The Dynein Gene Family in *Chlamydomonas reinhardtii*

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ABSTRACT

To correlate dynein heavy chain (*Dhc*) genes with flagellar mutations and gain insight into the function of specific dynein isoforms, we placed eight members of the *Dhc* gene family on the genetic map of Chlamydomonas. Using a PCR-based strategy, we cloned 11 *Dhc* genes from Chlamydomonas. Comparisons with other *Dhc* genes indicate that two clones correspond to genes encoding the alpha and beta heavy chains of the outer dynein arm. Alignment of the predicted amino acid sequences spanning the nucleotide binding site indicates that the remaining nine clones can be subdivided into three groups that are likely to include representatives of the inner-arm Dhc isoforms. Gene-specific probes reveal that each clone represents a single-copy gene that is expressed as a transcript of the appropriate size (>13 kb) sufficient to encode a high molecular weight Dhc polypeptide. The expression of all nine genes is upregulated in response to deflagellation, suggesting a role in axoneme assembly or motility. Restriction fragment length polymorphisms between divergent *C. reinhardtii* strains have been used to place each *Dhc* gene on the genetic map of Chlamydomonas. These studies lay the groundwork for correlating defects in different *Dhc* genes with specific flagellar mutations.

THE dynein ATPases are a family of motor enzymes that drive microtubule sliding in cilia and flagella and contribute to microtubule-based transport inside cells (reviewed in HOLZBAUR and VALLEE 1994; MITCHELL 1994). These enzymes convert the energy derived from nucleotide binding and hydrolysis into the directed movement of cellular cargoes toward the minus ends of microtubules. Cytoplasmic dynein appears to be relatively simple; it is composed of two identical dynein heavy chains (>500 kD) complexed with a cluster of intermediate (74 kD) and light intermediate (50–55 kD) chain components. On the other hand, the axonemal dyneins are much more diverse, and multiple dynein heavy chain (Dhc) isoforms have been identified in several species (GIBBONS *et al.* 1976; GIBBONS and GIBBONS 1987; GOLDSTEIN *et al.* 1982; BECKWITH and ASAI 1993; HAYS *et al.* 1994; STEPHENS and PRIOR 1995). This complexity is most clearly understood in Chlamydomonas, where biochemical and genetic analyses have identified three different Dhcs in the outer dynein arm (HUANG *et al.* 1979) and at least eight distinct Dhcs in the inner dynein arms (GOODENOUGH *et al.* 1987; PIPERNO *et al.* 1990; KAGAMI and KAMIYA 1992). The inner arm Dhcs are organized with various intermediate and light chains into seven different molecular complexes, one two-headed isoform (known as the I1 complex) and six single-headed isoforms (known as the I2 and I3 subspecies) (PIPERNO *et al.* 1990; KAGAMI and KAMIYA 1992).

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The presence of multiple dynein isoforms within the axoneme provides the cell with several potential mechanisms for affecting the flagellar waveform. Indeed, the study of flagellar mutations in Chlamydomonas has indicated that the outer and inner dynein arms have different functions in the generation of flagellar motility. Most mutants that lack the outer dynein arms swim with reduced beat frequencies (MITCHELL and ROSENBAUM 1985; KAMIYA 1988), whereas mutants that lack one or more inner arm isoforms have aberrant waveforms or are completely paralyzed (HUANG *et al.* 1979; BROKAW and KAMIYA 1987). These observations indicate that although the outer arms add power to the flagellar beat, the inner dynein arms are both necessary and sufficient to generate the flagellar waveform (BROKAW and KAMIYA 1987). Thus a critical step to understanding the mechanism of flagellar motility lies in elucidating the specific functions of the inner arm isoforms.

The complexity of inner arm composition also raises several important questions. First, what is the relationship between the multiple inner arm isoforms resolved by biochemical methods and numerous *Dhc* genes recently identified in several organisms by PCR procedures? Second, does each Dhc have a unique function or are some isoforms redundant? Finally, where are the multiple isoforms located in the flagellar axoneme, and how are they targeted to the appropriate site?

As a first step toward studying these questions, we have undertaken an extensive PCR screen to identify axonemal *Dhc* genes in Chlamydomonas. Although axonemal *Dhc* genes have recently been identified in other organisms (ASAI *et al.* 1994; GIBBONS *et al.* 1994; RASMUSSEN *et al.* 1994; TANAKA *et al.* 1995), Chlamydomonas

has several unique advantages for the study of axonemal dyneins. First, Chlamydomonas is haploid, and thus it is very easy to isolate motility mutations simply by screening for cells with altered swimming behaviors after mutagenesis. This strategy has identified mutations in >75 different flagellar loci (reviewed in DUTCHER 1995). Second, the ease of culture and the highly ordered structural organization of the flagellar axoneme mean that it is often possible to correlate motility phenotypes with both polypeptide deficiencies and structural defects. Finally, recent advances in the development of molecular techniques, such as RFLP mapping (RANUM *et al.* 1988), transformation (KINDLE 1990), and insertional mutagenesis (TAM and LEFEBVRE 1993), make it possible to correlate cloned genes with specific mutations. For instance, each of the outer arm *Dhc* genes (α, β, γ) has been linked to a specific outer arm mutation (SAKAKIBARA *et al.* 1991; PORTER *et al.* 1994; WILKERSON *et al.* 1994). However, there is still a large and growing collection of motility mutants for which the affected gene products have not yet been identified. These include several mutations that are known to disrupt the assembly of different inner arm isoforms (*e.g., pf23, pf9/ida1, ida2, ida3, ida5, ida6, pf2, pf3, sup-pf-3, sup-pf-5*) (HUANG *et al.* 1979; KAMIYA *et al.* 1991; PIPERNO *et al.* 1992, 1994; PORTER *et al.* 1992; KATO *et al.* 1993; GARDNER *et al.* 1994), as well as several other mutations that interfere with flagellar assembly (HUANG *et al.* 1977; ADAMS *et al.* 1982; HARRIS 1989) and/or alter the ability of cells to undergo phototaxis (HORST and WITMAN 1993; PAZOUR *et al.* 1995).

In this study, we report the identification and cloning of nine dynein-like sequences in Chlamydomonas. These sequences fall into three subgroups distinct from those associated with either cytoplasmic or outer arm dynein isoforms. Southern and Northern blot analyses suggest that the nine Dhcs are the products of distinct genes whose expression is enhanced by deflagellation. RFLP mapping procedures have been used to place the *Dhc* clones on the genetic map of Chlamydomonas. Several of these *Dhc* genes map near loci that have previously been shown to be involved in flagellar function.

MATERIALS AND METHODS

PCR amplification of *Dhc* genes: For the initial PCR reactions, four degenerate oligonucleotide primers were synthesized based on regions of amino acid sequence conservation between the sea urchin beta axonemal dynein (GIBBONS *et al.* 1991; OGAWA 1991) and Drosophila cytoplasmic dynein (LI *et al.* 1994). These primers correspond to amino acid sequences surrounding the predicted ATP hydrolytic site (GIBBONS *et al.* 1991; OGAWA 1991) and were used previously to isolate a family of *Dhc* genes from Drosophila (RASMUSSEN *et al.* 1994). The specific amino acids and the nucleotide sequence of each primer is listed below, written 5′ to 3′. Primer 1, a sense primer (ITPLTDR): AT(A/C/T)-AC(A/C/G/T)-CC(A/C/G/T)-CT(C/G/T)-AC(A/C/G/T)-GA(C/T)-(A/C)G. Primer 2, a sense primer (AGTGKTE): G C(A/C/G/T)-GG(A/C/T)-AC(A/C/G/T)-GG(A/C/T)-AA (A/G)-AC(A/C/G/T)-GA. Primer 3, an antisense primer (CFDEFNR): C(G/T)-(A/G)TT-(A/G)AA-(C/T)TC-(A/G)TC-(A/G)AA-(A/G)CA. Primer 4, an antisense primer (IFITMNP): GG(A/G)-TTC-AT(A/C/G/T)-GT(A/G/T)-AT(A/G)-AA(G/A/T)-AT. Oligonucleotide primers were purchased from Oligos, *etc.,* (Wilsonville, OR) or synthesized on an Applied Biosystems 392 DNA synthesizer (Foster City, CA) and generously supplied by T. HAYS, University of Minnesota.

To facilitate the recovery of all members of the dynein gene family, genomic DNA was used as the starting template for the PCR amplification. In this strategy, each dynein gene would be equally represented, regardless of the abundance of the corresponding mRNA. Genomic DNA (100–200 ng) prepared from a wild-type *C. reinhardtii* strain (137c) was added to 50–100 μl PCR reaction mixtures containing 0.2 mM dNTPs, 0.6–1.0 μM of each of two degenerate primers, 1× reaction buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton-X-100) and 0.025 U/μl *Taq* DNA polymerase (Promega Corp., Madison, WI). Following an initial 2-min denaturation step at 95°, the samples underwent 25 cycles of 50° for 2 min, 75° for 3 min, and 90° for 1 min, and then terminated with a single cycle of 50° for 2 min, 72° for 10 min.

The first rounds of PCR amplification utilized primers 1 and 4 in one reaction and primers 1 and 3 in a second reaction. Two microliters of amplified product then served as the starting template in a second round of reactions using internal primers. The first reaction was reamplified using primers 1 and 3 to yield a single product of ~230 bp. The second reaction was reamplified using primers 2 and 3 to yield two products of ~160 and ~300 bp. These products were recovered by ethanol-precipitation, resuspended in 20 μl TE, treated with T4 polynucleotide kinase and Klenow, and purified via low-melt agarose gels. The three fragments were then ligated separately into *Eco*RV-digested pBluescript KS (Stratagene, La Jolla, CA) and transformed into *Escherichia coli DH5α (F′)* (SAMBROOK *et al.* 1989). Eighteen transformants were picked from each ligation and rescreened by PCR using primers 2 and 3. Plasmid DNA was then prepared from PCR positive transformants (SAMBROOK *et al.* 1989).

Forty-seven of the PCR positive clones were sequenced, and seven different dynein-related sequences were identified. Only one sequence (*Dhc1*) was identified in the 230-bp fragment, whereas three different sequences were identified in the 160-bp fragment (*Dhc3* and the outer arm α*Dhc* and β*Dhc* genes). Three additional sequences were found in the 300-bp fragment (*Dhc2, Dhc4,* and *Dhc5*). All of the 300-bp products contained putative intron sequences in addition to dynein-related coding sequences.

To recover additional *Dhc* genes, a second PCR screen was performed at an annealing temperature of 59° using 10 μM of primer 1 and a modified version of primer 4 (3′ NEW, antisense for FITMNPG: CC(A/C/G/T)-GG(A/G)-TTC-AT(A/C/G/T)-GT(A/G/T)-AT(A/G)-AA). Four PCR products ranging in size from ~400 to ~850 bp were identified, subcloned, and screened as described above. Sequence analyses of 21 PCR products yielded two new dynein-related sequences (*Dhc6* and *Dhc7*) and longer versions of α*Dhc,* β*Dhc, Dhc2,* and *Dhc3.*

The number of times that each *Dhc* sequence was found among the different PCR products varied considerably for each *Dhc* gene; the final numbers are noted here in parenthesis: α*Dhc* (7), β*Dhc* (13), *Dhc1* (13), *Dhc2* (19), *Dhc3* (5), *Dhc4* (1), *Dhc5* (2), *Dhc6* (6), and *Dhc7* (2). We also failed to recover the γ*Dhc* sequence, although the degenerate primers used in these experiments would have been expected to amplify this gene (WILKERSON *et al.* 1994). These results indicate that the different PCR screens were not exhaustive, and that there may be additional *Dhc* genes present in the Chlamydomonas genome.

Isolation of genomic clones: To verify and extend each of

the *Dhc* sequences, longer clones were obtained by screening a large insert genomic library (Figure 1). This library was constructed by R. SCHNELL (University of Minnesota) in λFIXII (Stratagene, La Jolla, CA) using genomic DNA isolated from the *C. reinhardtii* wild-type strain 21gr, *mt+* (SCHNELL and LEFEBVRE 1993). Approximately 50,000 pfu were plated on the *E. coli* strain LE392 and then screened by hybridization of ^{32}P-labeled *Dhc* sequences to Magnagraph filter replicas (Micron Separations, Inc., Westboro, MA). Positive phage were plaque purified and rescreened two to three times by standard techniques (SAMBROOK *et al.* 1989). Phage DNA was isolated as described by CHISHOLM (1989), and insert DNA was released and mapped using the restriction enzymes *Not*I, *Sal*I, and *Sac*I. DNA fragments encoding the putative ATP hydrolytic domain were identified by probing Southern blots of restriction digests with ^{32}P-labeled PCR products or PCR primers tailed with terminal deoxynucleotidyl transferase (Gibco-BRL, Inc., Gaithersburg, MD) and α-^{32}P-CTP. These fragments were then subcloned into pBluescript II KS$^+$ and sequenced using the appropriate primers. Complete double-stranded DNA sequence was obtained for the region encoding the predicted ATP hydrolytic domain of Dhc1–Dhc7. When necessary, fragments of the gene adjacent to the region encoding the hydrolytic domain were also subcloned and used to identify gene-specific probes for use in expression and mapping studies.

