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The Light Chain p28 Associates with a Subset of Inner Dynein Arm Heavy Chains in *Chlamydomonas* Axonemes

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We show here that I2 and I3 inner dynein arm heavy chains of *Chlamydomonas* axonemes are resolved into two classes: one class associated with the protein p28 and the other associated with the protein caltractin/centrin.† We have determined the nucleotide sequence of the gene encoding p28, a light chain that, together with actin and caltractin/centrin, is associated with inner dynein arms I2 and I3 of *Chlamydomonas* axonemes. p28 is a novel protein with affinity for a subset of the inner dynein arm heavy chains, but with no apparent significant homologies to tubulin- or actin-binding proteins. An antiserum specific for p28 showed that p28 is present along the entire axoneme. The same antiserum coimmunoprecipitated p28, actin, and dynein heavy chains 2′ and 2. In contrast, an anti-caltractin/centrin antiserum coimmunoprecipitated caltractin/centrin, actin, and the heavy chains 2, 3, and 3′. It is likely that the dynein heavy chain 2 associated with p28, referred to as 2A, is a different polypeptide from dynein heavy chain 2 bound to caltractin/centrin, referred to as 2B. The complex formed by heavy chain 2B, actin, and caltractin/centrin is preferentially extracted by exposure to Nonidet P-40 and is missing in mutants lacking components 1 and 2 of the dynein regulatory complex.

INTRODUCTION

The bending of flagellar axonemes results from the activation of the dynein arms, mechanochemical structures found between the outer doublet microtubules. The dynein arms cause the doublet microtubule to which they are permanently attached to slide toward the minus end of the adjacent doublet (Fox and Sale, 1987; Sale and Satir, 1977). To generate axonemal waveforms, the activation of dynein arms must be tighly regulated for the following reasons: 1) the creation and propagation of the principal and reverse bends require a differential activation of the arms along the axoneme; 2) because the arms generate only minus-end–directed movement, topological reasons dictate that no sliding could occur without a radial regulation of the arms; 3) finally, the generation of a regular beat requires a temporal regulation.

The analysis of the movement of *Chlamydomonas* mutants lacking inner or outer dynein arms suggests that the control of the waveform is effected primarily through the inner dynein arms (Brokaw and Kamiya, 1987). Understanding how inner dynein arms are regulated is therefore crucial to the elucidation of the mechanism of flagellar movement. Two characteristics of inner dynein arms are essential in regard to their control: 1) there are more than five different types of inner dynein arms in the axoneme (Kagami *et al.*, 1990; Piperno *et al.*, 1990; Kamiya *et al.*, 1991; Kagami and Kamiya, 1992; Kato *et al.*, 1993) in contrast to outer dynein arms, whose molecular composition is homogeneous; and 2) inner dynein arms interact with a group of seven regulatory proteins, the "dynein regulatory com-

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† One of the I2 and I3 inner dynein arms light chains is referred to as caltractin/centrin (Huang *et al.*, 1988; Piperno *et al.*, 1992).

plex" (DRC),[1] that affects the binding to the axoneme and the activity of the dynein heavy chains (DHCs)‡ (Piperno *et al.*, 1992, 1994).

Inner dynein arms were grouped into three classes, named I1, I2, and I3, based on their longitudinal position relative to the radial spokes (Piperno *et al.*, 1990). Each 96-nm longitudinal segment of the axoneme contains one arm of each class. I1 arms are proximal to radial spokes S1, and I2 arms are located between radial spokes S1 and S2, whereas I3 arms are distal to radial spokes S2.

All I1 arms have the same composition: they contain two DHCs (1α and 1β), and light chains of molecular weight (MW) 140,000 and 110,000 (Piperno *et al.*, 1990; Porter *et al.*, 1992). The composition of inner dynein arms I2 and I3 is less well known, and varies along the length of the axoneme (Piperno and Ramanis, 1991). They are composed of the DHCs 2′, 2, 3, and 3′, and of four light chains: an 86,000 MW protein, actin, caltractin/centrin, and a 28,000 MW protein, p28. After extraction from the axonemes, the DHCs from inner dynein arms I2 and I3 are recovered in protein complexes with sedimentation coefficients near 11S. Each of these complexes contains a single DHC (Piperno *et al.*, 1990).

The diversity of I2 and I3 inner dynein arms may be greater than previously recognized. Although current electrophoretic techniques separate their DHCs into four bands (2′, 2, 3, and 3′), each band may contain several proteins. In addition, individual arms may contain various subsets of light chains. Separation of inner dynein arms by ion-exchange high pressure liquid chromatography suggested that there are at least five types of I2 and I3 arms (Kagami *et al.*, 1990; Kato *et al.*, 1993). The complexity of the composition of the I2 and I3 inner dynein arms is illustrated by the fact that the mutants *pf22*, *pf23*, *ida4*, *ida5*, and *ida6* are defective for five overlapping but different subsets of heavy or light chains (Kagami *et al.*, 1990; Kamiya *et al.*, 1991; Piperno and Ramanis, 1991; Kato *et al.*, 1993).

The binding of I2 and I3 inner dynein arms to the axoneme depends on the integrity of the DRC, a group of seven proteins, nonextractable by exposure to 0.55 M NaCl in the presence of ATP. Defects in the DRC also cause inefficient axonemal movement and suppress the paralysis of central pair and radial spoke mutants (Huang *et al.*, 1982; Piperno *et al.*, 1992, 1994). This suggests that radial spokes and central pair projections control the activity of the inner dynein arms through the DRC (Piperno *et al.*, 1992; Smith and Sale, 1992).

[1] Abbreviations used: DHC‡, dynein heavy chain; DRC, dynein regulatory complex; MW, molecular weight; PCR, polymerase chain reaction.

‡ The DHCs' nomenclature is based on their electrophoretic mobility as described by Piperno *et al.* (1992).

Here we present the results of a new study of I2 and I3 inner dynein arms that we initiated through the analysis of the light chain p28. Three main reasons led us to study p28: 1) p28 forms complexes with DHCs, actin, and caltractin/centrin, molecules whose functions were described. In contrast, the function of p28 was unknown and could be derived from its amino acid sequence. 2) p28 is missing in the inner dynein arm mutant *ida4* (Kamiya *et al.*, 1991; Piperno *et al.*, 1992). If p28 is the gene product of the *IDA4* locus, its function could be deduced from an analysis of the *ida4* mutant cells. 3) Finally, we wanted to determine whether p28 was found in association with all or only a subset of the DHCs 2′, 2, 3, and 3′.

Our results showed that only a subset of I2 and I3 inner arm DHCs bind to p28, and led us to revise the model of the organization of the inner dynein arm row.

MATERIALS AND METHODS

Strains Used, Cell Culture Conditions, and Axoneme Preparation

All cells were grown in liquid R medium (Harris, 1989) supplemented with 10 mM Tris-HCl, pH 7.0, at 25°C in constant light. For large scale cultures in 9-liter jars, the medium was aerated with a mixture of 5% CO_2 in air. Cells from large-scale cultures were concentrated 30-fold by tangential flow filtration with a Pellicon Cassette system (Millipore, Bedford, MA) and then collected by centrifugation.

To simplify the purification process of p28, we chose to use the strain *pf28pf30ssh1*, which lacks outer dynein arms and inner dynein arms I1 and has flagella of wild-type length. The mutation *ssh1* (for suppressor of shortness) suppresses the short-flagella phenotype of the parent strain *pf28pf30* without correcting its dynein deficiencies (Piperno, unpublished result). *pf28pf30ssh1* cells were grown in batches of 29 liters to a final concentration of 10^7 cells/ml.

Axoneme purification and separation of dynein arms was performed by scaling up a procedure previously described (Piperno and Ramanis, 1991). The dynein arms were separated by centrifugation in a 35-ml 5–20% sucrose gradient at 23,000 rpm for 40 h, in a SW27 rotor at 4°C. An aliquot of each fraction was analyzed by gel electrophoresis after trichloroacetic acid precipitation: ice-cold 100% trichloroacetic acid (w:v) was added to the aliquot to a final concentration of 10%. After 10 min on ice, the precipitate was collected by centrifugation in an Eppendorf centrifuge, washed briefly with ice-cold acetone, and redissolved in sodium dodecyl sulfate (SDS) electrophoresis buffer. The fractions containing the I2 and I3 inner dynein arms were identified by the presence of high MW chains, actin, p28, and caltractin/centrin. They were pooled and constitute the "I2-I3 pool" referred to in the text.

