants, daughter colonies on agar plates were transferred to 96-well plates, cultured for 1 or 2 d, and observed with an inverted microscope. It was easy to distinguish an outer arm mutant from a wild type, except when the cell density was exceedingly high. If temporary dikaryons between a pair of mutants did not appear to increase the beat frequency, their daughter cells from ~10 zygotes were cultured together in a well. The two strains were judged to be allelic if four to six wells of such cultures (i.e., cultures of >40 zygotes) did not contain a wild-type cell.

Examination of Axonemes

Flagella of mutants were isolated and demembranated by the method of Witman et al. (1978). SDS-PAGE was carried out as described by Pfister et al. (1982), using 3-5% acrylamide gradient and 3-8 M urea gradient. Gels were stained with silver (Merril et al., 1981).

Axonemes of exemplar strains of each complementation group were examined by thin-section electron microscopy. Specimens were fixed with 2% glutaraldehyde in the presence of 1% tannic acid, postfixed with 1% OsO4, dehydrated through a series of ethanol solutions, and embedded in Epon 812. Sections were double stained with uranyl acetate and lead citrate and observed with a JEM100C microscope (JEOL Co., Tokyo). The frequency of occurrence of outer arm-like structures was examined for >30 cross sections for each strain; when an outer doublet microtubule bore a structure which appeared much fainter or smaller than the normal outer arm, it was counted as 0.5 equivalent of a complete outer arm.

Swimming Velocity Measurement

Swimming velocity was measured with a dark-field microscope at 100× equipped with a TV camera. The TV images were superimposed on the monitor screen of a personal computer, which analyzed the position of cells as a function of time. Images of >50 cells were used to measure the average velocity of a given sample.

Results

Mutant Isolation and Genetic Analysis

35 motile strains that lacked the outer dynein arms were independently isolated from a total of ~4,000 colonies. The

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1. *Abbreviations used in this paper*: FFT, fast Fourier transformation; HMWs, high molecular weight peptides.

Table I. Classification of oda Mutants

Group	Strains
oda1	38‡*, 64, 99
oda2	41§*, 96, 133, 152, (pf28‖)
oda3	71, 73*, 104
oda4	7, 61, 62, 63, 75*, 76, 77, 78, 88, 122, 126, 129, 146, 149, 156, 207
oda5	94*
oda6	67, 95*
oda7	21*
oda8	142*, 153, 237
oda9	141*
oda10	225*

* Used in this study as the exemplar strain of each group.
‡ Kamiya and Okamoto, 1985.
§ Brokaw and Kamiya, 1987.
‖ Mitchell and Rosenbaum, 1985.

desired mutants were first selected by their slow swimming speed. Of ~100 mutants selected, the 35 described here were shown to lack outer dynein arms by SDS-PAGE analysis of axonemal proteins and 13 strains were shown to lack them by electron microscopy. Among the rest of the slow swimmers were mutants lacking a subset of inner dynein arms and mutants with decreased numbers of outer arms; mutants with partially assembled outer arms were not found.

The mutants missing outer arms were all found to be single-site mutations as judged by the 1:1 segregation pattern in crosses with the wild type. A qualitative complementation test in quadriflagellate zygotes and pairwise crosses, as described in Materials and Methods, defined 10 complementation groups; they were named oda1-10 after outer dynein arm (Table I). The mutant strains are identified by their isolation numbers. pf28 (Mitchell and Rosenbaum, 1985) belonged to oda2, as described by Brokaw and Kamiya (1987).

