

Reversion analysis of dynein intermediate chain function

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SUMMARY

The *ODA6* locus of *Chlamydomonas reinhardtii* encodes a 70 kDa intermediate chain protein of the flagellar outer row dynein ATPase, and mutations at this locus prevent assembly of the entire outer row dynein arm complex. To initiate a structure-function analysis of the 70 kDa protein, we used transformation with chimeric mutant/wild-type genes to localize the defect in one assembly mutation, *oda6-95*. Sequence analysis revealed a frame-shift mutation in codon 53, which is followed by a stop codon after 13 amino acids in the new reading frame. By selecting intragenic pseudorevertants of this mutation we obtained 11 new *oda6* alleles. Many of these pseudorevertants encode intermediate chain proteins that permit assembly of outer row arms but do not restore full wild-type motility. Revertant strains fall into two phenotypic classes, one with average beat frequencies of 54 Hz (similar to wild type) and one with average frequencies of 27 Hz (compared with 24 Hz for *oda6-95*) during normal forward swimming. Low beat

frequency strains also display abnormalities during photophobic reversal (symmetric waveform). Amplification and sequence analysis of revertant alleles indicated that each reversion caused a second frame-shift, within a 115 nt interval, which restored the original reading frame, and that phenotypic severity was related to both direction (5' or 3') and distance between the original mutation and the reversion event. On the basis of immunoblot analysis of outer arm proteins, we conclude that revertant motility defects do not correlate with deficits in assembly of a specific dynein heavy chain or intermediate chain polypeptide, and electron microscopy confirms that revertants have normal outer arm structures. These results suggest that the 70 kDa intermediate chain plays a direct role in outer arm function distinct from its role in the assembly process.

Key words: dynein mutants, outer dynein arm, flagellar motility, *Chlamydomonas*

INTRODUCTION

Dynein ATPases constitute a growing class of microtubule-associated molecular motors. Although first characterized as force generators in cilia and flagella, dyneins are also cytoplasmic motor proteins with potential roles in axonal transport (Vallee et al., 1988), intracellular organization (Scholey et al., 1984; Lacey and Haimo, 1992) and mitosis (Pfarr et al., 1990; Steuer et al., 1990).

While a great deal of progress has been made in determining the enzymatic, structural and mechanochemical properties of dyneins, much remains to be learned about the function of individual subunits within these large complexes. This lack of knowledge stems in part from the large size, biochemical complexity and isotopic diversity of these molecules. All dyneins studied to date contain two or three catalytic heavy chains of ~450 kDa, and as many as 12 smaller proteins whose functions are for the most part unknown. While the two heavy chains of some cytoplasmic dyneins may be identical (Vallee et al., 1988), each of the two (Tang et al., 1982) or three (Pfister et al., 1982; Piperno and Luck, 1979) heavy chains of the flagellar dyneins that have been extensively studied are unique pro-

teins, and sequence analysis has shown that an individual dynein heavy chain may have as many as four nucleotide binding site consensus motifs (Gibbons et al., 1991; Ogawa, 1991; Koonce et al., 1992; and D. R. Mitchell, unpublished observations), adding to overall complexity. In *Chlamydomonas reinhardtii*, outer row dynein arms have three catalytic subunits (alpha, beta and gamma) each containing a unique heavy chain (400-500 kDa) and one or more light chains (14-20 kDa) (Piperno and Luck, 1979; Pfister and Witman, 1984). Each outer row arm also has a non-catalytic subunit consisting of two intermediate chains of ~70 and 78 kDa (IC70 and IC78) and several additional light chains (Pfister and Witman, 1984; Mitchell and Rosenbaum, 1986; see Witman, 1989, 1992, for recent reviews).

Results of recent studies suggest that the intermediate chain subunit may play an important role in outer arm structure. The larger intermediate chain, IC78, can be crosslinked directly to tubulin in situ (King et al., 1991), suggesting that it is part of, or in close proximity to, a microtubule attachment site. The smaller intermediate chain, IC70, is required for outer arm assembly (Mitchell and Kang, 1991) and has been localized by immuno-electron microscopy with a monoclonal antibody to the base of

the tripartite 'bouquet' of isolated outer arm dynein particles (King and Witman, 1990). While a direct relationship between in situ outer arm structure and isolated dynein particle structure remains unclear, electron microscopic analysis of flagellar axonemes from wild-type cells (Goodenough and Heuser, 1984) and from heavy chain assembly mutants (Sakakibara et al., 1991) supports a model in which the large globular heavy chain domains that form the heads of in vitro particle bouquets are equivalent to the ends of in situ arms farthest from the A-tubule, while intermediate chains are located near the proximal, A-tubule attachment end. Taken together, the evidence suggests that the intermediate chain heterodimer forms a structural core important for attachment of each outer dynein arm to A-tubule sites of doublet microtubules.

To analyze further the role of intermediate chains in dynein assembly and function, we have selected new alleles at the *IC70* (*ODA6*) locus. Our allele selection scheme takes advantage of a built-in redundancy in the flagellar force-generating mechanism. Most mutations that prevent outer arm dynein assembly (*oda* mutations) result in flagella that beat, but at only 40% of the wild-type frequency. Mutations that disrupt any one of several inner arm dynein complexes affect flagellar waveform, but have minimal effects on beat frequency, while double mutants with assembly defects at both inner and outer arm loci are completely paralyzed. Since *Chlamydomonas* grows as a vegetative haploid, mutant alleles are fully expressed. By selecting motile revertants of a paralyzed strain containing an *oda6* allele (*oda6-95*) and an inner arm assembly mutation (*pf9-2*), we obtained intragenic pseudorevertants at the *oda6* locus that permit outer arm assembly, but fail to restore wild-type beat frequency. Several revertants were characterized at both molecular and phenotypic levels to correlate altered intermediate chain structure with changes in outer dynein arm function.

MATERIALS AND METHODS

Cells

The following *Chlamydomonas* stocks were used: *oda6-67* and *oda6-95* (Kamiya, 1988); *ida1-98* (Kamiya et al., 1991); *pf9-2* (Porter et al., 1992); *uni1-1* (Huang et al., 1982a); and wild-type strain 137c from J. Rosenbaum (Yale Univ.). All double mutant strains were constructed in this lab. *Chlamydomonas* genetic experiments followed standard procedures (Harris, 1989). Strains containing *ida1-98* were clonally selected at frequent intervals to eliminate spontaneous revertants.

