Genetic Analysis of Flagellar Length Control in *Chlamydomonas reinhardtii*: A New *Long-Flagella* Locus and Extragenic Suppressor Mutations

Catherine M. Asleson and Paul A. Lefebvre

Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108

Manuscript received June 9, 1997
Accepted for publication November 3, 1997

ABSTRACT

Flagellar length in the biflagellate alga *Chlamydomonas reinhardtii* is under constant and tight regulation. A number of mutants with defects in flagellar length control have been previously identified. Mutations in the three *long-flagella* (*lf*) loci result in flagella that are up to three times longer than wild-type length. In this article, we describe the isolation of long-flagellar mutants caused by mutations in a new *LF* locus, *LF4*. *lf4* mutations were shown to be epistatic to *lf1*, while *lf2* was found to be epistatic to *lf4* with regard to the flagellar regeneration defect. Mutations in *lf4* were able to suppress the synthetic flagella-less phenotype of the *lf1*, *lf2* double mutant. In addition, we have isolated four extragenic suppressor mutations that suppress the long-flagella phenotype of *lf1*, *lf2*, or *lf3* double mutants.

THE ability to assess and to regulate size is an important factor in many cellular processes. The active control of flagellar length in the biflagellate alga, *Chlamydomonas reinhardtii*, provides an excellent opportunity to use genetics to dissect a size control mechanism (Lefebvre *et al.* 1995). Active regulation of flagellar length is demonstrated by several lines of evidence. First, flagella are disassembled and reassembled to proper size at specific stages during both vegetative growth and sexual differentiation (Johnson and Porter 1968; Cavalier-Smith 1974). Second, cells are able to regrow new flagella to their original length when their flagella are amputated (Lefebvre *et al.* 1978). Third, a cell maintains its two flagella at the same length. This is demonstrated when a single flagellum is amputated from a biflagellate cell. The intact flagellum is disassembled while the amputated one is reassembled until both flagella reach equal length at which time they elongate together until the proper length is attained (Rosenbaum *et al.* 1969).

The most striking evidence for active length control comes from the phenotypes of mutants which have lost flagellar length control. Three *long-flagella* (*lf*) loci (McVittie 1972; Jarvik *et al.* 1976; Barsel *et al.* 1987) have been previously described. Mutations in *lf* loci result in cells with flagella as long as 40 μm, whereas wild-type cells never have flagella longer than 16 μm. The fact that these mutations disrupt an active mechanism controlling length is shown by the following experiment. During the sexual cycle, gametes of opposite mating type fuse, forming a quadriflagellate dikaryon cell with two flagella contributed from each parent. In quadriflagellate cells formed by mating a wild-type strain with a *lf* mutant strain, the two long flagella shorten in minutes to wild-type length, indicating that size control is rapidly imposed on the flagellar pair donated by the *long-flagella* parent (Starling 1969). It is important to note that in this experiment flagellar length is not averaged, but the preset wild-type length is enforced on the mutant flagella.

To identify new genes involved in flagellar length control and to uncover possible genetic interactions among length control genes, we have isolated mutants in five new loci. We have identified multiple alleles at a new *LF* locus, *LF4*, using DNA insertional mutagenesis (Tam and Lefebvre 1993). These new *lf* mutants are phenotypically similar to mutations in the other three *LF* genes, in that the *lf4* mutants assemble flagella that are two to three times wild-type length. However, all *lf4* mutants differ in two important ways from the previously described *lf* mutants. All *lf4* mutants are able to regenerate flagella rapidly after deflagellation, whereas *lf1* and several alleles of *lf2* regenerate flagella very slowly. Second, *lf4* mutants in double mutant combination with any of the other three *lf* loci have the long-flagellar phenotype, while any double mutant combination of *lf1*, *lf2*, or *lf3* alleles is flagella-less. *lf4* mutations were shown to be epistatic to *lf1*, while *lf2* was found to be epistatic to *lf4* with regard to the flagellar regeneration defects. In addition, mutations in *lf4* were able to suppress the flagella-less phenotype of the *lf1*, *lf2* double mutant.

Mutations in four *suppressor of long-flagella* (*slf*) loci were isolated using a screen that takes advantage of the synthetic flagella-less phenotype observed for double *lf* mutants. In addition to suppressing the flagella-less

Corresponding author: Catherine M. Asleson, Department of Plant Biology, 220 BioScience Center, University of Minnesota, 1445 Gortner Ave., St. Paul, MN 55108. E-mail: cathy@cbs.umn.edu

phenotype of double *lf* mutants, all *slf* mutants were able to suppress the flagellar length defect of single *lf* mutations to wild-type length. Three of the *slf* suppressors showed allele-specificity but not locus-specificity while suppression by *slf4-1(d)* was neither allele nor locus-specific. All *slf* mutations were found to be dominant for their suppression phenotype. The genes identified by these mutations may encode proteins whose products play a role in flagellar length regulation.

MATERIALS AND METHODS

Cell strains and media: The *C. reinhardtii* strains used in this study are listed in Table 1. All cells were grown under constant light (unless otherwise noted) in M (Minimal) medium (Harris 1989) or TAP medium (Gorman and Levine 1965).

Genetic analysis: Techniques for mating and tetrad analysis were as previously described (James *et al.* 1988). Meiotic progeny were scored for flagellar length by resuspending a single colony of cells in 100 μl of M medium in individual wells of a 96-well microtiter plate, and then growing the cells under constant light overnight. Cells were then fixed in 1% glutaraldehyde. For routine phenotypic analysis, fixed cells were examined by phase-contrast microscopy (×40 objective), and flagellar lengths were compared using an ocular micrometer. Flagellar lengths were also measured on a Zeiss Axioplat microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with an Argus 10 Image Analysis System. Flagellar regeneration was assayed using methods previously described (Lefebvre *et al.* 1978).

Diploids for complementation tests were constructed by mating strains containing the *lf* mutations of interest along with one of the complementing auxotrophic markers, *arg7* and *arg2*, and then selecting for growth on M medium. To confirm that putative diploid strains were in fact diploid, two additional phenotypes were scored. Diploid cells were identified as having the minus mating type and larger cell size than the haploid parents. Six diploid strains were measured for each complementation test.