Fig. 1 A is an SDS-PAGE pattern of axonemes of exemplar strains of the 10 different groups, showing that all the strains grossly lacked three HMWs present in wild-type axonemes. Patterns for the 10 strains were essentially identical except for variation in the bands of contaminating membrane proteins. The same pattern was observed with axonemes of a nonmotile mutant pf13a which is known to lack outer dynein arms (Huang et al., 1979; unpublished observation). In
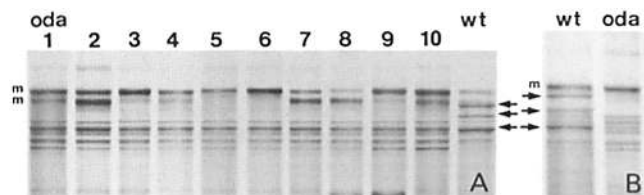

Figure 1. Portions of 3–5% SDS–urea–PAGE patterns showing the high mol wt bands of *oda* and wild-type axonemes. (*A*) Patterns of *oda*1–10 along with wild-type (*wt*) axonemes. (*B*) An example that shows exceptionally good separation. Wild-type and *oda*4 axonemes. *Arrows*, the three high mol wt chains of the outer arm. *m*, bands with contaminating membrane proteins. All were stained with silver.
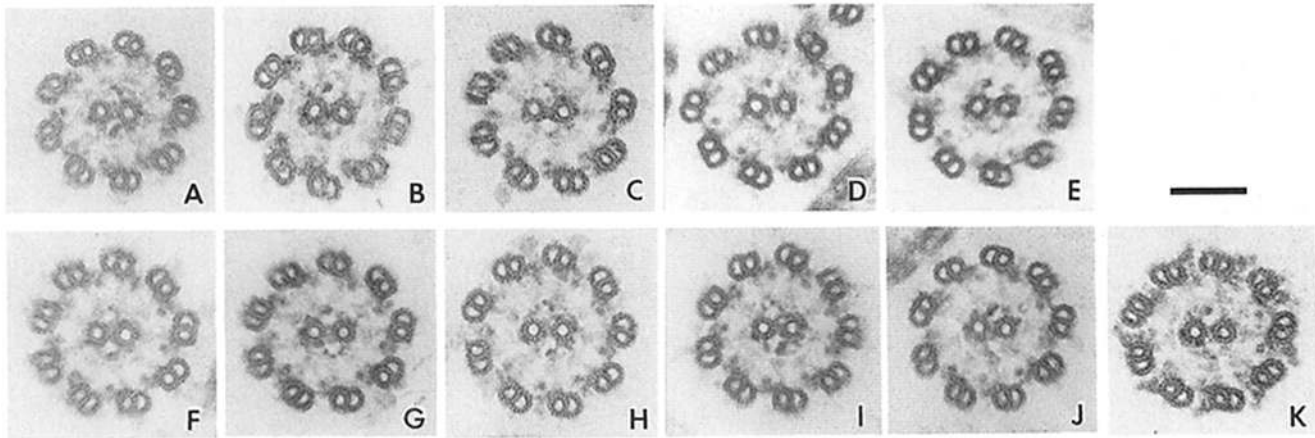
Figure 2. Cross sections of axonemes of exemplar strains. *A–J, oda*1–10, respectively; *K*, wild-type. Bar, 0.1 μm.

SDS-PAGE that showed very good separation, *oda* axonemes appeared to have six HMW bands (Fig. 1 *B*), although it is not known whether or not all of them were inner arm subunits. In addition to those bands, two faint bands often appeared at positions slightly below outer arm α-HMW, in both wild-type and *oda* axonemes. The origin of these bands is not known. Cross-section micrographs of the mutant axonemes demonstrated that all strains lacked outer dynein arms almost completely (Fig. 2); in a survey of 30–50 images of axonemes for each group, only 0.5–5.0% of the outer doublet microtubules were observed to have any structures attached to the outer arm site. Motility characteristics of these mutants were indistinguishable from those of previously described mutants *oda*38 (Kamiya and Okamoto, 1985; renamed *oda*1 in the present study) and *pf*28 (Mitchell and Rosenbaum, 1985); all showed reduced beat frequency and swimming velocity (27–30 Hz and 50–60 μm/s at 25°C, compared with 58–62 Hz and 150–170 μm/s of wild type) and had difficulty in swimming backwards.

