Domains in the 1α Dynein Heavy Chain Required for Inner Arm Assembly and Flagellar Motility in *Chlamydomonas*

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**Abstract.** Flagellar motility is generated by the activity of multiple dynein motors, but the specific role of each dynein heavy chain (Dhc) is largely unknown, and the mechanism by which the different Dhcs are targeted to their unique locations is also poorly understood. We report here the complete nucleotide sequence of the *Chlamydomonas* Dhc1 gene and the corresponding deduced amino acid sequence of the 1α Dhc of the I1 inner dynein arm. The 1α Dhc is similar to other axonemal Dhcs, but two additional phosphate binding motifs (P-loops) have been identified in the NH2- and COOH-terminal regions. Because mutations in Dhc1 result in motility defects and loss of the I1 inner arm, a series of Dhc1 transgenes were used to rescue the mutant phenotypes. Motile cotransformants that express either full-length or truncated 1α Dhcs were recovered. The truncated 1α Dhc fragments lacked the dynein motor domain, but still assembled with the 1β Dhc and other I1 subunits into partially functional complexes at the correct axoneme location. A nalysis of the transformants has identified the site of the 1α motor domain in the I1 structure and further revealed the role of the 1α Dhc in flagellar motility and phototactic behavior.

**Key words:** motors • dynein • flagella • phototaxis • inner arm

**The movement of cilia and flagella is powered by axonemal dyneins, a family of mechanoenzymes that convert the energy derived from ATP binding and hydrolysis into the sliding of adjacent outer doublet microtubules (Mitchell, 1994; Witman et al., 1994; Porter, 1996). A xonemal dyneins can be separated into two groups, the outer dynein arms and the inner dynein arms, which have different functions in generating and propagating the flagellar waveforms. The outer dynein arms provide power to the flagellar beat, as *Chlamydomonas* mutants lacking the outer arms generate normal waveforms, but swim with a reduced beat frequency (Mitchell and Rosenbaum, 1985; Brokaw and Kamiya, 1987). In contrast, the inner arms are essential for normal motility, as mutants with inner arm defects have near normal beat frequencies, but display aberrant waveforms (Brokaw and Kamiya, 1987). In contrast, the inner arms are essential for normal motility, as mutants with inner arm defects have near normal beat frequencies, but display aberrant waveforms (Brokaw and Kamiya, 1987).

The *Chlamydomonas* outer arm is one of the most well-characterized dynein complexes; it is composed of three dynein heavy chains (Dhc) (α, β, and γ), two intermediate chains (IC), and several light chains (LCs) (<20 kD), and repeats every 24 nm along the length of the axoneme (Witman et al., 1994). Sequence comparisons between outer arm Dhcs indicate that they share greatest similarity in the central and COOH-terminal regions (Mitchell and Brown, 1994, 1997; Wilkerson et al., 1994; Gibbons, 1995). This portion of the Dhc is thought to form a globular head domain that interacts transiently with the microtubule doublet during the cross-bridge cycle. The more variable NH2-terminal third of the Dhc is thought to form a flexible stem domain that extends to the base of the dynein arm and interacts with the isoform-specific IC and LC subunits (Sakakibara et al., 1993). The ICs are involved in the assembly of the outer arm complex (Mitchell and Rosenbaum, 1986; Mitchell and Kang, 1991) and its attachment to the outer doublet microtubules in an ATP-insensitive manner (S.M. King et al., 1991, 1995). The multiple LCs are thought to be involved in the regulation of motility, but their specific functions are largely unknown (Harrison and King, 1999).

The inner dynein arms share an overall structural similarity to the outer arms but are significantly more complex.
in both composition and function. Ion exchange chromatography and SDS-PAGE procedures have identified at least eight distinct inner arm Dhc s that are associated with specific ICs and LCs into seven different molecular complexes: one two-headed isoform (I1) and six single-headed isoforms (I2 and I3) (Goodenough et al., 1987; Kagami and Kamiya, 1992). These isoforms are arranged in complex groups along the length of the axoneme (Piperno et al., 1990; Piperno and Ramanis, 1991; Muto et al., 1991; Mas astronarde et al., 1992; Gardner et al., 1994; S.J. King et al., 1994), but the relationship between these different isoforms and the multiple Dhc genes (Porter et al., 1996, 1999) is almost completely unknown.

Very little is also understood about the mechanism by which any Dhc is targeted to its specific location within the axoneme. In this report, we focus on the role of the I1 Dhc in the assembly and targeting of the inner arm isoform known as the I1 complex. The I1 dynein provides several advantages for the study of dynein targeting. First, it is a relatively simple complex composed of two Dhc s (I1 and I2), three ICs of 140, 138, and 110 kD (Piperno et al., 1990; Smith and Sale, 1991; Porter et al., 1992) and three LCs of 14, 12, and 8 kD (Harrison et al., 1998). Second, the I1 complex has a well-defined axoneme location, proximal to the first radial spoke in each 96-nm repeat along the length of the axoneme (Piperno et al., 1990; Mas astronarde et al., 1992; Porter et al., 1992; Myster et al., 1997). Third, the I1 complex is an important target for the regulation of flagellar motility (Porter et al., 1992). A iterations in the phosphorylation state of I138 have been associated with changes in microtubule sliding velocities and phototactic behavior (Hamerber and Sale, 1997; King and Dutcher, 1997). Finally, progress in the cloning and mapping of Dhc genes has identified two sequences that encode the I1 Dhc s (Porter et al., 1996; Myster et al., 1997; Perrone, C.A., R. Bower, S.H., Myster, J.A., Knoth, and M.E. Porter, unpublished results). One of these sequences, Dhc1, maps to the PF9/IDA1 locus and encodes the I1 Dhc; mutations in this locus disrupt the assembly of the I1 complex and thereby alter flagellar motility (Myster et al., 1997). The availability of such mutations permits a functional analysis of the Dhc1 gene product in vivo.

To characterize the Dhc domains involved in the assembly and targeting of the I1 complex, we sequenced the complete Dhc1 transcription unit (~22 kb) and generated specific constructs of the Dhc1 gene. The constructs were used in cotransformation experiments to rescue the pf9 defects. These results report the first full-length inner arm Dhc sequence to be described in any organism, and the first reported rescue of a Dhc mutation in Chlamy domonas. Our analysis of the Dhc1 transfectants has also identified a subset of strains expressing truncated Dhc1 transcripts. The truncated transcripts encode NH2-terminal fragments of the I1 Dhc polypeptide that are capable of coassembly with other components of the I1 complex and rebinding to the proper axoneme location. These results indicate that domains within the NH2-terminal ~143 kD of the I1 Dhc are involved in the specific subunit interactions required for the assembly and targeting of the I1 complex. EM analysis of isolated axonemes has identified the position of the I1 Dhc motor domain within the structure of the I1 complex. The assembly of truncated I1 Dhc s in the flagella of the transformants also resulted in a new motility phenotype that has revealed the contribution of the I1 Dhc motor domain to flagellar motility and phototactic behavior. These findings have important implications for the regulatory mechanisms that control the activity of the I1 dynein motor.

**Materials and Methods**

**Origin of Genomic Clones and Sequence Analysis of the Dhc1 Gene**

35 kb of genomic DNA in the region of the Dhc1 gene was recovered from a large insert, wild-type (21gr) Chlamydomonas library. The position of the Dhc1 transcription unit within this region was determined by probing Northern blots of wild-type RNA with selected subclones, and the Dhc1 transcription unit was thereby narrowed down to ~22 kb of genomic DNA (see Fig. 1; Myster et al., 1997). Sequence information was obtained from both strands of subclones A – D, and G using a series of nested dele-
tions (Erase-a-Base System; Promega Corp.) and Sequenase 2.0 (A Mer sham Life Science, Inc.) following the manufacturer’s instructions. Subclones E and F were sequenced by the DNA Sequencing Facility (Iowa State University) on an A B1 Prism sequencer (Perkin Elmer Corp.).