Genetic screens: DNA insertional mutagenesis was performed as described (Tam and Lefebvre 1993; Smith and Lefebvre 1996, 1997). Single colonies were resuspended into 96-well culture plates and screened for aberrantly swimming cells using a stereomicroscope (Zeiss DR-C at ×80 magnification) because excessive flagellar length causes impaired motility. These aberrantly swimming strains were then fixed in 1% glutaraldehyde and then screened by phase-contrast microscopy (×400) to identify those mutants with longer than wild-type length flagella.

To screen for suppressor mutations, double mutant strains (*lf1-1 lf3-2*; *lf2-1 lf3-2*; *lf2-3 lf3-2*; and *lf1-1 lf3-1*) were constructed by genetic crosses. A single colony isolate of each double mutant was used to start a 4 liter culture in M medium. These cultures were grown on a 12-hr, light/dark cycle with constant aeration until late log phase (~3–5 × 10^6 cells/ml). Cells were collected by low-speed centrifugation (~2000 *g*) and resuspended to a density of 10^8 cells/ml. Each suspen-

TABLE 1

***Chlamydomonas reinhardtii* strains**

Strain	Genotype	Source
D68	*lf1-1 mt+*	(McVittie 1972)
C23	*lf2-2 mt−*	(McVittie 1972)
G51	*lf2-3*	(McVittie 1972)
E39	*lf3-2 mt−*	(Barsel *et al.* 1987)
H72	*lf3-3 mt−*	(Barsel *et al.* 1987)
F64	*lf4-1 ac17 mt+*	Dr. Elizabeth Smith
G39	*lf4-2 mt−*	Dr. Greg Pazour
G30	*lf4-3 mt−*	Dr. Greg Pazour
G32	*lf4-4 mt−*	Dr. Greg Pazour
G61	*lf4-5 mt+*	This study
G33	*lf4-6 mt−*	Dr. Greg Pazour
G31	*lf4-8 mt−*	Dr. Greg Pazour
G64	*lf4-7 ac17 mt+*	Andrea Kernan
E13	*slf1-1(d) lf1 lf3-2 mt−*	This study
F23	*slf1-1(d) mt+*	This study
F4	*slf2-1(d) lf1 lf3-2 mt−*	This study
D38	*slf2-1(d) mt−*	This study
F3	*slf3-1(d) lf1 lf3-wh8 mt−*	This study
D44	*slf3-1(d) mt*	This study
E30	*slf4-1(d) lf2-3 lf3-2 mt+*	This study
G24	*slf4-1(d) mt*	This study
L5	*nit1-305 apm1-19 mt+*	Dr. Lai Wa Tam
D48	*arg2 sr1 act1 mt+*	Dr. Mary Porter
H3	*arg7 mt+*	Dr. Lai Wa Tam
Y48	*ac17 nit1-e18 sr1 mt+*	Craig Amundsen
H30	*ery2b mt+*	Chlamydomonas Genetics Center
G69	*ac177 mt+*	Chlamydomonas Genetics Center
B31	*mam2*	Dr. Rogene Schnell

sion was placed in a small Petri dish, constantly stirred, and was irradiated for 180 sec with ultraviolet (UV) light from a General Electric G8T5 germicidal lamp at a distance of 18 cm. This UV exposure yielded ~30–50% survival. Immediately after mutagenesis, 200 μl aliquots of the cell suspension were added to 10 ml of M medium in glass culture tubes. The tubes were placed in the dark for 6 hr to prevent photorepair. The tubes were then placed in constant light for 2 wk, or until swimming cells could be seen at the meniscus of the medium. Phenotypic revertants were isolated by pipetting ~100 μl of liquid from the meniscus and plating cells to obtain single colonies (M medium). One colony was retained from each original culture tube to ensure that each phenotypic revertant examined was produced by an independent event.

RESULTS

Seven *lf* mutants generated by DNA insertional mutagenesis: A variety of *C. reinhardtii* flagellar mutants have been generated by DNA Insertional Mutagenesis (Tam and Lefebvre 1993). This procedure involves transforming *nit1* cells with a plasmid spMN24 that contains the structural gene for nitrate reductase (*NIT1*) (Fernandez *et al.* 1989). Nonhomologous integration of the plasmid into the genome results in a mutagenic event in which the insertion of the transgene is often accompanied by a deletion and/or rearrangement of the genomic DNA at the site of insertion.

Seven *lf* mutants were isolated by this lab and by others (Table 1) by first screening for cells with motility defects because the *lf4* mutation causes cells to swim poorly relative to wild-type cells. Next, motility-defective mutant strains were then individually screened using phase-contrast microscopy to identify those with extra-long flagella. While the length of the flagella on wild-type cells varies, no flagella exceeding 16 μm are ever observed in wild-type populations. In *lf* mutant populations, however, cells have flagella that are three times as long as the maximum wild-type value.

Each *lf* mutant was backcrossed to a wild-type strain, and the segregation of length phenotype in the F_1 progeny was analyzed. All seven mutants showed Mendelian segregation of the *lf* phenotype (two *lf*: two wild-type progeny per tetrad) indicating that in each case the length defect was caused by a single mutation (data not shown).

TABLE 2

Phenotype of stable diploids

lf mutant	G39	G30	G61	G33	G64	G31	WT
F64	long	long	long	ND	long	ND	wt
G39		long	long	ND	long	long	wt
G30			long	long	long	ND	wt
G61				long	long	long	wt
G33					long	ND	wt
G64						long	wt

Stable diploids were constructed using pairwise combinations of *lf* mutants, and the length of the flagella were determined.

ND, not determined.

TABLE 3

Linkage analysis

Parental strains	PD	NPD	TT
lf4 × *lf1*	34	17	60
lf4 × *lf2*	29	17	37
lf4 × *lf3*	33	43	38

PD = 4 *lf*: 0 WT; NPD = 2 WT: 2 unknown; TT = 2 *lf*: 1 WT: 1 unknown.

Seven new *lf* mutants define a single new complementation group, *LF4*: Stable vegetatively growing diploid strains can be constructed in *C. reinhardtii* by mating cells containing complementing auxotrophic markers (Ebersold 1956; James *et al.* 1989). All seven mutants were demonstrated to be recessive to the wild-type allele in stable diploid strains. Diploids were then constructed using all seven mutants in pairwise combinations. In every case tested, heterozygous diploid cells had long flagella (Table 2), indicating that the seven mutations define a single complementation group. Several strains containing mutant alleles from this complementation group were crossed to strains with mutations in one of the three previously identified *lf* loci: *lf1*, *lf2*, and *lf3*. Wild-type recombinants were recovered in all crosses (Table 3), demonstrating that the seven mutants are alleles of a new *lf* locus, *LF4*.

