Identification of *oda*6 as a *Chlamydomonas* Dynein Mutant by Rescue with the Wild-type Gene

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Abstract. We find that two *Chlamydomonas* outer arm dynein assembly loci, *oda*6 and *oda*9, are located on the left arm of linkage group XII, in the vicinity of the previously mapped locus for a 70,000 M_r dynein intermediate chain protein. Restriction fragment length polymorphism mapping indicates that this dynein gene is very closely linked to the *oda*6 locus. A cDNA clone encoding the 70,000 M_r protein was isolated, sequenced, and used to select genomic clones spanning the corresponding locus from both wild-type and *oda*6 libraries. When wild-type clones were introduced into cells containing an *oda*6 allele, the mutant phenotype was rescued, while no rescue was observed after transformation with *oda*6 clones. Genetic analysis further revealed that newly introduced gene copies were responsible for the rescued phenotype and thus confirms that *ODA*6 encodes the 70,000 M_r dynein intermediate chain protein. The inability of *oda*6 mutants to assemble any major outer arm dynein subunits shows that this protein is essential for assembly of stable outer dynein arms. This is the first use of transformation with a wild-type gene to identify the product of a *Chlamydomonas* mutant.

DYNEIN ATPases are exceptionally large (1-2 × 10⁶ M_r), multisubunit enzymes that translocate along microtubules. Flagellar dyneins generate force for flagellar beating, while the related cytoplasmic dyneins, such as MAP1C (Paschal et al., 1987), contribute to retrograde axonal transport (Paschal and Vallee, 1987) and other forms of intracellular motility (Scholey et al., 1984; Schroer et al., 1989) including, perhaps, mitotic chromosome movements (Steuer et al., 1990; Pfarr et al., 1990). One way to determine the structure-function relationships in such large multisubunit complexes is through the analysis of mutations that disrupt normal function of the complex, and much effort has been devoted to the isolation of flagellar dynein mutants, primarily in the single-celled green alga *Chlamydomonas reinhardtii* (Huang et al., 1979; Kamiya, 1988; Kamiya et al., 1989; Mitchell and Rosenbaum, 1985; Sakakibara et al., 1991). The recent development of a simple method for reliable high-frequency transformation of *Chlamydomonas* (Kindle, 1990) has now opened the way for molecular analysis of dynein mutants.

The outer row of dynein arms in *Chlamydomonas* is composed of three ∼400-kD heavy chains (alpha, beta, and gamma), two intermediate chains (80 and 70 kD), and at least eight light chains (10-30 kD) (Pfister et al., 1982; Piperno and Luck, 1979). A wide variety of mutations affecting flagellar structure and function have been isolated in this organism, including mutations at 12 loci that prevent formation of functional outer row dynein arms (Mitchell and Rosenbaum, 1985; Kamiya, 1988). All but one of these loci were identified on the basis of alleles that block the transport of dynein subunits into the flagellar compartment and/or prevent their assembly onto flagellar doublet microtubules, so that all three outer arm heavy chain subunits are missing from gels of flagellar proteins (outer arm dynein assembly, or *oda* mutants). The one exception, *sup-pf*1, was first identified as a suppressor of radial spoke and central pair defects in which the beta dynein chain has an altered electrophoretic mobility (Huang et al., 1982). Restriction fragment length polymorphism (RFLP)[1] analysis has confirmed that *sup-pf*1 is linked to the beta dynein gene (Ranum et al., 1988) and genetic analysis shows that *sup-pf*1 is allelic to assembly mutant *oda*4 (Luck and Piperno, 1989). The other eleven loci, whose gene products have not yet been identified, could potentially encode either additional key structural components of the complex or factors involved in dynein synthesis and/or assembly. Here we use genetic mapping and complementation with a genomic clone to identify one of these mutations, *oda*6, as a defect in the 70-kD intermediate chain gene.

Materials and Methods

Chlamydomonas Strains and Genetics

Chlamydomonas stocks were maintained by standard methods (Harris,

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[1]. *Abbreviation used in this paper*: RFLP, restriction fragment length polymorphism.

1989) on minimal medium I of Sager and Granick (1953). Strains SID2, gln1, and cor1 were obtained from Dr. P. A. LeFebvre (University of Minnesota), strain 137c was obtained from Dr. J. L. Rosenbaum (Yale University), and strains oda6-95, oda9-141, and idal-98 were generously provided by Dr. R. Kamiya (Nagoya University). Double mutant strain oda6-95,idal-98 (designated as oda6,idal throughout this publication) was constructed in our laboratory. Genetic crosses were performed by standard procedures, except that zygotes were germinated on medium solidified with 1.5% Gel Gro (ICN Biochemicals, Inc., Irvine, CA) rather than agar, and tetrads were dissected with the aid of a micromanipulator (Allen Benjamin Inc., Tempe, AZ). Map distances were calculated as $(NPD + 0.5T)/(PD + NPD + T)$ (Gowans, 1965).

RFLP Mapping

For RFLP mapping, oda mutants were crossed to wild-type strain SID2 (Gross et al., 1988), tetrads were dissected, and the products were scored by light microscopic observation as either fast swimming (wild type) or slow swimming (oda). DNA was isolated by the method of Weeks et al., 1986, digested with SalI, separated on agarose gels, and blotted to Nytran (S&S) following standard methods (Sambrook et al., 1989). Blots were probed with cDNA clone c70-16 (see below) labeled using a random prime kit from Boehringer Mannheim Biochemicals (Indianapolis, IN) and ^{32}P-dCTP. For most of the tetrads used in RFLP linkage studies, to minimize the required number of DNA samples, only DNA from the two slow-swimming or the two fast-swimming products were used for Southern blotting.