Transformation with chimeric genes

Chimeric genes were constructed as follows. A 6 kb *SacI* fragment spanning the wild-type *ODA6* allele (Mitchell and Kang, 1991), cloned into the *SacI* site of pGEM2, was digested to completion with *BamHI*, *HindIII* and *NheI*, and either digested to completion with *SmaI* or partially digested with *BglIII*. An *NheI* - *SmaI* fragment of 2097 bp, and *NheI* - *BglIII* fragments of 1857 bp, 1094 bp and 446 bp were gel-purified. The *oda6-95* allele, cloned as a 6 kb *SacI* fragment into pBluescript KS⁻ (Mitchell and Kang, 1991), was digested to completion with *NheI*, and partially digested with either *SmaI* or *BglIII*. Digestion products containing the vector were gel-purified and ligated with appropriate fragments

from the wild-type allele. Resulting chimeric genes were tested for correct size and vector before further use.

For transformation, paralyzed *oda6-95,ida1-98* cells grown in liquid MII medium (Sager and Granick, 1953) were pre-treated with autolysin to remove cell walls (Harris, 1989), and 1×10^8 cells were transformed with 1 μ g of plasmid by the glass bead method (Kindle, 1990). After transformation, cells were distributed into 5 tubes each containing 10 ml of MI medium (Sager and Granick, 1953) and motile transformants were selected from the tops of the tubes after 5-10 days in constant light. Each experiment included positive (complete wild-type gene) and negative (vector alone) controls.

Mutagenesis and selection of revertants

To select revertants of *oda6-95*, cells of a paralyzed double mutant *oda6-95,pf9-2* strain were mutagenized by ultraviolet irradiation as previously described (Mitchell and Rosenbaum, 1985). Irradiated samples were distributed into tubes of MI medium and examined periodically for the presence of motile cells. Samples from each tube that contained swimming cells were prepared for immunofluorescence microscopy (Sakakibara et al., 1991) using a mixture of three monoclonal antibodies directed against the alpha heavy chain, the beta heavy chain, and *IC70* outer arm dynein proteins (Mitchell and Rosenbaum, 1986). Five clones were selected from each tube containing cells with immunopositive flagella and were retested by immunofluorescence. A single positive clone corresponding to each original tube was then selected for further analysis.

Analysis of flagellar motility

Flagellar beat frequency of individual cells was determined by tuning the frequency of a Chadwick Helmholtz 8440 stroboscopic power supply attached to a 50 W xenon illuminator while observing the cells under dark-field illumination, as previously described (Mitchell and Kang, 1991). Each reported frequency represents the average of three determinations on separate samples, with 10 cells analyzed for each sample. To record flagellar waveform, each strain was crossed into a *uni1-1* background (Brokaw et al., 1982). Cells grown in liquid MI medium were suspended in MI medium containing 1 mM CaCl₂ and photographed with a Nihon Kohden model PC-2A camera mounted on a Zeiss Axioskop microscope. Images taken through a $\times 40$ oil immersion Plan Apo objective were recorded on 3200 ASA T-Max film (Kodak). To induce photophobic reversals, cells were pre-adapted to low-level red illumination (Kodak no.25 Wrattan filter) and were photographed during transition to high intensity white illumination.

Amplification and cloning of revertant alleles

DNA was prepared from each revertant by cesium chloride centrifugation (Weeks et al., 1986) and amplified on a Perkin-Elmer Cetus thermocycler using Taq polymerase. Primer 1 (GCAGCCAGGATGGAGATCTACC) contains the ATG initiation codon (underlined) and a naturally occurring *BglIII* site (bold) and extends from nt 543 to 564 of Fig. 2 (below). Primer 2 (CTCGGATCCTCTACGTGGCTCATCG) was constructed with an artificial *BamHI* site followed by the complement of nt 769 to 786 of Fig. 2. Amplification was accomplished using 1 μ g of revertant DNA and 100 pmol of each primer in a 100 μ l reaction. An initial cycle of 5 min at 95°C, 1 min at 50°C, 1.5 min at 72°C was followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, 1.5 min at 72°C. Amplified products (250 bp) were digested with *BamHI* and *BglIII*, gel-purified, and ligated into the *BamHI* site of pBluescript KS⁺ for sequencing.

Sequence analysis

Overlapping deletions of genomic clone pG70S (Mitchell and

Kang, 1991) were prepared in pBluescript with an Erase-a-base kit (Promega Biotec, Madison, WI). To sequence the *oda6-95* mutation, a 648 bp *Bgl*III fragment extending from nt 556 to nt 1204 of the mutant gene was subcloned in both orientations into the *Bam*HI site of pBluescript. All constructs were transformed into DIH101 and single-stranded DNA was produced by superinfection with R408 helper phage (Kidd et al., 1986) and sequenced by chain-termination reactions using a Sequenase kit from US Biochemicals (Cleveland, OH). Sequences were assembled and analyzed using the GCG programs (Devereux et al., 1984).

Flagellar fractionation and western blotting

Flagella were isolated from cells grown in liquid medium and deflagellated with dibucaine, as previously described (Mitchell and Rosenbaum, 1985). Purified flagella were demembrated by suspension in HMDEK (10 mM HEPES, pH 7.4, 4 mM MgSO₄, 1 mM DTT, 1 mM EDTA, 25 mM KCl) containing 0.1% Triton X-100. To purify the 18 S and 12 S dynein fractions used to characterize antiserum TD-1C, demembrated axonemes were extracted with 0.6 M KCl in HMDEK, and the extract was fractionated through a 0% to 30% sucrose gradient in HMDEK as previously described (Mitchell and Rosenbaum, 1986).

To prepare samples for electrophoresis, either sucrose gradient fractions, or flagellar pellets resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, were mixed with an equal volume of 2× SDS sample buffer. Proteins were separated on 4% to 10% acrylamide, 3 M urea gels using Tris-glycine buffers (Mitchell and Rosenbaum, 1986), and were transferred to Immobilon membranes (Millipore Corp., Bedford, MA) in Tris-glycine electrophoresis buffer containing 10% methanol. Transfers were stained for protein with amido black, and for outer arm dynein proteins with mouse monoclonal antibody C11.4 against the IC70 protein (Mitchell and Rosenbaum, 1986), or with rabbit antiserum TD-1C against the gamma heavy chain and IC78 (see below). Peroxidase-labeled goat anti-mouse or goat anti-rabbit second antibodies (Bio-Rad) were used at a 1:1000 dilution, and reactions were developed using 4-Cl-naphthol enhanced with *N,N*-dimethyl phenylenediamine (Kobayashi and Tashima, 1989).

