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Genetic Analysis of Long-Flagella Mutants of *Chlamydomonas reinhardtii*

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ABSTRACT

The length of the flagella of *Chlamydomonas reinhardtii* cells is tightly regulated; both short-flagella and long-flagella mutants have been described. This report characterizes ten long-flagella mutants, including five newly isolated mutants, to determine the number of different loci conferring this phenotype, and to study interactions of mutants at different loci. The mutants, each of which was recessive in heterozygous diploids with wild type, fall into three unlinked complementation groups. One of these defines a new gene, *lf3*, which maps near the centromere of linkage group I. The flagellar length distributions in populations of each mutant were broad, with the longest flagella measuring four times the length of the longest flagella seen on wild-type cells. Each of the ten mutants had defective flagellar regrowth after amputation. Some of the mutants showed no regrowth within the time required for wild-type cells to regenerate flagella completely. Other mutants had subpopulations with rapid regeneration kinetics, and subpopulations with no observable regeneration. The mutants were each crossed to wild type to form temporary quadriflagellate, dikaryon cells; in each case the long flagella were rapidly shortened in the presence of the wild-type cytoplasm, demonstrating that the mutants were recessive, and that length control could be exerted on already assembled flagella.

A number of lines of evidence indicate that the length of the flagella in the unicellular green alga *Chlamydomonas reinhardtii* is under active control. In a population every cell has two flagella of equal length, and the length of the flagella never exceeds a maximum length of 12 μm (for haploid cells of strain NO). The cell maintains a pool of assembly-competent flagellar protein, as detected by deflagellating cells in the absence of protein synthesis, and observing the regrowth of new flagella which are approximately one-half of the pre-deflagellation length (Rosenbaum, Moulder and Ringo 1969). Thus some partitioning mechanism maintains a certain length of assembled flagella, and accumulates the remainder of the assembly-competent protein into an intracellular pool. The equality of flagellar length between the two flagella on a single cell is also regulated. For example, if one of the two flagella is amputated, the remaining flagellum rapidly shortens, in some cases all the way into the cell body. The two flagella then grow out together to pre-deflagellation length (Rosenbaum, Moulder and Ringo 1969; Coyne and Rosenbaum 1970). Somehow the cell determines that the two flagella are not of equal length, and then repairs this defect. Flagellar length control and homeostasis clearly involve fundamental processes of intracellular signalling and macromolecular assembly and disassembly.

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Chlamydomonas mutants with altered length control have been described, including short-flagella (*shf*) mutants in three different genes (Jarvik *et al.* 1984; Kuchka and Jarvik 1987), and long-flagella (*lf*) mutants in two different genes (Randall *et al.* 1967; Randall 1969; McVittie, 1972a, b; Jarvik, Lefebvre and Rosenbaum 1976; Jarvik, Reinhart and Adler 1980). In this report we analyze ten different long-flagella mutants, including the five previously described *lf* mutants, and five newly isolated mutants. The new mutants were isolated in a screen for mutants with defective flagellar regeneration (Lefebvre *et al.* 1985). Complementation tests using stable diploids have been used to establish that the 10 *lf* mutants represent 3 different genetic loci—*lf1* (1 allele), *lf2* (5 alleles), and a new locus described in this work, *lf3* (4 alleles).

MATERIALS AND METHODS

Strains: The wild-type strains of *Chlamydomonas reinhardtii* used were the 137c derivatives NO mt^+ (Chlamydomonas Genetics Center number CC-620) and NO mt^- (CC-621); they were obtained from the Chlamydomonas Genetics Center, Duke University, Durham, North Carolina, as were the following strains: CC-48 (*arg-2*, mt^+), CC-51 (*arg-7*, mt^-), CC-1709 (*ery-3*, *pf-22*, mt^-) and the multiply marked strain CC-29 (*ac-17*, *can-1*, *nic-13*, *pf-2*, *y-1*, *pyr-1*, *msr-1*, *act-2*, *sr-1*, mt^-). Nitrate reductase deficient mutants *nit-3* and *nit-4*, used for diploid construction, were provided by Dr. Emilio Fernández, Department of Biochemistry, Córdoba, Spain. Sources of the long-flagella mutants used in this report are listed in Table 1. All *lf* mutants were backcrossed

TABLE 1

Long-flagella mutants used in this study

Mutant	Source
lf1	*Chlamydomonas* Genetics Center Stock No. CC-802[a]
lf2	*Chlamydomonas* Genetics Center Stock No. CC-803[a]
cs89	JON JARVIK[b]
185	JON JARVIK
218	JON JARVIK
BC1	This study
F8	This study
WH8	This study
WH9	This study
WS8	This study

[a] Isolated by A. MCVITTIE.

[b] Department of Biology, Carnegie-Mellon University, Pittsburgh, Pennsylvania.

to the NO strain a minimum of three times before use. An isogenic strain of NO mt^- was prepared by backcrossing NO mt^- to the NO mt^+ strain ten times, with NO mt^+ as the recurrent parent.

Media and culture conditions: Cultures were grown on minimal medium I (SAGER and GRANICK 1953); solid media were prepared by the addition of 1.5 or 2.0 g/liter of washed agar (Difco "Bacto agar" or GIBCO agar). Agar used in preparing plates for tetrad germination was washed overnight at least twice with stirring in deionized water at 4°. Cells were routinely grown at 24° under continuous light (approximately 1000 lux), either in 10-ml cultures in large (25-mm diameter), metal-capped tubes, or in 200–300 μl of media in wells of a 96-well tissue culture dish. Gametogenesis was induced by transferring cells to nitrogen-free medium "M-N" (KATES and JONES 1964) under continuous light at room temperature or 15°.

Mutant isolation and genetic analysis: New long-flagella mutants were identified among a collection of mutants with defects in the regeneration of flagella after deflagellation (LEFEBVRE *et al.* 1985). Cells were mutagenized with methylmethane sulfonate (MMS, Aldrich Chemicals) at a concentration of 0.03 M, essentially as described by HUANG, RIFKIN and LUCK (1977). Survival rates in three experiments averaged 64%, as determined by plating mutagenized cells on agar-containing medium. Tetrad analyses were performed at 24° using standard techniques (LEVINE and EBERSOLD 1960). Map distance was calculated using the formula (1/2 T + 3NPD)/(PD + NPD + T); centromere distance was calculated from crosses to the centromere-linked marker *ac-17* using the equation 1/2 T/(PD + NPD + T) (GOWANS 1965). Diploids were constructed using two different methods: either using complementing arginine auxotrophic mutants *arg-2* and *arg-7* (LOPPES, MATAGNE and STRIJKERT 1972; derived from EBERSOLD 1967) or using the method of FERNANDEZ and MATAGNE (1986), in which diploids were selected using complementing nitrate reductase deficient mutants. Diploids were identified using three criteria—growth on nitrate as sole nitrogen source (or on arginine-free media for arginine selection), minus mating type, and large cell size relative to haploid cells. For each diploid construction, at least four different diploid colonies were grown in liquid culture and analyzed.