Potential open reading frames were identified using the GCG program CodonPreference and a codon usage table compiled from the coding regions of 73 different Chlamydomonas nuclear sequences (Nakamura et al., 1997, available at http://www.dna.affrc.go.jp/~nakamura/codon.html). Potential splice donor and acceptor sequences within the open reading frames were identified based on splice junction consensus sequences found in Chlamydomonas nuclear genes (Mitchell and Brown, 1994; Ledizet and Piperno, 1995; Zhang, 1996; R. Schnell, personal communication).

In five regions of the Dhc1 gene, the presence of multiple potential splice donor or acceptor sequences did not allow a confident prediction of the putative exons. In those cases, the splice junctions were determined directly by sequence analysis of reverse transcriptase-PCR (RT-PCR) products generated from the Dhc1 transcript (see Fig. 1). Total RNA was isolated from wild-type cells 45 min after deflagellation, and then 5 μg of total RNA was reverse transcribed using either a random primer or a sequence-specific reverse primer and the Superscript Preamplification System (GIBCO BRL) according to manufacturer’s instructions. 5 μl of the resulting 25-μl cDNA product was used in a 100-μl PCR reaction with sequence specific primers. PCR reactions were performed using 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 2 mM deoxynucleotide triphosphates, 0.2 mM of each primer, and 2.5 U Taq polymerase (Life Technologies, Inc.). Some reactions also contained 3% DMSO. The PCR reactions were first denatured at 94°C for 3 min, followed by 30 cycles of 58°C for 1 min, 72°C for 3 min, and 94°C for 1 min, and then completed with a final cycle of 58°C for 1 min and 72°C for 5 min. The final reaction products were analyzed on agarose gels, and then purified using Wizard PCR prep (Promega Corp.) for direct sequencing with sequence-specific primers.

The proposed translation start site was determined by the recovery of an RT-PCR product using a forward primer downstream of the TATA box sequence and a reverse primer in exon 3. The resulting RT-PCR product contained stop codons in all three frames immediately preceding the proposed start codon.

The predicted amino acid sequence encoded by the Dhc1 gene was analyzed using the GCG program Motifs. The programs BEDFIT, Compare, and Pileup were used to compare the I1 Dhc sequence to Chlamydomonas outer arm Dhc sequences α, β, and γ (Mitchell and Brown 1994, 1997; Wilkerson et al., 1994) and the cytoplasmic Dhc from Dictyostelium (Koonce et al., 1992). Regions with the potential to form α-helical coiled coils were identified using the program COILS, version 2.2 (Lupas et al., 1991; Lupas, 1996).

**Cosmid Library Screening and Construction of pD1SA**

To identify clones that might contain a full-length Dhc1 gene, we screened two different Chlamydomonas cosmids libraries (Purton and Rochaix, 1994; H. Zhang et al., 1994) that were generously provided by S. Purton.
(University College, London) and D. Weeks (University of Nebraska). Because the Purton library contains the ARG7 gene within the cloning vector, noncotransformed cells can be used to directly transform arg7 strains. 10^6 independent clones from each library were screened on M. agarghnon (Micron Separations, Inc.) nylon membrane filters in duplicate with probes from the 5' and 3' ends of the Dhc1 gene (see Fig. 5 A). Probes used for hybridization were purified in low melting point agarose (GIBCO BRL) and radiolabeled with [35S]CTP and random hexamer primers using the Primemix kit (Stratagene). Conditions for hybridization and hybridization were as described previously (Porter et al., 1996; 1999; Myster et al., 1997). A fter single colony isolation, cosmid DNA was purified using alkaline lysis procedures and CsCl gradient centrifugation (Sambrook et al., 1989).

A truncated version of the Dhc1 gene was constructed by fusing sequence from the 5' end to sequences from the 3' end. To recover the 5' end, a 19-kb SalI fragment was subcloned to form the plasmid pSM8 (see Fig. 5 D). PSM8 contains ∼1.7 kb of genomic DNA located 5' of the coding region, but ends in the middle of the Dhc1 transcription unit. pSM8 was digested with SalI and A scl to release the Dhc1 gene as an 11-kb fragment that is truncated before the region encoding the ATP hydrolytic site (Pf). The 3' end of the Dhc1 gene was subcloned as a 4.3-kb SalI, EcoRI fragment to form the plasmid p343E, which was digested with SalI and A scl to release the region 5' of the AscI site. The SalI-A scl fragment from pSM8 was ligated into the digested p145E subclone to form the construct pD15A (see Fig. 5 D). pD15A joins sequences from the 5' end of the Dhc1 gene to the 3' end at the AscI site. It is predicted to encode the first 1,956 amino acids of the Dhc1 polypeptide.

Recovery of Bacterial Artificial Chromosome (BAC) Clones Containing the Dhc1 Gene

A modified pBELO BAC library containing Chlamydomonas genomic DNA was screened with selected subclones to identify large insert BAC clones containing the Dhc1 gene. This library was constructed by N. Haas and P. Lefebvre (University of M. Innesota, St. Paul, M.N.) using genomic DNA from the cell-wall less strain cw92 and is currently available from Genome Systems, Inc. BAC DNA was isolated from positive clones using a modified version of the manufacturer's protocol available from C. A munders (University of M. Innesota, St. Paul, M.N.) at the following URL: http://biocis.cbs.edu/~a.munders/chlamy/methods/bac.html. The final pellet of BAC DNA was resuspended in 200 μl of TE and stored at −20°C. To identify clones containing full-length Dhc1 genes, 5 μl of BAC DNA was digested with the restriction enzymes ScaI and analyzed on Southern blots using subclones from the 5' and 3' ends of the Dhc1 gene.

Nucleic Acid Analysis

Large-scale preparations of genomic DNA were isolated from wild-type and mutant transformant strains using CsCl gradients as described in Porter et al. (1996). A smaller scale mini-prep procedure (Newman et al., 1990) was used to isolate DNA samples from tetrader progeny and some of the transformants. Restriction enzyme digests, agarose gels, isolation of total RNA, and Southern Northern blots were performed as previously described (Porter et al., 1996; Myster et al., 1997).

Cell Culture, Mutant Strains, and Cotransformation Experiments

The strains used in this study are listed in Table I. A ll cells were maintained as vegetatively growing cultures at 22°C as previously described (Myster et al., 1997). The arg2 (E. versile, 1956) strains were grown on rich medium that contained reduced ammonium nitrate (one tenth the normal concentration), but was supplemented with 0.6 mg/ml arginine. The pF9-2 arg2 strain (Porter et al., 1992) was crossed to the outer arm mutant pF28 using standard genetic techniques (Levine and Ebersold, 1980; Harris, 1989) to obtain the triple mutant pf9-2 pf28 arg2. The pF9-2 pF28 arg2 strain assemblies small, immotile flagella, and requires arginine for growth. A fter growth in tris-acetate phosphate (TAP) media supplemented with 0.6 mg/ml arginine, this strain was cotransformed using the glass bead method (K. indle, 1990; Nelson et al., 1994) with various constructs of the Dhc1 gene (2-4 μg) and the plasmid pRG7.8, which contains a wild-type copy of the ARG7 (argininosuccinate lyase) gene (De-buchy et al., 1988). Cotransformed cells, were washed and plated on TAP media lacking arginine to select for arg+ transformants. A fter 10 days of growth, single arg+ colonies were picked into liquid media and tested for rescue of the flagellar assembly and motility defects.

Analysis of Motility

Positive transformants were picked into 96-well plates and screened for motility on an inverted microscope (Olympus CK). Wells containing motile cells were streaked for single colonies. The IC140 antibody was probed for the motile region of the IC140 strain (A. dross, G. Zell, Inc.) using a 40x objective and a 10x eyepiece. The phenotypes of motile transformants were further analyzed by measuring forward swimming velocities and beat frequencies as previously described (Porter et al., 1992; 1994; Myster et al., 1997).

Transformants were tested for their ability to phototax using two different assays. In the first assay (King and Dutcher, 1997), actively swimming cells were put in a dark box with a 3-cm-wide horizontal slit cut out along the bottom such that only the lower portion of a 10-ml suspension was illuminated. The box was placed ∼3 cm from a fluorescent light source for 40 min. Positively phototactic cells would concentrate in the lower, illuminated portion of the tube, whereas phototaxis defective cells would remain uniformly suspended throughout the tube. In the second assay, motile cells were transferred to a 96-well plate and placed on a dissecting microscope with substrate illumination. Thick posterboard was placed under the plate and positioned to cover half of the well. The location of the cells within the well was followed over a 60-min time course. Positively phototactic cells would move quickly to the side of the well exposed to light, whereas cells unable to phototax would remain uniformly distributed throughout the well.