***LF4* maps to linkage group XIV on the Chlamydomonas genetic map:** The *lf4* locus is linked to the linkage group XIV (LG XIV) marker *ery2b* (conferring erythromycin resistance) (O'Brien 1980). Results from two-factor crosses place *lf4* 6.7 cM from *ery2b*, and 25 cM from *mam2* (Table 4). The *LF4* centromere distance of

TABLE 4

Mapping data for *LF4*

	lf3-2[a]	*mam2*[a]	*ery2b*[b]
lf4-1	7:7:11		1:0
lf4-3	5:7:5		11:1
lf4-6	1:5:3		24:1
lf4-7	3:6:5	14:0:14	6:1
lf4-8	16:18:12		
	32:43:36		42:3
	16.2 cM	25.0 cM	6.7 cM

[a] Tetrad Data (PD:NPD:TT): TT/2[PD + NPD + TT] × 100 = Centromere Distance [TT + 6NPD]/2[PD + NPD + TT] × 100 = Map Distance.

[b] Random Progeny Data (Nonrecombinant progeny: Recombinant progeny): Recombinant Progeny/Total Progeny × 100 = Map Distance.

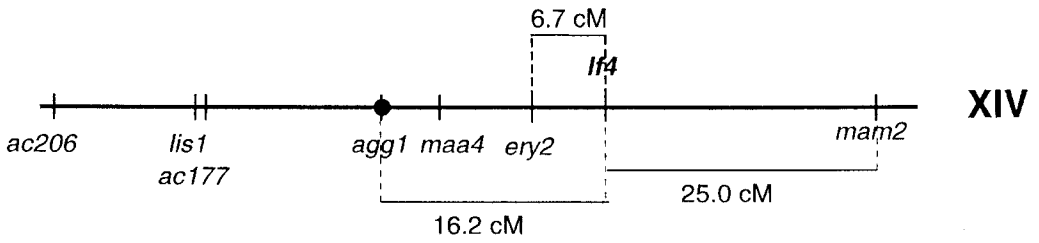

Figure 1.—Genetic map of Chlamydomonas LGXIV.

16.2 cM was determined from crosses with the centromere-linked marker, *lf3* (Table 4). These results place *LF4* on the right arm of LGXIV (Figure 1). Gene order was inferred from the centromere data, but not directly tested by three-factor crosses. The placement on LGXIV was confirmed by restricted fragment length polymorphism (RFLP) mapping (Ranum *et al.* 1988) using a fragment of DNA as a hybridization probe,

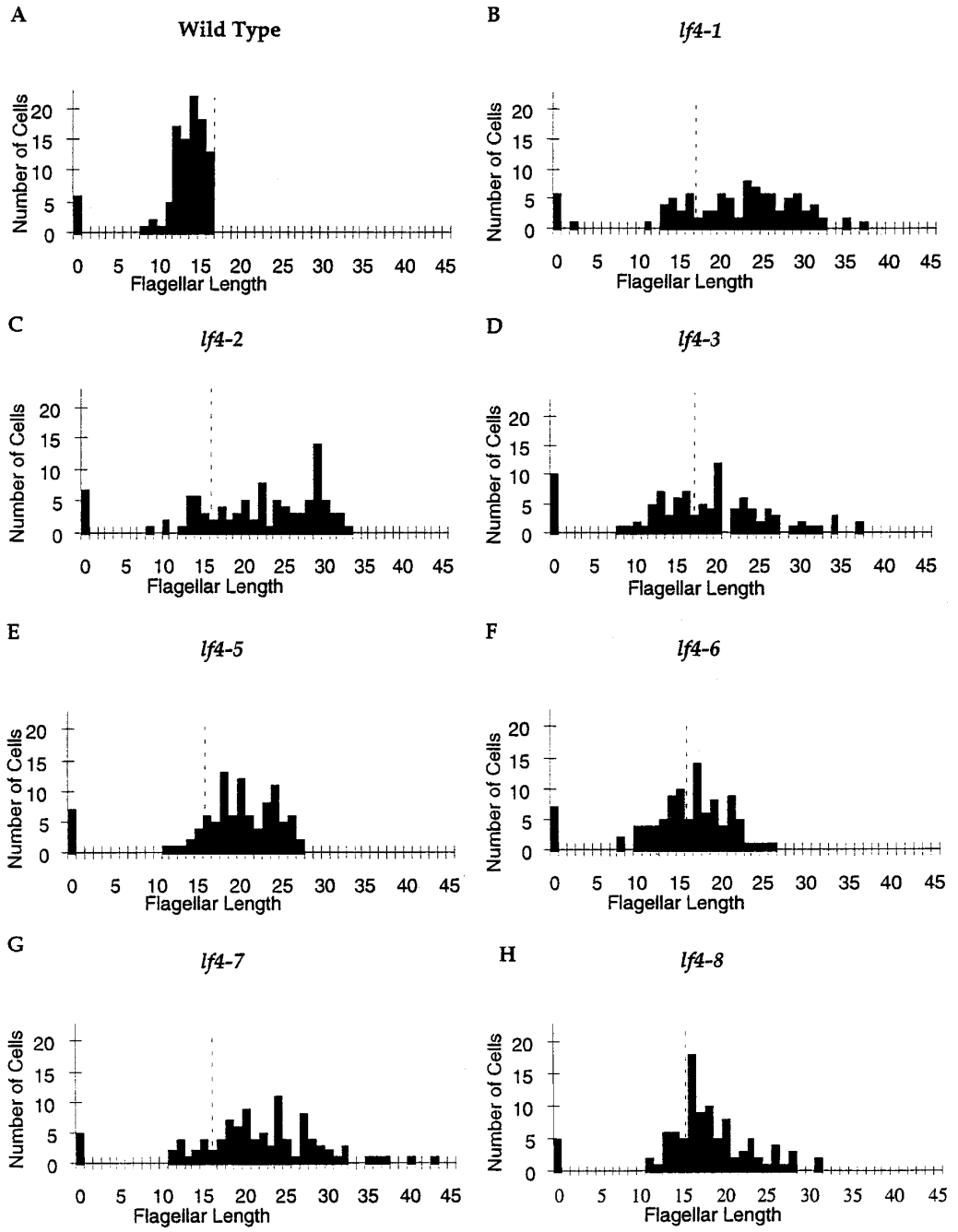

Figure 2.—Histograms of flagellar lengths (μm) within populations (n = 100 cells per panel). The dashed line indicates the maximum flagellar length of wild-type cells. Wild-type (A) and *lf4* mutant (B–H) populations.

flanking the site of plasmid insertion in *lf4-1* (data not shown).

