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Cytoplasmic Dynein Heavy Chain 1b Is Required for Flagellar Assembly in *Chlamydomonas*

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A second cytoplasmic dynein heavy chain (cDhc) has recently been identified in several organisms, and its expression pattern is consistent with a possible role in axoneme assembly. We have used a genetic approach to ask whether cDhc1b is involved in flagellar assembly in *Chlamydomonas*. Using a modified PCR protocol, we recovered two cDhc sequences distinct from the axonemal Dhc sequences identified previously. cDhc1a is closely related to the major cytoplasmic Dhc, whereas cDhc1b is closely related to the minor cDhc isoform identified in sea urchins, *Caenorhabditis elegans*, and *Tetrahymena*. The *Chlamydomonas cDhc1b* transcript is a low-abundance mRNA whose expression is enhanced by deflagellation. To determine its role in flagellar assembly, we screened a collection of stumpy flagellar (*stf*) mutants generated by insertional mutagenesis and identified two strains in which portions of the *cDhc1b* gene have been deleted. The two mutants assemble short flagellar stumps (<1–2 μm) filled with aberrant microtubules, raft-like particles, and other amorphous material. The results indicate that cDhc1b is involved in the transport of components required for flagellar assembly in *Chlamydomonas*.

INTRODUCTION

The dyneins are a large family of motor proteins that drive microtubule sliding in cilia and flagella and contribute to microtubule-based transport in eucaryotic cells (reviewed in Holzbaur and Vallee, 1994; Porter, 1996; Hirokawa *et al.*, 1998). These enzymes convert the energy derived from ATP binding and hydrolysis into the minus-end–directed movement of cellular cargoes along the surfaces of microtubules (Sale and Satir, 1977; Paschal and Vallee, 1987). The dyneins also play an important role in the spatial organization of microtubule arrays (Verde *et al.*, 1991; Li *et al.*, 1993; Dillman *et al.*, 1996; Koonce and Samso, 1996).

Dyneins have traditionally been separated into two distinct groups, axonemal and cytoplasmic. At least 11 different dynein heavy chain (Dhc)[1] subspecies are present in the inner and outer dynein arm structures of the flagellar axoneme, where they play highly specialized roles in the generation of the flagellar waveform (Kagami and Kamiya, 1992; reviewed in Asai and Brokaw, 1993; Gibbons, 1995; Porter, 1996). In contrast, a single cytoplasmic Dhc species has been implicated in a variety of microtubule-based movements, including vesicle transport, mitotic spindle assembly and positioning, nuclear migration, and chromosome movements (reviewed in Holzbaur and Vallee, 1994; Hirokawa *et al.*, 1998). Recently, a second cytoplasmic Dhc sequence, Dyh1b, was discovered in sea urchin embryos as a minor transcript whose expression could be stimulated by deciliation (Gibbons

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[1] Abbreviations used: cDhc, cytoplasmic dynein heavy chain; *che*, chemotaxis; cM, centiMorgan; GCG, Genetics Computer Group; IFT, intraflagellar transport; LC, light chain; *lf*, long flagella; *mt*, mating type; *osm*, osmotic avoidance; PBS, phosphate-buffered saline; RFLP, restriction fragment length polymorphism; RT, reverse transcription; *shf*, short flagellar; *stf*, stumpy flagellar.

et al., 1994). A homologous cDhc sequence has since been detected in a wide variety of cells and tissues (Tanaka *et al.*, 1995; Criswell *et al.*, 1996; Vaisberg *et al.*, 1996), although it appears to be most abundant in cells involved in some aspect of axoneme assembly (Tanaka *et al.*, 1995; Neesen *et al.*, 1997; Criswell and Asai, 1998). Immunolocalization studies have indicated that the cDhc1b polypeptide is concentrated in the apical cytoplasm of ciliated epithelial cells (Criswell *et al.*, 1996), but it can also be found in close association with the Golgi apparatus in human tissue culture cells (Vaisberg *et al.*, 1996). These studies have suggested that the Dyh1b/cDhc1b isoform might be involved in some aspect of membrane trafficking and/or ciliary and flagellar assembly.

Flagellar assembly has been most thoroughly studied in the biflagellate green alga *Chlamydomonas*. Both flagellar assembly and flagellar length are precisely regulated (Lefebvre and Rosenbaum, 1986; Tuxhorn *et al.*, 1998), and >33 different genetic loci that affect flagellar assembly have been identified (reviewed in Dutcher, 1989, 1995; Harris, 1989). Experimental deflagellation leads to the rapid induction of flagellar protein synthesis (Lefebvre *et al.*, 1978), and within 90 min, >250 flagellar proteins are assembled into two flagella, each 10–14 μm in length (reviewed in Lefebvre and Rosenbaum, 1986; Johnson and Rosenbaum, 1993). This process requires the rapid delivery of flagellar precursors to the anterior end of the cell, their specific sorting into the flagellar compartment, and their selective transport to the tips of the growing flagella, which is the site of flagellar assembly (Rosenbaum *et al.*, 1969; Witman, 1975; Johnson and Rosenbaum, 1992). Recently, the discovery of a bidirectional transport system within the flagellum (Kozminski *et al.*, 1993) has led to a search for motors that might mediate the process of intraflagellar transport (IFT). The initial studies identified several kinesin-related proteins associated with different classes of flagellar microtubules (Bernstein *et al.*, 1994; Fox *et al.*, 1994; Johnson *et al.*, 1994), including one isoform that is the gene product of the *FLA10* locus (Walther *et al.*, 1994). The FLA10 kinesin is required for both the maintenance of IFT and the incorporation of flagellar components onto preexisting flagella (Kozminski *et al.*, 1995; Piperno *et al.*, 1996; Cole *et al.*, 1998). The process of IFT appears to be widespread, because FLA10 kinesin–related proteins have also been implicated in the process of axoneme assembly in *Caenorhabditis elegans* sensory cilia (Shakir *et al.*, 1993; Tabish *et al.*, 1995), sea urchin blastula cilia (Morris and Scholey, 1997), and mouse embryonic cilia (Nonaka *et al.*, 1998).

Because the FLA10 kinesin–related proteins are plus-end–directed microtubule motors (Yamazaki *et al.*, 1995) and IFT is a bidirectional process, it was proposed that retrograde IFT must be driven by a minus-end–directed motor, such as cytoplasmic dynein, whose delivery to the distal end of the flagellum depended on FLA10 kinesin activity (Kozminski *et al.*, 1995). Indeed, studies in mammalian cells and *Drosophila* have indicated that cytoplasmic dynein is abundant in the testis and appears to be involved in some aspect of spermatogenesis and male fertility (Collins and Vallee, 1989; Rasmusson *et al.*, 1994; Gepner *et al.*, 1996). Recently, a dynein light chain (LC8) has been found to be essential for retrograde IFT in *Chlamydomonas* (Pazour *et al.*, 1998). Although LC8 has been associated with a number of different protein complexes (King and Patel-King, 1995; King *et al.*, 1996; Espindola *et al.*, 1996; Harrison *et al.*, 1998), the defect in retrograde IFT observed in the LC8 mutant strongly suggested that a cytoplasmic dynein motor was involved in both IFT and flagellar assembly (Pazour *et al.*, 1998).

In this study, we have asked whether a cytoplasmic Dhc has a role in flagellar assembly in *Chlamydomonas*. Previous PCR screens have identified nine different *Dhc* genes distinct from the outer arm *Dhc* genes described by others (Mitchell and Brown, 1994; Wilkerson *et al.*, 1994), but none of these sequences appeared to encode a cytoplasmic Dhc (Porter *et al.*, 1996). By modifying the PCR reaction conditions, we have now recovered four additional *Chlamydomonas Dhc* genes, two of which encode cytoplasmic Dhc sequences. One of these sequences, *cDhc1b*, is closely related to a *Dhc* gene that is required for the formation of sensory cilia in *C. elegans* (Grant, personal communication). The *Chlamydomonas cDhc1b* sequence is a relatively low-abundance transcript whose expression is stimulated in response to deflagellation. Restriction fragment length polymorphism (RFLP)-mapping procedures have indicated that the *cDhc1b* gene is closely linked to a locus implicated previously in flagellar assembly. To identify null alleles of the *cDhc1b* gene, we used the *cDhc1b* clones to screen a new collection of flagellar assembly mutants generated by insertional mutagenesis. Southern blot analysis of >70 flagellar mutants has identified two strains that are associated with significant deletions of the *cDhc1b* gene. Structural studies have revealed that these mutants typically assemble short flagellar stumps (<1–2 μm) filled with highly aberrant microtubules, raft-like particles, and other amorphous material. These studies indicate that the cDhc1b isoform plays an important role in flagellar assembly in *Chlamydomonas*. Because of the high degree of sequence conservation observed in cDhc1b sequences, it seems likely that cDhc1b isoforms may serve a similar function in other organisms.

MATERIALS AND METHODS

Cell Culture and Mutant Strains

All cells used in this study were maintained as vegetatively growing cultures at 21°C on rich medium containing sodium acetate (Sager

and Granick, 1953) as described previously (Porter *et al.*, 1992). Large-scale liquid cultures were supplemented with additional potassium phosphate as described by Witman (1986).

RNA Purification and Reverse Transcription (RT)–PCR Procedures

Total RNA was isolated from wild-type *Chlamydomonas* (137c, mt+) cells both before and 45 min after deflagellation induced by pH shock (Witman *et al.*, 1972) as described previously (Wilkerson *et al.*, 1994; Porter *et al.*, 1996). To remove minor amounts of contaminating genomic DNA, we treated the total RNA with RQ1 DNase (Promega, Madison, WI), extracted with phenol and chloroform, and recovered by ethanol precipitation. cDNA was made from 1 μg of total RNA using AMV reverse transcriptase and random primers (Promega). To control for residual genomic DNA contamination, a second set of reactions was performed without reverse transcriptase. The resulting cDNA products were then used as templates in a series of PCR reactions containing a sense primer based on the conserved amino acid sequence KTESVKA [5′-AAG-AC(CGT)-GAG-(AT)(GC)(CGT)-GT(CG)-AAG-GC-3′] and an antisense primer based on the amino acid sequence CFDEFNR [5′-TG(CT)T-TCGA(CT)GA(AG)TT(CT)AAC(CA)G-3′]. The PCR reactions contained 2 μl of cDNA, 0.2 mM dNTPs, 2 μM of each primer, 1× reaction buffer, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase in a total volume of 50 μl. These reactions were incubated at 95°C for 5 min, followed by 30 cycles of 58°C for 2 min, 72°C for 3 min, and 94°C for 1 min and 1 cycle of 58°C for 2 min and 74°C for 2 min, and then held at 4°C. The PCR products were run on a 1.5% agarose gel and compared against a 100-bp ladder (Life Technologies, Grand Island, NY). Products of the sizes predicted for mature transcripts were gel-purified and subcloned as described previously (Porter *et al.*, 1996). Twenty-one PCR positive clones were sequenced, and four different *Dhc* sequences were identified among the subclones: *cDhc1a* (10 copies), *cDhc1b* (4 copies), *Dhc10* (1 copy), and *Dhc3* (1 copy).

DNA Isolation and Southern Blot Analysis

Genomic DNA was isolated from wild-type and mutant *Chlamydomonas* cells as described in Johnson and Dutcher (1991) and modified in Porter *et al.* (1996). DNA samples (3–4 μg per lane) were digested with a series of restriction enzymes, separated on 0.8–1.0% agarose gels, and transferred overnight to either Zetabind (Cuno, Meriden, CN) or Magnagraph (Micron Separation Systems, Westboro, MA) membranes according to standard procedures (Sambrook *et al.*, 1989) and the manufacturer's instructions. DNA probes for hybridization were purified in low-melting point agarose and radiolabeled with [^{32}P]dCTP and random primers using either the Prime-it II labeling kit (Stratagene, La Jolla, CA) or the Rediprime labeling kit (Amersham, Uppsala, Sweden). Conditions for prehybridization and hybridization were as described previously (Porter *et al.*, 1996; Myster *et al.*, 1997).