The composition of the I2-I3 pool was identical to that shown previously (Piperno *et al.*, 1990). The major components are caltractin/centrin (MW 19,000), p28 (MW 28,000), actin (MW 42,000), tubulin (MW 55,000), and the DHCs (MW > 450,000). The 28,000 MW band was used as our starting material. Analysis of the I2-I3 pool by two-dimensional gel electrophoresis showed that it contains actually two proteins of MW 28,000. The major protein p28 is accompanied by a small amount of a more acidic protein, p28*, whose pI is approximately 0.4 lower. p28* likely is a post-translationally modified form of p28: 1) the amount of p28* in different mutants varies in proportion to the amount of p28; 2) p28 and p28* are both missing in the mutant *ida4* (Piperno *et al.*, 1992); and finally

3) p28* is recognized by the antibody we prepared against p28 (see below).

The Nonidet P-40 extraction of axonemes was as described (Piperno and Ramanis, 1991).

Protein Analysis

SDS gel electrophoresis was performed as described (Piperno, 1988; Piperno *et al.*, 1990, 1992). Four to eleven percent polyacrylamide gels were used throughout except that 7.5–15% polyacrylamide gels were used to separate p28 proteolytic fragments. Two-dimensional gel electrophoresis was as described (Piperno *et al.*, 1981, 1992).

Proteins were transferred electrophoretically to nitrocellulose or Immobilon membranes (Millipore) in a buffer consisting of 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11.0. Use of this buffer improved the transfer of p28 when compared with the Tris-glycine-SDS buffer we used previously (Piperno and Fuller, 1985). After transfer, the membranes were stained with 0.1% Ponceau S Red in 1% acetic acid, washed in several changes of distilled water, and allowed to air dry.

To partially digest p28 with V8 protease, the proteins of the I2-I3 pool were separated by electrophoresis and transferred to nitrocellulose. After staining with Ponceau S Red, the p28 band was cut out and the protein was eluted by boiling the membrane for 45 s in a small vol of 33 mM Tris-HCl, pH 8, 0.1% β-mercaptoethanol, 0.4% SDS. Approximately 10 μg of p28 in a vol of 85 μl was digested with 0.5 μg of V8 protease at 32°C for 2 h.

All polypeptides to be sequenced were transferred to Immobilon. The membrane was stained with 0.1% Coomassie blue in 50% methanol, 10% acetic acid and destained in 50% methanol, 10% acetic acid. The portion of the membrane containing the protein to be sequenced was excised. The amino acid sequencing was performed by Dr. Ronald Kohanski of the Mount Sinai School of Medicine. Amino acid sequences were determined by sequential Edman degradation. The initial sequencing yields were 4–8 pmol.

Western blot analysis was performed using a chemiluminescence kit from Amersham (Arlington Heights, IL), following the manufacturer's instructions. The primary antiserum was typically diluted 5,000-fold, and the secondary antibody was diluted 10,000-fold.

RNA and DNA Analysis

Standard molecular biology techniques were used throughout. All enzymes were from New England Biolabs (Beverly, MA) unless noted otherwise.

RNA was purified as described (Wilkerson *et al.*, 1994). The cells were deflagellated by the pH shock procedure, collected by centrifugation, and resuspended in R medium at 25°C with stirring in constant light. Cells were harvested and RNA was prepared 45 min after resuspension, at which point the flagella had regrown to approximately two-thirds of their original length. Polyadenylated RNA was purified by chromatography on prepacked oligo(dT) columns (Life Technologies, Gaithersburg, MD) following the manufacturer's instructions.

Chlamydomonas genomic DNA was purified as described (Walther *et al.*, 1994). Restriction digestions of genomic DNA were performed in a vol of 100 μl containing 5 μg of DNA and 50 U of the endonuclease. Incubation was at 37°C for 12–16 h.

RNA and DNA were blotted to GeneScreen Plus by capillarity following the membrane manufacturer's directions. The probe D16/Eco, which was used for all Northern and Southern blot analyses, corresponds to nucleotides 779-1335 of the sequence shown on Figure 2. D16/Eco was gel purified after digestion of clone D16 (see RESULTS section) with *Eco*RI and it was radioactively labeled by random-priming using the Prime-It II kit from Stratagene (La Jolla, CA). Prehybridizations and hybridizations were performed at 42°C in a solution containing 50% formamide. The blots were washed to a final stringency of 0.5× SSC, 0.1% SDS at 65°C. The radioactivity bound to the blots was detected and quantified with a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Analysis of the p28 gene nucleotide sequence made use of the GCG software package (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, Madison, WI).

Chlamydomonas DNA sequences found in the the GenBank database were analyzed using the GCG software package. Codon usage data were further analyzed using a custom-designed Microsoft Excel spreadsheet.

Polymerase Chain Reaction (PCR)

PCR was performed in a Perkin-Elmer Cetus Cycler Model 480, using Perkin-Elmer Cetus 500 μl tubes and Perkin-Elmer Cetus *Taq* polymerase (Norwalk, CT). The reaction mix contained 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 50 pmol of each primer. Mineral oil was used in the early stages of this work. Better yields and specificity were obtained later when AmpliWax PCR Gem 100 wax beads (Perkin-Elmer Cetus) were used instead of mineral oil. Typical cycling parameters were: 3 min at 95°C, followed by 30–40 cycles of 45 s at 95°C, 1 min 30 s at the hybridization temperature (48–55°C), and 3–5 min at 72°C. If the PCR products were to be cloned, this was followed by a one-time 10-min incubation at 72°C.

PCR products were cloned into the *Sma*I site of pBlueScriptII (Stratagene) by the "double Gene-Clean" procedure described in the manufacturer's instructions for GeneClean II (Bio 101, Vista, CA). Briefly, the fragments to be cloned are purified from an agarose gel using GeneClean II, repaired with DNA polymerase I Klenow fragment, and phosphorylated with T4 polynucleotide kinase. They were then purified again with GeneClean II and ligated to the vector.

The inverse PCR reaction was performed as described (Ochman *et al.*, 1989). Southern blot analysis had shown that the p28 gene is carried by a 2.9-kb *Kpn*I fragment. *Chlamydomonas* genomic DNA was digested to completion with *Kpn*I and subjected to agarose gel electrophoresis. The fragments of the right size class were purified using GeneClean II, and diluted to a final concentration of 240 ng of DNA in 140 μl of 1× ligation buffer. Eight hundred units of T4 DNA ligase were added and the mixture was incubated for 4.5 h at room temperature. An aliquot (containing approximately 30 ng of DNA) was used as template in a PCR reaction using the primers 100 and 1163. A product of the expected size (1.2 kb) was obtained and cloned into the *Sma*I site of pBlueScript.

The primers used were 14–26 nucleotides long. Primers mentioned in this paper are as follows: R stands for A or G; Y stands for C or T; and I stands for inosine. Their position in the sequence shown in Figure 2 is indicated between parentheses. A (+) indicates that the sequence is that of the coding strand. A (−) means that the sequence is that of the noncoding strand.

Y9203108: GAGAGAGAACTAGTCTCGAG$(T)_{18}$
Y9112147: GAGAGAGAACTAGTCTCGAGT

The T_{18} portion of Y9203108 hybridizes to the poly(A) tail of mRNAs and is used as a primer for cDNA synthesis. The cDNAs can then be amplified using Y9112147 and a gene-specific primer.

N3 (+): ATGATYCCICCICTITCITCICTIGT (482–507)
100 (−): TCTTGCCCGGCGTGCCTTTG (581–562)
ANT157 (−): GGCAGAATAGAGTTTAG (721–705)
350 (+): ATGAGCTCATCCGCCAGGTGACCAT (1051–1075)
fi1 (−): ACRTCICCCTCIAGCTGIGTRATICG (1357–1332)
1163 (+): CCTGGCGGTTGGTT (2294–2303)
1218 (−): GTTGCGGTCACGCCCCTTGCGTTGC (2341–2317)

DNA Sequencing

All DNA sequencing was performed with a Sequenase kit, version 1.0 or 2.0 (Amersham), using protocols provided by the manufacturer. Because all the DNA sequenced was synthesized by the *Taq* polymerase, care was taken to ensure that the sequence we deter-

mined was free of PCR mistakes. Several isolates of the cloned products were sequenced, the sequence of the genomic DNA was compared with that of the cDNA, and finally, uncloned PCR products were resequenced to definitely rule out cloning artifacts and PCR mistakes. To sequence uncloned PCR products, 0.5 pmol of purified DNA was mixed with 2.5 pmol of a given sequencing primer in a final vol of 10 μl of water and incubated at 100°C for 3 min before being cooled rapidly in an ice bath. This annealed template was then processed as usual for the sequencing reactions. Thirty percent formamide sequencing gels were sufficient to resolve most compressions. In one case (nucleotides 1855–1880), the sequencing reactions had to be performed in the presence of dITP.