Selection of cDNA and Genomic Clones

cDNA clones were selected from a lambda gt11 cDNA library (provided by Dr. S. Adair, Tufts University) constructed from wild-type strain NO-gamete mRNA, by hybridization with genomic clone Dal (Williams et al., 1986). To obtain large genomic clones, the insert from lambda gt11 cDNA clone Lc70-16 was subcloned into pBluescript KS+ and used to screen an EMBL4 genomic library (provided by J. Rosenbaum, Yale University) prepared from Sau 3A-digested DNA isolated from strain 137c+. A single EMBL4 clone, E70B2, was used for further study.

To clone the oda6-95 IC70 gene, a library of reduced complexity was constructed from oda6-95 DNA. DNA was digested with SacI, EcoRI, BamHI, and HindIII and run on a 1% agarose gel, and fragments of ~6.2 kb were electroeluted, ethanol precipitated, and cloned into the SacI site of pBluescript KS (−). This library was transformed into E. coli DH5alpha MCR (Gibco-BRL Laboratories, Grand Island, NY), and colony lifts were probed with cDNA insert c70-16. Several positives were selected and analyzed by restriction mapping, and clone pBoda6-c7 was chosen for further use.

Sequence Analysis

Overlapping deletions of insert c70-16 in pBluescript KS+ (Stratagene Cloning Systems, La Jolla, CA) were generated in both insert orientations by exonuclease III digestion (Henikoff, 1987) using an Erase-a-base kit from Promega Biotec (Madison, WI). Single-stranded sequencing templates produced by superinfection with R408 helper phage (Kidd et al., 1986) were sequenced with a Sequenase kit from United States Biochemical Corp. (Cleveland, OH). Sequences were assembled and analyzed using the GCG sequence analysis software programs, and the predicted amino acid sequence was compared with sequences from the Genbank (release 64.0) and National Biomedical Research Foundation (release 25.0) databases.

Transformation

Strains oda6,idal and oda9,idal were used as recipients for transformation. Vegetative cells were grown to 1×10^6 cells/ml in acetate-supplemented medium (medium II of Sager and Granick, 1953) and harvested by centrifugation, and cell walls were removed by two successive 30-min incubations in filter-sterilized gametic cell wall lysin (Harris, 1989). Spheroplasts were suspended in medium II and 1 ml was used per transformation. Transformation followed the method of Kindle, 1990: polyethylene glycol, DNA, and glass beads were added to the cells in a 15-ml screw cap centrifuge tube, and the tube was mixed on a Vortex Genie II at top speed for two 15-s pulses.

For initial experiments reported in Table II, 2×10^8 cells were used in each transformation, and cells were diluted with medium I and aliquoted into 60 tubes, each containing 1×10^6 cells and 4 ml medium I. After 1 wk under constant illumination, tubes were examined daily for the appearance of motile cells. For further analysis, single motile clones were selected from six different tubes.

For later experiments comparing wild-type clone pG70S and oda6-95 clone pBoda6-c7, the number of cells per transformation was reduced to 5 $\times 10^7$ and cells were distributed among 5 tubes rather than 60. Other aspects of the transformation and screening were not altered.

Frequency Determination

Beat frequency determinations were made on individual free-swimming cells suspended in a 20-μl sample between a glass slide and a cover slip supported along two edges with petroleum jelly. Cells were observed under dark-field illumination on a Zeiss Axioskop microscope, with stroboscopic illumination from a xenon flash tube powered by a Chadwick-Helmuth Strobex model 8440 frequency generator and power supply. Beat frequencies were determined by recording the strobe frequency required to create a "still" waveform image. Results were averaged from measurements on 15 cells per sample.

Results

Genetic Mapping

Genomic clones Dal and Da2, which encode part of the 70,000 M_r outer arm dynein intermediate chain, were previously selected by screening a lambda gt11 expression library with an mAb (Williams et al., 1986). One of these clones, Dal, was recently used to genetically map this intermediate chain gene to linkage group XII by RFLP analysis (Ranum et al., 1988). Recombination frequencies between Dal and each of the two Chlamydomonas beta tubulin genes, as well as between Dal and the centromere, placed the 70,000 M_r intermediate chain (IC70) gene ~22 map units from the centromere on the left arm of XII. While no motility mutant loci have been precisely mapped to this region, Kamiya (1988) determined that two oda loci, oda6 and oda9, are linked to a centromere marker on linkage group XII (pf27). To determine whether either of these oda mutations

Table I. Genetic Mapping of oda6 and oda9 on L. G. XII
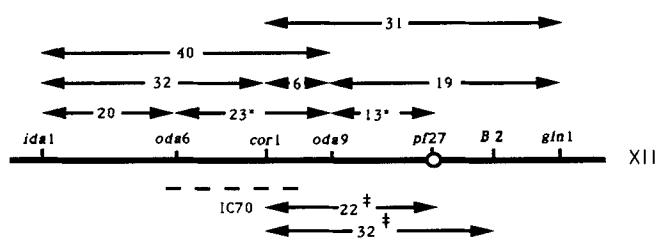
Experiment	Cross	Loci	P/N/T	Distance§
1	oda9 × gln1		24:0:8	12.5
2	idal × cor1		15:1:24	32.5
3	idal × oda6		76:0:52	20.3
4	idal × oda9		19:0:74	39.8
5	oda9,gln1 × cor1	oda9 vs. gln1	16:0:16	25.0
		cor1 vs. gln1	12:0:20	31.3
		oda9 vs. cor1	28:0:4	6.3

Genetic map distances of several loci on linkage group XII calculated from five crosses were used to construct the map shown at top. The dashed line indicates uncertainty in the location of IC70 determined by Ranum et al., 1988. Data from each cross is summarized in the table as tetrad ratios, where P = parental ditype, N = nonparental ditype, and T = tetratype.
* Data from Kamiya, 1988.
‡ Data from Ranum et al., 1988.
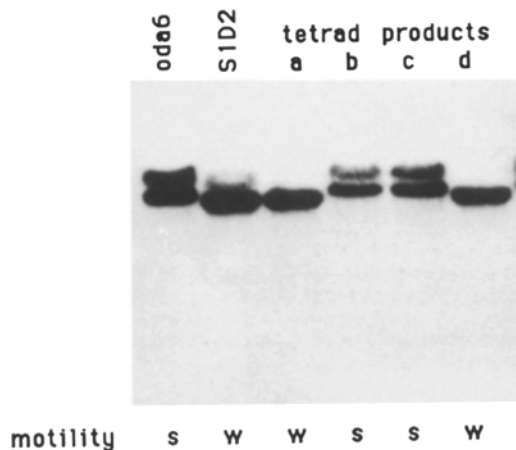§ Distance calculated as (n + .5T)/(P + N + T) × 100.