Flagellar length measurements: Cells were fixed in an equal volume of 10% glutaraldehyde aqueous solution; flagellar lengths were determined by phase contrast microscopy at ×400 using an ocular micrometer. In experiments using quadriflagellate dikaryon cells, the length of each of the four flagella was determined.

Flagellar amputation: Complete deflagellation (*i.e.*, removal of all flagella from all cells in a population) was achieved by pH shock, using a modified procedure from MCVITTIE (1972b). One-tenth volume of 0.3% acetic acid was added to 9/10 volume of the sample in medium I or M-N and cells were gently agitated. After 3 min, the mixture was diluted tenfold by addition of the appropriate medium, and the cells were pelleted by centrifugation at 2600 rpm for 1 min. The cells were resuspended in 1 ml of fresh medium, pelleted again for 1 min, and again resuspended in 1 ml of fresh medium. The cultures were placed in open 1.5-ml Eppendorf microfuge tubes under continuous illumination at room temperature. Aliquots of 10 μl were removed at intervals for flagellar length measurements. For determination of flagellar pool size, the same method was used, but cells were washed and resuspended in medium containing cycloheximide (10 μg/ml) to inhibit protein synthesis (ROSENBAUM, MOULDER and RINGO 1969).

Amputation of one of the pair of flagella to produce "long-zero" cells was performed by repeated passage of 1–2 ml of a culture through a 27 gauge needle. Comparison of the flagellar length distributions of the original long-flagella populations with the newly generated "long-zero" populations confirmed that the flagella were either intact or removed; flagella broken at intermediate lengths were not observed. Between 10% and 50% of the cells in the population were uniflagellate after this treatment. Individual "long-zero" cells could not be followed in these experiments. Instead, the frequencies of uniflagellate cells and cells with unequal length flagella were determined at various times after treatment. Flagellar length measurements of cells with unequal length flagella allowed us to monitor the resorption of the intact flagellum, and regrowth of the flagellum which had been amputated.

Dikaryon analysis: Dikaryons were obtained by mixing 50–100-μl aliquots of gametes of opposite mating types in 1.5-ml Eppendorf tubes, then gently agitating the mixtures. Mating efficiency was determined for each mixture by examining 10-μl aliquots of glutaraldehyde-fixed cells by phase contrast microscopy to determine the percentage of quadriflagellate dikaryons in the population. Flagellar length measurements in these and other experiments were performed immediately after fixation, to avoid the clumping of cells which occurs in fixed samples.

RESULTS

Isolation of new long-flagella mutants: Each of the five long-flagella mutants which were available at the beginning of this work had been isolated in screens for mutants with motility defects (MCVITTIE 1972b; JARVIK, LEFEBVRE and ROSENBAUM 1976; JARVIK, REINHART and ADLER 1980). Three of these mutants—*lf1*, *lf2* and cs89—had also been shown to regenerate flagella very slowly after deflagellation. As part of an effort to identify mutants with defects in the stimulation of flagellar protein synthesis after deflagellation (LEFEBVRE *et al.* 1985), we isolated more than 30 mutants which regenerated flagella slowly or not at all after amputation. Among these mutants, five were found to have longer flagella than wild-type. These five new mutants, and the five previously

TABLE 2

Pairwise complementation tests between long-flagella mutants

	Complementation group										
	lf1	lf2					lf3				Allele name
	lf1	lf2	BC1	cs89	F8	185	WH8	WH9	218	WS8	
lf1	–	+	+	+	+	+	+	+	+	+	*lf1*
lf2		–	–	–	–	–	+	+	+	+	*lf2-1*
BC1			–	–	–	–	+	+	+	+	*lf2-4*
cs89				–	–	–	+	+	+	+	*lf2-2*
F8					–	–	+	+	ND	+	*lf2-5*
185						–	+	+	+	ND	*lf2-3*
WH8							–	–	–	–	*lf3-1*
WH9								–	–	–	*lf3-2*
218									–	–	*lf3-3*
WS8										ND	*lf3-4*

Diploids were produced between the indicated pairs of mutants, and the flagellar lengths of the diploid cells were measured by phase contrast microscopy. + indicates complementation (diploids had wild-type length flagella). – indicates noncomplementation (diploids had long flagella). ND = not determined.

TABLE 3

***lf3* maps near the centromere of linkage group I**

pf-22 *lf3* C *ery-3* *msr-1*

Cross	Markers scored	Tetrad type: PD	NPD	T	Map distance (cM)
lf3-2 × CC-28	*lf, msr-1*	6	1	28	48.5
lf3-2 × CC-1709	*lf, ery-3*	18	1	23	34.5
lf3-2 × CC-1709	*lf, pf-22*	41	0	10	9.8
Centromere mapping:					
lf3-2 × CC-28	*lf, ac-17*	23	11	1	
Distance *lf3* to centromere:					1.4

msr-1 (methionine sulfoximine resistance), *ery-3* (erythromycin resistance), and *pf-22* (paralyzed flagella) are all on linkage group I. *ac-17* (acetate auxotroph) is tightly linked to the centromere of linkage group III.

isolated *lf* mutants, were characterized genetically and phenotypically as described below.

Dominance and complementation tests in diploids: Stable diploids in Chlamydomonas can be selected after mating cells containing complementing auxotrophic mutations (EBERSOLD 1967; LOPPES, MATAGNE and STRIJKERT 1972). Diploids were constructed between each of the long-flagella mutants and wild type, and the flagellar lengths of the resulting heterozygotes were measured. Each of the ten long-flagella mutants was fully recessive to wild type in diploids. The flagella of the diploids were less than 14 μm in length, the same maximum length found in homozygous wild-type diploids.

Because the mutants were recessive, complementation tests could be used to group the mutants into functionally distinct loci. Diploids were constructed between the different long-flagella mutants in pairwise crosses, and the flagellar lengths of the resulting cells were measured. The results of the complementation tests place the ten mutants into three complementation groups (Table 2). In every case scored as indicating complementation, no flagella longer than 14 μm were observed on the diploid cells. Four new alleles of *lf2* were identified: the previously described but unmapped mutants cs89 and 185, and two new mutants, BC1 and F8. No new alleles of *lf1* were found, so the *lf1* locus still consists of a single allele. Four of the mutants—218, WH8, WH9 and WS8—were placed in a new locus, *lf3*. Allele designations for all of these mutants are given in Table 2.

New long-flagella locus *lf3* maps near the centromere of linkage group I: Mutant WH9 (*lf3-2*) was used to map this locus by crosses to a multiply marked strain CC-29, which carries markers for ten of the Chlamydomonas linkage groups. *lf3-2* was found to be distantly linked to linkage group I marker *msr-1* (Table 3). Subsequent crosses were performed with markers *ery-3* and *pf-22*. The results of these crosses and crosses to the centromere-linked marker *ac-17* indicated that *lf3* maps within 2 cM of the centromere of linkage group I.

In setting up the crosses to *pf-22*, we noticed that the flagella of this paralyzed-flagella strain were short, with every cell in the population having flagella between 3 and 6 μm long (compared to a wild-type length of 12 μm). The short-flagella phenotype cosegregated with the paralyzed-flagella phenotype in each of the 51 tetrads examined in these mapping crosses. Double mutants of *pf-22* and *lf3-2*, present in the ten tetratype tetrads, had short, paralyzed flagella, the same phenotype seen for the flagella of single *pf-22* mutants. Thus *pf-22* is epistatic to the long-flagella mutation *lf3-2*.