To verify that the rescued motility in the transformants was due to expression of the Dhc1 gene, and not a reversion event at the PF9 locus, the motile transformants were backcrossed to a pF28 allelic (oda2), which lacks the outer arms but is wild-type at the PF9 locus (Kamiya, 1988). Tetrad progeny were recovered following standard genetic methods (Harris, 1989), and their motility phenotypes were scored using a phase-contrast microscope as described above.

Axoneme Isolation, Dynemin Extracts, and Sucrose Gradients

A xonemes were prepared from large-scale (5-40 liters) liquid cultures of vegetative cells using procedures described by Witman (1986) and S.M. King et al. (1986), as modified by Gardner et al. (1994) and Myster et al. (1997). Crude dynemin extracts were obtained by brief (∼30 min) high salt extraction of whole axonemes (Porter et al., 1992; Myster et al., 1997). To isolate I complexes, crude dynemin extracts were fractionated by sucrose density gradient centrifugation as previously described (Porter et al., 1992; Myster et al., 1997). A liquts of each fraction were analyzed by SDS-PAGE and Western blotting.

SDS-PAGE and Immunoblot Analysis

Protein samples from whole axonemes and sucrose gradient fractions were separated on 5% polyacrylamide gels using the Laemmli (1970) buffer system and either stained directly with Coomassie brilliant blue (R 250, Sigma Chemical Co.) or transferred to either nitrocellulose (Schleicher and Schull, K. gene) or Immobilon-P (Millipore) in 25 mM Tris, 192 mM glycine, and 12.5% methanol at 800 mA for 90 min at 4°C using a General electrotrob (Idea Scientific Co., C). Nitrocellulose blots were blocked in 1× PBS (0.58 M Na2HPO4, 0.017 M NaH2PO4, H2O, 0.68 M NaCl), 5% normal goat serum (Sigma Chemical Co.), and 0.05% Tween 20 (polyethylenesorbitor monolaurate), whereas the Immobilon-P membrane was blocked in 0.2% I-Block (Tropica) in 1× PBS and 0.5% Tween 20.

Four different antibody preparations were used to probe the blots. The 1× Dhc1 antibody has been previously described in detail and is highly specific for the Dhc1 gene product (Myster et al., 1997). This antibody was raised against the peptide sequence DGTCTVE TPE Q R G AT D, which corresponds to amino acids 1,059-1,073 of the 1× Dhc1 polypeptide. The 1× Dhc1 antibody was affinity-purified on Western blots of dynin extracts, and then used at a 1:30 dilution. The IC140 antibody was probed with P. Yang and W. Sale (E. mory University, A. ianza, G. A.). This antiserum was raised against a fusion protein containing a fragment of the 140-kD intermediate chain of the I complex (Yang and Sale, 1998), and it was typically used at a dilution of 1:3,000. The rabbit polyclonal antibody R5205, which was raised against a fusion protein of the human 14-kD dynine LC (S.M. King et al., 1996), was provided by S. King (University of Connecti-
The R5205 antibody cross-reacts with the 14-kD LC (Tctex1) of the I1 complex (Harrison et al., 1998) and was used at a dilution of 1:50. After incubation overnight at 4°C, the blots were washed in 1× PBS and 0.05% Tween 20. Immunoreactivity was detected using an alkaline phosphatase–conjugated secondary antibody, BCIP (5-bromo-4-chloro-3-indolyl phosphate), and NBT (nitro blue tetrazolium) following the manufacturer’s instructions (Sigma Chemical Co.). An mAb to tubulin (T5168; Sigma Chemical Co.) was used at a dilution of 1:1,000, and then detected using an HRP-conjugated secondary antibody, 4-chloro-1-naphthol, and hydrogen peroxide following the manufacturer’s protocol (Sigma Chemical Co.).

Electron Microscopy and Image Analysis

To view the I1 complex in strains with rescued motility, selected transformants were crossed to a pf9-3 strain to recover strains with rescued I1 complexes and the wild-type complement of outer dynein arms. Axonemes were prepared and processed for EM as previously described (Porter et al., 1992; Myster et al., 1997). Longitudinal images were selected, digitized, and averaged using the methods described in Mastronarde et al. (1992). Averages of individual axonemes were obtained by analyzing at least six 96-nm radial spoke repeats, and then averages from several axonemes were combined to obtain a grand average for each strain. The methods used to compute differences between two strains are described in detail in Mastronarde et al. (1992).

Recruitment of Dhc1 Transgene from G3 After Transformation

To identify the 3′ end of the Dhc1 transgene in the G3 transformant (which assembles the shortest 1a Dhc fragment), genomic DNA was isolated from wild-type and G3, digested with the restriction enzymes SacI and KpnI, and analyzed on Southern blots probed with Dhc1 subclones. A polymorphic 7.2-kb SacI-KpnI fragment was identified in G3 using subclone C. This polymorphic fragment was recovered from G3 genomic DNA by constructing a size-selected minilibrary, and then screening the library with subclone C. After single colony purification, the 3′ end of the truncated Dhc1 transgene was sequenced with Dhc1 specific primers to determine the predicted amino acid sequence at the COOH terminus of the 1a Dhc fragment.

Results

Sequence Analysis of the Dhc1 Transcription Unit

In previous work, we identified a null mutation in the Dhc1 gene that resulted in the failure to assemble the I1 inner arm complex into the flagellar axoneme (Myster et al.,...
The predicted amino acid sequence of the encoded Dhc1 contributes to dynein complex formation, we have now sequenced the entire Dhc1 transcription unit (Fig. 1a). A map of the deduced gene structure is shown in Fig. 1b. Sequence analysis of subclone A identified the 3' end of the neighboring gene (geranyl geranyl pyrophosphate synthase, ~800 bp of intervening sequence, and a TATA box sequence 144 bp upstream of the proposed translation start site of the Dhc1 gene (see Materials and Methods). All but the 5' sequence elements required for regulated Dhc1 expression should therefore be contained within an ~1-kb region. The next 20 kb of the sequence contains the coding region located within 29 exons. The 3' end of the gene is located in subclone G, which contains the last exon encoding the COOH-terminal 552 amino acids, a stop codon, and a consensus polyadenylation signal sequence (TGTA A) 471 bp downstream.

The predicted amino acid sequence of the encoded 1α Dhc contains 4,625 amino acid residues and corresponds to a polypeptide of 522,806 D (Fig. 2). A search for potential nucleotide binding sites within the 1α Dhc sequence identified six consensus or near consensus phosphate-binding (P-loop) motifs with the sequence A/GXXX-X-GKT/S (Walker et al., 1982). Four of the P-loop motifs (P1–P4) are located within the central region of the Dhc, and both spacing and sequences of these P-loops are similar among all Dhc sequences reported thus far (reviewed in Gibbons, 1995; Porter, 1996). Two additional P-loop motifs were identified in the NH2-terminal (Pn) and COOH-terminal (Pc) regions of the 1α Dhc respectively; these appear to be unique to the 1α Dhc (Fig. 2).

The predicted amino acid sequence of the 1α Dhc was compared with 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) and the cytoplasmic Dhc from Didymostelium (Koons et al., 1992). In each case, a high degree of sequence similarity was apparent over long stretches of the polypeptide, especially in the central and COOH-terminal thirds of the Dhc (28–38% identity, 58–67% similarity). However, the more variable NH2-terminal third of the 1α Dhc also shares significant homology with the β and γ Dhc of the outer arm (Fig. 3, ~24% identity, ~56% similarity). A alignment of the Dhc sequences using the GCG program PILEUP confirmed that the presence of conserved domains within the NH2-terminal region, but also revealed several short stretches of unique peptide sequence in the 1α Dhc, including the region previously used to generate a monospecific 1α Dhc antibody (Myster et al., 1997).