Phenotypic analysis of *lf4*: While flagella from a wild-type population may vary in length, no flagella exceed 16 μm in size (Figure 2A). In *lf* mutant populations, cells also have flagella with a wide variety of lengths, but many cells have flagella that exceed the wild-type maximum length, with some as long as 40 μm. The seven *lf4*

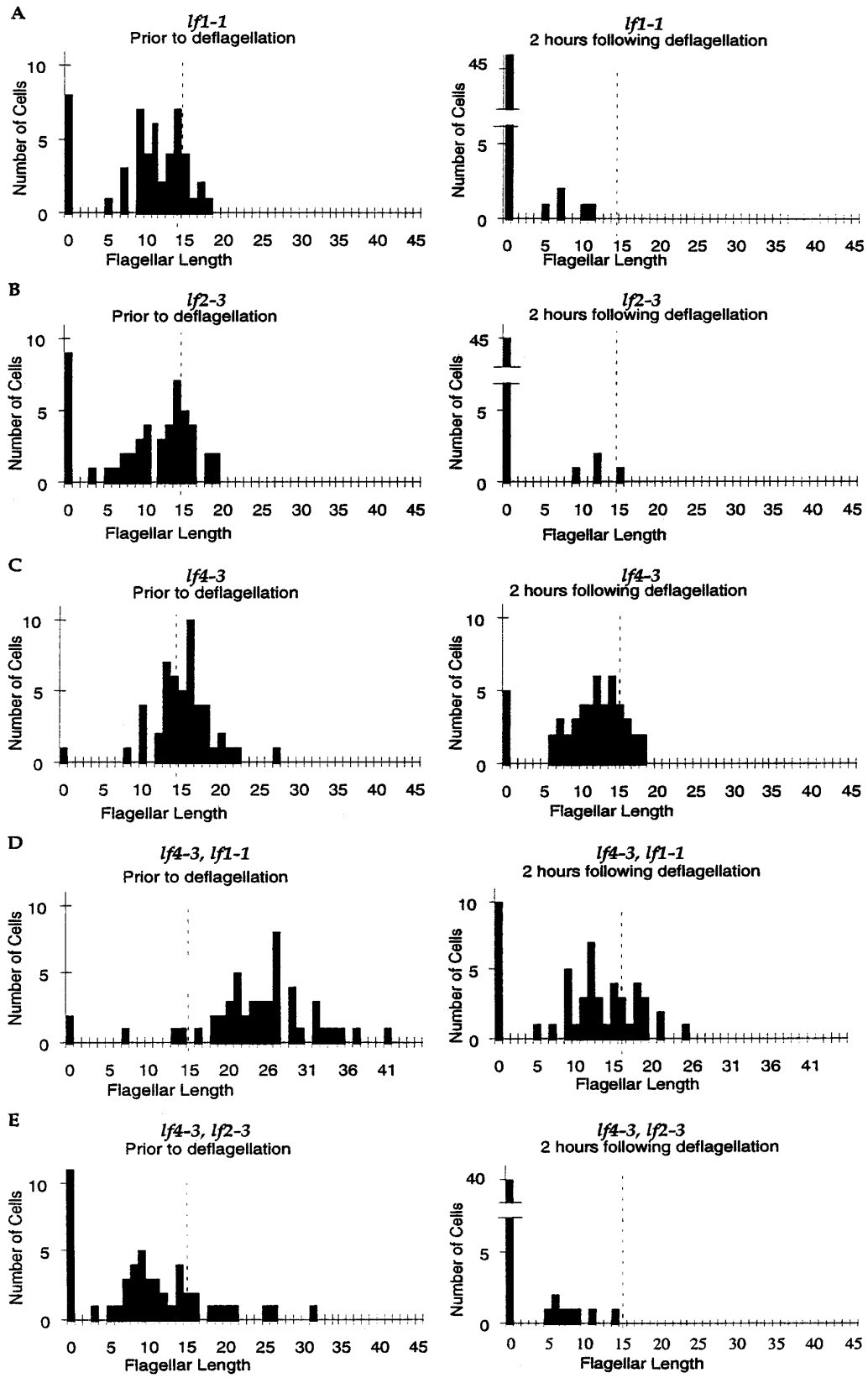

Figure 3.—Histograms of flagellar lengths (μm) within populations (n = 50 cells per panel) prior to and following deflagellation. The dashed line indicates the maximum flagellar length of wild-type cells. *lf1-1* (A), *lf2-3* (B), *lf4-3* (C), *lf4-3 lf1-1* (D), and *lf4-3 lf2-3* (E) populations.

mutant populations also exhibit this same excess flagellar length phenotype (Figure 2, B–H).

One perhaps counter-intuitive aspect of the phenotype of several of the original *lf* mutations is that they are also defective in their ability to regenerate flagella following amputation, and this additional phenotype always cosegregates with the length defect (Barsel *et al.* 1987). For example, both *lf1* and *lf2-3* are unable to regenerate flagella following amputation by mechanical shearing. When examined 2 hr after deflagellation, greater than 90% of the cells remained flagella-less (Figure 3, A–B).

All seven *lf4* alleles were examined for their ability to regenerate flagella. In all experiments, the cells were examined immediately following shearing in a homogenizer to confirm that 100% of the cells had been deflagellated. After 2 hr, the wild-type control cells had regrown full-length flagella as expected (Rosenbaum *et al.* 1969). The *lf4-3* allele was able to regenerate flagella within 2 hr following amputation when greater than 80% of the population had near wild-type length flagella (Figure 3C). During this same time period, all other *lf4* alleles also regenerated flagella to at least wild-type length, with some cells having flagellar lengths exceeding 16 μm. Thus, each *lf4* mutant regenerates flagella with at least wild-type rates of regeneration following amputation.