DNA sequencing and analysis: Double-stranded DNA templates were sequenced using either the PCR primers listed above or standard plasmid vector primers and Sequenase, version 2.0, according to manufacturer's instructions (United States Biochemical, Cleveland, OH). Sequence data was assembled and analyzed using the Genetics Computer Group software, version 8 (Madison, WI) (DEVEREUX *et al.* 1984) on a Sun Microsystems Solaris 2.4 computer available through the Advanced Biosciences Computing Center (University of Minnesota) or the MacVector Sequence Analysis Software Package (International Biotechnologies, Inc., Rochester, NY) on a Macintosh microcomputer. Potential open reading frames were identified using the GCG program CodonPreference and a codon usage table compiled from the coding regions of several different Chlamydomonas nuclear sequences (SILFLOW *et al.* 1985; WILLIAMS *et al.* 1989; SAVEREIDE 1991; MITCHELL and BROWN 1994; ZHANG 1996; R. SCHNELL, personal communication). Potential splice donor and acceptor sequences were also identified based on splice junction consensus sequences found in these same Chlamydomonas nuclear genes.

Preparation of genomic DNA: Genomic DNA was isolated from three different wild-type Chlamydomonas strains that are polymorphic at the DNA sequence level, *C. reinhardtii* (137c), *C. reinhardtii* (S1-D2), and *C. smithii* (CC-1373). Cells were grown in 5 liters of rich liquid media supplemented with additional potassium phosphate as described by WITMAN (1986). Cells were harvested with a Pellicon Cell Harvesting apparatus equipped with Durapore filter cassettes, resuspended in minimal media, and lysed overnight in 10 mM TrisHCl pH 7.6, 20 mM EDTA pH 8.0, 5% SDS, 1 mg/ml Pronase E at 50° with gentle agitation (JOHNSON and DUTCHER 1991). Ammonium acetate was added to a final concentration of 0.8 M, and the crude lysate was extracted once with phenol-chloroform. The aqueous phase was mixed with an equal volume of isopropanol to precipitate the genomic DNA. The pellet of DNA was washed twice with 70% ethanol and resuspended in 10 mM Tris-HCl pH 8.0, 10 mM EDTA. The crude DNA preparation was then further purified by banding on CsCl gradients (SAMBROOK *et al.* 1989).

Southern blot analysis: DNA samples (3–5 μg per lane) were digested with a series of restriction enzymes (*Pvu*II, *Pst*I, *Eco*RI/*Xho*I, *Sal*I, *Sac*I, *Sph*I, *Hind*III, and *Sma*I), separated on 0.8–1.0% agarose gels, and transferred overnight to nylon membranes (either Zetabind, Cuno, Inc., Meriden, CT or Magnagraph, Micron Separations, Inc., Westboro, MA) according to standard procedures (SAMBROOK *et al.* 1989). Zetabind membranes were washed 5 min in 2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate) at room temperature and air-dried, whereas Magnagraph membranes were washed in 5× SSC at 60°, baked in a vacuum oven at 80° for 1–2 hours, and then UV-crosslinked at a setting of 20,000 μjoules (Stratalinker, Stratagene, Inc., La Jolla, CA). Blots were prehybridized for a minimum of 4 hr at 65° in 6× SSPE (1× SSPE is 183 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA), 0.5% nonfat dried milk, 0.5% SDS, 50 mM Tris-HCl pH 7.5, 500 μg/ml salmon sperm DNA, and then hybridized overnight at 65° in the same solution plus 10% dextran sulfate and the appropriate ^{32}P-labeled DNA probe. Probes were radiolabeled with α-^{32}P-dCTP using the Prime-it II random primer labeling kit (Stratagene, Inc., La Jolla, CA) according to manufacturer's instructions. Probe templates were then purified on Sephadex-G-50 spin columns, denatured, and added to hybridization solutions. Blots were washed twice with 2× SSC, 0.1% SDS at room temperature for 5 min, followed by three 30-min washes with 0.1× SSC, 0.1% SDS at 65° (high stringency conditions). Low stringency hybridizations and washes were carried out at 45°. Autoradiograms were exposed in the presence of enhancer screens (Dupont Cronex, Wilmington, DE) at −80°. Blots were stripped for reprobing according to manufacturer's instructions (Zetabind) or by two washes in 0.2 N NaOH, 1% SDS at 42° for 20–25 min each (Magnagraph).

RNA preparation and Northern blot analysis: RNA for Northern blot analysis was prepared from wild-type Chlamydomonas (137c, *mt+*) cells both before and 45 min after deflagellation induced by pH shock (WITMAN *et al.* 1972; WILKERSON *et al.* 1994). Cells (2×10^9) were resuspended in 5 ml of minimal medium, added to 100 ml of lysis buffer (20 mM Tris-HCl pH 8.0, 20 mM EDTA, 5% SDS, and 1 mg/ml proteinase K) with gentle stirring, and incubated at room temperature for 2–4 hr. The lysate was then mixed with 10 ml of 3 M sodium acetate pH 5.2, followed by an equal volume of 1:1 phenol:chloroform and centrifuged at 3000 × *g* for 30 min. The aqueous phase was removed and mixed with an equal volume of isopropanol at room temperature to precipitate the RNA. After 15 min, the RNA was collected by centrifugation, washed with 80% ethanol, and resuspended in 5 ml of sterile dH_20. This solution was then mixed with 5 ml of 4 M LiCl and allowed to incubate at 4° overnight. The RNA was recollected by centrifugation, and the supernatant containing contaminating DNA and carbohydrate was gently removed. The RNA pellet was dissolved in sterile dH_20. Sodium acetate was added to 0.3 M, and the RNA was reprecipitated by the addition of 2.5 volumes of ethanol for long term storage at −80°.

For Northern blots, aliquots containing ~20 μg of total RNA were redissolved in sterile dH_20, mixed with sample buffer, and run on 1× MOPs, 18% formaldehyde, 0.75% agarose gels at 15–20 V overnight. Gels were washed briefly with 0.15 M NaCl, 0.05 N NaOH (one time for 5 min and one time at 10 min), neutralized with 0.15 M NaCl, 0.05 M TrisHCl pH 8.0 (three times at 10 min), and transferred in 10× SSC by capillary action to a Zetabind membrane. After a 5-min wash in 5× SSC, the membranes were air-dried and then UV-crosslinked at a setting of 20,000 μjoules (Stratalinker, Stratagene, Inc., La Jolla, CA). Prehybridization and hybridization conditions were identical to those described above for Southern blots. Control experiments in which the Northern blots were hybridized with a probe for the ribosomal protein S14 gene (NELSON *et al.* 1994) confirmed that equal amounts of RNA were loaded in all lanes.

RNA preparation and reverse transcription (RT)-PCR anal-

ysis of dynein sequences: RNA was isolated from 2×10^8 *C. reinhardtii* (137c) cells both before and 30 min after deflagellation using the guanidinium thiocyanate method (CHOMSZYNSKI and SACCHI 1987). To remove minor amounts of contaminating genomic DNA, total RNA was treated with RQ1 DNase (Promega Corp., Madison, WI), extracted with phenol:chloroform and recovered by ethanol precipitation. cDNA was made from one microgram total RNA using AMV reverse transcriptase (Promega Corp.) with an antisense primer corresponding to the sequence encoding the amino acids ITMNPGY, a highly conserved region present in all Chlamydomonas Dhc sequences identified thus far. The sequence of the RT primer is 5′-TA-(G/C/A)CC-(G/A)GG-GTT-CAT-GGT-(G/C)A-3′. To control for any residual genomic DNA contamination, a parallel set of reactions was carried out in which the reverse transcriptase was omitted.

Two microliters of the resulting cDNA products were then used as the template in a PCR reaction containing a sense primer corresponding to conserved amino acids in the P-loop region and a gene-specific antisense primer corresponding to a region of sequence divergence 5′ of the RT primer. Two different sense primers were used: a GKT-long primer corresponding to the amino acids TGKTETTK (5′-AC(C/T/G)-GGC-AAG-AC(C/G)-GAG-ACC-(A/G) (C/T) (C/G)-AAG-3′) and a GKT-short primer corresponding to the amino acids TGKTET (5′-AC(C/G/T)-GGC-AAG-AC(C/G)-GAG-ACC-3′). The amino acid residues used to design the gene-specific primers are shown in Figure 2, and the antisense nucleotide sequences corresponding to these residues are listed here. *Dhc1:* 5′-CGCGGGTCGATGCTAATC-3′; *Dhc2:* 5′-GTTGAG-GTCGATCTCCGTGTC-3′; *Dhc3:* 5′-ACGAGGGCAGCAGCC-GGAT-3′; *Dhc4:* 5′-CTTCAGCGGCAGTTCGGA-3′; *Dhc5:* 5′-GCATCTCCGTGCCCTCAA-3′; *Dhc6:* 5′-CCGCAGGCTCAG-CTTGGTG-3′; and *Dhc7:* 5′-TGGGCACCAGGCGGATCA-3′.

For *Dhc2–Dhc7,* these primers span regions of genomic DNA containing putative introns that should be spliced out of a mature mRNA transcript. Because *Dhc1* does not have an intron in this region, a second sense primer was designed to a region of amino-terminal sequence (CTGLFK) that is predicted to come together with the proper splicing of an intron. The nucleotide sequence for the *Dhc1*-splice primer is 5′-TGC-ACG-GGC-CTG-TTC-AAG-3′.

The PCR reactions consisted of 2 μl cDNA, 0.2 mM each dNTP, 200 nM GKT primer (either GKT-long or GKT-short), 200 nM sequence specific primer, 1× reaction buffer, 1.5 mM $MgCl_2$, and 2.5 units *Taq* polymerase (Promega Corp.) in a total volume of 50 μl. These reactions underwent 35 cycles of 94° for 1 min, 58° for 1 min and 72° for 1 min. The PCR products were run on a 1.5% agarose gel and compared against a 100-bp ladder (Gibco-BRL, Inc.). Products of the sizes predicted for mature transcripts were gel-purified, subcloned, and sequenced as described above.

Mapping strains: Because RFLPs with *Dhc* probes were observed infrequently between *C. reinhardtii* and *C. smithii* strains (Figure 7), it was necessary to rebuild the RFLP map of the Chlamydomonas genome using tetrad progeny derived from crosses between multiply marked *C. reinhardtii* strains and the polymorphic strain S1-D2. Three different mapping strains were used. The first strain (CC-28) contains the genetic markers *mt+, msr1, act2, nic13, pyr1, can1, sr1, ac17, y1,* and *pf2.* The progeny of 19 tetrads from a cross between CC-28 and S1-D2 were isolated and scored as described in GROSS *et al.* (1988). These progeny are known as the 400 series and were generously provided by P. LEFEBVRE and C. SILFLOW (University of Minnesota). The second strain (13-2) contains the genetic markers *mt+, ac17, act1, pf9-2, can1, sr1, ery1, y1,* and *maa4.* The progeny of 19 tetrads from a cross between 13-2 and S1-D2 were isolated and scored as described in DUTCHER *et al.* (1991). These progeny were generously provided by S. DUTCHER (University of Colorado). CC28 and 13-2 also contain two unlinked nitrate reductase mutations (*nit1* and *nit2*). Because *nit1* strains can grow on medium containing 2 mM nitrite as the sole nitrogen source, but *nit2* strains cannot (SCHNELL and LEFEBRVE 1993), we were also able to score the *nit2* marker in both sets of progeny. The third strain contains the inner arm mutation *ida2,* which has a slow swimming motility phenotype (KAMIYA *et al.* 1991). *ida2* was crossed to S1-D2 in this laboratory, and the progeny of 18 tetrads were isolated and scored by phase contrast microscopy. The three mapping strains therefore contain 16 different genetic markers that map to 13 of the 17 identified Chlamydomonas linkage groups (DUTCHER *et al.* 1991, see Table 1).

RFLP analysis: DNA was isolated from each tetrad member using a modified DNA miniprep procedure (JOHNSON and DUTCHER 1991). DNA samples (3 μg/lane) were digested overnight with either *Pst*I, *Pvu*II, or *Eco*RI/*Xho*I, separated on 0.8% agarose gels, and blotted to Magnagraph as described above. Blots of the appropriate enzyme digest were then hybridized overnight with ^{32}P-labeled probes as described above. Some of the molecular markers obtained from the Lefebvre/Silflow laboratories (see below) were labeled using digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN). These probes were provided in a hybridization solution containing 5× SSPE, 10× Denhardt's solution (0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidone), 50% formamide, 1% SDS, 300 μg/ml denatured salmon sperm DNA. The digoxigenin probes were hybridized at 42° overnight. Wash conditions were identical to those described above, but hybridization was detected using the Lumiphos detection system according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Filters were stripped and reused several times. Technical problems occasionally made it difficult to score all of the tetrad progeny. However, because the genetic and molecular markers segregated 2:2 in tetrad progeny, we could sometimes extrapolate the genotype(s) of one or two progeny from the genotypes of the other members of the tetrad.