The 1α Dhc sequence 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) (Fig. 4). One region located before P-loop 1 (residues 1,227–1,409) and a second after the P-loop 4 (residues 3,192–3,297, 3,400–3,494, and 3,701–3,789) show the highest probability of forming α-helical coiled coils. The presence of limited coiled-coil domains separating the central portion of the Dhc from the NH2-terminal and COOH-terminal regions has been observed in other Dhc sequences (Mitchell and Brown, 1994, 1997; Porter, 1996). These conserved structural domains are thought to play an important role in protein interactions within the dynein arms.

Isolation of Dhc1 Transgenes

To better understand how the specific domains of the 1α Dhc polypeptide might be involved in the assembly and activity of the inner arm dynein, we decided to analyze constructs of the Dhc1 gene in vivo in a pfp9 mutant background. Because of the large size of the Dhc1 gene (~21 kb), two cosmids libraries and one BAC library were screened with probes representing the 5' and 3' ends of the Dhc1 gene to improve the chances of recovering clones that contain the full-length gene (Fig. 5A). The first cosmid library yielded a single clone, cW1, which was positive with both probes, and further analysis proved to be lacking a small portion at the 3' end of the gene (Fig. 5B). Screening the second cosmid library resulted in the recovery of a single clone, cW1, which contained the complete Dhc1 gene. To understand how the Dhc1 gene product might contribute to dynein complex formation, we have now sequenced the entire Dhc1 transcription unit (Fig. 1a). A map of the deduced gene structure is shown in Fig. 1b. Sequence analysis of subclone A identified the 3' end of the neighboring gene (geranyl geranyl pyrophosphate synthase), ~800 bp of intervening sequence, and a TATA box sequence 144 bp upstream of the proposed translation start site of the Dhc1 gene (see Materials and Methods). All of the 5' sequence elements required for regulated Dhc1 expression should therefore be contained within an ~1-kb region. The next 20 kb of the sequence contains the coding region located within 29 exons. The 3' end of the gene is located in subclone G, which contains the last exon encoding the COOH-terminal 552 amino acids, a stop codon, and a consensus polyadenylation signal sequence (TGTA A) 471 bp downstream.

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Isolation of Dhc1 Transgenes

To better understand how the specific domains of the 1α Dhc polypeptide might be involved in the assembly and activity of the inner arm dynein, we decided to analyze constructs of the Dhc1 gene in vivo in a pfp9 mutant background. Because of the large size of the Dhc1 gene (~21 kb), two cosmids libraries and one BAC library were screened with probes representing the 5' and 3' ends of the Dhc1 gene to improve the chances of recovering clones that contain the full-length gene (Fig. 5A). The first cosmid library yielded a single clone, cW1, which was positive with both probes, and further analysis proved to be lacking a small portion at the 3' end of the gene (Fig. 5B). Screening the second cosmid library resulted in the recovery of a single clone, cW1, which contained the complete Dhc1 gene structure. (a) Partial restriction map of the Dhc1 gene. The black box labeled P1 represents the 227 bp fragment of the Dhc1 gene that was recovered in the first PCR screen for Dhc genes in Chlamydomonas (Porter et al., 1996). This sequence was used to screen a large insert genomic library and recover ~35 kb of genomic DNA surrounding the region encoding the hydrolytic ATP binding site (P1) (Porter et al., 1996; Myster et al., 1997). Indicated below are the approximate positions of selected SacI subclones (A–G) used as probes and other subclones (pSM8 and p145E) that were used to construct a truncated Dhc1 gene. (b) Intron/exon structure of the Dhc1 transcription unit. The approximate size and position of the 29 exons (open boxes) and 28 introns (intervening lines) predicted from the analysis of the Dhc1 nucleotide sequence are shown. A iso indicated are other features such as the TATA box, the regions encoding predicted P-loops, and a predicted polyadenylation signal at the 3' end of the gene. Bracketed regions numbered 1–5 indicate the position of the primers used for RT-PCR.
transcription unit as well as additional genomic sequences both 5' and 3' that might be required for proper expression in vivo (Fig. 5 C). Four larger clones (100–135 kb) containing the Dhc1 transcription unit were recovered from the BAC library; two of these clones were used in subsequent cotransformation experiments (Fig. 5 E). We also constructed a truncated version of the Dhc1 transgene known as pD1SA by fusing an 11-kb region encoding the NH2-terminal 1,956 amino acids to a 1-kb region containing the 3' end of the gene (Fig. 5 D). All of the Dhc1 transgenes were tested for their ability to rescue the pf9 mutant defects in vivo.

Rescue of pf9 Motility Defects by Transformation with Constructs of the Dhc1 Gene

Mutations at the PF9/IDA1 locus typically result in strains that have a slow, smooth swimming behavior (Kamiya et al., 1991; Porter et al., 1992). To increase the sensitivity of the screen for rescue of the pf9 mutant phenotype, we intro-
duced a second motility mutation into the pf9-2 background. pf28 is a mutation in the γDhc gene that results in the failure to assemble the outer dynein arms (Mitchell and Rosenbaum, 1985; Wilkerson et al., 1994). Cells carrying the pf28 mutation swim with a jerky phenotype that can be easily distinguished from the slow, smooth swimming behavior of the pf9 mutant cells. In addition, pf9-2 pf28 double mutants assemble short, paralyzed flagella and sink in liquid medium (Porter et al., 1992). This short, paralyzed flagellar phenotype makes it very straightforward to identify transformants that have rescued the pf9 mutant defects by screening for cells that assemble full-length, motile flagella and swim with a pf28-like motility phenotype.

The pf9-2 pf28 arg2 strain was first cotransformed with the selectable marker pARG7.8 and the cW1 cosmid containing the complete Dhc1 transcription unit. Positive transformants were selected by growth on solid medium lacking arginine, and then single colonies were picked into liquid media and screened for motility. Fig. 5 C illustrates the combined results of several independent cotransformation experiments with the cW1 cosmid. Although arg1 transformants were recovered at expected frequencies (Kindle, 1990), only 11 out of 2,880 transformants screened displayed any motility, for a frequency of rescue of 0.4%.

Moreover, the motility of the cW1 rescued strains was not the same as pf28 (see below and Table II).

Previous study of an outer arm mutation, oda4-s7, had indicated that the NH2-terminal third of the βDhc polypeptide is sufficient for assembly of a dynein complex (Sakakibara et al., 1993). To test if this is also true for the 1aDhc, we cotransformed the pf9 pf28 mutant with the smaller pD1SA construct, which encodes z40% of the 1aDhc sequence. These experiments yielded seven motile strains (Fig. 5 D), which represented only a modest (0.87%) increase in the frequency of rescue, but these rescues confirmed that truncated Dhc1 transgenes could restore partial motility.

To see if it was possible to completely rescue the motility defects, we also transformed the pf9 pf28 mutant with two BAC clones that contained the full-length Dhc1 gene located in the middle of z100–135-kb genomic inserts (Fig. 5 E). The frequency of rescue (~0.1%) was still quite low, but the motility phenotypes of the rescued strains were very similar to pf28 (see below).

The recovery of motile isolates after cotransformation could also be due to an intragenic reversion event at the PF9 locus during the course of transformation and/or se-
Figure 4. Secondary structure of the 1α Dhc. Shown here is a graphical representation of the regions of the 1α Dhc predicted to form α-helical coiled coils, as determined by the program COILS version 2.2 (Lupus et al., 1991; Lupus, 1996). The six regions of the sequence predicted to encode P-loops are identified by arrows. The position of the peptide sequence (amino acids 1,059-1,073) used to generate an isoform specific antibody (Myster et al., 1997) is also indicated.

Motility Phenotypes of the Dhc1 Transformants

Although the frequency of rescue was low, it was clear that the rescued motility was due to the presence of the different Dhc1 transgenes, and so the motility phenotypes of the Dhc1 transformants were analyzed in greater detail. More specifically, we measured the flagellar beat frequency, the forward swimming velocity, and the ability to phototax (Table II). Transformants with complete rescue of the pf9 mutation would be expected to have a swimming phenotype nearly identical to that of pf28. The flagellar beat frequencies of the Dhc1 transformants were almost identical to the beat frequency of pf28, but measurements of forward swimming velocities clearly indicated that most of the transformants swam more slowly than pf28 (Table II). In particular, the swimming velocities of the rescued strains obtained by transformation with the cosmid clones and pD15A were slower than those obtained by transformation with the BAC clones. These results suggested that there were still some inner arm defects in most of the Dhc1 transformants.

