Molecular Biology of the Cell
Vol. 6, 1549–1562, November 1995

The Dynein Genes of *Paramecium tetraurelia*: The Structure and Expression of the Ciliary β and Cytoplasmic Heavy Chains

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Submitted April 10, 1995; Accepted August 24, 1995
Monitoring Editor: Thomas D. Pollard

The genes encoding two *Paramecium* dynein heavy chains, DHC-6 and DHC-8, have been cloned and sequenced. Sequence-specific antibodies demonstrate that DHC-6 encodes ciliary outer arm β-chain and DHC-8 encodes a cytoplasmic dynein heavy chain. Therefore, this study is the first opportunity to compare the primary structures and expression of two heavy chains representing the two functional classes of dynein expressed in the same cell. Deciliation of paramecia results in the accumulation of mRNA from DHC-6, but not DHC-8. Nuclear run-on transcription experiments demonstrate that this increase in the steady state concentration of DHC-6 mRNA is a consequence of a rapid induction of transcription in response to deciliation. This is the first demonstration that dynein, like other axonemal components, is transcriptionally regulated during reciliation. Analyses of the sequences of the two *Paramecium* dyneins and the dynein heavy chains from other organisms indicate that the heavy chain can be divided into three regions: 1) the sequence of the central catalytic domain is conserved among all dyneins; 2) the tail domain sequence, consisting of the N-terminal 1200 residues, differentiates between axonemal and cytoplasmic dyneins; and 3) the N-terminal 200 residues are the most divergent and appear to classify the isoforms. The organization of the heavy chain predicts that the variable tail domain may be sufficient to target the dynein to the appropriate place in the cell.

INTRODUCTION

Dynein is the molecular motor that converts ATP hydrolysis into directed translocation along microtubules (see Holzbaur and Vallee, 1994, and Mitchell, 1994, for recent reviews). In situ, dynein is a protein oligomer of M_r 1200–2000K composed of two or three heavy chains and several intermediate and light chains. Although the smaller subunits perform important regulatory functions, isolated heavy chains (M_r ~500K) can produce microtubule movements in vitro (Sale and Fox, 1988; Moss *et al.*, 1992; Mazumdar *et al.*, 1994). Each heavy chain has two structural domains that perform distinct functions: the catalytic head domain, which contains the MgATPase and ATP-sensitive microtubule-binding activities; and the flexible tail domain, which tethers via accessory proteins the dynein to its molecular cargo. An important problem is to determine precisely the way the long heavy chain folds into the functional domains. This is not known, although evidence suggests that the catalytic domain includes the central one-third of the protein, which has four P-loop sequences, and may extend to the C-terminus, and the tail domain includes the N-terminal ~1200 residues (Mocz and Gibbons, 1993; Sakakibara *et al.*, 1993; reviewed in Vallee, 1993).

The many different cellular functions of dynein are effected by specific combinations of heavy chain isoforms, which can be divided into two functional classes: axonemal and cytoplasmic. There are at least eight or nine functionally distinct axonemal dynein heavy chains–two or three from the outer arm and six or more from the inner arms (Piperno *et al.*, 1990; Kagami and Kamiya, 1992; Mastronarde *et al.*, 1992). Axonemal

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dynein specialization occurs in the motor domain: all axonemal dyneins carry an outer doublet microtubule as their cargo, but each arm is specialized to generate precise mechanical force so that the dynein arms working together initiate and propagate ciliary bends (Asai and Brokaw, 1993; Brokaw, 1994). In contrast to axonemal dynein, cytoplasmic dynein carries a diverse array of molecular cargoes (Schnapp and Reese, 1989; Verde *et al.*, 1991; Corthesy-Theulaz *et al.*, 1992; Lin and Collins, 1992; Aniento *et al.*, 1993; Vaisberg *et al.*, 1993; Saunders *et al.*, 1995; Wang *et al.*, 1995) and is probably less specialized in force production. The current view is that cytoplasmic dynein is a homodimer of the heavy chain MAP1C (Vallee *et al.*, 1988; Neely *et al.*, 1990). An important problem is to determine the mechanism by which each dynein isoform is targeted to the specific place in the cell where it will perform its task.

RNA-based polymerase chain reaction (RNA-PCR) utilizing degenerate oligonucleotide primers has been used to amplify the highly conserved region around the catalytic P-loop (Asai *et al.*, 1991; Asai and Criswell, 1995; Gibbons *et al.*, 1992). This method has defined the families of dynein heavy chain isoforms expressed in several model systems, including sea urchin (Gibbons *et al.*, 1994), *Drosophila* (Rasmusson *et al.*, 1994), *Chlamydomonas* (Porter *et al.*, 1992; Wilkerson *et al.*, 1994), and *Paramecium* (Asai *et al.*, 1994). It appears that each protein isoform is encoded by a separate gene.

We are studying the dynein genes expressed in *Paramecium* to learn about the intracellular targeting of specific dynein isoforms. *Paramecium* is a large, single-celled organism that can be genetically manipulated and is receptive to DNA-mediated transformation. *Paramecium* axonemal dyneins include several inner arm dyneins and an outer arm dynein composed of three heavy chains, called α, β, and γ. The three outer arm heavy chains can be resolved on sucrose density gradients (Travis and Nelson, 1988; Larsen *et al.*, 1991; Beckwith and Asai, 1993). These heavy chains have been mapped by the vanadate-mediated UV photolytic reactions V1 and V2 (Lee-Eiford *et al.*, 1986; Tang and Gibbons, 1987). Only the outer arm β-chain undergoes a nucleotide-*in*dependent vanadate-mediated photolysis reaction at the V1 site, and this unique property unambiguously identifies the β-chain (Beckwith and Asai, 1993). *Paramecium* cytoplasmic dynein, which is biochemically distinct from axonemal dynein, is thought to be responsible for the recycling of intracellular membranes to the oral apparatus (Schroeder *et al.*, 1990; Fok *et al.*, 1994).

Paramecium expresses at least 12 dynein heavy chains (Asai *et al.*, 1994), and we have focused on two of these, DHC-6 and DHC-8. In this paper, we report the complete sequences of DHC-6 and DHC-8. Sequence-specific antibodies demonstrated that DHC-6 encodes the ciliary β heavy chain and that DHC-8 encodes cytoplasmic dynein. The steady state level of DHC-6 mRNA, but not DHC-8 mRNA, increases after deciliation. This increase is due to an increase in DHC-6 gene transcription. This presents a unique opportunity to compare two classes of dynein expressed in the same cell.

MATERIALS AND METHODS

Cell Line and Cultivation

Homozygous stock 51 *Paramecium tetraurelia* was cultured in 0.25% wheat grass medium (Pines International, Lawrence, KS) buffered with sodium phosphate. Before addition of paramecia, the sterile medium was inoculated with a nonpathogenic strain of *Klebsiella pneumoniae* (ATCC 27889), which served as a nutrient source.