To assess linkage, pairwise crosses of the exemplar strains of Table I were made. As shown in Table II, recombination analysis indicated that these mutants were derived from distinct genetic loci, all different from *pf*13 and *pf*22. Linkages exist between *oda*6 and *oda*9, between *oda*7 and *oda*8, and

between *oda*7 and *pf*22. In crosses with other flagellar mutants, *oda*6 and *oda*9 were found linked with *pf*27 (Huang et al., 1981); parental ditype/nonparental ditype/tetratype equals 8:1:20 for *oda*6 × *pf*27 and 26:0:9 for *oda*9 × *pf*27. These results indicate that *oda*7 and *oda*8 are on linkage group I, ∼39 and 54 cM from *pf*22; and *oda*6 and *oda*9 are on linkage group XII, ∼36 and 13 cM from *pf*27, respectively (Harris et al., 1987).

Temporary Dikaryon Rescue

When gametes of opposite mating types are mated, they form quadriflagellated zygotes which swim for 2–3 h before the flagella are resorbed. As in the case of other motility mutants (Luck et al., 1977), zygotes between many, but not all, combinations of different *oda* mutants recovered a higher level of motility during this period as a result of complementation. In most cases, enhanced motility in heterozygotes could be readily discerned by simply comparing their swimming velocity with the velocity of homozygotes.

Complementation in temporary dikaryons can be assessed semiquantitatively by analyzing the flagellar beat frequency of zygotes after 1.5–2 h of mating. The beat frequency was measured by analyzing the vibration of the cell body with an

Table II. Tetrad Analysis Between Different oda Strains

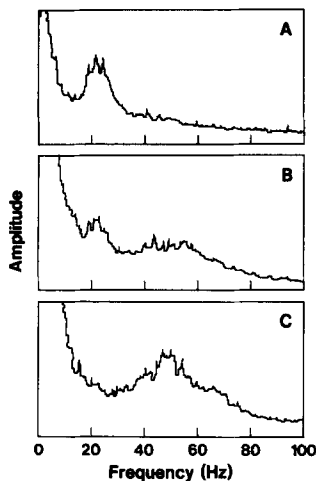|	*oda*1	*oda*2	*oda*3	*oda*4	*oda*5	*oda*6	*oda*7	*oda*8	*oda*9	*oda*10
*oda*1										
*oda*2	6:9:0*									
*oda*3	0:2:18	2:2:15								
*oda*4	2:2:16	0:3:11	3:3:7							
*oda*5	7:7:0	7:4:5	1:3:7	3:1:22						
*oda*6	3:4:12	3:2:16	1:0:9	3:4:18	2:2:9					
*oda*7	3:1:8	3:4:5	1:3:8	4:2:6	3:1:5	3:1:8				
*oda*8	2:2:19	3:3:14	5:5:8	4:4:12	3:2:8	2:2:11	51:0:22			
*oda*9	5:5:2	6:5:0	1:0:9	0:3:9	2:8:3	34:0:30	1:2:12	3:3:18		
*oda*10	4:4:14	6:3:7	4:3:15	0:3:7	1:6:4	1:1:9	2:1:9	2:4:11	1:2:7	
*pf*13a	2:5:7	2:5:11	1:1:7	1:1:15	3:6:9	0:2:13	2:2:13	0:2:9	4:2:8	1:3:7
*pf*22	6:6:0	6:4:5	3:1:11	3:4:12	9:5:2	1:6:8	18:0:65	8:2:48	5:6:7	4:2:5

* Numbers of parental ditype/nonparental ditype/tetratype.

Figure 3. FFT spectra from microscopic images of swimming zygotes. (A) oda1⁺ × oda1⁻ homozygotes; (B) oda1⁺ × oda2⁻ heterozygotes; (C) wt⁺ × wt⁻ homozygotes. Each spectrum was taken 2 h after the onset of mating.

whereas those involving oda2 tended to show poor recovery. However, pairs such as oda1 × oda3 and oda5 × oda8 were not rescued clearly, even though zygotes involving each of these strains usually underwent good frequency recovery.

