TEMPERATURE-SENSITIVE MUTATIONS AFFECTING FLAGELLAR ASSEMBLY AND FUNCTION IN *CHLAMYDOMONAS REINHARDTII*

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ABSTRACT

A series of conditional mutants of the algal, biflagellate *Chlamydomonas reinhardtii* with temperature-sensitive defects in flagellar assembly and function were isolated. The genetics and phenotypes of 21 mutants displaying a rapid alteration in flagellar function upon shift from the permissive (20°C) to the restrictive (32°C) temperatures are described. These mutants designated as "*drop-down*" or *dd*-mutants have been placed in four categories on the basis of their defective phenotypes: (*a*) *dd-assembly* mutants - the preformed flagella are resorbed at 32°C and reassembly of flagella is inhibited; (*b*) *dd-fragile flagella* mutants - the flagella are lost by detachment at 32°C, but can be reassembled; (*c*) *dd-motility* mutants - the flagella are retained at 32°C, but are functionally defective; (*d*) *dd-lethal* mutants - display combined defects in flagellar function and cell growth. Tetrad analysis of the mutants back-crossed to wild-type, recombination analysis of intermutant crosses, and complementation tests in the construction of heterozygous diploid strains indicate that at least 14 nuclear genetic loci are represented among the 21 mutants. The availability of temperature-sensitive mutations affecting the assembly and function of the flagellum suggests that the morphogenesis of this complex eukaryotic organelle is amenable to genetic dissection.

In an attempt to study the genetic control of flagellar assembly and to investigate interrelationships between cellular microtubules and those of the flagellar axoneme, we have undertaken the isolation of mutants of *Chlamydomonas reinhardtii* showing defects in flagellar assembly and function. The feasibility of this approach was demonstrated by Randall and his colleagues who have made extensive investigations of nonconditional mutants of this organism showing alterations of flagellar length and motility (18–20, 28). In contrast to these investigators, we have chosen to search for conditional (temperature-sensitive) mutants for several reasons: (*a*) Genetic analysis by recombination or by complementation in diploids in *Chlamydomonas* requires the presence of flagella. Since mutations for some of the structural components of the flagellum might result in the nonassembly of flagella, only in the conditional state is genetic analysis of such lesions possible. (*b*) If genes exist which determine structural or regulatory components common to axonemal and cytoplasmic microtubules, mutants affecting these sites might be lethal. (*c*) With thermal-sensitive mutants, careful phenotypic observations during temperature shifts give important clues which may help the search for a molecular diagnosis. (*d*) At

permissive temperatures, mutant gene products can be expected to occupy the normal site and could be isolated for molecular analysis.

As first shown by Lewin (16) and more recently by Randall and his colleagues (for a recent review, see reference 21), *Chlamydomonas* is an ideal organism for these studies. Mutants showing altered flagellar activity may be recovered in a sedimenting pellet and may be identified during growth on solid medium by their characteristic colony morphology (18). The genetics of this organism have been well studied (15) and mutants may be characterized by recombination analysis in mating cells or by complementation analysis in temporary dikaryons (26) and stable diploids (4, 5). The structure of the organism's two flagella have also been well described (9, 23) as have the microtubular systems which are present during interphase, mitosis, and cytokinesis (12). Flagella may be experimentally amputated and their subsequent regeneration used to study morphogenesis (19).

In this paper, we describe the types of thermal sensitive mutants obtained in an extensive search using three methods of chemical mutagenesis and several protocols for enrichment and selection. In the studies to be described, 20°C was chosen as the permissive temperature and 32°C as the nonpermissive condition.

MATERIALS AND METHODS

Strains and Culture Media

Wild-type *C. reinhardtii* strains 89 (mt^-) and 90 (mt^+) were obtained from the Culture Collection of Algae at Indiana University. Wild-type strain 137c which was used for most of the mutagenesis experiments was kindly supplied by Margaret Hudock.

The minimal culture medium used for all vegetative cultures and maintenance of stocks is based on minimal I of Sager and Granick (25) in which ferric chloride was replaced by 0.01 g/liter ferric citrate and 0.01 g/liter citric acid. In more recent experiments, following the recommendation of Z. Ramanis (personal communication), the original medium has been further altered: $MgSO_4$ is reduced to one-half the concentration of the original formula, and $CaCl_2$ to one-third. When it was desirable to use acetate-supplemented medium, we used Tris-acetate phosphate medium (TAP) (8). Solid media were prepared by the addition of appropriate quantities of agar (Difco Laboratories, Detroit, Mich.).

Chemical Induction of Mutants

Three methods of mutagenesis have been used according to the following protocols:

METHOD 1: MUTAGENESIS WITH *N*-METHYL-*N'*-NITRO-*N*-NITROSOGUANIDINE (MNNG) DURING RANDOM EXPONENTIAL GROWTH AND AT HIGH KILLING EFFICIENCY: Cells were inoculated into minimal medium at a density of 2×10^4 cells/ml and allowed to grow overnight at 20°C in constant light without aeration. The cells were then sedimented by centrifugation and concentrated 12-fold by resuspension in sterile 0.1 M citrate buffer, pH 5. MNNG (Aldrich Chemical Co., Milwaukee, Wis.) (4 mg/ml in citrate buffer, sterilized by Millipore filtration) was added to a final concentration of 50 μg/ml. After 15 min at room temperature with occasional shaking, the cells were sedimented, washed in growth medium, resedimented, and finally resuspended in approximately one-half of the initial volume. In four experiments, this procedure yielded 1.1–2.3% survivors.

METHOD 2: MUTAGENESIS WITH MNNG IN SYNCHRONIZED CULTURES DURING THE TIME OF NUCLEAR DNA REPLICATION: Cells were synchronized by growth in minimal medium, with forced aeration of CO_2 in compressed air, on an alternating light/dark cycle of 12 h. Following the procedures of Lee and Jones (13), the cells were exposed to MNNG at approximately 2 h into the dark cycle when 2–3% of the cells appeared as division forms. Cultures at a density of $1–2 \times 10^5$ cells/ml were centrifuged and resuspended in 0.02 M citrate buffer, pH 5.0, at a 10-fold higher density. MNNG was added to a final concentration of 5 μg/ml and the suspension stirred for 10 min at 20°C. Cells were washed in growth medium for two cycles, and suspended in 50% the initial volume. In three mutant runs the procedure resulted in 70–75% survivors.

METHOD 3: MUTAGENESIS WITH METHYL-METHANO SULFATE (MMS): Following the procedure of Lopez (17), light-synchronized cells at a density of $\sim 5 \times 10^5$ were collected 3–4 h into the light cycle, and resuspended in 0.02 M phosphate buffer, pH 7.0, at a density of 1.0×10^6 cells per ml; 0.1 vol of 0.13 M MMS (Aldrich Chemical Co.) was added and the mixture held at 22°C for 4 h with constant stirring. Aliquots of 2.5 ml of the cell suspension were added to 250 ml of minimal medium. The mutagenic efficiency of the three protocols was tested by the rate of induction of streptomycin-resistant clones (at 50 μg/ml) in a wild-type strain (see Table I).

Enrichment Techniques

After mutagenized cells were allowed to undergo one or two division cycles to express their phenotypes, efforts were made to enrich the cell suspensions for a class of mutants which would have normal motility at 20°C and abnormal motility at 32°C. The general plan for enrichment involved a two-step series—the first step at 20°C to discard nonswimmers and the second at 32°C to collect nonswimmers. To improve the selectivity of the second step, we turned after the first few experiments to the use of

TABLE I

Frequency of Streptomycin-Resistant Mutants after Chemical Mutagenesis

Mutagenesis method	Clones viable	Resistant clones/10^6 survivors
	%	
(1) MNNG 50 μg/ml—exponential growth	2.2	282
(2) MNNG 4 μg/ml—synch. cells dark cells	72	169
(3) MMS 0.013 M—synch. cells light cycle	78	63

After mutagenic treatment as indicated, strain 137c cells were plated at ~10^6 cells/dish on 20 ml of TAP medium containing yeast extract and 1.5% agar. After 24-h growth at 25°C in the light, the plates were overlayed with 10 ml of the same medium containing streptomycin 150 μg/ml; growth was continued for 5-6 days. In control experiments, the spontaneous frequency of resistant clones was $3.4/10^6$ survivors. Scoring of resistant clones was rechecked by streaking on streptomycin-containing plates. For details of mutagenesis methods 1-3, see Materials and Methods.

light-synchronized cultures, so that enrichment techniques could be applied during the early light cycle when the population of nonswimming cells would be least likely to include cells in the process of cell division. The following methods were used to obtain each step of enrichment.