Figure 1. Genomic Southern blot demonstrating the RFLP used to show tight linkage between *oda6* and the IC70 gene. A blot of SalI-digested *oda6* DNA, wild isolate S1D2 DNA, and DNA from tetrad progeny from an *oda6* × S1D2 mating was probed with cDNA clone *c70-16*. Swimming phenotype is indicated below each lane as slow (*s*) or wild type (*w*).

could be allelic to the IC70 gene, we first mapped them relative to three other linkage group XII markers, *glnl*, *corl*, and *idal*. As shown in Table I, both *oda6* and *oda9* map on XII left, with *oda9* proximal to *oda6*. The data of Ranum et al. (1988) place the IC70 locus in the region of both assembly loci.

Further tests of linkage were performed by crossing *oda6* and *oda9* strains with wild-type strain S1D2, which displays many RFLPs with respect to the lab strain, 137c, that is used as the background for most mutational analysis. Meiotic products (tetrads) were dissected and scored for motility phenotype (both *oda6* and *oda9* cells swim at approximately half of the wild-type speed) and for inheritance of a SalI restriction site polymorphism at the IC70 locus. Lanes 1 and 2 of Fig. 1 show the pattern of hybridization of IC70 cDNA clone pBC70-16 (see below) to DNA isolated from each parental strain and digested with SalI, and demonstrate the presence of a clear polymorphism detectable with this enzyme. The RFLP patterns of progeny from an *oda6*×S1D2 tetrad are shown in lanes 3-6. Motility phenotypes of these progeny, indicated at the bottom of each lane, show that in this tetrad the IC70 RFLP pattern has not recombined with the *oda6* mutation. Southern blots from an additional 13 tetrads revealed no recombinants between IC70 and *oda6*, indicating linkage to within three map units, while similar blots from an *oda9* cross produced two recombinants among four tetrads (data not shown).

Selection of IC70 cDNA Clones and Sequence Analysis

To determine whether *oda6* could be a mutation in the IC70 gene, we wished to test the ability of a full-length IC70 clone to complement the *oda6* mutation by transformation. Since preliminary evidence suggested that our available genomic IC70 clone, Da1, represented only a partial gene fragment, we first selected cDNA clones that could be used to map the complete transcription unit. An amplified lambda gt11 cDNA library was screened with Da1, and five positives were

selected. L*c*70-13 contained a 2.1-kb insert, while L*c*70-16 and four other apparently identical clones contained 2.6-kb inserts (Fig. 2 *A*). Since the L*c*70-16 insert hybridizes to a single 2.6-kb mRNA (Fig. 2 *B*), we judged it to be close to full length. This insert was subcloned in both orientations into pBluescript KS+, nested deletions were generated by the Henikoff exonuclease III procedure (Henikoff, 1987), and both strands were sequenced.

Sequence analysis of *c*70-16 revealed a 2,603-bp insert containing a single large open reading frame of 1,701 nt, beginning with an ATG at nt 313 and ending with a TAA stop codon at nt 2,014 (Fig. 3). Near the 3' end of the insert is a perfect copy of the putative *Chlamydomonas* polyadenylation signal, TGTAA, followed by 14 nt and a short poly A tract. While there are several ATG codons near the 5' end of the insert, only the ATG at position 313 is followed by a long open reading frame, and codon usage for this ORF adheres to the codon bias typical of both flagellar (Williams et al., 1989) and nonflagellar (Goldschmidt-Clermont and Rahire, 1986) *Chlamydomonas* genes, with only 2.8% of the codons containing A residues in the third position. Furthermore, the predicted amino acid sequence between residues 332 and 350 is identical to the directly determined sequence of a cyanogen bromide fragment of the 70,000 M_r dynein protein (personal communication, S. King and G. Witman, Worcester Foundation). A search of the NBRF and EMBL databases revealed no obvious homologies with previously reported sequences.
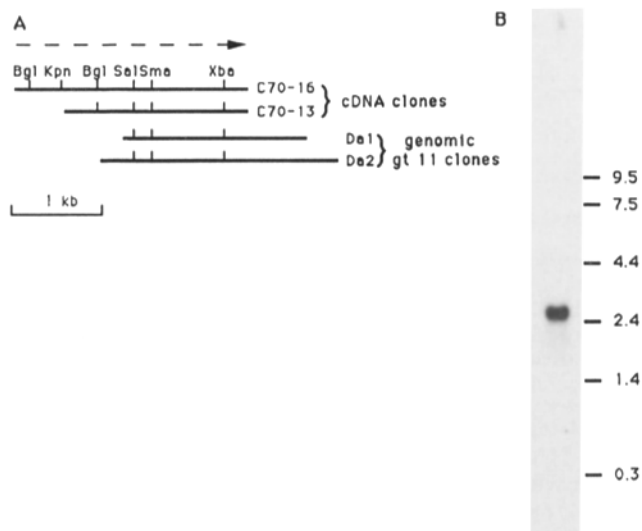

Figure 2. IC70 genomic and cDNA clones. (*A*) Restriction map of IC70 genomic and cDNA clones. Genomic clones Da1 and Da2 were previously selected from an expression library with an mAb (Williams et al., 1986); cDNA clones *c*70-13 and *c*70-16 were selected from a lambda gt11 cDNA library by hybridization with genomic clone Da1. The arrow indicates the direction of transcription, as determined by sequence analysis and by orientation of insert Da1 in lambda gt11. The largest cDNA insert, *c*70-16, is ∼2.6 kb. (*B*) Northern blot of total *Chlamydomonas* RNA probed with clone Da1, showing hybridization to a single band of ∼2.6 kb. The sizes in kb of RNA standards (BRL RNA ladder) are shown along the right margin.