Freshly prepared gametic cells of pf13a and pf22 were completely nonmotile. After gametes of the two strains were mixed and left for 2–2.5 h, some zygotes were observed to swim at low speed. The fraction of motile zygotes was <1/10 of the total zygotes, indicating a low efficiency of rescue in zygotes between these strains.

Discussion

35 strains of mutants lacking entire outer dynein arms were isolated and found to be derived from 10 different genetic loci, distinct from pf13 and pf22 (Huang et al., 1979). These results indicate that at least 12 genes are responsible for the occurrence of the outer dynein arm.

This and other studies have provided clues to the genetic loci for all but two oda mutations. In addition to the evidence for linkage between oda7, oda8, and pf22, and between oda6, oda9, and pf27, I have obtained data indicating oda10 is closely linked with pf3 on linkage group VIII (Harris et al., 1987; parental ditype/nonparental ditype/tetratype = 132:0:5). Furthermore, Mitchell has found pf28 (oda2) is linked with ery1 on the linkage group XI (pf28 × ery1 = 13:0:14) (Dr. D. Mitchell, personal communication), and Luck and Piperno (1988) have found oda4 is located at sup_pf1, which is on linkage group IX (see below). Finally, Ramanis has found oda5 maps to linkage group VII, 9 cM from pf17 (oda5 × pf17 = 49:0:11) and ∼18 cM from the centromere (oda5 × ac17 = 15:12:15) (Dr. Z. Ramanis, personal communication). More precise mapping of all oda mutations is in progress.

The 10 oda strains can swim at low speed, whereas pf13(a) and pf22(a) are nonmotile (Huang et al., 1979). The fact that no oda strains are allelic to these nonmotile strains indicate that oda and pf13/pf22 may constitute two intrinsically different classes of mutants missing outer arms. Brokaw and Kamiya (1987) have reported that gametic cells of pf13a obtained from old agar-plate cultures display flagellar beating, although they are nonmotile when prepared from fresh cultures. Flagellar beating in old pf13a gametes is similar to,

FFT analyzer, since the cell body vibrates at the frequency of flagellar beating (Kamiya and Hasegawa, 1987). Fig. 3 shows an example of such analysis, in which the power spectrum from oda1 × oda2 zygotes was compared with the spectra from oda1 × oda1 and wt × wt homozygotes. The spectra from the homozygotes showed a single peak at ∼22 Hz (oda1 × oda1) and 49 Hz (wt × wt). The spectrum from oda2 × oda2 homozygotes was essentially identical with that of oda1 × oda1 (not shown). However, the spectrum from oda1 × oda2 had two peaks: one at 22 Hz and another broad one at 40–60 Hz. The former peak was interpreted as representing the frequency of cells that did not undergo complementation (possibly because of failure of mating), and the latter peak as the beat frequency of zygotes that underwent complementation.

Such assessment of complementation was carried out for zygotes between examples from all of the 10 complementation groups and two nonmotile strains. Table III lists the median frequency of the peak of zygotes as read in FFT patterns similar to the one shown in Fig. 3, showing that different combinations of odas gained different degrees of higher beat frequency after 2 h of mating. Zygotes involving oda1, oda3, oda5, and oda8 tended to undergo clear frequency recovery,

Table III. Beat Frequency in Temporary Dikaryons Between Different oda Strains (Hz)*

	oda1	oda2	oda3	oda4	oda5	oda6	oda7	oda8	oda9	oda10	pf13a
oda1	24										
oda2	45	23									
oda3	28	39	23								
oda4	45	31	40	23							
oda5	40	41	40	47	24						
oda6	41	28	40	35	47	24					
oda7	43	20	40	29	42	35	25				
oda8	40	44	44	46	33	43	42	24			
oda9	50	27	49	44	42	26	37	38	24		
oda10	38	42	39	39	24	39	45	26	46	24	
pf13a	38	25	39	26	41	30	29	42	33	36	—
pf22	42	26	42	24	40	26	34	41	36	32	19

* Median values of the peak in FFT spectra from temporary dikaryons between the 12 strains listed; when two peaks appeared as in Fig. 3 B, the higher one was regarded as representing the frequency of zygotes. The beat frequency in wild-type homozygotes was ∼50 Hz. Temperature, 20°C.