TECHNIQUE 1: ENRICHMENT FOR SWIMMERS AT 20°C: (*a*) Cells were held in 250-ml conical centrifuge bottles (Bellco Glass Company, Vineland, N. J.) for 16 h, before collection of the supernate by siphonage under pressure of Millipore-filtered compressed air. (*b*) Cells in 250-ml centrifuge bottles were centrifuged for 30 min at 1,400 rpm in an IEC PR-2 centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.). Without disturbing the small pellets, the bottles were held at 20°C for ~30 min and swimming cells recovered by siphonage or by careful decanting. (*c*) Cell suspensions (250–300 ml) were placed in a separatory funnel and held at 20°C for 2–3 h, and the lower 50 ml were discarded.

TECHNIQUE 2: ENRICHMENT FOR NONSWIMMERS AT 32°: (*a*) Cells were held in 250-ml centrifuge bottles for 16 h at 32°C, the supernates were removed by siphonage and discarded. (*b*) Cells were exposed to 32°C for 1.5–3 h, pelleted in centrifuge bottles (see 1 *b* above) and held at 32°C for 30–60 min to allow swimmers to escape. The supernate was discarded. (*c*) Cells were deflagellated by the method of Watson and Hynes (28) and regrowth was allowed to take place for 1 h at 32°C. The suspension was then processed as in 2 b above or by incubation in a 4 × 25-cm cylindrical column for 3–4 h, after which the lowest 2–5 ml was withdrawn. (*d*) Cells were deflagellated as in 2 c, suspended at a density of ~7.5×10^6 in minimal medium to which 3% Ficoll had been added, and allowed to regenerate at 32°C for 30 min. The 20-ml cell suspension was then placed as an underlay in a 4 × 25-cm column containing 250 ml of minimal medium (32°C). The column was opaque in its lower two-thirds, and swimming cells were observed to accumulate within minutes in the upper lighted portion. After 1–1.5 h at 32°C the lowest 5 ml was collected.

Cloning and Testing Clones

Aliquots of cell suspensions made after the second step of enrichment were spread on minimal medium 1.5% agar plates. Since these suspensions are enriched in dead and dying cells, the particle count had to be corrected by careful microscope examination of the cells. Prediction of viability was difficult and in practice it was necessary, after correcting for cells which appeared to be damaged by phase microscopy, to assume that 50–75% of the remaining cells would not survive. Plates were grown at 20°C under high light intensity.

In some cases a selection step was added at the time of cloning. Instead of the procedure just described, cells were spread on TAP medium 2.5% agar plates and allowed to grow at 32°C under high light intensity. Under these conditions clones of nonmotile cells can be distinguished by their tight colony morphology.

After growth on plates, colonies were picked up, transferred to small tubes containing 3–4 ml of minimal medium, and grown at 20°C to a density approximating 10^5 cells/ml. Cultures in which actively swimming cells formed a layer at the surface were transferred at 32°C and the behavior at 6 h and 24 h was observed.

Genetic Analysis by Recombination

Standard procedures (6) were used for genetic analysis. Because of the temperature-sensitive motility phenotype of the mutants, gametogenesis, mating, and zygote maturation were allowed to occur at 20°C. Strains to be crossed were grown on minimal medium 1% agar plates for 5–7 days. For gametogenesis, the cells were suspended in nitrogen-free medium (minimal medium without NH_4NO_3) for ~ 6 h. After the gametes were allowed to differentiate, they were mated for ~ 2 h and subsequently plated on minimal medium 4% agar plates. The plates were incubated for 18–22 h in the light and then placed in the dark for zygote maturation.

For tetrad analysis, individual zygotes were dissected on TAP medium 1.5% agar plates. Zoospore colonies produced by the meiotic products of the zygotes were individually tested for their swimming behavior at 20 and 32°C. Each daughter clone was inoculated into a small test tube containing 4 ml of medium and grown at 20°C. The test tubes were transferred to 32°C and the motility characteristics of the clones evaluated after incubation at the high temperature for 6–24 h.

To determine recombination frequencies between two mutants in larger numbers of zygotes, we utilized the method of zygote plating (14). Mature zygotes were plated on TAP medium 2.5% agar plates. The products from each zygote were allowed to grow as a single colony

at 25°C. In each experiment at least 90% of the plated zygotes germinated and gave rise to testable colonies. Zygote colonies containing meiotic products resulting from a recombination event were identified by the presence of a proportion of wild-type swimmers in liquid cultures at 32°C.

Construction of Diploid Strains

Selected mutants were crossed to *arg-2* and *arg-7* mutants mapping close together on linkage group I to obtain double mutants for construction of diploids (5). Mating mixtures of appropriate mutants were plated on TAP medium 4% agar plates, incubated at 20°C, and illuminated at ~ 2,000 lux. After 5 days of growth several presumptive diploid clones from each mating were isolated.

Confirmation of the diploid status of the strains was obtained by the following criteria: (*a*) lack of arginine requirement for growth, (*b*) mating type determination as minus (5), (*c*) detection of 2 N DNA/cell by the method of Burton (1) as modified by Sueoka et al. (27), and (*d*) recovery of parental phenotypes in back-crosses of the diploids to *wt-137*c.

Flagellar Amputation and Quantitation of Regeneration

Light-synchronized cultures in log phase of growth (density ~5 × 10^5 cells/ml) were deflagellated by the method of Watson and Hynes (29) between the first 2–4 h of the light cycle and concentrated to a density of ~ 2 × 10^6 cells/ml in culture media for the period of regeneration. Regenerating cultures were incubated in temperature-controlled water baths, illuminated at ~ 2,000 lux.

Samples of regenerating cultures were taken at regular intervals and fixed by the addition of equal volumes of 1% glutaraldehyde in 0.01 M sodium phosphate buffer, pH 7.2. Fixed samples were stored in the cold until the end of the experiment. Two methods for quantitation of flagellar regeneration were utilized. (*a*) Under conditions in which regeneration occurred relatively synchronously among cells within a population, average flagellar lengths ± 1 SD were determined by direct measurement of both flagella on 50–100 cells. The flagella were measured under a phase microscope at a magnification of 1,500 with an ocular micrometer. Flagellar lengths refer to the distance between the flagellar tip and the outer limit of the cell wall. (*b*) Under conditions in which regeneration did not occur with a high degree of synchrony among cells within a population, the regenerative capacity of the populations was quantitated by scoring the number of cells which appeared, at the light microscope level, to have completed flagellar reassembly. For each time point the percentage of fully-deflagellated cells was determined by observations made on at least 500 cells.

Electron Microscopy

Cell suspensions were initially fixed by the addition of 12% glutaraldehyde in 0.02 M sodium cacodylate buffer, pH 7.2, to give a final concentration of ~2.5% glutaldehyde. A brief 5-min exposure to glutaraldehyde at room temperature, the samples were pelleted, resuspended in a combination of cold 1.5% glutaraldehyde and 0.5% osmium tetroxide in 0.01 M sodium cacodylate buffer, pH 7.2, and fixed for 1 h on ice. After fixation, the cells as a pellet were dehydrated through a graded series of ethanol, passed through propylene oxide, and embedded in Epon 812. Sections cut on a Porter-Blum-MT-2 were stained with uranyl acetate and lead citrate (22) and examined in a Siemens Elmiskop I or Philips 300 electron microscope.

RESULTS AND DISCUSSION

Classification of Conditional Motility Mutants

During the course of these investigations, a series of conditional mutants of *C. reinhardtii* with thermosensitive defects in their motility properties were isolated. At the permissive temperature of 20°C, all of the mutants resemble wild-type strains and preferentially swim in a thin layer at the top surface of liquid cultures. However, at the restrictive temperature of 32°C they are unable to swim normally and are found pelleted at the bottom of liquid culture tubes.

In screening the conditional mutants at the phenotypic level, we noted that the mutants generally fell into two discrete classes. One group, which we have classified as 1° conditional swimming mutants, are characterized by a rapid loss of wild-type motility upon shift from 20 to 32°C. The other group, designated as 2° conditional swimming mutants, display a more delayed response to temperature shift.

1° CONDITIONAL SWIMMING MUTANTS: These mutants designated "*drop-down*" or *dd* mutants begin to pellet within 1 h of exposure to the high temperature and are completely pelleted by 4–6 h. Phenotypically, the *dd* mutants fall into four categories on the basis of the effect of high temperature on the functional and structural stability of the flagella, on their ability to assemble flagella, and on their viability. *dd-assembly mutants (dd-a mutants):* Flagella preformed at 20°C are resorbed upon exposure to 32°C, and reassembly of flagella is inhibited at the restrictive temperature. *dd-fragile flagella mutants (dd-frag mutants):* Flagella are lost by detachment but can be

reassembled at 32°C. *dd-motility mutants (dd-m mutants):* Flagella are retained at 32°C, but are functionally defective. *dd-lethal mutants (dd-l mutants):* Mutants displaying combined defects in flagellar function and cell growth.