Specificity of the antibodies used in this study is illustrated in Fig. 1. Antiserum TD-1C, a gift from J. T. Travis (SUNY Albany), was produced by immunizing a rabbit with sucrose gradient-purified *Tetrahymena thermophila* 21 S dynein (J. T. Travis, personal communication). When used on western blots of *Chlamydomonas* outer arm dynein proteins, TD-1C reacts exclusively with the 78 kDa intermediate chain present in the faster-sedimenting (18 S) outer arm dynein fraction (Fig. 1, lane 3) and the gamma heavy chain present in the slower sedimenting (12 S) fraction (Fig. 1, lane 4). This antiserum does not cross-react with any other *Chlamydomonas* dynein proteins on western blots of total flagellar proteins (Fig. 1, lane 6), although another serum sample from the same animal reacted weakly with a 140 kDa inner arm dynein protein (data not shown). Minor bands below the gamma chain on the blot in lane 6 are not consistently present and are most likely to be proteolytic fragments of the gamma chain. Monoclonal C11.4 reacts exclusively with IC70 on blots of total flagellar protein (Fig. 1, lane 7), as previously described (Mitchell and Rosenbaum, 1986). Lanes 5, 6 and 7 of Fig. 1 are the same piece of Immobilon, which was stained with amido black, photographed and stripped in 95% ethanol before successive reactions with C11.4 and TD-1C.

Electron microscopy

Demembrated flagellar axonemes were pelleted and fixed in 2% glutaraldehyde, 50 mM sodium phosphate, pH 7.4, for 18 h at 4°C, postfixed in 1% osmium tetroxide for 1 h at room temperature, dehydrated in a graded ethanol series, and embedded in Epon

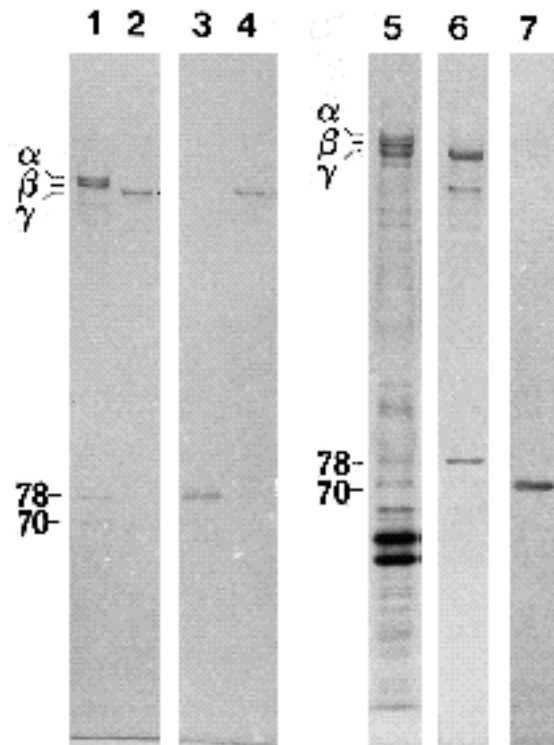


Fig. 1. Immunoblot characterization of antibodies. Lanes 1-4, blots of purified 18 S dynein (lanes 1 and 3) and 12 S dynein (lanes 2 and 4) were stained for protein with amido black (lanes 1 and 2) or reacted with polyclonal antibody TD-1C (lanes 3 and 4). Lanes 5-7, blots of total flagellar proteins were stained with amido black (lane 5), reacted with polyclonal TD-1C (lane 6), or reacted with monoclonal C11.4 (lane 7). The locations of outer arm dynein heavy chains (alpha, beta and gamma) and intermediate chains (IC78 and IC70) are indicated to the left of lanes 1 and 5. TD-1C reacts with the gamma chain (lanes 4 and 6) and IC78 (lanes 3 and 6), while C11.4 reacts exclusively with IC70 (lane 7). All proteins were separated on 4% to 10% acrylamide SDS-urea gels and transferred to Immobilon.

812. Thin sections were stained with uranyl acetate and lead citrate, and viewed in a JEOL 100-CX electron microscope. Images were recorded at an original magnification of ×53,400.

RESULTS

Sequence analysis of the *IC70* gene

We previously determined that the *Chlamydomonas oda6-95* mutation, which prevents outer arm assembly, is a defect in the *IC70* gene (Mitchell and Kang, 1991). Although wild-type and *oda6-95* genomic clones were characterized in that study, only wild-type cDNA sequences were reported. In order to further characterize the mutant locus, we first sequenced portions of genomic clone G70S (Mitchell and Kang, 1991) that span the complete wild-type gene, and compared them with the cDNA sequence. Regions of the wild-type gene sequence relevant to the experiments described in this paper, extending from the beginning of cDNA clone c70-16 (Mitchell and Kang, 1991) to codon 239, are presented in Fig. 2. Since a precise transcription initiation site has not been determined for this gene,