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but somewhat poorer than, that in *oda* mutants. It is possible that *pf*13/*pf*22 mutations cause a second defect in addition to lack of the outer arm. However, no apparent difference between the motile and nonmotile strains has been found in one-dimensional and two-dimensional SDS-PAGE analyses of flagellar proteins (Luck and Piperno, 1988).

It is remarkable that no mutants having partial structures of outer dynein arms have been found, although mutants lacking a subset of inner dynein arms and mutants with incomplete numbers of outer arms have been isolated in addition to *oda*s. This situation with outer arm mutants is different from that of radial-spoke mutants, in which case several types of strains with different degrees of deficiency exist (Luck et al., 1977; Huang et al., 1981). One possible explanation for the absence of partial assembly mutants is that the screening of slow-swimming mutants precluded them; if partial assembly mutants tended to grow at the upper part of test tube cultures, swam at high speed, or had paralyzed flagella, they may well have escaped the screening. However, it is difficult to believe that all possible partial assembly mutants are of such phenotypes.

A more plausible explanation is that some or all of the genetic loci identified in this study code for subunits of the outer dynein arms, and that lack of a single subunit results in failure of assembly or transportation of the entire outer arm. Support for the idea that some of the *oda* mutants are deficient in structural genes for dynein subunits was obtained by Luck and Piperno (1988); they found that *oda*4 is allelic to $sup_{pf}1$, a mutant that has a aberrant outer arm β-HMW with reduced mobility probably due to mutation in the structural gene for this protein (Huang et al., 1982). It is interesting to note that there are ~12 subunits of the outer dynein arm (Luck and Piperno, 1988), which is the same as the number of the genetic loci of outer arm mutants identified in the present study. Although this agreement may be a mere coincidence, there is a possibility that *oda*1–10, *pf*13, and *pf*22 mutations cover a large part of the outer arm structural genes. However, it is also possible that some mutants may have defects in synthesis, processing, or transportation of subunits.

The flagellar beat frequency in temporary dikaryons between most, but not all, combinations of different *oda* gametes increased to a level close to that of the wild type within 2 h of mating. This indicates that the outer dynein arm can be transported and attached to the correct site on the outer doublet microtubules independently of the flagellar growth event.

Why dikaryons of some pairs did not recover a higher level of motility, while those of other pairs readily did so, is not known. One possibility is that some mutants produce aberrant outer arm components, which interfere with assembly of a functional outer arm even when a complete set of components is present in zygotes. Strains that generally display poor rescue ability (e.g., *oda*2) may produce such aberrant components. However, it is difficult to envisage what is happening in zygotes such as *oda*1 × *oda*3, which did not display a clear rescue although each of these strains rescued other combinations of zygotes. In these cases, the reason for the rescue failure can be understood only when the gene products of these loci are known; these complementation data can then provide a basis for clarifying the assembly pathway of the outer dynein arm.

The 10 groups of *oda* mutants will be useful for future studies on the assembly, structure, and function of inner and outer dynein arms, as has been found with *pf*13(a), *pf*22(a), and *pf*28 in biochemical and physiological studies (Huang et al., 1979; Mitchell and Rosenbaum, 1985; Goodenough et al., 1987; Piperno, 1988; Brokaw and Kamiya, 1987). For example, isolation of intragenic revertants from these strains may give rise to strains with variant outer dynein arms and thereby enable us to infer the function of various dynein subunits. Recently, gene clones for the α and β chains of outer arm dynein were obtained (Williams et al., 1986; Mitchell, D. R., and B. D. Williams, unpublished observations). These studies indicate that the structural and functional properties of dynein subunits can be studied with the power of genetic engineering in the near future. With the development of the transformation technique with *Chlamydomonas*, the availability of the 12 kinds of outer arm mutants will become more important for studies along these lines.

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