2° CONDITIONAL SWIMMING MUTANTS: These mutants show no immediate swimming defects upon shift from 20 to 32°C; loss of wild-type motility is detected only after exposure to 32°C for 12 h or more. Four basic phenotypes have been identified. *Conditional Flagellar Motility Mutants:* Flagella are present but functionally defective at 32°C. In some of the mutants falling into this category the delay in expression of the motility defect appears to be associated with assembly of defective flagella after cell division at 32°C. This is supported by the observation that when the mutants grown at 20°C are experimentally deflagellated and allowed to regenerate at 32°C, the defect in flagellar motility is immediately expressed. *Conditional Flagellaless Mutants:* Flagella are not formed in the daughter cells produced at 32°C. To determine whether the flagellar nonassembly phenotype in these mutants is expressed independent of growth and cell division at 32°C, the ability of the mutants grown at 20°C to regenerate flagella at the high temperature was tested. Each of the mutants has been found to undergo at least three consecutive cycles of flagellar regeneration at 32°C, suggesting that inhibition of flagellar formation may be dependent upon division at the restrictive temperature. *Conditional Lethal Mutants:* Prolonged exposure to 32°C results in cell death. Loss of wild-type flagellar function precedes cell death. *Conditional Palmella Mutants:* At 32°C, nonmotile clumps of cells surrounded by thick cell walls are formed.

In our preliminary analysis of the conditional mutants, we attempted to select for in-depth study a group most likely to include thermosensitive alterations for flagellar proteins. Some of these mutations might be expected to result in an instability or loss of function of the preformed flagella upon transfer from the permissive to the restrictive temperature. Since all the mutants falling into the *dd* category exhibit this phenotype, they were chosen for detailed study. It is also possible that a lesion for intrinsic components of the flagellum might only be revealed upon flagellar assembly at the high temperature, with preassembled structures remaining stable upon temperature shift-up. In an attempt to identify such mutants among those falling into the 2° conditional category, we examined the response of the mutants to experimental deflagellation and incubation at 32°C, as noted above. One potentially interesting group of motility defective mutants, in which the lesions are expressed immediately upon regeneration at 32°C, has been identified and is currently under study. Applying the same test to the 2° conditional flagellaless mutants, we have not yet identified a strain in which the assembly defect is expressed during flagellar regeneration at 32°C. In our studies to date, all conditional mutants which are unable to regenerate at the restrictive temperature also show instability of preformed flagella at 32°C (*dd-a* mutants).

In this paper the isolation, phenotypic characteristics, and genetics of 21 genetically stable and phenotypically nonleaky 1°C conditional motility mutants (*dd* mutants) will be discussed.

Isolation of dd Mutants

The 21 *dd* mutants which are described in this paper were isolated from six different mutagenesis experiments in which three methods of chemical mutagenesis and several protocols for enrichment were utilized (Table II). In experiments I through V*a* exposure of mutagenized populations to the restrictive temperatures for any prolonged period of time was avoided so as not to preclude the possibility of recovering conditional motility mutants with concomitant defects in cell division. In experiments V*b* and VI, a selection step was introduced which categorically eliminated the possibility of recovering such mutants. In these experiments the mutagenized populations were grown at the restrictive temperature on agar plates, and only those clones displaying a "heaped" morphology characteristic of nonmotile strains (see Fig. 1) were screened for temperature-sensitive motility defects.

In each mutant run, approximately 2–5% of all the clones which were tested for swimming behavior in liquid test tubes at 20 and 32°C were retained as presumptive temperature-sensitive motility mutants. Out of a total of 534 such clones, only 21 have been identified as genetically and phenotypically stable mutants falling into the *dd* category. Outside of clones which retested as wild-type or conditional auxotrophs, the remaining clones which are not included in this summary fall into the following categories: (*a*) conditional motility mutants displaying a delayed response to

TABLE II

Summary of Mutant Search Experiments

Exp.	Mutagenesis method	Enrichment technique	Selection step	Clones screened	Clones retained as presumptive ts mutants	*dd* mutants
I	1	1*b*, 2*b*	0	3,243	65	1 *dd-frag*-1
II	1	1*b*, 2*b*	0	3,884	132	3 *dd-m*-5 *dd-m*-7 *dd-a*-6
III	1	1*b*, 2*b*	0	5,328	155	3 *dd-a*-13 *dd-l*-14 *dd-m*-16
IV	2	1*c*, 2*c*	0	1,220	38	2 *dd-a*-103 *dd-m*-104
V*a*	2	1*c*, 2*d*	0	1,082	38	2 *dd-l*-108 *dd-a*-111
V*b*	2	1*c*, 2*d*	+	1,237	68	6 *dd-m*-100 *dd-m*-101 *dd-m*-106 *dd-m*-107 *dd-m*-112 *dd-m*-113
VI	3	1*a*, 2*a*	+	1,264	38	4 *dd-a*-211 *dd-a*-223 *dd-a*-224 *dd-m*-200
Totals				17,258	534	21

The numbers in columns for mutagenesis methods and enrichment techniques refer to numbered descriptions in Materials and Methods. In experiment V*a* and V*b*, cells were treated as a single batch until the final cloning step where half were plated on TAP medium, 2.5% agar with growth at 32°C (exp. V*b*) and half were cloned at 20°C (exp. V*a*).

temperature shift; (*b*) conditional motility mutants in which defective swimming is detected in a large proportion of the cells at 20°C; (*c*) conditional motility mutants in which the mutation is not expressed in all the cells at 32°C, (*d*) conditional motility mutants displaying heterogeneous phenotypes.

In addition to these mutants, large numbers of presumptive nonconditional mutants characterized by defects in swimming at both 20 and 32°C were obtained. In recent studies (G. M. Adams, unpublished observations) it has been noted that included among this class are mutants which may be regarded as thermosensitive, in that expression of the genetic lesions is quantitatively or phenotypically different at the two temperatures. Such mutants represent a potentially interesting group which has yet to be studied.

Segregation Patterns of dd Mutants

Each of the 21 *dd* mutants has been backcrossed to wild-type strains and the segregation patterns for their thermosensitive motility phenotypes examined by tetrad analysis. As seen in Table III, in all cases segregation of mutant: wild-type behavior at 32°C was 2:2, consistent with Mendelian segregation of single gene mutations. The multiple thermosensitive traits which are characteristic of the seven *dd-a* mutants and two *dd-l* mutants were found to invariably segregate together, suggesting that in each instance the traits are the pleiotropic expression of single mutations.

dd-assembly Mutants

PHENOTYPIC CHARACTERISTICS: The *dd-a* mutants display a phenotype which comes closest to that anticipated for a thermosensitive lesion affecting intrinsic structural components of the flagellum. In each of the seven mutants falling into this category, a single gene mutation not only imposes a functional and structural instability to preformed flagella at 32°C but also interferes with the ability of the mutants to assemble flagella at the restrictive temperature.

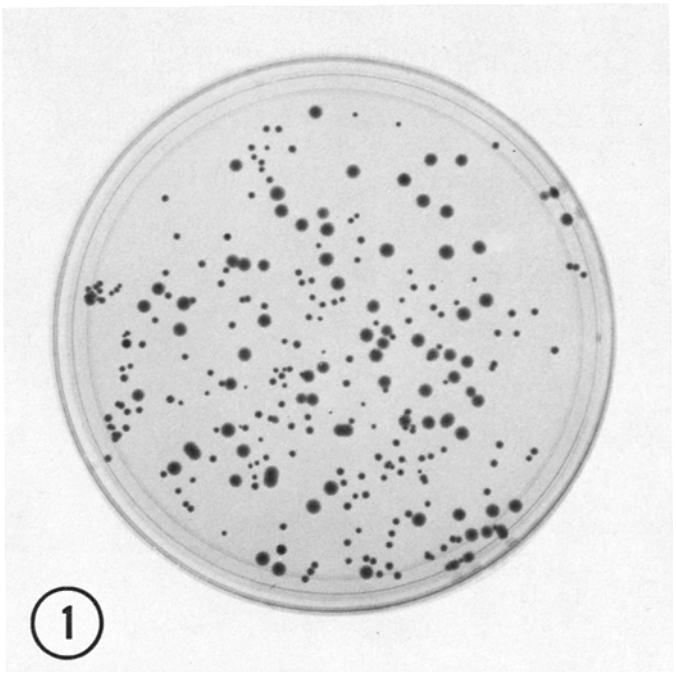

FIGURE 1 Photograph illustrating the difference in colony morphology between wild-type and nonmotile strains. A mixture of wild-type strain 137c and the temperature-sensitive mutant *dd-a*-13 was plated at a 1:3 ratio and grown at 32°C. The mutant colonies are easily distinguished from the wild-type clones by their smaller and denser appearance.

