The *Chlamydomonas reinhardtii ODA3* Gene Encodes a Protein of the Outer Dynein Arm Docking Complex

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Abstract. We have used an insertional mutagenesis/gene tagging technique to generate new *Chlamydomonas reinhardtii* mutants that are defective in assembly of the outer dynein arm. Among 39 insertional *oda* mutants characterized, two are alleles of the previously uncloned *ODA*3 gene, one is an allele of the uncloned *ODA*10 gene, and one represents a novel *ODA* gene (termed *ODA*12). *ODA*3 is of particular interest because it is essential for assembly of both the outer dynein arm and the outer dynein arm docking complex (ODA-DC) onto flagellar doublet microtubules (Takada, S., and R. Kamiya. 1994. *J. Cell Biol.* 126:737–745). Beginning with the inserted DNA as a tag, the *ODA*3 gene and a full-length cDNA were cloned. The cloned gene rescues the phenotype of *oda*3 mutants. The cDNA sequence predicts a novel 83.4-kD protein with extensive coiled-coil domains. The ODA-DC contains three polypeptides; direct amino acid sequencing indicates that the largest of these polypeptides corresponds to ODA3. This protein is likely to have an important role in the precise positioning of the outer dynein arms on the flagellar axoneme.

Dyneins are ubiquitous molecular motors that generate force against microtubules to produce many different types of cellular movements, including vesicle transport, localization of the Golgi apparatus, nuclear migration, spindle formation and orientation, possibly some types of chromosome movements, and beating of cilia and flagella (Holzbauer and Vallee, 1994). Both the cytoplasm (Vaisberg et al., 1996) and ciliary/flagellar axonemes (Witman et al., 1994) contain multiple isoforms of dynein, and the maintenance of many essential cell functions undoubtedly depends on the correct attachment of a specific dynein isoform to a specific cell structure. Hence, the mechanisms by which dyneins are targeted to and bind cell organelles are of considerable interest (Vallee and Sheetz, 1996).

An ideal system for studying targeting of dynein isoforms to specific attachment sites is the flagellum of the green alga *Chlamydomonas reinhardtii*, which is amenable to genetic (classical and molecular), biochemical, and physiological approaches. In *C. reinhardtii*, as in most other organisms, the flagellar dyneins make up biochemically distinct structures known as the outer dynein arms and the inner dynein arms, each of which is anchored to a specialized site on the A-tubule of the doublet microtubule (see Fig. 1). Both types of dynein arms interact transiently with the B-tubule of the apposing doublet microtubule to generate interdoublet sliding that is the basis for flagellar bending (for review see Witman, 1990). The outer dynein arms, which repeat with a 24-nm spacing along the doublet microtubules, are believed to contribute as much as four-fifths of this sliding force (Brokaw, 1994).

The outer dynein arm of *C. reinhardtii* has been extensively investigated and consists of at least 15 polypeptides, including three dynein heavy chains (DHCs[1]: DHCα, DHCβ, DHCγ), two intermediate chains (ICs: IC69, IC78), and 10 light chains (LCs) (Huang et al., 1979; Piperno and Luck, 1979; Pfister et al., 1982; King and Witman, 1989) (see Fig. 1 *A*). Each DHC consists of a globular head, containing one or more ATP-hydrolytic sites, and a flexible stem, which extends toward the base of the dynein (Witman et al., 1983). The two ICs are associated with each other and with several of the LCs to form an IC/LC complex (Mitchell and Rosenbaum, 1986; King et al., 1991) that is located at the base of the DHC stems (King and Witman, 1990). One component of this complex, IC78, is in direct contact with α-tubulin in the axoneme (King et al., 1991) and probably plays an important role in binding the outer dynein arm to the A-tubule of the doublet microtu-

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1. *Abbreviations used in this paper*: DHC, dynein heavy chain; IC, dynein intermediate chain; LC, dynein light chain; ODA-DC, outer dynein arm docking complex; RFLP, restriction fragment length polymorphism.

bule (King et al., 1995). This structure and biochemistry are remarkably similar to the structure and biochemistry of cytoplasmic dynein, which has DHCs, ICs, and LCs that are homologous to those of *C. reinhardtii* flagellar outer arm dynein (Paschal et al., 1992; Mitchell and Brown, 1994; Wilkerson et al., 1994; King and Patel-King, 1995). Moreover, in both outer arm and cytoplasmic dynein, the ICs interact directly with the structure to which the dynein attaches (King et al., 1991, 1995; Vaughan and Vallee, 1995). As a result, the outer dynein arm of *C. reinhardtii* has been a very useful model for studying how dyneins in general are targeted to specific attachment sites (Paschal et al., 1992; King et al., 1995).

Since the outer dynein arm attaches to a precisely defined site on the doublet microtubule, it is important to understand what is structurally or biochemically unique about that site. Recently, Takada and Kamiya (1994) demonstrated the existence of a factor that assembles onto the outer dynein arm binding site in the absence of arms in vivo, and that promotes functional reconstitution of outer dynein arms onto armless axonemes in vitro. This factor therefore has the properties expected for an outer dynein arm docking complex (ODA-DC). The putative ODA-DC is visible in certain outer arm–less mutants as a small projection at the site where the outer dynein arm normally would be attached to the doublet microtubule (Takada and Kamiya, 1994; compare Fig. 1 *C* to 1 *D* in this report). It is composed of three polypeptides of ~105, 62.5, and ~25 kD (Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation).