Molecular markers for RFLP mapping: To expand coverage over the Chlamydomonas genome, 26 molecular markers were also used to probe the RFLP mapping filters (see Table 1). Three molecular markers on linkage group XIX (*Gulliver* G, *Gulliver* H, and GP37), along with two other *Gulliver* elements (band b and band d) had been previously mapped on the CC-28 progeny by L. RANUM (1989). The remaining molecular markers were obtained as plasmids or digoxigenin-labeled probes from individuals in the laboratories of C. SILFLOW and P. LEFEBVRE (University of Minnesota). Three cDNA clones (pcf6-8, pcf6-187 and pcf8-13) were isolated by SCHLOSS *et al.* 1984. pcf8-13 is a constitutively expressed transcript; pcf6-8 corresponds to an unknown transcript that accumulates after deflagellation (SCHLOSS *et al.* 1984), and pcf6-187 corresponds to the transcript for the radial spoke polypeptide that is the gene product of the *PF1* locus (CURRY *et al.* 1992). The RsaI clone corresponds to the transcript for another radial spoke polypeptide that is the gene product of the *PF26* locus (RANUM *et al.* 1988; WILLIAMS *et al.* 1989). The pEA4-2 clone is a genomic fragment of the alpha *Dhc* gene, which has been mapped to the *ODA11* locus (SAKAKIBARA *et al.* 1991). The genomic clone pMN24, which corresponds to the *NIT1* locus (FERNANDEZ *et al.* 1989), was provided by R. SCHNELL, along with a probe (pMN84) for a dispersed repetitive element known as *Tcr1* (SCHNELL and LEFEBVRE 1993). The p5E8 clone corresponds to the *VFL1* locus (TAM and LEFEBVRE 1993). The markers designated GP are cloned *Pst*I fragments of genomic DNA; those designated CN are random cDNA clones (see Table 1). These clones specify loci that have been placed on the *C. reinhardtii/C. smithii* RFLP map (C. SILFLOW, personal communication, see Chlamydomonas database, http://www.botany.duke.edu/DCMB/chlamy.htm).

RESULTS

Cloning of dynein heavy chain (*Dhc*) genes in Chlamydomonas: Sequence analysis of axonemal and cytoplasmic *Dhc* genes in other organisms has identified several regions of amino acid homology (reviewed in GIBBONS 1995). In particular, the region surrounding the first P-loop, which is thought to correspond to the primary ATP hydrolytic site, is highly conserved. Based on this conservation, five degenerate oligonucleotide primers were used in nested combinations to isolate PCR products from Chlamydomonas DNA ranging in size from 160 to 850 bp (see MATERIALS AND METHODS for details).

Sequence analysis of 68 subclones identified nine dynein-related sequences that contain the conserved ATP hydrolytic site. Two of the sequences correspond to the genes encoding the alpha and beta Dhcs of the outer dynein arm (MITCHELL and BROWN 1994), but the other sequences appear to represent seven different *Dhc* genes. To verify and extend these sequences, longer clones were recovered from a genomic library. For each gene, overlapping phage clones spanning 20–30kb of DNA around the region encoding the putative ATP hydrolytic site were isolated, subcloned, and partially sequenced (Figure 1). The predicted amino acid sequences through the hydrolytic domain of the *Dhc* clones are shown in Figure 2.

The genomic clones recovered for *Dhc1–Dhc7* appear to contain small introns occuring at semi-conserved positions within the coding sequence. These introns were identified by sudden changes in codon bias and by the presence of Chlamydomonas consensus splice sites (see MATERIALS AND METHODS). To verify expression and confirm both the splice sites and the predicted amino acid sequences of the *Dhc* genes, the corresponding cDNA clones were amplified from total RNA using sequence specific primers (see Figure 2) and RT-PCR procedures (see below). RT-PCR products of the appropriate size were cloned, and individual colonies were sequenced. This analysis confirmed the splice sites and amino acid sequences shown in Figure 2.

Sequence analysis of the RT-PCR products also resulted in the recovery of two additional dynein-related sequences, *Dhc8* and *Dhc9*. These two cDNAs were obtained from the pool of RT-PCR products amplified with the *Dhc5* primer. Because Southern blot analysis suggests that the *Dhc8* and *Dhc9* sequences are derived from two additional *Dhc* genes (see below), the predicted amino acid sequences of these two clones are included in Figure 2.

Comparisons between the deduced amino acid sequences of the Chlamydomonas *Dhc* genes: Comparisons between the nine Dhc sequences recovered in the PCR screens and the three outer arm Dhc sequences identified by others (MITCHELL and BROWN 1994; WILKERSON *et al.* 1994) demonstrate that, within an 120 amino acid region surrounding the ATP hydrolytic site, the Chlamydomonas Dhc sequences are very highly conserved. For example, Dhc1–Dhc9 share 60–80% amino acid identity with the beta Dhc of the outer arm. However, further comparison between Dhc1–Dhc9 indicates that these sequences are even more closely related to one another than to the three Dhcs of the outer arm. As shown in Figure 3, the Dhc sequences can be divided into at least three subgroups. One group contains four genomic clones (Dhc2, Dhc4, Dhc5, and Dhc6) and the two cDNAs (Dhc8 and Dhc9). Interestingly, the four genomic clones in this group share a conserved exon splice site (see Figure 2). The Dhc3 and Dhc7 sequences represent a second group of closely related genes that also share another conserved exon splice site (see Figure 2). The Dhc1 sequence is similar to Dhc3 and Dhc7, but other evidence suggests that it may represent a third subclass of Dhc sequence (see below). Surprisingly, none of the Chlamydomonas sequences identified thus far shows significant homology to any of the cytoplasmic *Dhc* genes cloned in other organisms. These results suggest that the *Dhc1–Dhc9* sequences correspond to structural genes for axonemal Dhcs and encode representatives of the I1, I2, and I3 inner arm isoforms identified by biochemical methods (KAGAMI and KAMIYA 1992). Consistent with this interpretation, the Chlamydomonas Dhc sequences do share significant sequence homologies with axonemal Dhc sequences identified by expression analysis in sea urchin, Drosophila, Paramecium, and rat (ASAI *et al.* 1994; GIBBONS *et al.* 1994; RASMUSSEN *et al.* 1994; TANAKA *et al.* 1995, see DISCUSSION).

Size of the *Dhc* gene family in Chlamydomonas: To estimate the size of the *Dhc* gene family in Chlamydomonas, we performed genomic Southern blot analysis. Genomic DNA from two polymorphic Chlamydomonas strains was digested with a series of restriction enzymes, gel fractionated, blotted to reuseable nylon membranes, and hybridized with each *Dhc* PCR product. Figure 4 illustrates the pattern of restriction fragments obtained with the enzymes *Sma*I, *Pst*I, and *Sph*I after hybridization at both high and low stringency with a 227-bp probe corresponding to a conserved region of the *Dhc1* sequence. These enzymes do not cut the *Dhc1* gene in the region represented by the probe. At high stringency (Figure 4, left panel), the *Dhc1* probe hybridizes to one major band in each lane and approximately four or five minor bands. At lower stringency (Figure 4, right panel), however, the *Dhc1* probe cross-hybridizes to at least 14 or 15 restriction fragments. We therefore stripped and reprobed this blot at high stringency with the other *Dhc* clones (data not shown). Using this strategy, we were able to identify the genomic restriction fragments corresponding to 11 *Dhc* genes (including the outer arm α*Dhc* and β*Dhc* genes). Our results suggest that the Chlamydomonas genome contains approximately 14–15 closely related *Dhc* genes, including at least three to four *Dhc* sequences that have not yet been identified (see Figure 4, right panel).

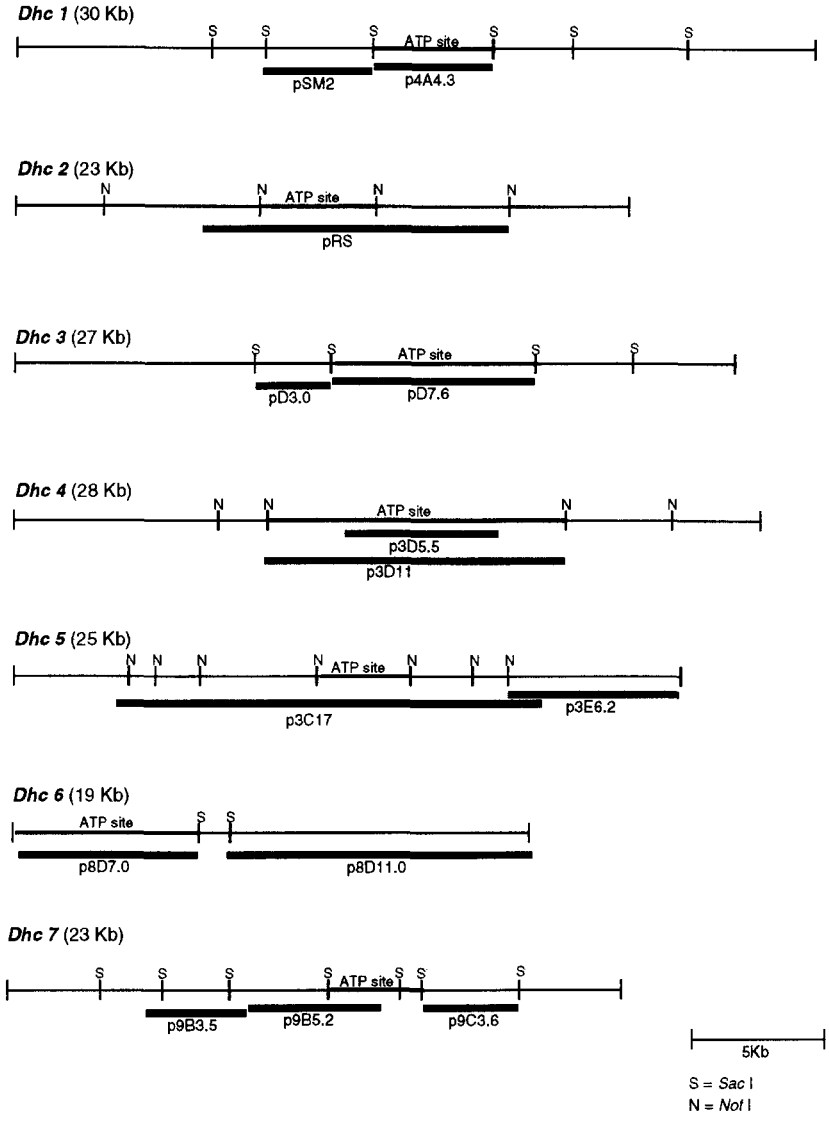

FIGURE 1.—Isolation of genomic clones encoding seven different Dhc sequences. PCR products representing seven different *Dhc* genes were used to screen a large insert genomic library. Overlapping phage clones were recovered for each gene and analyzed by restriction digests with the enzymes *SacI*, *SalI*, and *NotI*. The restriction fragments encoding the ATP hydrolytic domain were subcloned and sequenced to verify the identity of each gene. The top line represents the genomic DNA recovered for each *Dhc* gene. The positions of the subclones used for expression and mapping studies are indicated by the black bars below.

Expression of *Dhc* genes: Previous study has shown that large transcripts (>13 kb) encoding the outer arm Dhcs are enriched in RNA preparations isolated from deflagellated cells (MITCHELL 1989; WILKERSON *et al.* 1994). To determine if the *Dhc* genes identified in this study show a similar pattern of transcript accumulation, we analyzed RNA isolated before and after deflagellation by both Northern blots and RT-PCR procedures. For *Dhc1–Dhc7*, fragments ranging in size from 3 to 17 kb were subcloned from a large insert genomic library (see Figure 1) and tested on Southern blots at high stringency to identify probes that are gene-specific. The gene-specific probes were then hybridized under identical conditions to Northern blots containing 20 μg of total RNA isolated before and 45 min after deflagellation induced by pH shock. As shown in Figure 5, each clone hybridized to a large transcript (>13 kb) that is enriched in RNA isolated from deflagellated cells.

The pattern of transcript accumulation is particularly interesting for two *Dhc* genes. Transcripts corresponding to the *Dhc3* gene could only be detected after prolonged exposures and appear to be much less abundant than other *Dhc* transcripts (Figure 5). In addition, the *Dhc4* probe hybridized with approximately equal intensity to two large transcripts that are both >13 kb (Figure 5). Whether these two transcripts represent alternatively spliced variants of the *Dhc4* gene or incomplete mRNA processing of a single *Dhc4* transcript is unknown. Regardless, all of the transcripts are large enough to encode Dhc polypeptides, and, as expected, all of the transcripts are upregulated upon deflagellation. These results, together with the analysis of the deduced amino acid sequences, suggest that the encoded Dhcs are involved in axoneme assembly and/or motility.