Preparation of an Antiserum against Escherichia coli–synthesized p28

The preparation of the p28-maltose binding protein (MBP) fusion protein, its expression in XL1Blue cells, and its purification and cleavage with factor Xa were all performed as recommended by the manufacturer (New England Biolabs). A DNA fragment containing the quasi-complete cDNA sequence of p28 (clone R16; see the RESULTS section) was excised from pBlueScriptII by digestion with *Hin*dIII and *Bam*HI and ligated to the plasmid pMALc2 (New England Biolabs) digested with the same enzymes. Both DNA pieces were purified with GeneClean II before ligation. This cloning procedure resulted in the presence of seven extraneous amino acids ahead of the N-terminal methionine of p28. Except for this minor modification, the protein synthesized by the bacteria should have had precisely the same sequence as true p28.

The fusion protein expressed had the expected MW of 70,000 (the MBP has an MW of 42,000 and p28 has an MW of 28,000). The fusion protein was soluble and represented approximately 50% of the total cell protein after induction by IPTG. It was purified by affinity chromatography on amylose and cleaved with factor Xa. Two products (MBP and p28) were obtained, with the correct molecular weights. p28 was separated from MBP by adsorption chromatography on a 1-ml hydroxyapatite column: after the digestion with factor Xa, the digestion mix was applied to the column, which was then washed extensively with 50 mM Tris-HCl, pH 8, 100 mM NaCl. The proteins were then eluted by applying a 6-ml, 0.025–0.5 M sodium phosphate gradient at pH 6.8. The vast majority of p28 eluted before MBP. Fractions containing p28 were applied to an amylose column to remove the last traces of MBP. An aliquot of purified p28 was analyzed by SDS-polyacrylamide gel electrophoresis; no remaining MBP was detectable by Coomassie blue staining.

A rabbit was first immunized with 300 μg of *E. coli*–synthesized p28 mixed with Freund's complete adjuvant. The rabbit was then reinjected monthly with 300 μg aliquots of purified p28 mixed with incomplete adjuvant. The first positive serum was obtained 2 mo after the first injection and the final bleeding occurred 6 mo after the first injection.

Immunofluorescence Localization of p28

Immunofluorescence staining of *Chlamydomonas* axonemes was performed as previously described (LeDizet and Piperno, 1986), with two modifications: 1) cells were fixed in 3% formaldehyde, 0.1 M sodium phosphate, pH 6.8, before deposition on glass coverslips; and 2) the fluorescent signal was detected by confocal microscopy.

Immunoprecipitation of Dynein Arm Heavy Chains

^{35}S-labeled axonemes (Piperno and Ramanis, 1991) were suspended in 0.1 M Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 1.5 mM EGTA. The dynein arms were detached from the axonemes by exposure to 0.55 M NaCl in the presence of Mg^{2+} and ATP as described (Piperno *et al.*, 1994). The insoluble material was separated from the "high salt extract" by centrifugation at 14,000 rpm in an Eppendorf centrifuge.

Five to twenty-five microliters of high salt extract were used per reaction, in a total vol of 40 μl containing 25 mM Tris-HCl, pH 7.5, and 0.5% Nonidet P-40. Antiserum (0.5 μl) was added and the reaction was incubated for 1 h at 4°C with gentle mixing. Thirty microliters of Protein A-agarose beads (Boehringer Mannheim, Indianapolis, IN) per reaction were washed three times in 1.0 ml of wash solution (150 mM NaCl, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 7.5), resuspended in their original volume of wash solution, and added to the reaction mixture. After a 1-h incubation as before, the beads were collected by a 10 s centrifugation in an Eppendorf centrifuge and washed four times for 20 s in wash solution. The proteins bound to the beads were detached by boiling in 10–50 μl of SDS electrophoresis buffer (1.1% SDS, 40 mM Tris borate, pH 8.65, 1 mM EDTA, 1% [v:v] β-mercaptoethanol, 6% sucrose, 0.02% bromophenol blue), and the stripped beads were pelleted by centrifugation. The supernatant was analyzed by electrophoresis on SDS polyacrylamide gels. The dried electrophoretograms were analyzed quantitatively with a PhosphorImager (Molecular Dynamics).

RESULTS

Isolation of Overlapping PCR Clones Spanning the p28 Gene

Our first goal was to isolate and analyze the p28 gene to characterize the protein referred to as p28. To this effect, we followed a three-step procedure: 1) we determined two partial amino acid sequences from purified p28; 2) we obtained a quasi-full length cDNA clone; and 3) we isolated overlapping clones containing the entire p28 gene. Figure 1 shows the position of these clones within the p28 gene.

To determine the N-terminal sequence of p28, we separated the proteins of the I2-I3 pool by one-dimen-

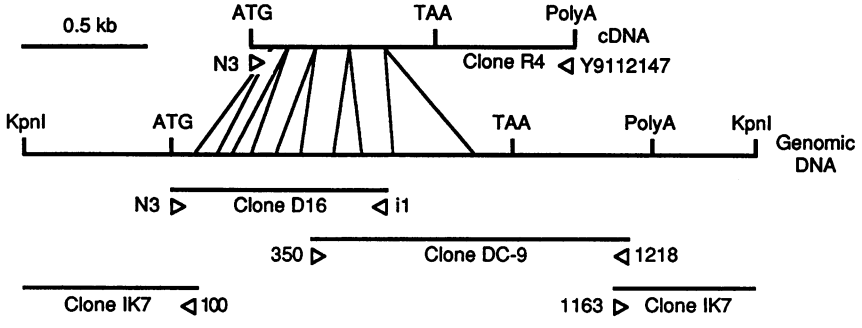

Figure 1. Schematic map of the p28 gene and the PCR clones R4, D16, DC-9, and IK7. Features shown are: ATG, the putative initiation codon; TAA, the termination codon; PolyA, the polyadenylation site; *Kpn*I, the *Kpn*I sites at either end of the sequence shown in Figure 2. The primers used are named under the corresponding clones.

sional gel electrophoresis and transferred the proteins to an Immobilon membrane, which was then stained. We obtained the following amino acid sequence from the p28 band: MIPPLSSLVRYDNXXLK (X indicates an amino acid that could not be identified). Therefore the N-terminus of p28 is partially or totally unblocked.

We then subjected p28 to a partial digestion with V8 protease, separated the proteolytic fragments by gel electrophoresis, and transferred them to an Immobilon membrane. Two main products were visible after Coomassie blue staining: one had an MW of 28,000 and its N-terminal sequence was identical to that of p28. The other fragment had an apparent MW of 12,000 and its N-terminal sequence was SRITQLEGDVXLLERQVQE. Therefore, the first product was uncut p28 and the second was an internal proteolytic fragment of p28.

To obtain a quasi-full length cDNA clone of p28, we designed the primer N3 corresponding to the amino acids MIPPLSSLV, which are found at the N-terminus of the mature p28 protein. We reverse transcribed polyadenylated RNA extracted form wild-type cells 45 min after deflagellation, using Y9213108 as primer. The subsequent PCR amplification used an aliquot of the cDNAs synthesized as template and the primers N3 and Y9112147.

The largest PCR amplification product had a length of 1.4 kb. We cloned it into pBlueScriptII and determined its nucleotide sequence. The two isolates mentioned in this report are R4 and R16. Their nucleotide sequences are identical. The first 26 nucleotides of these clones are derived from the sequence of N3. The rest of the sequence represents a quasi full-length p28 cDNA. Three arguments show that this clone was indeed derived from a p28 cDNA: 1) it contains an open reading frame encoding a protein of the correct molecular weight and isoelectric point; 2) immediately after the N3 sequence, it contains a DNA sequence coding for the amino acids that were determined experimentally from the N-terminus of p28; 3) finally, it contains an internal DNA sequence coding for the amino acid sequence of the internal V8 peptide derived from p28.

We then determined the nucleotide sequence of the genomic region encoding p28. The sequence of a 2922 nucleotide *Kpn*I fragment carrying the p28 gene is shown in its entirety on Figure 2. It was derived from the sequences of three overlapping clones, D16, DC-9, and IK7, whose positions are indicated in Figure 1.

The clone D16 was obtained by PCR amplification of wild-type genomic DNA with the primers N3 and I1. Primer I1 is complementary to the nucleotide sequence encoding amino acids RITQLEGDV found in the p28 internal proteolytic peptide. The clone D16 has a length of 854 nucleotides, corresponding to nucleotides 482-1335 of the sequence shown in Figure 2.