We next tested if the Dhc1 transformants had recovered the ability to phototax. King and Dutcher (1997) have previously used a photoaccumulation assay to demonstrate that pf9 mutant cells do not phototax effectively. Using similar conditions, we have found that pf28 cells, which lack outer arms but have the full complement of inner dynein arms, are able to phototax, as assayed by their tendency to become concentrated in the illuminated portion of a tube within 40 min of exposure to a directional light source (Table II). However, all of the Dhc1 transformants obtained with the cosmid clones remained equally distributed between the illuminated and darkened regions of the tube. To confirm these findings by direct observation of individual cells, the ability to phototax was also monitored in 96-well plates over a 60-min time course (see Materials and Methods). In the absence of outer arms, the Dhc1 transformants obtained with the cosmid clones remained

| Strain name | Dhc1 construct | Swimming velocity | Beat frequency | Ability to phototax |
|-------------|----------------|------------------|---------------|-------------------|
| pf28        | N.A.           | 51.5 ± 6.9       | 21.8 ± 1.1    | +                 |
| C1          | cW1            | 37.8 ± 9.1       | 18.8 ± 0.9    | -                 |
| E2          | cW1            | 36.1 ± 5.5       | 18.7 ± 1.7    | -                 |
| F2          | cW1            | 42.4 ± 8.7       | 19.3 ± 0.7    | -                 |
| F3          | cW1            | 35.2 ± 5.4       | 18.9 ± 1.6    | -                 |
| F4          | cW1            | 36.5 ± 6.1       | 21.2 ± 1.4    | -                 |
| F5          | cW1            | 37.5 ± 7.0       | 20.2 ± 1.9    | -                 |
| F7          | cW1            | 37.0 ± 8.2       | 19.5 ± 1.1    | -                 |
| G3          | cW1            | 34.9 ± 5.7       | 21.0 ± 2.3    | -                 |
| G4          | cW1            | 41.2 ± 5.6       | 22.3 ± 2.1    | -                 |
| G9          | cW1            | 41.2 ± 9.9       | 20.3 ± 1.3    | -                 |
| G11         | cW1            | 41.1 ± 8.4       | 19.5 ± 1.5    | -                 |
| H10         | pD15A          | 40.9 ± 9.2       | 19.7 ± 0.9    | -                 |
| A2          | cA1            | 29.6 ± 4.1       | 15.9 ± 1.3    | -                 |
| 2A-1B       | N24-1          | 45.6 ± 7.7       | N.D.          | +                 |
| 2B-4E       | N24-1          | 46.4 ± 5.8       | N.D.          | +                 |
| 4B-C2       | J1-5           | 44.6 ± 6.0       | N.D.          | +                 |
| 4B-G2       | J1-5           | 45.4 ± 6.9       | N.D.          | +                 |
| 5B-G10      | J1-5           | 44.8 ± 4.1       | N.D.          | +                 |

N.A., not applicable; ND, not determined.

Table II. Motility Phenotypes of Dhc1 Transformants
nomic DNA both upstream and downstream of the cross to presence of outer arms, two strains, G4 and E2, were effectively as wild type. These results suggest that the outer arms, the two strains could photoaccumulate as ef-
fectively as wild type. These results suggest that the outer arms can compensate in some way for the phototaxis de-
efects in the well within 15 min. These results indicated that this group of Dhc1 transformants does not phototax as effect-
ively as pf28 control cells.

To examine the motility of the transformants in the presence of outer arms, two strains, G4 and E2, were crossed to pf9-3 and tetrad products containing the Dhc1 transgene in a wild-type outer arm background (G4+ OA and E2+ OA) were recovered. The two strains have beat frequencies almost identical to wild type, but their swim-
motility velocities are intermediate in speed between pf9 and wild type (Table II). Moreover, in the presence of the outer arms, the two strains could photoaccumulate as effec-
tively as wild type. These results suggest that the outer arms can compensate in some way for the phototaxis de-
efects in the Dhc1 transformants.

Truncated 1α Dhc1 Transformants

The swimming behavior of the Dhc1 transformants obtained with the cosmid clones demonstrated that the intro-
duction of these Dhc1 clones resulted in only a partial res-
cue of the pf9 motility defects. Given the large size of the Dhc1 transcription unit (~22 kb), we were initially con-
cerned that these transgenes might not be expressing wild-
type levels of the Dhc1 gene product. To address this ques-
tion, we isolated axonemes from the Dhc1 transformants and analyzed the components of the I1 complex. Previous work has shown that the I1 complex is composed of eight polypeptides, two Dhcs (1α and 1β), three ICs (IC140, IC138, and IC110) (Smith and Sale, 1991; Porter et al., 1992; Myster et al., 1997), and three LCs (LC8, LC12, and LC14) (Harrison et al., 1998). The Dhc1 gene encodes the 1α Dhc, which can be identified on Western blots using an-
tibody directed against a peptide epitope in the NH2-ter-

equally distributed in both the illuminated and darkened portions of the microtiter well. Conversely, the majority of pf28 cells became concentrated on the illuminated side of the well within 15 min. These results indicated that this group of Dhc1 transformants does not phototax as effect-
ively as pf28 control cells.

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tibody directed against a peptide epitope in the NH2-ter-
minal region (Myster et al., 1997). Fig. 6 A shows three Coomassie blue–stained polyacrylamide gels containing whole axonemes isolated from several mutant strains: the 11 motile Dhc1 transformants obtained with the cW1 cosmid, the 4 motile Dhc1 transformants recovered with the cA1 construct, and the 5 transformants recovered with the Dhc1 BAC clones. Fig. 6 B shows the corresponding Western blots probed with the 1α Dhc antibody. The 1α Dhc antibody identified the z520 kD 1α Dhc in the pf28 control sample, but polypeptides significantly smaller than the 1α Dhc were identified in all of the motile strains obtained by transformation with the Dhc1 cosmids. In contrast, all of the axoneme samples prepared from rescued strains obtained by transformation with the Dhc1 BAC clones contained full-length 1α Dhc polypeptides. These results indicated that the partial rescue phenotype seen with the Dhc1 cosmid clones was not due to low levels of expression of a full-length 1α Dhc, but instead due to the expression of truncated 1α Dhcs, ranging in size from z165 to z300 kD.

To determine if other I1 subunits were associated with the truncated 1α Dhc, Western blots of isolated axonemes from Dhc1 transformants, split into triplicate for separation on 5% polyacrylamide gels, and then either stained with Coomassie blue or transferred to membranes and incubated with the affinity-purified 1α Dhc antisera or the IC140 antisera. (A, left panel) A 5% polyacrylamide gel loaded with 20-μg whole axonemes from pf28, pf9 pf28, and the 11 motile Dhc1 transformants generated by transformation with the cW1 cosmid. The additional bands in the pf9 pf28 sample are most likely contaminating flagellar membrane proteins. (Middle panel) A 5% gel loaded with whole axonemes from pf28, pf9-3, and the four rescued strains generated by transformation with the cA1 cosmids. (Right panel) A 5% gel loaded with whole axonemes from pf28 and the five rescued strains obtained with the BAC clones. (B) Duplicate immunoblots probed with the affinity-purified 1α Dhc antibody. (C) Duplicate immunoblots probed with the IC140 antisera. Control blots probed with tubulin antibodies confirmed that roughly equivalent amounts of flagellar protein were loaded in each lane (data not shown).

Assembly of I1 Complexes in Dhc1 Transformants

To confirm that the other polypeptide subunits were assembled into an I1 complex, we isolated whole axonemes from large-scale cultures of two Dhc1 transformants, E2 and G4, as well as from control pf28 cells. Partially purified I1 complexes were obtained by high salt extraction of the isolated axonemes followed by sucrose density gradient centrifugation. The resulting fractions were analyzed by both SDS-PAGE and Western blotting. Fig. 7 A shows the 19S region of a sucrose gradient that was loaded with the Dhc1 BAC clones contained full-length 1α Dhc polypeptides. These results indicated that the partial rescue phenotype seen with the Dhc1 cosmids was not due to low levels of expression of a full-length 1α Dhc, but instead due to the expression of truncated 1α Dhcs, ranging in size from ~165 to ~300 kD.