Construction of a SacI *Minilibrary*

To facilitate the specific recovery of the *cDhc1b* gene, an ~7.7-kb *Sac*I genomic fragment spanning the region that encodes the proposed ATP-binding site was isolated from a genomic minilibrary. Twenty-five micrograms of genomic DNA were digested with the restriction enzyme *Sac*I and size-fractionated on a 0.8% agarose gel. The region between 6 and 9 kb was cut into seven 1-mm slices, and the DNA was extracted with phenol and chloroform, ethanol precipitated, and resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). An aliquot of each slice (one-tenth volume) was rerun on a second gel, transferred to a Magnagraph membrane, and hybridized overnight with the 150-bp fragment corresponding to the *cDhc1b* PCR product. The three fractions with the strongest signals were pooled and ligated overnight into *Sac*I-digested pBluescript II. Two microliters of the ligation mixture were transformed into the *Escherichia coli* strain DH5αF′ using a BTX electroporator (Biotechnologies and Experimental Research, San Diego, CA) and following the manufacturer's protocols. The transformed cells were plated on LB-Amp, and ampicillin-resistant colonies were transferred to Magnagraph membranes and hybridized overnight with the 150-bp *cDhc1b* PCR product. A single positive clone containing a 7.7-kb *Sac*I fragment of the *cDhc1b* gene was identified out of 5000 colonies. The 7.7-kb *Sac*I subclone was purified by CsCl centrifugation and used to screen a large insert genomic library as described below.

Recovery of Large Insert Genomic Clones

Each *Dhc* sequence was used to screen a λFIX library containing wild-type (21gr) genomic DNA (Schnell and Lefebvre, 1993) as described previously (Porter *et al.*, 1996; Myster *et al.*, 1997). The resulting phage clones were mapped with the restriction enzymes *Not*I and *Sac*I, and the appropriate fragments were subcloned and sequenced to confirm the identity of the *Dhc* clones. Initial screens with the *cDhc1b* PCR product resulted in the recovery of phage clones containing either the *cDhc1a* gene or a new axonemal *Dhc* sequence, *Dhc11* (see Figure 1). Rescreening the library with the 7.7-kb *Sac*I subclone permitted the specific recovery of seven phage clones that span an ~23-kb region of genomic DNA containing the *cDhc1b* gene.

Subcloning and Sequencing of Genomic DNA

Restriction fragments from the phage clones were subcloned into pBluescript KSII (Stratagene), and plasmid DNA was purified using either CsCl centrifugation, Wizard Maxi-Preps (Promega), or Quantum Preps (Bio-Rad, Riverside, CA). Selected subclones were sequenced by primer walking using ABI Prism Sequencers (Perkin Elmer, Norwalk, CT) available in the DNA Sequencing Facility (Iowa State University, Ames, IA) or the Microchemical Facility (University of Minnesota, Minneapolis, MN). The sequence data were assembled and analyzed using both the MacVector software, version 3.0, and the Genetics Computer Group (GCG; Madison, WI) sequence analysis programs, version 9.0, available through the Advanced Biosciences Computing Center (University of Minnesota, St. Paul, MN).

Potential open-reading frames were identified using the GCG program Codon Preference and a codon usage table based on *Chlamydomonas* nuclear sequences (see Myster *et al.*, 1997; Nakamura *et al.*, 1997) (Myster, Knott, and Porter, unpublished results). Potential splice donor and acceptor sites were identified on the basis of the consensus sequences found in *Chlamydomonas* nuclear genes (Mitchell and Brown, 1994; LeDizet and Piperno, 1995; Zhang, 1996; Myster *et al.*, 1997) (Myster, Knott, and Porter, unpublished results; Schnell, University of Arkansas, personal communication). All splice junctions were confirmed directly by sequence analysis of RT–PCR products derived from the *cDhc1b* transcript.

cDNA was made from 5 μg of total RNA using Superscript II reverse transcriptase and random hexamers (Life Technologies). PCR reactions were then performed using sequence-specific primers and the Expand PCR kit containing both *Taq* DNA polymerase and *Pwo* DNA polymerase (Boehringer-Mannheim, Indianapolis, IN). All reactions were initiated by a single cycle of 94°C for 3 min, 51°C for 1 min, and 74°C for 3 min, followed by 29 cycles of 94°C for 1 min, 51°C for 2 min, and 74°C for 5 min. The PCR products were analyzed on a 1.5% agarose gel, and RT–PCR products of the appropriate size were purified using 0.8% low-melt agarose gels and Wizard PCR Preps (Promega) for direct sequencing with sequence-specific primers.

The proposed translation start site was identified by the recovery of a RT–PCR product using a forward primer located downstream of the TATA box sequence and a reverse primer designed in exon 2. Sequence analysis of the resulting product identified stop codons in

all three frames preceding an ATG, which was thereby designated the translation start site.

The predicted amino acid sequence of the *cDhc1b* gene was compared with other Dhc sequences using the GCG programs Bestfit, Compare, and Pileup. Potential nucleotide-binding sites were identified using the GCG program Motifs, and regions with the potential to form α-helical coiled-coils were identified using the program COILS, version 2.2 (Lupus *et al.*, 1991; Lupus, 1996).

Northern Blot Analysis

Aliquots containing 20 μg of total RNA were size fractionated on 0.75% agarose–formaldehyde denaturing gels and then transferred to either a Zetabind or Magnagraph membrane as described previously (Porter *et al.*, 1996). RNA was immobilized on the membrane by baking at 80°C for 2 h and UV irradiation at 20,000 μJ (Stratalinker II; Stratagene). Prehybridization and hybridization conditions were as described previously (Porter *et al.*, 1996; Myster *et al.*, 1997). To ensure that the signals were gene specific, we obtained probes for hybridization from the 5′ end of the *Dhc* gene. To control for equal loading of the RNA samples, we also hybridized blots with a probe corresponding to a fragment of the *CRY1* gene, which encodes the ribosomal S14 protein (Nelson *et al.*, 1994), as described previously (Porter *et al.*, 1996; Myster *et al.*, 1997; Perrone *et al.*, 1998).

RFLP Mapping

To identify a potential RFLP that might be used as a molecular marker for mapping the *cDhc1b* gene, we screened selected subclones by hybridization on Southern blots of genomic DNA isolated from two *C. reinhardtii* strains, 137c and S1-D2, that are polymorphic at the DNA sequence level (Gross *et al.*, 1988). A specific RFLP could be observed using genomic DNA that was double digested with *Eco*RI–*Xho*I and a 3.1-kb *Sac*I fragment derived from the 5′ end of the *cDhc1b* gene. The 3.1-kb *Sac*I fragment was next hybridized to a series of mapping filters containing genomic DNA that had been isolated from tetrad progeny derived from crosses between multiply marked *C. reinhardtii* strains and S1-D2. The segregation pattern of the *cDhc1b* gene was then analyzed with respect to 42 genetic and molecular markers covering all of the known *Chlamydomonas* linkage groups. The mapping filters and the associated genetic and molecular markers are described in detail by Porter *et al.* (1996).

Isolation of Stumpy Flagella Mutations by Insertional Mutagenesis

The strain A54-e18 (*ac17, nit1-1, sr1*) was provided by R. Schnell and P. Lefebvre (University of Minnesota, St. Paul, MN). This strain contains an ~10-kb deletion in the nitrate reductase (*NIT1*) gene and can be transformed with the plasmid pMN56. Approximately 20,000 nit+ transformants were generated as described by Nelson *et al.* (1994). After growth on selective medium for 10 d, positive transformants were picked into liquid medium and analyzed for flagellar assembly defects by phase-contrast light microscopy. Approximately 100 transformants were chosen for further study by electron microscopy (Dentler, unpublished results). A similar number of strains with potential flagellar assembly defects was also isolated by transformation of a *nit1-305* strain with the pMN24 plasmid. These strains were generously provided by K. Kozminski and J. Rosenbaum (Yale University, New Haven, CT) and were further analyzed by both light and electron microscopy (Dentler, unpublished results).

Electron Microscopy

For structural studies of flagellar mutants, cells were grown under a 12:12 h light/dark cycle in 100-ml liquid cultures of M or R medium with air bubbling (Harris, 1989). Immotile cells were harvested from the bottom of the culture flasks with a large bore pipette and then concentrated in polypropylene tubes using an IEC clinical centrifuge at speed #3 for 3 min. The cells were resuspended in M medium containing 2% glutaraldehyde, fixed for 1 h at room temperature, pelleted again, and then resuspended in 100 mM Na cacodylate, pH 7.2, and 2% glutaraldehyde for fixation overnight at 4°C. The next day, cells were washed three times in fresh 100 mM cacodylate buffer and then post-fixed in cacodylate buffer containing 1% OsO_4 for 30–60 min on ice. After three washes with distilled water, the cells were resuspended in 1% aqueous uranyl acetate for 3–12 h at room temperature. The samples were then dehydrated in an acetone series and embedded in BEEM capsules using Embed 812 resin (Electron Microscopy Services, Fort Washington, PA). Thick (~200 nm) sections were cut on a Dupont (Wilmington, DE) MT6000 microtome, stretched with xylene vapors, and then picked up on naked 300-mesh grids. Sections were stained with 1% uranyl acetate in 50% methanol, followed by lead citrate (Hyatt, 1970), and then imaged with a JEOL 1200EXII microscope operating at 125 kV.

Extraction of Flagellar Stumps

For studies of extracted cells, wild-type and mutant strains were grown and collected as described above, resuspended in buffer (20 mM HEPES, pH 7.5, 3 mM $MgSO_4$, 1 mM EGTA), put on ice, and then diluted with an equal volume of the above buffer containing 1% Nonidet P-40 and 6 mM EGTA. After incubation on ice for 5 min, the extracted cells were pelleted, resuspended in 100 mM Na cacodylate, pH 7.2, containing 2.5% glutaraldehyde, and fixed overnight at 4°C. In some experiments, cells were extracted with 4% Nonidet P-40 for up to 30 min before fixation. All samples were then rinsed, post-fixed, stained, and embedded as described above. Thin sections, ~30–40 nm, were cut and stained as described above and then observed at 80 kV.

Immunofluorescence Microscopy

Cells were fixed and stained using the methods described by Sanders and Salisbury (1995). Cells were attached to polyethyleneamine-coated coverslips, fixed with cold methanol, air-dried, and rehydrated in phosphate-buffered saline (PBS). Cells were incubated with mouse anti-β-tubulin (diluted 1:500) and mouse anti-kinesin II (K2.4; diluted 1:200) antisera for 1–2 h at 37°C. Coverslips were washed with PBS and incubated with Alexa 594-labeled anti-mouse antibodies (Molecular Probes, Eugene, OR) for 1 h at 37°C. Coverslips were rinsed in PBS and mounted on slides with Gelvatol antibleach solution (Rodriguez and Deinhardt, 1960). Cells were examined with a Zeiss (Thornwood, NY) WL epifluorescence microscope, and images were captured using a DAGE SIT camera, Image Σ frame averaging computer, and Macintosh 6500 computer equipped with a Scion video board (Scion, Frederick, MD). Some cells were viewed and photographed with a Bio-Rad MRC 1000 confocal microscope.

The tubulin antibody was raised against bovine brain β-tubulin and was generously provided by Dr. R. Himes (University of Kansas, Lawrence, KS). The anti-kinesin II antibody (K2.4) was raised against the 85-kDa subunit of sea urchin kinesin II (Cole *et al.*, 1993; Henson *et al.*, 1997) and was generously provided by Dr. J. Scholey (University of California, Davis, CA). The K2.4 antibody specifically cross-reacts with the 90-kDa FLA10 kinesin subunit in *Chlamydomonas* (Cole *et al.*, 1998).