The clone DC-9 covers the 3′ region of the gene. It was obtained by PCR amplification of genomic DNA using two primers (350 and 1218) derived from the cDNA sequence found in clone R4. DC-9 has a length of 1291 nucleotides, corresponding to nucleotides 1051–2341 of the sequence shown in Figure 2. The sequence of the clones D16 and DC-9 is identical to that of R4 (with the addition of five introns), which confirms the accuracy of the R4 sequence.

To characterize the regions immediately upstream and downstream from the previous clones, we made use of the inverse PCR technique (see MATERIALS AND METHODS). The clone obtained (IK7) contains nucleotides 1–581 and 2294–2922.

Structure of the p28 Gene and Analysis of the Encoded Protein

The methionine found at the N-terminus of the mature p28 protein is encoded by the ATG codon at position 482. This ATG is preceded by an in-frame stop codon at position 446, and there is no other ATG between nucleotide 446 and 482. Although this strongly suggests that the ATG at position 482 is the initiation codon, there could be an intron between nucleotides 446 and 482, and the mature p28 could be derived from a slightly larger protein. The size of the p28 mRNA is approximately 1.45 kb (shown in the accompanying paper). Two polyadenylation sites were identified by cDNA sequencing, at positions 2459 and 2475, downstream from a canonical *Chlamydomonas* polyadenylation signal (TGTAA) at position 2442. Barring the presence of an intron upstream of the initiation codon, this would place the transcription start site near nucleotide 380.

Comparison of the genomic DNA sequence with that of cDNAs indicated that the coding sequence of p28 is interrupted by five introns, at positions 584, 728, 912, 1149, and 1377. The lengths of the introns are 67, 69, 84, 105, and 321 nucleotides, respectively. The sequences of the splice sites conform to the eukaryotic consensus.

The nuclear genes of *Chlamydomonas reinhardtii* exhibit a well-documented bias favoring codons ending in G or C. Table 1 shows a codon usage table for all *C. reinhardtii* nuclear genes found in the GenBank database (as of 1/22/95, 95 sequences, 36787 codons) and the codon usage of the p28 gene (254 codons). Clearly, the codon usage of the p28 gene is biased in a fashion that is similar to that of other *Chlamydomonas* nuclear genes. The bias is, however, less strict than in previously sequenced genes (see for example the amino acids D and G).

We studied this codon usage bias quantitatively by calculating the "standardized synonymous codon

Figure 2. Sequence of a 2.9-kb *Kpn*I fragment carrying the gene encoding p28 of *C. reinhardtii*. The conceptual translation of the exons is provided below the nucleotide sequence. The amino acids underlined were determined experimentally from purified p28. The sequence is available from GenBank/EMBL databases (accession number Z48059).

bias'' (B) (Long and Gillespie, 1991) of nuclear protein-coding *Chlamydomonas* genes found in the GenBank database. To ensure the validity of the evaluation of B, we analyzed only sequences longer than 94 codons (89 sequences total). B varies from 0 (no usage bias) to 1 (only one codon is used per amino acid.) B is essentially independent of the nature of the bias or of the amino acid composition. Figure 3 shows how many *Chlamydomonas* nuclear sequences have a given B value. The B value for p28 is 0.316, which means that the p28 gene is the least biased of all *Chlamydomonas* nuclear sequences determined until now. In the case of *Chlamydomonas*, we also found that the codon usage bias favors codons ending in C, while selecting against codons ending in A or T. Using the fraction of codons ending in G or C as a measure of codon usage bias also shows that the p28 gene is among the least biased of all *Chlamydomonas* genes in its codon usage.

Southern blot analysis showed that there is only one p28 gene in the *Chlamydomonas* genome. Figure 4 shows the hybridization pattern obtained with the D16/Eco probe (see MATERIALS AND METHODS). The pattern is typical of a single copy gene.

p28 Is a Novel Protein Containing a Large α-Helical Domain

The DNA sequence shown in Figure 2 encodes a protein of MW 28,652 with a pI of 7.13, in good agreement with the mobility of p28 in two-dimensional gel electrophoresis. The protein encoded contains the two amino acid sequences that were determined experimentally from purified p28 (underlined in Figure 2). In each case, one amino acid is different from that determined experimentally. Although this could reflect a difference between wild-type and *pf28pf30ssh1*

Table 1. Codon usage bias in the p28 gene

Aa	Codon	C.r.	p28	Aa	Codon	C.r.	p28	Aa	Codon	C.r.	P28
A	GCG	26.8	23.8	K	AAG	98.6	85.7	S	AGT	1.8	9.1
	GCA	5.0	23.8		AAA	1.4	14.3		AGC	28.5	45.5
	GCT	15.2	9.5	L	TTG	2.0	3.8		TCG	30.2	18.2
	GCC	53.0	42.9		TTA	0.2	0.0		TCA	2.1	0.0
C	TGT	4.2	0.0		CTG	81.6	57.7		TCT	8.4	18.2
	TGC	95.8	100.0		CTA	1.5	19.2		TCC	29.0	9.1
D	GAT	12.6	45.5		CTT	3.5	0.0	T	ACG	22.6	43.8
	GAC	87.4	54.5		CTC	11.3	19.2		ACA	3.4	6.3
E	GAG	97.5	96.3	N	AAT	5.3	20.0		ACT	9.6	12.5
	GAA	2.5	3.7		AAC	94.7	80.0		ACC	64.3	37.5
F	TTT	11.7	25.0	P	CCG	23.9	42.9	V	GTG	68.7	53.3
	TTC	88.3	75.0		CCA	3.7	14.3		GTA	1.0	6.7
G	GGG	3.4	21.4		CCT	10.9	14.3		GTT	5.7	13.3
	GGA	2.1	21.4		CCC	61.6	28.6		GTC	24.6	26.7
	GGT	14.8	7.1	Q	CAG	94.8	85.7	Y	TAT	5.4	14.3
	GGC	79.7	50.0		CAA	5.2	14.3		TAC	94.6	85.7
H	CAT	8.7	0.0	R	AGG	3.3	5.9	End	TGA	4.5	0.0
	CAC	91.3	100.0		AGA	0.5	0.0		TAG	18.2	0.0
I	ATA	0.8	8.3		CGG	8.3	23.5		TAA	77.3	100.0
	ATT	23.9	41.7		CGA	1.2	0.0				
	ATC	75.3	50.0		CGT	10.3	17.6				
					CGC	76.4	52.9				

The column C.r. shows the relative frequency of the different codons for each amino acid in all *Chlamydomonas reinhardtii* nuclear sequences present in the GenBank database (95 sequences, 36787 codons as of 1/22/95). The column p28 shows the relative frequency of the different codons in the gene encoding p28 (254 codons).

proteins, it is more likely due to mistakes made during the protein sequencing process.

The internal protein sequence that we determined experimentally begins at amino acid number 175. The p28 proteolytic fragment whose N-terminus was sequenced was therefore generated by a V8 protease cut after the glutamate residue at position 174. The fragment containing the residues 175–253 has an MW of 9,100, well below that predicted from its electrophoretic mobility (12,000). This discrepancy may be due to its basic isoelectric point (predicted pI of 8.4) or because its high α-helical content causes it to adopt an extended configuration; computer-based predictions of secondary structure suggest that the residues 87–119, and 153–253 are likely to adopt an α-helical configuration. The algorithm of Lupas *et al.* (1991) further indicates that the residues 161–207 and 210–237 have a probability greater than 0.99 of forming a coiled coil structure (using a window of 28 residues).

Homology searches between p28 and previously sequenced proteins indicated that p28 is a novel protein, not previously studied. The C-terminal part of p28 contains many short stretches with high homology to α-helical domains found in proteins such as myosin heavy chains or α-tropomyosin; however, this is likely due to structural constraints rather than to a common function or origin. In particular, p28 does not appear to contain any of the motifs that have been implicated in binding to tubulin, actin, or Ca^{2+}. One region that may function as a tubulin-binding motif is a stretch of basic residues at positions 23–34. This 12-residue stretch harbors five positive charges, and might mediate an interaction with the acidic protein tubulin. Interestingly, the N-terminus of caltractin/centrin contains a similar positively charged stretch; in *Chlamydomonas* caltractin/centrin (Huang *et al.*, 1988), residues 4–19 carry six net positive charges. In *Scherffelia dubia* (Bhattacharya *et al.*, 1993) and human (Lee and Huang, 1993) caltractin/centrins, the positive charge (+6) of this region is preserved, although the amino acid sequence is different.

p28 Is Located along the Entire Length of the Axoneme

We prepared an antiserum against p28, to confirm the identity of the gene we isolated, to localize p28 within the axoneme, and to determine the composition of the dynein arms containing p28.