At the descriptive level, the thermosensitive phenotypes of the seven *dd-a* mutants are very similar. When the mutants are grown at 20°C, the cells are flagellated and highly motile. However, within 1 h of exposure to the high temperature the cells visibly begin to settle, eventually to form a compact pellet at the bottom of the test tube. Light microscope examination of mutant populations at 32°C suggests that the initial alteration in swimming is associated with some dysfunction in the motility properties of the preformed flagella. Although we have made no attempt to define this defect in flagellar activity, cells with full-length flagella are observed in the earliest pelleted populations (Fig. 2 *a*).

The preformed flagella are not only functionally defective at 32°C but structurally unstable. Over a period of several hours at 32°C the percentage of cells with full-length flagella progressively decreases with the appearance of cells with shortened equal-length flagella, shortened unequal-length flagella, single elongated flagella, and no visible flagella. The rate at which the preformed flagella are lost, presumably by length regression, varies among the mutants, but, in each case, within 4–10 h of exposure to 32°C, virtually all the cells in mutant populations lack visible flagella (Fig. 2 *b*).

Following the loss of the preformed flagella, the reassembly of flagella does not occur as long as the mutants are maintained at the high temperature. If the mutants are allowed to divide at 32°C the daughter cells which are formed invariably lack flagella.

In terms of their cell division rates, each of the *dd-a* mutants closely resembles wild-type strains at both the permissive and restrictive temperatures. A common characteristic of the mutants grown at 32°C is the slow release of mitotic products from the mother cell wall. In addition to single nonflagellated cells, clumps of two, four, and eight cells are common (Fig. 2 *c*). This characteristic has also been described for nonconditional "flagellaless" mutants of *C. reinhardtii* (7, 17), suggesting that the slow release of daughter cells is a consequence of the nonassembly phenotype of the mutants. Within 6–24 h after return to the permissive temperature, the majority of cells in mutant populations are flagellated single cells (Fig. 2 *d*).

Although the seven *dd-a* mutants display similar

TABLE III

Segregation Patterns of the dd-Phenotypes in Back-Crosses to Wild Type

	Mutagenesis experiment	Tetrads showing 2:2 segregation of *dd*-phenotype	Aberrant tetrads	Total tetrads scored
dd-assembly mutants				
dd-a-6	II	360	4	364
dd-a-13	III	323	2	325
dd-a-103	IV	107	1	108
dd-a-111	V *a*	114	0	114
dd-a-211	VI	116	0	116
dd-a-223	VI	129	0	129
dd-a-224	VI	110	0	110
dd-fragile flagella mutants				
dd-frag-1	I	29	0	29
dd-motility mutants				
dd-m-5	II	121	0	121
dd-m-7	II	56	0	56
dd-m-16	III	93	0	93
dd-m-100	V *b*	14	0	14
dd-m-101	V *b*	22	0	22
dd-m-104	IV	37	1	38
dd-m-106	V *b*	20	0	20
dd-m-107	V *b*	14	0	14
dd-m-112	V *b*	12	1	13
dd-m-113	V *b*	12	0	12
dd-m-200	VI	20	0	20
dd-lethal mutants				
dd-l-14	III	64	1	65
dd-l-103	V *a*	10	1	11

phenotypes at 32°C, they differ in the threshold temperature at which the genetic lesions are expressed. In three of the mutants, *dd-a*-111, *dd-a*-211, and *dd-a*-223, the majority of cells are nonflagellated if grown at 24°C. In *dd-a*-6, this occurs at around 28°C. Only in *dd-a*-13, *dd-a*-103, and *dd-a*-224 does 32°C appear to be close to the limiting temperature at which expression of the mutations is detected.

FLAGELLAR REGENERATION CHARACTERISTICS: One of the properties of *Chlamydomonas* is its ability to rapidly regenerate flagella following experimental deflagellation. The effects of temperature shifts on the regeneration characteristics of the *dd-a* mutants have been examined. These studies have been used to assay the temperature-sensitivity of the mutant gene products which give rise to the nonassembly phenotypes of the mutants. The results of this analysis are summarized in Table IV.

When the mutants grown at 20°C are chemically deflagellated by the method of Watson and Hynes (28) and incubated at the permissive temperature, flagellar regeneration occurs in all the mutants. In three of the mutants, *dd-a*-13, *dd-a*-103, and *dd-a*-224, the rate of flagellar elongation at 20°C is similar to that of wild-type strains and is essentially completed by 60–90 min. In comparison, in *dd-a*-6, *dd-a*-111, *dd-a*-211, and *dd-a*-223 regeneration at 20°C does not occur synchronously among the cells within a population, and several hours are required for the majority of cells within a culture to reassemble full-length flagella. It should be noted that, for these mutants, poor regeneration at 20° correlates with the lower threshold for thermosensitive loss of swimming described in the preceding section.

TEMPERATURE SHIFT-UP EXPERIMENTS

In wild-type strains the kinetics of flagellar elongation have been shown to be temperature-dependent, proceeding more rapidly at 32°C than

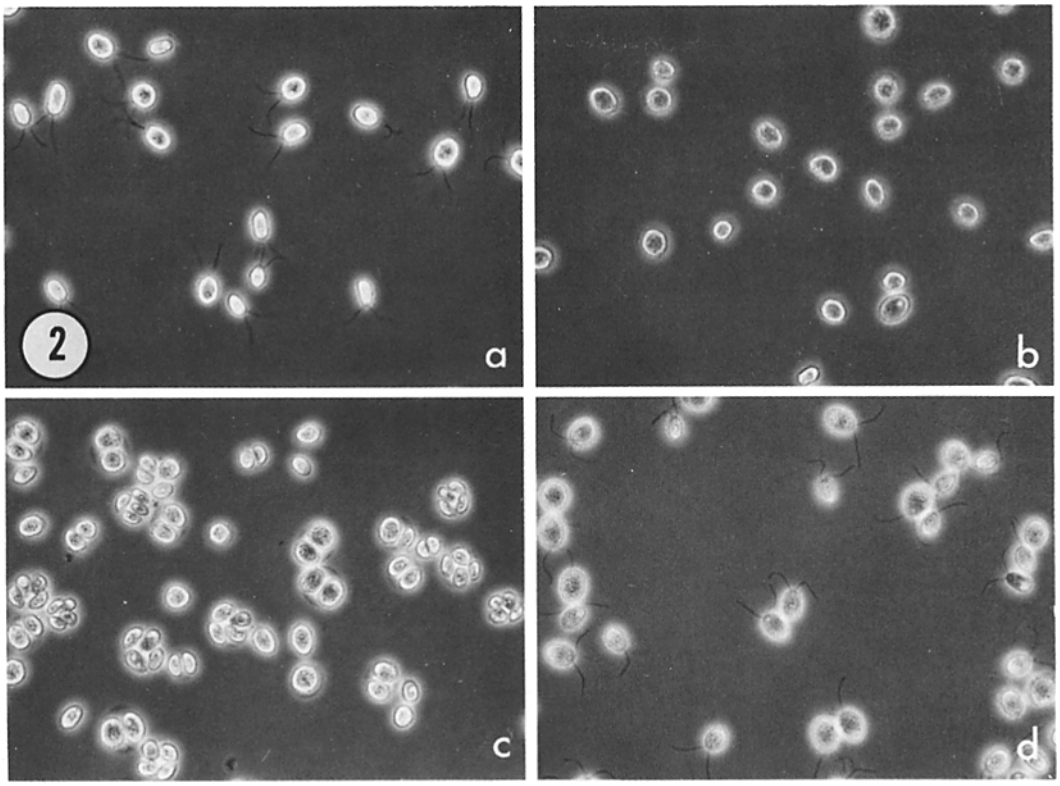

FIGURE 2 Phase-contrast light micrographs of *dd-a*-13, illustrating the effect of exposure to 32°C on the *dd*-assembly mutants. × 400. (*a*) 1 h after shift from 20°C to 32°C, the cells have still retained their flagella. (*b*) After exposure to 32°C for 6 h, the cells have lost their flagella by resorption. (*c*) After incubation at 32°C for 24 h, single nonflagellated cells and clumps of nonreleased daughters are seen. (*d*) 6 h after return to the permissive temperature, the cells have assembled flagella and are released from the maternal cell walls.

TABLE IV

Flagellar Regeneration Characteristics of the dd-assembly Mutants

	20°C	32°C	Regeneration kinetics at 20°C after exposure to 32°C.
dd-a-6	Slow	Immediate and complete inhibition	Increasing lag with increasing preexposure to 32°C
dd-a-13	Normal	"	Kinetics similar to controls not exposed to 32°C
dd-a-111	Very slow	"	"
dd-a-211	Very slow	"	"
dd-a-223	Very slow	"	"
dd-a-103	Normal	Regeneration of unstable flagella. Complete inhibition after 8-h exposure to 32°C.	"
dd-a-224	Normal	Regeneration of unstable flagella. Complete inhibition after 30-min exposure to 32°C.	"

at 20°C (18). The regeneration characteristics of the mutants at 32°C differ markedly from those of wild-type strains. When the first five mutants listed in Table IV are incubated at the high temperature, flagellar regeneration is immediately and completely inhibited. Expression of the nonassembly phenotype in these mutants is clearly not dependent on prior exposure to the restrictive temperature. This rapid inhibition of flagellar assembly is observed even if exposure to 32°C occurs after flagellar regeneration has been initiated at 20°C. This effect of temperature shift is illustrated for one of the five mutants, *dd-a*-13, in Fig. 3.