To verify that the signals observed on the Northern blots were due to specific hybridization with the transcript of interest and not cross-hybridization to other *Dhc* transcripts, we assayed for the presence of each sequence using the more sensitive procedure of RT-PCR. Total RNA was isolated from wild-type cells before and after deflagellation and then converted to cDNA using an antisense primer corresponding to the conserved amino acid sequence ITMNPG. This primer is degenerate and should therefore prime the synthesis

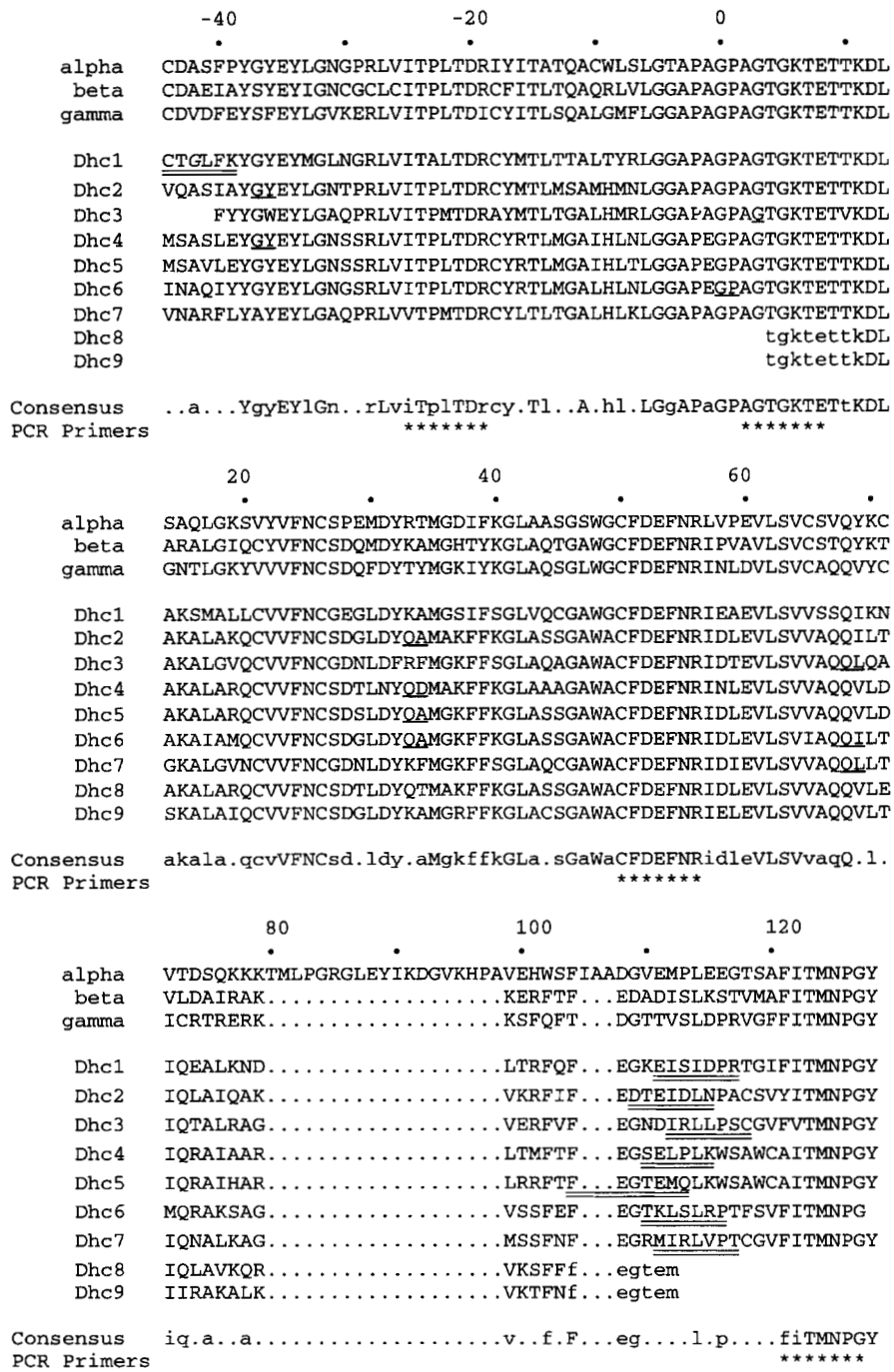

FIGURE 2.—The dynein heavy chain (*Dhc*) gene family in Chlamydomonas. The deduced amino acid sequences of 12 *Dhc* genes in the region surrounding the conserved ATP hydrolytic site, also known as the first P-loop, are shown here. The outer arm Dhc sequences (α, β, and γ) previously identified by MITCHELL and BROWN (1994) and WILKERSON *et al.* (1994) are included for comparison. In the consensus line below, capital letters indicate amino acid residues that are conserved in all family members, lowercase letters indicate those conserved in at least six of the clones, and periods indicate nonconserved residues. The positions of exon splice sites are shown by a single underline (_). Sequences used to design gene-specific primers are indicated by a double underline (_). Because the *Dhc8* and *Dhc9* sequences were recovered in the RT-PCR screen, the residues identified in lowercase correspond to the amino acids encoded by the PCR primers. These residues have not yet been confirmed by the sequence analysis of larger genomic clones. The *Dhc1–Dhc9* sequences have been deposited in GenBank under the accession numbers U61364–U61372. The outer arm *Dhc* sequences are listed under the accession numbers L26049, U02963, and U15303, respectively.

of cDNAs for all of the *Dhc* genes if they are expressed as RNA. The resulting cDNAs were then used as templates in a series of PCR reactions using the appropriate gene-specific primers (see MATERIALS AND METHODS and Figure 2). These primers were chosen such that they span regions of genomic DNA containing putative introns that would be spliced out of a mature mRNA transcript. As shown in Figure 6, RT-PCR products of the size expected for a properly processed trancript are seen in each reaction. Consistent with the Northern data, the RT-PCR products were more abundant in RNA samples prepared from deflagellated cells (data not shown). Control reactions in which reverse transcriptase was omitted during the initial cDNA synthesis confirmed that these products were derived from mRNA and not from contaminating genomic DNA. To ensure that each RT-PCR product corresponded to the appropriate *Dhc* gene, at least two independent subclones were sequenced for each reaction. This analysis confirmed that all of the *Dhc* genes are expressed as mature transcripts.

Identification of RFLPs: The results of our expression studies suggest that Dhc1–Dhc9 play some role in axoneme assembly or motility. Because previous work in Chlamydomonas has identified >75 genetic loci that contain mutations which affect flagellar function (HAR-

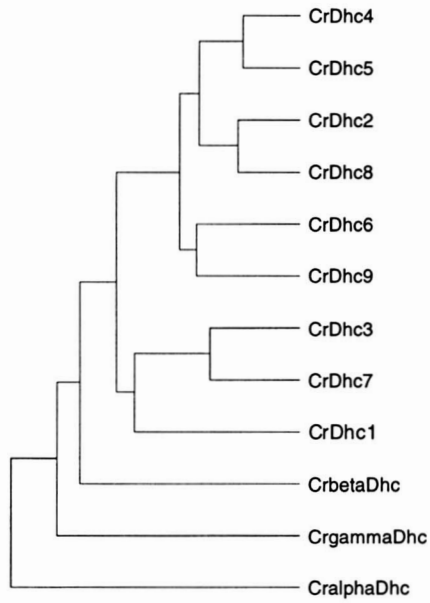

FIGURE 3.—Diagrammatic alignment of the Dhc sequences. The Dhc sequences shown in Figure 2 were aligned using the GCG program Pileup. Dhc1–Dhc9 cluster into groups distinct from the outer arm Dhcs.

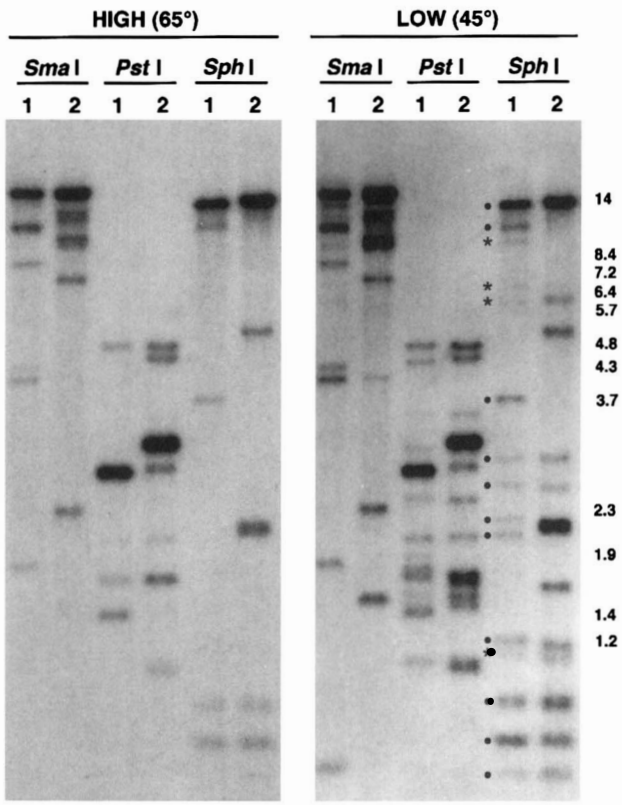

FIGURE 4.—Southern blot analysis of Chlamydomonas DNA probed with the *Dhc1* PCR product. Genomic DNA isolated from two polymorphic wild-type *C. reinhardtii* strains, 137c (1) and S1-D2 (2), was digested with the restriction enzymes *Sma*I, *Pst*I, and *Sph*I, fractionated on a 1% agarose gel, blotted, and hybridized with a 227-bp PCR product representing the highly conserved ATP hydrolytic site of the *Dhc1* gene. The panel on the left was hybridized at high stringency (65°), and the panel on the right at low stringency (45°). This blot was then stripped and reprobed at high stringency with each of the *Dhc* sequences to identify the corresponding restriction fragments. The restriction fragments identified as specific *Dhc* genes in the *Sph*I digest are indicated by black dots. Unidentified bands are marked with *.

RIS 1989; DUTCHER 1995), we were interested in testing whether any of the *Dhc* sequences might be linked to any of the previously identified flagellar loci. However, before we could place the *Dhc* genes on the genetic map of Chlamydomonas, a specific RFLP had to be identified for each gene. A physical map of the Chlamydomonas genome had previously been constructed using two polymorphic strains, *C. reinhardtii* (137c) and *C. smithii* (RANUM *et al.* 1988). Because this map contains over 200 molecular and genetic markers (C. SILFLOW, personal communication), we first screened genomic DNA isolated from *C. reinhardtii* (137c) and *C. smithii* strains for the presence of RFLPs that could be used as molecular markers to map the *Dhc* genes. Unfortunately, RFLPs were infrequently observed between these two strains using the *Dhc* probes (data not shown). We therefore analyzed DNA from a third Chlamydomonas strain, S1-D2, for the presence of *Dhc* RFLPs. As shown in Figure 7, RFLPs between 137c and S1-D2 are readily observed using four different restriction enzymes (*Eco*RI, *Sal*I, *Sma*I, and *Sph*I) and a 6.2-kb probe corresponding to *Dhc5*. Similar results were observed with probes for the other *Dhc* genes (Figure 8 and data not shown). These results demonstrated the need to map the *Dhc* genes using tetrad progeny derived from crosses with the S1-D2 strain.

Construction of a RFLP map: Three strains of *C. reinhardtii* that carry 16 different genetic markers from 13 of the 17 known linkage groups were crossed to the wild-type strain, S1-D2. Eighteen tetrads were dissected from each cross, and the segregation of the different genetic markers in the resulting tetrad progeny were scored as previously described (see MATERIALS AND METHODS). Analysis of the pattern of segregation of the genetic markers indicated that each marker segregated 2:2, and that markers on different linkage groups assorted independently (GROSS *et al.* 1988, and data not shown).

To provide coverage over the remainder of the Chlamydomonas genome, DNA was isolated from the tetrad progeny, digested with restriction enzymes known to reveal RFLPs, transferred to Southern blots, and hybridized with 26 different molecular markers. The pattern of segregation of each molecular marker was analyzed with respect to the genetic markers and the other molecular markers (data not shown but available upon request). A complete listing of the genetic and molecular markers used in this study and their positions on the different linkage groups is provided in Table 1. Using this collection of markers, linkage can be detected over ~90% of the Chlamydomonas genome (C. SILFLOW, personal communication).

Because we analyzed marker segregation in tetrad

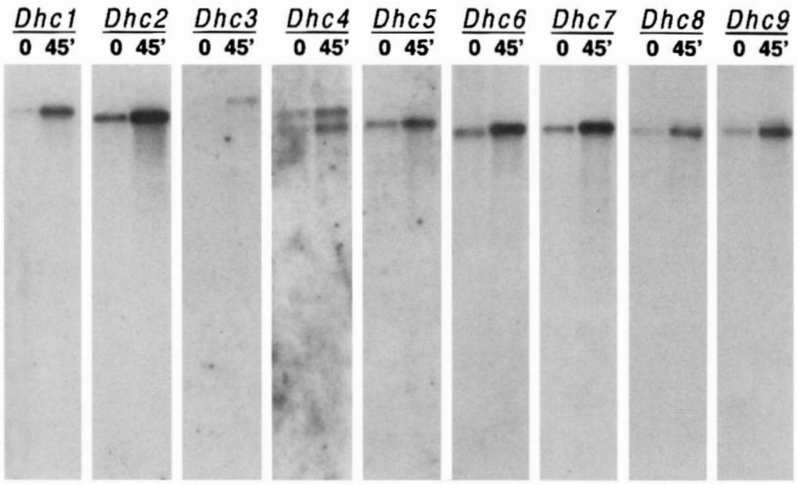

FIGURE 5.—Northern blot analysis of *Dhc* transcripts. Twenty micrograms of total RNA isolated before (0) and after (45′) deflagellation of wild-type cells was fractionated on 0.8% agarose-formaldehyde gels, blotted, and hybridized with probes for each of the *Dhc* genes. The probes for *Dhc1–Dhc7* were the subclones from the large insert genomic library shown in Figure 1: *Dhc1* (pSM2), *Dhc2* (pRS), *Dhc3* (pD3.0), *Dhc4* (pD311), *Dhc5* (p3C17), *Dhc6* (p8D7.0 and p8D11), *Dhc7* (p9B3.5, p9B5.2, and p9C3.6). The probes for *Dhc8* and *Dhc9* were the 260-bp cDNA clones recovered from the RT-PCR screen. The exposure times of the autoradiograms varied for each probe.

progeny, we were also able to estimate the approximate centromere distance for each marker by determining the frequency of tetratype tetrads with respect to the centromere-linked markers *ac17* and *y1* (see Table 1). This information allowed us to compare the centromere distances observed in the above crosses against the published data for crosses within *C. reinhardtii* strains (HARRIS 1989; DUTCHER *et al.* 1991) or crosses between *C. reinhardtii/C. smithii* strains (RANUM *et al.* 1988). The centromere distances shown in Table 1 are within 10 cM of previous estimates. The positions of the genetic and molecular markers are therefore consistent with other versions of the genetic map.