Genomic Library Screening and DNA Sequencing

A wild-type stock 51 *P. tetraurelia* genomic library in λEMBL4 (Scott *et al.*, 1993) was screened by standard methods (Sambrook *et al.*, 1989). Probes were labeled 180-bp dynein fragments obtained by reverse transcriptase (RT)-PCR (Asai *et al.*, 1994). Overlapping genomic clones spanning the complete DHC-6 and DHC-8 dynein genes were subcloned into phagemids pUC118 and pUC119. Each subcloned segment was sequenced in both orientations using single-stranded DNA templates (Sequenase 2.0, United States Biochemicals, Cleveland, OH).

Ribonuclease Protection Assay and 5′ and 3′ RACE

Ribonuclease protection assay (Ausubel *et al.*, 1987) was used to define the 5′ end of DHC-6. Templates were transcribed in vitro using T7 RNA polymerase (United States Biochemicals) with [α^{32}P]ATP. The resulting RNA probe was hybridized overnight at 30°C with 1 μg poly(A)$^+$ RNA or 10 μg total RNA isolated from *P. tetraurelia*. The hybridized product was digested with RNAse Plus (a mixture of RNAse A and RNAse T1; 5 Prime-3 Prime, Boulder, CO) and the protected products were analyzed on a polyacrylamide/urea gel. The 3′ end of DHC-6 was determined by a procedure in which cDNA synthesis was primed with a poly(T) antisense oligonucleotide and PCR was performed using the poly(T) primer and a sense strand primer located upstream of the putative 3′ end of the DHC-6 transcript. The amplified product was sequenced as described below.

The 5′ end of DHC-8 was determined by rapid amplification of cDNA ends, or 5′ RACE (Frohman *et al.*, 1988; Loh *et al.*, 1989). cDNA was synthesized with Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) in which 1 μg poly(A)$^+$ RNA was primed with 25 pmol of an antisense oligonucleotide primer located downstream of the putative 5′ end. The resulting cDNA was modified at its 5′ end by the addition of a poly(G) tail using 0.1 mM GTP and 20 U of terminal deoxynucleotidyl transferase (Promega, Madison, WI). PCR was then performed with a nested antisense primer and a poly(C) sense primer and the amplified product was sequenced as described in the RT-PCR section below. The 3′ end of DHC-8 was identified by its close similarity with the end of rat brain MAP1C (Mikami *et al.*, 1993) and by the presence of two closely spaced in-frame stop codons.

RNA-directed PCR and Direct Sequencing of PCR Products

RNA-directed PCR was used in the 5′ and 3′ RACE procedures and to identify the introns in DHC-6 and DHC-8. Approximately 1 μg of *P. tetraurelia* poly(A)$^+$ RNA was reverse transcribed (Superscript, Life Technologies) at 40°C for 100 min using 100 pmol of an anti-

sense oligonucleotide primer. As a control, 1 μg of RNA was mock reverse transcribed. One-tenth of the first strand cDNA was amplified with 7.5 U of *Taq* polymerase using 100 pmol of each primer, 1.5 mM Mg^{2+}, and 0.2 mM dNTPs. The DNA was amplified for 30 cycles in which each cycle included 1 min at 94°C, 2 min at 50–55°C, and 2 min at 72°C. The gel-purified double-stranded PCR product was sequenced directly using 0.5 pmol DNA and 0.5 pmol oligonucleotide primers with Sequenase 2.0 (United States Biochemicals).

RNA Blotting and In Vitro Nuclear Run-on Transcription Assays

RNA (Strohman *et al.*, 1977) and nuclei (Gilley *et al.*, 1990) were isolated at various times from 12–20 liters of *P. tetraurelia*, growing in log phase, after deciliation (Machemer and Ogura, 1979). Transcriptional activity was measured using the run-on assay (Gilley *et al.*, 1990). Filters containing RNA samples for analysis were prepared by standard methods (Sambrook *et al.*, 1989). Ten micrograms of each RNA sample were applied to Maximum Strength Nytran (Schleicher & Schuell, Keene, NH) using a vacuum slot-blotter and the RNA was affixed to the membrane using UV light.

Filters containing DNA for hybridization to labeled RNAs in the nuclear run-on transcription assay were prepared according to our standard methods (Leeck and Forney, 1994). Cloned DNAs used in this study were as follows: the 2.2-kb *Eco*RI fragment of *Paramecium* α-tubulin (pTc2; the gift of John Preer, Jr., Indiana University); the 5-kb *Hin*dIII–*Sal*I fragment of DHC-6 (pDHC6–5HS; see Figure 1); the 5-kb *Bam*HI fragment of DHC-8 (pDHC8–5B; see Figure 1); the 0.4-kb *Eco*RI fragment of *Paramecium* calmodulin (pCamtel; Kanabrocki *et al.*, 1991); and lambda phage DNA. Approximately 10^7 cpm of labeled RNA was incubated with the membranes.

For the RNA slot-blots and the nuclear run-on transcription assays, the DNA and RNA samples were applied to Nytran membranes as described above and then hybridized as described elsewhere (Leeck and Forney, 1994). The membranes were washed three times, 30 min per wash, with 0.2× SET, 25 mM phosphate buffer, 0.1% sodium pyrophosphate, and 0.1% SDS at 65°C, 68°C, and 71°C. The intensities of the signals reported in Figure 4 were measured using a Phosphorimager SF instrument and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To control for possible variations in sampling, the intensities of the dynein and tubulin signals were normalized to the calmodulin signal. This enabled the comparison among the dyneins and tubulin, but did not necessarily provide an absolute measure of the amounts of the mRNAs. Thus, the results in Figure 4 are presented as relative amount of mRNA with respect to a mock deciliated control (control relative amount = 1).

Sequence-specific Antibodies and Western Blotting

Three new antibodies were produced during this study. Peptides 6–491 (residues 491–509 of DHC-6), 6–3261 (residues 3261–3288 of DHC-6), and 8–524 (residues 524–539 of DHC-8) (see Figure 2) were synthesized with an N-terminal cysteine through which the peptide was coupled to porcine thyroglobulin using sulfo-SMCC (Pierce, Rockford, IL). The peptide-thyroglobulin conjugates were utilized as antigens in rabbits.

Paramecium outer arm axonemal dyneins were obtained by a high salt extraction of demembranated cilia and subjected to V1 photolysis in the presence and absence of ATP by our standard methods (Beckwith and Asai, 1993). *Paramecium* cytoplasmic dynein was partially purified by ATP-sensitive binding to exogenously added taxol-stabilized microtubules (Fok *et al.*, 1994). SDS-PAGE, transfer to nitrocellulose, probing with the antibodies, and development of the Western blots using alkaline phosphatase were performed by our standard methods (Beckwith and Asai, 1993). The JR antiserum, which is specific for the cytoplasmic dynein catalytic P-loop motif B, has been described elsewhere (Asai *et al.*, 1994; Tjandra *et al.*, 1994).