RESULTS

Recovery of New Dhc Sequences in Chlamydomonas

To recover the *cDhc* genes from *Chlamydomonas*, we designed a series of oligonucleotide primers based on regions of sequence conservation surrounding the primary nucleotide-binding site (P-loop 1) in cDhc se-

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cDhc1a    ITPLTERAFSTMMAAVHLHYGGAPEGPAGTGKTETVKELAKCLGKQCVVFNTTEQLESGHLTRLLMGIISTGAWACFDEFNR
cDhc1b    YTPLTDKCYLTLTQGMALGYGGNPYGPAGTGKTESVKALGQALARQVLVFNCDEEFDFKSMGRIFVGLVKCGAWGCFDEFNR
Dhc3      ITPMTDRAYMTLTGALHMRLGGAPAGPAGTGKTETVKDLAKALGVQCVVFNCGDNLDFRFMGKFFSGLAQAGAWACFDEFNR
Dhc10     ITPLTDRCYMTLGAAMFTRRGGNPLGPAGTGKTETVKDFGKALARYVIVFNCSDGVDYKMTGKMFSGLAQTGAWACLDEFNR
Dhc11     ..........................GTGKTETTKDLAKAVAIQCVVFNCSDGLDYLAMGRFFKGLAQTGAWACFDEFNR
consensus iTPLTdrcy.Tl.aam.l.yGGnP.GPAGTGKTEtvKdl.kalarq.vVFNcs.g.dyk.mgr.f.GlaqtGAWaCfDEFNR
primers                                *******                                *******
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Figure 1. Identification of additional *Dhc* genes in *Chlamydomonas.* The deduced amino acid sequences of four new *Dhc* sequences in the region surrounding the conserved ATP hydrolytic site (P-loop 1) are shown. *cDhc1a*, *cDhc1b*, and *Dhc10* were initially recovered in the RT–PCR screen, whereas *Dhc11* was identified during subsequent screening of a genomic library (see MATERIALS AND METHODS for details). *Dhc3* is an axonemal *Dhc* sequence (Porter *et al.*, 1996) that was fortuitously isolated in the PCR screen.

quences in other organisms. These primers were then used to amplify the *Dhc* sequences present in cDNA prepared from vegetatively growing, nondeflagellated cells. A specific PCR product of the expected size (~150 bp) was observed using a sense primer based on the amino acid sequence KTESVKA and an antisense primer based on the amino acid sequence CFDEFNR. The 150-bp product was subcloned, and 21 different reaction products were sequenced, yielding four distinct *Dhc* sequences (see MATERIALS AND METHODS). Comparison of the predicted amino acid sequences with that of other *Dhc* genes revealed that two of the sequences, *cDhc1a* and *cDhc1b*, are related to *cDhc* genes identified in other organisms (Figures 1 and 2). The other two sequences correspond to axonemal *Dhc* genes, *Dhc3* and *Dhc10*, that were fortuitously amplified along with the cytoplasmic *Dhc* sequences (Porter *et al.*, 1996) (our unpublished results). To verify and extend the *Dhc* sequences, we recovered longer clones from a genomic library (see MATERIALS AND METHODS). Because of the high degree of sequence conservation within the P-loop 1 region, the library screen yielded another axonemal *Dhc* sequence, *Dhc11*. The predicted amino acid sequences through the hydrolytic domain of the *Dhc* clones are shown in Figure 1.

Comparison with Cytoplasmic Dhc Sequences in Other Organisms

Previous studies in other organisms have shown that the cytoplasmic Dhc sequences fall into two distinct groups (Gibbons *et al.*, 1994; Gibbons, 1995; Tanaka *et al.*, 1995). The major cytoplasmic Dhc sequence is ubiquitously expressed in all eucaryotic organisms (reviewed in Gibbons, 1995). The minor cytoplasmic Dhc sequence, known as DYH1b, DLP4, cDHC1b, or Dhc2, has thus far only been detected in those organisms that assemble cilia or flagella at some stage during their life cycle (Gibbons *et al.*, 1994; Tanaka *et al.*, 1995; Vaisberg *et al.*, 1996; Vaughan *et al.*, 1996; Neesen *et al.*, 1997). Comparison of the two *Chlamydomonas* cytoplasmic *Dhc* sequences with that of other cytoplasmic *Dhc* genes confirms that the *Chlamydomonas* sequences also fall into these two groups (Figure 2). The *Chlamydomonas* cDhc1a sequence is most similar to the cytoplasmic Dhc sequences identified in the budding yeast *Saccharomyces cerevisiae* (Eschel *et al.*, 1993; Li *et al.*, 1993) and the fission yeast *Schizosaccharomyces pombe* (West and McIntosh, personal communication), whereas

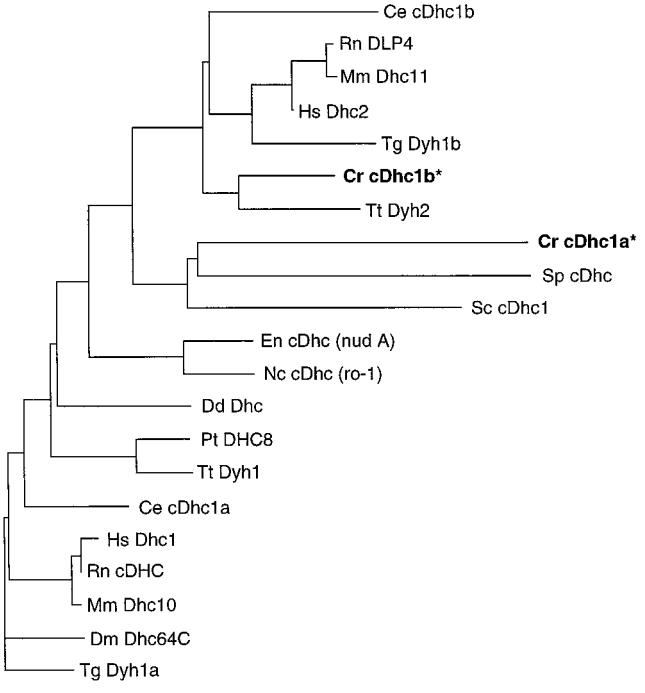

Figure 2. Diagrammatic alignment of cytoplasmic Dhc sequences. The *Chlamydomonas reinhardtii* (Cr) cytoplasmic Dhc sequences were aligned with cytoplasmic Dhc sequences identified in other organisms using the program CLUSTAL W (Thompson *et al.*, 1994) and were displayed using the program DRAWGRAM from the Phylip package, version 3.57c (Felsenstein, 1998). The abbreviations and GenBank accession numbers for the sequences are as follows: *Caenorhabditis elegans* (Ce), L33260 and Z75536; *Dictyostelium discoideum* (Dd), Z15124; *Drosophila melanogaster* (Dm), L23195; *Emericella nidulans* (En), U03904; *Homo sapiens* (Hs), L23958 and U20552; *Mus musculus* (Mm), Z83808 and Z83809; *Neurospora crassa* (Nc), L31504; *Paramecium tetraurelia* (Pt), L17132; *Rattus norvegicus* (Rn), D13893, L08505, and D26495; *Saccharomyces cerevisiae* (Sc), Z21877 and L15626; *Schizosaccharomyces pombe* (Sp), AB006784; *Tetrahymena thermophilia* (Tt), AF025312 and AF025313; and *Tripneustes gratilla* (Tg), Z21941 and U03969.

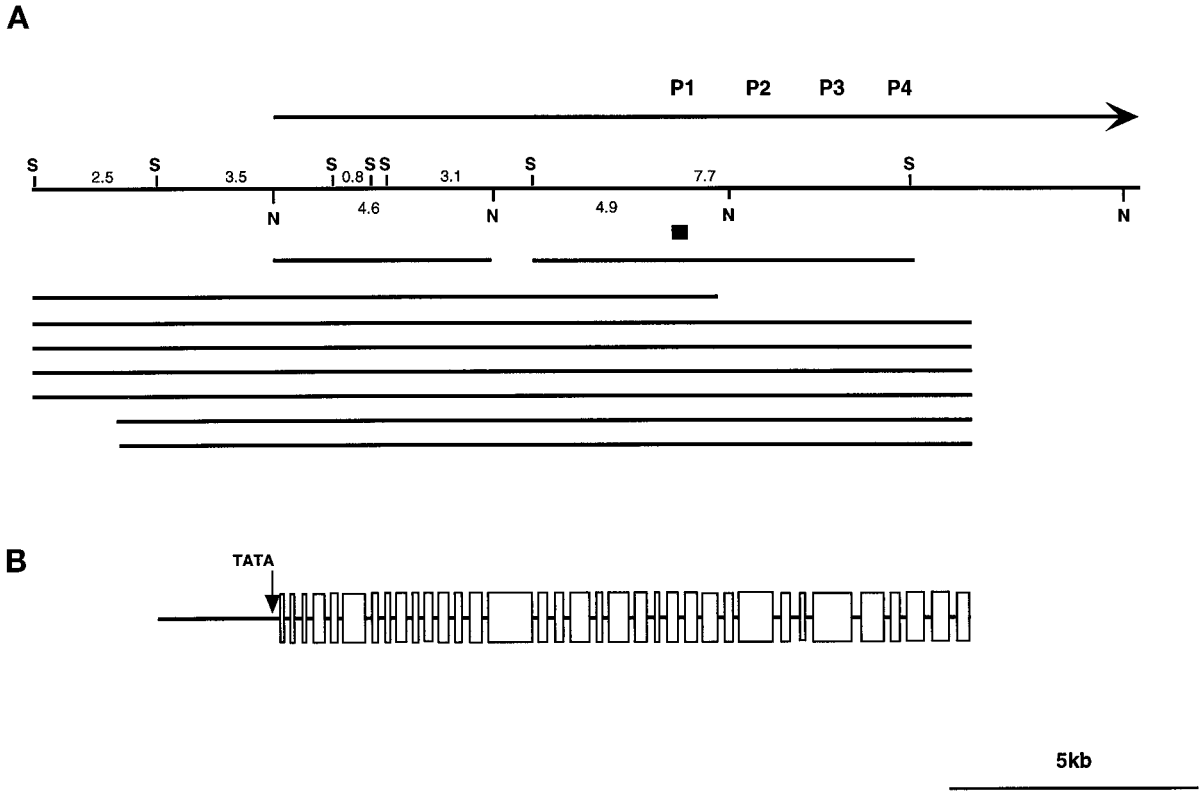

Figure 3. Recovery of the *cDhc1b* transcription unit. (A) A partial restriction map of the genomic DNA region containing the *cDhc1b* transcription unit. Also indicated on the diagram are the 150-bp fragment recovered in the PCR screen, the ~7.7-kb *Sac*I fragment cloned from the size-fractionated minilibrary, the ~4.6-kb *Not*I fragment used as a Northern probe, and the approximate positions of the seven phage clones obtained from the large insert genomic library. S, *Sac*I sites; N, *Not*I sites. (B) Intron–exon structure of the N-terminal and central region of the *cDhc1b* gene. A diagram of the relative sizes of the introns and exons is shown. All splice sites were confirmed by RT–PCR. Also indicated are the approximate positions of the TATA box sequences and the proposed translation start site.

the *Chlamydomonas* cDhc1b appears to be most closely related to the cDhc1b isoforms identified in *C. elegans*, sea urchin, and *Tetrahymena* (Gibbons *et al.*, 1994; Wilson *et al.*, 1994; Lee *et al.*, 1999). Because both the sea urchin and *C. elegans* cDhc1b isoforms have been proposed to function in some aspect of flagellar assembly (Gibbons *et al.*, 1994) (Grant, personal communication), we were interested in characterizing the *Chlamydomonas cDhc1b* gene further.

Recovery and Sequence Analysis of the cDhc1b *Gene*

To obtain longer clones of the *cDhc1b* gene, we screened a series of genomic libraries (see MATERIALS AND METHODS) and eventually recovered seven phage clones spanning >23 kb of genomic DNA (see Figure 3). Restriction mapping and sequence analysis of selected subclones indicated that the 23-kb region contained ~8 kb of genomic DNA located 5′ of the proposed translation start site and ~14.5 kb of the *cDhc1b* transcription unit. A partial restriction map of the region containing the *cDhc1b* gene and a diagram of the associated subclones used in this study are shown in Figure 3.