Double mutant phenotypes of *lf4* with other *lf* loci: As previously described, any double mutant combination of *lf1*, *lf2*, or *lf3* alleles results in a flagella-less phenotype (Barsel *et al.* 1987). A similar phenotype has also been seen with many combinations of *short-flagella* mutants as well (Jarvik *et al.* 1984; Kuchka and Jarvik 1987). In contrast, double mutants containing any allele of *lf4* and any of the other three *lf* loci have the *lf* phenotype (Figure 3, D–E).

Because *lf1* and *lf2-3* cells are defective in flagellar regeneration, while *lf4* mutants regenerate normally, it was possible to determine the epistatic relationship among these mutants with regard to flagellar regeneration. The double mutants *lf4-3 lf1-1*, and *lf4-3 lf2-3*, were constructed and examined for the ability to regenerate flagella. The double mutant *lf4-3 lf1-1* was able to regenerate flagella following amputation (Figure 3D), whereas the double mutant, *lf4-3 lf2-3* was unable to regenerate flagella following deflagellation (Figure 3E). The regeneration phenotype of the *lf4-3 lf1-1* double mutant combination is the same as the *lf4-3* mutant alone (Figure 3C). The *lf4-3 lf2-3* double mutant displayed the same regeneration phenotype as the *lf2-3* mutant alone (Figure 3B). Thus, with regard to the flagellar regeneration phenotype, *lf4* is epistatic to *lf1*, and *lf2* is epistatic to *lf4*.

Triple mutant phenotype: A triple *lf1-1 lf2-3 lf4-3* mutant was constructed by crossing a *lf1-1 lf4-3* double mutant and a *lf2-3 lf4-8* double mutant. Only progeny with long flagella were recovered, indicating that the triple mutant combination results in a *long-flagella* phenotype (data not shown). Thus the *lf4* mutation can act as an extragenic suppressor of the flagella-less phenotype of the double *lf1 lf2-3* mutant, as well as functioning as a suppressor of the *lf1-1* regeneration defect.

Genetic screen for extragenic suppressors of *lf* mutants: To isolate extragenic suppressors of *lf* mutations, we capitalized on the synthetic flagella-less phenotype of double *lf* mutant strains. We were able to obtain phenotypic revertants of double *lf* mutants by isolating cells that swim up from a pellet of nonmotile cells. These revertants were then backcrossed to determine whether the phenotypic reversion was because of a reversion of one of the two *lf* loci, or whether it was because of an extragenic suppressor mutation. A similar screen was used to obtain suppressors of *short-flagella* mutations (Kuchka and Jarvik 1987).

Several double *lf* mutant strains were used as parents for the isolation of revertants: *lf1-1 lf3-2*; *lf2-1 lf3-2*; *lf2-3 lf3-2*; *lf1-1 lf3-1*. Cultures grown from a single colony were mutagenized with UV light (James *et al.* 1989), split into 10 separate 10 ml cultures, incubated in the dark for 6 hr to prevent photorepair, and then grown under constant light. Initially, all of the cells in the culture were nonmotile, accumulating at the bottom of the culture tube. After several weeks of growth, motile cells were observed at the top of the culture tube. In cultures not exposed to UV light, spontaneous motile revertants were also obtained. These motile revertants were isolated by pipetting the swimming cells from the meniscus. The cells were then plated to obtain single-colony isolates, and only one isolate from each 10 ml culture was characterized further.

A total of 49 swimming revertant strains were isolated from the four different double mutant strain backgrounds (Table 5). Of these independent revertants, 44 strains were isolated from UV-mutagenized double mutant populations, and five revertants arose in nonmutagenized cultures. Some of the revertant strains had cells with flagella of wild-type length and the rest had cells with long flagella. For example, 13 different swimming revertants were isolated from *lf1-1 lf3-2* double mutant populations; two contained spontaneous mutations and 11 were from UV-mutagenized

TABLE 5

Swimming phenotypic revertants

Double mutant genotype	Revertant phenotype: long flagella	Wild-type flagella
lf1-1 lf3-2	11 (9UV)	2 (2 UV)
lf2-1 lf3-2	6 (1 UV)	
lf2-3 lf3-2	10 (8 UV)	4 (4 UV)
lf1-1 lf3-1	13 (12 UV)	3 (3 UV)
Totals	40	9

cultures. Eleven of the 13 had long flagella, while only two revertants had flagella of wild-type length.

Each revertant strain was backcrossed to wild type to determine whether the phenotypic reversion was caused by the action of a new extragenic suppressor mutation, or by an intragenic event involving one of the two original *lf* mutations. If motility was restored by an intragenic reversion event, the resulting backcross tetrads should each contain two progeny with wild-type flagellar length and two progeny with long flagella. On the other hand, if phenotypic reversion was caused by an extragenic suppressor mutation, then the segregation patterns in the tetrads from the backcross would be complex. In this case, three genes would segregate in the cross, producing eight different genotypes among the progeny. One of these eight genotypes would contain the original two *lf* mutations, along with the wild-type allele at the suppressor locus, producing the flagella-less phenotype. Therefore, the presence of an extragenic suppressor mutation in the motile revertant strain was indicated by the identification of flagella-less cells among the progeny of a backcross.

It was expected that most revertants with a *long-flagella* phenotype would result from an intragenic event, either true reversion or intragenic suppression. Surprisingly, among the six different long-flagellar revertants that were analyzed further by backcrosses, the segregation data indicated that only two were caused by intragenic events. That is, when the motile revertant strain was crossed to a wild-type strain, all of the resulting tetrads contained two wild-type progeny and two progeny with long flagella. From this result, we can conclude that in these two revertants either an intragenic event or tightly linked extragenic event occurred. In the other four revertants with long flagella, the backcross segregation pattern was more complex and flagella-less progeny were generated. These results suggest that an extragenic event occurred to cause the phenotypic reversion in the parental strains.

Six revertants with wild-type flagellar length were also backcrossed. All six crosses resulted in complex segregation patterns that included tetrads containing flagella-less progeny. From these data we conclude that all six revertants with wild-type length flagella contain extragenic suppressor mutations.

Extragenic suppressor mutations were isolated from each of the four double *lf* mutant backgrounds: Four revertant strains with wild-type flagellar length, E13, F4, F3, and E30, were analyzed in greater detail after they were shown to contain extragenic mutations. The extragenic suppressor mutations present in these strains were designated *slf*. The genotypes and phenotypes of the triple mutants are listed in Table 6.