The low abundance of p28 in axonemes precluded purifying enough protein to use as an immunogen. Instead, we synthesized a bacterial maltose-binding protein-p28 fusion protein and cleaved it with factor Xa (see MATERIALS AND METHODS). The p28 part of the fusion protein was used to immunize a rabbit.

To test the specificity of the antiserum obtained, we separated axonemal proteins by one-dimensional gel

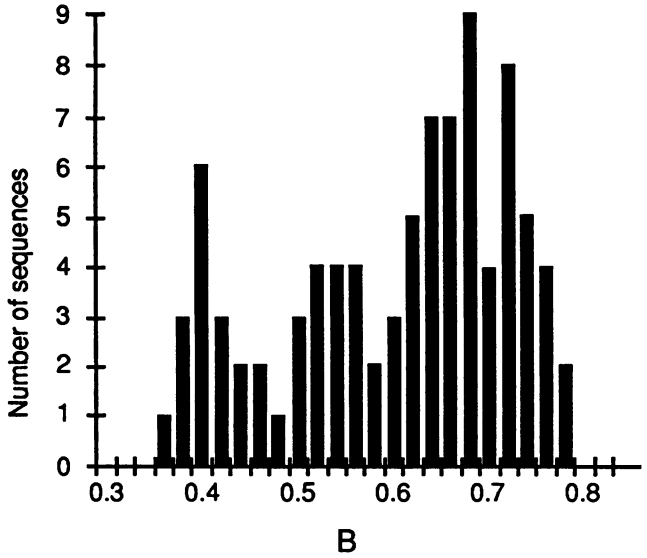

Figure 3. Codon usage bias in nuclear genes of *C. reinhardtii*. The "standardized synonymous codon bias" B (Long and Gillespie, 1991) was evaluated for all previously sequenced protein-coding nuclear genes of *C. reinhardtii* longer than 94 nucleotides (89 sequences as of 1/22/95). A highly biased codon usage bias is reflected by a high value of B, which varies from 0 to 1. Shown here is the number of sequences having a given B value. The p28 gene has a B value of 0.316.

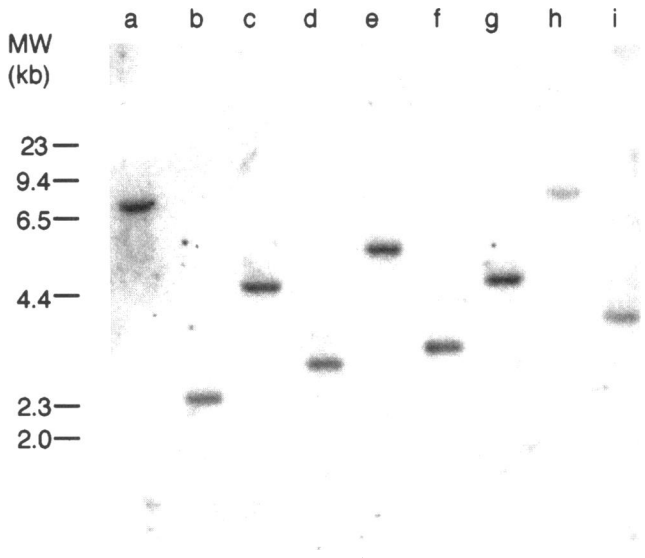

Figure 4. Southern blot analysis of the p28 gene. Five micrograms (per lane) of *C. reinhardtii* DNA was digested and blotted as described in MATERIALS AND METHODS. The blot was hybridized with the D16/Eco probe and washed at high stringency. Lanes a–i contain DNA that was digested with, respectively, *Apa*I, *Apa*LI, *Eco*RV, *Hin*dIII, *Hin*cII, *Kpn*I, *Sal*I, *Stu*I, and *Sty*I. The position of the MW markers is indicated on the left.

electrophoresis, transferred them to Immobilon, and immunostained them with the anti-p28 antiserum. Figure 5 (panel B, first lane) shows that the antiserum recognizes a single protein, with an MW of 28,000. After two-dimensional gel electrophoresis, the antibody detects two proteins with an MW of 28,000. Their location in the electrophoretogram clearly identifies them as p28 and p28*. As expected, no reaction is observed with proteins prepared from *ida4* axonemes (Figure 5, panel B, second lane). These results together prove that the antiserum specifically recognizes p28 in *Chlamydomonas* axonemes and confirm that the gene isolated is indeed that of p28.

We used the anti-p28 antiserum to localize p28 by immunofluorescence and found that it was present along the entire length of *Chlamydomonas* axonemes. Within the cell body, the only region stained was the anterior part of the cell surrounding the basal bodies. No staining was observed in *ida4* cells, which confirms the specificity of the antibody reaction. No staining was observed if either the primary or the secondary antibody was omitted.

Association with p28 or Caltractin/Centrin Defines Different Sets of Inner Dynein Arm Heavy Chains

To determine whether p28, caltractin/centrin, and actin were associated with every DHC found in I2 and I3 arms, we used the antiserum against p28 to immunoprecipitate protein complexes derived from inner dynein arms of wild-type and *ida4* axonemes. Nearly all dynein arms are severed from the axonemes by exposure to high salt in the presence of Mg^{2+} and ATP. After this procedure, the heavy chains of I2 and I3 arms are found in complexes with sedimentation co-

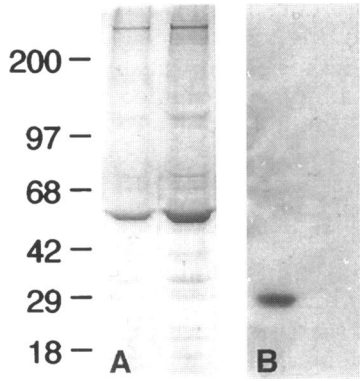

Figure 5. Western blot analysis of axonemal proteins with an antiserum against p28. Panel A shows a Coomassie blue–stained electrophoretogram of axonemal proteins prepared from wild-type cells (first lane) and *ida4* cells (second lane). Panel B shows the chemiluminescent signal obtained after immunostaining of identical samples blotted to an Immobilon membrane. The position of the MW markers (in kilodaltons) is indicated on the left.

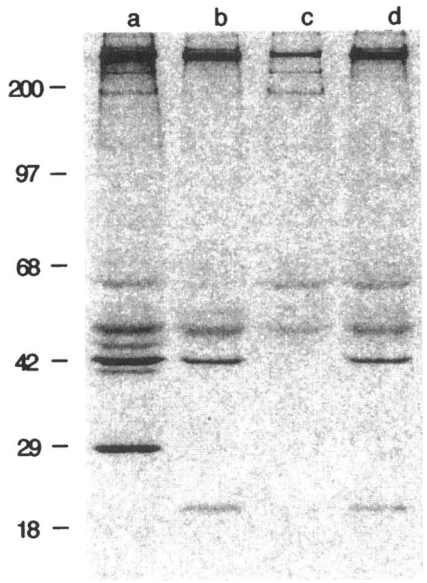

Figure 6. Low MW components of the dynein complexes immunoprecipitated with the antisera against p28 (lanes a and c) or against caltractin/centrin (lanes b and d). The complexes analyzed were isolated from radioactively labeled wild-type (lanes a and b) or *ida4* cells (lanes c and d). The proteins in the immunoprecipitates were separated by electrophoresis on a 4–11% gradient polyacrylamide gel, which did not resolve dynein heavy chains from one another. The position of MW markers (in kilodaltons) is indicated on the left.

efficients near 11S. Each of these complexes contains one DHC.

Lane a of Figure 6 shows the composition of the immunoprecipitate from wild-type dyneins as analyzed by electrophoresis on a 4–11% polyacrylamide gel. The DHCs are not resolved from one another on this type of gel. The anti-p28 antiserum precipitated p28, actin, and DHCs, but not caltractin/centrin.

To determine whether caltractin/centrin was dissociated from the heavy chains during the precipitation, we applied an anti-caltractin/centrin antiserum (kindly provided by Dr. Bessie Huang, Scripps Research Institute, La Jolla, CA) to a second aliquot of the wild-type dynein extract. The anti-caltractin/centrin antiserum precipitated caltractin/centrin, actin, and DHCs but not p28 (Figure 6, lane b). This result proves that p28 and caltractin/centrin are part of different protein complexes in the dynein extract. When dynein arm components from the mutant *ida4* were immunoprecipitated with the anti-p28 antiserum, only traces of tubulin and other proteins contaminating the immunoprecipitate are seen (Figure 6, lane c). This was expected because *ida4* is devoid of p28. The anti-caltractin/centrin antibody precipitated a seemingly identical set of proteins as the wild-type strain (Figure 6, lane d).