Sequence analysis of both genomic DNA and RT–PCR products derived from the *cDhc1b* transcript indicated that the 5′ end of the *cDhc1b* gene is located within a 3.5-kb *Sac*I subclone (see Figure 3). This region also contains several TATA and tub box sequences (Brunke *et al.*, 1984; Davies and Grossman, 1994) that are presumably required for the regulated transcription of the *cDhc1b* gene. On the basis of the analysis of the RT–PCR products, the remaining 14.5 kb of the *cDhc1b* transcription unit contains ~70% of the coding region located in 35 exons ranging in size from 71 to 905 bp. The predicted amino acid sequence obtained thus far (see Figure 4) corresponds to 3074 amino acids out of an expected ~4200 residues and extends from the N terminus through to the central region containing the predicted motor domain (Koonce and Samso, 1996; Gee *et al.*, 1997).

A search for potential nucleotide-binding sites in the *Chlamydomonas cDhc1b* amino acid sequence identified

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   1  MSSDSRKTFVVTTIACAIAPAGQQDQYAAYLGQDENGAIASFLDSTQNTLQAGVSGLGTGQLQLKLSNAAEFPEGCEFSVVLSKLRAGPIRTEDVPAGVA  100
 101  VATVAHSPLSSLYHTLKDVYSPLIKSQTAEGVPVLDKRLSELLAQVQAGLGTAVRKGVPASAQEVADPNQAPLQEVVTPLDEINFWAELTNSPNAGPIKS  200
 201  SALQVSSALEPLRQSFEQLSSDAPEGEGGSIGWEGAKELVDLTTNALRAAWPVKLPIGGWAMGQKRADNFLRVVGAALATYVQRRVDRLAAAGRGDLWSA  300
 301  PFGEVRAVLVGASGLMTRWVQESTALVEDWRLGVDTGGHDWEGAAFADAYVRSFQERLEEIYNMREMVDELAKLVGREEAGSLGVQQVFAPFQGLQALQV  400
 401  SDFNTHVWKAAHEDFERRMGPIEQRISQKLKELFASVIIPSLTSAIGPGRGGDRAAAGGSLIQPQQVFAEIKRYSSLMGRKNIASALQSEKETLAKQVDR  500
 501  HLDSVQAEFDAHRDSATGGAKVAPVGRVTASIVERIMWCMQTLQKLGKVSEVLKHMLRGGVAGDEGSGGAALRNTLGVVSELQKEVEIFRKEQYSAWEEW  600
 601  MSEELGEMANWKNSKLMTFDSQNSHVKTHFNDQLVLLLREVRQLQSLGFGVRKDILNEVEIANKFYRYGMVLKQRANFYNNIATEMVQCQKPMMLKDALD  700
 701  FEKVLMNPKDAQGKEITWRNAAALDGYVRRLNEVADRLAEKNRTLRKWHSVLSDKVVTLAGTDLVRHKDKWAAGVKEMREIFGRLEAEGYSRESQQVWRQ  800
 801  HWDFQLYKALEVQYLSGLEMINKTLPEVEVKMVFRQHRLQYDPPLEELRIRHVKDLLNTFLGLPLRMKGVSDLSERPGFFRPIVDANPTGIARVYAAAES  900
 901  LFTQLADELKKYQDWMVLGTLDLDEFADANLLEVSDWELNFRMLKAASRDAEKLPNEIRIECYKVSLAPVKGSIDEHMKKLQDTLVASLRRKTVAEKDQI 1000
1001  EDFMKNGRELFTRQANTVEDIGLAGQEAKGLTAKLAEVQAARRRIDEKNKLLRQMAGGGRDAAFAVVDLTEVNNSWDAFTNQLQQFDAHLEEQKGNLAVQ 1100
1101  ISRQLEEFKGKVAGMNSRWQELKPKSGPSGNPAVVLAKIQEYANAIKELREESAKLYKEAEAFKIDVPDFELMTEMETDVMATKAHWDRYADFLRERDEM 1200
1201  ANRDWLSMRDQVWKIEDFLAKWTKATAGKSDDPIAVILTQEIDNYTLCLPHLKSCLRGAGWEDTHWNQLFGLLGMKTSGPAAVSKETVTLTHFLEKADLV 1300
1301  VKHADVIKSLDAQAQGEAVIRKALTELKMWGMAREFTFTESTQSVAGRQRRTPLIKEWRDAMTEVGDNQSLVASLKQSSYYNMFKDEVSSWENKLSFLQE 1400
1401  GLTLLNQIQRKWVYLEPIFGRGALPSQQQRFRNVDEEFRRTMTSLESTKKVVTFADIPGIRDKLPQMAQQLDVCQRALADFLEEKRSQFPRFYFLGDDDL 1500
1501  LEILGQARNPAVIQSHLKKLFAGIQKVKFSQDQSTIQAMQSMEGEVVDLAPTVRITEQIETWLGDLTRSMKNTLQQQNEVLCAGRMNDEFRAAASQCLQL 1600
1601  KEAVAFTEKAEVALKAGSSGLAKLVTEMRAQLMKLTGSDFTGHHLLQLKKQALVLDFIHYCDVAEYLAKDKIGGTTEWGWTRQLRYYHRAEGSVKVAMAE 1700
1701  ATFDYTWEYQGNAAKLVYTPLTDKCYLTLTQGMALGYGGNPY GPAGTGKT ESVKALGQALARQVLVFNCDEEFDFKSMGRIFVGLVKCGAWGCFDEFNRL 1800
1801  DEEVLSAVSQQIQTIQLALKEGAKTMMFMDKTVEVDKNAGIFVTLNPAGKGYGGRSKLPDNLKQLFRSIAMTVPNNELIAEVLLLSEGFNHAKDLARKLV 1900
1901  SLFSLSRELLSPQQHYDWGLRALKTVLGIAGRELRDARKAGQNVDAEIEAEIIIRSVAATKLPTLTFDDNSRFKALINDLFPGAKLTDARNEALEKALAE 2000
2001  AAAACKMELTQQQIDRMLQLHLACEQRIGVIIV GPSGSGKS TLWELLEKAYERLGRKPIVYKMNPKAMPRQQLLGSMNMDTREWSDGVLTAAARKVVKEP 2100
2101  LEQRSWIICDGDVDPEWIESLNSVLDDNRLLTMPNGERIQFANNVNFIFECHSLEFASPATVSRCGMLFMSDEAMEVERMLQRWLKVQATDNGDPGQMQS 2200
2201  WMNDFFDKAFQWALSHPRAVETTKGGILDSGLSHLKLGPGSKQEFMAGLCRGLGSNMNPDIRNQFYNDMARMSGEGGIMDVGVATDPLIVLGDELRERGM 2300
2301  DEADGGLVVTPEVTQNLLMMAPWFKNRDPFLVV GPEGCGKG ALLDYCFKRIMGVQVAVVNCSAQTSAANVVQKLVQVCGKPVTTTSGKALRPPDNTRVIL 2400
2401  YLKDLNLPRPDKYNTCQLISFLQQLIAHHGYYDENLDFIRVERVQIVGSMTPPGSVGRHALSTRFTALVRIVTMGYPDRENLATIYTNMAQRVLANSKTA 2500
2501  SSVSPAALSKAMLEVYSSVRERFTPNDYPHYEFNARELSDWINGIQRYSLEGGLTLVQAIAHEGLRVFRDRLVGDHQEQFTSMLYGTLTSLLGYKPDATP 2600
2601  WYTSTLGASAEERISGDLTKIKMLRWEQDTFAELVAEKLKGYEREHKELNLLLFPEVLERVSRFDRVLSQQGGSLLLC GNSGVGRR SLMLLLAYMHNMDF 2700
2701  ITPKMTKNYDLKSFRNDLKEVLRRAGVEAKPVMLFLEDHQLVNNAFLELVNSLLSGGEVPGLFTPEELAKELAPLDKARDEDPLYTGPSNSYAFFSYRIR 2800
2801  RNLHIVVSMDPSNEMFRSRCEANPALFTRCSVQWLEGWSVKGLQQIAAARLTELVESSPELMKLGRDKLINHMIHIHASSGSQTTREYLALVSLYGQIYN 2900
2901  RKRTQVLEQQNFLKGGLGKLAEAAVTVDTLSAEAEKQRVVLKAKQAEADEALVHIQDSMLKAADRRKEVEVLKKRTAIEEVEMKERRVKVEEELSEVQPL 3000
3001  IDAARKAVGNIKKDNIAEIRSLKMPPDAIRDVLEGVLMVLGQQDTSWNNMKTFLGKGSVKDDIINYDAHKITPE 3074
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Figure 4. Partial amino acid sequence of *cDhc1b.* The deduced amino acid sequence of the N-terminal and central region of the *cDhc1b* gene is shown. The four conserved P-loop motifs are indicated in bold letters. EMBL accession number AJ132478.

four consensus or near consensus phosphate-binding (P-loop) motifs with the sequence GXXXGKT/S (Walker *et al.*, 1982) in the central region of the polypeptide (Figure 4). These four P-loops (P1–P4) are spaced ~300 amino acids apart at conserved positions relative to other Dhc sequences. The amino acid sequence around P1 is the most highly conserved among all Dhc sequences, consistent with the proposal that this P-loop corresponds to the primary ATP hydrolytic site (Gibbons, 1995). Comparison with cDhc1b-related sequences in other organisms (Gibbons *et al.*, 1994; Wilson *et al.*, 1994; Lee *et al.*, 1999) indicates that the region around P2 is more conserved than that around either P3 or P4; this differs from previous observations with cDhc1a-related sequences, in which P3 is more highly conserved, or with axonemal Dhc sequences, in which P4 is more highly conserved (reviewed in Gibbons, 1995).

The predicted amino acid sequence of the *cDhc1b* gene was also analyzed using programs that predict secondary structure to identify regions with the potential to form α-helical coiled-coil domains (Lupus *et al.*, 1991; Lupus, 1996). As indicated in Figure 5, one region before the first P-loop (residues 1023–1056 and 1133–1172) and another region after P4 (residues 2926–3000) show a high probability of forming coiled-

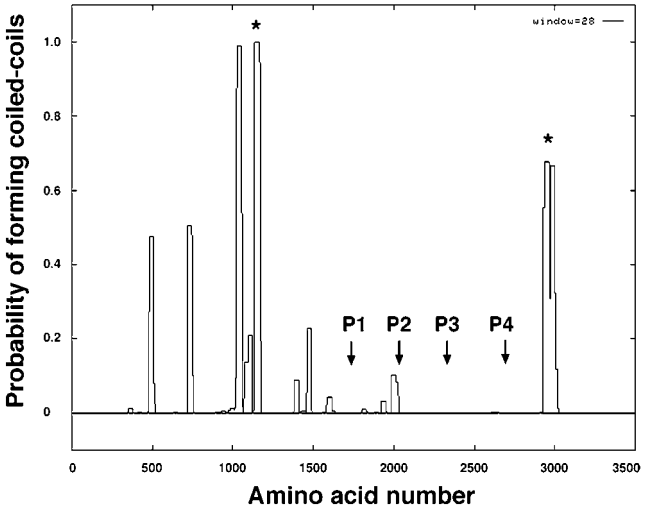

Figure 5. Structural domains within the cDhc1b polypeptide. The probability of forming regions of α-helical coiled-coil structure was determined using the program COILS (Lupus *et al.*, 1991; Lupus, 1996). Peaks of high probability that are also encoded by homologous regions in other Dhc sequences are indicated by the asterisks (see Mitchell and Brown, 1994, 1997).

coil domains. The presence of predicted coiled-coil domains separating the central region containing the four P-loop sequences from both the N-terminal and C-terminal regions has also been observed in many other Dhc sequences (Mitchell and Brown, 1994, 1997).