RESULTS

The Characterization of the Paramecium DHC-6 and DHC-8 Genes

The clones of DHC-6 and DHC-8 were obtained from a lambda phage library of *Paramecium tetraurelia* genomic DNA by screening with the short P-loop containing fragments cloned after RNA-directed PCR (Asai *et al.*, 1994). The DHC-6 and DHC-8 genes are diagrammed in Figure 1. The 5′ end of the DHC-6 transcript was identified by RNAse protection assay and occurs approximately 22 nucleotides from the initiation codon. The 5′ end of the DHC-8 transcript was identified by 5′ RACE and occurs 23 or 24 nucleotides from the initiation codon. 16,557 bp of the DHC-6 gene were sequenced; this includes 5′- and 3′-untranslated regions of 1,483 and 1,204 bp, respectively, and a coding region of 13,870 bp. 15,228 bp of the DHC-8 gene were sequenced; this includes 5′- and 3′-untranslated regions of 371 and 1,210 bp, respectively, and a coding region of 13,647 bp. These sequences have been deposited in the GenBank database (accession numbers U19464 and U20449).

Four introns in DHC-6 and one intron in DHC-8 were identified by analysis of the genomic sequence and confirmed by direct sequencing of RNA-PCR products. In DHC-6, the introns begin at nucleotides 128, 339, 513, and 13,468 (where nucleotide 1 is the A

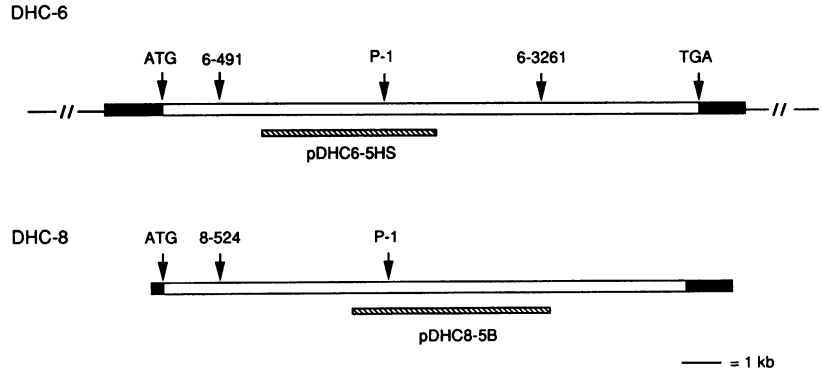


Figure 1. Diagrams of the DHC-6 and DHC-8 genes. Sequenced portions (16,557 bp of DHC-6 and 15,228 bp of DHC-8) are indicated by boxes: coding regions are unshaded; noncoding regions are darkened. The 5-kb probes pDHC6–5HS and pDHC8–5B used in the Northern blotting and nuclear run-on transcription assays are shown as hatched bars. The locations of translation initiation codons, translation termination codons, the three peptide epitopes (6–491, 6–3261, and 8–524), and the catalytic P-loops (P-1) are marked.

of the translation initiation codon); in DHC-8, the intron begins at nucleotide 170. As in other *Paramecium* genes (Russell *et al.*, 1994) the identified introns are short (23–28 nucleotides) and conform to the GT-AG rule for splicing (reviewed in Moore *et al.*, 1993). It is possible that other introns, which do not alter the reading frames, are present in both genes. Except for the first introns in the β genes from *Chlamydomonas* and *Paramecium*, the introns in DHC-6 and DHC-8 do not align with the positions of the introns found in the heavy chain genes from other organisms, including *Chlamydomonas* (Mitchell and Brown, 1994), *Aspergillus* (Xiang *et al.*, 1994), and *Caenorhabditis* (Lye *et al.*, 1995).

The deduced amino acid sequences of DHC-6 (4588 residues) and DHC-8 (4540 residues) were aligned (PC/Gene, IntelliGenetics, Mountain View, CA) and the aligned sequences are shown in Figure 2. Both sequences possess the canonical pattern of four P-loops found in all dynein heavy chains. The greatest similarities occur near the P-loops. Secondary structure analysis (PC/Gene, Intelligenetics) reveals several regions in both deduced sequences that have a high probability of forming coiled-coils. These regions are clustered in the N-terminal ~1800 residues and in the C-terminal ~1500 residues in a pattern very similar to what has been reported for other dynein heavy chains (Mitchell and Brown, 1994). There are two regions in each dynein that could form extended coiled-coils. These regions are residues 3109–3211 and 3346–3437 in DHC-6 and residues 3076–3174 and 3302–3373 in DHC-8.

Sequence-specific Antibodies Demonstrate that DHC-6 Encodes Ciliary β-Chain and DHC-8 Encodes Cytoplasmic Dynein

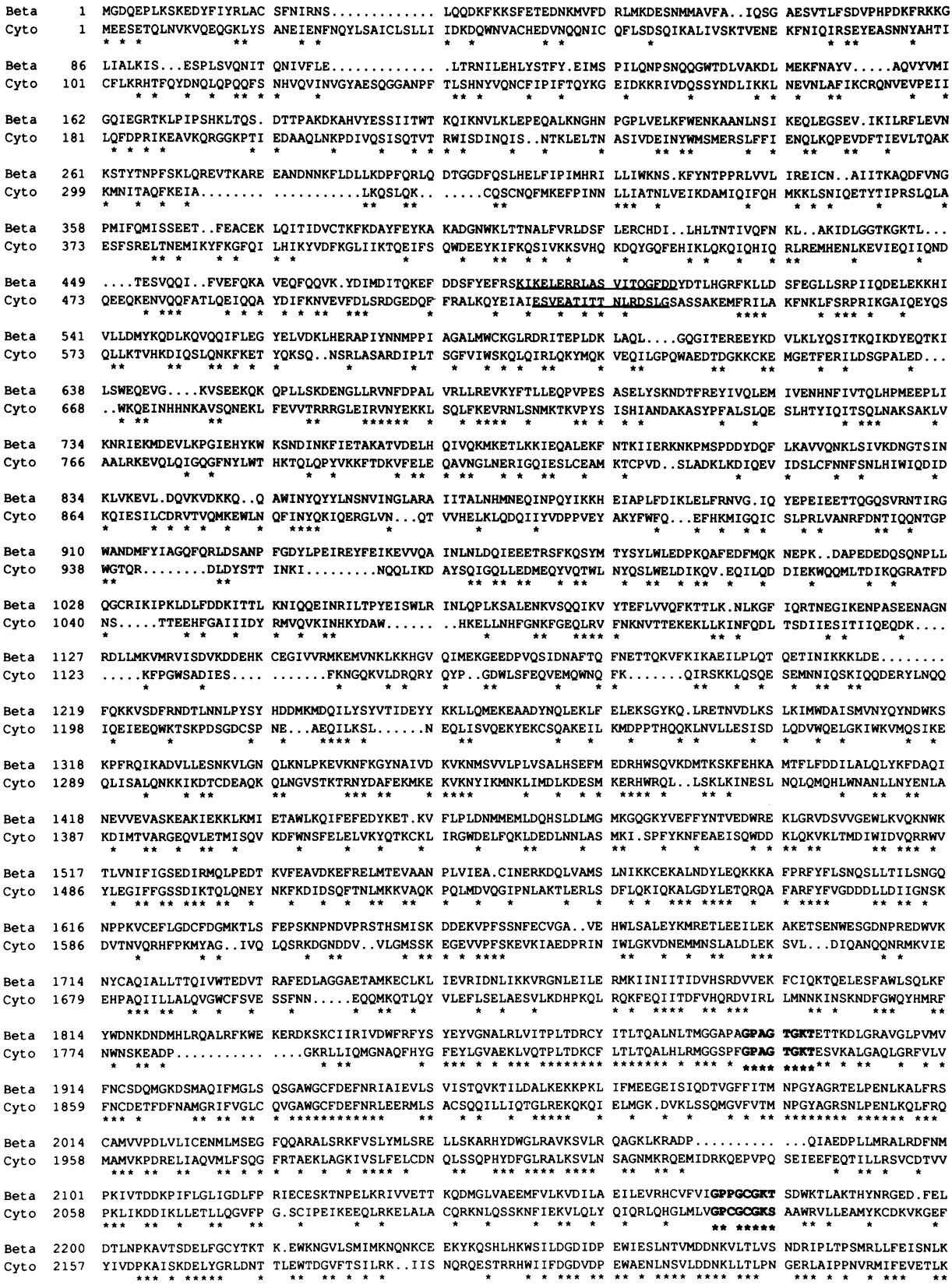
Three peptide haptens were synthesized from the DHC-6 and DHC-8 sequences. The peptide sequences are indicated in Figure 2. Epitope 6–491 and Epitope 8–524 correspond to amino acids 491–509 in DHC-6 and 524–539 in DHC-8, respectively, and are derived from homologous positions in the two proteins. Epitope 6–3261 was derived from the predicted loop flanked by the two coiled-coil domains predicted from the DHC-6 sequence, and corresponds to residues 3261–3288.

