Analysis of Flagellar Size Control Using a Mutant of *Chlamydomonas reinhardtii* with a Variable Number of Flagella

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ABSTRACT A mutant of *Chlamydomonas reinhardtii* with a variable number of flagella per cell has been used to investigate flagellar size control. The mutant and wild-type do not differ in cell size nor in flagellar length, yet the size of the intracellular pool of flagellar precursor protein can differ dramatically among individual mutant cells, with, for example, triflagellate cells having three times the pool of monoflagellate cells. Because cells of the same size, but with very different pool sizes, have flagella of identical length, it appears that the concentration of the unassembled flagellar precursor protein pool does not regulate flagellar length. The relation between cell size, pool size, and flagellar length has also been investigated for wild-type cells of different sizes and ploidies. Again, flagellar length appears to be maintained independent of pool size or concentration.

Size control is an essential concern for any cell, and yet the way in which a cell controls size is not understood in even a single case. The importance of a size regulation system is obvious for growing cells, because growth and division must be precisely coordinated to maintain cell size from generation to generation (3, 4, 26) Whatever the molecular mechanism of size control may be, a number of investigations clearly show that it determination is a homeostatic phenomenon in which cell size anomalies, whether induced naturally (9) or experimentally (20), are corrected.

Like total cell size, organelle size must also be controlled to maintain balanced cell growth, or to express particular differentiated states. The flagella of the biflagellate unicellular eucaryote *Chlamydomonas reinhardtii* are organelles whose size and number are closely regulated. Amputation of one or both flagella triggers a host of complex events, including commencement of flagellar assembly through the mobilization of an intracellular flagellar precursor protein pool (21), transcription of specific flagellar protein genes (12, 25), translation of the newly transcribed messages (12), use of the newly synthesized protein to continue flagellar regeneration (11, 21), cessation of net flagellar assembly at the appropriate length (11, 21), replenishment of the intracellular pool (11), and cessation of specific transcription/translation (11). As a consequence of these events, the original state of the cell is restored within 90 min of amputation. The complex response by *C. reinhardtii* to flagellar amputation may involves flagellar size detection both at its beginning, when the detection of flagellar shortness may lead to the mobilization of the pool for assembly, and at its end, when the detection of flagellar full-length may lead to the cessation of net flagellar assembly.

Flagellar size control in *Chlamydomonas* is not confined to periods of flagellar growth or regeneration; rather, it appears to be a constant cellular activity. Thus, if the flagella are caused to resorb partially and then are released from resorption conditions, normal length is quickly restored (11). Or, when quadriflagellate dikaryons are constructed by mating wild-type with certain long (6) or short (7) flagellamutants, normal length control is rapidly imposed, with the long or short flagella being adjusted to wild-type length. Therefore, it appears that the cell constantly monitors the length of its flagella and takes appropriate action if the length is not right.

In this paper we describe experiments using a mutant with an abnormal flagellar number, and using cells that differ from one another in size but not in flagellar number, to test the hypothesis that the cell regulates flagellar length by controlling the level of some assembly-limiting component of the precursor protein pool. The results show that pool size and flagellar length are not dependent on one another in any simple way, thereby suggesting that the hypothesis is invalid. Other recent results from our laboratory, based on experiments using a short-flagella mutant, indicate the same thing, and further suggest that flagellar length is not dictated by some intrinsic property of flagellar assembly whereby longer flagella assemble
less readily (7). The basis for flagellar length control thus remains an intriguing mystery.