To examine the flagellar phenotype of the *slf* mutants, revertant strains were crossed to a wild-type strain. The genotypes of each of the four meiotic products from any tetrad with two flagella-less progeny could be inferred. The two flagella-less progeny were presumed to contain the original two *lf* mutations, along with the wild-type allele at the suppressor locus. The remaining two progeny from this tetrad would therefore contain the *slf* mutation along with the wild-type alleles at the two *lf* loci. By examining tetrads that contained at least two flagella-less progeny, the phenotype of cells containing the *slf* mutation alone was determined. It was established that all cells containing any of the four *slf* mutations alone had wild-type length flagella (Table 6).

As discussed above, some *lf* mutations, especially *lf1* and *lf2* alleles, have defects in flagellar regeneration after amputation. To determine whether the *slf* mutations share this phenotype, the kinetics of flagellar regeneration was examined for each *slf* mutant. We found that after 2 hr, all *slf* mutants regenerated flagella to wild-type length (data not shown) as well as wild-type cells. Thus, none of the four *slf* mutants were defective in flagellar regeneration.

Characterization of suppression: All four *slf* mutations were examined for the ability to suppress single *lf* mutations. Each suppressor was crossed to *lf1* and to multiple alleles of both *lf2* and *lf3*. All four *slf* mutations suppressed the length defects of *lf1*, *lf2-3*, and *lf3-2*. They differed in their ability to suppress *lf2-2* and *lf3-3*. For example, while *slf1-1(d)* was able to suppress *lf3-2*, it did not suppress the *lf3-3* length defect. In contrast, *slf4-1(d)* was able to suppress both *lf3-2* and *lf3-3*. None of the *slf* mutations were able to suppress the length defects of either *lf4-1* or *lf4-8* (Table 7).

Although the *slf* mutations suppressed defects in flagellar length control, it was possible that the defects

TABLE 6

Extragenic suppressors of *long-flagella* mutations

Triple mutant strain	Genotype of triple mutant	Phenotype of triple mutant	Spontaneous UV-induced	Phenotype of *slf* mutation	Dominant / recessive suppression
E13	*lf1-1 lf3-2 slf1-1(d)*	Wild type	Spontaneous	Wild type	Dominant
F4	*lf2-1 lf3-2 slf2-1(d)*	Wild type	UV	Wild type	Dominant
F3	*lf1-1 lf3-1 slf3-1(d)*	Wild type	UV	Wild type	Dominant
E30	*lf2-3 lf3-2 slf4-1(d)*	Wild type	UV	Wild type	Dominant

TABLE 7

Suppression of flagellar length defect

	slf1-1(d)	*slf2-1(d)*	*slf3-1(d)*	*slf4-1(d)*
lf1-1	+	+	+	+
lf2-3	+	+	+	+
lf2-2	−	+	−	+
lf3-2	+	+	+	+
lf3-3	−	−	−	+
lf4-1	−	−	−	ND
lf4-8	−	−	ND	−

ND, not determined.

TABLE 8

Mapping data

	lf1	*lf2*	*lf3*	*arg7/arg2*	*ery2b*
slf1	6:3:10	9:5:16	26:0:24	17:0:1	
slf2	4:2:24	9:7:19	4:13:11		7:2:11
slf3	4:9:12	1:2:6	3:6:12		
slf4	2:1:10	1:1:12	23:0:18	19:20[a]	

Tetrad data = PD:NPD:TT.
[a] Random progeny data = nonrecombinants:recombinants.

in regeneration seen for *lf1* and *lf2* mutations would not be suppressed. To test this possibility, specific *slf*, *lf* double mutants, in which the *lf* allele is defective in flagellar regeneration, were examined for the suppression of the regeneration defect. For all cases in which the suppressor mutation could suppress the flagellar length defect, it also suppressed the flagellar regeneration defect (data not shown).

The *suppressor of long-flagella* mutations are dominant in stable diploids: Because the *slf* mutants had no other phenotype than their ability to suppress *lf* mutations, diploids were constructed that were heterozygous for the *slf* mutation being tested and homozygous for the *lf3-2* mutation. Using these strains, we were able to determine whether *slf* mutations were dominant or recessive. Each heterozygous *slf* diploid strain had wild-type length flagella, indicating that all four *slf* extragenic suppressor mutations were dominant for the wild-type allele with regard to their ability to suppress *lf3-2* (Table 6).

Genetic locations of *slf* mutations: Two of the *slf* loci have been placed on the Chlamydomonas genetic map. The loci, *slf4* and *slf1*, are both linked to *lf3* on linkage group I (LGI) (Table 8). The *slf1* locus maps 24 cM from *lf3*, and 2.8 cM from the *arg7/arg2* locus (Figure 4). The *slf4* locus is 22 cM from *lf3*, but linkage was not detected with *arg7/arg2*; *slf4* is presumably on the opposite arm of LGI (Figure 4).

Although *slf2* and *slf3* were not mapped, neither of these *slf* loci correspond to a known *lf* locus based on the following observations. Neither *slf2* nor *slf3* is linked to *lf1* on LGII, to *lf2* on LGXII/XIII or to *lf3* on LGI. The *slf2* locus is also unlinked to *ery2b*, an erythromycin-resistance marker linked to the *LF4* locus on LGXIV. Linkage to *LF4* could not be assayed directly because neither extragenic suppressor mutation suppressed *lf4*. These data eliminate the possibility that *slf2-1(d)* is a mutation of any of the four *lf* loci.

DISCUSSION

All seven *lf* insertional mutations examined define a single new complementation group, *LF4*. While the purpose of this study was to identify new genes involved in flagellar length control, it was expected that insertional alleles of *lf1*, *lf2*, and *lf3* would also have been isolated by this screen. There are several possibilities to explain the absence of insertion mutations affecting these loci.

The first possibility is that the process of insertional mutagenesis generates predominantly null mutations, and null mutants of *lf1*, *lf2*, and *lf3* may not have the *lf* phenotype. Tam and Lefebvre (1993) examined three flagellar insertion mutants at the molecular level. They found that not only were the mutations generated by the insertion of plasmid DNA, but this insertion was accompanied by deletions and rearrangements of the flanking genomic region. The previously identified alleles of *lf1*, *lf2*, and *lf3* were generated with either chemical mutagens or UV irradiation. While nothing is known about the nature of the genetic lesions in these mutants, it is entirely possible that the null phenotype at these loci may be different from the *long-flagella* phenotype. If so, then insertional mutagenesis that produces mainly null mutations will not be useful for identifying mutations in *lf1*, *lf2*, and *lf3*.