Antibodies were raised to each of the three peptides. Competitive solid-phase binding assays demonstrated that the three antibodies were peptide-specific and did not cross-react with the inappropriate peptide. The three antibodies were utilized in Western blotting experiments. Figure 3, A and B, shows *Paramecium* ciliary outer arm dynein, intact and after the V1 photolysis reaction in the absence and presence of ATP. Epitope 6–491 is near the N-terminus and should reside on the LUV-1 fragment after photolysis, and this was the result obtained (Figure 3, lane 3). Epitope 6–3261 should reside on the HUV-1 fragment, and this was the result obtained (Figure 3, lane 6). The photolysis reaction in the absence of added nucleotide occurs only in the β heavy chain (Beckwith and Asai, 1993), and the results demonstrate that epitopes derived from DHC-6 are on the β-chain (Figure 3, lanes 2 and 5). *Paramecium* cytoplasmic dynein (Figure 3C) and total ciliary dynein (Figure 3D) were probed with the antibody raised against epitope 8–524. The antibody reacted with cytoplasmic dynein (Figure 3, lane 9), but not with ciliary dynein (Figure 3, lane 11). The HM_r band reactive with anti-epitope 8–524 is the same as that which reacts with JR antibody (Figure 3, lane 8), which is specific for the catalytic P-loop sequence motif B and is reactive with all cytoplasmic dyneins examined (Tjandra *et al.*, 1994). Our preliminary data indicate that the slower-migrating band present in crude cytoplasmic dynein, seen in lane 7 of Figure 3C, corresponds to ciliary dynein precursor residing in the cell body; the SDS-PAGE does not resolve multiple heavy chain isoforms in this putative precursor band (see Fok *et al.*, 1994). These results demonstrate that DHC-6 encodes ciliary β heavy chain and DHC-8 encodes cytoplasmic dynein.

Axonemal Dynein Expression Is Transcriptionally Induced during Reciliation

The steady state concentration of axonemal dynein mRNA increases in response to deciliation/deflagellation where it has been examined, including in *Chlamydomonas* (Williams *et al.*, 1986) and sea urchin embryos (Gibbons *et al.*, 1992, 1994). In *Paramecium*, mRNA from DHC-6 but not DHC-8 was shown to be substantially increased during reciliation (Asai *et al.*, 1994). To investigate the cellular mechanism underlying the change in ciliary dynein mRNA, run-on transcription experiments with isolated nuclei were performed. Paramecia were sampled at different times after deciliation and divided into two portions. From one portion mRNA was isolated for quantification on slot blots. The second portion was detergent extracted for nuclear run-on experiments. Probes for the slot blots were pDHC6–5HS and pDHC8–5B (see Figure 1), *Paramecium* α-tubulin DNA, and the *Paramecium* calmodulin gene (see MATERIALS AND METHODS for details). These same DNA samples were immobilized to filters for the nuclear run-on transcription assays.

Figure 2 (following pages). Deduced amino acid sequences of DHC-6 and DHC-8. The alignment of the two sequences is shown. Identical or conserved residues (A and G, D and E, F and Y, I and L, K and R, N and Q, and S and T) are indicated with an *. The four central P-loops are in bold. The three peptide epitopes are underlined. The nucleotide sequences of DHC-6 and DHC-8 have been deposited in the GenBank database (accession numbers U19464 and U20449, respectively).



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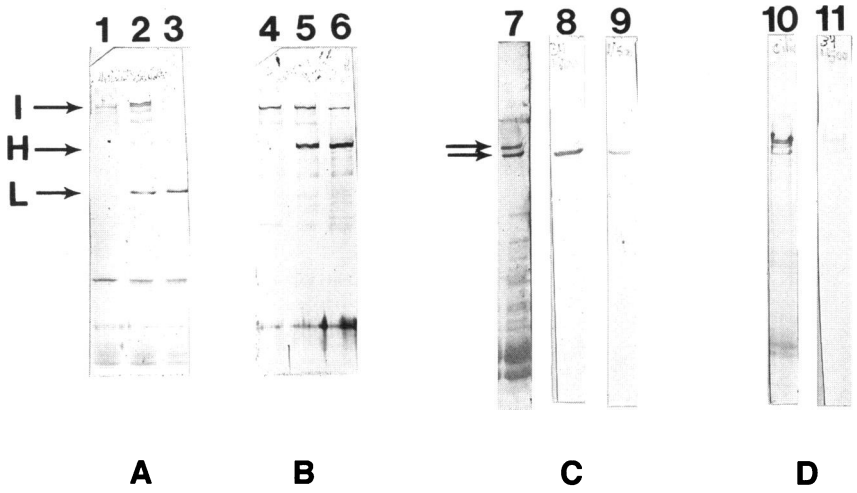