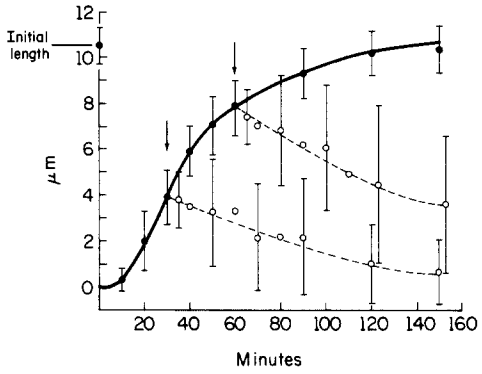

FIGURE 3 The effect of temperature-shift to 32°C on the course of flagellar regeneration in *dd-a*-13. ●, the kinetics of flagellar regeneration at 20°C, as determined by measurement of average flagellar lengths ± 1 standard deviation (⊢—⊣). ○, the effect of temperature shift-up to 32°C after regeneration at 20°C for 30 min and 60 min (arrows).

After flagellar assembly has been allowed to occur at 20°C for 30 min or 60 min a shift to 32°C results in a rapid cessation of further elongation, followed by a gradual decrease in flagellar lengths. The marked increase in the standard deviation for average flagellar lengths after shift to 32°C reflects the asynchrony among cells within a population in which regression in flagellar lengths occurs. These observations indicate that the temperature-induced alteration in the mutant gene products which interferes with flagellar assembly occurs rapidly upon shift from 20 to 32°C. The fact that inhibition of assembly occurs even after flagellar elongation has been initiated at 20°C suggests that the inability of the mutants to generate flagella at the high temperature resides in a defect in the assembly process itself rather than in some initiation step.

In contrast to the first five mutants listed in Table IV, without prior incubation at 32°C *dd-a*-103 and *dd-a*-224 are initially able to regenerate flagella at the high temperature. However, these flagella formed at 32°C are unstable and are gradually resorbed. In *dd-a*-224, complete inhibition of flagellar assembly occurs only after a brief exposure of ~30 min to the restrictive temperature; in *dd-a*-103, a more prolonged exposure of ~8 h is required for expression of the assembly defect.

As has been discussed by Jarvik and Botstein (11), temperature-sensitivity can be an expression of the temperature-sensitive denaturation of mutant gene products at the time of synthesis or the temperature lability of formed products. In analyzing the phenotype of *dd-a*-103, we explored the possibility that the mutation affects a structural component of the flagellum which is conditionally denatured only during its synthesis at the restrictive temperature.

In *Chlamydomonas* a limited precursor pool of flagellar proteins has been shown to exist (24). To test the possibility that the delay in expression of the nonassembly phenotype of *dd-a*-103 might reflect the time required for the dilution of any preformed pool with defective product synthesized at 32°C, we examined the ability of the mutant to undergo repeated cycles of flagellar regeneration at 32°C. The premise was that inhibition of flagellar assembly at 32°C might occur more rapidly with depletion of any preformed pool. *dd-a*-103 was not found to possess the predicted characteristics; the mutant undergoes at least three consecutive cycles of regeneration at 32°C before the nonassembly phenotype is expressed. It therefore seems likely that a nonflagellar protein may be responsible for the *dd-a*-103 phenotype.

TEMPERATURE SHIFT-DOWN EXPERIMENTS

The ability of the mutants to recover from temperature inhibition of flagellar assembly has also been examined. The recovery kinetics of the first five mutants listed in cultures at 32°C for varying periods of time and observing the rate at which the mutants regenerated flagella upon temperature shift-down to 20°C. In the case of *dd-a*-224 and *dd-a*-103, the cultures were preincubated at 32°C for at least 30 min or 8 h, respectively, deflagellated, and then shifted to 20°C.

Among the seven mutants, two distinct types of recovery kinetics have been observed. In *dd-a*-6 the initiation of regeneration at 20°C following exposure to 32°C occurs only after a distinct lag period, the duration of which is dependent on the time spent at the high temperature.

This characteristic of the mutant is illustrated in Fig. 4, in which the time course for the appearance of fully-flagellated cells at 20°C following exposure to 32°C for 10 min to 6 h is plotted. If the criterion used to define the time in which flagellar regeneration has begun is that at least 10% of the cells within a population have flagella of any discernible lengths, the duration of the lag period is observed to increase from 60 min to 4 h after incubation at 32°C for 10 minutes to 6 hours. In the samples preincubated at 32°C for 10 min, 2 h,

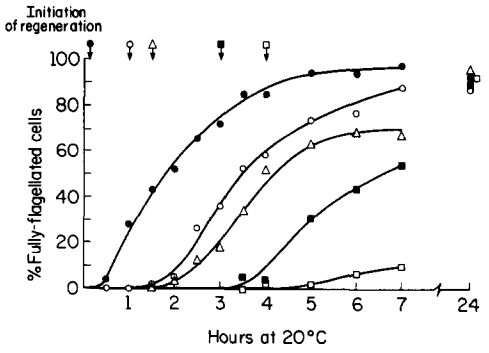

FIGURE 4 Flagellar regeneration at 20°C in *dd-a*-6 following incubation of deflagellated cultures at 32°C for varying periods of time. ●, no preincubation at 32°C; ○, 10-min exposure to 32°C, △, 2-h exposure to 32°C; ■, 4-h exposure to 32°C; □, 6-h exposure to 32°C. Arrows, times at which the initiation of flagellar regeneration is observed following incubation at 32°C for the specified periods of time.

and 4 h, the rate at which cells that have completed flagellar regeneration accumulate following the initial lag period does not differ significantly from that of the control population. In the case of the cells exposed to 32°C for 6 h, a decrease in the time course for the appearance of fully-flagellated cells is evident. This apparent decline may not be significant, however, since these cells which were light-synchronized are entering the division cycle. Regardless of the exposure time to the restrictive temperature (up to 96 h has been tested), *dd-a*-6 is able to completely recover within 24 h at 20°C at which time the vast majority of cells within a population are fully-flagellated.

The recovery kinetics of *dd-a*-6 suggests that the effect of high temperature on the mutant gene product which interferes with flagellar assembly is not rapidly temperature-reversible, and that the effect of exposure to 32°C is in some way cumulative.

In contrast to the recovery pattern observed in *dd-a*-6, the other 6 *dd-a* mutants initiate flagellar regeneration upon temperature shift-down without a distinct lag period. This suggests that during exposure to 32°C the mutant gene products in these strains are not irreversibly altered. Under these circumstances, we would expect that flagellar assembly upon temperature shift-down is not dependent on protein synthesis. This has been tested directly for one of these mutants, *dd-a*-13.

In Fig. 5 the effects of 10 μg/ml cycloheximide, a concentration which immediately and completely inhibits protein synthesis in *Chylamydomonas reinhardtii* (24), on the recovery kinetics of *dd-a*-13 are recorded. The top curve represents the assembly kinetics of the mutant at 20°C in the absence of the inhibitor. In the presence of cycloheximide, regeneration follows similar kinetics but plateaus after elongation to approximately 6 μm. These observations are consistent with previous studies on flagellar regeneration in *Chylamydomonas* (24) which suggest that at the time of flagellar amputation a precursor pool of flagellar proteins exists. In *dd-a*-13 the pool appears to be sufficiently large enough to allow for the regeneration of approximately one-half initial flagellar lengths. If deflagellated cultures of the mutant are incubated at 32°C for 1 or 4 h, and if cycloheximide is added and the cultures are shifted to 20°C, not only is flagellar regeneration immediately initiated but the degree of elongation is significantly enhanced over that of the control population. After 1 and 4 h at 32°C, flagellar elongation at 20°C plateaued at 7.2 μm and 9.0 μm, respectively. The fact that inhibition of protein synthesis does not interfere with the ability of the mutant to recover from exposure to 32°C indicates that the *dd-a*-13 mutant gene product must be highly temperature-reversible. The observation that the mutant regenerates longer flagella in the presence of cycloheximide after exposure to the

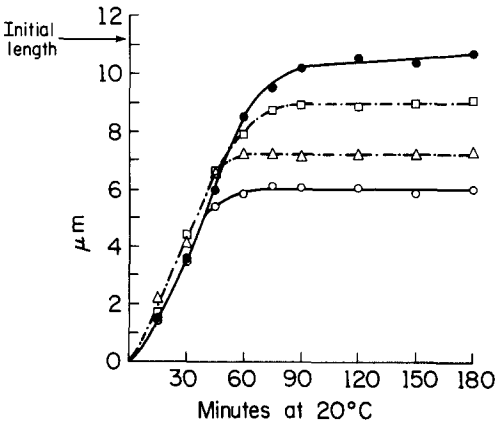

FIGURE 5 The effects of cycloheximide (10 μg/ml) on flagellar regeneration in *dd-a*-13, as determined by measurement of average flagellar lengths. ●, regeneration at 20°C in the absence of cycloheximide; ○, regeneration at 20°C in the presence of cycloheximide; △, regeneration at 20°C in the presence of cycloheximide following incubation at 32°C for 1 h; □, regeneration at 20°C in the presence of cycloheximide following incubation at 32°C for 4 h.

high temperature suggests that, while the mutant gene product interferes with assembly at 32°C, the synthesis and accumulation of the precursor pool of flagellar proteins occurs[1] and that this pool is rapidly available for flagellar assembly upon shift-down to the permissive temperature.