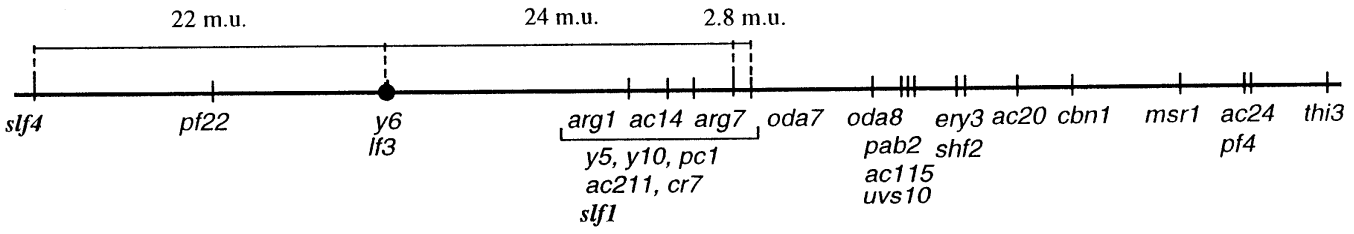

Figure 4.—Genetic map of Chlamydomonas LGI.

A second possibility is that the *LF4* sequence is a frequent target for insertional mutagenesis because it is a physically large locus relative to *lf1*, *lf2*, and *lf3*. Therefore, the *LF4* gene would be more likely to be disrupted by the random insertion of plasmid DNA than would *LF1*, *LF2*, or *LF3*. Alternatively, the *LF4* locus could represent a hot-spot for these integration events. These possibilities will be examined as a consequence of the molecular characterization of this locus. We have obtained clones of genomic DNA from the region flanking the insertion in *lf4-1*. These genomic clones are being used to examine the genomic DNA in all *lf4* alleles to characterize the genetic lesions in these mutants.

Two classes of *lf* mutations: *A priori*, it is likely that two types of length-control mutations will be uncovered. First, mutations in the cellular mechanisms that detect the length of the flagella would cause the cells either to assemble flagella of an improper size or to lose control of size altogether. Second, mutations in genes that regulate the assembly of the flagella, probably under the control of the detection mechanism, could result in improper flagellar size. For example, if a mutation causes cells to be unable to turn off assembly once it was initiated, flagella of excess length would be produced. It is possible that the length-control loci may be separated into these two classes.

Flagellar assembly occurs under two conditions. During each cell cycle, flagella must be reassembled because Chlamydomonas cells resorb their flagella before division. In addition, cells can regrow their flagella after amputation. Some *lf* mutations separate the cellular control for these two assembly events because *lf1-1* and *lf2-3* are obviously able to assemble flagella as part of the cell cycle, albeit to an incorrect size, but these mutants are unable to regenerate their flagella after amputation. Once assembly in these mutants finally begins, flagella of wild-type length or greater are regenerated. Thus, *lf1* and *lf2* mutants are not defective in flagellar assembly *per se*, but in the regulation of flagellar assembly, particularly in response to amputation. This observation suggests that *LF1* and *LF2* may normally function to regulate flagellar assembly rather than to monitor flagellar length.

Genetic interactions between *long-flagella* loci: The synthetic phenotype of double *lf* mutants (*lf1*, *lf2*, and *lf3*) suggests that the gene products either interact to form a functional complex or function independently of each other in redundant pathways. In contrast, the *lf4* mutations do not exhibit synthetic interactions with the other three *lf* loci. Double mutants containing an *lf4* allele all have the lf phenotype of the single mutant (Figure 3). Analysis of double mutants established that *lf4* is epistatic to *lf1*, and that *lf2* is epistatic to *lf4* with respect to flagellar regrowth. If the synthetic *lf1, lf2* double mutant phenotype is the result of the *lf1* and *lf2* products failing to form an interaction necessary for function, one would predict that *lf4* would show the same epistatic relationship to both genes. The fact that the epistatic interactions of *lf4* with *lf1* and *lf2* differ suggests that *lf1* and *lf2* may function in redundant or parallel pathways.

Extragenic suppressor mutations: Extragenic suppressors can be grouped into three classes: informational, interactive, and bypass. The four *slf* extragenic suppressors are unlikely to be interactive suppressors because they do not act in an allele-specific and locus-specific fashion; therefore, the *slf* mutants are probably either informational or bypass suppressors of *long-flagella* mutations. Several observations are consistent with the possibility that the *slf* mutants are informational suppressors. The *slf* mutants have wild-type flagellar length and are able to regenerate flagella with wild-type kinetics following deflagellation. The lack of any other flagellar phenotype beside the suppression is consistent with an informational suppressor model. In addition, the fact that all four *slf* mutations are dominant is consistent with the possibility that they are informational suppressors.

Other characteristics of the suppressor mutations do not readily fit an informational suppressor model. For example, if one of the *slf* mutations is an amber suppressor, it will only suppress mutations caused by an in-frame UAG codon. If a second *slf* mutation is an ochre suppressor, it will only suppress mutations caused by an in-frame UAA codon. The set of alleles that each suppressor can suppress should be unique and nonoverlapping. The data in Table 7 show that the sets of alleles suppressed by each *slf* mutation have some alleles in common and some alleles that are unique to a particular *slf* mutation. For instance, *slf1-1(d)* can only suppress *lf1-1*, *lf2-3*, and *lf3-2*, but not *lf2-2* and *lf3-3*, whereas *slf4-1(d)* can suppress all five of these alleles. This overlap in suppression sets can be explained if some *slf* mutations act as informational suppressors and others function as bypass suppressors. In addition, these suppressors are unlikely to be omnipotent suppressors because they cannot suppress the *arg2*/*arg7* mutation used to build the stable diploids, nor can *sfl1* or *slf3* suppress *pf14*, an ochre mutation (data not shown) (Williams *et al.* 1989).