Small amounts of unidentified proteins can be seen in Figure 6, lane a. These are likely to represent proteolytic fragments of DHCs that are formed during the immunoprecipitation. They are missing in Figure 6, lane c, which contains only traces of DHCs. If these proteins were actual components of the p28-containing complexes, they should be detectable in the "I2-I3 pool." Our unpublished results and those of Piperno *et al.* (Figure 2 of Piperno *et al.*, 1990) indicate that this is not the case.

We estimated the stoichiometry among the immunoprecipitated proteins by assuming that all components are labeled by ^{35}S to the same specific radioactivity. We then calculated the ratio of the radioactivity contained in p28, caltractin/centrin, actin, and DHCs after normalization for the sulphur content of each protein. p28 contains seven atoms of sulphur (this work) whereas the caltractin/centrin mRNA encodes a protein containing eight atoms of sulphur (Huang *et al.*, 1988). *Chlamydomonas* actin is likely to contain approximately 20 atoms of sulphur (there are 20 atoms of sulphur in *Saccharomyces cerevisiae* actin and 21 in *Arabidopsis* actin, for instance), whereas inner dynein arm heavy chains probably contain 200 (outer dynein arm heavy chains β and γ, which are slightly larger, contain 217 and 208). With these assumptions, the molar ratio p28:actin:heavy chains in the immunoprecipitates was approximately 1:0.7:0.5. The p28-containing complexes may therefore be composed of one heavy chain, one or two molecules of actin, and two molecules of p28. The same analysis with caltractin/centrin-containing complexes suggests a composition of one heavy chain, one molecule of actin, and one molecule of caltractin/centrin (the molar ratio caltractin/centrin:actin:heavy chains is approximately 1:0.8:0.8). A portion of p28 and/or caltractin/centrin may have dissociated from the complexes and be found in the immunoprecipitates as a monomer. Therefore, the molar ratios presented above may overestimate the amount of p28 and caltractin/centrin in the protein complexes.

We then sought to characterize the DHCs present in the immunoprecipitates. To this end, we analyzed immunoprecipitates similar to the previous ones on a 3.6–5% gradient polyacrylamide gel that resolves DHCs from one another. Each DHC was identified by comparing its mobility to that of the heavy chains found in a high salt extract of wild-type axonemes (Figure 7, lane a). The complexes immunoprecipitated by the anti-p28 antiserum contain heavy chains 2′ and 2 (Figure 7, lane b), whereas the complexes immunoprecipitated by the anti-caltractin/centrin antiserum contain heavy chains 2, 3, and 3′ (Figure 7, lane c). When dynein extracts from the mutant *ida4* were analyzed, no heavy chain was specifically precipitated by the anti-p28 antiserum (Figure 7, lane d) whereas the anti-caltractin/centrin antibody precip-

itated heavy chains 2, 3, and 3′ (Figure 7, lane e). The association of p28 with heavy chains 2′ and 2 was expected: the mutant *ida4*, which lacks p28, is also lacking heavy chain 2′ and is deficient for heavy chain 2 (Piperno *et al.*, 1992).

A small amount of other DHCs can be seen in the precipitates shown in Figure 7. Their position in the electrophoretogram identifies them as DHCs 1α and 1β from inner dynein arms I1 or DHCs from outer dynein arms. Two results show that these DHCs are nonspecifically precipitated during our procedure. First, they are present in the anti-p28 immunoprecipitate of dynein complexes prepared from *ida4* cells, which lack p28 (Figure 7, lane d). Second, p28 is not a component of inner dynein arms I1 or the outer dynein arms; when dynein arm heavy chains isolated from wild-type cells were separated by sucrose gradient centrifugation, no p28 could be detected by immunoblotting in the 20–22S fractions that contain the I1 arms and part of the outer arms. All of p28 was confined to the 11S fractions, which contain heavy chains from I2 and I3 arms.

Analysis of the association of p28 and caltractin/centrin with the DHCs of inner dynein arms I2 and I3 suggested a grouping into the following three sets: DHC 2′, associated with p28; DHCs 3 and 3′, associated with caltractin/centrin; and DHC 2, associated with both. We will refer to DHC 2 associated with p28 as 2A, and to DHC 2 associated with caltractin/centrin as 2B. Each electrophoretic band may contain different DHCs. As a consequence, it is possible that DHCs 2A and 2B are different polypeptides, as the following experiments suggest.

When axonemes are prepared by exposing the cells to 0.035% Nonidet P-40, only dynein arms in the proximal region of the axoneme remain bound to the outer doublet microtubules. Piperno and Ramanis (1991) showed that the DHCs in these arms are 1α, 1β, 2, and 3′. We sought to determine whether the heavy chain 2 that remains bound in these conditions was associated with p28, caltractin/centrin, or both. To this end, we prepared axonemes from wild-type cells and from the mutant *ida4* by exposure to Nonidet P-40 and extracted the arms that remained bound by exposure to high salt in the presence of ATP. Lanes a and d of Figure 8 show that the heavy chains present in wild-type axonemes prepared by exposure to Nonidet P-40 are 1α, 1β, 2, and 3′, as expected. The residual heavy chain 2 is 2A, because it could be immunoprecipitated by the anti-p28 antiserum (Figure 8, lane b) but not by the anti-caltractin/centrin antiserum, which precipitated only heavy chain 3′ (Figure 8, lane c). In agreement with the observations that heavy chain 2B is extracted from axonemes prepared by exposure of the cells to Nonidet P-40 and that the mutant *ida4* has only heavy chain 2B, we found that *ida4* axonemes prepared by Nonidet P-40 retain heavy chain 3′ but neither 2A nor 2B (Figure 8, lane e). This experimental approach also revealed that *ida4* axonemes are defective for a polypeptide larger than DHCs. This polypeptide is marked by an asterisk (Figure 8, lane d).

These results indicate that dynein arms containing heavy chain 2B are selectively extracted by exposure to Nonidet P-40. The molecular basis for the extraction of some but not all DHCs by exposure to Nonidet P-40 is unknown. However, the extractibility of heavy chain 2B cannot solely derive from its association with caltractin/centrin, because heavy chain 3′ is not extracted. The extractibility of a heavy chain therefore reflects properties of the heavy chain itself. This in turn strongly suggests that heavy chains 2A and 2B are different polypeptides.

The Binding of Heavy Chain 2B to the Axoneme Requires the Presence of DRC Components 1 and 2

The evidence that heavy chains 2A and 2B may belong to different dynein arms prompted us to determine

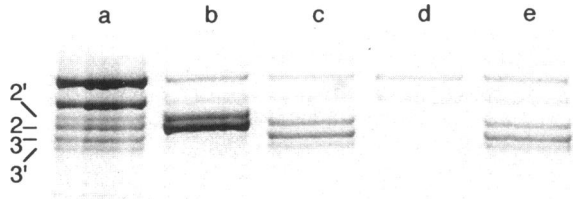

Figure 7. High MW components of the dynein complexes immunoprecipitated with antisera against p28 (lanes b and d) and against caltractin/centrin (lanes c and e). The complexes analyzed were isolated from radioactively labeled wild-type (lanes b and c) or *ida4* cells (lanes d and e). The proteins in the immunoprecipitates were separated by electrophoresis on a 3.6–5% gradient polyacrylamide gel to resolve dynein heavy chains from one another. The heavy chains from a high salt extract of wild-type axonemes are shown as a reference (lane a). The positions of dynein heavy chains 2′, 2, 3, and 3′ are indicated on the left.

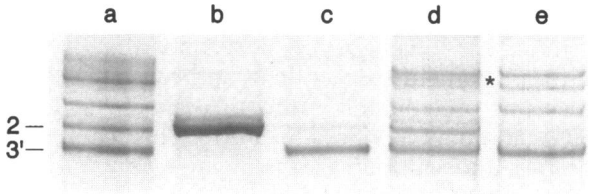

Figure 8. Analysis of the dynein heavy chains found in axonemes prepared by exposure to Nonidet P-40. The proteins were isolated from radioactively labeled wild-type (lanes a, b, c, and d) or *ida4* cells (lane e). Lanes a, d, and e show the dynein heavy chains present in the axonemes. Dynein complexes in the high salt extract prepared from wild-type cells were immunoprecipitated with antisera against p28 (lane b) or caltractin/centrin (lane c). An asterisk located between lane d and e marks the position of a polypeptide present in wild-type but missing in *ida4* axonemes.

how they were affected by defects in the DRC, a complex regulating the binding and the activity of inner dynein arms in the axoneme. The results presented above indicated that the *ida4* mutation causes the absence of heavy chain 2A (associated with p28). Piperno *et al.* (1992) showed that double mutants carrying the *ida4* mutation and some DRC mutations completely lack heavy chain 2. This suggested to us that some DRC mutants might be deficient in heavy chain 2B (that is, heavy chain 2 associated with caltractin/centrin).