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1  ATTGGAATTGCACCGAATAGCTTGACCTGTTATAAGGCCCTTCCGGGAACACCTCGGCA 60
61  CGCGCTTTACTACTGCTTTATTGACCCGAATGGCTGGGCCACACATAATAGCTAGCAGCAT 120
121 GAGAACACTAGACGCTGCTGGGCATTGCTGAACACAGTCGgtgagtgagaaattcgaga 180
181  gccgggcaagccggggtcaagcaggccatgccgcgctgttagcaatgttctatcgtgct 240
241  agtaaggtttgcagccagctcgagctcagggcgctccagttggagtgagtcaccggg 300
301  gaccatgttggtcgctgttttggctattcgcgactgggtattcaagcgtgctgaggggaaa 360
361  ctatatggtcttccagcagtagcgggtttctggaactgcagGTCACCCGTCACCGAGCAGC 420
421  AGCGCAGCTGGGGGGCCGGTTCAGCAGCGTGCCACGACCGGAACTCGCGCCCGCGTGC 480
481  CCACACGGAGGCAGCAGCAGCAGCGGTTAGCAGAAACCCCTCCGATCACTGCGTAGGAGCT 540
541  AAGCAGCCAGGATGGAGATCTACCATCAATATATTAAGTCGCGCAAGCAGTTGGCCGCT 600
1  M E I Y H Q Y I K L R K Q F G R F
601  TTCCAAGTTTGGGGATGAGGGCAGCGAGATGCTTGGGCACATCCGCCCAACGAGGACC 660
18  P K F G D E G S E M L A D I R P N E D H
661  ACGCAAGGAGTACATCCCGCGCAACCCCGTCACGACGGTGACGAGTGCGTGCOCGGAGA 720
38  G K E Y I P R N P V T T V T Q C V P E M
721  TGTCCGAGCATGAGGGCAACACCAACCGCGGTGATCCTGGTCAACAGGCGATGAGCCAGC 780
58  S E H E A N T N A V I L V N K A M S H V
781  TAGAGGGCGGGTGGCCCAAGGATGTTGACTACACCGAAGCGGAGCACACGATCCCGTACC 840
78  E G G W P K D V D Y T E A E H T I R Y R
841  GCAAGAAGTGGAGAAGGATGAGGACTACATCCGTCAGTTGTCAGCTGGGCTCGTCAG 900
98  K K V E K D E D Y I R T V V Q L G S S V
901  TGGAGGACCTGATCAAGCAGAACACCGCGGTGGACATCTACCAGGAGTACTTCCAGAACG 960
118  E D L I K Q N N A V D I Y Q E Y F T N V
961  TGACGATGGACCACACGTCGGAGGCACCGCACCTGAAGACGGTGACGGTGTCAAGAAC 1020
138  T M D H T S E A P H V K T V T V F K D P
1021  CTAACAACATCAAGCGCAGCGCCTCCTACGTCAACTGGCACCCAGAGCGGCTCCGTGCCA 1080
158  N N I K R S A S Y V N W H P D G S V P K
1081  AGGTGGTGGTGGCGTACTCGATCCTGAGTCCAGCAGCAGCGCGCGGATGCGCTCA 1140
178  V V V A Y S I L Q F Q Q Q P A G M P L S
1141  GCTCGTACATATGGGACGTGAACAACCCCAACACCGCGAGTACGAGATGGTCCCCACCA 1200
198  S Y I W D V N N P N T P E Y E M V P T S
1201  GCCAGATCTGTGCGCAAGTTCAACCTCAAGTggtgggaagcggctgcccggggggtgag 1260
218  Q I C C A K F N L K
1261  ggagtcggggaggggagggattgcccgtcaagggacgcggggcaaggataagggggaag 1320
1321  ggatgcggggcagggcaggggtgctggggcaacagtcggggaatgggtggcttggaac 1380
1381  ggccgcgtgtggtgttacoggtggcgctcctcaagcatctagocagccgaaggtgcttc 1440
1441  ctggcagctgggagtaggcaatatcatcccgtgcgcaagaatctgctcctcgcogcttc 1500
1501  gccccgatccgcttctcaccgttccagctcttggcgctccagctctcaccctcccta 1560
1561  gctcccgtccctccctccctccctccctccctccctccctccctccctccctccctccctcc 1620
1621  tctctccgcagGACAACAACCTTGTGGCGGGCCAGTACAACGGC.....
228  D N N L V G A G Q Y N G.....

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Fig. 2. Nucleotide and translated amino acid sequence of the 5 portion of the *IC70/ODA6* gene. Exons are shown in upper case introns in lower case, and deduced amino acids in single letter code. Nucleotide numbering is based on the beginning of cDNA clone c70-16 (Mitchell and Kang, 1991) and may not reflect the true transcription initiation site. The mutation in *oda6-95* involves the three underlined nucleotides at position 710-712 and results in translational termination at a TGA codon (italicized) at position 751-753 (see text for details).

sequence numbering is based on the 5' end of the cDNA clone. As shown in Fig. 2, and diagrammatically in Fig. 3, the *IC70* gene contains two introns, one of 240 bp within the 5' untranslated region and another of 400 bp between the codons for amino acids 227 and 228. All four splice junctions are consistent with *Chlamydomonas* splice donor and acceptor consensus sequences. No deviations from the previously reported cDNA sequence were observed except that the C reported as the first base of cDNA clone c70-16 (Mitchell and Kang, 1991) is not present in the genomic sequence and presumably was introduced during cDNA library construction.

oda6-95 mutation

To localize the *oda6-95* mutation, plasmids containing chimeric genes were constructed in which restriction fragments of the cloned mutant gene that extended various distances 3' of an *NheI* site in the first exon were replaced by equivalent fragments from the wild-type gene. Chimeric genes were then introduced into a paralyzed *oda6-95,ida1-98* double mutant strain to test their ability to complement the *oda6-95* mutation and restore motility (Mitchell and Kang, 1991). This assay exploits the interaction between inner and outer arm assembly defects that results in paralysis of double mutants, even though single mutations disrupting either inner or outer row dyneins do not prevent motility (Huang et al., 1982a). As shown schematically in Fig. 3, replacement of a 446 bp *NheI* - *BglIII* fragment, which extends just 5 bp beyond the ATG initiation codon, was not able to repair the mutant gene. Replacement of larger fragments extending from the same *NheI* site to any of three sites further 3' converted the *oda6-95* allele to wild-type.

A 648 bp *BglIII*-*BglIII* fragment spanning the apparent mutant locus was subcloned in both orientations into the *Bam*HI site of pBluescript KS⁺ and its sequence was determined. The only sequence variation from wild-type occurred at codons 53 and 54, where TGC GTG had changed to TG-TGG (bases in bold type are altered by the mutation, and - indicates a deletion). The overall effect of

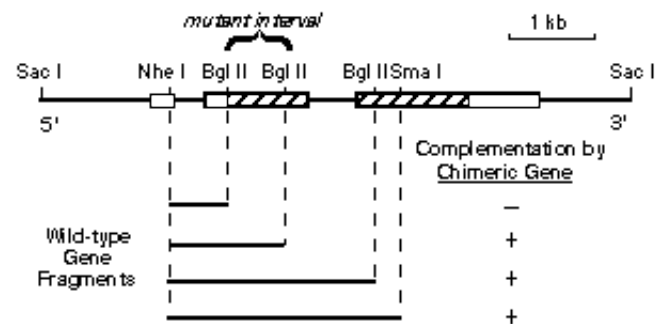


Fig. 3. Location of the mutation in *oda6-95*. The restriction map indicates the position of exons (boxed) and coding regions (hatched) within mutant genomic clone pBoda6-c7, and the location of restriction sites used to generate chimeric genes. Fragments of pBoda6-c7 were replaced by the indicated wild-type fragments and chimeric genes were tested for their ability to rescue (complement) the *oda6-95* mutation. Complementation is shown by a + to the right of the fragment.

this mutation is a shift in reading frame, which results in translational termination at a TGA codon (nt 751-753 of Fig. 2) after the incorporation of only 13 new amino acids. The *oda6-95* allele is thus predicted to encode a peptide that represents less than 10% of the full-length (567 amino acid) wild-type IC70 protein.