CATTGGAATTGCACCGAATAGCTTGACCTGTTATAAGGCCCTTCCGGGAACTACCTCGGCACGCGCTTTACTACGTGTTTATTGCACCGAATGGCTGGGCCCACATAATAGCTAGCAGCA 120

TGAGAACACTAGAGCGTGCTGGGCATTGCTGAACACAGTCGGTCACCCGTCACCAGGCACGAGCGCAGCTGGGGGGCCGCGTTCAGCAGCGTGCCACGCACGGGAACTCGCGCCCGGCGT 240

CCCACACGGAGGCAGCAGAGCAGCGGTTAGCAGAACCCCTCCGATCACTGGCGTAGGAGCTAAGCAGCCAGGATGGAGATCTACCATCAATATATTAAGCTGCGCAAGCAGTTTGGCCGC 360
                                                                 M E I Y H Q Y I K L R K Q F G R   16

TTTCCAAAGTTTGGGGATGAGGGCAGCGAGATGCTTGCGGACATCCGCCCCAACGAGGACCACGGCAAGGAGTACATCCCGCGCAACCCCGTCACGACGGTGACGCAGTGCGTGCCGGAG 480
F P K F G D E G S E M L A D I R P N E D H G K E Y I P R N P V T T V T Q C V P E   56

ATGTCGGAGCATGAGGCGAACACCAACGCGGTGATCCTGGTCAACAAGGCGATGAGCCACGTAGAGGGCGGGTGGCCCAAGGATGTTGACTACACGGAAGCGGAGCACACGATCCGGTAC 600
M S E H E A N T N A V I L V N K A M S H V E G G W P K D V D Y T E A E H T I R Y   96

CGCAAGAAGGTGGAGAAGGATGAGGACTACATCCGTACAGTTGTCCAGCTGGGCTCGTCAGTGGAGGACCTGATCAAGCAGAACAACGCGGTGGACATCTACCAGGAGTACTTCACGAAC 720
R K K V E K D E D Y I R T V V Q L G S S V E D L I K Q N N A V D I Y Q E Y F T N   136

GTGACGATGGACCACACGTCGGAGGCACCGCACGTGAAGACGGTGACGGTGTTCAAAGACCCTAACAACATCAAGCGCAGCGCCTCCTACGTCAACTGGCACCCAGACGGCTCCGTGCCC 840
V T M D H T S E A P H V K T V T V F K D P N N I K R S A S Y V N W H P D G S V P   176

AAGGTGGTGGTGGCGTACTCGATCCTGCAGTTCCAGCAGCAGCCGGCCGGCATGCCGCTCAGCTCGTACATATGGGACGTGAACAACCCCAACACGCCCGAGTACGAGATGGTGCCCACC 960
K V V V A Y S I L Q F Q Q Q P A G M P L S S Y I W D V N N P N T P E Y E M V P T   216

AGCCAGATCTGCTGCGCCAAGTTCAACCTCAAGGACAACAACCTTGTTGGCGCGGGCCAGTACAACGGCCAGCTGGCCTACTTCGACGTGCGCAAGGGCAACGGCCCCGTGGAGGCCACG 1080
S Q I C C A K F N L K D N N L V G A G Q Y N G Q L A Y F D V R K G N G P V E A T   256

CCCATCGACATCAGCCACCGCGACCCCATCTACGACTTTGCCTGGCTGCAGTCCAAGACCGGGACCGAGTGCATGACCGTATCCACGGACGGCAACGTGCTGTGGTGGGACCTGCGCAAG 1200
P I D I S H R D P I Y D F A W L Q S K T G T E C M T V S T D G N V L W W D L R K   296

ATGAATGAGTGCGTGGAGAACATGCCGCTCAAGGAGAAGAACTCGGAGACCACGGTGGGCGGCGTGTGCCTTGAGTACGACACCAACGCCGGCCCAACCAACTTCATGGTGGGCACGGAG 1320
M N E C V E N M P L K E K N S E T T V G G V C L E Y D T N A G P T N F M V G T E   336

CAAGGCCAGATCTTCTCCTGCAACCGCAAGGCCAAGAACCCGGTCGACCGCGTCAAGTACGTGCTCAGTGGCCACCACGGCCCCATCTACGGCCTGCGGCGCAACCCCTTCAACTCCAAG 1440
Q G Q I F S C N R K A K N P V D R V K Y V L S G H H G P I Y G L R R N P F N S K   376

TACTTCCTGTCCATCGGCGACTGGACGGCGCGCGTGTGGGTGGAGGACACGGCGGTCAAGACGCCCATCCTCACCACCAAGTACCACCCCACCTACCTCACCGGCGGCACCTGGTCGCCC 1560
Y F L S I G D W T A R V W V E D T A V K T P I L T T K Y H P T Y L T G G T W S P   416

TCGCGCCCCGGGCGTGTTCTTCACCATCAAGATGGACGGCGCCATGGACGTGTGGGACCTGTACTACAAGCACAACGAGCCTACGCTCACGGTGCAGGTGTCGGACCTGGCGCTCACGGCC 1680
S R P G V F F T I K M D G A M D V W D L Y Y K H N E P T L T V Q V S D L A L T A   456