To determine if other I1 subunits were associated with the truncated 1α Dhc, Western blots of isolated axonemes were probed with an antiserum raised against the 140-kD intermediate chain (Yang and Sale, 1998). This antibody detects the IC140 in wild-type axonemes, but not in I1 mutant axonemes. As shown in Fig. 6 C, the IC140 antibody recognized a single polypeptide of ~140 kD in pf28 and each rescued transformant, but did not detect the IC140 in any of the pf9 mutant strains. Similar results were seen using the antibody directed against the 14-kD Tctex1 light chain (data not shown).
with the 1α Dhc antibody identified this novel band as the truncated 1α Dhc (Fig. 7 B, blot). Identical results were observed with dynein extracts isolated from the E2 strain (data not shown). The truncated 1α Dhc in the Dhc1 transformants therefore form stable complexes with the other polypeptides of the I1 complex, but the resulting mutant complexes sediment more slowly than wild-type complexes.

Structural Analysis of Axonemes from Dhc1 Transformants Reveals Defects in the I1 Complex

To analyze the structure of the I1 complex in the Dhc1 transformants, we prepared purified axonemes from wild-type and mutant strains for thin section EM. To facilitate the analysis of the images, the transformants G4 and E2 were crossed to a pf9-3 strain to recover the Dhc1 transgene in a wild-type outer arm background (G4+OA and E2+OA, see Materials and Methods). Fig. 8 a shows the grand average of the 96-nm repeat from wild-type axonemes, which lacks lobe 2 of the I1 structure.

Previous work has shown that the inner dynein arms repeat as a complex group of structures every 96 nm in register with the radial spokes (Muto et al., 1991; Mastroberardino et al., 1992). The I1 complex is a trilobed structure located proximal to the first radial spoke (S1) in each 96-nm repeat (Fig. 8 b, lobes 1–3) (Piperno et al., 1990; Mastroberardino et al., 1992; Myster et al., 1997). These three lobes are missing in pf9-3 axonemes (Fig. 8, c and d), which lack the I1 complex (Myster et al., 1997). Fig. 8 e and g, show the grand averages of the axonemes from the G4+OA and E2+OA strains, which contain I1 complexes with truncated 1α Dhcs. Lobes 1 and 3 of the I1 complex are present in the axonemes from these samples, but lobe 2 is still missing. Difference plots between these images and wild-type confirms that the loss of lobe 2 is the only significant defect in the two Dhc1 transformants (Fig. 8, f and h).

These images demonstrate that the I1 complex is assembled and targeted to the appropriate axoneme location. In addition, these images suggest that the region of the 1α Dhc that is missing in the Dhc1 transformants corresponds to lobe 2 of the I1 structure.

The Dhc1 Transcripts in the Cosmid Transformants Lack the 3’ End of the Gene

Although the initial cotransformation experiments involved the use of a full-length or near full-length Dhc1 cosmid clones, all of the motile transformants recovered with these clones assemble partially functional I1 complexes with truncated 1α Dhcs (Fig. 6 and Table II). To understand how the 1α Dhc fragments were related to the Dhc1 sequence, we analyzed the Dhc1 transcripts from several of the rescued transformants on Northern blots. Total RNA was first isolated from the G4+OA and E2+OA strains, which contain the Dhc1 transgene in the pf9-3 null mutant background. This background facilitated our analysis because the pf9-3 mutation is a large deletion (~13 kb) in the Dhc1 gene and does not generate an endogenous Dhc1 transcript (Myster et al., 1997).

Fig. 9 A shows a partial restriction enzyme map of the Dhc1 gene and the subclones that were used as probes to analyze the Dhc1 transcripts. A s shown in Fig. 9 B, probe A 3’, which spans the Dhc1 transcription start site, identified a single, large (>13 kb) transcript in wild-type RNA. However, in G4+OA and E2+OA strains, the transcripts recognized by the A 3’ probe were significantly smaller than the wild-type Dhc1 transcript, but these smaller transcripts were still upregulated in response to deflagellation (compare lanes 0 and 45). Identical results were observed with the next two subclones, probes B and C. Probe D, which includes the conserved region encoding the primary ATP hydrolytic site, hybridized to the truncated transcripts in E2+OA, but it did not recognize the truncated transcripts in G4+OA. Probes E–G did not hybridize with any transcripts in the transformants (Fig. 9 B, right panel, and data...
The Dhc1 transcripts in G4 and E2 are therefore truncated from the 3' end of the Dhc1 gene, and G4 is truncated before E2.

To characterize the Dhc1 transcripts present in other transformants, RNA was isolated from three additional strains: G3, G9, and A2. G3 and G9 are the cW1 transformants that assemble the smallest and largest 1α Dhc fragments respectively, whereas A2 is a cA1 transformant that

G4+OA and E2+OA. Probes B and C gave similar results. Probe D (middle panel) hybridized to the truncated Dhc1 transcript in E2+OA, but not in G4+OA. (Probe D, which corresponds to the most highly conserved region of the Dhc1 gene, also cross-hybridized weakly with other Dhc transcripts in these samples.) Probe G (right panel) did not detect any transcripts in either G4+OA or E2+OA, and similar results were seen with probes E and F. (C) Northern blots of total RNA from wild-type, G3, A2, and G9 isolated 45 min (45) after deflagellation. Each probe hybridized to a single transcript in wild-type (wt). Probe A3' (left panel) also hybridized to truncated Dhc1 transcripts that are upregulated in response to deflagellation in the pf9-2 background of the transformants (Myster et al., 1997). Probe C (left panel) hybridized to the truncated Dhc1 transcripts in G3, A2, and G9, but probes D-F (middle panel) did not.
assembles the largest (>300 kD) 1α Dhc fragment obtained thus far (Fig. 6). As shown in Fig. 9 C, in each strain, probe C hybridized to a truncated transcript that is significantly smaller than the endogenous Dhc1 transcript derived from the pf9-2 mutant background. However, probe D, which corresponds to the region encoding the ATP hydrolytic site, failed to hybridize with the truncated transcripts present in the G3, A2, and G9 samples. Similar results were seen with probes E and F (data not shown). Therefore, all three strains encode 1α Dhc fragments that are truncated before the proposed motor domain.

**Recovery of a Modified Transgene from a Dhc1 Transformant**

To identify the sites where the Dhc1 cosmid clones were being modified during transformation, we isolated genomic DNA from the transformants and analyzed the structure of the integrated Dhc1 transgenes on Southern blots. Fig. 10 A shows a blot of SacI digested genomic DNA that was hybridized with a probe for subclone C. As expected, this probe hybridized to the endogenous Dhc1 gene present in the pf9-2 mutant background of the transformants. However, for each transformant, a second polymorphic band could be detected using either probe C (Fig 10 A) or probe D (data not shown). From these and other blots, we concluded that the Dhc1 cosmids were being rearranged during integration into genomic DNA. In addition, the region of the Dhc1 gene encoding the conserved motor domain appeared to be the most common target for disruption during these integration events.

Because the G3 transformant assembles the smallest 1α Dhc fragment identified thus far (Fig. 6), we recovered the modified Dhc1 transgene using probe C to screen a mini-library made from genomic DNA of the G3 transformant (see Materials and Methods). The Dhc1 transgene was then sequenced with Dhc1 specific primers to identify the junction between the Dhc1 sequence and the site of integration in G3 genomic DNA. The Dhc1 sequence in G3 is fused to an unidentified DNA sequence, and the resulting hybrid gene is predicted to encode up to amino acid residue 1,249 of the 1α Dhc, followed by the addition of 17 novel amino acids before encountering a stop codon (Fig. 10 B). Sequence analysis of an RT-PCR product derived from G3 RNA has confirmed the presence of this hybrid transcript. The polypeptide encoded by the modified transgene would, therefore, correspond to a 1α Dhc fragment of ~143 kD that is truncated just COOH-terminal to the epitope recognized by the 1α Dhc antibody (Fig. 10 C). The recovery of this fragment in G3 axonemes (Fig. 6) reveals that the NH2-terminal coiled-coil domains of the 1α Dhc are not required for I1 complex assembly.