Selection of revertants

We reasoned that by selecting revertants of this allele we might obtain new alleles (intragenic pseudorevertants) that only partially restored normal structure and activity to this protein. Examination of the *oda6-95* sequence shows that a second frame-shift occurring anywhere between an out-of-frame TGA stop codon at nt 617 and the new TGA stop codon at nt 751 could restore the correct reading frame without introducing another premature termination signal. To select such revertants, we constructed a new paralyzed double-mutant strain containing *oda6-95* and *ida1* allele *pf9-2* (Porter et al., 1992). While the paralyzed *oda6-95,ida1-98* strain used above for complementation studies could also have been used for revertant selection, we found that *pf9-2* had a lower reversion rate than *ida1-98*, and hence favored selection of new mutations at the *oda6* locus (data not shown). *oda6-95,pf9-2* cells were mutagenized by ultraviolet irradiation, and revertants to a motile phenotype were selected as swimming cells in tubes of liquid medium. A similar screen was used to select revertants and suppressors of *pf9-2* (Porter et al., 1992). In two screens of 10^7 cells distributed into 60 tubes, motile revertants were observed in every tube. Because such swimmers could result from reversion of *pf9-2*, reversion of *oda6-95*, or extragenic suppression of either locus, samples of each revertant population were tested for the presence of assembled outer arm dynein proteins by immunofluorescence microscopy using a mixture of three monoclonal antibodies directed against the alpha, beta and IC70 proteins. A total of 16 tubes were identified as containing cells with assembled outer arm dynein, and colonies with immunofluorescence-positive flagella were obtained from 14 of these tubes. All 14 revertants were crossed to an *ODA6,pf9-2* strain, and the absence of paralyzed products among the progeny of at least six tetrads was taken to indicate that each was likely to represent an intragenic pseudoreversion rather than an extragenic suppressor.

On the basis of differences in swimming speed and beat frequency, revertant strains were divided into two major classes. Three revertants (*oda6-r88*, *-r111* and *-r113*) displayed beat frequencies and swimming velocities significantly lower than those of *ODA6,pf9-2* cells, while the remaining 11 strains (*oda6-r11*, *-r12*, *-r13*, *-r14*, *-r15*, *-r17*, *-r19*, *-r24*, *-r55*, *-r75* and *-r123*) did not (data not shown). All three low-frequency revertants and eight of the 11 high-frequency revertants were crossed into a wild-type background for further characterization of their biochemical, molecular and motile properties.

Beat frequency analysis

Flagellar beat frequencies of wild-type, *oda6-95* and 11 revertant strains are illustrated in Fig. 4. Eight revertants have beat frequencies between 48 and 60 Hz, and can be grouped into a high-frequency class (class I). Of these, only



Fig. 4. Beat frequency analysis of wild-type (wt), *oda6-95* (*oda*), and revertant strains. Eight of the revertants can be grouped in a high-frequency class, and the remaining three in a low-frequency class. All of the revertants differ significantly from *oda6-95*, and all but *r13*, *r15*, *r17* and *r19* differ significantly from wild type (Dunnett's test, $P = 0.01$). Each bar represents the mean \pm s.d. for 24 measurements.

four (*oda6-r11*, *-r24*, *-r75* and *-r123*) have average beat frequencies that differ significantly from wild type (Dunnett's test, $P = 0.01$). Two of the high-frequency revertants, *oda6-r15* and *oda6-r17*, were later revealed to have identical genotypes (see below). The remaining three revertants (*oda6-r88*, *-r111*, *-r113*) display average frequencies that differ significantly from both *oda6-95* (24 Hz) and wild type (60 Hz), but not from each other, and hence are grouped into a low-frequency class (class II). The overall average beat frequency for this class was 27 Hz.

Revertant sequence

To determine the specific defects responsible for reductions in beat frequency, we sequenced the relevant region of the *oda6* gene in 10 of the 11 revertants. A 250 bp DNA fragment was amplified by PCR, cloned into pBluescript, and sequenced, as described in Materials and Methods. For the eleventh revertant, *oda6-r113*, we have been unable to clone the amplified region for reasons not understood at present. The resulting sequences, and a schematic diagram of their predicted translation products, are shown in Fig. 5. Data for *oda6-r17* have been omitted because the sequence was repeatedly identical to that obtained from *oda6-r15*, using templates amplified from at least two different preparations of genomic DNA. For each revertant reported in Fig. 5, a second mutation has occurred in the interval between nt 640 and nt 753, which effectively shifts the reading frame back to wild-type before reaching the TGA codon that terminates translation of the *oda6-95* transcript. These new mutations create regions of predicted abnormal amino acid sequence of one (*oda6-r13*, *-r15*) to 23 (*oda6-r88*) residues. Eight reversions do so by deleting two additional base pairs, while a single reversion (*oda6-r24*) contains a four-base insertion. The two reversions in class II (*oda6-r88* and *-r111*) are located 21 and 23 codons 5' to the original *oda6-95* muta-