MATERIALS AND METHODS

Strains and Culture Conditions

Wild-type strains used were the 137c derivatives NO mt- and NO mt+ provided by Dr. U. Goodenough. Strains CC47 (arg-2 mt+) and CC51 (arg-7 mt+), which were used for diploid construction, and strain CC530 (ac-17 mt+), which was used for centromere mapping, were provided by the Chlamydomonas Genetics Center, Duke University, Durham, N. C. Cells were grown at 25°C in liquid media. Each tube was observed under a dissecting microscope (8). Strains showing slow or no motility phenotype; some cells were nonmotile whereas others showed aberrant swimming. On closer inspection, the mutant was found to have a variable number of flagella per cell, with the number of flagella on individual cells varying from zero to six. A frequency distribution for cells of different flagellar number is shown in Fig. 1. This distribution was found to be the same for vegetative cells or for gametes. A Nomarski differential interference contrast micrograph showing two mutant cells, one with two flagella and one with four flagella, is shown in Fig. 2; note that, as in wild-type cells, all the flagella of the mutant originate at the anterior end of the cell. Cell size in a growing population is variable but no more so than for wild-type, ranging from ~100 μm² in small cells to ~600 μm² in large cells. There is no apparent relationship, however, between cell size and flagellar number—i.e., a large cell is no more likely to have excess flagella than a small cell. It is important to point out that, although mutant 220 is abnormal with respect to flagellar number, it is quite normal with respect to flagellar length. The original vfl-2-220 isolate was used in all experiments reported here, except where noted otherwise.

Genetic Analysis

34 mutant × wild-type tetrads were dissected, and in each the variable flagellar number trait segregated 2:2. Thus, the mutant phenotype appears to represent the expression of a single mendelian mutation. We have named the gene carrying the mutation vfl-2 (for variable number of flagella), and we denote the mutation under study here as vfl-2-220. To determine centromere linkage, a vfl-2-220 mt+ strain was crossed to a ac-17 mt- strain, and 25 tetrads were dissected and analyzed. The ac-17 marker is tightly linked to its centromere on linkage group III. Of the 25 tetrads, 6 were parental ditype (PD), 8 were nonparental ditype (NPD), and 11 were tetratype (T). vfl-2 is thus unlinked to ac-17, and is ~22 map units from its own centromere. Based on this analysis, vfl-2 appears to be distinct from vfl-1, which was isolated by M. Adams and D. Luck and which has been located 3 map units from the centromere on the right arm of linkage group VII (M. Adams, personal communication).

Single Cell Pedigree Analysis

Single vfl-2-220 cells were immobilized in 0.5% agar, and flagellar number was recorded before and after cell division.

RESULTS

Isolation and Initial Characterization of a Mutant with Variable Flagellar Number

Wild-type C. reinhardtii was mutagenized with ultraviolet light, and individual clones were screened for aberrant motility. Mutant 220 was first identified on the basis of a complex frequency distribution of flagellar number on vegetative mutant cells. Cells with five or six flagella were present at a frequency of ~0.1%. A grand total of 2,162 cells were scored. In wild-type cultures, cells with two flagella typically make up >95% of the total and cells with 1 and 3 flagella always make up <1%.
The results for 15 randomly chosen cells are shown in Table I. There seems to be no general rule of segregation for flagellar number in the mutant cells. In just these 15 cases, cells with 2 flagella gave rise to daughters or granddaughters with 0, 1, 2, 3, or 4 flagella; cells with 1 flagellum produced cells with 0, 1, or 2 flagella; cells with 3 flagella gave progeny with 0 or 2 flagella; and cells with no flagella gave rise to progeny with 0, 1, or 2 flagella. These data demonstrate that it is possible to obtain the entire spectrum of flagellar number classes from a single vfl-2-220 cell in just a few generations.

Two properties of mutant populations are consistent with the above observations. First, all clones derived from single mutant colonies show identical distributions of flagellar number classes; it is not possible to clone a line that is pure with respect to flagellar number. Second, when mutant cultures are grown continuously over periods of many weeks with repeated dilution to fresh medium, the distribution of flagellar number classes remains constant.