Mutational loss of the outer dynein arm in *C. reinhardtii* usually results in a slow, jerky swimming phenotype (Kamiya, 1988). Such cells are viable and easily detected in mutant screens, so the outer dynein arm is readily studied by genetic methods (for a recent review see Kamiya, 1995). Currently, mutations at 10 independent loci (*ODA*1–*ODA*10)[2] are known that result in loss of the outer dynein arm and jerky swimming at a rate approximately one-third that of wild-type cells (Table I). A mutation at an 11th locus (*ODA*11) results in loss of part of the outer arm and a reduction in swimming speed to approximately two-thirds the wild-type rate (Sakakibara et al., 1991). Mutations at two other loci (*PF*13 and *PF*22) result in loss of outer arms and paralyzed flagella (Huang et al., 1979); the reason for the complete loss of motility in these mutants is not yet understood. Several of the *ODA* loci have been shown to encode structural components of the outer dynein arm. *ODA*2 encodes DHCγ (Wilkerson et al., 1994), *ODA*4 encodes DHCβ (Sakakibara et al., 1993), *ODA*6 encodes IC69 (Mitchell and Kang, 1991), *ODA*9 encodes IC78 (Wilkerson et al., 1995), and *ODA*11 encodes DHCα (Sakakibara et al., 1991). Therefore, with the apparent exception of DHCα, defects in any one of the outer arm DHCs or ICs result in loss of the complete outer dynein arm. *oda*1 and *oda*3 lack the ODA-DC in addition to the outer dynein arm (Takada and Kamiya, 1994); *ODA*1 recently has been shown to encode the 62.5-kD polypeptide of the ODA-DC (Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation). Therefore, defects in an ODA-DC component also can lead to loss of the outer dynein arm. The bases for the loss of outer arms in the other *oda* mutants and in *pf* 13 and *pf* 22 are not yet known.

The development of techniques for the efficient transformation of the nuclear genome in *C. reinhardtii* (Kindle, 1990) now makes it possible to use insertional mutagenesis (Tam and Lefebvre, 1993; Gumpel and Purton, 1994) to study the polypeptides necessary for outer dynein arm assembly and binding to the doublet microtubule. When *C. reinhardtii* is transformed, the transforming DNA usually is inserted into the genome by nonhomologous recombination, causing disruption or deletion of any gene at the site of insertion. For molecular genetic studies, this has two major benefits. First, if a gene already has been cloned, insertional mutants of that gene can be identified easily by restriction fragment length polymorphism (RFLP) analysis; this technique was used previously to identify mutants with defects in the *IC78* gene (Wilkerson et al., 1995). Second, for insertional mutants defective in genes that have not yet been cloned, it is possible to use the inserted DNA as a tag to clone host DNA near the site of insertion, and then use that DNA to select wild-type genomic DNA clones containing the gene of interest. That the correct gene has been cloned can be confirmed by rescuing the mutant by transformation with the cloned wild-type DNA.

Here we report the use of these two powerful approaches to generate and identify insertional alleles for previously known but still uncharacterized *ODA* loci, as well as to identify a new *ODA* locus. One of the insertional mutants was defective in the *ODA*3 gene and hence was of particular interest because that gene is necessary for assembly of the ODA-DC (Takada and Kamiya, 1994). Start-

*Table I. Chlamydomonas Outer Dynein Arm Mutants**

Mutant	Protein affected	Reference‡
*oda*1	62.5 kD	1, 2
*oda*2 (*pf* 28)	DHCγ	1, 3, 4
*oda*3	105 kD	1, this study
*oda*4 (*oda*4-s7, sup_{pf}1)	DHCβ	1, 5, 6, 7, 8
*oda*5	unknown	1
*oda*6	IC69	1, 9
*oda*7	unknown	1
*oda*8	unknown	1
*oda*9	IC78	1, 10
*oda*10	unknown	1
*oda*11	DHCα	11
*oda*12	unknown	this study
pf 13	unknown	12
pf 22	unknown	12

*Table updated from Kamiya (1988, 1995).

‡1: Kamiya (1988). 2: Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman (manuscript in preparation). 3: Mitchell and Rosenbaum (1985). 4: Wilkerson et al. (1994). 5: Luck and Piperno (1989). 6: Mitchell and Brown (1994). 7: Huang et al. (1982). 8: Sakakibara et al. (1993). 9: Mitchell and Kang (1991). 10: Wilkerson et al. (1995). 11: Sakakibara et al. (1991). 12: Huang et al. (1979).

2. To conform to recommendations for a standard genetic nomenclature in *Chlamydomonas* (Dutcher, 1995), genetic loci and the wild-type allele for those loci are denoted using uppercase italic letters, followed by a number when there is more than one locus with the same name (e.g., *ODA*3); mutant alleles are designated in lowercase italic (e.g., *oda*3); different mutant alleles at the same locus are distinguished by a number separated from the locus designation by a hyphen (e.g., *oda*3-4); phenotypes are indicated by an uppercase letter followed by lowercase letters and a plus or minus (e.g., Oda−), and gene products are designated using uppercase roman letters (e.g., ODA3).

ing with the inserted DNA as a tag, we cloned wild-type genomic DNA containing the *ODA*3 gene and showed that it could rescue both the new *oda*3 insertional mutant and a preexisting *oda*3 mutant. An *ODA*3 cDNA clone was isolated and sequenced; the sequence is predicted to encode a novel 83.4-kD protein with three long coiled-coil domains. Protein sequencing and in vitro translation experiments independently demonstrated that the *ODA*3 gene product is the ~105,000-M_r polypeptide of the ODA-DC. Since ODA3 is predicted to have coiled-coil domains of similar length to those of the 62.5-kD ODA-DC polypeptide (Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation), it is possible that these two proteins interact to form a coiled-coil structure that precisely positions outer dynein arms in the axoneme.