Double mutant strains: Double mutants were constructed with various combinations of alleles of *lf1*, *lf2* and *lf3*, to examine possible interactions between the mutations. Initially we observed that such double mutants were flagella-less. Each double mutant, when grown without bubbling in a small tube (10 ml or less) had more than 98% aflagellate cells (Table 4). However, when these same double mutants were grown in cultures with aeration (75-ml cultures bubbled with air), most of the cells in each population were flagellated (Table 4). Surprisingly, a large percentage of the cells in each population had only one flagellum. In the double mutant *lf2-1 lf3-2*, for example, of 102 cells examined, 61 had a single flagellum, whereas only 26 had two flagella. In every case, except for the double mutant *lf1 lf2-4*, more than half of the flagellated cells had only one flagellum.

The unusual phenotype of having only one flagellum has previously been observed for the *uni* mutants,

TABLE 4

Phenotypes of double *lf* mutants

	Without aeration				With aeration			
Double mutant	Total	0 flag	1 flag	2 flag	Total	0 flag	1 flag	2 flag
lf1 lf3-2	122	120	1	1	193	23	89	81
lf1 lf2-4	386	369	6	11	99	33	29	37
lf2-1 lf3-1	358	330	16	12	100	44	45	11
lf2-1 lf3-2	416	385	28	3	102	15	61	26
lf2-4 lf3-1	393	389	1	3	100	69	25	6

For each double mutant, the number of cells with 0 flagella, 1 flagella, or 2 flagella was scored for fixed samples using phase contrast microscopy. Cells without aeration were grown in 10 ml of medium in large (22-mm diameter) culture tubes; cells with aeration were grown in 75 ml of medium in 200-ml flasks, bubbled with air.

which map to a single locus (HUANG *et al.* 1982). These mutants have been shown to have ultrastructural defects in the basal bodies, specifically in the transition zone between the shaft of the flagellum and the basal body. One interesting characteristic of the *uni* mutants is that the single flagellum is found in a defined relationship to an asymmetrically located organelle in the cell body, the eyespot. That is, the eyespot is always found on only one side of the cell, and the single flagellum of *uni* mutant cells is found on the opposite side of the cell from the eyespot (the "*trans*" configuration) in more than 90% of the uniflagellate cells. We examined the position of the eyespot relative to the single flagellum of the five double mutants shown in Table 4, and found no preference for the *cis* or *trans* position of the flagellum relative to the eyespot (data not shown). Thus the defect which results in a single flagellum being formed on cells of *uni* mutants is likely to be different than the defect causing uniflagellate cells to occur in double *lf* mutant populations.

Phenotypic characterization of long-flagella mutants: Each of the ten long-flagella mutants was characterized as described below, to compare the flagellar phenotypes of the mutants within each complementation group, and between representatives of each complementation group.

1. *Flagellar length.* The flagellar length of both gametic and vegetative cells of wild-type (NO) and each of the 10 *lf* mutants was measured, and the results are presented as the histograms in Figure 1. The maximum flagellar length observed in wild type was 12 μm. For each of the mutants but one (vegetative cells of *lf2-2*), most of the cells in each population had flagella longer than 12 μm. All of the *lf* mutants had some cells with flagella longer than 24 μm, and some had flagella as long as 45 μm (almost four times normal length). The different mutants showed different distributions of flagellar lengths, but in each case the distributions were broader than those of wild-type cells. In addition, each of the mutants had longer flagella as gametes (*solid bars*, Figure 1) than as vegetative cells (*hatched bars*, Figure 1). This difference was especially pronounced for mutant *lf2-2*, whose long-flagella phenotype was almost entirely gamete-conditional. In *lf2-2*, most vegetative cells had flagella of wild-type length, but most gametes had long flagella, up to three times normal length.

2. *Flagellar regeneration.* Wild-type Chlamydomonas cells rapidly regrow new flagella after amputation, which can be induced by pH shock or mechanical shearing (ROSENBAUM, MOULDER and RINGO 1969; LEFEBVRE *et al.* 1978). Three previously described mutants, *lf1*, *lf2-1* and *lf2-2*, were shown to regenerate flagella very slowly (RANDALL *et al.* 1967; MCVITTIE 1972b; JARVIK, LEFEBVRE and ROSENBAUM 1976). To determine whether defective flagellar regeneration always accompanies the long-flagella phenotype, we measured the flagellar regeneration of all of the mutants in our collection (Figure 2).

Flagellar regeneration in each of the *lf* mutants differed from wild-type in two ways: (1) regeneration was asynchronous for each of the *lf* mutants; (2) after amputation none of the *lf* mutants recovered the original percentage of flagellate cells present before deflagellation. The different mutants showed a wide range of regeneration kinetics, from nearly wild-type regeneration to almost complete lack of regeneration. For example, mutant *lf1* showed extreme regeneration defects (Figure 2A) (MCVITTIE 1972b). No cells regenerated flagella of measurable length for at least 120 min after deflagellation, by which time wild-type cells had regenerated nearly full-length flagella. By 240 min, only 10% of the *lf1* cells had begun to regrow flagella, and the longest of these were only 10 μm. Even after 28 hr (not shown) the mean flagellar length of an *lf1* population was less than 70% of the pre-deflagellation value. After 28 hr, the percentage of flagellate *lf1* cells ranged from 6 to 49% in different experiments; the percentage of these cells exceeding wild-type length (12 μm) ranged from 33 to 62%.

In contrast, the four mutants in the *lf3* locus regenerated flagella with nearly normal kinetics. In fact, for *lf3-3* (Figure 2A) regeneration occurred in parallel in wild-type and mutant cells, but by 240 min, at which time regeneration in wild-type cells plateaued at 12 μm, some cells in the *lf3-3* population had regenerated flagella as long as 21 μm. Initiation of flagellar regeneration in the *lf3-3* cells was asynchronous, however, such that by the time some of the cells had regenerated flagella longer than wild-type, others had not begun to regenerate.