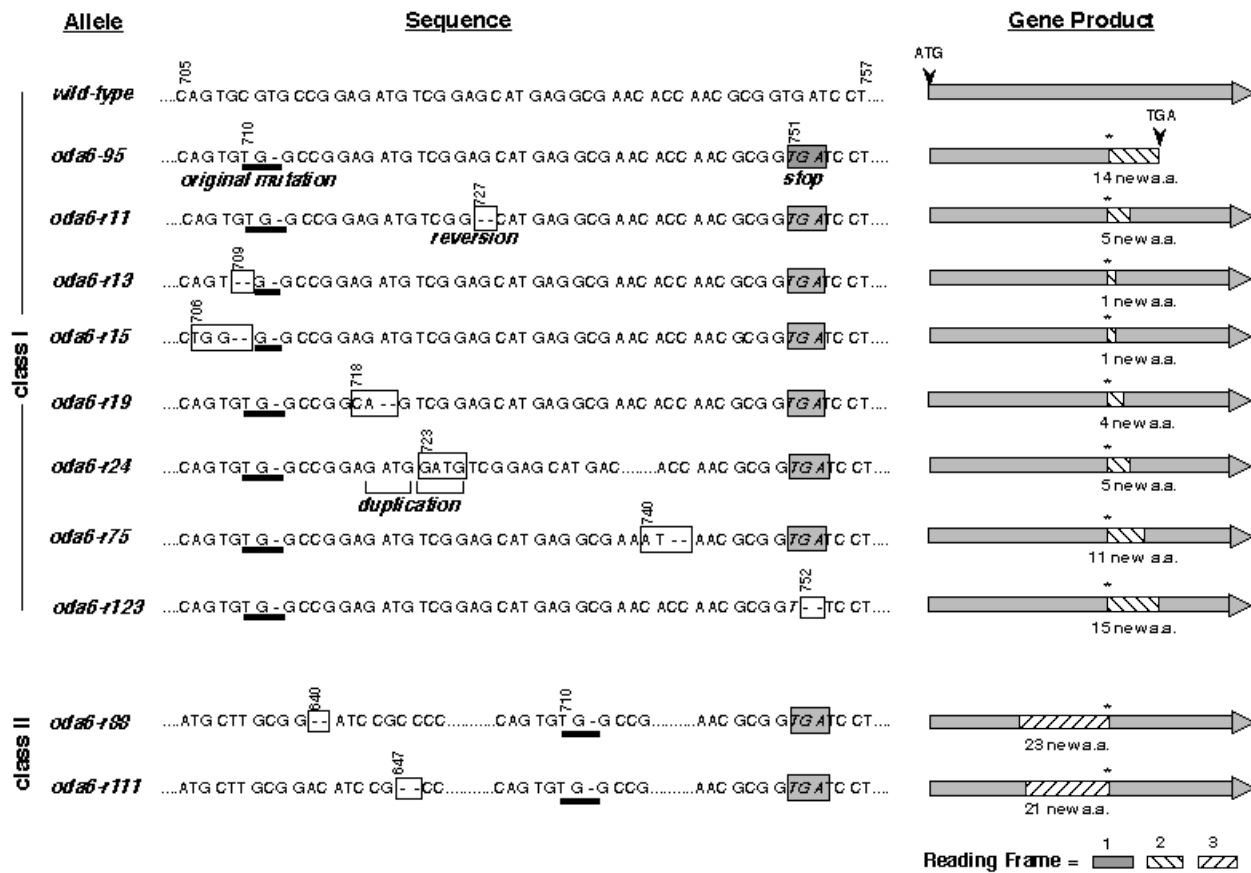


Fig. 5. Sequence of *oda6-95* intragenic pseudorevertants. Observed sequence alterations for the original mutation (underlined) and each revertant allele (boxed) are displayed in column two, where - - indicates deleted bases. The out-of-frame TGA that acts as a stop codon for the *oda6-95* allele is enclosed in a shaded box, and nucleotide numbers refer to nucleotide positions from Fig. 2. Column three shows the predicted effect of each mutation on translation, where the frameshift introduced by *oda6-95* is indicated by an asterisk.

tion, while those in class I lie from two codons 5 (*oda6-r15*) to 13 codons 3 (*oda6-r123*) of the original mutation. Note that the *oda6-r123* reversion event has precisely removed the last two nucleotides of the TGA stop codon, creating the greatest possible sequence alteration 3 from the original mutation.

Motility analysis

During the course of beat frequency observations, abnormalities in photophobic reversal responses were also noted. Specifically, all three class II revertants stopped swimming forward but failed to move in the reverse direction when presented with a sudden increase in white light intensity. Only occasional, low-amplitude bends were observed during these photophobic responses. In contrast, all class I revertants responded similarly to wild-type cells by briefly swimming backward with large-amplitude, symmetric flagellar bends. Representative alleles *oda6-r88* and *oda6-r123*, which encode intermediate chains with the longest frameshifts 5 and 3 from the original *oda6-95* mutation, were examined in greater detail. For these experiments, uni-flagellate cells were produced by crossing each allele into a *uni1-1* background (Brokaw et al., 1982), and 'wild-type' refers to *uni1-1* cells that are wild-type at the *ODA6* locus.

Flagellar waveforms were recorded with stroboscopic illumination on continuously moving film (see Materials and Methods for details).

Wild-type cells display an asymmetric flagellar waveform during normal forward motility (Fig. 6A), but switch to a symmetric waveform during the photophobic reversal response (Fig. 6B). Even with only a single flagellum, wild-type cells are frequently propelled backward during this reversal episode, as seen by the change in relative positions of the cell body and stationary debris (bright spot) in Fig. 6B. Similar records of *oda6-r88* and *oda6-r123* strains (Fig. 6C-F) reveal waveforms during forward swimming (Fig. 6C,E) that are similar to those of wild-type cells (Fig. 6A), but appear slightly less asymmetric, as seen most clearly by the reduction in curvature of the principal bend (arrows in Fig. 6A,C,E). Major differences were not observed between *oda6-r88* and -r123 in forward swimming waveform. During photophobic reversal *oda6-r88* generates only low bend amplitude and sporadic beating (Fig. 6D), while *oda6-r123* (Fig. 6F) responds by propagating symmetric flagellar bends similar to those of wild-type cells (Fig. 6B).