**Flagellar Regeneration in vfl-2-220 and Wild-type Cells**

Cultures of vfl-2-220 and wild-type cells were deflagellated and the kinetics of regeneration, with and without new protein synthesis, were determined. Flagellar number, as well as flagellar length, was scored for each mutant cell examined. The results are plotted in Fig. 3. They can be easily summarized by stating that neither the kinetics of regeneration nor the final lengths achieved show any dependence on flagellar number. Furthermore, the distribution of flagellar number classes in the mutant cultures was the same before and after regeneration, suggesting that each cell regenerates the same number of flagella with which it began.

To examine whether or not the partial regeneration that takes place in the absence of protein synthesis is due to the exhaustion of some limiting pool component, two refflagellation experiments were performed. In the first experiment, cells were deflagellated, allowed to regenerate for 120 min (i.e., to completion), deflagellated a second time, and, finally, allowed to regenerate for an additional 180 min. Regenerations were carried out in the presence and absence of cycloheximide. Cycloheximide, when present, was added at the time of the first deflagellation and was maintained continuously in the culture thereafter. It was found that the cells in cycloheximide failed to regenerate at all after the second deflagellation, suggesting that they had fully exhausted some component necessary for assembly. They had not lost the potential to regenerate, however, because if they were washed free of cycloheximide after the second deflagellation, they regenerated full length flagella.

A refflagellation experiment similar to the one just described was also performed, but here the second deflagellation was scheduled after just 20 min of regeneration, at a time before the flagella had stopped growing. This time, flagellar regeneration occurred after both deflagellations in the presence of cycloheximide, and the length achieved after the second regeneration had reached completion was such that the total amount of flagellar growth from both regenerations was very nearly equal to the amount achieved when the first regeneration was allowed to go to completion (Table II). These results again suggest that the flagellar length achieved in cycloheximide is determined by some limiting component of the unassembled precursor protein pool, and they show that more than one initiation of flagellar assembly can take place in the absence of protein synthesis. The possibility that the above results represent a time-dependent decay, in the presence of cycloheximide, of cellular regeneration capacity—a decay that would occur with or without flagellar growth—was ruled out in the following experiment. NO m^7 cells were preincubated for 1 h in cycloheximide medium, deflagellated, and monitored for flagellar regeneration in the same medium. Regeneration was observed, and its extent was comparable to that of nonpreincubated controls. (Preflagellation lengths: 11.0 ± 1.5 μm with preincubation and 11.4 ± 1.2 μm without. Flagellar lengths 90 min after deflagellation: 6.0 ± 1.2 μm with preincubation and 5.0 ± 1.5 μm without.)
FIGURE 3 Flagellar regeneration kinetics of mutant vfl-2-220 cells with 1, 2, or 3 flagella in the presence (dashed lines) and absence (solid lines) of cycloheximide. (○) cells with 1 flagellum; (□) cells with 2 flagella; (△) cells with 3 flagella. Each point represents measurements of 20 cells. Standard deviations of all points were <1.8 μm.

TABLE II Flagellar Lengths Attained After One or Two Deflagellations in Cycloheximide

| Final length at 180 min | Length 20 min after deflagellation | Deflagellation at 0 and again | Deflagellation only at 0 |
|------------------------|-----------------------------------|-----------------------------|-------------------------|
| Cell Type              |                                   |                             |                         |
|                        | Original length | μm | μm | μm | μm | μm |
|                        | μm | μm | μm | μm | μm |
| With cycloheximide     |                                   |                             |                         |
| Wild type              | 9.4 ± 1.2* | 1.3 ± 1.0 | 1.6 ± 1.1 | 3.0 ± 1.3 |                         |
| vfl-2-220 monoflagellates | 9.7 ± 1.3 | 1.9 ± 0.8 | 2.4 ± 1.0 | 4.1 ± 1.2 |                         |
| vfl-2-220 biflagellates | 9.7 ± 1.3 | 1.6 ± 0.6 | 2.5 ± 0.6 | 3.7 ± 1.0 |                         |
| vfl-2-220 triflagellates | 9.4 ± 1.6 | 1.7 ± 0.8 | 2.1 ± 0.7 | 3.5 ± 1.1 |                         |
| Without cycloheximide  |                                   |                             |                         |
| Wild type              | 9.4 ± 1.2 | 2.2 ± 1.1 | 7.8 ± 1.2 | 8.5 ± 1.2 |                         |
| vfl-2-220 monoflagellates | 9.7 ± 1.3 | 2.4 ± 1.2 | 6.4 ± 1.3 | 8.9 ± 1.3 |                         |
| vfl-2-220 biflagellates | 9.7 ± 1.3 | 2.3 ± 0.5 | 8.3 ± 1.3 | 8.4 ± 1.0 |                         |
| vfl-2-220 triflagellates | 9.4 ± 1.6 | 2.0 ± 0.6 | 9.1 ± 1.5 | 8.6 ± 1.5 |                         |