Different mutant alleles of the *lf2* locus showed substantially different rates of flagellar regeneration (Figure 2B). At one extreme, *lf2-1* regenerated very

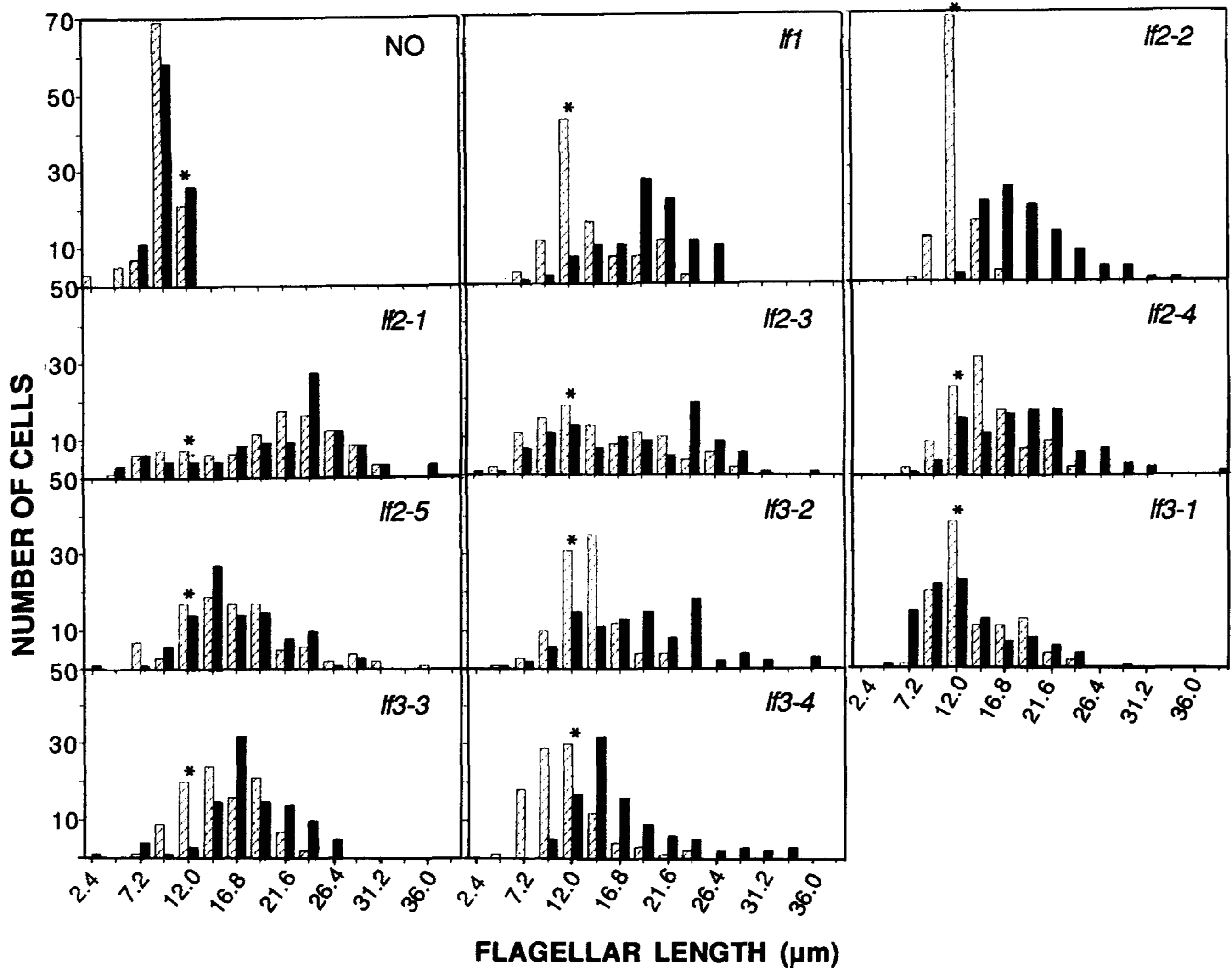

FIGURE 1.—Flagellar lengths of vegetative and gametic cells of wild-type (NO) and ten long-flagella mutants. The lengths of the flagella of 100 vegetative cells (*hatched bars*) and 100 gametic cells (*solid bars*) were determined for glutaraldehyde-fixed samples of each population using phase contrast microscopy; flagellar lengths were scored with an ocular micrometer (at ×400 magnification). Cells without measurable flagellar length were not included in this figure. The maximum flagellar length observed on wild-type cells (12 µm) is marked by an asterisk (*) in each panel.

poorly. Cells exhibited lag periods of at least 240 min; within 480 min after amputation, the maximum percentage of flagellate cells in any population was 15%. Neither vegetative cells nor gametes elongated to pre-deflagellation lengths within 24 hr (Figure 2B) (S.-E. BARSEL, unpublished observations) in agreement with earlier observations (MCVITTIE, 1972b). On the other extreme, individual *lf2-5* gametes (Figure 2B) were able to regenerate flagella as rapidly as wild-type gametes. In fact, by 240 min after deflagellation, more than half of the cells in a population of *lf2-5* gametes had regenerated flagella *longer* than the longest flagella in wild-type cells. The only regeneration defect observed for *lf2-5* was the asynchronous initiation of regeneration. Although some cells regenerated flagella rapidly, others in the population did not begin regeneration until 60 min post-deflagellation. Although the flagellar regeneration characteristics of *lf* mutants differ, excess flagellar length and slow flagellar regeneration were not always correlated.

Pool size of unassembled flagellar proteins in *lf* mutants: Wild-type Chlamydomonas contain a cytoplasmic pool of flagellar precursors sufficient to assemble approximately one-half length flagella after deflagellation in the absence of protein synthesis (ROSENBAUM, MOULDER and RINGO 1969). One possible explanation for at least part of the excess flagellar length attained by *lf* mutants might be that they do not maintain a pool of unassembled flagellar proteins; that is, all available flagellar proteins would be assembled into long flagella. We examined the size of the precursor pools in the *lf* mutants by deflagellating cells and allowing regeneration in the presence of cycloheximide (data not shown). Wild-type cells (strain NO^-) had a pool sufficient to