**Discussion**

16 different Dhc genes have been identified in Chlamydomonas: 2 cytoplasmic Dhc sequences (Pazour et al., 1999; Porter et al., 1999), 3 genes that encode the Dhcs of the outer dynein arm (Mitchell and Brown, 1994, 1997; Wilkerson et al., 1994), and 11 other Dhc genes whose expression patterns are consistent with gene products that are involved in flagellar function (Porter et al., 1996, 1999; Wilkerson et al., 1994), and 11 other Dhc gene products (Pazour et al., 1999).
Sequence Analysis of Dhc1 Transcription Unit

As described in Fig. 1, we have determined the nucleotide sequence for the complete Dhc1 transcription unit and used this information to obtain the predicted amino acid sequence of the 1α Dhc (Fig. 2). To our knowledge, this is the first full-length, inner arm Dhc sequence to be reported in any organism. Comparisons to other full-length Dhc sequences indicate that the 1α Dhc is most similar to the β and γ Dhcs of the outer arm, and that these sequence similarities extend into the NH2-terminal region (Fig. 3). A second NH2-terminal region of the Dhc is thought to be involved in the association with isofrom specific IC and LC subunits. These observations suggest that the 1α Dhc and the β and γ Dhc contain conserved sites for the binding of accessory subunits. Indeed, recent studies have revealed that the 11 complex and the outer arm do contain similar IC and LC components, such as the β-kD LC, the Tcp1, and Tcex2 LCs, and a family of WD-repeat containing ICs (Patel King et al., 1997; Harrison et al., 1998; Yang and Sale, 1998). However, a novel feature of the 1α Dhc sequence is the presence of a P-loop motif (Pn) at amino acid residues 960–967 (Fig. 2). A weakly conserved P-loop motif has also been identified within the first 200 residues of the β Dhc (Mitchell and Brown, 1994; Kandl et al., 1995). Whether the Pn sequence in the 1α Dhc is a bona fide nucleotide binding site is unknown, but the future sequence analyses of 1α Dhc homologues in other organisms should indicate whether the Pn motif is a conserved feature of this class of Dhc.

The central and COOH-terminal thirds are the most highly conserved regions of the 1α Dhc, and our transformation experiments are consistent with previous proposals that this region corresponds to the dynein motor domain (Sakakibara et al., 1993; Koonce and Samso, 1996; Gee et al., 1997). The amino acid residues around P1 (the primary ATP hydrolytic site) and P4 are highly conserved with other axonemal Dhcs, whereas the sequences around P2 and P3 are less well conserved (Gibbons et al., 1994; Gibbons, 1995). For example, P3 does not strictly conform to the P-loop consensus sequence GXX XG KS/T (Walker et al., 1982), as the glycine in position 6 is substituted by an alanine in the 1α Dhc sequence, but the same amino acid substitution was found in the γ Dhc sequence (Wilkerson et al., 1994).

The COOH-terminal third of the 1α Dhc also contains a small region ~340 amino acids downstream from P4 that is predicted to form a limited coiled-coil domain (Fig. 4). A similar region in cytoplasmic Dhc sequences has been identified recently as the stalk structure that extends from the globular head domain and forms the microtubule binding site (Koonce, 1997; Gee et al., 1997; Gee and Vallee, 1998). Mutations in this region of the outer arm β Dhc can have dramatic effects on flagellar motility (Porter et al., 1994). Interestingly, a sixth P-loop motif (Pc) has been identified in the 1α Dhc sequence, ~280 amino acids downstream from the proposed microtubule binding site. The function of this sixth P-loop in the 1α Dhc is unknown, but its position downstream from the microtubule binding site is intriguing. Recent sequence analysis has suggested that all dyneins may contain six ATPase-like repeat regions: the four central P-loops previously identified and two additional, less well conserved repeats after the COOH-terminal coiled-coil domains (Neuwald et al., 1999).

Rescue of the pf9 Mutant Phenotype by Transformation with Dhc1 Constructs

Using the Dhc1 sequence information, we recovered two BAC clones and two cosmid clones containing full-length or near full-length Dhc1 genes, and then used these constructs to rescue the pf9 motility defects (Fig. 5). 20 independent transformants with rescued motility were recovered. Backcrossing the transformants confirmed that the rescued motility was due to the presence of the Dhc1 transgene and not to a reversion event at the PF9 locus. However, analysis of the Dhc1 transformants produced two unexpected results. First, the frequency of rescue (~1%) was much lower than previously observed with other flagellar genes (5–10%) (Dienert et al., 1990; Kindle, 1990; Tam and Leftube, 1993), and second, the 1α Dhc were truncated in most of the motile transformants recovered thus far.

One reason that the cotransformation frequencies were so low might be due to the large size of the Dhc1 transcription unit, which could make the Dhc1 transgenes more susceptible to damage during the transformation protocol. Exogenous DNA sequences often undergo deletions as they integrate into the Chlamydomonas genome (Tam and Leftube, 1993; Smith and Leftube, 1996, 1997; Ootoulis et al., 1997). The recovery of truncated 1α Dhc fragments (Fig. 6) indicated that the Dhc1 cosmid were being truncated during the transformation protocol, and both Southern and Northern blot analyses of the rescued transformants (Fig. 9 and 10) have confirmed that deletions from the 3′ end of the Dhc1 cosmids did occur. The presence of additional genomic DNA flanking the Dhc1 transcription unit in the BAC clones may have served to protect the Dhc1 transgenes and thereby permitted the full-length rescues observed with these clones (Figs. 5 and 6).

Another reason for the low frequency of rescue might be the relatively small amount of genomic DNA present on the 5′ end of the Dhc1 gene in certain constructs (Fig. 5). If this region was randomly deleted, the resulting cotransformants would not retain the sequences necessary for expression of the Dhc1 transcript and rescue of the mutant phenotype. Recent experiments with constructs encoding the IC140 subunit have indicated that sufficient DNA upstream from the 5′ end of the IC140 gene is essen-
The NH$_2$-terminal 143-kD of the 1$\alpha$ Dhc Is Sufficient for Complex Assembly

The second unexpected result was the frequency with which we recovered motile transformants expressing only NH$_2$-terminal fragments of the 1$\alpha$ Dhc (Fig. 6 B). Northern and Southern blot analyses have shown that the rescued strains retained the 5' sequence elements required for regulated expression of the Dhc1 transcripts, but several of these strains lacked the 3' end of the transgene (Figs. 9 and 10). Therefore, the truncated 1$\alpha$ Dhc represent those NH$_2$-terminal fragments that were competent to assemble with other subunits into the I1 complex (Fig. 6, B and C, and Fig. 7).

The observation that none of the 1$\alpha$ Dhc fragments is smaller than $\sim$143 kD may indicate that this is the shortest NH$_2$-terminal fragment capable of complex assembly. Studies of outer arm mutants have identified a novel \textbeta Dhc mutation with similar properties. The od4-s7 mutant expresses a 160-kD fragment of the \textbeta Dhc that is capable of coassembly with other outer arm subunits at the correct axoneme location (Sakakibara et al., 1993). Likewise, low level expression of cytoplasmic Dhc constructs in Dictyostelium indicates that a 158-kD NH$_2$-terminal fragment is also capable of complex assembly (Koonce and Knecht, 1998).

Although the NH$_2$-terminal third of the 1$\alpha$ Dhc is the most variable region, secondary structure programs have identified a region just before P1 that is predicted to form a limited coiled-coil domain (Fig. 4). This domain, which has been identified in nearly all Dhc sequences to date (Mitchell and Brown, 1994, 1997), has been proposed as a potential region that might mediate interactions between the Dhcs and their associated ICs and LCs. To determine if this coiled-coil domain is required for assembly of the I1 complex, we recovered the Dhc1 transgene from the G3 transformant, which expresses the shortest 1$\alpha$ Dhc fragment (Fig. 6 B). Sequence analysis of the truncated Dhc1 transgene demonstrated that the 1$\alpha$ Dhc sequence terminates before the region predicted to form the NH$_2$-terminal coiled-coil domain (Fig. 10). Given that this 1$\alpha$ Dhc fragment still assembles with other I1 components into the flagellar axoneme (Fig. 6), other sites within the NH$_2$-terminal region must be required for complex formation. We plan to analyze additional Dhc1 constructs to further delineate the domains required for specific subunit interactions and complex assembly.