The predicted amino acid sequence of the *cDhc1b* gene was compared with several other full-length or near full-length Dhc sequences, including the three Dhc sequences (α, β, and γ) that form the outer dynein arm in *Chlamydomonas* (Mitchell and Brown, 1994, 1997; Wilkerson *et al.*, 1994), the 1α and 1β Dhcs of the I1 inner dynein arm (Myster, Knott, Bower, Perrone, and Porter, unpublished results), and cytoplasmic Dhc sequences from several organisms (Koonce *et al.*, 1992; Eschel *et al.*, 1993; Li *et al.*, 1993; Mikami *et al.*, 1993; Vaisberg *et al.*, 1993, 1996; Zhang *et al.*, 1993; Gibbons *et al.*, 1994; Plamann *et al.*, 1994; Wilson *et al.*, 1994; Xiang *et al.*, 1994; Lye *et al.*, 1995; Lee *et al.*, 1999). In each case, a high degree of sequence similarity was evident over long stretches of the central region of the Dhc (Figure 6) (our unpublished results). However, the *Chlamydomonas* cDhc1b also shares significant sequence homology in its N-terminal region with the cDhc1b sequence identified in *C. elegans* (Figure 6). Because the N-terminal region is thought to be important in the association of a Dhc with its specific intermediate and light chain subunits (Mocz and Gibbons, 1993; Sakakibara *et al.*, 1993), these observations suggest that the *Chlamydomonas* and *C. elegans* cDhc1b sequences could assemble into motor complexes containing related accessory subunits and perform similar functions.

Expression of the cDhc1b *Transcript in* Chlamydomonas

Although the *Chlamydomonas cDhc1b* gene was recovered by RT–PCR using RNA isolated from nondeflagellated cells, previous work has indicated that the

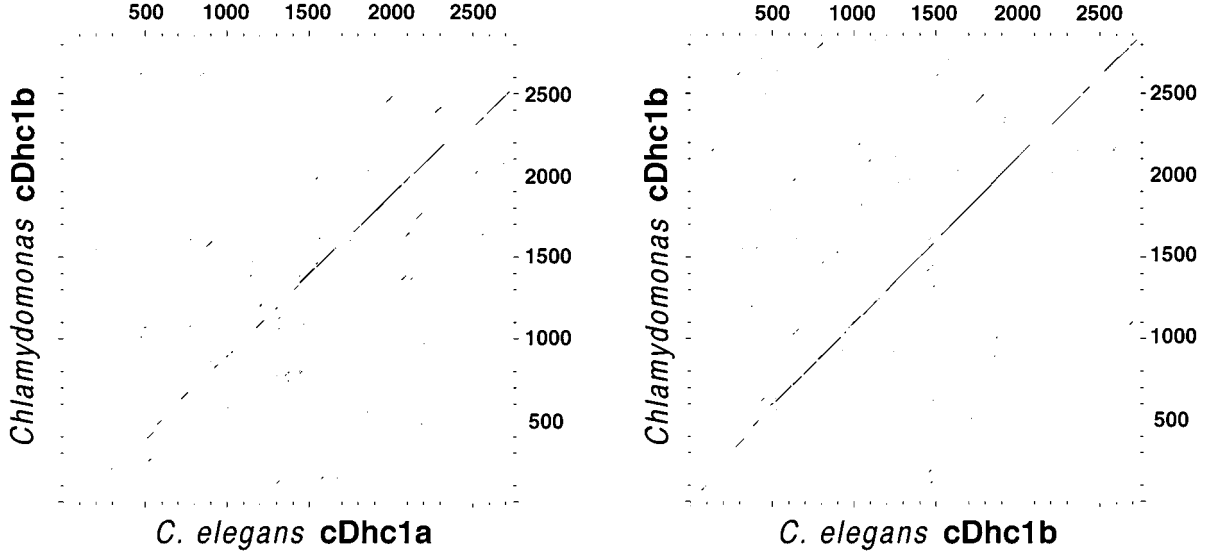

Figure 6. Pairwise comparison of Dhc sequences. The *Chlamydomonas* cDhc1b polypeptide was compared with other full-length Dhc sequences using the GCG program Compare with a window size of 50 and a stringency of 22. Shown are the plots against the two *C. elegans* cytoplasmic dynein sequences, cDhc1a (Lye *et al.*, 1995) and cDhc1b (Wilson *et al.*, 1994). The GenBank accession numbers for the *C. elegans* sequences are L33260 and Z75536, respectively.

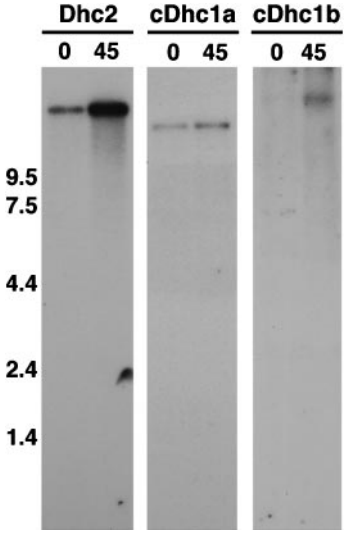

Figure 7. Expression of *Chlamydomonas Dhc* genes in response to deflagellation. Shown are autoradiograms of Northern blots loaded with total RNA isolated from wild-type cells before (0) and 45 min after (45) deflagellation. Left, the blot was hybridized with a control probe for the axonemal sequence *Dhc2* and was exposed overnight. Middle, the blot was hybridized with an ~6.0-kb *Sac*I fragment from the 5′ end of the *cDhc1a* transcription unit and was exposed for 20 d, although faint signals could be observed on shorter exposures. Right, the blot was hybridized with a 4.6-kb *Not*I fragment isolated from the 5′ end of the *cDhc1b* transcription unit (see Figure 3) and was exposed for 10 d. All probes were labeled to the same specific activity, and all of the *Dhc* transcripts are estimated to be >13 kb in length (see also Porter *et al.*, 1996).

expression of the sea urchin *cDhc1b* gene can be upregulated in response to deciliation (Gibbons *et al.*, 1994). We therefore isolated RNA both before and after deflagellation of *Chlamydomonas* cells and analyzed the expression of the *cDhc1b* transcript on Northern blots. Because we were concerned about possible cross-hybridization between the *cDhc1b* sequence and the abundant axonemal *Dhc* transcripts present in deflagellated cells (Mitchell, 1989; Wilkerson *et al.*, 1994; Porter *et al.*, 1996), we used a restriction fragment derived from the 5′ end of the gene as the hybridization probe. This fragment encodes the divergent N-terminal region (see Figure 3). As shown in Figure 7, the expression of the *Chlamydomonas cDhc1b* transcript (>13 kb) is stimulated by deflagellation. The signal both before and after deflagellation is significantly weaker than that observed with a control probe for an axonemal *Dhc* transcript (*Dhc2*), as indicated by the difference in exposure times (see Figure 7 legend), but the *cDhc1b* transcript is consistently more abundant after deflagellation than is the *cDhc1a* transcript (see Figure 7) (our unpublished results). These results indicated a potential role for *cDhc1b* in either flagellar motility or assembly.

Identification of cDhc1b *Mutations*

To determine whether the *cDhc1b* gene might be linked to a previously identified flagellar mutation, we used RFLP-mapping procedures to place the sequence on the genetic map of *Chlamydomonas* (see MATERIALS AND METHODS). As shown in Figure 8, the *cDhc1b* gene is closely linked to the mating type (*mt*) locus on linkage group VI. This location places the *cDhc1b* gene in close proximity to the reported map position of a temperature sensitive, flagellar assembly mutation, *fla6* (Adams *et al.*, 1982). We have been unable to analyze this linkage further in a direct cross with *fla6* because the original mutation has apparently reverted (Bower and Porter, unpublished results), but because of these observations, we decided to screen a new collection of flagellar assembly mutants generated by insertional mutagenesis with the goal of identifying other potential *cDhc1b* mutations.

Transformation of *Chlamydomonas* cells with exogenous DNA containing a selectable marker is a highly

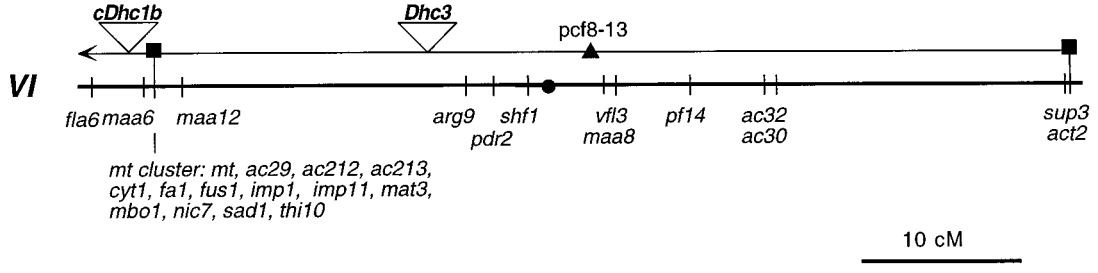

Figure 8. Genetic map position of the *cDhc1b* gene. The genetic map of linkage group VI (redrawn from Harris, 1989; Porter *et al.*, 1996) is shown on the bottom line. The approximate map locations of the *Dhc* clones (open triangles) and another molecular marker (black triangle) are shown on the top line. The black squares indicate the genetic markers in the *C. reinhardtii* strain that were used to anchor the two maps relative to one another. The black circle marks the position of the centromere. The parental ditype:nonparental ditype:tetratype ratios and estimated map distances in centiMorgans (cM) are as follows: *cDhc1b* versus *mt* (22:0:1; 2.2 cM), *cDhc1b* versus *Dhc3* (11:0:6; 17.6 cM), *cDhc1b* versus pcf8–13 (8:0:22; 36.7 cM), and *cDhc1b* versus *act2* (3:0:11; 39.3 cM). The distance from the centromere was estimated using the centromere-linked markers *ac17* (4:5:21; 35 cM) and *y1* (4:4:20; 35.7 cM). Additional mapping data for the linkage group VI markers are provided in Porter *et al.* (1996).

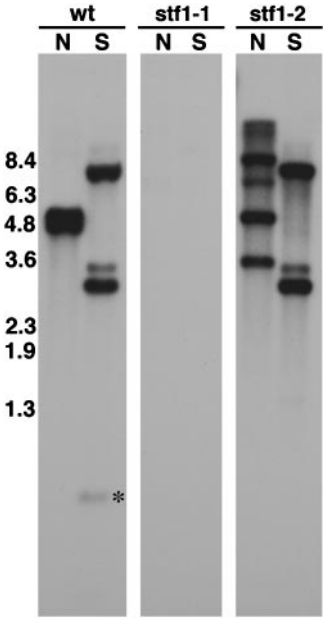

Figure 9. Identification of *cDhc1b* mutations. Shown are autoradiograms of three duplicate Southern blots containing genomic DNA isolated from wild-type (wt) and two stumpy flagellar mutants, *stf1-1* and *stf1-2*. Four micrograms of genomic DNA were digested with the restriction enzymes *Not*I (N) and *Sac*I (S), separated on a 0.8% agarose gel, transferred to a Magnagraph membrane, and hybridized overnight with the 4.6- and 4.9-kb *Not*I fragments of the *cDhc1b* gene (see Figure 3). No cross-hybridizing sequence is detected in the *stf1-1* genomic DNA, whereas an RFLP in one of the *Not*I fragments is detected in *stf1-2* genomic DNA. Longer exposures indicated that the *stf1-2* genomic DNA is missing the two *Sac*I fragments of 0.8 and 0.3 kb located within the wild-type 4.6-kb *Not*I fragment (see Figure 3). Hybridization with probes for the *NIT1* gene indicated the presence of a single plasmid insert in each strain and the hybridization of this sequence to the polymorphic *cDhc1b* restriction fragments in *stf1-2* (Bower and Porter, unpublished results).

efficient method for the recovery of new mutations that affect either flagellar assembly or flagellar motility (Tam and Lefebvre, 1993). The selectable marker integrates nonhomologously into genomic DNA, and as a result, the site of the new mutation is often marked by plasmid sequences that can be used as a molecular tag for the recovery of flanking genomic DNA. In addition, plasmid insertion is often accompanied by deletion or rearrangement of the host cell DNA, and the resulting mutant phenotype is very stable. Finally, if cloned genes corresponding to a potential mutant locus are available, it is relatively straightforward to identify mutations in the gene of interest simply by screening genomic DNA from the mutants on Southern blots and looking for changes in the restriction pattern of the gene. We have used this approach previously to identify mutations in several genes that encode subunits of the axonemal dyneins (Myster *et al.*, 1997; Perrone *et al.*, 1998) (Perrone and Porter, unpublished results).