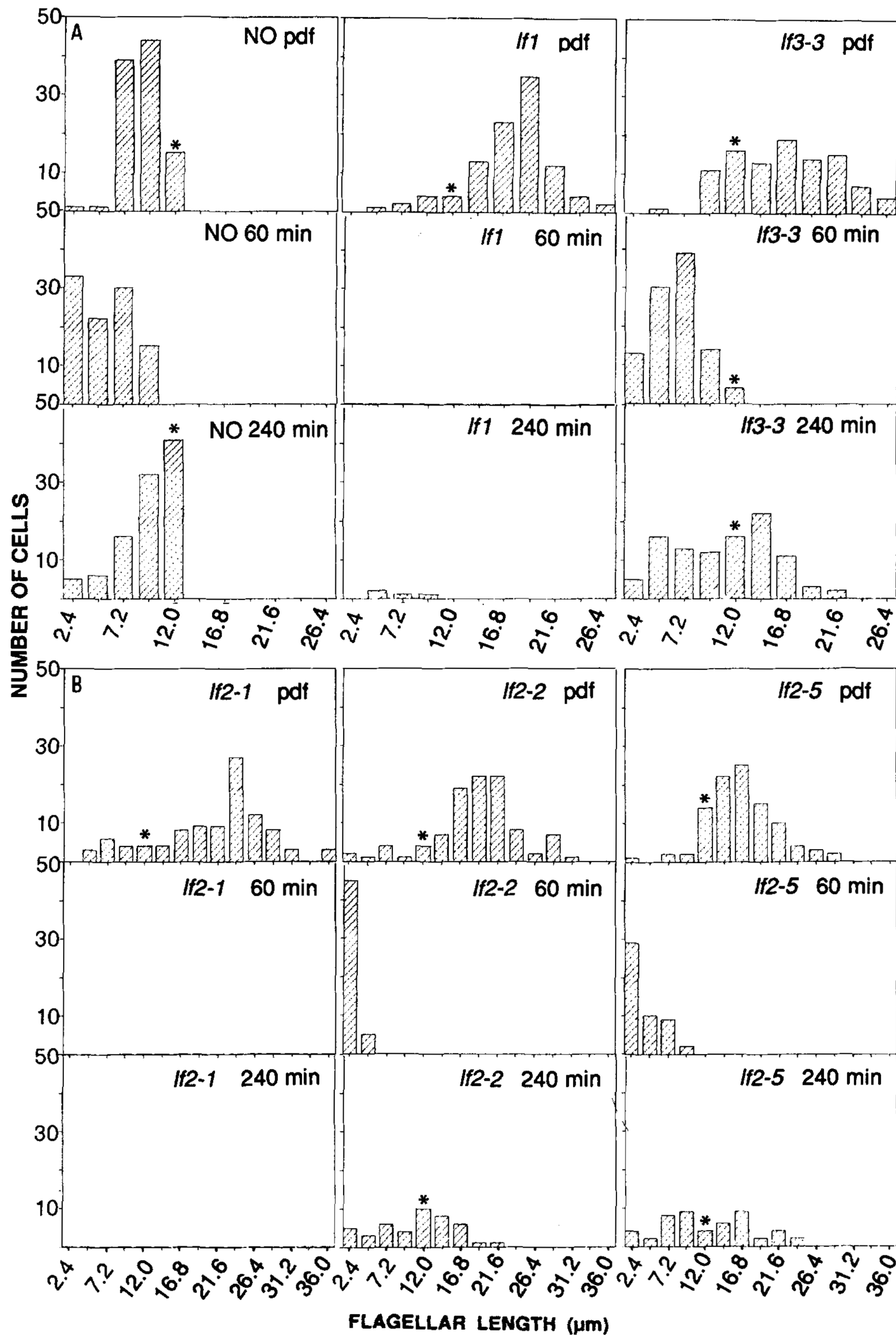

FIGURE 2.—Flagellar regeneration in gametes of wild-type (NO) and five *lf* mutants. A, Flagellar regeneration of NO, *lf1*, and *lf3-3*. The lengths of the flagella of 100 cells were measured at three times: pre-deflagellation ("pdf"), 60 and 240 min after deflagellation. In this figure, cells with no measurable flagellar length were not included in the histograms. In panel "*lf1*-60 min," for example, 100 cells were examined, and none of these had measurable flagella. B, Flagellar regeneration of three mutants at the *lf2* locus: *lf2-1*, *lf2-2* and *lf2-5*. Flagellar lengths were obtained for 100 cells for the "pdf" time points, and for 50 cells for the 60- and 240-min time points. The maximum flagellar length observed on wild-type cells (12 μm) is marked by an asterisk (*) in each panel.

regenerate 2.4–4.8 μm flagella in vegetative cells. Because *lf1* and *lf2-1* regenerated so slowly, it was impossible to determine the pool size by measuring flagellar regeneration in the presence of cycloheximide. The eight *lf* mutants which could regenerate some flagellar length within 120 min each contained measurable pools of unassembled flagellar proteins. The smallest pools observed were sufficient to assemble 2.4 μm flagella (*lf2-5, lf3-2*). The largest pools were sufficient to assemble 2.4–4.8 μm flagella, with occasional flagella as long as 9.6 μm being observed (*lf2-4, lf3-3*). Thus the *lf* mutants, like wild type, maintained unassembled flagellar protein precursor pools in their cytoplasm, indicating that loss of length control does not require the assembly of the total amount of flagellar proteins present in the cell.

"Long-zero" responses of long-flagella mutants: Chlamydomonas actively maintains its two flagella at the same length. If one of the pair of flagella on a cell is amputated, the intact flagellum shortens rapidly while the amputated flagellum is replaced by regeneration, a phenomenon called the "long-zero" response (ROSENBAUM, MOULDER and RINGO 1969). Simultaneous resorption and regeneration continue until the flagella achieve equal length, at which time they elongate together to normal length. If resorption of the intact flagellum after amputation of its mate requires the action of the same mechanism used to control flagellar length, then *lf* mutants might be defective in this response. The observation that *lf* mutants can have unequal length flagella has been reported by MCVITTIE (1972b). The response of *lf* mutants to the amputation of one flagellum was analyzed to determine whether they responded by rapidly resorbing the remaining flagellum.

Four mutants, representing all three *lf* loci, were examined: *lf1, lf2-1, lf2-3* and *lf3-3*. In each case, the cells responded immediately to the amputation of one flagellum by shortening the remaining flagellum. The single flagellum remaining on wild-type cells rapidly shortened from a mean starting length of 8.6 to 5.3 μm within 15 min (Figure 3A). Each of the *lf* mutants resorbed its intact flagellum to wild-type length or less within 20 min. For example, in *lf2-1* (Figure 3C) the mean length of the single flagellum on uniflagellate cells was 16.0 μm at time 0. Within 20 min these flagella had shortened to a mean length of 9.6 μm. Thus within 20 min after amputation, *lf2-1* resorbed 7.2 μm of its remaining flagellum into the cell. Similarly, *lf1* rapidly shortened its remaining flagellum after "long-zero" amputation: immediately after amputation the mean length of the single flagellum was 17.8 μm, and it shortened to 12.2 μm within 15 min (Figure 3B). Comparably rapid rates of resorption of the single flagellum were seen for *lf2-3* and *lf3-3* (data not shown). Clearly the defect in *lf* mutants which causes them to lose control of flagellar length does not prevent them from rapidly responding to the loss of one flagellum by resorbing the other. Thus both the signal to begin resorption and the mechanism which shortens the flagella must be unimpaired in each of the *lf* mutants.

In all four of the mutants, the rate of regeneration of the amputated flagellum clearly exceeded the rate of regeneration measured after both flagella were amputated (compare Figures 2 and 3). The most dramatic difference in the rate of regeneration was apparent in *lf2-1*. When both flagella were removed from cells of *lf2-1*, no regrowth was seen within 240 min (Figure 2B). Within 80 min after amputation of one flagellum, however, the mean length of the regenerating flagella was 4.8 μm; the range was 0–12 μm on different cells in the population (Figure 3C). Similarly, *lf1* (Figure 3B) regenerated no measurable flagellar length within 60 minutes after removal of both flagella (Figure 2A), but within 45 min after amputation of one flagellum, regrowth to a mean length of 4.8 μm was observed. Thus for *lf1* and *lf2-1* (Figure 3, B and C), as well as for *lf2-3* and *lf3-3* (data not shown), the rate of regeneration of the missing flagellum in a long-zero experiment exceeded the rate of regeneration when both flagella were amputated. We can conclude that the regrowth of a single flagellum is somehow different (at least in *lf* mutants) from the regrowth of two flagella; the difference may involve the reuse of flagellar proteins from the resorbing flagellum.

To determine whether the resorbed flagellar components were used to assemble the regenerating flagellum, mutant *lf1* was deflagellated by mechanical shearing in the presence of cycloheximide (10 μg/ml). *lf1* was chosen because it did not have a detectable flagellar precursor pool, and because amputation of both flagella of this mutant did not result in regrowth within 120 min. Thus any regeneration of the amputated flagellum in *lf1* in the absence of new protein synthesis was probably due to reutilization of flagellar proteins from the resorbing flagellum. Resorption of the intact flagellum and regeneration of the amputated flagellum were found to be comparable in the presence and absence of new protein synthesis (data not shown). Thus *lf1* "long-zero" cells appear able to reuse the resorbed flagellar components to regenerate (at least partially) a new flagellum.