Outer arm assembly

The polypeptide composition of assembled outer arms in

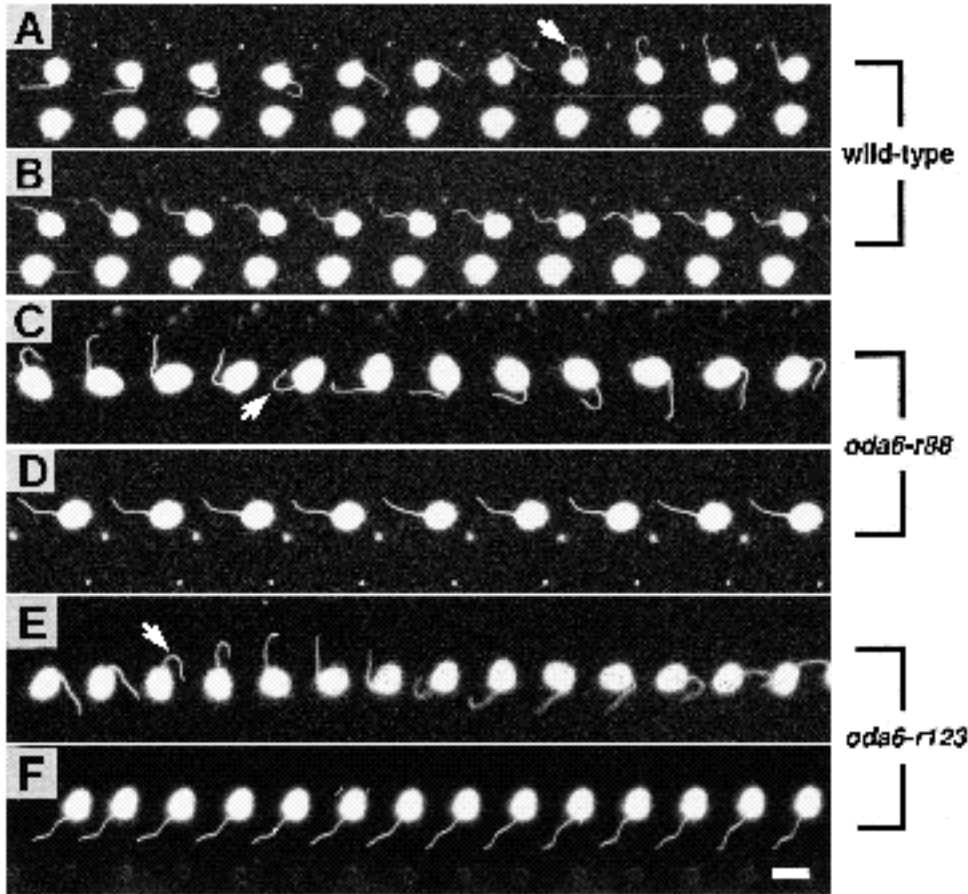


Fig. 6. Stroboscopic analysis of flagellar waveform. Uniflagellated cells were photographed on continuously moving film during episodes of forward-swimming waveform (A,C,E) or photophobic reversals induced by a transition from dim red light to bright white light (B,D,F). The same wild-type cell was filmed during (A) forward and (B) reverse motility; flash rate 62 Hz, film transport rate 20 cm/s. Two different *oda6-r88* cells are shown during (C) forward, and (D) reverse motility; flash rate 33 Hz, film transport 10 cm/s for C, flash rate 50 Hz, film transport 20 cm/s for D. The same *oda6-r123* cell is shown during (E) forward and (F) reverse motility; flash rate 40 Hz, film transport 10 cm/s. Forward beat frequencies were 65 Hz (A), 41 Hz (B), and 45 Hz (C). Reverse beat frequencies were not determined. Arrows in A,C,E indicate principal bends that illustrate the reduction in principal bend angle typical of *oda6* revertants.

representative pseudorevertant strains was compared with that of wild-type outer arms by SDS PAGE and immunoblot, to see whether assembly of the intermediate chains or any of the three outer arm heavy chain subunits was altered by these IC70 defects. Total flagellar protein samples were separated by SDS-PAGE and either stained for protein (Fig. 7A), or transferred and reacted with antibodies specific for three different outer row dynein polypeptides (Fig. 7B-D). All three outer arm dynein heavy chains (alpha, beta and gamma) are missing from *oda6-95* flagella (Fig. 7A, lane 2) but are present in flagella of wild-type (Fig. 7A, lane 1) and both pseudorevertant strains (Fig. 7A, lanes 3 and 4).

Because the gamma heavy chain is partially obscured by inner arm heavy chains, which comigrate with the gamma heavy chain in this gel system, and the intermediate chains are also not readily identifiable on one-dimensional gels of whole flagella, separate panels were probed with antibody-

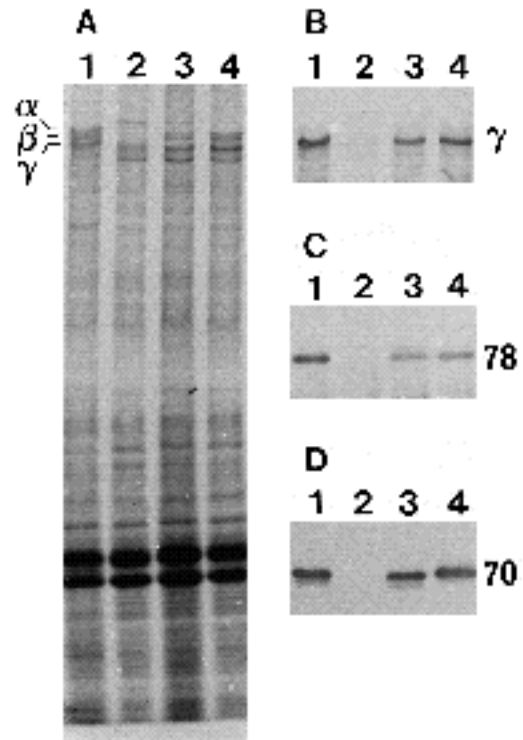


Fig. 7. Western blot analysis of outer arm dynein assembly. Total flagellar protein samples separated by SDS-PAGE and transferred to Immobilon were stained for protein with amido black to resolve the alpha and beta heavy chains (A) or immunostained to reveal the gamma heavy chain (B), 78 kDa intermediate chain (C), or 70 kDa intermediate chain (D). For each panel, flagellar samples were from wild-type (lane 1), *oda6-95* (lane 2), *oda6-r88* (lane 3) or *oda6-r123* (lane 4) cells. All five major outer arm proteins are missing from *oda6-95* flagella, but are present in both -r88 and -r123 revertant flagella.

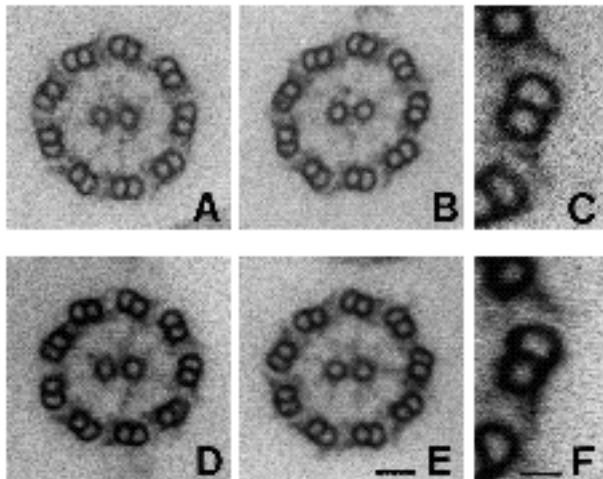


Fig. 8. Cross-sections of wild-type (A-C) and *oda6-r88* (D-F) axonemes reveal no major differences in outer arm dynein structures. (C) and (F) are enlargements of portions of (B) and (E), respectively. Bars: 50 nm for A,B,D,E; 25 nm for C,F.

ies to reveal the presence and relative amounts of each of these proteins. The gamma heavy chain (Fig. 7B) IC78 (Fig. 7C) and IC70 (Fig. 7D) proteins are all present in both revertants (lanes 3 and 4 of each panel). While the total amounts of protein loaded in each lane of Fig. 7A are approximately equal, the amount of each outer arm protein present in revertant samples (lanes 3 and 4 of each panel) is slightly reduced from the amount present in the wild-type sample (lane 1 in each panel). A slight reduction in the apparent size of the IC70 protein was also observed in samples from *oda6-r88* flagella, and is presumed to reflect changes in primary sequence specific to this reversion.

Outer arm structure

Although *oda6-r88* revertant flagella contain outer arm polypeptides, their assembly into typical outer arm structures cannot be assayed through biochemical analyses. Wild-type and revertant axonemes were therefore prepared for thin-section electron microscopy and cross-sections were scored for the presence and appearance of outer arm images. As shown by the images in Fig. 8, revertant axonemes (Fig. 8D-F) contain outer arms that display no discernible structural abnormalities when compared with wild-type axonemes (Fig. 8A-C). The average number of outer arms per *oda6-r88* cross-section was reduced, however, in agreement with the gel analysis shown in Fig. 7. While all cross-sections of wild-type axonemes had outer arm images on eight of the nine outer doublets, as previously reported for this species (Hoops and Witman, 1983), outer arms were frequently missing on two or three doublets of *oda6-r88* axonemes (av. 6.2 arms per cross-section; $n=40$). Positions lacking arm images formed no regular pattern.