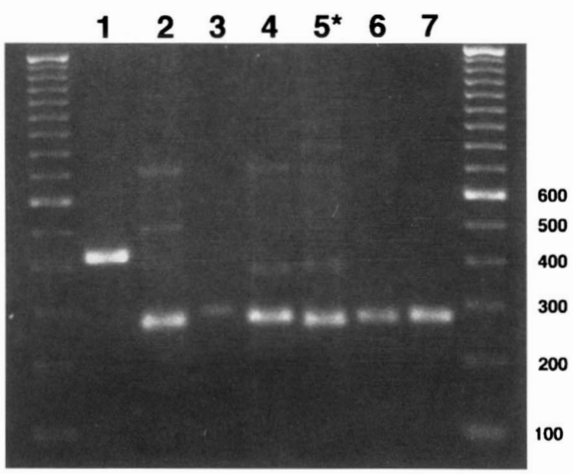

FIGURE 6.—RT-PCR analyses confirm that all *Dhc* genes are expressed as mature transcripts. One microgram of total RNA from deflagellated cells was converted to cDNA using an antisense primer corresponding to the conserved amino acid sequence ITMNPGY. Two microliters of the resulting cDNA products were then used as templates in a series of PCR reactions containing gene-specific primers that were chosen because they spanned regions of genomic DNA containing putative intron sequences (see Figure 2). The final reaction products were loaded on a 1.5% agarose gel (lanes 1–7) and stained with ethidium bromide. Lane 1 contains the RT-PCR product amplified with two *Dhc1* gene-specific primers. Lanes 2–7 contain the RT-PCR products amplified with a conserved sense primer corresponding to TGKTET(TK) and the appropriate gene-specific antisense primers for *Dhc2–Dhc7*, respectively. The major band in each lane was subcloned and sequenced to verify that the reaction product corresponded to the appropriate *Dhc* gene. The *Dhc5* reaction product (marked with *) contained two additional *Dhc* sequences (see text).

Mapping of *Dhc* clones: To place the *Dhc* genes on the genetic map, the RFLPs identified in Figure 8 were used as molecular markers whose pattern of segregation in the tetrad progeny was compared to that of the other 42 genetic and molecular markers described in Table 1. Eight of the *Dhc* genes show unique and consistent linkage to specific subsets of molecular and genetic markers (see Table 2). For each *Dhc* clone, linkage was confirmed with at least two independent markers per linkage group. Only one *Dhc* clone, *Dhc8,* failed to show close linkage to any of the markers tested and remains

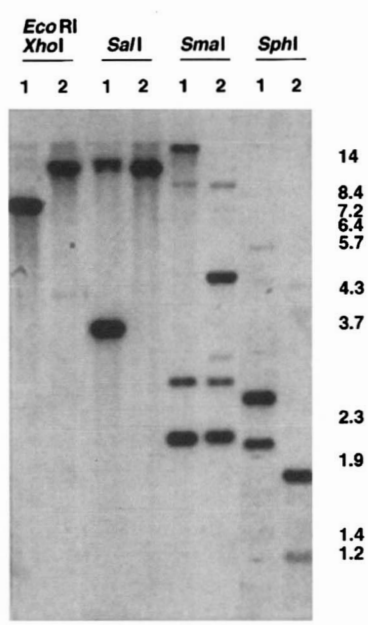

FIGURE 7.—Identification of RFLPs between two Chlamydomonas strains. Genomic DNA from two different Chlamydomonas strains, 137c (lanes 1) and S1-D2 (lanes 2), was digested with the restriction enzymes *Eco*RI/*Xho*I, *Sal*I, *Sma*I, and *Sph*I, fractionated on a 0.8% agarose gel, blotted, and hybridized with an 6.2-kb fragment of the *Dhc5* gene (p3E6.2). RFLPs are seen with all four enzymes.

TABLE 1

Genetic and molecular markers used for RFLP mapping

Linkage group	Arm	Test markers	*ac17* PD:NPD:TT	Centromere distance (cM)	*y1* PD:NPD:TT	Centromere distance (cM)
I	Unknown	GP387	6:3:1	5.0	1:5:2	12.5
	Right	*msr1*	1:0:17	47	0:2:17	45
II	Left	GP130	4:4:6	21	0:1:4	40
	Right	GP366	4:9:3	9.4	3:1:0	<10
	Right	*act1*	5:6:7	19	3:1:1	10
III	Left	*nit2*	12:0:12	25	0:3:10	38
	Centromere	*ac17*			3:15:4	10
IV	Right	*pyr1*	6:8:3	8.8	6:7:4	11.8
V	Right	*PF1*	4:5:9	25	4:1:5	25
	Right	*PF26*	4:6:15	30	4:2:8	29
VI	Left	*mt*	4:2:23	40	3:1:12	40
	Right	pcf8-13	17:10:3	5.0	7:6:2	6.6
	Right	*act2*	4:3:11	31	2:3:13	36
VII	Left	*ODA11*	3:2:26	42	1:2:11	39
	Right	*can1*	4:11:3	8.3	2:3:0	<8.3
	Right	GP202	8:8:10	19	5:3:5	19
VIII	Left	*VFL1*	6:7:0	<3.6	0:3:0	
IX	Left	*sr1*	10:8:7	14	9:7:4	10
	Right	*NIT1*	5:12:12	21	4:2:10	31
X	Left	*nic13*	4:8:6	16.6	8:4:5	14.7
	Left	Tcr1-A	13:12:2	3.7	5:7:2	7.1
XI	Centromere	*pf2*	0:6:3	17	7:0:1	6.3
	Right	*ery1*	9:6:1	3.1	4:0:1	10
XII/XIII	Left	*pf9*	2:2:14	39	0:0:5	50
	Left	band d	2:0:7	39	1:1:7	39
	Right	CNC63	5:4:7	22	1:2:2	20
XIV	Left	Tcr1-B	3:4:8	27	1:1:3	30
	Left	Tcr1-C	7:6:11	23	4:4:5	19
	Left	Tcr1-D	12:12:4	7.1	6:9:2	5.9
	Left	GP324	12:12:2	3.8	6:6:2	7.1
	Right	*maa4*	6:6:5	14.7	2:3:0	<8.3
XV	Left	Tcr1-F	8:11:9	16.1	4:5:8	24
	Left	*ida2*				
XVI/XVII	Unknown	GP61	1:8:16	32	7:0:8	27
	Centromere	*y1*	3:15:4	10		
XVIII	Right	pcf6-8	5:3:7	23	1:1:3	30
XIX	Left	*Gulliver* G	1:1:6	43	0:0:7	50
	Left	GP37	0:3:5	31	1:0:6	43
	Left	*Gulliver* H	0:4:4	25	3:1:4	25
	Left	ef3a	0:4:4	25	3:1:4	25
	Left	GP50	4:7:1	4.2	0:2:0	
Unknown Group B	Unknown	GP228	4:4:7	23	2:1:2	20

The approximate map locations of the molecular markers used in this study have been established as described previously (see RANUM *et al.* 1988; RANUM 1989; SAKAKIBARA *et al.* 1991; C. SILFLOW, personal communication, see Chlamydomonas database). GP228 represents a collection of linked molecular markers that have not yet been assigned to a linkage group. Although recombination frequencies can differ in crosses between polymorphic strains, both the order of the markers and the map distances between markers are in good agreement with other versions of the map (RANUM *et al.* 1988; GROSS *et al.* 1989; HARRIS 1989; DUTCHER *et al.* 1991).

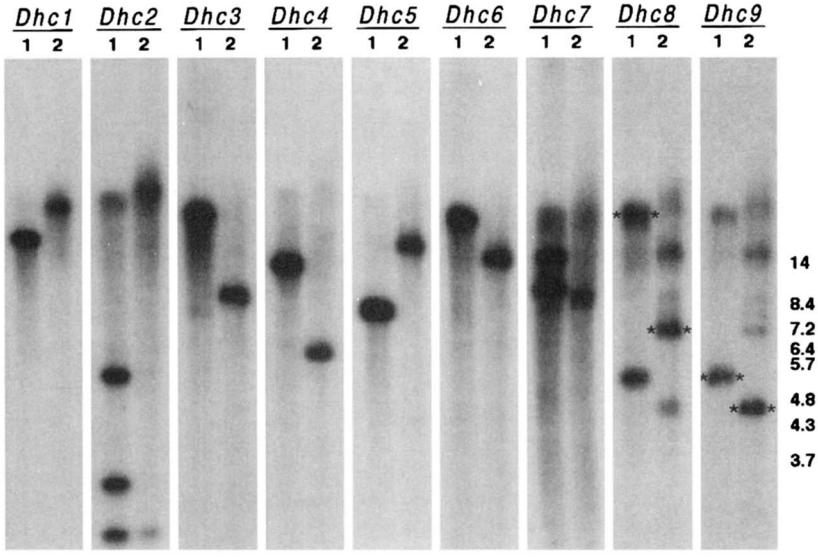

FIGURE 8.—RFLPs used in the mapping of the *Dhc* genes. Genomic DNA was isolated from the two polymorphic strains 137c (lanes 1) and S1-D2 (lanes 2), digested with the restriction enzymes *Eco*RI/*Xho*I, fractionated on a 0.8% agarose gel, blotted, and hybridized with subclones representing each of the *Dhc* genes. The clones for *Dhc1*–*Dhc7* were gene-specific probes (see Figure 1) obtained from the large insert genomic library: *Dhc1* (p4A4.3), *Dhc2* (pRS), *Dhc3* (pD7.6), *Dhc4* (p3D5.5), *Dhc5* (p3E6.2), *Dhc6* (p8D7.0), *Dhc7* (p9B5.2). *Dhc8* and *Dhc9* were mapped using the cDNA clones obtained in the RT-PCR screen. Although these two clones are not strictly gene-specific, the two genes could be mapped by following the most prominent restriction fragments (marked with *) that segregated 2:2 in the tetrad progeny.

unmapped (data not shown). These results, together with the additional data on centromere distance, place eight of the *Dhc* genes on six different linkage groups (Figure 9).

Dhc1 maps to the left arm of linkage group XII/XIII based on its tight linkage to the inner arm mutation *pf9-2* (<3 cM) and the molecular marker known as *Gulliver* element band d (RANUM 1989; PORTER *et al.* 1992). Consistent with this location, *Dhc1* is a centromere distal marker, as evidenced by its high tetratype frequencies with respect to the centromere markers *ac17* and *y1*. These data place the *Dhc1* gene in the

TABLE 2

RFLP mapping of *Dhc* genes

Gene	Linkage group	Linkage data			Centromere distance		
		Markers	PD:NPD:TT	cM	Markers	PD:NPD:TT	cM
Dhc1	XII/XIII	*pf9*	16:0:0	<3.0	*ac17*	3:2:24	41
		Band d	4:0:1	10	*y1*	0:0:15	50
Dhc2	II	GP130	3:0:10	38	*ac17*	3:4:22	38
		GP366	6:0:10	31	*y1*	2:2:12	37.5
		act1	10:0:6	18.8			
Dhc3	VI	*mt*	10:0:12	27	*ac17*	11:5:9	18
		pcf8-13	19:0:9	16	*y1*	5:3:6	21
		act2	2:0:11	42			
Dhc4	II	GP130	6:0:9	30	*ac17*	9:11:7	13
		GP366	15:0:1	3.1	*y1*	6:4:3	12
		act11	14:0:3	8.8			
		Dhc2	12:1:14	33			
Dhc5	II	GP130	6:0:7	27	*ac17*	7:13:7	13
		GP366	14:0:1	3.3	*y1*	8:4:3	10
		Dhc4	24:0:0	<2.0			
		act1	10:0:4	8.3			
		Dhc2	11:1:15	38			
Dhc6	V	*PF1*	14:0:5	13.2	*ac17*	10:7:12	21
		PF26	16:0:6	13.6	*y1*	3:3:8	29
Dhc7	XV	TCR1-F	21:0:5	9.6	*ac17*	11:11:4	7.6
		ida2	17:0:1	2.8	*y1*	4:7:4	13
Dhc8	Unknown				*ac17*	11:11:3	6
					y1	3:5:2	10
Dhc9	XVI/XVII	GP61	11:0:15	28.8	*ac17*	7:18:3	5.5
		y1	14:0:1	3.3	*y1*	14:0:1	3.3