Figure 3. Western blots demonstrate that DHC-6 is ciliary β-chain and DHC-8 is cytoplasmic dynein heavy chain. (A and B) *Paramecium* ciliary outer arm dynein was electrophoresed on 6% SDS-PAGE gels (Dreyfuss *et al.*, 1984), transferred to nitrocellulose, and probed with antibodies to epitopes 6–491 (A) and 6–3261 (B). Lanes 1 and 4, intact outer arm dyneins; lanes 2 and 5, outer arm dyneins photolyzed in the presence of Mg^{2+}, 50 μM vanadate, and no added nucleotide; lanes 3 and 6, outer arm dyneins photolyzed in the presence of Mg^{2+}, 50 μM vanadate, and 50 μM ATP. The antibodies each recognized intact dynein heavy chain (I). Anti-epitope 6–491 reacted with the LUV1 product (L), and anti-epitope 6–3261 reacted with the HUV1 product (H). This pattern of reactivity is the expected result. The V1 treatment in the absence of added nucleotide (lanes 2 and 5) results in the specific photolysis of *Paramecium* β-chain (Beckwith and Asai, 1993). Western blots were developed with alkaline phosphatase. (C) Crude *Paramecium* cytoplasmic dynein was electrophoresed and transferred to nitrocellulose. Lane 7 is a Ponceau S–stained blot of the crude dynein; several protein bands are visible. Lanes 8 and 9 are nitrocellulose strips identical to the one shown in lane 7, probed with JR antiserum (lane 8) and with anti-epitope 8–524 (lane 9). The JR antiserum was raised against the cytoplasmic dynein P-loop sequence (Tjandra *et al.*, 1994). Of the two high molecular weight bands seen in lane 7 (arrows), the faster migrating band is cytoplasmic dynein. Western blots were developed with alkaline phosphatase. (D) Total ciliary dyneins were electrophoresed and transferred to nitrocellulose. Lane 10 is a Ponceau S–stained strip that shows the prominent dynein heavy chains. Lane 11 was probed with anti-epitope 8–524 and developed with alkaline phosphatase.

The relative expression of DHC-6, DHC-8, and tubulin was determined on RNA slot blots and normalized to the calmodulin signal. The specificity of the dynein probes under the hybridization conditions used was confirmed in Southern blots in which the same dynein probes hybridized only with single, unique bands. The apparent concentrations of DHC-6 and tubulin mRNAs substantially increased after deciliation, but DHC-8 mRNA did not change, which is the result obtained previously (Asai *et al.*, 1994). These data are summarized in Figure 4A. The nuclear run-on assays showed that DHC-6 and tubulin were transcriptionally induced, but DHC-8 was not (Figure 4B). The results shown in Figure 4 are from samples taken from one culture of cells; thus, the Northern and run-on transcription data for each timepoint were obtained from the same sample. The samples from this culture were independently measured twice: by autoradiography followed by densitometric scanning of the x-ray film, and by phosphorimaging. The results from both sets of measurements were qualitatively the same; the data in Figure 4 are based on the phosphorimager measurements. The entire experiment was repeated with a second culture of cells and confirmed the results shown in Figure 4, except that the second peak at 90 min for DHC-6 in the run-on experiment was not as large. The kinetics of tubulin transcriptional induction were similar to what was reported for tubulin induction in *Tetrahymena* (Soares *et al.*, 1993). These results demonstrate that the increase in the steady state concentrations of DHC-6 and tubulin mRNAs was due at least in part to an induction of transcription of these genes in rapid response to deciliation.

The Greatest Sequence Divergence among Dynein Heavy Chains Occurs in the Tail Domains

The dynein heavy chain can be divided into two functional domains. The catalytic domain, which includes the four central P-loops, is presumed to fold into the globular head and produces the motive force along the microtubule. The tail domain, thought to be comprised of the N-terminal ~1200 residues, forms the short flexible tail that interacts with other proteins including intermediate chains (King *et al.*, 1991) and dynactin (Gill *et al.*, 1991; Lees-Miller *et al.*, 1992) to tether dynein to its cargo and to regulate dynein activity. The tail domain of the β-chain mediates the assembly of the other heavy chains to form the outer dynein arm (Sakakibara *et al.*, 1993). A comparison of the available dynein sequences, divided into catalytic

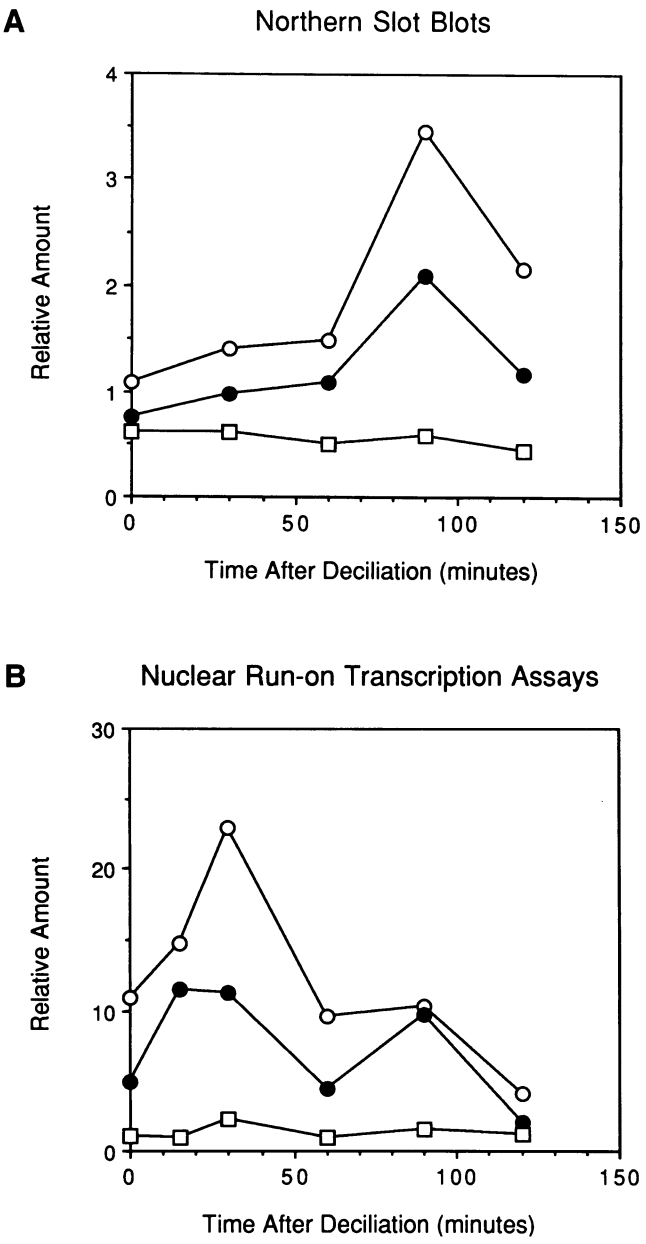

Figure 4. DHC-6 is transcriptionally activated in response to deciliation. Paramecia in log phase growth were deciliated and sampled at various times during reciliation. (A) RNA from each sample was probed on a slot-blot as described in MATERIALS AND METHODS. To control for variation in the amount of RNA in each slot, the signals were normalized at each time point to the intensity of the calmodulin signal and reported as the amount relative to the mock deciliated control. (B) Nuclei, from the same samples from which RNA was isolated for the slot-blot, were utilized in in vitro nuclear run-on transcription assays as described in MATERIALS AND METHODS. Intensities were normalized to the calmodulin transcription signal and the results are reported as the amount of transcription relative to the mock deciliated control. In both panels: tubulin, open circles; DHC-6, filled circles; DHC-8, open squares.

and tail domains, is summarized in Table 1. All dyneins are more similar to one another in their catalytic domains than in their tail domains. Although the catalytic domains of the cytoplasmic dyneins are more similar to one another than to the axonemal dyneins, there is significant sequence conservation in the catalytic domains of dyneins irrespective of functional class. Class distinctions are apparent when examining the tail domains, where it is clear that the sequences have diverged between the cytoplasmic and axonemal heavy chains.