Materials and Methods

Strains

Chlamydomonas reinhardtii strains used in this study (and their relevant genotypes) include: g1 (*nit*1, *NIT*2, *agg*1, *mt*+) and 1330.1 (*nit*1, *NIT*2, *ac*14, *agg*1, *mt*−), both selected for ease of transformation; B214 (*nit*1, *NIT*2, *ac*17, *agg*1, *mt*−) (Pazour et al., 1995); CC2229 (*oda*1, *nit*1, *nit*2, *AC*17, *mt*−); CC2231 (*oda*2); CC2233 (*oda*3-1, *nit*1, *nit*2, *AC*17, *mt*−); CC2235 (*oda*4); CC2237 (*oda*5); CC2239 (*oda*6); CC2241 (*oda*7); CC2243 (*oda*8); CC2245 (*oda*9); CC2247 (*oda*10); CC2673 (*oda*11); CC1030 (*pf*13); CC1382 (*pf*22); CC124 (*nit*1, *nit*2); 137C; 209A (*oda*3-1, *nit*1, *NIT*2) and 210A (*oda*3-1, *nit*1, *NIT*2), both derived from a g1 × CC2233 cross. A *Chlamydomonas smithii* strain (CC1373) that is interfertile with *C. reinhardtii* also was used. Strains with "CC" numbers and 137C are from the *Chlamydomonas* Genetics Center, Department of Botany, Duke University, Durham, NC. Insertional mutants generated by transformation of g1 and 1330.1 were assigned "V" and "F" numbers, respectively. One of these (V40) had a disruption of the *ODA*3 gene and was termed *oda*3-4 (*oda*3-4::pUC119-*NIT*1); this strain was crossed to B214 and CC124 to create strains 27D (*oda*3-4::pUC119-*NIT*1; *NIT*2, *ac*17, *mt*+) and 98A (*oda*3-4::pUC119-*NIT*1; *nit*2), respectively.

Growth Media

Chlamydomonas strains were grown in the following media: M (Sager and Granick [1953] medium I modified to contain 0.0022 M KH_2PO_4 and 0.00171 M K_2HPO_4), R (M medium supplemented with 0.0075 M sodium acetate), M−N (M medium without nitrogen), M−N/5 (M−N medium diluted 5× except for K_2HPO_4, which was increased 2×), M−N/5+SA (M−N/5 medium supplemented with 0.1% sodium acetate), M−N+KNO_3 (M−N medium supplemented with 0.0375% $KNO_3 \cdot H_2O$), SGII/NO_3 (Sager and Granick [1953] medium II modified to contain 0.003 M KNO_3 as the sole nitrogen supply), and SGII/NO_2 (Sager and Granick [1953] medium II modified to contain 0.0045 M $NaNO_2$ as the sole nitrogen supply).

Insertional Mutagenesis

Strains (g1 and 1330.1) defective in nitrate reductase were transformed with the cloned *NIT*1 gene using the glass bead method (Kindle, 1990) as described in Pazour et al. (1995). Essentially, cell walls were removed with gametic autolysin (obtained by mixing gametes of strains 137C and CC124), and cells were vortexed with 0.3 g of glass beads (0.5 mm; Thomas Scientific, Swedesboro, NJ), 5% polyethylene glycol (average mol wt 8,000; Sigma Chemical Co, St. Louis, MO), and linearized plasmid pGP505 (Pazour et al., 1995) for 20–45 s. After vortexing, the cells were plated on SGII/NO_3 solid medium. Individual transformants were picked into liquid SGII/NO_3 and screened using a photoaccumulation assay described in Pazour et al. (1995). Cell lines were exposed to directional bright light for 3 min, and those that did not accumulate opposite the light source were retained as possible mutants. Retained cell lines were examined by light microscopy to ascertain whether they were motility or phototaxis mutants. Mutants with an Oda− phenotype (i.e., mutants that swim in a slow-jerky manner about one-third the speed of wild-type cells) were examined further by EM, complementation in stable diploids, and RFLP analysis using DNA probes of cloned *ODA* genes. DNA probes used included inserts of pcγ1 (*ODA*2) (Wilkerson et al., 1994), pEB1.2 (*ODA*4–5′ end) and pBB4.2a (*ODA*4–3′ end) (Mitchell and Brown, 1994), pBC70-16 (*ODA*6) (Mitchell and Kang, 1991), pc78k3 (*ODA*9) (Wilkerson et al., 1995), and pBA1.3a (*ODA*11–5′ end) and pBA4.7 (*ODA*11–3′ end) (Mitchell and Brown, 1994).

EM

Cells were fixed in glutaraldehyde as described in Hoops and Witman (1983) and processed as described in Wilkerson et al. (1995).