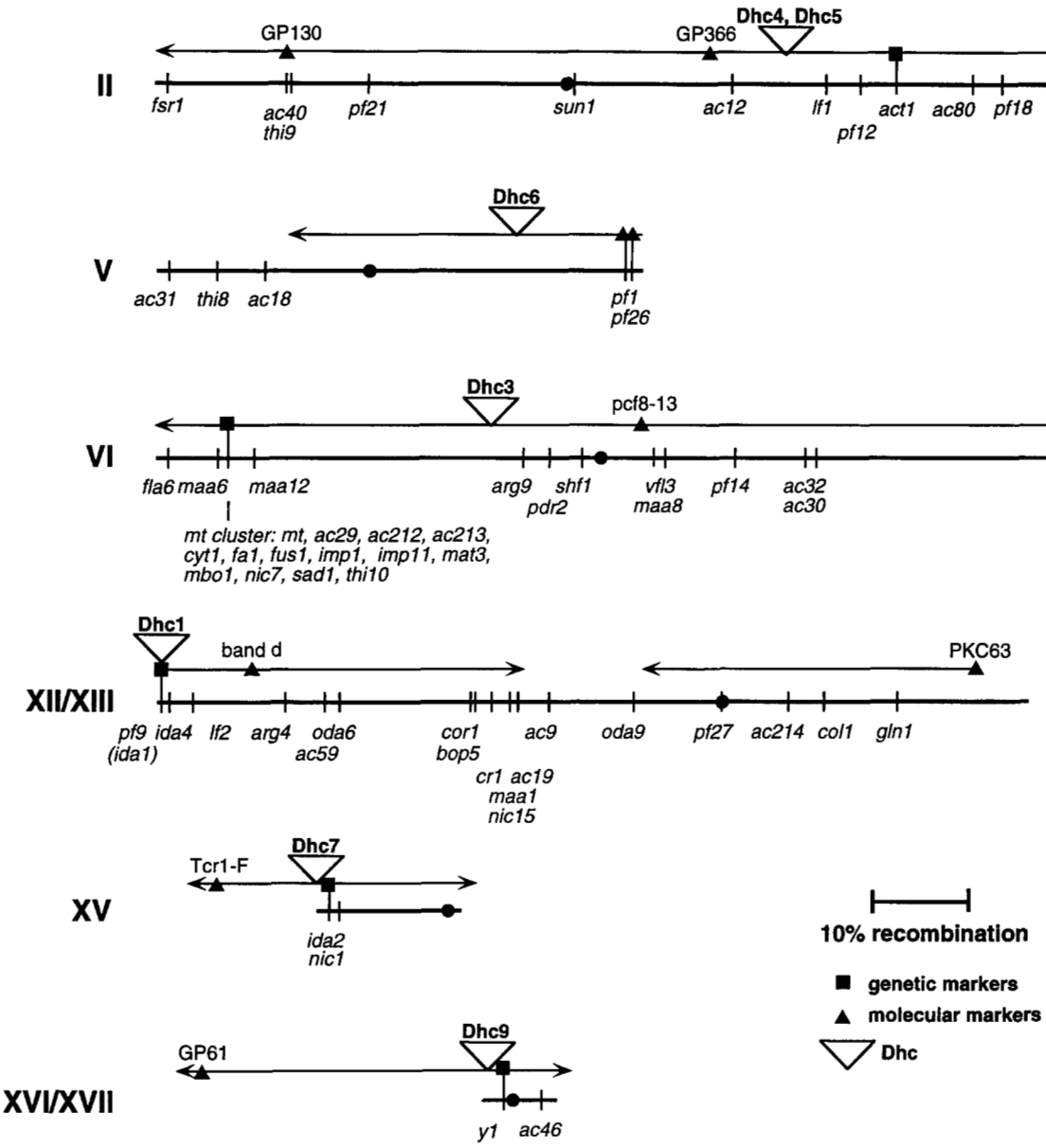

FIGURE 9.—Genetic map locations of *Dhc* genes. The genetic maps of six Chlamydomonas linkage groups (redrawn from HARRIS 1989; DUTCHER *et al.* 1991) are shown on the bottom lines. The approximate map locations of the *Dhc* clones (△) and other molecular markers (▲) are shown on the upper lines. ■ indicate the genetic markers in the multiply marked *C. reinhardtii* strains that were used to anchor the two maps relative to one another. ● mark the position of each centromere. The arrows on the upper lines indicate the linkage detection limits for the markers used in this study. Because the molecular map is based on crosses with the S1-D2 strain, it is not precisely colinear with the genetic map below.

vicinity of three closely linked flagellar loci, *PF9 (IDA1), IDA4,* and *LF2,* but cannot discriminate between these three loci as possible *Dhc* genes.

Dhc2 maps to the right arm of linkage group II, ~18.8 cM distal from the genetic marker *act1*. The only flagellar mutation that has been mapped to this region is *pf18* (EBERSOLD *et al.* 1962). *pf18* mutants lack the central pair microtubules, but no dynein defect has been described (ADAMS *et al.* 1981).

Two genes, *Dhc4* and *Dhc5,* are closely linked to one another (<2 cM apart). They map to linkage group II, between the molecular marker GP366 and the genetic marker *act1*. These data place the *Dhc4* and *Dhc5* genes in the vicinity of three flagellar loci, *LF1, PF12,* and *MIA1*. The *lf1* mutation results in unusually long flagella (MCVITTIE 1972a,b), the *pf12* mutation in an abnormal swimming phenotype (EBERSOLD *et al.* 1962; MCVITTIE 1972a), and the *mia1* mutation in phototaxis defects (S. KING and S. DUTCHER, personal communication).

The *Dhc3* gene maps to linkage group VI, between the mating type (*mt*) locus and the gene encoding a constitutively expressed transcript represented by the cDNA clone pcf8-13 (RANUM *et al.* 1988). Analysis of recombinant tetrads for each marker on linkage group VI suggests that the *Dhc3* gene is located on the left

arm and pcf8–13 is on the right. These data place the *Dhc3* gene in the vicinity of the *SHF1* locus. *shf1* cells have short flagella and a slow swimming motility phenotype (JARVIK *et al.* 1984; KUCHKA and JARVIK 1987).

Dhc6 maps to the right arm of linkage group V, ~20 cM from two radial spoke genes, *PF1* and *PF26*. No other flagellar mutations have been mapped to linkage group V.

Dhc7 maps to linkage group XV, within 3 cM of the inner arm locus *IDA2*. Analysis of 18 tetrads identified one tetratype tetrad in which a recombination event was observed between the *Dhc7* probe and the site of the *ida2* mutation. These results would appear to suggest that *Dhc7* and *ida2* represent two different loci. However, because Chlamydomonas *Dhc* genes are typically 20–30 kb in size (MITCHELL 1989; MITCHELL and BROWN 1994), and the probe used in this experiment covers only a 5.2-kb central region of the *Dhc7* gene, we cannot exclude the possibility that the tetratype tetrad represents a cross-over event occurring within the *Dhc7* gene, between the site of the *ida2* mutation and the region represented by the 5.2-kb probe.

Dhc9 maps to linkage group XVI/XVII, based on linkage to the genetic marker *y1* and the molecular marker GP61. No flagellar mutations have been mapped to this linkage group.

In summary, six *Dhc* genes are linked to genetic loci that have previously been shown to be involved in flagellar function; two *Dhc* genes map to regions of the genome where no flagellar mutation has yet been identified, and one *Dhc* gene remains unmapped.

DISCUSSION

In this study, we have identified nine *Dhc* genes whose expression is enhanced by deflagellation. Two of the *Dhc* genes characterized in this study were previously recovered as partial cDNAs in a PCR screen of a Chlamydomonas cDNA library (WILKERSON *et al.* 1994). All nine Dhc sequences are likely to be involved in axoneme assembly or motility, but they are clearly distinct from the outer arm Dhcs (MITCHELL and BROWN 1994; WILKERSON *et al.* 1994). However, only eight Dhc polypeptides have been identified as inner arm dynein isoforms (KAGAMI and KAMIYA 1992). These results suggest that some of the *Dhc* genes may encode additional inner arm Dhc isoforms that were not resolved in earlier studies. Alternatively, some of these Dhc sequences may be associated with other structures in the flagellar axoneme besides the inner dynein arms. Interestingly, several kinesin-related polypeptides have recently been identified as components of the central pair apparatus in Chlamydomonas (BERNSTEIN *et al.* 1994; FOX *et al.* 1994; JOHNSON *et al.* 1994), where they may play a role in central pair rotation. Another kinesin-related polypeptide is associated with outer doublet microtubules (FOX *et al.* 1994; WALTHER *et al.* 1994) and basal bodies (VASHISHTHA *et al.* 1996), where it has been implicated in both intraflagellar transport and flagellar assembly (WALTHER *et al.* 1994; KOZMINSKI *et al.* 1995; VASHISHTHA *et al.* 1996). It is therefore reasonable to suppose that some of the Dhc sequences identified in this study may play a role in axoneme assembly or motility distinct from that of an inner arm dynein.

Comparison of the Chlamydomonas Dhc sequences with Dhc sequences identified in other organisms suggests that we have recovered most, but not all members of the Chlamydomonas *Dhc* gene family. All nine of the Chlamydomonas sequences have closely related homologues in organisms as diverse as Drosophila, Paramecium, sea urchin, and rat (ASAI *et al.* 1994; GIBBONS *et al.* 1994; RASMUSSEN *et al.* 1994; TANAKA *et al.* 1995; see figure 10). The results of the Southern blot analysis further suggest that there are at least three or four additional *Dhc* genes (Figure 4). One of these genes corresponds to the *gDhc* gene of the outer dynein arm, which was not recovered in our PCR screen, but has previously been identified by expression cloning (WILKERSON *et al.* 1994). We have also not yet identified the Chlamydomonas homologue of the major cytoplasmic Dhc isoform (Figure 10). As the cytoplasmic Dhc sequence appears to be ubiquitious, even in organisms that do not assemble motile flagellar axonemes (KOONCE *et al.* 1992; ESHEL *et al.* 1993; LI *et al.* 1993; PLAMANN *et al.* 1994; XIANG *et al.* 1994; LYE *et al.* 1995), it seems probable that this gene is present in the Chlamydomonas genome and will likely be recovered in future PCR screens. Regardless, the 12 Dhc sequences identified thus far appear to represent the majority of the *Dhc* gene family in this organism.

Phylogenetic comparisons between Dhc sequences from different organisms suggest that the branch that contains the Chlamydomonas Dhc1 sequence represents one subdivision of the *Dhc* gene family (see GIBBONS *et al.* 1994; TANAKA *et al.* 1995; GIBBONS 1995). This branch includes the Drosophila Dhc98D sequence, the sea urchin Dhc4 and Dhc5C sequences, the rat Dhc2 and Dhc10 sequences, and the Paramecium Dhc5 sequence (see Figure 5 in TANAKA *et al.* 1995; and Figure 10, this study). Interestingly, the Chlamydomonas *Dhc1* gene maps to a region of linkage group XII/XIII that contains three flagellar loci, *LF2, PF9* and *IDA4*. *lf2* mutations result in the assembly of abnormally long flagella (MCVITTIE 1972a,b). Mutations at the *PF9* locus (*e.g.*, *pf9, ida1,* and *pf30*) disrupt the assembly of the I1 inner arm complex, which contains two Dhc polypeptides, 1-alpha and 1-beta (PIPERNO *et al.* 1990; KAMIYA *et al.* 1991; PORTER *et al.* 1992). *ida4* mutations disrupt the assembly of three single-headed I2 inner arm subspecies (KAGAMI and KAMIYA 1992; MASTRONARDE *et al.* 1992). Recent work has demonstrated that the *IDA4* locus encodes the structural gene for a 28 kD dynein light chain known as p28 (LEDIZET and PIPERNO, 1995). It is therefore plausible that the *Dhc1* gene corresponds to either the *LF2* locus or the *PF9* locus and encodes either a Dhc involved in flagellar assembly or one of the two I1 inner

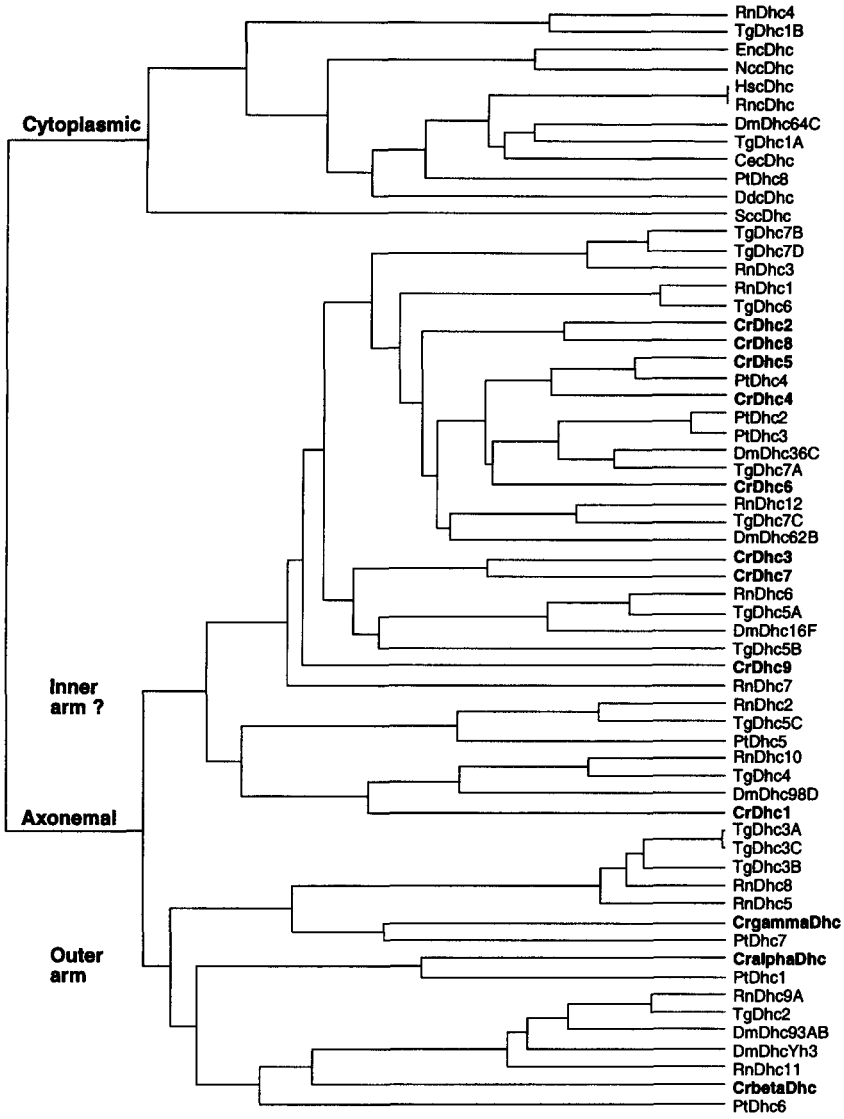

FIGURE 10.—Alignment of the deduced amino acid sequences of *Dhc* genes identified in several organisms. *Dhc* gene families have been identified in four other organisms (ASAI *et al.* 1994; GIBBONS *et al.* 1994; RASMUSSEN *et al.* 1994; TANAKA *et al.* 1995). To examine the relationship between these *Dhc* genes and the Chlamydomonas sequences, a section of ~117 amino acid residues surrounding the highly conserved ATP hydrolytic site was aligned using the GCG program PILEUP. The GenBank accession numbers for the sequences used are as follows: *Tripneustes gratilla* (Tg): U03969–U03981, X59603, Z21941; *Drosophila melanogaster* (Dm): L23195–L23201; *Paramecium tetraurelia* (Pt): L16962, L17050, L17132, L18801–L18804; *Rattus norvegicus* (Rn): L08505, D13896, D26492–D26504; *C. reinhardtii* (Cr): L26049, U02963, U15303. Also included in the alignment are cytoplasmic Dhc sequences identified in several other species (KOONCE *et al.* 1992; ESHEL *et al.* 1993; LI *et al.* 1993; MIKAMI *et al.* 1993; VAISBERG *et al.* 1993; ZHANG *et al.* 1993; PLAMANN *et al.* 1994; XIANG *et al.* 1994; LYE *et al.* 1995) as follows: *Caenorhabditis elegans* (Ce), L33260; *Dictyostelium discoideum* (Dd), Z15124; *Emericella nidulans* (En), U03904; *Homo sapiens* (Hs), L23958; *Neurospora crassa* (Nc) L31504; *Saccharomyces cerevisiae* (Sc), Z21877, L15626.