To identify a potential *cDhc1b* mutation, we screened DNA samples isolated from >70 different flagellar assembly mutants by hybridization with different fragments of the *cDhc1b* gene. As shown in Figure 9, we have thus far recovered two independently isolated, stumpy flagellar (*stf*) mutants with different defects in the *cDhc1b* gene. The *stf1-1* strain is associated with a deletion of >15 kb of genomic DNA, which includes at least two-thirds of the *cDhc1b* coding region. The *stf1-2* strain is missing ~1 kb of genomic DNA located in the 5′ end of the coding region (see Figure 9 legend). Defects of this magnitude in the N-terminal region of a Dhc are likely to be null mutations. Hybridization of the blots with control probes for other *Dhc* sequences has confirmed that the RFLPs observed are specific to the *cDhc1b* gene and are not caused by problems with the loading or digestion of the DNA samples (Wysocki and Porter, unpublished results). Hybridization with probes for the *NIT1* gene used as the selectable marker has also demonstrated that both mutants contain only a single plasmid insert (Bower and Porter, unpublished results) (see Figure 9). The *stf1-1* and *stf1-2* strains therefore contain bona fide *cDhc1b* mutations that are associated with plasmid insertions.

cDhc1b Mutants Assemble Short, Defective Flagella

Short flagellar stumps were observed on both *stf1* mutants using differential interference contrast microscopy. Examination of thin-sectioned cells by transmission electron microscopy revealed that both *stf1* strains fall into a class of stumpy mutants whose flagella are shorter than 1–2 μm in length (Figure 10). The basal body and transition zone structures appeared identical to those found in wild-type cells (Figure 10, D–I), but the microtubules within the flagellar stumps were extremely short (0.5–1.0 μm) and aberrantly organized. When viewed in cross section (Figure 10, A–C), it was evident that most *stf1* flagella did not contain the typical "9+2" array of microtubules, although a few examples could be found. In most flagella, one or more singlet microtubules could be seen, usually collapsed into the center of the flagellum (Figure 10, B and C). When viewed in longitudinal section (Figure 10, D–H), the flagellar microtubules extended from the basal body microtubules, but many appeared to end in open, slightly frayed sheets of protofilaments (Figure 10, F and G). Few convincing examples of a normal central pair apparatus were found, although some flagella contained microtubules with free proximal ends, similar to central pair microtubules (Figures 10E and 11C). Others contained a core of amorphous material similar in appearance to that found in central pair mutants (Figure 10D). No clearly

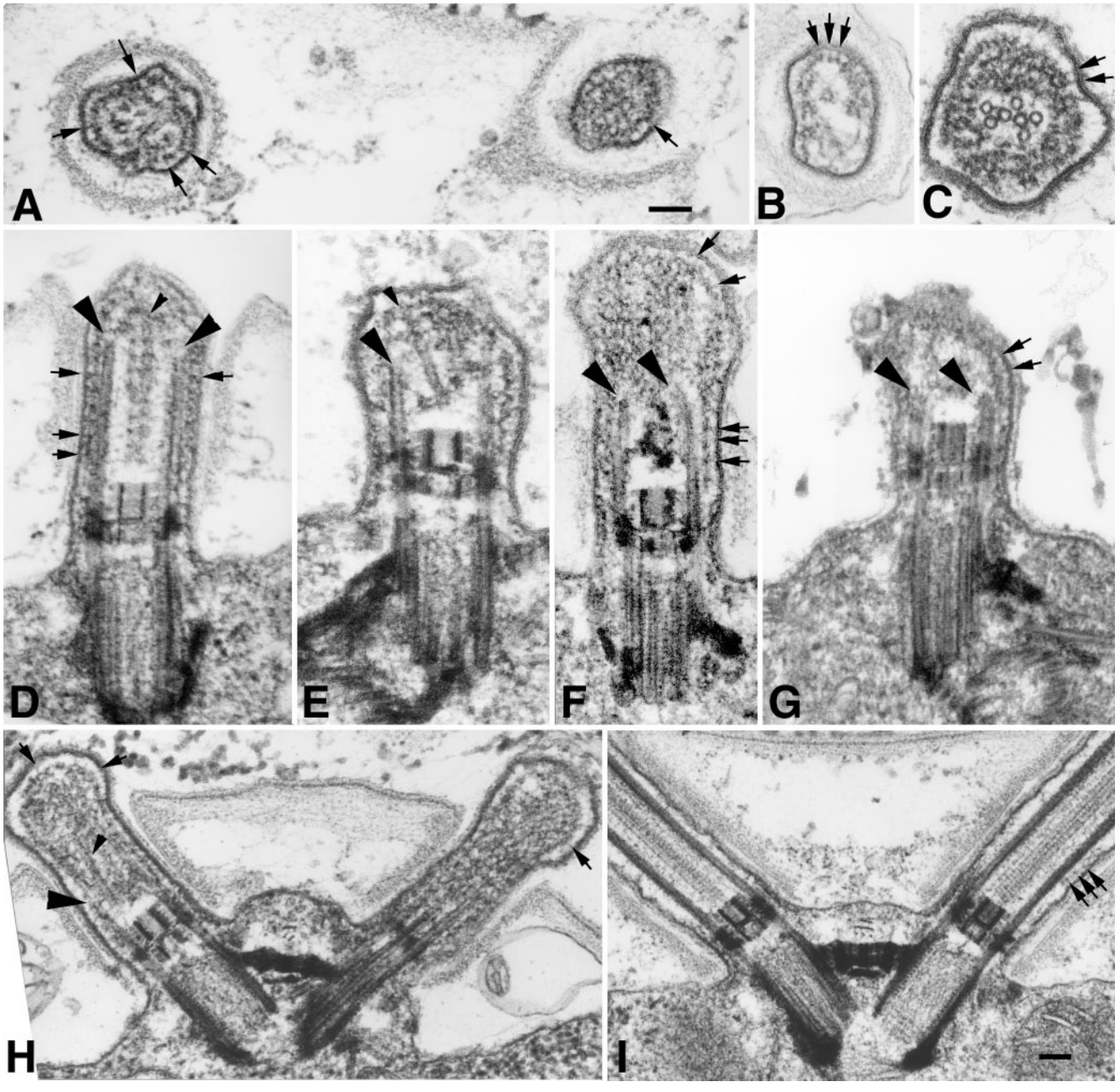

Figure 10. Electron microscopic analysis of stumpy flagellar mutants (A–H) and wild-type *Chlamydomonas* cells (I). Cross sections of flagella on a single *stf1* cell (A) and on individual cells (B and C) typically reveal few complete microtubules and no outer doublet microtubules. Compared with the wild-type flagella (I), the flagella on the *stf1* mutants rarely extend beyond the cell wall (D and H). The transition zones and basal bodies appear normal in the mutants, but the flagellar microtubules are short and often terminate as open-ended or filamentous structures (see large arrowheads). Occasionally, microtubules extend from a distal cap-like structure and terminate with the free ends just distal to the basal cup (D, E, and H, small arrowheads). Flagella are filled with electron dense material, and stalked bead structures line the flagellar membrane (small arrows). Bars, 0.1 μm.

defined capping structures were observed, but the distal ends of the central microtubules were embedded in an amorphous substance (Figures 10E and 11C), similar to the cap material seen in growing, wild-type flagella (Dentler, unpublished results).

The flagella of the *stf1* mutants were also filled with an electron dense, amorphous matrix that surrounded the microtubules (Figures 10 and 11). Numerous spherical particles with short stalks were found adjacent to flagellar membrane, and free particles of a

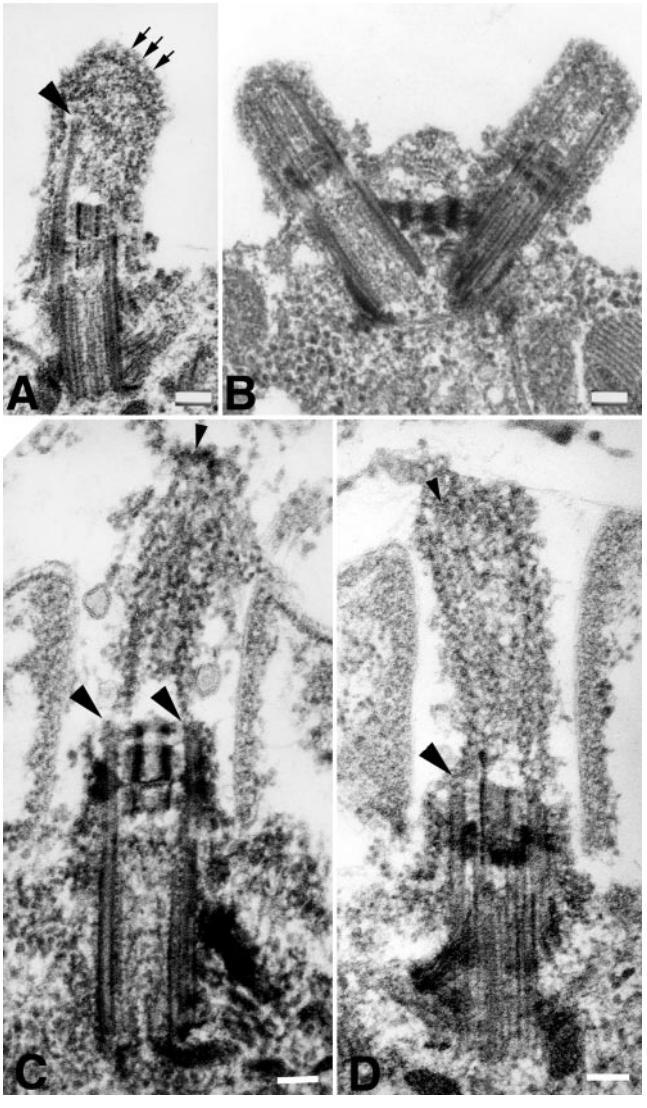

Figure 11. Electron microscopic analysis of detergent-extracted *stf1* mutants. Whole cells were extracted with 0.5% Nonidet P-40 for 5 min (A and B) or 2% Nonidet P-40 for 30 min (C and D) before fixation. Both the cell and flagellar membranes have been removed, but amorphous granular and filamentous material remain associated with the flagellar stumps. Filamentous structures extend from the ends of the flagellar or basal body microtubules (A, C, and D, large arrowheads). In some flagella, the filaments associated with the microtubules coalesce at the distal tip (A, small arrows). In others, microtubules with free proximal ends are occasionally found linked to a distal cap-like structure (C, small arrowhead). Bars, 0.1 μm.

similar size also filled the flagellar matrix (Figure 10, A–H, small arrows). In longitudinal sections, the particles often appeared in rows below the flagellar membrane (Figure 10, D–H), similar to the raft particles associated with IFT in wild-type flagella (see Figure 10I) (see Kozminski *et al.*, 1995).

To determine whether the amorphous granular material was directly associated with the microtubules or whether it was simply excess flagellar raft material that filled the matrix, we extracted cells with detergent before fixation (Figure 11). Initial extractions for 2–10 min with 0.5% Nonidet P-40 completely removed the flagellar membrane and most of the stalked bead structures but left a large amount of material still firmly attached to the flagellar stumps (Figure 11, A and B). Microtubules and amorphous material filled the matrix, and the flagella appeared nearly identical to that in control *stf1* cells whose membrane was intact. Organized arrays of raft particles were not observed, although some raft-like structures were visible (Figure 11A, arrows). Thus, the amorphous material seen in the *stf1* flagellar stumps is not simply soluble matrix protein.

Additional extraction of the cells with 2% Nonidet P-40 for 30 min removed more granular material and clearly revealed the filamentous material extending from the flagellar and basal body microtubules (Figure 11, C and D, large arrowheads). In some flagella, the filaments coalesced at the distal tips (see Figure 11C, small arrowhead), in association with cap-like material. Additional extraction of the cells with 2 mM Mg-ATP did not release either the matrix material or the filaments associated with microtubules (Dentler, unpublished results).