DNA and RNA Isolation

Chlamydomonas miniprep DNA was obtained by digesting ~0.3 ml of packed cells with 0.5 ml of 1 mg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) in 5% sodium lauryl sulfate, 20 mM EDTA, and 20 mM Tris, pH 7.5, at 50°C overnight. NH_3 acetate was added to 1.5 M, and the DNA was extracted once with phenol/chloroform (1:1) and once with chloroform, precipitated with ethanol, and then resuspended in 1 mM EDTA and 10 mM Tris, pH 8.0 (Pazour et al., 1995). *Chlamydomonas* mRNA was obtained from wild-type (g1) cells before deflagellation (control) and 30 min after deflagellation. Deflagellation was carried out by pH shock (Witman et al., 1972) to induce transcripts of flagellar genes (Lefebvre and Rosenbaum, 1986). Total RNA was obtained and poly A^+ mRNA was isolated using oligo(dT)-cellulose spin columns (mRNA Separator Kit; Clontech, Palo Alto, CA). Gel electrophoresis, Southern blotting, and Northern blotting were performed using standard procedures (Sambrook et al., 1987).

Genetic Analysis

Mating and tetrad analysis were performed using techniques described in Levine and Ebersold (1960) and Harris (1989). Meiotic products were separated using a glass needle held by a micromanipulator (1-axis, 36S Tilt Platform; Newport Corp., Irvine, CA). Products were scored for motility (Oda+/−) by light microscopy; for *NIT*1 by comparing growth on solid SGII/NO_3 and M; for *NIT*2 by comparing growth on solid SGII/NO_2, SGII/NO_3, and M; for *AC*17 by comparing growth on solid M and R; and for the presence of pUC119 sequences by Southern blot analysis.

Complementation in Stable Diploids

Stable diploids can be selected in *Chlamydomonas* by mating complementing auxotrophs and by plating on selective medium. In this study known *oda* mutants unable to grow on NO_3 as the sole nitrogen source were mated to new *oda* mutants that could use NO_3 but required acetate for growth (*ac*17 or *ac*14 mutants). Mated cells were plated on medium without acetate but with NO_3 as the sole nitrogen source. This selects for stable diploids and haploid progeny that have recombined to become Nit+/Ac+. To distinguish diploids from haploids, selected lines were examined by RFLP analysis using a probe that detects mating type (Ferris and Goodenough, 1994). Haploids have one of two bands while diploids have both bands.

Cloning and Sequencing of ODA3

To generate a λ phage library of genomic *oda*3-4 DNA, genomic DNA was partially digested with Sau3AI, and 14–20-kb fragments were isolated by sucrose gradient centrifugation. Fragments were ligated into λDASH II and packaged using Gigapack II extract (Stratagene, La Jolla, CA).

To obtain sequences flanking the site of pGP505 insertion, phage clones containing pUC119 were isolated from the above library. These clones were digested with a variety of restriction enzymes, and Southern blots were probed with pGP505 to identify DNA fragments that did not contain either pUC119 or *NIT*1. These DNA fragments potentially represented genomic DNA closely linked to the inserted DNA. Two such fragments were obtained but neither showed an RFLP between *oda*3-4 and its wild-type parent (g1). To determine if they were nevertheless linked to the site of insertion, they were used as RFLP probes to score progeny of a cross between *C. reinhardtii* (*oda*3-4) and *C. smithii.* One fragment (termed ODA3 probe) detected a BamHI RFLP that segregated with the *oda*3-4 motility defect (12 parentals, 0 nonparentals), indicating that it was tightly linked to *oda*3-4. The ODA3 probe was then used to isolate wild-type genomic cosmid clones that spanned the *ODA*3 locus. These clones were from an unamplified cosmid library constructed from genomic DNA

that was size fractionated after partial digestion with Sau3A (Pazour, G.J., and G.B. Witman, unpublished data).

Rescue of *oda*3 was achieved by cotransforming strains 98A, 209A, and 210A with the cosmid or plasmid clone being tested and a selectable marker. Cotransformation was performed using the glass bead method as described above. Transformants were scored for Oda+/− phenotype by light microscopy and, in selected cases, for outer dynein arms by EM.

To obtain cDNA clones, a 1-kb HindIII fragment (see Fig. 3) was used to probe a λZAPII cDNA library (Wilkerson et al., 1994). Positive cDNA clones were sequenced using the 7-deaza Sequenase kit (United States Biochemical Corp., Cleaveland, OH); double-stranded DNA was denatured with NaOH, while single-stranded DNA was produced using VCSM13 helper phage. Primers used for sequencing were selected using the PRIMER3 program (S. Rozen and H.J. Skaletsky. 1996. Primer3. http://www-genome.wi.mit.edu/genome_software/other/primer3.html).

Computational Analysis

The Genetics Computer Group suite of programs (Devereux et al., 1984) was used for sequence assembly and protein structure predictions; NEWCOILS (Lupas et al., 1991; Lupas, 1996*a*) was used to predict coiled-coil regions; and BLAST (Altschul et al., 1990) was used to search for related sequences. The ExPASy molecular biology server (http:\\expasy.hcuge.ch\) was used to obtain the theoretical isoelectric point and mass of the *ODA*3 gene product (Bellqvist et al., 1993). The PROSITE database was used to determine possible sites for posttranslational modifications (Bairoch et al., 1995).

In Vitro Transcription and Translation

T3 RNA polymerase was used to generate synthetic RNA from XhoI-digested pK101. RNA was translated in vitro using a reticulocyte lysate system (Promega, Madison, WI) including [^{35}S]methionine. In vitro translation products were separated by SDS-PAGE (7.5% separating gel) and detected by autoradiography.