Drug was added at time of first deflagellation and was maintained in cultures thereafter. Each point represents measurements of 20 cells.

* Lengths expressed ± SD.

Measurements of Flagellar Length, Pool Size, and Cell Size in Diploid and Haploid Cells

The sizes of the flagellar precursor protein pools for diploid and haploid vegetative and gametic cells of different cell volumes were determined. Cell body size was scored along with flagellar length before and after deflagellation in the presence and absence of cycloheximide. Table III shows average length values for cells of different sizes, and Fig. 4 shows representative data from the same experiments displayed in scatter plot form. Although there is a rather broad distribution of cell size in both haploid and diploid cells, diploid vegetative and gametic cells, and the same can be said for haploid and diploid vegetative cells. Nor is there a consistent difference in pool size between haploid and diploid vegetative cells of the same size. There may be some positive correlation between cell size and pool size, particularly for gametes, but the scatter in the data is such that this relationship cannot be used to predict the pool size of any particular cell (Fig. 4).

DISCUSSION Flagellar Length Is Not Dependent on Pool Size

The eukaryotic axoneme is extremely complex, containing well in excess of 100 structural proteins (19). However, because...
Several models for flagellar length determination have been proposed which hypothesize that the flagellar assembly process is intrinsically more difficult, and therefore slower, at longer flagellar lengths (1, 14). These models have the virtue of accounting for the well-established fact that flagellar regeneration kinetics are deceleratory. However, recent experiments from our laboratory using a short-flagella mutant of C. reinhardtii suggest that these models are inadequate to explain length regulation (7). The results presented here also argue against a model of the sort just mentioned. It could be imagined that, if assembly of longer flagella is intrinsically less favorable than assembly of shorter flagella, flagellar length would be proportional to the size or concentration of the pool. If this were the case, then monoflagellate vfl-2 cells, whose pools are only one-third the size of those of triflagellate cells, should have considerably shorter flagella. In fact, they do not.

The Extent of Regeneration in Cycloheximide Really Indicates the Quantity of Some Limiting Component

We have presented evidence here (Table II) indicating that the premature cessation of flagellar growth characteristic of regeneration in cycloheximide represents the exhaustion of some pool component. In the absence of new protein synthesis, cells regenerate to about one-half length. If deflagellated again, they are incapable of a second regeneration, as though some limiting component was used up during the first. If, on the other hand, the second deflagellation occurs when the flagella have grown to only one-quarter length, they do regenerate a second time, but now to a final length of one-quarter. These results are exactly what is expected if there is a pool that limits the extent of flagellar growth, because \( \frac{1}{4} + \frac{1}{4} = \frac{1}{2} \). The results are not consistent with an alternative possibility: that in the absence of protein synthesis the usual length determining system operates, but that for unknown reasons it regulates length to an abnormal, short value. If this were the case, then the flagella should again grow to one-half length; in fact, they do not. The results also indicate that the length "regulation" observed in the presence of cycloheximide is not analogous to that which occurs when protein synthesis is allowed, because under those more normal conditions, pool exhaustion clearly does not limit flagellar growth (21).