TTCGCGGTGCAGGAGAGCGGCGGCACGGTGGCGGTGGGCACCAGCGACGGCTGTACCTCGGTGCTGCAGCTCAGCACGGGCCTGTCCGAGGCCTCGCCAGCGGGAGAAGGCCAACATCAAC 1800
F A V Q E S G G T V A V G T S D G C T S V L Q L S T G L S E A S P A E K A N I N   496

GCCATGTTCGAGCGCGAGACCACGCGCGAGAAGAACCTGGAGAAGGCCATCAAGGAGGCCAAGGTGAAGGCGCGCAAGGAGCAGGGCCGGCGCGACGAGGTGAAGGACAACGTGACGGAG 1920
A M F E R E T T R E K N L E K A I K E A K V K A R K E Q G R R D E V K D N V T E   536

GAGCAGCTCAAGGCGCTGGAGGACGAGTTCTTTAAGACCACCGACCCGGCGGTGGGCGGTGGCTATGGCGCAGGGGAAGGCGCGGCGGCGGAGTAAGGTGGCGGACCAAGGCGGCGGGCG 2040
E Q L K A L E D E F F K T T D P A V G G G Y G A G E G A A A E * 567

GGGGAGTAGGGGAAGTGGTGTGGAGGGAGGAAGGTGCCGGCGTTGTGGTGCAGGCGGGCGGGTTGAGGTGTGGCGGACTGGGCTCGCATGGCCTGCGGCTGCGGTGGGCGGCGTGCGTTG 2160

GTGGCGGGTGTGCTGTGCGGCAAGCATGCGTCAGTCGTTTCTCTCGTGCCGTGTACATGTCTGTGCCACCGCAGTGCGTTTGTCATGGGAGTGCGGAGAAACGAACTCACGACATGACAC 2280

TTTGTGCGGCGGTGCTCGAGTGGCCTTACATTAGGACTGACATTCCTTGTCACCGAATCGTGACTGGACGCTGAACTACTTGACTGTTAGGCATGGCGTGTCTAGACCAAAAAGTATGCT 2400

GACGCTGCAAGGCATTTCATGTAGCTAGGCCTAAGTGGGACTGGAAAGCGGTGGGGGTAAGGTGCCGCTATTGAAGACGACATGGGGTTGTGGTATGATTGGCGGCCGCCAGTCACGCTG 2520

AATGAATACACGATGCAGCCAAAGAGGTATCGGTGTGAACACTCGGAGGAGGCGGTGC<u>TGTAA</u>GTTTGCTACACTCGAAAAAA 2603

Figure 3. Sequence of cDNA clone c70-16. The predicted amino acid sequence is shown beginning at the presumed translational start at nt 313. The 1,701-nt open reading frame terminates with TAA and is followed by a 590 nt 3' untranslated region. A single copy of the putative *Chlamydomonas* polyadenylation signal, TGTAA, appears near the 3' end and has been underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number X55382.

The predicted 567 a.a. sequence encodes a 63,400 M_r protein with a pI of 5.2, while the native IC70 protein has an M_r of 68,000–73,000 and a pI of ~5.9, as estimated from two-dimensional gel electrophoresis of flagellar protein fractions (Williams et al., 1986; Piperno and Luck, 1979). We previously demonstrated that in vitro translation in a reticulocyte lysate of mRNA hybrid selected with IC70 clone Dal generates a protein of identical charge and size to the endogenous flagellar protein, not the smaller and more acidic gene product predicted from the cDNA sequence (Williams et al., 1986). While additional sequence analysis of independently isolated cDNA or genomic clones will be required to rule out a possible cDNA cloning artifact, our data suggest that the IC70 protein may run anomalously on two-dimensional gels. Similar discrepancies between pre-

dicted and observed gel mobility characteristics have been reported for a *Chlamydomonas* flagellar radial spoke protein (Williams et al., 1989).

Selection of Wild-type IC70 Genomic Clones

An EMBL4 genomic library was screened with c70-16, and several overlapping or identical inserts were selected. EMBL4 clone E70B2 was selected for further use because restriction maps indicated that it extends for several kilobases both 5' and 3' of the transcription unit (Fig. 4). A 6.2-kb Sac-I fragment that also spans the IC70 coding region was subcloned from E70B2 into the SacI site of plasmid vector pGEM2 to create pG70S. Both E70B2 and pG70S were used in the transformation experiments described below.

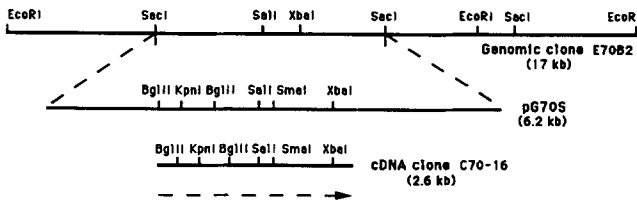To determine gene copy number, Southern blots of DNA

Figure 4. Restriction map of the IC70 locus. The map of lambda clone E70B2 (*top*) shows only EcoRI, SacI, SalI, and XbaI sites, while the expanded map of 6.2 kb SacI–SacI subclone pG70S (*middle*) also includes those BglII, KpnI, and SmaI sites that occur in cDNA clone c70-16 (*bottom*). The broken arrow indicates the direction of transcription of the IC70 gene.

isolated from wild-type cells were probed with cDNA clone c70-16. As shown in Fig. 5, this clone hybridized to a single band in five of six digests. Two bands appear only after digestion by SalI, which is consistent with the location of a SalI site within the clone used as hybridization probe. These results demonstrate that the IC70 locus previously mapped to linkage group XII by RFLP analysis (Ranum et al., 1988) consists of a single copy of the IC70 gene.