Isolation and Direct Protein Sequencing of the ~105-kD ODA-DC Protein

Axonemes were isolated from CC2239 (*oda*6) as described in Witman (1986), preextracted with 0.5 M potassium acetate, and then further extracted with 0.6 M KCl (Nakamura et al., 1997). The latter extract was subjected to 5–20% sucrose density gradient centrifugation under Mg^{2+}-free conditions (Takada et al., 1992). The 7S fraction (Takada and Kamiya, 1994) was collected and the ~105-kD protein of the ODA-DC was separated from other proteins by electrophoresis in a 5–20% gradient polyacrylamide gel (Takada, S., C.B. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation). The gel was blotted to a polyvinylidene difluoride membrane and stained with Ponceau S. The portion containing the ~105-kD protein was excised and digested with trypsin. The resultant peptides were separated by HPLC; selected peptides were then directly sequenced in an amino acid sequencer (model 477a; Applied Biosystems, Foster City, CA).

Results

Generation of oda Mutants by Insertional Mutagenesis

To obtain motility mutants by insertional mutagenesis, *nit*1 cell lines of *C. reinhardtii* (g1 or 1330.1) were transformed with the plasmid pGP505 (Pazour et al., 1995), which contains the cloned *NIT*1 gene in the pUC119 vector (Fernandez et al., 1989). Transformants were picked into liquid selective medium and examined for motility defects (see Materials and Methods). Motility mutants were obtained that exhibited a wide range of phenotypes, including slow-jerky swimmers, slow-smooth swimmers, uniflagellate cells, aflagellate cells, cells with paralyzed flagella, and cells with long flagella. The slow-jerky swimmers were of particular interest for this project, as this phenotype (termed Oda−) is indicative of a defect in the outer dynein arm (Kamiya, 1991).

Insertional mutagenesis in *C. reinhardtii* occurs via nonhomologous recombination and usually results in deletions, insertions, and/or rearrangements (Tam and Lefebvre, 1993), all of which should be detectable by RFLP analysis using appropriate probes. Therefore, to determine if any of the insertional Oda− mutants had defects in genes encoding the previously cloned outer dynein arm heavy or intermediate chains, Southern blots of the Oda− mutants were probed with DNA clones encoding portions of DHCγ (*ODA*2), DHCβ (*ODA*4), IC69 (*ODA*6), IC78 (*ODA*9), and DHCα (*ODA*11). The results indicated that 12 of the Oda− mutants were defective in *ODA*4, while 15 were defective in *ODA*9 (data not shown; see, e.g., Fig. 8 in Wilkerson et al., 1995).

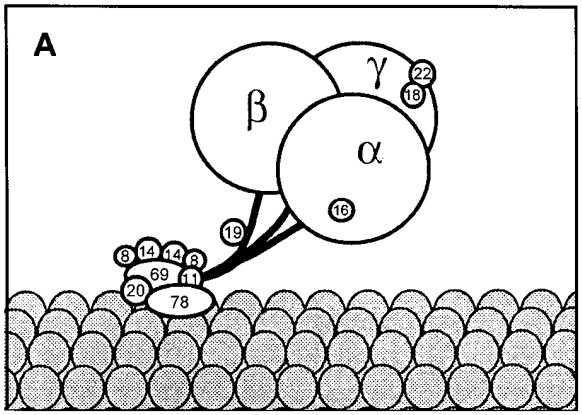
The remaining Oda− mutants did not exhibit RFLPs when probed with DNA encoding the outer dynein arm heavy and intermediate chains. These mutants therefore are potentially defective in uncloned genes necessary for outer arm assembly or function. To determine if these mutants were actually missing their outer arms, they were examined by EM. The vast majority had specific defects in this structure. Most completely lacked outer arms (Fig. 1, *C* and *D*) and therefore are "complete" *oda* mutants. In seven cell lines, the arms were missing on only some of the doublet microtubules (data not shown); these are termed "partial" *oda* mutants. A few cell lines had apparently intact outer arms and are not currently considered to be *oda* mutants.

Taken together, the data from the RFLP analysis and EM indicated that 18 *oda* mutants (five of which were partial) were obtained from 2,978 transformants examined in one transformation experiment (V series), and 21 *oda* mutants (two partial) were obtained from 6,903 transformants examined in another transformation experiment (F series) (Table II).

Identification of an oda3 Insertional Mutant

The five complete *oda* insertional mutants that did not show an RFLP when probed with DNA encoding cloned outer dynein arm heavy or intermediate chain genes were tested to determine if they would complement those existing *oda* and related *pf* mutants whose defective gene products are unknown. Stable diploids were generated by mating haploid insertional mutant cell lines with *oda*1, *oda*3, *oda*5, *oda*7, *oda*8, and *oda*10 cell lines; F series mutants also were mated to *pf*13 and *pf*22. RFLP analysis using a probe that is closely linked to the mating-type locus (Ferris and Goodenough, 1994) confirmed that colonies selected for further analysis were true stable diploids containing both mating-type loci (e.g., Fig. 2 *A*). One cell line (V40) did not complement the motility defect of *oda*3 (Fig. 2 *A*). Complementation studies with another cell line (F60) were not complete when the ODA3 probe became available (see below); RFLP analysis using this probe revealed that F60 had a defect in the *ODA*3 gene (results not shown). Thus, V40 and F60 represent new alleles of *ODA*3; since there already were three mutant alleles of *ODA*3 (Kamiya, 1988), V40 and F60 have been named *oda*3-4 and *oda*3-5, respectively. A third mutant (V87.2) did not complement *oda*10 and therefore appears to be a new *ODA*10 allele.