GENETIC ANALYSIS: To test for possible linkage or allelism, we intercrossed the 7 *dd*-assembly mutants in all possible pairwise combinations. The frequency of recombination was examined by tetrad analysis, and verified for larger numbers of zygotes by the zygote plating method. The results of this analysis are summarized in Table V. In all of the crosses except for 4, the frequency of recombinant tetrads and zygotes was found to be greater than 50%, indicating that four out of the seven *dd-a* mutants are distantly linked or unlinked to one another.

As seen in Table VI, the results from pairwise crosses between *dd-a*-6, *dd-a*-211, and *dd-a*-223 suggest that each of the 3 mutants is closely linked to the other. Since *dd-a*-211 and *dd-a*-223 were isolated from the same mutagenesis experiment and have been found to be phenotypically indistinguishable, it is likely that they represent daughters of the same mutant clone. *dd-a*-6 and *dd-a*-223 have been found to be noncomplementing in isolated heterozygous mutant diploids, indicating that these two independently isolated mutants are allelic. Although the two mutants are functionally noncomplementing alleles, the fact that they differ in their range of temperature-sensitivity suggests that they represent lesions for different sites within the same gene.

The results from tetrad analysis and zygote plating tests of crosses between *dd-a*-13 and *dd-a*-111 also indicate close-linkage between this pair of mutants (Table VI). Complementation has been observed in diploid strains carrying both the *dd-a*-13 and *dd-a*-111 markers, demonstrating that, although the two mutants are closely linked, they are nonallelic. Based on the results from tetrad analysis (Table VI), *dd-a*-13 and *dd-a*-111 are linked by a distance of approximately 3.3 map units.

[1] Enhancement of the length-limiting precursor pool of flagellar protein(s) which occurs during exposure of *dd-a*-13 to 32°C does not appear to be a consequence of the mutation or the assembly-inhibited state of the mutant. Wild-type strain 137c has been observed to regenerate longer flagella at 20°C in the presence of cycloheximide following exposure to 32°C.

In addition to selection of diploid strains to test for complementation between closely-linked mutants, diploid strains were also constructed to test for expression of each of the five nonallelic mutations (*dd-a*-6, *dd-a*-13, *dd-a*-103, *dd-a*-111, and *dd-a*-224) in the presence of their wild-type alleles. In each instance, expression of their thermosensitive phenotypes was not observed, indicating that each of the mutations is recessive to its wild-type allele.

The recombination analysis and complementation tests which have thus far been completed indicate that at least five distinct genetic loci are represented among the seven *dd-assembly* mutants. Of the five genes hit, only one was hit more than once. With this small number of mutants, it is difficult to accurately predict the number of genes which can mutate to produce the *dd-a* phenotype. Recently, by the utilization of different methods for enrichment and selection, an additional 15 mutants falling into the *dd-assembly* category have been isolated in our laboratory (G. M. Adams, unpublished results). One of the mutants appears to be an allele for *dd-a*-13. Once genetic analysis has been completed on the remaining mutants, we should have a clearer indication of the number of different gene mutations which can give rise to the *dd-a* phenotype.

SHIFT IN TEMPERATURE-SENSITIVITY OF RECOMBINANTS CARRYING 2 *dd-assembly* MARKERS

In our tetrad analysis of several of the intercrosses made between pairs of *dd-a* mutants, an unexpected observation was made on the behavior of the double mutant recombinants grown at 20°. In contrast to the parental haploids, the recombinants were found to lack flagella at 20°C, and only by growing the strains at lower temperatures are the majority of cells flagellated and motile. In Table VII, those crosses which consistently yielded double mutant recombinants (from both tetratype and nonparental tetrads) displaying this shift in the temperature threshold for expression of the *dd-a* phenotype are indicated as forming pellets (P) at 20°C. This synergy of temperature-sensitivity suggests that the presence of two specific *dd-a* markers in a haploid cell are in some way additive. One explanation for these observations is that, although the mutations are genetically independent, some functional relationship exists between the mutant gene products.

ULTRASTRUCTURAL ANALYSIS: The mor-

TABLE V

Summary of Recombination Analysis on Pairwise Crosses Between dd-assembly Mutants

	dd-a-6	*dd-a*-211	*dd-a*-223	*dd-a*-13	*dd-a*-111	*dd-a*-103	*dd-a*-224
dd-a-6		+	+	–	–	–	–
dd-a-211			+	–	–	–	–
dd-a-223				–	–	–	–
dd-a-13					+	–	–
dd-a-111						–	–
dd-a-103							–
dd-a-224							

+ Crosses which yielded less than 5% recombinant zygotes, indicating linkage.
– Crosses which yielded greater than 50% recombinant zygotes, indicating nonlinkage or distant linkage.

TABLE VI

Results of Recombination Analysis and Allelism Testing between Linked dd-assembly Mutants

	Tetrad analysis, PD:NPD:TT	Zygote plating, recombinant/total	Diploid test*
dd-a-6 × *dd-a*-211	20:0:0	1/267	–
dd-a-6 × *dd-a*-223	75:0:0	0/150	Noncomplementing
dd-a-211 × *dd-a*-223	9:0:0	–	–
dd-a-13 × *dd-a*-111	30:1:0	9/388	Complementing

* To test for allelism, stable heterozygous mutant diploids were constructed as described in Materials and Methods. Each of the mutations tested in this manner is recessive to its wild-type allele as determined by construction of stable diploids (*dd-a*-6, *dd-a*-13, *dd-a*-111) and/or temporary dikaryons (26) (*dd-a*-6, *dd-a*-13, *dd-a*-223).

phology of each of the *dd-a* mutants grown at 32°C has been examined at the electron microscope level. Except for the absence of normal flagellar structures, the mutants have been observed to be morphologically wild-type.

Although the *dd-a* mutants are unable to assemble flagella at 32°C following the loss of the preformed flagella, they have been found to possess the normal complement of basal bodies and wild-type transition zone structures (for example, compare Fig. 6*a* to Figs. 6*b*, 6*c*, and 6*d*). The morphology of the region beyond the transition zone has been found to vary among mutants and, to some degree, within a single mutant population. Some examples of the ultrastructure of this region from mutants grown at 32°C are found in Figs. 6*b*, 6*c*, and 6*d*.

For each of the mutants, images have been observed such as that seen in Fig. 6*b*, in which there is an absence of any structure past the transition zone other than the surrounding cell membrane. In one of the mutants, *dd-a*-6, it is not uncommon to observe the presence of a cap of amorphous dense material lying distal to the transition zone (Fig. 6*c*). A frequent observation made on preparations of *dd-a*-13 and *dd-a*-111 grown at 32°C is the presence of short flagellar stubs measuring not more than 0.5 μm in length (Fig. 6*d*). In both mutants, these 32°C-generated stubs consist of the normal nine outer-doublets, but a formed central pair of microtubules is absent (compare Figs. 6*d* and 6*a*). In what way these morphological observations made on the *dd-a* mutants may be symptomatic of the genetic lesions is unknown at this time. It is interesting to note, however, that in contrast to previously isolated nonconditional flagellaless mutants of *Chlamydomonas*, in which defects in the basal body or transition zone structures have been observed (7, 17), none of the conditional *dd-a* mutants display similar morphological alterations.

dd-fragile flagella Mutant

One mutant, *dd-frag*-1, has been isolated in

TABLE VII

Motility Phenotype at 20°C of Haploid Recombinants Carrying 2 dd-assembly Markers

	dd-a-6	*dd-a*-211	*dd-a*-223	*dd-a*-13	*dd-a*-111	*dd-a*-103	*dd-a*-224
dd-a-6		–	–	P	P	P	P
dd-a-211			–	P	P	P	P
dd-a-223				P	P	P	P
dd-a-13					S	S	P
dd-a-111						P	P
dd-a-103							S
dd-a-224							

P, form pellets at 20°C; S, swimmers at 20°C; – Crosses from which no recombinants were recovered due to close-linkage.

which shift from 20°C to 32°C results in a rapid loss of the preassembled flagella, but unlike the *dd-a* mutants, this mutant loses its flagella by detachment rather than resorption. Within 1 h of exposure to 32°C, cells with single elongate flagella and those with a complete absence of flagella appear within the mutant population. At the same time, flagella are found free in the culture medium. By 6 h at 32°C, the majority of cells are nonflagellated. In contrast to the *dd-a* mutants, following the loss of the performed flagella, flagellar assembly does occur at 32°C in *dd-frag*-1. Like the flagella formed at 20°C, the 32°C-generated flagella are unstable at the high temperature and rapidly lost by detachment.