arm Dhcs. Indeed, recent work from our laboratory has confirmed that the *PF9* locus is the *Dhc1* structural gene (MYSTER *et al.* submitted for publication).

The remaining Chlamydomonas Dhc sequences (Dhc2–Dhc9) form two distinct but closely related groups and presumably include representatives of the I2 and I3 inner arm Dhcs. These sequences can be subdivided based on sequence similarities and the presence of conserved intron locations within their coding sequences. One group of sequences is represented by *Dhc3* and *Dhc7*. These two genes, along with *Dhc6*, share an intron splice site at a conserved position just downstream from the region encoding the CFDEFNR consensus sequence. *Dhc6* is also related to a second group of *Dhc* genes, *Dhc2*, *Dhc4*, and *Dhc5*. These four genes share another intron splice site located just upstream from the region encoding the CFDEFNR consensus sequence. Additional sequence information will be required to more fully understand the evolutionary relationships between the *Dhc* genes, but these preliminary observations suggest that Dhc2–Dhc7 are derived from one or two ancestral *Dhc* genes.

Analysis of *Dhc* transcripts by both Northern blots and RT-PCR procedures demonstrates that all of the *Dhc* genes identified in this study are upregulated in response to deflagellation. However, individual transcripts appear to vary in abundance, as evidenced by the relatively weak signals obtained with the *Dhc3* probe (see Figures 5 and 6). Although we cannot exclude the possibility that the weaker signals on the blots are due to an abundance of intron sequences in the subclone used as the hybridization probe, *Dhc3* may represent a Dhc isoform that is present in more limited amounts than the other Dhc polypeptides. The *Dhc3* gene has also been mapped to the left arm of linkage group VI, between the mating type locus and its centromere. This position is based on the analysis of recombinant tetrads for the four markers tested on linkage group VI (*e.g.*, *mt*, *Dhc3*, pcf8-13, and *act2*). As this region of the map is not densely populated with molecular markers (C. SILFLOW, personal communication), we are not able to estimate the location of *Dhc3* more precisely. However, the slow swimming, short flagellar mutation *shf1* has also been mapped to this region (JARVIK *et al.* 1984).

Interestingly, previous work has suggested that certain Dhc isoforms are restricted to either the proximal or distal regions of the flagellar axoneme (PIPERNO and RAMINIS 1991; GARDNER *et al.* 1994). A direct test of linkage between the *Dhc3* gene and the *shf1* mutation will be an important next step toward identifying a possible function for the *Dhc3* gene. For instance, *Dhc3* could represent a low abundance Dhc isoform that is targeted to a specific region of the flagellar axoneme and is missing in *shf1* flagella.

Three closely related *Dhc* sequences, *Dhc2, Dhc4,* and *Dhc5,* all map to linkage group II. *Dhc2* is linked to *pf18,* a central pair mutation that disrupts the assembly of 23 axonemal polypeptides (ADAMS *et al.* 1981; DUTCHER *et al.* 1984). Whether this linkage is coincidental or suggestive of a previously unrecognized central pair-associated Dhc isoform remains to be determined. *Dhc4* and *Dhc5* are tightly linked to one another and map to a region that contains three closely linked flagellar loci, *LF1, PF12,* and *MIA1.* Whether any of these loci are good candidates for *Dhc* genes is also an open question. *lf1* mutations, like the *lf2* mutations described above, result in abnormally long flagella (MCVITTIE 1972a,b). Limited biochemical analysis of *lf* axonemes has not revealed any clear polypeptide defects, but Dhcs have not been specifically examined (BARSEL 1987; P. LEFEBVRE, personal communication). *pf12* mutants swim with an aberrant motility phenotype (MCVITTIE 1972a,b), but we have not uncovered any obvious inner arm dynein defects, either by FPLC analysis of dynein extracts or by ultrastructural analysis of *pf12* axonemes (GARDNER, O'TOOLE, and PORTER, unpublished results). *mia1* mutants are defective in phototaxis, and preliminary results suggest that the phototaxis phenotype can be correlated with changes in the phosphorylation state of an inner arm dynein intermediate chain (S. KING and S. DUTCHER, personal communication). Given the close proximity between the two *Dhc* genes (Table 2) and the three flagellar loci (HARRIS 1989; S. KING and S. DUTCHER, personal communication), RFLP mapping of additional tetrad progeny is not a practical strategy for determining if any of these loci might be a *Dhc* gene. Future experiments to identify a *Dhc* locus may therefore require the rescue of a mutant phenotype by transformation with a wild-type copy of a *Dhc* gene.

The *Dhc7* gene maps to linkage group XV, ~3 cM from the *ida2* mutation. *ida2* mutations, like the *pf9 (ida1)* mutations described above, disrupt the assembly of the I1 inner arm complex (KAMIYA *et al.* 1991). However, the presence of a tetratype tetrad indicates that a recombination event has taken place between the site of the *ida2* mutation and the 5.2-kb subclone used to map the *Dhc7* gene. Whether this tetrad represents a recombination event occuring within a *Dhc* gene, such as has been observed previously with the outer arm *beta Dhc* gene (PORTER *et al.* 1994), or alternatively, a recombination event between two different dynein related genes, is still unknown. We are currently trying to resolve this question by walking in both directions from the region encoding the predicted ATP binding site and probing the mapping filters with additional subclones of the *Dhc7* gene.

The *Dhc8* sequence is tightly linked to its centromere (Table 2), but appears unlinked to any of 46 genetic or molecular markers tested on the RFLP mapping filters in this study (data not shown). Because several of these markers are themselves centromere-linked (see Table 1), these results would appear to exclude linkage groups I–IV, VI–VIII, X-XI, XIV-XVI/XVII, and XIX as the possible locus of the *Dhc8* gene. In addition, linkage groups IX and XII/XIII are represented by markers on both arms (Table 1) that are also unlinked to the *Dhc8* clone. The *Dhc8* gene may therefore be located on an unmarked region of linkage group V or XVIII, or alternatively identify a new linkage group in Chlamydomonas. Mapping of additional molecular markers will be required to further clarify the position of the *Dhc8* gene.

Two of the *Dhc* genes (*Dhc6* and *Dhc9*) map to regions of the genome where no flagellar mutation has yet been identified. These observations may mean that the functions of these *Dhc* genes are either subtle or redundant, and a specific *Dhc* mutation would simply not have been recovered in previous screens for flagellar mutants. Alternatively, these *Dhc* genes may perform some essential function that cannot be revealed by mutagenesis of a haploid organism. However, it is important to note that there are a large number of flagellar mutations that have not been definitively placed on the genetic map, and this number is growing rapidly with the advent of insertional mutagenesis procedures (TAM and LEFEBVRE 1993; PAZOUR *et al.* 1995).

In summary, we have identified a family of *Dhc* genes in Chlamydomonas that are highly homologous to *Dhc* genes in other organisms. Expression studies indicate that these genes appear to play a role in axoneme assembly or motility. Moreover, several of these genes map near previously identified flagellar loci. In the future, the isolation of "tagged" motility mutants that can be analyzed with these *Dhc* probes should identify *Dhc* mutations and clarify their relationship to the previously identified flagellar loci. In addition, the recovery of full-length genomic clones will also permit the identification of *Dhc* loci by the rescue of a mutant phenotype upon transformation with a wild-type copy of the appropriate *Dhc* gene.

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LITERATURE CITED

ADAMS, G. M. W., B. HUANG, G. PIPERNO and D. J. L. LUCK, 1981 Central-pair microtubular complex of Chlamydomonas flagella: polypeptide composition as revealed by analysis of mutants. J. Cell Biol. **91:** 69–76.

ADAMS, G. M. W., B. HUANG and D. J. L. LUCK, 1982 Temperature sensitive assembly-defective flagella mutants of *Chlamydomonas reinhardtii.* Genetics **100:** 579–586.

ASAI, D. J., S. M. BECKWITH, K. A. KANDL, H. H. KEATING, H. TJANDRA *et al.,* 1994 The dynein genes of *Paramecium tetraurelia.* Sequences adjacent to the catalytic P-loop identify cytoplasmic and axonemal heavy chain isoforms. J. Cell Sci. **107:** 839–847.

BARSEL, S. E., 1987 Isolation and characterization of long flagellar mutants of *Chlamydomonas reinhardtii.* Ph.D. thesis, University of Minnesota.

BECKWITH, S. M., and D. J. ASAI, 1993 Ciliary dynein of *Paramecium tetraurelia:* photolytic maps of three heavy chains. Cell Motil. Cytoskel. **24:** 29–38.

BERNSTEIN M., P. L. BEECH, S. G. KATZ and J. L. ROSENBAUM, 1994 A new kinesin-like protein (Klp1) localized to a single microtubule of the Chlamydomonas flagellum. J. Cell Biol. **125:** 1313–1326.

BROKAW, C. J., and R. KAMIYA, 1987 Bending patterns of Chlamydomonas flagella: IV. Mutants with defects in inner and outer dynein arms indicate differences in dynein arm function. Cell Motil. Cytoskel. **8:** 68–75.

CHISHOLM, D., 1989 A convenient procedure for obtaining DNA from bacteriophage lambda. BioTechniques **7:** 21–23.

CHOMCZYNSKI, P., and N. SACCHI, 1987 Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry **162:** 156–159.

CURRY, A. M., B. D. WILLIAMS and J. L. ROSENBAUM, 1992 Sequence analysis reveals homology between two proteins of the flagellar radial spoke. Mol. Cell Biol. **12:** 3967–3977.

DEVEREUX, J., P. HAEBERLI and O. SMITHIES, 1984 A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:** 387–395.

DUTCHER, S. K., 1995 Flagellar assembly in two hundred and fifty easy to follow steps. Trends Genet. **11:** 398–404.

DUTCHER, S. K., B. HUANG and D.J.L. LUCK, 1984 Genetic dissection of the central pair microtubules of the flagella of *Chlamydomonas reinhardtii.* J. Cell Biol. **98:** 229–236.

DUTCHER, S. K., J. POWER, R. E. GALLOWAY and M. E. PORTER, 1991 Reappraisal of the genetic map of *Chlamydomonas reinhardtii.* J. Hered. **82:** 295–301.

EBERSOLD, W. T., R. P. LEVINE, E. E. LEVINE and M. A. OLMSTED, 1962 Linkage maps in *Chlamydomonas reinhardi.* Genetics **47:** 531–543.

ESHEL, D., L. A. URRESTARAZU, S. VISSERS, J.-C. JAUNIAUX, J. C. VAN VLIET-REEDIJK *et al.,* 1993 Cytoplasmic dynein is required for normal nuclear segregation in yeast. Proc. Natl. Acad. Sci. USA **90:** 11172–11176.

FERNANDEZ, E., R. SCHNELL, L. P. W. RANUM, S. C. HUSSEY, C. D. SILFLOW *et al.,* 1989 Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardtii.* Proc. Natl. Acad. Sci. USA **86:** 6449–6453.

FOX, L., K. SAWIN and W. SALE, 1994 Kinesin related proteins of the eukaryotic flagellum. J. Cell Sci. **107:** 1545–1550.

GARDNER, L. C., E. O'TOOLE, C. A. PERRONE, T. GIDDINGS and M. E. PORTER, 1994 Components of a "dynein regulatory complex" are located at the junction between the radial spokes and the dynein arms in Chlamydomonas flagella. J. Cell Biol. **127:** 1311–1325.

GIBBONS, B. H., and I. R. GIBBONS, 1987 Vanadate-sensitized cleavage of dynein heavy chains by 365-nm irradiation of demembranated sperm flagella and its effect on the flagellar motility. J. Biol. Chem. **262:** 8354–8359.