Transformation

Double mutant strains containing an outer dynein arm assembly mutation (either *oda6* or *oda9*) and an inner dynein arm assembly mutation (*ida1*) were constructed as backgrounds for selecting transformants. Strains containing only *ida1* are easily distinguished from strains containing only *oda* mutations on the basis of flagellar beat frequency, which is wild type (~60 Hz) in *ida1*, but reduced to ~24 Hz in *oda* strains (Brokaw and Kamiya, 1987). While each mutation alone causes a reduction in swimming speed to ~50% of the wild-type value, the two mutations in combination result in complete flagellar paralysis (Kamiya et al., 1989). Complementation or reversion of the *oda6* mutation should therefore rescue paralyzed double mutants back to a motile
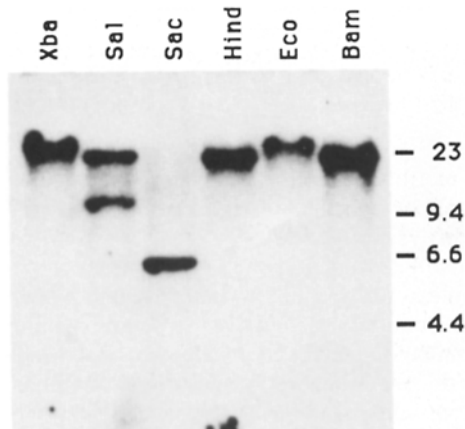

Figure 5. Genomic Southern blot demonstrating that IC70 is a single-copy gene. DNA from strain 137c was digested with the indicated enzymes and probed with cDNA clone c70-16. This probe contains XbaI and SalI, but no SacI, HindIII, EcoRI, or BamHI sites (see Fig. 2 *A*). Only one XbaI band is visible in this exposure. The size in kb of lambda HindIII standards is indicated along the right margin.

Table II. Transformation with IC70 Clones

DNA (insert)	Recipient strain	Tubes with swimmers (#Obs/60 tubes)
pGEM (vector)	*oda6, ida1*	0
E70B2 (17 kb)	*oda6, ida1*	38
pG70S (6 kb)	*oda6, ida1*	60
pG70S (6 kb)	*oda9, ida1*	0

The indicated recipient strains were transformed as described in Materials and Methods with either 12 μg (E70B2) or 8 μg (pGEM, pG70S) of DNA.

phenotype characteristic of cells containing *ida1* alone. To test this screen, double mutants were suspended in liquid medium either with or without prior exposure to a mutagenizing dose of ultraviolet irradiation. The results indicated that motile revertants could be selected at an efficiency of 10^{-6} by this screen after UV mutagenesis, while spontaneous reversion rates of both the *oda6*-95 and *ida1*-98 alleles were $<10^{-8}$ (data not shown).

For transformation, double mutant strains *oda6,ida1* and *oda9,ida1* were treated with gametic autolysin to remove cell walls, and spheroplasts were transformed by the glass bead method of Kindle (1990). After transformation, cells were distributed into 60 tubes, each containing ~3 × 10⁶ cells in 4 ml of minimal medium. Tubes were visually examined after a 2-wk incubation, and single clones were isolated from tubes containing motile cells. As summarized in Table II, transformation of *oda6,ida1* with either the complete 17-kb lambda clone E70B2 or 6-kb subclone pG70S resulted in the appearance of motile cells, while no motile cells were recovered after transformation of *oda9,ida1*, or after transformation of *oda6,ida1* with vector alone. Assuming the distribution of motile cells among the 60 tubes follows a Poisson distribution, where P_0 (the probability that a tube contains 0 swimmers) is related to u (the average number of swimmers per tube) by the formula $P_0 = e^{-u}$, then in the experiment of Table II, E70B2 generated 60 swimmers (if $u = 1$, $P_0 \times 60$ tubes = 22 tubes with no swimmers), while pG70S generated a minimum of 300 swimmers (if $u = 5$, $P_0 \times 60$ tubes = 0.4 tubes with no swimmers).

In every case tested, motile clones displayed the slow swimming speed and near wild-type beat frequency typical of the *ida1* phenotype, consistent with complementation, reversion, or suppression of *oda6*-95. Swimming phenotype was initially scored by visual discrimination of "vibrational" swimming, indicative of low flagellar beat frequency, vs. "smooth" swimming, associated with a higher beat frequency. Direct measurement then confirmed that the beat frequency of a randomly selected transformant (51 ± 3 Hz, $n = 15$) was not statistically different from that of *ida1*-98 (52 ± 3 Hz) or of four other transformants. In contrast, the beat frequency of an *oda6*-95 strain under the same conditions was 28 ± 2 Hz, while that of wild-type strain 137c was 58 ± 3 Hz.

Five putative pG70S transformants were tested for the presence of extra gene copies by Southern blot analysis. Since XbaI cuts once within the fragment used as probe, Southern blots of XbaI + EcoRI digests should reveal two bands for every gene copy. As shown in Fig. 6, the endogenous gene (lane WT) is cut by EcoRI + XbaI into fragments of ~25 kb (*A*) and 5.4 kb (*B*), while all five transformants

(lanes *1–5*) contain additional IC70-specific sequences. Integration of intact pG70S should result in new bands of 4.3 kb (*C*) and 1.9 (*D*); other bands presumably result from rearrangements or from integration of fragments of pG70S. The observed patterns suggest the presence of two to four additional IC70 gene copies in each transformant examined.

Further evidence that the introduced gene copies can complement *oda6* was obtained from genetic analysis of transformants. Since transformation of *Chlamydomonas* by the presently available methods results primarily or exclusively in integration by nonhomologous rather than site-specific recombination (Kindle et al., 1989; Diener et al., 1990), introduced gene copies should be integrated at random locations and should segregate away from the original *oda6-95* mutation during meiosis. If introduced genes are complementing the original mutation, then some meiotic products will display the mutant phenotype. If an intragenic reversion has eliminated the original mutation, it will not be uncovered in a backcross, while if an extragenic suppressor has been generated, it will segregate from the original mutation but will not cosegregate with introduced gene copies. Six pG70S transformants including the five analyzed in Fig. 6 were crossed with an *ODA6,ida*1-98 strain, and in every case paralyzed progeny were obtained, indicating that the original *oda6-95* allele was still present in the transformed cells (Table III). Furthermore, in products from a cross of

Figure 6. Genomic Southern blot of transformants. Lane WT contains DNA from wild-type strain 137c, while lanes 1–5 are DNA samples of five independently isolated transformants selected as swimming cells in the nonmotile *oda6,ida*1 background after transformation with pG70S. All samples are digested with EcoRI + XbaI and probed with the pG70S insert. Fragments A (25 kb) and B (5.4 kb) are derived from the endogenous gene, as indicated on the restriction map of the IC70 locus on linkage group XII (*middle*), while fragments C (4.3 kb) and D (1.9 kb) would be expected from integration of the complete pG70S insert (*bottom*). An intact copy of band D has not been integrated in transformants 1 and 4, but all of the transformants appear to contain two to four extra gene copies.