We prepared high salt extracts from the DRC mutants *pf2*, *pf3*, $sup_{pf}3$, $sup_{pf}4$, and $sup_{pf}5$ and immunoprecipitated their dynein arms with the anti-caltractin/centrin antiserum. The results shown in Figure 9 indicate that heavy chain 2B is entirely missing in *pf3* and $sup_{pf}5$, deficient in *pf2* and $sup_{pf}3$, and present in wild-type–like amounts in $sup_{pf}4$. In contrast, the anti-p28 antiserum precipitated heavy chains 2′ and 2A in similar amounts from each of the DRC mutants. These new results explain the complete absence of heavy chain 2 in the double mutants *ida4pf3* and $ida4sup_{pf}5$ (Piperno *et al.*, 1992).

The finding that the binding of heavy chain 2B is specifically affected by some DRC mutations again suggests that 2A and 2B are different polypeptides. Furthermore, it shows that the binding of 2B is more sensitive to DRC defects than the binding of other heavy chains. *pf3* and $sup_{pf}5$ are the only mutants lacking DRC components 1 and 2. The binding of heavy chain 2B to the axoneme therefore requires DRC components 1 and 2, but is not sensitive to defects of DRC components 5 and 6, which are missing in each DRC mutant including $sup_{pf}4$.

The mutants *pf3* and $sup_{pf}5$ represent different loci but are defective for the same subset of DRC components. The DRC component 1 is the putative gene product of *PF3* (Piperno *et al.*, 1994). The absence of heavy chain 2B in *pf3* and $sup_{pf}5$ axonemes prompted us to determine whether 2B is the putative gene product of $SUP_{PF}5$. We applied the dikaryon rescue procedure (Luck *et al.*, 1977; Piperno *et al.*, 1994) to both mutants and immunoprecipitated the DHCs extracted from *pf3*xwild-type and $sup_{pf}5$xwild-type dikaryons. We found that the heavy chain 2B was rescued from the cellular pool of both mutants. Therefore heavy chain 2B is not the gene product of the $SUP_{PF}5$ locus.

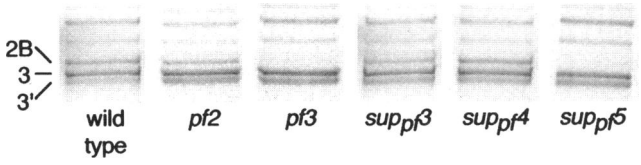

Figure 9. Analysis of the dynein heavy chains found in the axonemes of various DRC mutants. Shown are the heavy chains found in the dynein complexes immunoprecipitated by the antiserum against caltractin/centrin. The arms analyzed were isolated from radioactively labeled wild-type, *pf2*, *pf3*, $sup_{pf}3$, $sup_{pf}4$, and $sup_{pf}5$ cells. Dynein heavy chains 2B, 3, and 3′ are identified on the left.

DISCUSSION

The p28 Gene

The procedure we followed to isolate the gene for p28 requires the purification of a few micrograms of the protein of interest. It should be of use for other flagellar proteins, because most of the structural components of the axoneme are more abundant than p28, which represents less than 0.1% of the mass of the axoneme. It should be noted that we did not use any libraries of cloned sequences, which eliminates the concerns of rarity or absence of specific sequences from libraries.

Besides the problems linked to the purification of the protein of interest, this method is limited by two factors: one is the reliability of the determination of amino acid sequences; the other is the ability to design degenerated oligonucleotides corresponding to the sequence encoding the amino acid sequences determined experimentally. In this work, the oligonucleotide design was complicated by the fact that the p28 gene is the least biased in its codon usage of all *C. reinhardtii* nuclear sequences determined so far. For this reason, we used inosine-containing oligonucleotides, after several unsuccessful attempts at designing partially degenerated oligonucleotides.

The significance, if any, of the low codon usage bias of the p28 gene is difficult to assess. Although this hypothesis has rarely been tested experimentally, codon usage bias is supposed to reflect the selection of codons optimizing translational efficiency (Bulmer, 1991). A positive correlation between degree of codon usage bias and level of expression was found in *Drosophila* (Shields *et al.*, 1988), in yeast (Bennetzen and Hall, 1982), and in various prokaryotes, but not in mammals. It appears that in *Chlamydomonas*, the nuclear sequences with the most extreme codon usage bias are those encoding proteins ultimately imported into the chloroplast. The 26 sequences of this type have B values ranging from 0.570–0.792 (with one exception), with an average of 0.682. The 13 histone genes are also strongly biased, with B values ranging from 0.533–0.794 with an average of 0.675. Genes encoding axonemal proteins exhibit more variation: while the four tubulin genes have a strong codon usage bias (B values of 0.71, 0.72, 0.73, and 0.74), other genes are less biased. Excluding the tubulin genes, the 11 genes encoding axonemal components have B values ranging from 0.39–0.64 with an average of 0.56. It would be interesting to determine whether in *Chlamy-*

domonas the translation rate of individual genes is indeed affected by the codon usage bias. Until such a phenomenon is shown to occur, it is difficult to know whether the low degree of codon usage bias of the p28 gene (B value of 0.316) means that this protein is translated less efficiently than others.

Whether or not the codon usage bias in *Chlamydomonas* influences translation rates, it should be noted that it is not the result of a global mutational drift causing a higher abundance of G and C throughout the genome or parts thereof. For a given gene, there is no correlation between the degree of bias of exons and the G+C content of introns. This means that the high G+C content of the coding sequences results from a selective pressure acting on the exons but not on the introns.

Identification of the DHCs Associated with p28 and Caltractin/Centrin

Although the function of p28 could not be deduced from its amino acid sequence, quantitative analysis of the immunoprecipitated proteins showed that 60–80% of the heavy chain 2′ and 2A are precipitated by the antiserum anti-p28. These arms therefore require p28 to assemble or to bind to the microtubule doublets. This view is supported by their complete absence in the mutant *ida4*, a null mutant of p28 (see accompanying manuscript). The function of p28 may be to promote the stable assembly of the p28-containing dynein arms or their binding to the axoneme.

An important outcome of this work was the identification of the DHCs associated with the light chains p28 and caltractin/centrin. Heavy chain 2′ is found to be exclusively bound to p28 whereas heavy chains 3 and 3′ are found to be exclusively bound to caltractin/centrin. Heavy chain 2 appears to be associated with p28 or with caltractin/centrin. However, two arguments presented above suggest that the heavy chain 2 bound to p28 (2A) may be a different polypeptide from the heavy chain 2 bound to caltractin/centrin (2B): 1) exposure to Nonidet P-40 selectively extracts heavy chain 2B from the axoneme; and 2) heavy chain 2B is specifically absent from the axonemes of mutants lacking DRC components 1 and 2.

Our finding that the anticaltractin/centrin antibody immunoprecipitates only 15–20% of heavy chains 2B, 3, and 3′ could mean that the association of these heavy chains with caltractin/centrin is optional. An alternative explanation is that the affinity of the antibody for caltractin/centrin is not sufficient to precipitate all the caltractin/centrin-containing complexes.

Other investigators partially separated dynein arm complexes by ion-exchange chromatography (Kagami and Kamiya, 1992; Kato *et al.*, 1993; Gardner *et al.*, 1994). Complexes derived from inner dynein arms were resolved in eight fractions termed a through g. A direct comparison of our results with the chromatographic data is complicated by three factors: 1) the chromatographic system used does not allow a complete resolution of all dynein complexes from each other; 2) each fraction also contains numerous other polypeptides that are not part of the dynein complexes; and 3) caltractin/centrin was not identified in electrophoretograms of fraction components and was not visualized by immunostaining. These limitations notwithstanding, our results are consistent with the chromatographic data.

A 28,000 MW protein (very likely, p28) is present in complexes found in the three fractions a, c, and d. These species, absent in extracts from *ida4* axonemes, also contain actin and DHC 2′ (fractions a and d) or 2 (fraction c). Ultraviolet-vanadate cleavage showed that DHC 2′ found in fraction a is different from that found in fraction d. However, these two polypeptides comigrate electrophoretically.

Fraction e contains DHC 2 and actin, but no 28,000 MW protein. We propose that the complexes found in fraction e contain DHC 2B, actin, and also caltractin/centrin. This hypothesis is made extremely likely by the recent finding that the chromatographic species e is missing in *pf3* and deficient in *pf2* and $sup_{pf}3$ (Gardner *et al.*, 1994); we showed here that the complexes consisting of DHC 2B, actin, and caltractin/centrin are missing in *pf3* (and $sup_{pf}5$) and deficient in *pf2* and $sup_{pf}3$.