The lack of allele-specificity for suppression is also consistent with the possibility that some, or all, of the *slf* mutations operate by bypassing the function of the mutant *lf* loci because bypass suppressors may or may not be allele-specific. We do know that it is possible to obtain bypass suppressors of flagellar length control mutations because the *lf4* mutations function as putative bypass suppressors. *lf4* mutations can suppress both the flagellar regeneration defect of *lf1* and the synthetic flagella-less phenotype of *lf1-1 lf2-3*. These mutations appear to function as bypass suppressors for two reasons. First, the data are consistent with the possibility that the *lf4* gene functions in the same pathway as the *lf1* gene. While the original three *lf* loci have synthetic double

mutant phenotypes, the *lf4* alleles have no synthetic interactions with the other *lf* loci. This lack of interaction is consistent with the hypothesis that *lf4* functions in the same pathway as the other *lf* loci. The *lf4 lf1* double mutant has the same flagellar-regeneration phenotype as the *lf4* single mutant; therefore, *lf4* is epistatic to *lf1*. In other words, mutations in *lf4* are able to suppress the regeneration defect of *lf1*. If the suppression was due to a restoration of an interaction between the *LF4* and *LF1* gene products, then the extent of suppression would be highly dependent on specific alleles of *lf4* tested. Because suppression of *lf1* was not found to be specific for individual *lf4* alleles, these mutations most likely act as bypass suppressors of the flagellar regeneration defect. These results fit with a model in which the *lf4* gene product acts downstream of *lf1*.

Second, as discussed earlier, the *lf4* mutations are probably null alleles. The lack of a functional *lf4* gene product is also consistent with a model of bypass suppression. Therefore, it appears that both *lf4* and some *slf* mutations may be functioning as bypass suppressors of the original three *lf* loci.

The authors thank Andrea Kernan, Greg Pazour, and Elizabeth Smith for graciously providing *lf* mutant strains. We thank Craig Amundsen and Shinichiro Enomoto for critical readings of the manuscript. This work was supported by National Institute of General Medical Sciences grant 34437 to P.A.L. C.M.A. was supported by National Science Foundation grant DIR-9113444.

LITERATURE CITED

Barsel, S.-E., D. E. Wexler and P. A. Lefebvre, 1987 Genetic analysis of *long-flagella* mutants of *Chlamydomonas reinhardtii*. Genetics **118:** 637–648.

Cavalier-Smith, T., 1974 Basal body and flagellar development during the vegetative cell cycle and the sexual cycle of *Chlamydomonas reinhardtii*. J. Cell Sci. **16:** 529–536.

Ebersold, W. T., 1956 Crossing over in *Chlamydomonas reinhardii*. Am. J. Bot. **43:** 408–410.

Fernandez, E., R. Schnell, L. Ranum, S. Hussey, C. Silflow *et al.*, 1989 Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA **86:** 6449–6453.

Gorman, D. S., and R. P. Levine, 1965 Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA **54:** 1665–1669.

Harris, E., 1989 The *Chlamydomonas* Sourcebook. Academic Press, San Diego.

James, S. W., L. P. W. Ranum, C. D. Silflow and P. A. Lefebvre, 1988 Mutants resistant to anti-microtubule herbicides map to a locus on the *uni* linkage group in *Chlamydomonas reinhardtii*. Genetics **118:** 141–147.

James, S. W., C. D. Silflow, M. D. Thompson, L. P. W. Ranum and P. A. Lefebvre, 1989 Extragenic suppression and synthetic lethality among *Chlamydomonas reinhardtii* mutants resistant to anti-microtubule drugs. Genetics **122:** 567–577.

Jarvik, J. W., P. A. Lefebvre and J. L. Rosenbaum, 1976 A cold-sensitive mutant of *Chlamydomonas reinhardtii* with aberrant control of flagellar length. J. Cell Biol. **70:** 149a.

Johnson, U. G., and K. R. Porter, 1968 Fine structure of cell division in *Chlamydomonas reinhardtii*. J. Cell Biol. **38:** 403–425.

Kuchka, M. R., and J. W. Jarvik, 1987 Short-flagella mutants of *Chlamydomonas*. Genetics **115:** 685–691.

Lefebvre, P. A., S. A. Nordstrom, J. E. Moulder and J. L. Rosenbaum, 1978 Flagellar elongation and shortening in *Chlamydomonas:* IV. Effects of flagellar detachment, regeneration and resorption on the induction of flagellar protein synthesis. J. Cell Biol. **78:** 8–25.

Lefebvre, P. A., C. M. Asleson and L.-W. Tam, 1995 Control of flagellar length in *Chlamydomonas*. Seminars in Developmental Biology **6:** 317–323.

McVittie, A. C., 1972 Flagellum mutants of *Chlamydomonas reinhardtii*. J. Gen. Microbiol. **71:** 525–540.

O'Brien, S. J. (Editor), 1980 *Genetic Maps: A Complilation of Linkage and Restriction Maps of Genetically Studied Organisms*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ranum, L. P. W., M. D. Thompson, P. A. Lefebvre and C. D. Silflow, 1988 Mapping flagellar genes in *Chlamydomonas* using restriction fragment length polymorphisms. Genetics **120:** 109–122.

Rosenbaum, J. L., J. E. Moulder and D. L. Ringo, 1969 Flagellar elongation and shortening in *Chlamydomonas:* the use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. J. Cell Biol. **41:** 601–619.

Smith, E. F., and P. A. Lefebvre, 1996 *PF16* encodes a protein with armadillo repeats and localizes to a single microtubule of the central apparatus in *Chlamydomonas* flagella. J. Cell Biol. **132:** 359–370.

Smith, E. F., and P. A. Lefebvre, 1997 *PF20* gene product contains WD repeats and localizes to the intermicrotubule bridges in *Chlamydomonas* flagella. Mol. Biol. Cell **8:** 455–467.

Starling, D., 1969 Complementation tests on closely linked flagellar genes in *Chlamydomonas reinhardtii*. Genet. Res. **14:** 343–347.

Tam, L.-W., and P. A. Lefebvre, 1993 Cloning of flagellar genes in *Chlamydomonas reinhardtii* by DNA Insertional Mutagenesis. Genetics **135:** 375–384.

Williams, B. D., M. A. Velleca, A. M. Curry and J. L. Rosenbaum, 1989 Molecular cloning and sequence analysis of the Chlamydomonas gene coding for radial spoke protein 3: flagellar mutation pf-14 is an ochre allele. J. Cell Biol. **109:** 235–245.

Communicating editor: V. G. Finnerty