These relationships are further illustrated in the paired sequence comparisons plotted in Figure 5. Even dyneins from two different classes–e.g., *Paramecium* ciliary β-chain and *Paramecium* cytoplasmic dynein–are similar through portions of the catalytic domain. Comparison between dyneins of the same class–e.g., the β chains from *Paramecium* and sea urchin, and the cytoplasmic dyneins from *Paramecium* and rat brain–reveals extensive similarity except in the most N-terminal ~200 residues. Our unpublished parsimony analysis indicates that the N-terminal 200 residues of the three sequenced β chains (from sea urchin, *Chlamydomonas*, and *Paramecium*) are more similar to one another than any is to the corresponding region of γ chain or any cytoplasmic dynein. Thus, dynein sequences diverge in the N-terminal 1200 residues and are most divergent in the N-terminal 200 residues.

DISCUSSION

In this paper, we present the sequences of two dynein heavy chains expressed in *Paramecium tetraurelia*. Sequence-specific antibodies were used to demonstrate that DHC-6 encodes axonemal β heavy chain and DHC-8 encodes cytoplasmic dynein heavy chain. The cloning of two dynein heavy chains, representing two functional classes of dynein from the same organism, enabled us to compare their expression in response to deciliation. For other axonemal genes, including genes encoding tubulin and radial spokes, there is a transcriptional induction in response to deciliation (Lefebvre *et al.*, 1980; Schloss *et al.*, 1984; Johnson and Rosenbaum, 1993). In *Paramecium* and sea urchin, the only other organism for which there are probes for both axonemal and cytoplasmic genes, there is a significant increase in the steady state concentrations of mRNA encoding ciliary dynein but not cytoplasmic dynein (Asai *et al.*, 1994; Gibbons *et al.*, 1994). However, until this study, we did not know if the change in dynein mRNA concentration in any organism was a consequence of an increase in transcription. The present study is the first demonstration that the induction of axonemal dynein, like tubulin and radial spokes, is a transcriptional response to decilia-

Table 1. Pairwise % similarity comparisons of dynein tail and catalytic domains

Tail domains

	DHC-6	SU β	Chlamy β	Chlamy γ	DHC-8	Dicty	Dro cyto	MAP1C	C eleg	SU cyto	N crassa	Asp	Yeast
DHC-6	—												
SU β	**54.4**	—											
Chlamy β	**59.4**	**56.0**	—										
Chlamy γ	47.5	47.7	48.6	—									
DHC-8	45.2	45.1	43.8	44.5	—								
Dicty	45.6	44.5	43.5	43.9	**56.9**	—							
Dro cyto	43.2	43.5	44.3	43.1	**56.8**	**61.6**	—						
MAP1C	44.5	45.2	46.0	45.6	**58.7**	**63.8**	**81.7**	—					
C eleg	42.3	41.9	44.4	44.7	**52.9**	**55.9**	**66.9**	**68.2**	—				
SU cyto	NA	NA	NA	NA	**NA**	**NA**	**NA**	**NA**	**NA**	—			
N crassa	45.6	42.9	43.9	42.5	**54.2**	**56.1**	**59.3**	**61.7**	**55.0**	**NA**	—		
Asp	45.6	43.2	43.8	44.2	**54.6**	**58.3**	**59.1**	**62.7**	**55.5**	**NA**	**76.9**	—	
Yeast	45.4	43.6	41.8	42.7	**47.0**	**45.1**	**45.7**	**47.2**	**46.0**	**NA**	**47.2**	**46.9**	—

Catalytic domains

	DHC-6	SU β	Chlamy β	Chlamy γ	DHC-8	Dicty	Dro cyto	MAP1C	C eleg	SU cyto	N crassa	Asp	Yeast
DHC-6	—												
SU β	**68.4**	—											
Chlamy β	**68.5**	**69.9**	—										
Chlamy γ	61.4	60.3	59.8	—									
DHC-8	56.2	60.2	56.3	59.5	—								
Dicty	58.0	59.1	56.7	61.4	**76.1**	—							
Dro cyto	56.7	60.1	58.3	58.9	**75.4**	**79.2**	—						
MAP1C	56.8	59.3	55.4	59.6	**76.3**	**79.6**	**89.7**	—					
C eleg	56.4	59.0	54.9	56.5	**73.9**	**75.7**	**83.5**	**82.8**	—				
SU cyto	58.2	59.3	55.7	59.7	**77.6**	**79.8**	**89.4**	**91.3**	**83.1**	—			
N crassa	55.2	56.7	55.7	58.3	**73.5**	**76.9**	**79.7**	**80.4**	**77.7**	**79.6**	—		
Asp	56.2	57.3	56.2	57.3	**75.2**	**76.8**	**79.7**	**80.3**	**76.5**	**80.2**	**88.6**	—	
Yeast	53.6	52.8	51.1	50.4	**62.9**	**64.9**	**65.1**	**64.1**	**62.6**	**64.4**	**64.6**	**64.3**	—

The N-terminal ~1200 residues (for DHC-6, residues 1–1250) of 12 dynein heavy chain sequences were aligned and then compared with one another by the Bestfit program (GCG, University of Wisconsin). The degree of sequence similarity in the N-terminal tail domains in each pairwise combination is shown in the upper half of Table 1. With the exceptions of the *Chlamydomonas* axonemal γ chain and yeast cytoplasmic dynein, there is a clear clustering of the tail domains into an axonemal group and a cytoplasmic group. The comparisons among the β chains and the comparisons among the cytoplasmic dyneins are indicated in bold font. In the lower half of Table 1, the pairwise comparisons of the catalytic domains is shown. The catalytic domain includes all four P-loops; for DHC-6, it is residues 1850–2943 (see Gibbons *et al.*, 1994). There is a clear separation between the three β chains and the nine cytoplasmic dyneins (bold font). Dynein heavy chain sequences and their abbreviations used in this table are as follows: DHC-6, *Paramecium* β (this study; accession number U19464); SU β, sea urchin β (Gibbons *et al.*, 1991; X59603); Chlamy β, *Chlamydomonas* β (Mitchell and Brown, 1994; U02963); Chlamy γ, *Chlamydomonas* γ (Wilkerson *et al.*, 1994); DHC-8, *Paramecium* cytoplasmic (this study; U20449); Dicty, *Dictyostelium* (Koonce *et al.*, 1992; Z15124); Dro cyto, *Drosophila* cytoplasmic (Li *et al.*, 1994; L23195); MAP1C, rat cytoplasmic (Mikami *et al.*, 1993; L08505); C eleg, *Caenorhabditis rhabditis* (Lye *et al.*, 1995; L33260); SU cyto, sea urchin 1a (Gibbons *et al.*, 1994; Z21941); N crassa, *Neurospora crassa* (Plamann *et al.*, 1994; L31504); Asp, *Aspergillus nidulans* (Xiang *et al.*, 1994; U03904); Yeast, *Saccharomyces cerevisiae* (Eshel *et al.*, 1993; Z21877).

tion. The sequenced 5′-untranslated regions of DHC-6 and DHC-8 are where we expect to find the regulatory elements governing dynein gene expression in *Paramecium*.