Finally, DHC 3′ is found in fraction b, whereas DHC 3 is found in fraction g. Both fractions also contain actin, but no protein with an MW of 28,000. Our results predict that they also contain caltractin/centrin.

Localization of the p28-containing Complexes in the Inner Dynein Arm Row

Several authors have shown that the inner dynein arm row is organized as repeats of a basic 96-nm unit (Goodenough and Heuser, 1985; Piperno *et al.*, 1990; Muto *et al.*, 1991). We do not know the original location of the complexes containing p28, actin, and heavy chains 2′ and 2A within each repeat. These complexes, absent in the mutant *ida4*, have the composition that had been proposed for the inner arms I2 (Piperno *et al.*, 1990; Piperno and Ramanis, 1991). According to the model described by Piperno and Ramanis (1991), these arms are located in the I2 segment, between the radial spokes S1 and S2. The I3 segment, in contrast, was predicted to contain homodimers of heavy chain 3 or homodimers of heavy chain 3′. However, when other investigators analyzed the mutant *ida4* by computer-based averaging of electron micrographs and compared it to wild type, they found that *ida4* axonemes were deficient in three parts of the 96-nm repeats, in

both the I2 and I3 positions (Mastronarde *et al.*, 1992; Gardner *et al.*, 1994; King *et al.*, 1994).

This new result clearly contradicts the Piperno and Ramanis model, which predicts that the defects in the *ida4* mutant should be restricted to the I2 segment. We therefore reinterpreted the previous data that supported the Piperno and Ramanis model, in view of the results presented here and the quantitative analysis of electron micrographs performed by others (Mastronarde *et al.*, 1992; Gardner *et al.*, 1994; King *et al.*, 1994). Figure 10 shows our current understanding of the organization of a typical 96-nm long repeat of the inner dynein arm row. The radial spokes S1 and S2 delineate the I1, I2, and I3 segments. The proximal part of the axoneme is directed to the left of the drawing. The shape, number, and position of the various densities/complexes are derived from the diagram shown by Gardner *et al.* (1994).

Each I1 segment contains an I1 arm, a heterodimer consisting of one DHC 1α and one chain 1β, whereas the I2 and I3 segments contain heavy chains 2′, 2A, 2B, 3, and 3′. We propose that each I2 and I3 segment contains two single DHCs, one associated with p28 and the other associated with caltractin/centrin. The previous hypothesis that the heavy chains in the I2 and I3 segments form dimers was solely based on their appearance in metal replicas of unfixed axonemes (Goodenough and Heuser, 1985) and could be mistaken. We propose instead that DHCs are present in the I2 and I3 segments as monomers.

The presence of two DHCs in each of the I2 and I3 segments is suggested by the appearance of the inner dynein arm row in electron micrographs and is supported by the fact that the amount of heavy chains 2′, 2A, 2B, 3, and 3′ in the axoneme is approximately double the amount of heavy chains 1α and 1β (Piperno and Ramanis, 1991).

We further suggest that p28-associated DHCs may be found at the base of radial spokes S1 and S2 (Figure 10, regions filled in black). Analysis of electron micrographs of *ida4* axonemes showed deficiencies in three (and not two) regions of the 96 nm repeat (Gardner *et al.*, 1994); the third structure shown to be missing is filled in dark gray in Figure 10.

One explanation of the discrepancy is that *ida4* lacks structures other than p28-containing arms. When *ida4* axonemes are prepared by exposure to Nonidet P-40 and compared with wild-type axonemes, a polypeptide larger than DHCs is found to be missing. This polypeptide is marked by an asterisk in Figure 8. Preliminary evidence indicates that this polypeptide is not extracted by exposure to high salt in the presence of ATP. Therefore, this protein does not behave as a dynein arm heavy chain. It is, however, larger than a DHC and its absence could be noticed by electron microscopy as much as that of a single DHC associated with light chains.

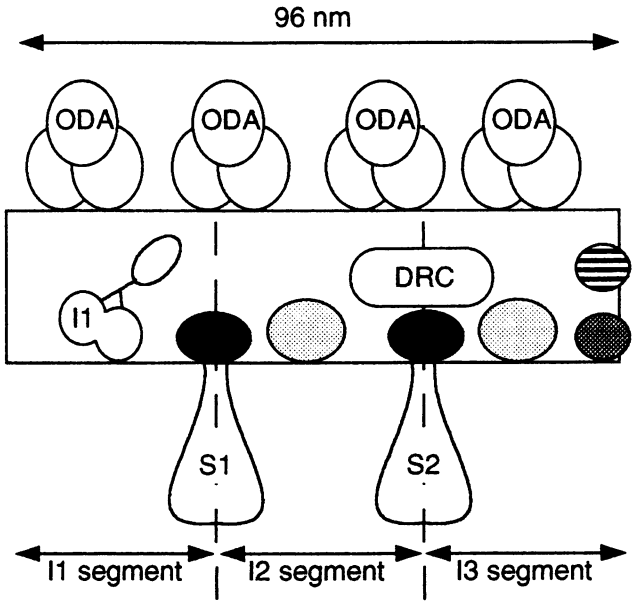

Figure 10. Model of the organization of a 96-nm repeat unit of the inner dynein arm row of *Chlamydomonas* axonemes. This model is based on previous models (Piperno and Ramanis, 1991; Gardner *et al.*, 1994; King *et al.*, 1994). The structures shown are: ODA, outer dynein arm; S1 and S2, radial spokes S1 and S2; I1, I1 inner dynein arm (trilobed), containing heavy chains 1α and 1β; black areas, dynein heavy chain 2′ or 2A, associated with p28; light-gray areas, dynein heavy chain 2B or 3 or 3′, associated with caltractin/centrin; DRC, dynein regulatory complex; dark-gray area, unknown, missing in *ida4* axonemes (see text for details); horizontally striped area, unknown.

The position of the DRC was reproduced from the data of others (Mastronarde *et al.*, 1992; Gardner *et al.*, 1994; King *et al.*, 1994). The DRC is located in proximity of both p28-containing and caltractin/centrin-containing arms. This could explain the generalized deficiency of DHCs 2′, 2, 3, and 3′ exhibited by DRC mutants (Piperno *et al.*, 1992).

DHC 2B and Suppression of Flagellar Paralysis

Over the past several years, several mutants have been characterized that suppress the paralysis caused by the absence of radial spokes or central pair complex, without restoring the missing structure. These mutants are *sup*$_{pf}$*1*, *sup*$_{pf}$*2*, *sup*$_{pf}$*3*, *sup*$_{pf}$*4*, *sup*$_{pf}$*5*, *pf2*, and *pf3* (Huang *et al.*, 1982; Piperno *et al.*, 1992, 1994; Porter *et al.*, 1994). No common biochemical defect was ever found among these mutants: the *sup*$_{pf}$*1* mutation affects the β-chain of outer dynein arms (Porter *et al.*, 1994), whereas no deficiency was found in the axonemes of *sup*$_{pf}$*2* mutant cells. The other five mutants lack subsets of a group of seven proteins termed DRC. Most DRC mutants fail to assemble a normal complement of inner dynein arms (Piperno *et al.*, 1992, 1994). The lack of a common biochemical defect means that

the suppression of flagellar paralysis can be effected through several mechanisms.

Our finding that the mutants *pf3* and $sup_{pf}5$ totally lack heavy chain 2B prompted us to investigate whether such a deficiency was common to other suppressor mutants. We found that 2B is deficient in *pf2* and $sup_{pf}3$ and is present in normal amounts in the suppressor mutants $sup_{pf}2$ (not shown) and $sup_{pf}4$. Therefore, the absence of 2B is not necessary to suppress the flagellar paralysis of central pair and radial spoke mutants. *pf3* and $sup_{pf}5$ are the only DRC mutants lacking DRC components 1 and 2, which suggests that these components are required for the binding of heavy chain 2B and possibly directly interact with it. The low abundance of heavy chain 2B (comparable with that of heavy chain 3′) may mean that it is restricted to a longitudinal segment of the axoneme, or to only a few of the outer doublet microtubules. This in turn may point to the location of DRC components 1 and 2 within the axoneme.

In summary, this work showed that the DHCs 2′, 2A, 2B, 3, and 3′ bind either p28 or caltractin/centrin but not both. Further work, presented in a companion paper, showed that p28 is the gene product of the *IDA4* locus. In *ida4* mutants, the absence of p28 leads to the absence of the heavy chains normally associated with it, namely 2′ and 2A. This indicates that p28 is necessary for the assembly of a subset of inner dynein arms or for their binding to the microtubule doublets.

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