Although the extent of regeneration in the absence of new protein synthesis appears to reflect the amount of some limiting protein, or proteins, in the pool, it obviously says nothing about the specific identity of the protein(s). Indeed, we know of no data that point to any particular protein as being limiting, nor is it known whether one, or many, of the flagellar proteins exist in the pool in potentially limiting amounts.

No Simple Relation Exists Between Cell Size, Flagellar Length, and Pool Size

Additional evidence that flagellar length is not determined by pool size comes from measurements of cell size, flagellar length, and pool size for cells of different sizes, ploidies, and states of differentiation (Table III). In summary, we find no simple relation between the three quantities. For example, small haploid gametes (50–150 \( \mu \text{m}^3 \) cell volumes) have 25.6 \( \mu \text{m} \) of flagella (2 flagella, each 12.8 \( \mu \text{m} \) long) and 7 \( \mu \text{m} \) of pool (2 \( \times 3.5 \mu \text{m} \)) whereas diploid gametes of the same size have 22.8 \( \mu \text{m} \) of flagella and 10.6 \( \mu \text{m} \) of pool. Thus, the cells with the longer flagella have the smaller pools. In contrast, large (450–550 \( \mu \text{m}^3 \)) haploid vegetative cells have 22.4 \( \mu \text{m} \) of flagella and 13 \( \mu \text{m} \) of pool, whereas diploid vegetative cells of the same size have 24.2 \( \mu \text{m} \) of flagella and 14.4 \( \mu \text{m} \) of pool. Here the cells with the longer flagella also have the larger pools. Although none of the differences observed is dramatic, we feel that these

![Figure 4](image-url)
data provide some support for the notion that pool size does not determine flagellar length.

The Relation of vfl-2 to Other Known Genes Influencing Flagellar Number

As was mentioned in Results, a mutation conferring variable flagellar number was isolated by Adams and Luck and given the name vfl-1. This mutation was mapped to the right arm of linkage group VII, three map units from the centromere. The mutation described here, vfl-2-220, is unlinked to its centromere, and so it is not an allele of vfl-1. In addition, the phenotypes of the two differ. The flagellar number distribution in vfl-1 cultures closely fits a poisson distribution (M. Adams, personal communication), whereas the distribution in a vfl-2 culture clearly does not (Fig. 1). A few other reports of Chlamydomonas mutants with abnormal flagellar number exist (15, 24), but these mutants differ dramatically from vfl-2 in phenotype in that their primary defects appear to be in cell division, so that cultures contain large multinucleate, multiflagellate cells. Vfl-2 cells, in contrast, are normal in size, and, based on microscopical observations, they are mononucleate.

Could the Pool be Compartmentalized

The existence of a flagellar precursor pool is well-established but nothing is known about its location. In interpreting the results reported in this paper, we have assumed, implicitly or explicitly, that pool volume is proportional to cell volume, so that a pool of a given size would be more concentrated in a small cell than in a large cell. If this is not the case, then, by definition, the pool (i.e., the length-limiting pool component(s), if they exist) must be compartmentalized. It could be, for example, that each flagellum has its own pool, so that a triflagellate cell would have three times the pool size of a monoflagellate, while having an identical concentration of subunits in the pool. If such individual pools exist, however, their respective contents must be rather free to mix. Thus, when one of the two flagella of a cell is amputated, the remaining flagellum is resorbed as the lost one regenerates, and the resorbed material from the one is apparently used in the assembly of the other (21). Also, in many mutant/wild-type or mutant/mutant quadriflagellate dikaryons generated by mating, it has been demonstrated that protein from the pool donated by one parent appears in the flagella donated by the other (5, 19).

Support for this work was provided by grant PCM-7823253 from the National Science Foundation. J. W. Jarvik is the recipient of Research Career Development Award K04 AM00710 from the National Institutes of Health.

Received for publication 15 June 1981, and in revised form 24 August 1981.

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