Common Features in all Dynein Heavy Chain Sequences

Including the two *Paramecium* sequences presented here, there are now twelve complete dynein heavy chain sequences reported. These are as follows: the axonemal β heavy chains from sea urchin (Gibbons *et al.*, 1991; Ogawa, 1991), *Chlamydomonas* (Mitchell and Brown, 1994), and *Paramecium* (this report); the axonemal γ heavy chain from *Chlamydomonas* (Wilkerson *et al.*, 1994); and the cytoplasmic dyneins from *Dictyostelium* (Koonce *et al.*, 1992), rat brain (Mikami *et al.*, 1993; Zhang *et al.*, 1993), *S. cerevisiae* (Eshel *et al.*, 1993; Li *et al.*, 1993), *Aspergillus* (Xiang *et al.*, 1994), *Neurospora* (Plamann *et al.*, 1994), *Drosophila* (Li *et al.*, 1994), *C. elegans* (Lye *et al.*, 1995), and *Paramecium* (this report). Importantly, the two *Paramecium* sequences provided us the unique opportunity to study sequence divergence between dynein functional classes uncompli-

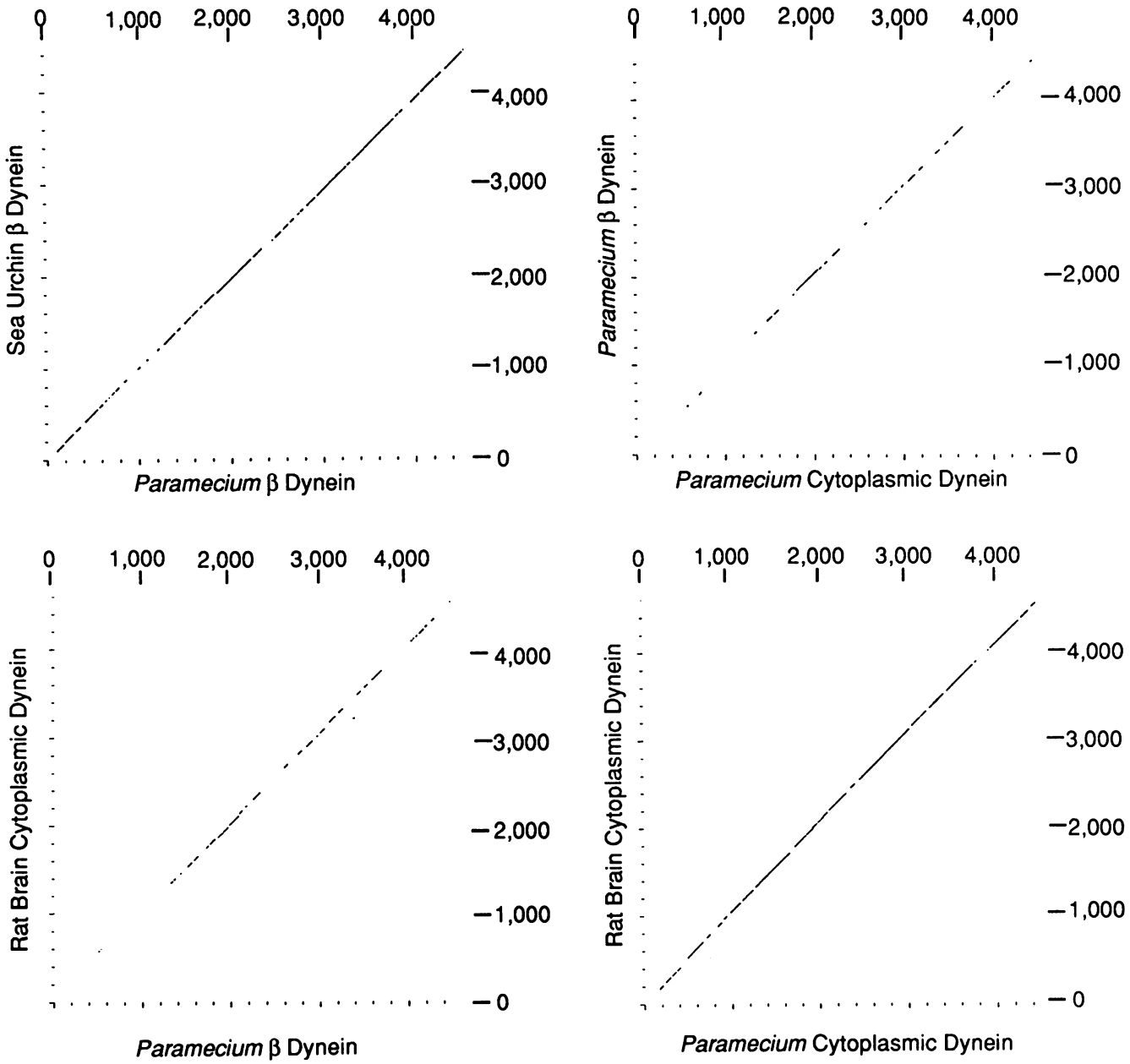

Figure 5. Pair-wise comparisons of some dynein heavy chain sequences. The deduced protein sequences of *Paramecium* β chain and *Paramecium* cytoplasmic dynein heavy chain were compared with one another and to rat brain cytoplasmic dynein (Mikami *et al.*, 1993); *Paramecium* β chain was also compared with sea urchin β chain (Gibbons *et al.*, 1991). The comparisons were made by the Compare program (GCG, University of Wisconsin), using a window of 50 residues and a stringency of 30 (i.e., at least 30 of 50 are identical for a dot to be plotted). The comparison between *Paramecium* axonemal and cytoplasmic dyneins reveals similarities in the catalytic domain. The divergence in the 1200-residue N-terminal tail domain between axonemal and cytoplasmic dyneins is apparent. The two β chains are very similar throughout their entire lengths; the two cytoplasmic dyneins are very similar except in the N-terminal 200 residues.

cated by potential differences due to species and tissue variation.