The remaining two complete *oda* insertional mutants



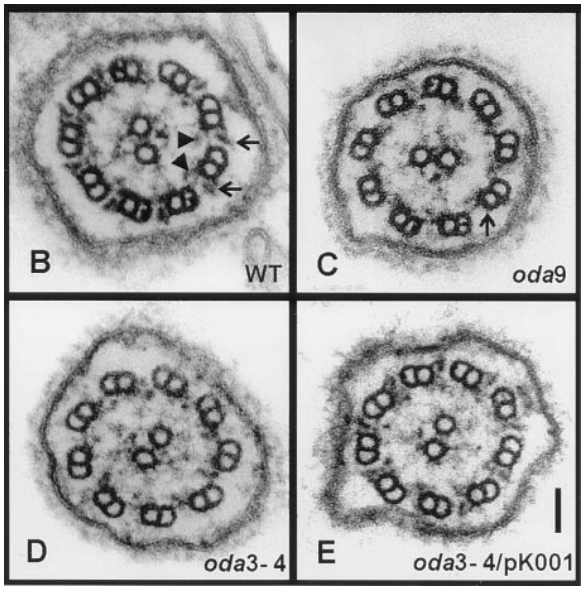


Figure 1. Structure of outer dynein arm. (*A*) Diagrammatic representation of *C. reinhardtii* outer dynein arm, which consists of three DHCs (α, β, and γ), two ICs (*69* and *78*), and at least 10 LCs (*8*, *11*, *14*, *16*, *18*, *19*, *20*, and *22*) (modified from King et al., 1995). (*B–E*) Electron micrographs of cross-sections of *C. reinhardtii* flagella. (*B*) Wild-type (g1). Note outer dynein arms (*arrows*) and inner dynein arms (*arrowheads*). The arms occupy precise positions on the A-tubule of the doublet microtubule. (*C*) *oda*9 insertional mutant lacking outer dynein arms (Wilkerson et al., 1995). Tiny projections at sites normally occupied by outer dynein arms are putative ODA-DCs (*arrow*). (*D*) *oda*3-4 insertional mutant (V40) lacking outer dynein arms. The profiles of the doublet microtubules at the sites normally occupied by the outer dynein arms are rounder than in *oda*9, indicating that this mutant lacks the ODA-DC (Takada and Kamiya, 1994). (*E*) *oda*3-4 insertional mutant rescued by transformation with the cosmid pK001. The outer dynein arms are completely restored.

complemented all of the uncharacterized preexisting *oda* cell lines, suggesting that these insertional mutants represented novel *oda* genes. However, it also was possible that the RFLP analysis failed to reveal defects in outer dynein arm heavy and intermediate chain genes because the defect is caused by a small disruption not detectable by RFLP analysis. To distinguish between these possibilities,

Table II. Distribution of oda Insertional Mutants

Locus	V series*	F series‡
*ODA*1	0	0
*ODA*2	0	0
*ODA*3	1 (V40)	1 (F60)
*ODA*4	3	10
*ODA*5	?	?
*ODA*6	0	0
*ODA*7	?	?
*ODA*8	?	?
*ODA*9	8	7
*ODA*10	1 (V87.2)	0
*ODA*11	0	0
*ODA*12	?	1 (F56)
Unknown§	5	2
Total	18	21

*From 2,978 Nit+ transformants.
‡From 6,902 Nit+ transformants.
§Seven insertional mutants, all partial *oda*s, have yet to be categorized. It has not been determined whether any of these are alleles of *oda*5, *oda*7, *oda*8, and *oda*12; question marks have therefore been placed at appropriate positions in the table.

these two mutants were tested further to determine if they would complement *oda*2, *oda*4, *oda*6, *oda*9, and *oda*11 in stable diploids. One of the insertional mutants (F56) complemented all of these strains, indicating that it represents a new *ODA* gene; the mutant allele has been named *oda*12. The other mutant complemented all alleles tested except for *oda*4, indicating that it is an *ODA*4 allele that had been missed by the RFLP analysis. The seven partial *oda* mutants have not yet been completely characterized by complementation analysis; some of these may represent mutant alleles of additional new *ODA* genes.

Defects in *ODA*3 result in loss of both the outer dynein arm and the ODA-DC (Takada and Kamiya, 1994; see Fig. 1, *C* and *D*). Further work therefore concentrated on the *oda*3-4 insertional mutant, as it provided an opportunity to learn more about a component necessary for assembly of the ODA-DC.

oda3-4 Is Tagged with pUC119

An advantage of insertional mutagenesis is that the mutated gene frequently is tagged with the inserted DNA, facilitating the cloning of the disrupted gene. To determine if the *oda*3-4 allele is tagged, the mutant was crossed to a wild-type cell line (B214), and meiotic progeny were scored for motility (Oda+/−), ability to grow on medium containing nitrate as the sole nitrogen source (Nit+/−), and the presence of pUC119 sequences in the genome (Fig. 2 *B*). In all cases, the Oda− phenotype segregated with both the Nit+ phenotype and pUC119, indicating that the *oda*3-4 allele is tagged with both *NIT*1 and pUC119.