Table III. Backcross of pG70S Transformants

Transformant	Motile/paralyzed products		
	4:0	3:1	2:2
1	0	5	1
2	0	2	0
3	0	1	2
4	0	5	0
5	2	6	0
6	2	5	1

Six putative transformants (*oda6, ida*1, pG70-n) were crossed with *ida*1, and tetrad products of each cross were scored as motile or paralyzed. The appearance of paralyzed products among the progeny of all six crosses indicates that motility of putative transformants is not due to intragenic reversion of the *oda6* mutation.

transformant *#3* with an *oda6-95,IDA*1 strain, the introduced pG70S sequences cosegregate with the *ODA6+* phenotype (Fig. 7), confirming that pG70S is complementing the *oda6-95* mutation.

Transformation with an *oda6 IC70* Clone

When wild-type and *oda6-95* alleles of the IC70 locus were compared by probing genomic Southern blots with pG70S, no differences in restriction fragment size were observed (not shown). To eliminate the possibility that *oda6* was closely linked but not allelic to IC70, and was merely complemented by overexpression of the nearby IC70 gene, we cloned the IC70 gene from *oda6* cells and tested its ability to complement the mutation. If *oda6* were not an IC70 mutation, overexpression of the IC70 allele cloned from *oda6* cells should also rescue the mutation. A plasmid minilibrary of ~6.2 kb SacI fragments was prepared from DNA isolated from an *oda6-95* strain, and clone pBoda6-c7 was selected. No differences were observed in the location of restriction sites in the pBoda6-c7 and pG70S inserts when 10 enzymes known to cut the wild-type gene were used.

In two separate experiments, 5×10^7 *oda6,ida*1 cells were transformed with 5 μg of either pG70S or pBoda6-c7 and distributed into five tubes. Each time, swimming cells were observed in all five tubes containing cells transformed with pG70S after 5–7 d, while no swimming cells were observed in tubes containing cells transformed with pBoda6-c7.

Discussion

Because flagellar motility depends on the coordinate action of a large number of components, it has been an obvious target of mutational analysis. Flagellar mutations have been described that selectively disrupt assembly of part or all of the central pair complex, radial spokes, inner dynein arms, outer dynein arms, basal bodies, and even structures within the doublet microtubules (reviewed in Dutcher and Lux, 1989). Primarily by electrophoretic analysis of flagellar proteins from intragenic pseudorevertants and temporary dikaryons, the gene products of many of these mutations have been identified (Luck et al., 1977), but for reasons that are not clearly understood, these methods have not been successfully applied to mutations that disrupt outer arm dynein assembly. Gene products have thus far been identified only for *oda4*, which encodes the beta-heavy chain (Huang et al., 1982; Luck and Piperno, 1989), and for *oda6* (this study).
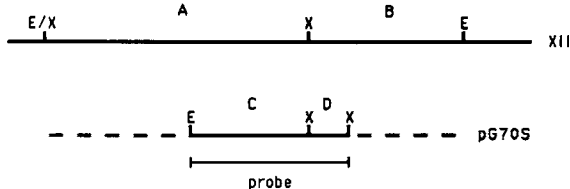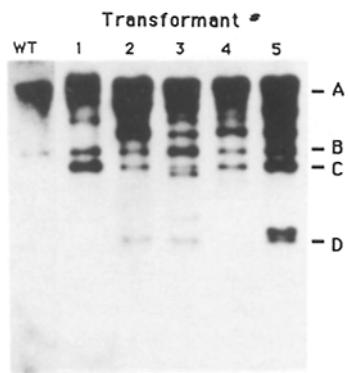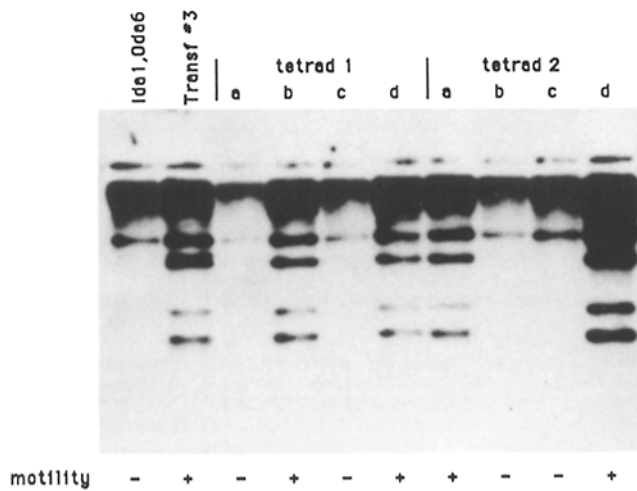
Figure 7. Genomic Southern blot demonstrating cosegregation of integrated gene copies and the wild-type *ODA6+* phenotype. DNA from the paralyzed recipient strain (lane *1*), motile transformant #3 (lane 2), and two tetrads from a cross between transformant #3 and an *oda6-95* strain (lanes *3–10*), was digested with EcoRI + XbaI and probed with pG70S, as in Fig. 6. Motility was scored by measuring flagellar beat frequency, and is listed below each lane as wild-type (+) or reduced to levels typical of oda mutants (−).