There is remarkable conservation among all of the dynein sequences, especially in the catalytic domains. All dyneins contain four equally spaced P-loops, called P-1, -2, -3, and -4, in the middle portion of the sequence. P-1 is very near the V1 photolysis site and is the catalytic P-loop. P-4 is near the V2 photolysis site, which may identify the adenine-binding portion of the ATP-binding domain (Tang and Gibbons, 1987). All

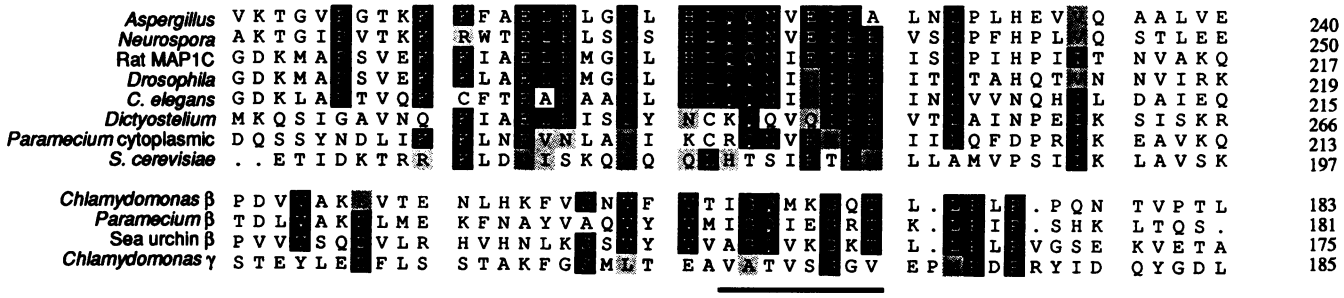

Figure 6. A portion of the alignment of twelve dynein heavy chains reveals the existence of a possible P-loop near the N-terminus of all β heavy chains. An alignment of the 1200-residue tail domains of 12 dynein heavy chains was made utilizing the Pileup program (GCG, University of Wisconsin) and printed using the Prettybox program. Shown here is a small portion of that alignment. The position in the deduced protein sequence of each entry is indicated by the number at the far right, which identifies the last residue in each peptide. There is a sequence, which is underlined, present in all β chains but not present in γ chains or in cytoplasmic dyneins that weakly conforms to the consensus P-loop sequence GXXXXGKT.

dynein sequences predict two closely-spaced α-helical regions that could form extended coiled-coils between P-4 and the C-terminus. The hypothesis that this region might interact with other proteins (Asai and Brokaw, 1993) is supported by analysis of two *Chlamydomonas* mutations affecting dynein regulation, which occur in this region of the β heavy chain (Porter *et al.*, 1994). In unpublished experiments, we have utilized synthetic peptides and the antibody to epitope 6–3261 to show that the coiled-coil domain probably does not interact directly with microtubules.

Features that Discriminate between Axonemal and Cytoplasmic Dynein Heavy Chains

All dyneins have the same catalytic P-loop sequence, GPAGTGKT. However, immediately adjacent to the P-loop are two different sequence motifs, A and B, which discriminate between axonemal (motif A) and cytoplasmic (motif B) heavy chains. This observation has been detailed elsewhere (Asai and Brokaw, 1993) and tested with an antibody specific for motif B (Asai *et al.*, 1994; Tjandra *et al.*, 1994). In addition to these sequence patterns near the first P-loop, the different dyneins also segregate into axonemal and cytoplasmic classes by virtue of their P-3 and P-4 sequences. With two exceptions, all of the complete and partial dynein heavy chain sequences that have been extended through P-4 fall into two patterns. All axonemal dyneins have an invariant P-4 sequence (GVGGSGKQ) but their P-2 and P-3 sequences are not completely conserved. In contrast, all cytoplasmic dyneins have an invariant P-3 sequence (GPPGSGKT) but different P-2 and P-4 sequences. The two exceptions identified thus far occur in sea urchin dyneins: 1b possesses motif B near P-1 but does not conform to other cytoplasmic dyneins in its P-3 sequence; and 7a contains motif A near P-1 but its P-4 sequence is different from other axonemal dyneins (Gibbons *et al.*, 1994). Because both 1b and 7a are induced upon deciliation in sea urchin embryos, they may be axonemal dyneins that do not conform to the sequence patterns noted above, or they may be cytoplasmic dyneins that are utilized to carry materials to the growing cilia. Sequence differences in the catalytic domain, including the motifs A and B near P-1 and the P-3 and P-4 sequences, may affect the enzymatic and force-producing activities of dyneins and be partly responsible for the differences observed between axonemal and cytoplasmic dynein (Pallini *et al.*, 1983).

A second feature that distinguishes dynein isoforms is a sequence resembling a P-loop occurring near the N-terminus of all β heavy chains sequenced but in no other dyneins (first identified in Gibbons *et al.*, 1991). An alignment of all of the available dynein heavy chain sequences in this region is shown in Figure 6. In addition, in the *Paramecium* β-chain, there is a sixth sequence resembling a P-loop, which occurs at residues 440–447: LGGTKGKT. Unlike the four centrally located P-loops that appear to be involved in the motor activity of dynein, these additional putative P-loops may have no functional significance. The kinetics of binding to ATP analogues suggests that axonemal dynein may possess multiple nucleotide-binding sites (Kinoshita *et al.*, 1994). Biochemical evidence showing the ability of these putative P-loops to bind phosphate or a phosphate analogue would support the idea that this sequence is important in the regulation of dynein activity.

The analysis of dynein sequences reveals that the tail domain, comprising the N-terminal ~1200 residues, is more divergent than the rest of the heavy chain. Alignment of the available tail domain sequences reveals several short regions where there is a sequence similarity pattern that distinguishes the cytoplasmic dyneins from the axonemal heavy

chains (e.g., a small portion of the alignment is shown in Figure 6). The class-dependent sequence divergence in the tail domain is consistent with the hypothesis that it is this domain that interacts with other subunits and helps specify attachment of dynein to its molecular cargo.

The most divergent portion of the dynein heavy chain sequence occurs in the N-terminal ~200 residues. A parsimony analysis of the most N-terminal sequences shows that there is significant divergence in this region, even among dynein heavy chains from the same class. These differences may correspond to the isoform-specific interaction with other proteins to tether the dynein to a particular molecular cargo. Expression of modified dynein genes, an experiment that is reasonably straightforward in *Paramecium*, may reveal the functional significance of different regions of the heavy chain. We are currently constructing and expressing in *Paramecium* truncated versions of the β-chain in experiments aimed at identifying regions of the heavy chain that direct the intracellular targeting of dynein.

ACKNOWLEDGMENTS

Jifan Chen provided extensive technical assistance in this work. We gratefully acknowledge Drs. Ching Kung, Chuck Leeck, John Preer, and Hali Wang for providing us with reagents and good advice. John Wilder assisted in the production of the peptide-directed antibodies, and Brent Branstetter and Zsuzsa Hamburger contributed to Figure 3. Jason Curtis assisted in the parsimony analysis of the sequences. K.A.K. was partially supported by a Purdue Research Foundation fellowship. This project was supported by a grant from the National Institutes of Health (GM-49889) to D.J.A. and J.D.F. This is publication number 14735 from the Purdue Agricultural Experiment Station.

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