DISCUSSION

The *oda6-95* mutation

To complement previous phenotypic analyses of dynein mutants, we have characterized a dynein intermediate chain mutation at the molecular level and used it as the starting material for creating new mutant alleles with unique phenotypic properties. Comparison of wild-type genomic and cDNA sequences revealed the presence of two introns in the *oda6* gene, one within the 5' untranslated region and a second in the coding region (Fig. 2). We localized the *oda6-95* mutation to coding regions within the second exon by transformation of mutant cells with chimeric mutant/wild-type genes (Fig. 3), and then sequenced the region to determine that the mutation is a 3-base rearrangement/deletion in codons 53 and 54 of the IC70 structural gene that results in a shift in translational reading frame. The predicted protein product of the *oda6-95* gene terminates after an additional 13 amino acids in the new frame.

The complete absence of major outer arm proteins in *oda6-95* flagella (Kamiya, 1988, and Fig. 7) confirms that the IC70 polypeptide is essential for outer arm assembly and suggests that the N-terminal 10% of the protein is insufficient to supply that essential function. We have not determined whether the predicted *oda6-95* product is stable and accumulates in the cytoplasm or in the flagellar compartment, or is rapidly degraded after synthesis. Because this mutant product would not include the anti-IC70 monoclonal C11.4 epitope, it would not have been detected in our immunoblot or immunofluorescence experiments.

Selection of pseudorevertants

Eleven intragenic pseudorevertants of *oda6-95* were selected and phenotypically characterized (Fig. 4). The primary screen to select these revertants was based on suppression of the nonmotile phenotype that results from genetic interactions between inner- and outer-arm assembly mutants, rather than directly on restoration of outer arm assembly or function. A similar screen has been used to select revertants and suppressors of *pf9-2* (Porter et al., 1992). This was followed by an immunofluorescent screen for assembled outer arm components and a genetic screen for intragenic revertants. By using this multi-step procedure we were able to obtain intragenic pseudorevertants with a wide range of genotypic and phenotypic defects. Genotypic analysis of several revertants (Fig. 5) reveals that most, like the original *oda6-95* mutation, are either simple deletions or small rearrangements accompanied by a deletion. These similarities could reflect either the specific mutagen used (254 nm ultraviolet irradiation) or the phenotypic selection applied.

Revertant phenotypes

The majority of outer arm dynein mutations described to date interfere with the assembly of most or all of the proteins normally present in outer row arms, and hence have very similar motility phenotypes (Brokaw and Kamiya, 1987). Three exceptions are *sup1*, which alters the beta heavy chain but does not block assembly (Huang et al., 1982b); *oda11*, which blocks assembly of the alpha subunit (consisting of the alpha heavy chain and one tightly associated light chain), but not the rest of the outer arm com-

plex (Sakakibara et al., 1991); and the *oda6-95* revertants described here. Beat frequency is only reduced by 15% in *oda11* (Sakakibara et al., 1991) and by 46% in *sup1* (Brokaw et al., 1982), as opposed to 60% in strains with no assembled outer arms such as *oda6-95* (Brokaw and Kamiya, 1987; Mitchell and Rosenbaum, 1985), suggesting that different outer arm heavy chain subunits do not contribute equally to outer arm function. The *oda6-95* revertants of class II, exemplified by *oda6-r88*, resemble most closely the original *oda6-95* mutation with respect to beat frequency during forward swimming (Fig. 4), and bend amplitude during photophobic reversal (Fig. 6), but differ in their genetic interactions with inner arm loci, since double mutants between these revertants and *pf9-2* have full-length, motile flagella, while double-mutants between *oda6-95* and *pf9-2* have stubby, paralyzed flagella. One interpretation would be that although the *oda6-r88* IC70 protein interferes with outer arm function, double mutants between *oda6-r88* and *ida* loci are motile because they retain an outer arm structure (Fig. 8). Similar double mutants between inner arm mutations (*ida1-98* or *pf9-2*) and either of the two other outer arm mutations that do not completely block assembly (*sup1* or *oda11*) also remain motile (Porter et al., 1992, and D. R. Mitchell, unpublished observations), supporting the hypothesis that non-motility in *ida,oda* double mutants is related to absence of both outer and inner arm structures rather than lack of an outer arm contribution to motility per se.

While both of the class II revertants for which sequence information has been obtained result from new mutations 5' to the original *oda6-95* defect (Fig. 5), we have not determined how the sequence changes associated with these mutations cause the observed decrease in beat frequency. Although heavy chain and intermediate chain assembly are not prevented, the possibility remains that a light chain fails to assemble in class II revertants. Further biochemical analyses are underway to test this hypothesis. Alternatively, the class II reversion defects could merely affect dynein assembly onto outer doublets, and the absence of about 22% of the outer arms in *oda6-r88* flagella might directly interfere with the normal function of the remaining outer arms. This would be consistent with the data of King et al. (1991), who showed that IC78 can be cross-linked to alpha tubulin in situ, and suggested that the IC70/IC78 heterodimer acts as an outer doublet attachment subunit. However, we think this is unlikely to be responsible for the reduced beat frequency of class II revertants. Sakakibara and Kamiya (1989) have shown that when normal motility is restored by adding wild-type dyneins to *oda* flagella, increases in beat frequency parallel the fraction of outer arms restored. A 22% reduction in outer arms should therefore have a very modest effect on beat frequency.

As a third possibility, the IC70 protein may be involved directly in regulating and coordinating the action of one or more of the three catalytic outer arm subunits, and our class II reversions might interfere with an interaction necessary for this function. We have previously shown that both the beta heavy chain and IC78 interact with IC70 (Mitchell and Rosenbaum, 1986), but further biochemical analysis will be needed to show whether one of these interactions is specifically altered by the sequence defects in class II revertants.

Comparisons of the *Chlamydomonas* IC70 sequence with that of a 74 kDa cytoplasmic dynein intermediate chain has shown that the C-terminal halves of these two intermediate chains are similar, but the N-terminal regions lack significant homology (Paschal et al., 1992). Since one obvious difference between cytoplasmic and flagellar dyneins is the association of the former with vesicles and the latter with doublet microtubules, this non-homologous N-terminal intermediate chain domain could play a role in specifying the correct target for dynein association, while the homologous domains are more likely to be involved in a common function such as heavy chain/intermediate chain interactions. The region altered by our *oda6-95* revertants lies in the non-homologous N-terminal portion of the molecule, and might therefore be expected to play a predominantly structural role in dynein attachment to outer doublet A-tubules. However, the effects on flagellar motility of mutations such as *oda6-r88* indicate that this N-terminal domain may be important for some aspects of force generation as well.

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