We previously selected and characterized a genomic clone, Da1, encoding part of the *Chlamydomonas* 70-kD dynein intermediate chain (Williams et al., 1986), and this clone was used by Ranum et al. (1988) to map the corresponding genetic locus to a region of the left arm of linkage group XII, ~22 map units distal to the centromere. Mutations affecting outer dynein arms are absent from published maps of the corresponding interval of linkage group XII (and of linkage group XIII, which may not actually be a separate linkage group from XII; Dutcher et al., 1991). We have now mapped two oda loci, *oda6* and *oda9*, to this general region, and have determined their locations relative to the IC70 gene.

RFLP analysis with cDNA clone pBc70-16 and the two *oda* loci on linkage group XII revealed that IC70 had recombined with *oda9*, but not with *oda6*. Only 14 tetrads from the cross between *oda6* and S1D2 were examined, so that linkage better than three map units could not be proven, but since further RFLP analysis could merely reduce the possible interval between IC70 and *oda6* without proving identity, we turned to other methods to extend this analysis. Southern blots indicated that *Chlamydomonas* contains only a single copy of the IC70 gene, eliminating the possibility that *oda6* and IC70 could be closely related genes resulting from a recent gene duplication. To further rule out the remote possibility that *oda6* represented a mutation in a closely-linked, unrelated gene, and not in the IC70 structural gene, we cloned full-length copies of the wild-type and *oda6-95* IC70 genes and tested their ability to complement the *oda6* mutation by transformation.

A 17-kb genomic fragment spanning the wild-type IC70 locus, as well as a 6-kb SacI subclone of the wild-type gene, were each capable of complementing the *oda6* mutation when introduced into *oda6,ida1* cells by glass bead–mediated transformation. Transformation events that successfully complemented the *oda6* mutation were selected directly by

screening for motile cells, and in all cases examined, motile clones contained one or more newly introduced gene copies. Proof that introduced gene copies were responsible for loss of the mutant phenotype included genetic crosses in which the new wild-type locus cosegregated with the introduced sequences. Additional crosses in which the original mutant locus was uncovered indicated that swimming cells were not spontaneous revertants of *oda6-95*.

Similar transformation experiments with the *oda6-95* IC70 gene failed to complement the mutation, indicating that the *oda6-95* mutation resides within the 6.2-kb SacI fragment used in these experiments. Preliminary transformation experiments with chimeric genes suggest that the *oda6-95* defect is located in the 5′-half of the IC70 transcription unit (Y. Kang and D. Mitchell, unpublished observations), but further molecular analysis will be needed to determine the exact nature of this mutation.

This is, to our knowledge, the first report of a full-length sequence for any dynein protein, so the apparent lack of homology with other published sequences is perhaps not unexpected. Cross-reactivity of antibodies against this *Chlamydomonas* protein with outer arm dynein proteins of similar size in sea urchin and trout spermatozoa (King et al., 1990) indicates that at least portions of the primary structure have been conserved across a considerable evolutionary distance. Whether this conservation extends to intermediate chains of flagellar inner row dynein arms or cytoplasmic dyneins is a question of considerable interest that must await further study.

The 70-kD intermediate chain is clearly needed for assembly of other outer arm dynein proteins, since none of the major outer arm dynein subunits are present in flagella isolated from cells containing either of two *oda6* alleles (Kamiya, 1988). The precise reason for lack of outer arm assembly in these mutants has not been determined. If attachment of outer arm complexes to doublet microtubules is directly mediated by the 70-kD intermediate chain, then its absence or alteration would necessarily prevent other proteins from assembling, and would result in an oda phenotype. Models of dynein morphology based both on electron microscopic images of extracted dynein particles and on biochemical dissociation experiments place a heterodimer of the 70- and 80-kD intermediate chains at the "base" of the tripartite *Chlamydomonas* outer arm dynein (King et al., 1990; Mitchell and Rosenbaum, 1986; King and Witman, 1990), but unfortunately, although the data show that intermediate chains are at one end of the extracted dynein complex, the relationship between the morphology of dyneins in vitro and in situ remains poorly understood. Electron microscopic analysis of in situ dyneins both in wild-type cells (Goodenough and Heuser, 1984) and in alpha heavy chain assembly mutant *oda11* (Sakakibara et al., 1991), suggests that globular heavy chain head domains are located closer to the site of force generation on the B subfiber than to the structural attachment site on the A subfiber, but an exact location of intermediate chains within the in situ outer row arm remains to be determined.

An alternative hypothesis to explain the assembly phenotype of *oda6* would be that dynein proteins normally associate into complexes in the cytoplasm and attach to outer doublets as complete outer arm units, in which case the functional absence of any member of the complex could prevent

an entire unit from assembling. This idea is supported by the observation that mutations at all of the other *oda* loci, with the exception of *oda*11, result in similar assembly defects. It also provides an explanation for the inability of mutations at some of these loci to complement mutations at other oda loci in temporary diploids (Kamiya, 1988), since the normal proteins contributed by each haploid cell would be sequestered into incomplete complexes and hence be unable to associate into a functional dynein arm. Further knowledge of the gene products of other *oda* loci, and of the location of dynein polypeptides within the in situ outer arm structure, should begin to resolve these questions.

The ability to use transformation with a wild-type gene to identify the gene product of a *Chlamydomonas* mutation, as reported here, demonstrates the expanding repertoire of techniques available for molecular and genetic analyses of cellular processes in this organism. It should now be possible to use complementation with a genomic library to clone the remaining *oda* loci and to determine whether they encode additional known dynein subunits, previously unidentified structural components of the flagellum essential to dynein assembly, or proteins that act in nonstructural roles to aid in the assembly process. Analysis of the functions of individual dynein subunits in force generation should then be amenable to dissection through transformation of null mutants with in vitro-mutagenized genes.

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