GIBBONS, B. H., D. ASAI, W.-J. TANG, T. S. HAYS and I. R. GIBBONS, 1994 Phylogeny and expression of axonemal and cytoplasmic dynein genes in sea urchins. Mol. Biol. Cell **5:** 57–70.

GIBBONS, I. R., 1995 Dynein family of motor proteins: present status and future questions. Cell Motil. Cytoskel. **32:** 136–144.

GIBBONS, I. R., E. FRONK, B. H. GIBBONS and K. OGAWA, 1976 Multiple forms of dynein in sea urchin sperm flagella, pp. 915–932 in *Cell Motility,* edited by R. GOLDMAN, T. POLLARD and J. ROSENBAUM. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

GIBBONS, I. R., B. H. GIBBONS, G. MOCZ and D. J. ASAI, 1991 Multiple nucleotide binding sites in the sequence of dynein β heavy chain. Nature **352:** 640–643.

GOLDSTEIN, L. S. B., R. W. HARDY and D. L. LINDSLEY, 1982 Structural genes on the Y chromosome of *Drosophila melanogaster.* Proc. Natl. Acad. Sci. USA **79:** 7405–7409.

GOODENOUGH, U. W., B. GEBHART, V. MERMALL, D.R. MITCHELL and J.E. HEUSER. 1987 High pressure liquid chromatography fractionation of Chlamydomonas dynein extracts and characterization of inner-arm dynein subunits. J. Mol. Biol. **194:** 481–494.

GROSS, C. H., L. P. W. RANUM and P. A. LEFEBVRE, 1988 Extensive restriction fragment length polymorphisms in a new isolate of *Chlamydomonas reinhardtii.* Curr. Genet. **13:** 503–508.

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

HAYS, T. S., M. E. PORTER, M. MCGRAIL, P. GRISSOM, P. GOSCH *et al.,* 1994 A cytoplasmic dynein motor in Drosophila: identification and localization during embryogenesis. J. Cell Sci. **107:** 1557–1569.

HOLZBAUR, E. L. F., and R. B. VALLEE, 1994 Dyneins: molecular structure and cellular function. Annu. Rev. Cell Biol. **10:** 339–372.

HORST, C., and G. B. WITMAN, 1993 *ptx1,* a nonphototactic mutant of Chlamydomonas, lacks control of flagellar dominance. J. Cell Biol. **120:** 733–741.

HUANG, B., M. R. RIFKIN and D. J. L. LUCK, 1977 Temperature sensitive mutations affecting flagellar assembly and function in *Chlamydomonas reinhardtii.* J. Cell Biol. **72:** 67–85.

HUANG, B., G. PIPERNO and D. J. L. LUCK, 1979 Paralyzed flagellar mutants of *Chlamydomonas reinhardtii.* J. Biol. Chem. **254:** 3091–3099.

JARVIK, J. W., F. D. REINHART, M. R. KUCHKA and S. A. ADLER, 1984 Altered flagellar size-control in *shf-1* short-flagella mutants of *Chlamydomonas reinhardtii.* J. Protozool. **31:** 199–204.

JOHNSON, D. E., and S. K. DUTCHER, 1991 Molecular studies of linkage group XIX of *Chlamydomonas reinhardtii:* evidence against a basal body location. J. Cell Biol. **113:** 339–346.

JOHNSON, K., M. A. HAAS and J. L. ROSENBAUM, 1994 Localization of a kinesin-related protein to the central pair apparatus of the *Chlamydomonas reinhardtii* flagellum. J. Cell Sci. **107:** 1551–1556.

KAGAMI, O., and R. KAMIYA, 1992 Translocation and rotation of microtubules caused by multiple species of Chlamydomonas inner arm dynein. J. Cell Sci. **103:** 653–664.

KAMIYA, R., 1988 Mutations at twelve independent loci result in the absence of outer dynein arms in *Chlamydomonas reinhardtii.* J. Cell Biol. **107:** 2253–2258.

KAMIYA, R., E. KURIMOTO and E. MUTO, 1991 Two types of Chlamydomonas flagellar mutants missing different components of inner-arm dynein. J. Cell Biol. **112:** 441–447.

KATO, T., O. KAGAMI, T. YAGI and R. KAMIYA, 1993 Isolation of two species of *Chlamydomonas reinhardtii* flagellar mutants, *ida5* and *ida6,* that lack a newly identified heavy chain of the inner dynein arm. Cell Struct. Funct. **18:** 371–377.

KINDLE, K., 1990 High frequency nuclear transformation of *Chlamydomonas reinhardtii.* Proc. Natl. Acad. Sci. USA **87:** 1228–1232.

KOONCE, M. P., P. M. GRISSOM and J. R. MCINTOSH, 1992 Dynein from Dictyostelium: primary structure comparisons between a cytoplasmic motor enzyme and flagellar dynein. J. Cell Biol. **119:** 1597–1604.

KOZMINSKI, K. G., P. L. BEECH and J. L. ROSENBAUM, 1993 The *Chlamydomonas* kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. Proc. Natl. Acad. Sci. USA **90:** 5519–5523.

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

LEDIZET, M., and G. PIPERNO, 1995 *ida4-1, ida4-2,* and *ida4-3* are intron splicing mutations affecting the locus encoding p28, a light chain of *Chlamydomonas* axonemal inner dynein arms. Mol. Biol. Cell **6:** 713–723.

LI, M.-G., M. MCGRAIL, M. SERR and T. S. HAYS, 1994 Drosophila cytoplasmic dynein, a microtubule motor that is asymmetrically localized to the oocyte. J. Cell Biol. **126:** 1475–1494.

LI, Y.-Y., E. YEH, T. HAYS and K. BLOOM, 1993 Disruption of mitotic spindle orientation in a yeast dynein mutant. Proc. Natl. Acad. Sci. USA **90:** 10096–10100.

LYE, R. J., R. K. WILSON and R. H. WATERSON, 1995 Genomic structure of a cytoplasmic dynein heavy chain gene from the nematode *Caenorhabditis elegans.* Cell Motil. Cytoskel. **32:** 26–36.

MASTRONARDE, D. N., E. T. O'TOOLE, K. L. MCDONALD, J. R. MCINTOSH and M. E. PORTER, 1992 Arrangement of inner dynein

arms in wild-type and mutant flagella of Chlamydomonas. J. Cell Biol. **118:** 1145–1162.

McVittie, A., 1972a Flagellar mutants of *Chlamydomonas reinhardii.* J. Gen. Microbiol. **71:** 525–540.

McVittie, A., 1972b Genetic studies on flagellum mutants of *Chlamydomonas reinhardii.* Genet. Res. Camb. **19:** 157–164.

Mikami, A., B. M. Paschal, M. Mazumdar and R. B. Vallee, 1993 Molecular cloning of the retrograde transport motor cytoplasmic dynein (MAP 1C). Neuron **10:** 787–796.

Mitchell, D. R., 1989 Molecular analysis of the alpha and beta dynein genes of *Chlamydomonas reinhardtii.* Cell Motil. Cytoskel. **14:** 435–445.

Mitchell, D. R., 1994 Cell and molecular biology of flagellar dyneins. Int. Rev. Cytol. **155:** 141–180.

Mitchell, D. R., and J. L. Rosenbaum, 1985 A motile Chlamydomonas mutant that lacks the outer dynein arms. J. Cell Biol. **100:** 1228–1234.

Mitchell, D. R., and K. S. Brown, 1994 Sequence analysis of the Chlamydomonas alpha and beta dynein heavy chain genes. J. Cell Sci. **107:** 635–644.

Nelson, J. A. E., P. B. Savereide and P. A. Lefebvre, 1994 The *CRY1* gene in *Chlamydomonas reinhardtii:* structure and use as a dominant selectable marker for nuclear transformation. Mol. Cell. Biol. **14:** 4011–4019.

Ogawa, K., 1991 Four ATP-binding sites in the midregion of the β heavy chain of dynein. Nature **353:** 643–645.

Pazour, G. J., O. A. Sineshchekov and G. B. Witman, 1995 Mutational analysis of the phototransduction pathway of *Chlamydomonas reinhardtii.* J. Cell Biol. **131:** 427–440.

Piperno, G., and Z. Ramanis, 1991 The proximal portion of Chlamydomonas flagella contains a distinct set of inner dynein arms. J. Cell Biol. **112:** 701–709.

Piperno, G., Z. Ramanis, E. F. Smith and W. S. Sale, 1990 Three distinct inner dynein arms in Chlamydomonas flagella: molecular composition and location in the axoneme. J. Cell Biol. **110:** 379–389.

Piperno, G., K. Mead and W. Shestak, 1992 The inner dynein arms I2 interact with a "dynein regulatory complex" in Chlamydomonas flagella. J. Cell Biol. **118:** 1455–1463.

Piperno, G., K. Mead, M. Ledizet and A. Moscatelli, 1994 Mutations in the "dynein regulatory complex" alter the ATP-insensitive binding sites for inner arm dyneins in Chlamydomonas axonemes. J. Cell Biol. **125:** 1109–1117.

Plamann, M., P. F. Minke, J. H. Tinsley and K. S. Bruno, 1994 Cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi. J. Cell Biol. **127:** 139–149.

Porter, M. E., J. Power and S. K. Dutcher, 1992 Extragenic suppressors of paralyzed flagellar mutations identify loci that alter the inner dynein arms. J. Cell Biol. **118:** 1163–1176.

Porter, M. E., J. A. Knott, L. C. Gardner, D. R. Mitchell and S. K. Dutcher, 1994 Mutations in the *SUP-PF-1* locus of *Chlamydomonas reinhardtii* identify a regulatory domain in the β-dynein heavy chain. J. Cell Biol. **126:** 1495–1507.

Ranum, L. P. W., 1989 Mapping nuclear genes in Chlamydomonas using restriction fragment length polymorphisms. Ph. D. Thesis, University of Minnesota.

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

Rasmussen, K., M. Serr, J. Gepner, I. Gibbons and T. S. Hays, 1994 A family of dynein genes in *Drosophila melanogaster.* Mol. Biol. Cell **5:** 45–55.

Sakakibara, H., D. R. Mitchell and R. Kamiya, 1991 A Chlamydomonas outer arm mutant missing the alpha heavy chain. J. Cell Biol. **113:** 615–622.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual,* Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Savereide, P. B., 1991 The molecular basis of resistance to the protein synthesis inhibitor crytopleurine in *Chlamydomonas reinhardtii.* Ph. D. thesis, Univeristy of Minnesota.

Schloss, J. A., C. D. Silflow and J. L. Rosenbaum, 1984 mRNA abundance changes during flagellar regeneration in *Chlamydomonas reinhardtii.* Mol. Cell Biol. **4:** 424–434.

Schnell, R. A., and P.A. Lefebvre, 1993 Isolation of the Chlamydomonas regulatory gene *NIT2* by transposon tagging. Genetics **134:** 737–747.

Silflow, C. D., R. L. Chisholm, T. W. Conner and L. P. W. Ranum, 1985 The two alpha-tubulin genes of *Chlamydomonas reinhardi* code for slightly different proteins. Mol. Cell Biol. **5:** 2389–2398.

Stephens, R. E., and G. Prior, 1995 Dynein inner arm heavy chain identification in cAMP-activated flagella using class-specific polyclonal antibodies. Cell Motil. Cytoskel. **30:** 261–271.

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

Tanaka, Y., Z. Zhang and N. Hirokawa, 1995 Identification and molecular evolution of new dynein-like protein sequences in rat brain. J. Cell Sci. **108:** 1883–1893.

Vaisberg, E. A., M. P. Koonce and J. R. Mcintosh, 1993 Cytoplasmic dynein plays a role in the mammalian mitotic spindle. J. Cell Biol. **123:** 849–858.

Vashishtha, M., Z. Walther and J. L. Hall, 1996 The kinesin-homologous protein encoded by the Chlamydomonas *FLA10* gene is associated with basal bodies and centrioles. J. Cell Sci. **109:** 541–549.

Walther, Z., M. Vashishtha and J. L. Hall, 1994 The Chlamydomonas *FLA10* gene encodes a novel kinesin-homologous protein. J. Cell Biol. **126:** 175–188.

Wilkerson, C. G., S. M. King and G. B. Witman, 1994 Molecular analysis of the g heavy chain of Chlamydomonas outer arm dynein. J. Cell Sci. **170:** 497–506.

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 *pf14* is an ochre allele. J. Cell Biol. **109:** 235–245.

Witman, G. B., 1986 Isolation of Chlamydomonas flagella and flagellar axonemes. Meth. Enzymol. **134:** 280–290.

Witman, G. B., K. Carlson, J. Berliner and J. L. Rosenbaum, 1972 Chlamydomonas flagella I. Isolation and electrophoretic analysis of microtubules, membranes, matrix, and mastigonemes. J. Cell Biol. **54:** 507–539.

Xiang, X., S. Beckwith and R. N. Morris, 1994 Cytoplasmic dynein is involved in nuclear migration in *Aspergillus nidulins.* Proc. Natl. Acad. Sci. USA **91:** 2100–2104.

Zhang, D., 1996 Regulation of nitrate assimilation in *Chlamydomonas reinhardtii.* Ph.D. Thesis, University of Minnesota.

Zhang, Z., Y. Tanaka, S. Nonaka, H. Aizawa, H. Kawasaki *et al.,* 1993 The primary structure of rat brain (cytoplasmic) dynein heavy chain, a cytoplasmic motor enzyme. Proc. Natl. Acad. Sci. USA **90:** 7928–7932.

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