Dikaryon analysis: *in situ* complementation: The sexual cycle of *C. reinhardtii* offers an opportunity to use an unusual type of phenotypic complementation to study flagellar mutants (STARLING and RANDALL, 1971; LUCK *et al.* 1977). When gametes of opposite mating type are mixed, cell fusion occurs and temporary dikaryon cells with four flagella are formed. The nuclei of these dikaryons remain separate for several hours (CAVALIER-SMITH 1974). Full-length flagella persist on the dikaryon cell for 2–3 hr, and

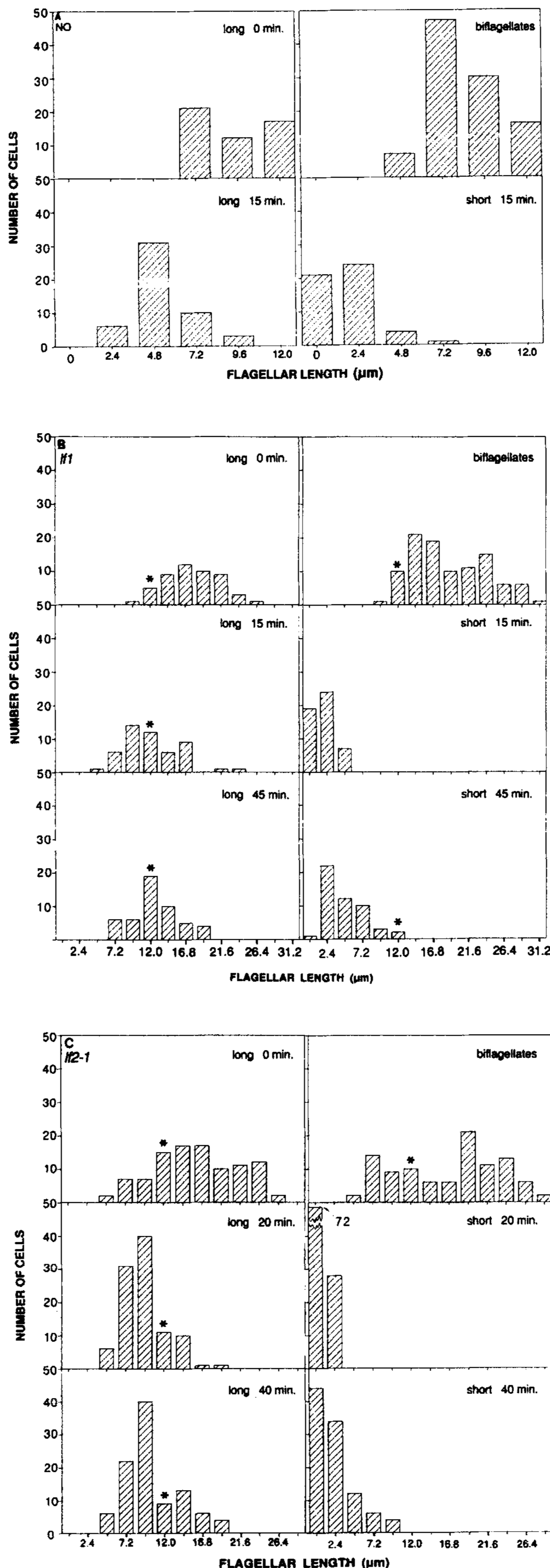

FIGURE 3.—"Long-zero" experiments: amputation of one of the two flagella. A, Wild-type (NO) (n = 50 for each time point); B, *lf1* (n = 100 for each time point); C, *lf2-1* (n = 100 for each time point). Populations of cells were repeatedly passed through a syringe needle, which amputated 1 of the 2 flagella from as many as 30% of the cells (see MATERIALS AND METHODS). Cells with unequal length flagella were scored in fixed samples by measuring the length of the longer ("long") and the shorter ("short") flagella on each cell. Samples were taken immediately after passage through the needle ("0 min") and at different times later—15, 20, 40, or 45 min in the different samples. The length distribution of the flagella from cells immediately before passage through the needle is also shown ("biflagellates"). The maximum flagellar length observed on wild-type cells (12 μm) is marked by an asterisk (*) in each panel.

are then resorbed as zygote maturation proceeds. Complementation of flagellar defects can be visualized as a gain of flagellar function in the dikaryon (*e.g.*, if paralyzed flagella begin to beat). We performed dikaryon analyses to determine whether the length control defect in long-flagella mutants could be repaired in dikaryons formed during matings with wild-type or with other *lf* mutants.

Long-flagella mutants are recessive to wild type in dikaryons: Each of the long-flagella mutants was mated to wild-type gametes to produce quadriflagellate dikaryons which had one pair of long flagella and one pair of wild-type flagella. For each of the 10 mutants, the long flagella resorbed to wild-type length within 60 or 90 min after mating. For example, when *lf2-1* was crossed to wild type (Figure 4A), the long flagella contributed by *lf2-1* shortened to nearly wild-type length within 90 min. The longest flagella observed in dikaryons of *lf2-1* × NO at 15 and 90 min after mating are compared in Figure 4A. Within 15 min after mating, 80% of the dikaryons had one flagellar pair that exceeded wild-type length (12 μm); 40% of the extra-long flagella were longer than 24 μm (twice wild-type length). By 90 min, however, all flagella were wild-type length on at least 80% of the dikaryons (Figure 4A). On the remaining 20% of the dikaryons, the longest flagella observed were less than 20 μm in length. Clearly the long flagella from the *lf2-1* cell shortened rapidly after mating to wild type, indicating that the mutation is recessive, and that length control was established *in situ*. The wild-type cytoplasm somehow established length control by actively shortening long flagella to wild-type length. Similar results were seen for each of the *lf* mutants crossed to wild type (data not shown); each of the mutants was recessive to wild type, and length control was demonstrated by the shortening of long flagella in dikaryons.