Electron microscope examination of *dd-frag*-1 exposed to 32°C has not revealed any distinct alteration in the structure of the flagellum or basal body complex which might account for the fragility of the flagellum. In wild-type strains of *Chlamydomonas reinhardtii* flagellar detachment can be a facile response to a variety of unfavorable conditions. For example, in cultures which have reached the stationary phase of growth, cyclic loss of flagella by detachment followed by reassembly is commonly observed. Under these circumstances, mutants displaying the phenotype of *dd-frag*-1 seem to be less likely to represent lesions directly affecting flagellar function.

dd-motility Mutants

The *dd-m* mutants represent a class of thermosensitive mutants in which the genetic lesions appear to specifically affect the motility properties of the flagellum. At the restrictive temperature the flagella are retained and assembled with kinetics similar to that of wild-type strains. Each of the mutants also displays wild-type growth characteristics; their division rates at 32°C are enhanced over those at 20°C.

The 11 *dd-motility* mutants fall into 3 phenotypic classes:

(*a*) *dd-m-5, dd-m-104, dd-m-112, dd-m-200.* At the restrictive temperature, these mutants display uncoordinated, rapid movements of the flagella. The pattern of abnormal flagellar activity is similar whether the cells are shifted from 20°C to 32°C or following assembly of flagella at 32°. Recovery of wild-type motility rapidly occurs with shift to the permissive temperature and does not appear to require reassembly of flagella at 20°C.

(*b*) *dd-m-100, dd-m-101, dd-m-106, dd-m-107.* When these mutants are shifted to the restrictive temperature, the 20°C pre-assembled flagella beat in an erratic, uncoordinated manner. Following cell division or flagellar regeneration at 32°C, the flagella are essentially immobile. Recovery of wild-type flagellar function occurs only following

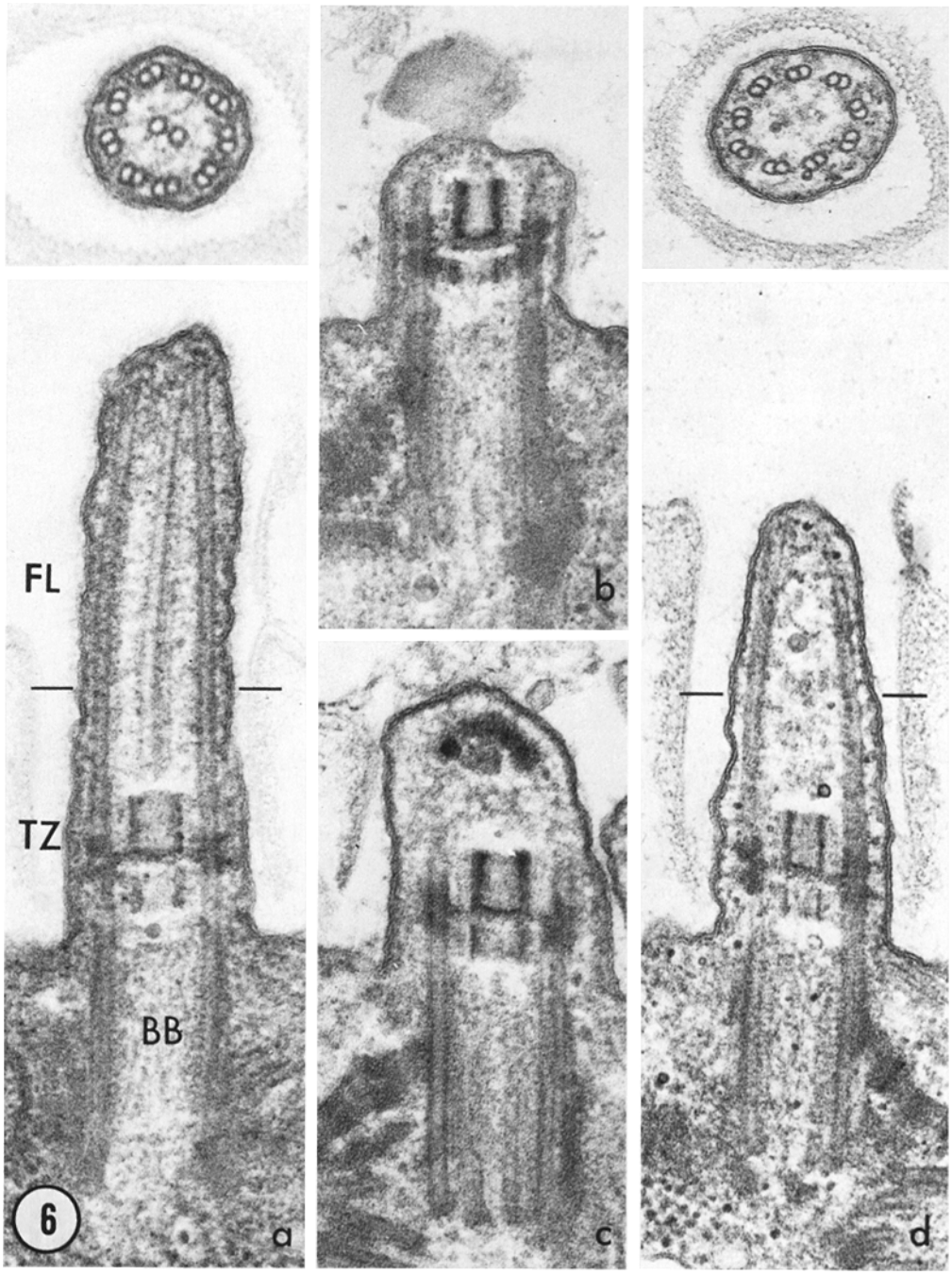

FIGURE 6 Electron micrographs illustrating the morphology of the flagellar region in *dd-assembly* mutants grown at 32°C. × 90,000. (*a*) A mid-sagittal section through the flagellum and basal body of a wild-type cell fixed during the process of regeneration. *BB*, basal body; *TZ*, transition zone; *FL*, flagellum proper. A cross-sectional image of the flagellum at the level indicated by the lines is seen at top. The normal complement of nine outer-doublet and two central-pair microtubules is seen. (*b, c, d*) Similar planes of section through the same region from *dd-assembly* mutants grown at 32°C. (*b*) *dd-a*-103, axonemal structures beyond the transition zone are absent. (*c*) *dd-a*-6, distal to the transition zone is an accumulation of dense, amorphous material. (*d*) *dd-a*-13, a short flagellum extends beyond the transition zone. A cross-sectional image of the 32°C-generated flagellar stub at the level indicated by the lines is seen at the top. The absence of formed central-pair microtubules is evident.

cell division or flagellar regeneration at the permissive temperature.

(c) *dd-m-7, dd-m-16, dd-m-113*. At 32°C these mutants display slow spiral swimming. In addition to this abnormal motility property, the flagellar length at both 20°C and 32°C is variable; cells with flagella distinctly shorter than wild-type flagella are found in mutant populations at both temperatures. Recovery of wild-type motility requires flagellar assembly at 20°C.

To test for linkage and possible allelism between the 11 *dd-m* mutants, we intercrossed the mutants in all pairwise combinations. Recombination frequencies were determined by tetrad analysis and/or the zygote plating method. The results of this analysis are summarized in Table VIII. Except for those crosses indicated by the letter "L", nonlinkage or distant linkage was indicated by a frequency of greater than 50% recombinant zygotes.

In each pairwise cross between *dd-m*-100, *dd-m*-101, *dd-m*-106, and *dd-m*-107 no recombinant zygotes were identified out of a total of at least 100 zygotes tested (Table IX), indicating that each of the mutants is closely linked to the other. The fact that they are phenotypically similar suggests that they are probably allelic. Since all four mutants were isolated from the same mutagenesis experiment, it is possible that two or more of the mutants represent replicas of a single mutant.

The results from recombination analysis (Table IX) suggest that *dd-m*-5 and *dd-m*-104 which were isolated from different mutagenesis experiments, but which are phenotypically similar are closely linked and probably alleles. The same is true for *dd-m*-7 and *dd-m*-16. When *dd-m*-200 was crossed to *dd-m*-7 or *dd-m*-16, approximately 16% of the zygotes tested contained wild-type recombinants, indicating that *dd-m*-200 is closely linked to the *dd-m*-7 and *dd-m*-16 locus but probably represents a mutation for a different site.