Cloning of the ODA3 Gene and Rescue of oda3 Mutants

To obtain DNA at or near the *ODA*3 locus, a phage library was constructed using genomic *oda*3-4 DNA, and clones containing pUC119 sequences were selected. Using RFLP analysis (see Materials and Methods), a DNA frag-

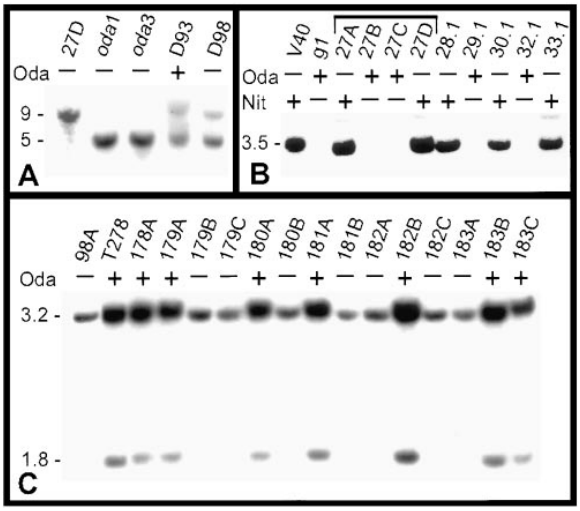


Figure 2. Genetic analysis of *ODA*3. (*A*) Complementation in stable diploids demonstrates that V40 is an *oda*3 allele (referred to as *oda*3-4). Cell line 27D, a plus mating type (*mt*+) derivative of the insertional mutant V40, was mated to *oda*1 (*mt*−) and *oda*3-1 (*mt*−) mutants to generate stable diploids D93 and D98, respectively. Complementation (Oda+ phenotype) was observed in D93 but not in D98, indicating that the *ODA*1 gene is functional in V40, while the *ODA*3 gene is defective. To confirm that cell lines D93 and D98 were true stable diploids, genomic DNA from the indicated cell lines was digested with BamHI and probed in Southern blots with a 1.6-kb XbaI fragment of λ phage QK7 that is tightly linked to the *mt* locus. *mt*+ and *mt*− correlate with hybridization to ~9- and ~5-kb fragments, respectively, when using this probe. Hybridization to both ~9- and ~5-kb fragments in D93 and D98 indicates that these cell lines contain both mating types and thus are stable diploids. (*B*) Linkage between inserted DNA and Oda− phenotype in *oda*3-4. The insertional mutant *oda*3-4 (*V40*) (Oda− and Nit+ phenotypes) was crossed to B214 (Oda+ and Nit− phenotypes). Progeny were scored for motility (Oda+/−) and ability to grow on nitrate as sole nitrogen source (Nit+/−). Genomic DNA was isolated from the progeny, from *oda*3-4 (*V40*), and from the untransformed parental cell line (*g1*). The DNA was digested with PstI and probed in Southern blots with pUC119, which contains only the plasmid sequence used for transformation. The pUC119 sequence (3.5-kb fragment) is present in *oda*3-4 but not g1; in the progeny, the plasmid sequence segregates with the Oda− and Nit+ phenotypes, indicating that the Oda− phenotype is due to the inserted DNA. Data are shown for four products (*27A–27D*, *bracketed*) from one tetrad and individual products (*28.1–33.1*) from five other tetrads. (*C*) Linkage between rescuing DNA (pK001) and restored Oda+ phenotype. Cell line 98A (a *nit*2 derivative of *oda*3-4) was cotransformed with pMN68 (containing *NIT*2) and the cosmid pK001. One of the rescued transformants, T278, was crossed to *oda*3-4, and progeny were scored for motility (Oda+/−). Genomic DNA from 98A, T278, and the progeny was digested with PvuII and probed in Southern blots with the ODA3 probe, which flanked the original insertion in *oda*3-4 and is contained in pK001. The probe hybridizes to an ~3.2-kb endogenous fragment, as well as to new fragments of ~3.2 and ~1.8 kb in T278. The new bands cosegregate with the Oda+ phenotype in the progeny from the cross of T278 and *oda*3-4, indicating that the Oda+ phenotype is linked to stably inserted pK001 DNA. Data are shown for 14 meiotic products (*178A–183C*) from six different zygotes.

ment (termed ODA3 probe) flanking the site of plasmid insertion was identified and used to screen a wild-type genomic cosmid library. Five positive cosmid clones were selected.

To determine if any of the cosmid clones contained a functional copy of the *ODA*3 gene, *oda*3-4, *nit*2 cells were cotransformed with cosmid DNA and cloned *NIT*2 DNA (Schnell and Lefebvre, 1993) as a selectable marker. Two of the cosmid clones rescued the motility defect of the *oda*3-4 mutant; one of these, pK001, which contained a >40-kb insert (Fig. 3), was selected for further studies. EM of several cell lines rescued with this cosmid revealed that the outer dynein arms were fully restored (Fig. 1 *E*), indicating that pK001 contained the *ODA*3 gene. RFLP analysis of DNA from products of a cross between *oda*3-4 and a rescued strain (T278) showed that the pK001 DNA segregated with the Oda+ phenotype (Fig. 2 *C*), confirming that the rescued phenotype was linked to the inserted cosmid.

To locate the *ODA*3 gene on pK001, the cosmid was digested with several restriction enzymes and the resultant fragments were inserted into plasmid vectors (Fig. 3). These subclones were then assayed in cotransformation experiments (with *NIT*1 as the selectable marker) for the ability to rescue the motility phenotype of strains 209A and 210A, which contain the original *oda*3-1 allele (Kamiya, 1988) in a *nit*1, *NIT*2 background. The smallest fragment that was capable of rescue (pK144; Fig. 3) was ~10 kb long. EM of several of the rescued transformants confirmed that the outer dynein arms were restored (data not shown). pK145 and pK147, which overlapped either side of pK144, were unable to rescue the mutants. These results indicated that the *ODA*3 gene was contained in pK144 and probably spanned the XhoI site near the center of this fragment. Rescue of the original *oda*3-1 allele also provided independent evidence that pK144 contained the bona fide *ODA*3 gene.