Phenotypic complementation of flagellar length in mutant/mutant heterokaryons: To determine whether complementation between different *lf* mutants could occur in dikaryons, all possible pairwise crosses of the ten *lf* mutants were performed, and the length of the four flagella in the resulting dikar-

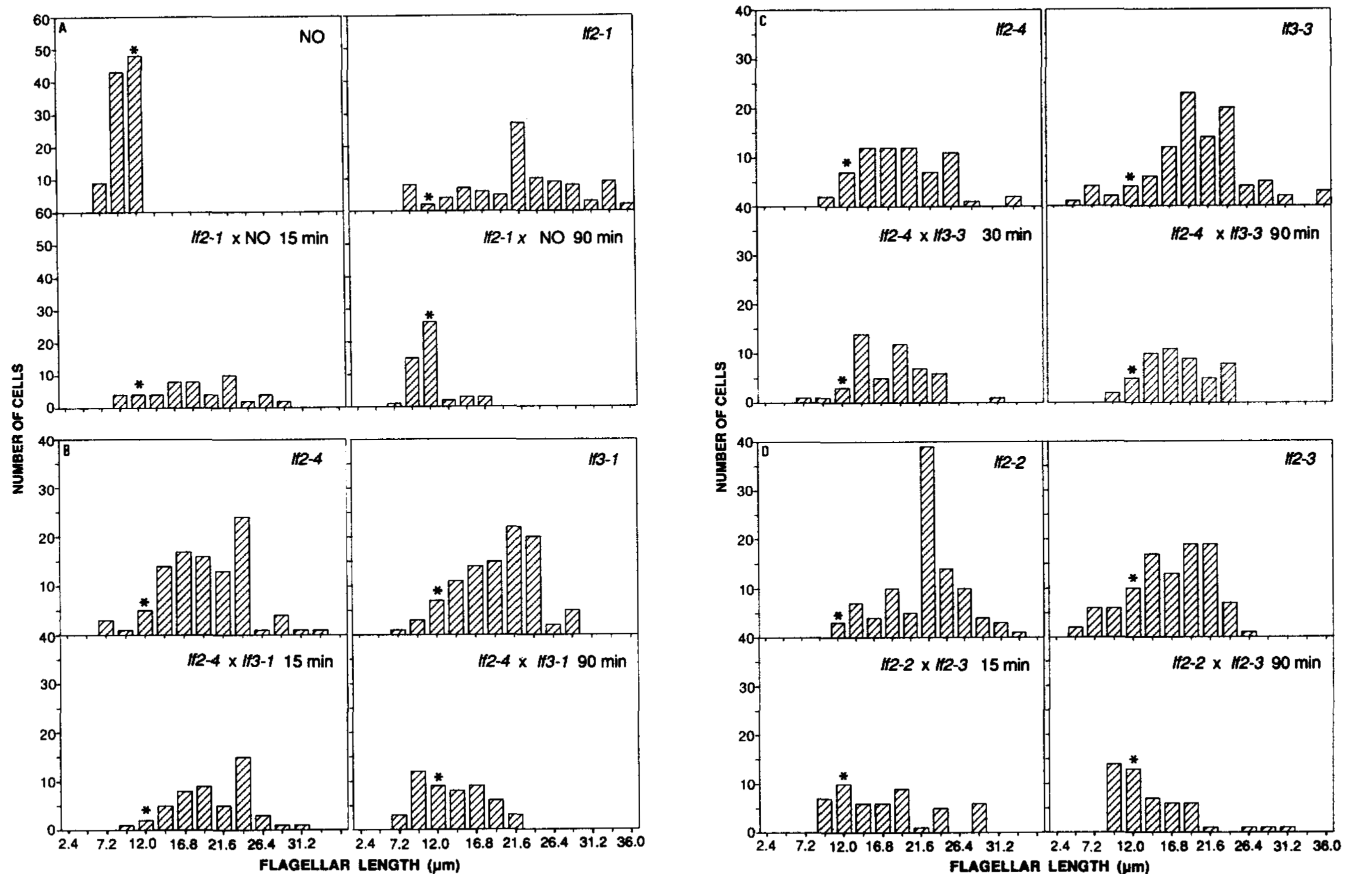

FIGURE 4.—Flagellar lengths in quadriflagellate dikaryon cells formed during mating. Four different matings are shown in panels A–D. For each cross, the flagellar lengths of each of the two parents are shown in the top two panels (n = 100). Mating rapidly results in the formation of dikaryon cells with four flagella, two from each parent. The bottom two panels for each cross show the lengths of the longest flagellum (n = 50) on the dikaryons at two different times after mating. A, *lf2-1* × wild-type (NO); B, *lf2-4* × *lf3-1*; C, *lf2-4* × *lf3-3*; D, *lf2-2* × *lf2-3*. The maximum flagellar length observed on wild-type cells (12 μm) is marked by an asterisk (*) in each panel.

yons was measured at various times after mating. We expected that crosses between *lf* mutants in different genes would produce dikaryons in which all four flagella shortened to wild-type length. This expectation was based on two observations: (1) the long flagella on each of the mutants rapidly shortened to wild-type length in dikaryons formed by mating to wild type (see above); and (2) in stable diploids constructed by crossing two *lf* mutants in different genes, the two flagella were wild-type length, indicating that the mutations could complement (Table 2). When crosses between *lf* mutants in different genes were performed, however, shortening of the four long flagella to wild-type length was not consistently observed. Crosses between different mutants produced a range of results from no shortening to partial shortening of the long flagella in the dikaryons. To determine whether the partial phenotypic complementation was statistically significant, data were analyzed by a one-tailed MANN-WHITNEY Rank Sum Test (MANN and WHITNEY 1947).

An example of statistically significant ($P < 0.05$) partial flagellar shortening is shown in Figure 4B, in which dikaryons formed between *lf2-4* and *lf3-1* were examined. Note that by 15 min after mating, the two longest flagella were greater than wild-type length in more than 90% of the dikaryons, and the longest flagella observed were longer than 31 μm. By 90 min, the longest flagella were of wild-type length or less on more than 40% of the dikaryons, and the longest flagella observed were only 22 μm in length. Thus some shortening of the long flagella was observed, indicating that partial complementation had occurred, but the majority of dikaryon cells from this cross had flagella longer than wild type after 90 min.

The most surprising result from this analysis was the frequent observation of crosses between mutants in different genes in which no significant ($P < 0.05$) flagellar shortening was observed. An example of such a cross, between *lf2-4* and *lf3-3*, is shown in Figure 4C. The distribution of flagellar lengths on dikaryons at 30 min after mating was very similar to the lengths seen 90 min after mating, suggesting that few of the dikaryons underwent significant flagellar shortening within 90 min. This non-complementation, however, was found only in dikaryons; stable diploids made between any pair of nonallelic mutants showed full complementation, *i.e.*, flagella of wild-

type length. Furthermore, the noncomplementation seen in dikaryons between mutants in different genes did not result from a resistance to shortening of the long flagella because, as seen earlier (*e.g.*, Figure 4A), flagella from each of the mutants shortened rapidly after mating to wild-type cells.

Mating and cell fusion were not synchronous in these experiments, as the mating reaction requires interaction between pairs of flagella, and long-flagella mutants do not mate as efficiently as wild type. Thus the 90-min time point probably included some dikaryons in which cell fusion had occurred only a few minutes before. Such dikaryons would not have had time to shorten their flagella before they were fixed. This experimental complication would lead us to underestimate the amount of flagellar shortening observed in the intermutant crosses. Even with this experimental limitation, however, rapid complementation between *lf* mutants in different genes as assayed by dikaryon analysis did not occur consistently.

Some flagellar shortening in crosses between different mutants from the same locus was seen. The most pronounced example of shortening seen in a dikaryon formed between different alleles in the same locus, *lf2-2* and *lf2-3*, is shown in Figure 4D. This flagellar shortening could represent partial interallelic complementation; the biological significance of such partial flagellar shortening, however, is not clear in view of the fact that 2 self-crosses (*lf2-4* and *lf3-1*) exhibited statistically significant ($P < 0.05$) flagellar shortening.