In addition to the flagella, the *stf1* mutants were analyzed for other microtubule-related structural defects. Examination of wild-type and mutant cells by thin-section transmission electron microscopy indicated no morphological differences in the organization of the Golgi apparatus (Dentler, unpublished results). Examination of cytoplasmic microtubule arrays using both conventional and confocal immunofluorescence microscopy revealed that the *stf1* mutants contained apparently normal arrays of basal body rootlet microtubules. However, the number of cytoplasmic microtubules was lower in the *stf1* mutants than in wild type, and in many mutant cells, the cytoplasmic microtubules were shorter than those in wild type (see Figure 12, A and B). In wild-type cells, microtubule arrays extending from the basal bodies were easily observed in all focal planes (Figure 12A), but in the *stf1* mutants, the microtubule arrays were only evident around the edges of the cells (Figure 12B). Staining wild-type cells (Figure 12C) with an antibody specific for the FLA10 subunit indicated that the kinesin II complex was present throughout the cell body but concentrated in the basal body region, consistent with previous reports (Vashishtha *et al.*, 1996; Cole *et al.*, 1998). Staining of *stf1* mutant cells with the same antibody revealed that the FLA10 kinesin II complex was more concentrated in the anterior region of the cell (Figure 12D).

DISCUSSION

Recovery of Additional Dhc *Sequences*

In this study, we report the recovery of four *Dhc* sequences in *Chlamydomonas* that are distinct from the

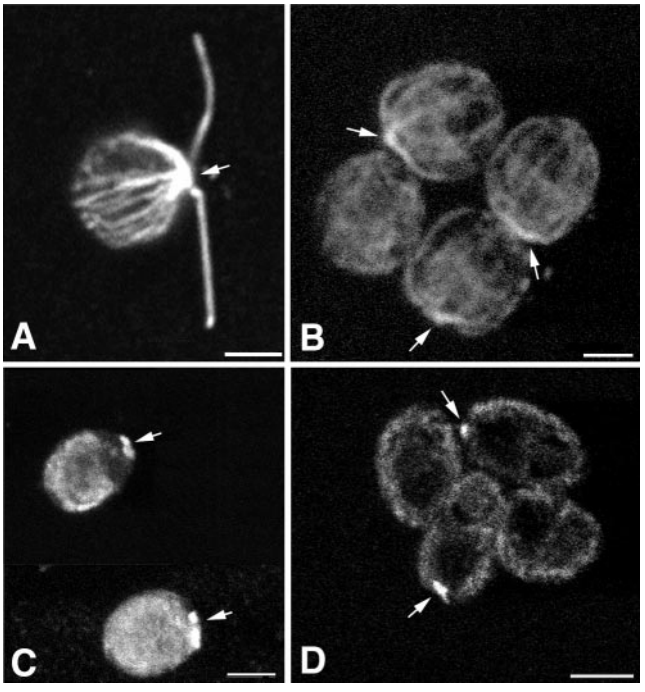

Figure 12. Immunofluorescence microscopy of wild-type (A and C) and *stf1* mutant (B and D) cells stained with monoclonal antibodies to β-tubulin (A and B) or one of the kinesin II subunits (C and D). Bars, 2 μm.

three outer arm *Dhc* genes (Mitchell and Brown, 1994, 1997; Wilkerson *et al.*, 1994) and the nine putative inner arm genes (*Dhc1-Dhc9)* identified previously (Porter *et al.*, 1996). Sequence comparisons indicate that two genes (*Dhc10* and *Dhc11*) are closely related to the axonemal *Dhc* sequences, and consistent with this hypothesis, the expression of both genes is enhanced by deflagellation (Knott and Porter, unpublished results). More recent work has demonstrated that *Dhc10* encodes an inner arm Dhc (Perrone and Porter, unpublished results). The two remaining sequences (*cDhc1a* and *cDhc1b*) are more similar to the cytoplasmic Dhc sequences identified in other organisms (Figures 1 and 2). Sequence data beyond the region represented by the PCR primers have shown that the *cDhc1a* gene also encodes a dynein sequence identified previously as pcr4 (Wilkerson *et al.*, 1994) (Bower and Porter, unpublished results; Witman, personal communication). Together with previous estimates based on Southern blot analyses (Porter *et al.*, 1996), these observations indicate that the *Chlamydomonas* genome contains ~16 different *Dhc* genes. The size of the *Dhc* gene family in *Chlamydomonas* is comparable with that found in other species such as sea urchin (14), *Paramecium* (12), *Drosophila* (>7), rat (13–15), mouse (11), and humans (>8) (Asai *et al.*, 1994; Gibbons *et al.*, 1994; Rasmusson *et al.*, 1994; Tanaka *et al.*, 1995; Andrews *et al.*, 1996; Vaisberg *et al.*, 1996; Vaughan *et al.*, 1996; Neesen *et al.*, 1997). The remarkable conservation of the *Dhc* gene family between such diverse organisms is consistent with the proposal that the *Dhc* gene family diverged into a small number of groups relatively early in the evolution of eucaryotes, but after these groups were established, they remained largely unchanged (Gibbons, 1995).

Alignment of the region encoding the ATP hydrolytic domain suggests that the *cDhc1a* sequence is the *Chlamydomonas* homologue of the major cytoplasmic dynein isoform (Figure 1). This isoform is the only *Dhc* sequence that has been identified thus far in both budding and fission yeast, the slime mold *Dictyostelium*, and filamentous fungi, where it plays important roles in the assembly and positioning of the mitotic spindle, nuclear migration, and vesicle transport (Koonce *et al.*, 1992; Koonce and Samso, 1996; Koonce and Knecht, 1998; Eschel *et al.*, 1993; Li *et al.*, 1993; Plamann *et al.*, 1994; Xiang *et al.*, 1994; Inoue *et al.*, 1998; Pollock *et al.*, 1998) (West and McIntosh, personal communication). Because of these observations, we would predict that the *cDhc1a* sequence might be involved in cell division and/or the positioning of basal body structures in *Chlamydomonas.* Although *cDhc1a*-related sequences are abundant in the testes of both *Drosophila* and vertebrates (Collins and Vallee, 1989; Rasmusson *et al.*, 1994; Criswell and Asai, 1998), our Northern blot analyses indicate that the *Chlamydomonas cDhc1a* sequence is a relatively low-abundance transcript whose expression is not dramatically altered by deflagellation (Figure 8), consistent with what has been observed during ciliogenesis in other organisms (Asai *et al.*, 1994; Gibbons, *et al.*, 1994; Kandl *et al.*, 1995; Andrews *et al.*, 1996). Whether the *cDhc1a* sequence plays a role in flagellar assembly remains to be determined. Recent studies on the associated 8-kDa dynein LC have demonstrated that this polypeptide is required for flagellar assembly and retrograde IFT (Pazour *et al.*, 1998), but no *cDhc1a* defects have thus far been detected in the present collection of the flagellar assembly mutants (Wysocki, Porter, and Dentler, unpublished results). Further insight into the functions of the *cDhc1a* sequence in *Chlamydomonas* will require both more information about its subcellular location and the identification and characterization of a specific *cDhc1a* mutation. We are obtaining N-terminal sequence for production of an isoform-specific antibody and screening additional insertional mutants with our *cDhc1a* clones to address these questions.

Alignment of the *Chlamydomonas* cDhc1b sequence with that of other cytoplasmic *Dhc* genes has identified homologues in sea urchin (Dyh1b), rat (DLP4), humans (Dhc2), mouse (Dhc11), and the worm *C. elegans* (Dhc1b) (Gibbons *et al.*, 1994; Wilson *et al.*, 1994; Tanaka *et al.*, 1995; Vaisberg *et al.*, 1996; Neesen *et al.*, 1997). These sequence similarities extend into the N-terminal region of the polypeptide (Figure 6). Be-

cause the N-terminal region is thought to be involved in the association of the Dhc with isoform-specific intermediate and light chains (Sakakibara *et al.*, 1993), these observations suggest that the cDhc1b-related sequences are likely to be assembled into similar multisubunit complexes, but nothing is yet known about the cDhc1b-associated subunits in any organism. Sucrose density gradient centrifugation of the dynein isoforms in rat testis indicates that the cDhc1b heavy chain does not cosediment with any of the intermediate chain or LC subunits typically found in association with the cDhc1a isoform, including the 8-kDa LC (Criswell and Asai, 1998) (Vaisberg, Grissom, and McIntosh, personal communication). Thus it is not clear how the flagellar assembly defects observed in the 8-kDa dynein LC mutants (Pazour *et al.*, 1998) may be related to those observed with the *cDhc1b* mutants. Additional work is clearly needed to characterize the components of the cDhc1b motor complex.

The cDhc1b Mutant Phenotype in Chlamydomonas

To test the possible role of *cDhc1b* in flagellar function in *Chlamydomonas*, we used gene-specific probes to place the *cDhc1b* gene on the genetic map and to screen collections of flagellar mutants generated by insertional mutagenesis. The *cDhc1b* gene maps near the reported position of the *FLA6* locus (Adams *et al.*, 1982) (Figure 8), but because the original *fla6* strain is no longer available, we were unable to determine directly whether *fla6* is a temperature-sensitive mutation in the *cDhc1b* gene. However, using Southern blot analyses, we have identified two flagellar assembly mutants associated with significant deletions in the *cDhc1b* gene (Figure 9). Although the size of the deletion varies between the two strains, in both cases, the region encoding the N-terminal portion of the Dhc has been disrupted, and the resulting mutant phenotype is the same. The basal body and transition zone structures are wild-type in appearance, but most of the microtubules distal to the basal bodies within the flagellar stumps are highly aberrant (Figure 10). Doublet microtubules were rarely found, and most flagella contained fewer than seven singlet microtubules. The singlet microtubules present were abnormally short and often ended in open sheets resembling the protofilaments seen at the ends of microtubules assembled in vitro. Detergent extraction of *stf1* mutants revealed filamentous structures, possibly incomplete microtubules, continuous with some of the basal body microtubules (Figure 11). The microtubule-capping structures normally observed in wild-type flagella (Dentler, 1980; Dentler and LeCluyse, 1982) were not found at the ends of the microtubules in the *stf1* flagella. However, amorphous material was observed at the distal ends of *stf1* microtubules (Figures 10 and 11), and this material is similar in appearance to that seen at the distal ends of microtubules in growing cilia that are shorter than 2 μm in length (Portman *et al.*, 1987).

The *stf1* mutant phenotype is quite distinct from the short flagellar (*shf*) mutants, which assemble outer doublet and central pair microtubules but fail to reach wild-type lengths (Jarvik and Chojnacki, 1985; Kuchka and Jarvik, 1987; Pazour *et al.*, 1998) (Dentler, unpublished results), but very similar to other stumpy flagellar mutants, which lack normal microtubule arrays (McVittie, 1972; Jarvik and Chojnacki, 1985). Whether the *stf1* mutants represent new alleles of the other stumpy flagellar mutant strains remains to be determined.

Another striking feature of the *stf1* mutant phenotype in *Chlamydomonas* is that the flagellar matrix is filled with an amorphous, electron dense material. Similar material has been described in other stumpy mutants (McVittie, 1972; Jarvik and Chojnacki, 1985), where it was presumed to represent unassembled flagellar protein. Small particles resembling the raft structures associated with IFT (Kozminski, *et al.*, 1993, 1995; Cole *et al.*, 1998) are also found in the *stf1* mutants (Figure 10). Although the biochemical composition of the matrix is largely unknown, its appearance resembles the material found in the LC8 mutant *fla14*, which includes some unassembled flagellar precursors and raft particle polypeptides (Cole *et al.*, 1998; Pazour *et al.*, 1998). However, on the basis of our morphological analysis of detergent-extracted cells, a significant amount of matrix material does remain associated with the extracted flagellar stumps (Figure 11). These observations suggest that the *stf1* flagella lack some component(s) critical for flagellar microtubule assembly or stability.