Although complementation tests are needed to confirm the results from recombination analysis, the data indicate that the 11 *dd-m* mutants repre-

TABLE VIII

Summary of Recombination Analysis on Pairwise Crosses between dd-motility Mutants

	dd-m-5	*dd-m*-104	*dd-m*-7	*dd-m*-16	*dd-m*-200	*dd-m*-100	*dd-m*-101	*dd-m*-106	*dd-m*-107	*dd-m*-112	*dd-m*-113
dd-m-5		+	–	–	–	–	–	–	–	–	–
dd-m-104			–	–	–	–	–	–	–	–	–
dd-m-7				+	+	–	–	–	–	–	–
dd-m-16					+	–	–	–	–	–	–
dd-m-200						–	–	–	–	–	–
dd-m-100							+	+	+	–	–
dd-m-101								+	+	–	–
dd-m-106									+	–	–
dd-m-107										–	–
dd-m-112											–
dd-m-113											

* Crosses which yielded less than 20% recombinant zygotes, indicating linkage.
– Crosses which yielded greater than 50% recombinant zygotes, indicating nonlinkage or distant linkage.

TABLE IX

Results of Recombination Analysis on Linked dd-motility Mutants

	Tetrad analysis PD:NPD:TT	Zygote plating recombinant/total	Recombinant zygotes
			%
dd-m-100 × *dd-m*-101	–	0/113	0
dd-m-100 × *dd-m*-106	–	0/212	0
dd-m-100 × *dd-m*-107	–	0/151	0
dd-m-101 × *dd-m*-106	–	0/242	0
dd-m-101 × *dd-m*-107	–	0/132	0
dd-m-106 × *dd-m*-107	–	0/212	0
dd-m-5 × *dd-m*-104	24:0:0	0/295	0
dd-m-7 × *dd-m*-16	12:0:0	0/81	0
dd-m-7 × *dd-m*-200	–	16/101	15.8
dd-m-16 × *dd-m*-200	10:0:0	36/210	16.4

sent mutations for at least six distinct genetic loci which give rise to thermosensitive defects in flagellar motility.

All of the *dd-m* mutants have been examined at the ultrastructural level for possible morphological defects in the structure of the flagellar apparatus. In both thin-section and negatively stained preparations, we have been unable to detect any distinct alterations in the axonemal 9 + 2 complement of microtubules and their associated structures, or in the morphology of the basal body complex.

dd-lethal Mutants

One of the aims of this project has been to identify conditional mutants of *Chlamydomonas* which are likely to reflect genetic lesions for the structure and assembly of microtubules. In *Chlamydomonas,* microtubules represent the major structural components of the intranuclear mitotic spindle as well as the flagellar axoneme. With the possibility that a common genetic pool of tubulin peptides exists for these two systems, particular attention has been given to identifying mutants with combined defects in flagellar function and cell division.

During the course of these investigations a number of strains have been isolated whose phenotypes include both abnormal motility and altered growth characteristics at the high temperature. Two conditional lethal mutants whose motility characteristics upon shift from 20°C to 32°C place them in the *dd*-category will be described briefly. At the outset, it should be noted that neither of these mutants possesses those characteristics (i.e., G-2 mitotic arrest and flagellar nonassembly at the high temperature) which would indicate a genetic lesion for a common tubulin pool. Failure to find such a mutant may indicate that the number of mutational events which we have observed is yet too small, that microtubules of the two systems are genetically independent or that mutations affecting shared peptides of axonemal and cellular microtubles are immediately lethal.

dd-l-108: When grown at the permissive temperature, *dd-l*-108 closely resembles wild-type strains; the cells are flagellated and highly motile. The cloning efficiency of the mutant on semi-solid media and its growth rate in liquid cultures at 20°C are comparable to those of wild-type strains.

The temperature-sensitive flagellar characteristics of *dd-l*-108 are similar to those displayed by *dd-frag*-1. When the mutant grown at 20°C is shifted to 32°C, the preassembled flagella are rapidly lost by detachment and found free in the culture medium. Within 1 h of exposure to the high temperature, virtually all the cells lack flagella. Following the loss of the preformed flagella, the cells are able to initiate flagellar reassembly at 32°C, but as the flagella elongate they are lost by detachment.

dd-l-108 was initially identified as a lethal mutation because of its inability to form clones at 32°C under conditions which promote rapid growth of wild-type strains (on minimal media or acetate and yeast extract-supplemented agar plates grown under constant light conditions). When the effect of exposure to 32°C on the growth characteristics of *dd-l*-108 was examined in more detail, it became apparent that expression of the lethal phenotype is dependent on the conditions under which the mutant is grown. When *dd-l*-108 is grown in liquid cultures under constant light conditions, shift from 20°C to 32°C results in a rapid inhibition of cell division. Inhibition of cell division is correlated with the formation of densely green, large, spherical cells. In marked contrast, if *dd-l*-108 is grown at 32°C on an alternating 12-h light/dark regime (in which populations are synchronized and the growth rate is regulated), cell division occurs normally and lethality is not observed.

The fact that expression of the temperature-sensitive nondivision phenotype of *dd-l*-108 occurs only under conditions that promote rapid growth and cell division suggests that the mutation affects some synthetic or metabolic property of the cell, rather than specifically interfering with the mitotic process.

dd-l-14: When grown under continuous light at the permissive temperature, *dd-l-14* grows more slowly than wild type and reaches stationary phase at a lower density; the cells are flagellated and highly motile. Cloning efficiency at 20° on semi-solid media is comparable to that of wild-type strains.

The temperature-sensitive flagellar characteristics of *dd-l-14* resemble those of the *dd-a* mutants; no flagellar regeneration occurs at 32°.

Growing at 32° under continuous light, daughter cells fail to be released from the maternal cell walls, and subsequent divisions of daughters are asynchronous. Thus, at 24 and 18 h of growth, huge clumps of cells of widely varying sizes are present. By 72 h many cells are dead, and after longer periods of culture the entire population dies. The cloning efficiency of individual daughter cells at 24 and 48 h has been studied with the use of media from mating cells (3) to disrupt maternal cell walls. In both cases, plating efficiency is indistinguishable from that of wild-type cells, and no evidence for decreased cell viability at these time points has been obtained. Growth at 32°C under alternating 12-h light-dark periods results in the same type of cellular morphology, but there is much less lethality even upon prolonged culture. Electron microscope examination of *dd-l-14* cells undergoing synchronous division at 32°C reveals the presence of the normal complement of microtubules associated with the mitotic spindle and the cleavage furrow.

SUMMARY

With the aim of identifying potentially useful mutants for studies on the structure and assembly of the eukaryotic flagellum, a search for conditional mutants of *C. reinhardtii* defective in cell motility was undertaken. A series of 21 genetically stable and phenotypically nonleaky temperature-sensitive motility mutants have been isolated. Each of these mutants designated as "*drop-down*" or *dd-* mutants displays a rapid impairment of flagellar function upon shift from the permissive (20°C) to the restrictive (32°C) temperatures. The mutants have been placed into four categories on the basis of their defective phenotypes.

Among this series of mutants, those which have been categorized as *dd-a* mutants appear to be the most likely candidates for lesions directly affecting flagellar structure and assembly. In each of the seven *dd-a* mutants, a single Mendelian-segregating gene mutation affects the functional and structural stability of the preformed flagella and interferes with flagellar assembly at the restrictive temperature. Recombination analysis and complementation tests indicate that five distinct genetic loci are represented by the seven mutants. The flagellar regeneration characteristics of the mutants in response to temperature shifts have been examined. These studies have helped to define the thermosensitivity of the mutant gene products. We are currently examining the possibility that some of the *dd-a* mutants are conditionally defective for intrinsic structural proteins of the flagellum. At the same time, consideration is being given to the possibility that some of the mutants may contain mutations for nonflagellar proteins which influence the structural stability and assembly/disassembly cycle of the flagellum (2).

In addition to the *dd-a* mutants, 11 mutants (representing at least six distinct genetic loci) have been isolated in which the genetic lesion appears to specifically affect the motility properties of the flagellum. Although electron microscope examination has yet to reveal any distinct alteration in the structure of the flagellum which might account for the defects in flagellar activity, further analysis of these mutants may prove fruitful for studies on the motility function of the flagellum.

Of the remaining three mutants, one is characterized by fragile flagella (*dd-frag-1*) and 2 display defective flagellar function coupled with abnormal growth properties at the high temperature (*dd-l-108*) and (*dd-l-14*). Analysis of the phenotypes of these three mutants suggests that they are likely to represent mutations affecting flagellar function only indirectly.

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