DISCUSSION

The genetic complexity of the mechanisms controlling the length of Chlamydomonas flagella is illustrated by the fact that at least three genes can be mutated to produce a long-flagella phenotype, and at least three others can be mutated to produce a short-flagella phenotype. No locus has yet been shown to produce long-flagella and short-flagella mutations. Long-flagella mutants show defects not only in flagellar length control, but also in the regeneration of flagella after amputation. In addition, these mutants often have two unequal length flagella on a single cell (McVittie 1972b).

Not enough *lf* or *shf* mutants have yet been analyzed to conclude that all potential flagellar length control loci have been discovered. However, the fact that five different alleles of *lf2*, and four different alleles of *lf3* have been found suggests that either these loci contain hotspots for mutation, or that there may not be more than a few, if any, unidentified genes which can be mutated to produce a long-flagella phenotype. The fact that only one allele of *lf1* has been found, while multiple alleles of *lf2* and *lf3* have been identified, may provide a clue as to the nature of the *lf1* mutation. If, for example, *lf1* represented a gain-of-function mutation, while *lf2* and *lf3* represented null mutations, then *lf1* mutants would be expected to occur infrequently relative to mutants in the other two *lf* genes.

Although the site of action of the *lf* gene products is not known, two results suggest that they may be structural components of the flagella or basal bodies, or else nonstructural components needed for flagellar assembly: (1) each of the *lf* mutants shows defects in flagellar regrowth after amputation; and (2) double *lf* mutants are flagellaless, or have predominantly one flagellum, depending on growth conditions. The regeneration defects seen in *lf* mutants differed among the mutants. For all four *lf3* mutants, the defect appeared to involve asynchronous initiation of regeneration, as subpopulations which began regeneration early after deflagellation regrew extra-long flagella with kinetics at least as fast as wild type. *lf1* and several *lf2* mutants, however, not only initiated regeneration slowly and asynchronously, but also showed very slow rates of flagellar elongation after initiation. Although the defect causing *lf* mutants to have regeneration defects is not known, excess flagellar length is clearly not a consequence of slow regeneration, as many mutants with very slow flagellar regeneration have flagella of wild-type length (Lefebvre *et al.* 1985; P. A. Lefebvre, unpublished observations).

The uniflagellar phenotype observed in double *lf* mutants may suggest the involvement of the basal bodies in the control of flagellar length, as the known mutants with such a phenotype, the *uni* mutants, have been shown to have defects in basal body structure (Huang *et al.* 1982). Unlike the asymmetry seen in the *uni* mutants, in which the single flagellum is found in a particular orientation relative to the eyespot, the double *lf* mutants show no preference for the *cis* or *trans* position. Another difference is seen in the fact that the double *lf* mutants only have flagella when grown with aeration, whereas the *uni* mutants show no such requirement. The flagellaless phenotype of double *lf* mutants grown without aeration has proven to be very useful in isolating revertants. Revertants are readily isolated from mutagenized double mutant cultures by simply removing swimming cells from the top of culture tubes. Among such revertants we have isolated extragenic suppressors of the *lf* mutants (M. Lavoie and P. Lefebvre, unpublished observations). These revertants and suppressors are currently being analyzed to identify other loci which interact with the known *lf* genes. Interestingly, double short-flagella mutants are also predominantly flagella-less, and from such strains Kuchka and Jarvik (1987) have isolated revertants and suppressors of *shf* mutants.

One characteristic of wild-type cells retained by

each of the long-flagella mutants is the ability to respond to certain stimuli by rapidly resorbing their flagella. If one of the flagella of any *lf* mutant cell was amputated, the other flagellum immediately began to shorten, as previously seen in wild-type cells (ROSENBAUM, MOULDER and RINGO 1969). For each of the *lf* mutants, the long flagellum remaining after amputation resorbed to wild-type length or less within 20 min. Thus the mutants were able to detect that one flagellum was amputated, and responded by shortening the remaining flagellum. These results indicate that the length control defect in *lf* mutants is neither a simple inability to resorb flagella, nor an inability to detect inequality of flagellar length within a pair. The "long-zero" studies of mutants *lf1* and *lf2-1* also demonstrate that even though these mutants regenerate flagella very poorly, they are capable of rapid flagellar growth. Biflagellate cells of these mutants did not begin to regrow flagella for at least four hours after both flagella were amputated. However, when only one flagellum was amputated these mutants regenerated significant flagellar length within 20 min. Thus the mutants were capable of rapid flagellar regeneration when one of the two flagella was resorbed into the cell. Perhaps in *lf2-1* the total loss of some flagellar protein when both flagella were removed prevented initiation of flagellar assembly onto the basal bodies, but the resorbed material from the surviving flagellum in a long-zero experiment allowed assembly to begin.

The fact that length control is maintained by an active process is dramatically shown in dikaryon experiments. For each of the 10 mutants we have studied the long flagella of the *lf* mutants resorbed to normal length within minutes after cell fusion during mating to a wild-type cell. Somehow the wild-type cell "enforced" normal length control, and rapidly induced the resorption of long flagella to wild-type length. Similarly, JARVIK *et al.* (1984) found that when mutants with short flagella were mated to wild type, the short flagella rapidly elongated to wild-type length. In the crosses reported here, the wild-type flagella did not change length while the long flagella were shortening. Thus in dikaryons of *lf* mutants and wild type, flagellar length control is not the result of a simple averaging process in which the total flagellar protein in the dikaryon is distributed equally to the four flagella. Instead, the results indicate that Chlamydomonas is able to monitor the length of its flagella, and rapidly bring long flagella to wild-type length if some necessary component(s) is provided by the wild-type cell.

Although the mutants were clearly recessive to wild type in dikaryons, mutants in different genes did not necessarily complement rapidly or completely. For example, when *lf2-4* was mated with *lf3-3* (Figure 4C), the flagella in the dikaryon shortened very little within 90 min, and no statistically significant ($P < 0.05$) flagellar shortening was observed within 90 min after mating. These mutants, located in different genes, were each fully recessive to wild type, in both dikaryons and stable diploids. This noncomplementation was commonly observed in dikaryons formed between *lf* mutants in different genes, although some shortening of the long flagella was observed in certain crosses. In dikaryons which did show partial complementation, the final average flagellar length exceeded the longest wild-type length (12 μm), but was significantly shorter than the average flagellar length of the initial dikaryon population (Figure 4B). One possible explanation for this observation could be that cells can resorb only a certain amount of flagellar protein into the cell body. If four extra-long flagella must be resorbed to wild-type length, then a large amount of unassembled flagellar proteins would need to be brought into the cell body. This explanation seems unlikely in view of the rapid and complete resorption of long flagella in crosses to wild-type cells, regardless of the initial length of the long flagella. Another explanation could be that at least one of the parent cells in the mating must be wild type for length control to be established in the dikaryon. If, for example, length control involves a multiprotein complex, and this complex cannot be readily assembled after mating, then length control would not be established in the time course of our dikaryon experiments (within 2 hr). In the crosses of *lf* mutants to wild type, the already assembled complex would be contributed by the wild-type cell, and length control would thus be possible.

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