Comparison with cDhc1b Defects in Other Organisms

The phenotype of the *Chlamydomonas cDhc1b* mutants is also similar to the phenotype of several sensory cilia mutants in *C. elegans* (reviewed in Bargmann, 1993; Mori and Ohshima, 1997). These mutants fail to assemble the nonmotile cilia located at the distal end of their sensory neurons. Such structural defects alter the ability of the sensory neurons to monitor the local environment, leading to defects in such behaviors as chemotaxis (*che*) and osmotic avoidance (*osm*) (Bargmann, 1993; Starich *et al.*, 1995). One of these genes, *osm-3*, encodes a FLA10-related kinesin homologue (Shakir *et al.*, 1993; Tabish *et al.*, 1995), whereas two others, *osm-1* and *osm-6*, encode homologues of the raft particle polypeptides (Collet *et al.*, 1998; Cole *et al.*, 1998) (Stone and Shaw, personal communication). A fourth sensory cilium mutant, *che-3*, encodes the *C. elegans* homologue of the *cDhc1b* gene (Grant, personal communication), and its phenotype is particularly striking. In *che-3*, the sensory cilia are shortened, the

microtubule structures are highly aberrant, and the distal tips of the neurons become filled with an amorphous matrix material (Lewis and Hodgkin, 1977; Albert *et al.*, 1981; Perkins *et al.*, 1986). Analysis of several green fluorescent protein–labeled proteins in the *che-3* mutant neurons has revealed that the matrix material includes homologues of the raft particle polypeptides (OSM-6) as well as ciliary membrane receptors (ODR-10) that accumulate in the distal tips at levels significantly above those observed in wild-type neurons (Collet *et al.*, 1998; Dwyer *et al.*, 1998). The accumulation of material in the *che-3* mutant neurons differs from what has been observed in other sensory cilia mutants such as *osm-3* (Collet *et al.*, 1998). These results demonstrate that most of the components of the sensory cilia are transported from the cell body to the site of assembly in *che-3* neurons, but once there, they fail to be incorporated into a functional cilium.

The observation that *cDhc1b* mutations disrupt axoneme assembly in both *Chlamydomonas* flagella and *C. elegans* sensory cilia suggests that the cDhc1b isoform may participate in the formation of axoneme structures in a variety of tissues. These include the motile axonemes in sperm flagella and ciliated epithelia, as well as the nonmotile axonemes in vertebrate photoreceptors and inner ear kinocilia. An involvement of cDhc1b in axoneme assembly would be consistent with its high level of expression in respiratory epithelia and the testis (Tanaka *et al.*, 1995; Criswell *et al.*, 1996; Criswell and Asai, 1998; Neesen *et al.*, 1997). A minus-end–directed dynein motor would also complement the plus-end–directed activity of the FLA10-related kinesin II motors (Cole *et al.*, 1993; Kondo *et al.*, 1994; Walther *et al.*, 1994) that have been found in association with these structures (Kondo *et al.*, 1994; Yamazaki *et al.*, 1995; Beech *et al.*, 1996; Henson *et al.*, 1997).

Observations on *Dyh2* knockouts in *Tetrahymena* indicate that the cDhc1b homologue is not required for axoneme assembly in all cells (Lee *et al.*, 1999). *Tetrahymena Dyh2* mutant cells can regenerate motile cilia with apparently normal kinetics and without any visible defects in ciliary ultrastructure. However, if the cortical microtubule cytoskeleton is disrupted in the *Dyh2* mutants during deciliation, the basal body rows become disorganized, and cilia regenerate randomly over the cell surface (Lee *et al.*, 1999). These apparent discrepancies in the mutant phenotypes may reflect a difference in the dynamic behavior of cilia and flagella in the two organisms (see discussion in Lee *et al.* [1999]). *Tetrahymena* cilia are relatively stable organelles that do not shorten or elongate during the life cycle, whereas *Chlamydomonas* flagella assemble and disassemble with each cell cycle, as well as adjust their lengths in response to a variety of environmental conditions (Johnson and Porter, 1968; Lefebvre and Rosenbaum, 1986; Tuxhorn *et al.*, 1998). These differences indicate that it will be important to evaluate the function of the cDhc1b isoform in the context of several different cell types.

The phenotype of the *cDhc1b* mutations in *Chlamydomonas* is not strictly limited to a defect in flagellar assembly. Although we have observed no gross defects in cell size or shape, as might have been predicted from the phenotype of the *Dyh2* knockouts in *Tetrahymena* (Lee *et al.*, 1999), the cytoplasmic microtubule array does appear to be altered in the *stf1* mutants (Figure 12). As analyzed by immunofluorescence, both the number and length of the cytoplasmic microtubules appear to be reduced, although further work will be needed to quantify these defects. In addition, the FLA10 kinesin II complex appears to be more concentrated in the anterior region of the mutant cells as compared with wild type (Vashishtha *et al.*, 1996; Cole *et al.*, 1998), which may reflect an accumulation of the kinesin II in the flagellar stumps (Figure 12D). However, no significant changes in the appearance of the Golgi apparatus were observed in the *stf1* mutants, as might have been expected from studies in cultured mammalian cells in which Dhc2 colocalizes with markers for the Golgi apparatus and microinjection of a Dhc2 antibody leads to fragmentation of the Golgi (Vaisberg, *et al.*, 1996). Whether these discrepancies reflect a bona fide difference in the function of the Dhc2/cDhc1b homologues in these two cell types or simply the presence of multiple cDhc1b-like isoforms in vertebrates that perform specialized functions (Criswell and Asai, 1998) remains to be determined.

The Role of cDhc1b in Flagellar Assembly

Within the past few years, it has become clear that motor activities are essential for flagellar assembly. Flagellar assembly requires the delivery of flagellar precursors to the distal ends of the flagellar microtubules (Johnson and Rosenbaum, 1992; Piperno *et al.*, 1996), and plus-end–directed, kinesin II complexes seem to be essential for axonemal growth (Walther *et al.*, 1994; Kozminski *et al.*, 1995; Morris and Scholey, 1997; Nonaka *et al.*, 1998). What then would be the role of a minus-end–directed motor such as cytoplasmic dynein? We suggest three possibilities: 1) to transport flagellar precursors to the basal body region or flagellar base, 2) to recycle cargoes (rafts) carried to the flagellar tips by the FLA10 kinesin II, and/or 3) to carry signals from the flagellar tip so the cell can monitor flagellar length (see Figure 13).

In most ciliated cells, the basal bodies are associated with the minus ends of a cytoplasmic microtubule array that extends its plus ends into the cell body. Because microtubule-based transport appears to be essential for moving axonemal precursors within the flagellum (Kozminski *et al.*, 1995; Piperno *et al.*, 1996;

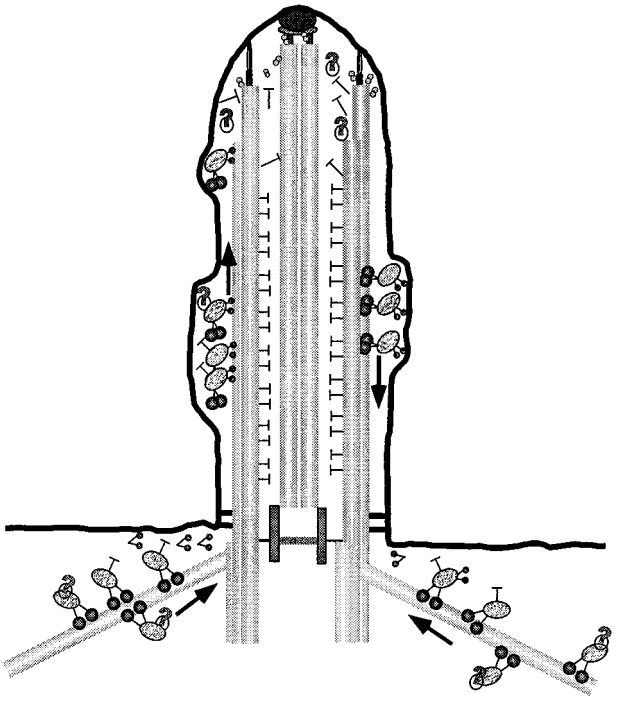

Figure 13. Model for cDhc1b activity in flagellar assembly and maintenance. Transport of IFT particles toward the flagellar tips (the microtubule plus ends) is mediated by FLA10 kinesin II motors (small, paired spheres). Cytoplasmic dynein (large, paired spheres) may be required for the minus-end–directed transport of flagellar components along cytoplasmic microtubules to the base of the flagellum. Alternatively, cytoplasmic dynein may be required to transport IFT particles from the flagellar tip to the flagellar base or for the recycling of IFT particles in the cytoplasm to pick up new components. Although the nature of material transported up the flagellum is unknown, possible components include radial spokes (T-shaped structures), unidentified complexes (large question marks), and/or signaling components involved in regulating flagellar length.

Morris and Scholey, 1997), it seems reasonable to propose that some of these components are also transported along cytoplasmic microtubules to the basal body region by a cytoplasmic dynein. The accumulation of material in the *stf1* mutant flagella indicates that not all flagellar components require dynein 1b–mediated transport to the basal body region, but the absence of normal flagellar microtubules also suggests that one or more components essential for flagellar assembly are not present in the mutant flagella. Cytoplasmic dynein 1b could therefore be involved in the delivery of some essential but as yet unidentified components from the cell body to the basal body region. Because microtubule caps are always present on doublet and central pair microtubules in full-length cilia and flagella (Dentler, 1990) and because the caps form as cilia and flagella grow beyond 2 μm, it is reasonable to suggest that part of the *stf1* defect might be the inability to assemble the microtubule caps. Whether this is due to a failure in the transport of cap components remains to be determined.

Cytoplasmic dynein 1b could also complement the activity of a plus-end–directed FLA10 kinesin II and serve as the retrograde motor for IFT. Disrupting the retrograde motor would lead to an excess of both the FLA10 kinesin II motor and associated raft components in the flagellar compartment, as was observed in *fla14* (Pazour *et al.*, 1998). The failure to recycle these components back to the cell body could eventually result in a "traffic jam" that blocks flagellar assembly. However, a raft-recycling defect may not completely explain the *stf1* mutant phenotype, because so few intact microtubules are observed in the *stf1* flagella. One might predict that a recycling defect would produce short flagella with normal axonemes, similar to that seen in the dynein LC mutant *fla14* (Pazour *et al.*, 1998). Still, the loss of a dynein heavy chain subunit may have a more drastic effect on retrograde IFT that results in the excess or deficiency of some component that affects microtubule stability.

If flagellar assembly requires the retrograde movement of some component or signal that allows the cell to monitor flagellar assembly or length, a defect in this signaling pathway might also led to a block in flagellar assembly (reviewed in Lefebvre and Rosenbaum, 1986; Johnson and Rosenbaum, 1993). Studies of the long flagella (*lf*) mutants in *Chlamydomonas* have revealed that the flagellar length control is a dynamic process requiring the interaction of several components (McVittie, 1972; Barsel *et al.*, 1987). Single *lf1*, *lf2*, or *lf3* mutants assemble flagella nearly twice the wild-type length, but double *lf* mutants or null mutants of the *LF* loci fail to assemble flagella (Barsel *et al.*, 1987) (Tam and Lefebvre, personal communication). Other experiments have shown that *Chlamydomonas* can adjust the lengths of its flagella in response to changes in its environment (Dentler and Adams, 1992; Tuxhorn *et al.*, 1998). Interestingly, the sequence analysis of raft polypeptide homologues has indicated that some of these proteins contain PxxP motifs that might interact with SH3 domains and could potentially be involved in signal transduction (Wick *et al.*, 1995; Collet *et al.*, 1998; Cole *et al.*, 1998). In addition, recent work has demonstrated that a G protein α subunit is involved in the specification of sensory cilia morphology in *C. elegans* (Roayaie *et al.*, 1998), and G protein subunits have also been detected in flagellar membrane preparations from the green alga *Gonium pectorale* (Haller and Fabry, 1998). How such signaling pathways may be related to the process of intraflagellar transport and dynein motor activity remains to be determined. To better understand the specific role of the cDhc1b motor, we need additional information on its subcellular location in the cell body and/or the flagellum. The identifica-

tion of the specific cargoes of the cDhc1b motor may also provide new insights into the mechanism by which the cDhc1b isoform contributes to the process of flagellar assembly.

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