Assembly of the Dynein Motor Domain

If the Dhc1 transgenes were deleted randomly from the 3' end, we would expect to recover a broad distribution of Dhc fragments ranging in size from the minimum required to assemble the I1 complex to nearly full-length. Therefore, why are almost all of the 1$\alpha$ Dhc fragments smaller than $\sim$217 kD (Fig. 6 B)? One possibility may be that larger 1$\alpha$ Dhc fragments are unstable and prevent assembly of the I1 complex into the axoneme. Studies in Dictyostelium have shown that constructs of cytoplasmic Dhc lacking significant portions of the COOH terminus are expressed poorly as compared with other constructs that contain the entire motor domain (Koonce, 1997). Alternatively, the presence of larger 1$\alpha$ Dhc fragments with partial motor domains may inhibit flagellar motility. If so, we would not recover such transformants in our screen, which was based on the rescue of a motility defect. Indeed, Northern blot analysis of the two transformants (A2 and G9) that assemble the largest 1$\alpha$ Dhc fragments demonstrates that the sequences encoding the dynein motor domain are not present in the associated Dhc1 transcripts (Fig. 9). The NH$_2$-terminal regions of these larger 1$\alpha$ Dhc fragments must, therefore, be fused to other protein sequences. Interestingly, the absence of motile transformants with partial motor domains is consistent with previous reports that nucleotide binding by the cytoplasmic Dhc is inhibited by deletion of the COOH terminus, leading to the formation of rigor complexes (Gee et al., 1997).

Implications for the Structure of the I1 Complex

Structural studies of the isolated I1 complex by negative staining or rotary shadowing have shown that it is a two-headed isoform (Goodenough et al., 1987; Smith and Sale, 1991) but the physical relationship between the globular heads seen in situ and the structural domains identified in situ has been unknown. Because some of the Dhc1 transformants assemble I1 complexes that lack central and COOH-terminal regions of the 1$\alpha$ Dhc (Fig. 7), we can now identify the position of the 1$\alpha$ motor domain within the I1 structure. EM analysis of axonemes isolated from the E2 and G4 transformants has revealed that lobe 2 of the I1 structure corresponds to the missing 1$\alpha$ motor domain (Fig. 8). Lobe 2 is close to the first radial spoke, in a position that may permit direct signaling between the radial spoke and the 1$\alpha$ Dhc motor domain. We predict that the remaining two lobes of the I1 structure represent the positions of the 1\beta Dhc motor domain and stem region containing the I1 ICs and LCs respectively. We are currently transforming other I1 mutants with the genes for other I1 subunits to identify the polypeptide components that are located within these structural domains (Perrone et al., 1998; Perrone, C.A., E. O'Toole, and M.E. Porter, work in progress).

The Role of the 1$\alpha$ Dhc Motor Domain in Motility and Phototaxis

The recovery of transformants missing only the 1$\alpha$ Dhc motor domain but containing the other components of the I1 complex has allowed us to analyze the specific role of the 1$\alpha$ Dhc motor domain in flagellar motility. I1 mutants lacking both the 1$\alpha$ and 1$\beta$ Dhc have a slow, smooth swimming phenotype with an altered flagellar waveform (Kamiya et al., 1991; Porter et al., 1992). Measurements of swimming velocities reveal that the Dhc1 transformants with truncated 1$\alpha$ Dhcswim faster than I1 mutants but slower than control strains containing both Dhc (Table II). The 1$\alpha$ Dhc motor domain, therefore, contributes directly to force production during motility.

The I1 complex is also an essential component of the phototaxis response in Chlamydomonas. Strains that have defects in outer dynein arms, the dynein regulatory complex, or other inner arm isoforms can phototax, but mu-
tants lacking the I1 complex cannot (King and Dutcher, 1997). A analysis of other phototaxis mutants that retain the I1 complex reveals that the phosphorylation state of IC138 is altered (King and Dutcher, 1997). In vitro sliding assays have shown that the phosphorylation state of the IC138 affects microtubule sliding velocities (Haberacher and Sale, 1997). These results suggest a model in which the phosphorylation state of the IC138 modulates the activity of the I1 Dhc motor domains.

To assess the specific role of the 1α Dhc motor domain in phototaxis, we compared the swimming behavior of the Dhc1 transformants to that of control cells in response to a directional light source. pf28 cells were clearly phototactic, but the Dhc1 transformants with truncated 1α Dhcs remained uniformly dispersed during the time course of our assays. These observations indicate that the motor activity of the 1α Dhc contributes to phototaxis, at least in the absence of the outer arms. However, if the outer arms were present, the Dhc1 transformant strains could undergo phototaxis, whereas I1 mutant strains could not (Table II). This difference in behavior in the presence or absence of outer arms suggests that there are cooperative interactions between the I1 complex and the outer arms during the phototaxis response.

Previous studies have demonstrated that differences in the activity of the cis and trans flagellum are the basis of the phototaxis response (Kamiya and Witman, 1984; Rüffer and Nultsch, 1991; Horst and Witman, 1993; Witman, 1993). This differential activity includes both differences in beat frequency between the two flagella as well as differences in flagellar waveforms (Sakakibara and Kamiya, 1989; Rüffer and Nultsch, 1991). The outer dynein arms are responsible for generating the differences in beat frequency observed between cis and trans flagella, as the cis-trans frequency differential is lost in mutants that lack the outer arm or the outer arm α Dhc (Sakakibara and Kamiya, 1989; Sakakibara et al., 1991). More recent work has demonstrated that this differential beat frequency depends on the presence of the docking structure that facilitates the attachment of the outer arm to its specific binding site on the A-tubule (Takada and Kamiya, 1997). The differential in beat frequency is not essential for the phototaxis response (Rüffer and Nultsch, 1991), as outer arm mutant cells such as pf28 are capable of phototaxis (Table I). However, it is clear that the cis-trans differences in beat frequency and flagellar waveform must be coupled in some way, because phototaxis mutants such as pbx1 are defective in both (Rüffer and Nultsch, 1997; Takada and Kamiya, 1997). In this context, it appears that regulation of the I1 complex is important for generating the asymmetries in flagellar waveform between the cis and trans flagella that contribute to phototaxis (King and Dutcher, 1997), whereas regulation of the outer arm contributes to the differential in flagellar beat frequency (Takada and Kamiya, 1997). Phototaxis can occur in the absence of the outer arms, but not the I1 complex (King and Dutcher, 1997; Table II). However, if both the outer arms and part of the I1 complex are present (such as in G4+OA or E2+OA), then their combined activity can apparently compensate for the absence of the 1α Dhc motor domain.

These observations raise several interesting questions about the mechanism by which changes in the phosphorylation state of IC138 might contribute to phototaxis. For example, where is IC138 located in the axoneme relative to the motor domains of the two I1 Dhcs and the three outer arm Dhcs? Is it in lobe 3 of the I1 structure, which is also in close proximity to at least one outer arm per axoneme repeat? Does IC138 interact directly with either the 1α or 1β Dhc? We are planning to address these questions by analyzing subunit interactions within the I1 complex. Other important questions concern the identity and location of the axonemal kinases and phosphatases that modulate the phosphorylation state of IC138. From other laboratories has indicated that many of these regulatory components are tightly bound to the flagellar axoneme (Haberacher and Sale, 1997; King and Dutcher, 1997; Yang, P., and W.S. Sale. 1998. The M, 140,000 intermediate chain of Chlamydomonas flagellar inner arm dynein is a WD-repeat containing protein implicated in dynein arm anchoring. Mol. Biol. Cell. 9:3335–3349), but the specific enzymes that act on either the outer arm or the I1 IC138 in situ have not yet been identified. The future identification and localization of these regulatory components will provide new insights into the pathway that governs the activity of the multiple dynein motors during flagellar motility and phototaxis.

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