Isolation of ODA3 cDNA

A 1-kb HindIII fragment (Fig. 3) that spans the central XhoI site in pK144 was used to probe Northern blots of total *C. reinhardtii* mRNA isolated from wild-type cells that were not deflagellated, and from cells that had been deflagellated and allowed to regenerate their flagella for 30 min to induce mRNAs encoding flagellar proteins. The probe hybridized with an ~3.1-kb mRNA that was induced after deflagellation (Fig. 4 *A*). As the *ODA*3 mRNA was expected to be one of a relatively small subset of transcripts induced during flagellar regeneration, this further suggested that the 1-kb HindIII fragment encoded part of the *ODA*3 gene. The 1-kb HindIII fragment therefore was used to probe *C. reinhardtii* cDNA libraries, yielding cDNA clones of ~3.0 and ~2.4 kb. Sequence analysis indicated that these clones were the same at their 3′ ends.

ODA3 cDNA Sequence

The ~3.0-kb clone was sequenced in both directions and found to contain a 1,797-bp open reading frame. The putative initiation codon was preceded by two in-frame stop codons located 45 and 99 nucleotides upstream (Fig. 5). The open reading frame ended with an amber stop codon

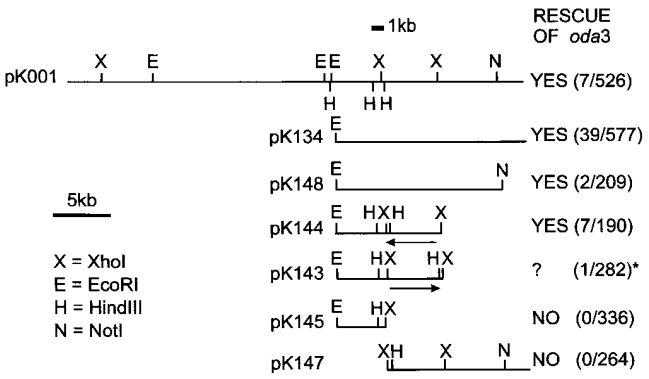


Figure 3. Restriction maps of cosmid pK001 insert and plasmid inserts, and ability of the inserts to rescue motility in *oda*3. The number of Oda+ transformants out of the total number of Nit+ transformants examined is given in parentheses. The pK144 insert was the smallest piece of genomic *C. reinhardtii* DNA to rescue *oda*3. A 1-kb HindIII fragment was used to isolate cDNA clones. (*Asterisk*) A plasmid (pK143) was constructed that contained an insert with an inverted 6-kb XhoI fragment (*arrow pointing right*). In cotransformation experiments using pK143, one cell line was generated in which the rate of swimming was restored to near wild-type levels. EM indicated that outer dynein arms also were restored. However, in crosses to g1, mixed motility phenotypes were recovered, and the results could not be interpreted in terms of Mendelian segregation of a single gene (data not shown). Further study will be necessary to understand the basis for apparent rescue in this transformant.

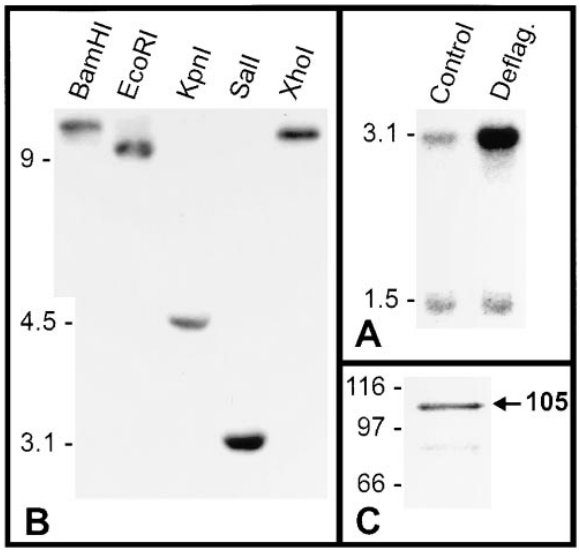


Figure 4. Expression of *ODA*3. (*A*) The *ODA*3 gene is induced after deflagellation. Northern blot of *C. reinhardtii* wild-type (g1) mRNA isolated from nondeflagellated cells (*Control*) and from cells 30 min after deflagellation (*Deflag.*). The blot was probed on two different occasions and the resultant autoradiographs were superimposed. The 3.1-kb band represents mRNA hybridizing to the 1-kb HindIII fragment of pK001 (Fig. 3), which contains part of the *ODA*3 sequence; this mRNA is strongly induced in the deflagellated cells. The 1.5-kb band represents mRNA hybridizing to a *PTX*2 cDNA clone (pG557 insert, provided by G.J. Pazour); *PTX*2 is not induced by deflagellation (Pazour, G.J., and G.B. Witman, manuscript in preparation) and is used here to demonstrate that similar amounts of mRNA were loaded into each lane. (*B*) The *ODA*3 gene occurs once in the *C. reinhardtii* genome. Southern blot of *C. reinhardtii* wild-type (g1) genomic DNA digested with five different restriction enzymes and probed with a full-length *ODA*3 cDNA clone (pK101 insert). A single band was detected in each lane. (*C*) ODA3 produced by translation in vitro migrates as an ~105,000-M_r protein. Synthetic mRNA was prepared from *ODA*3 cDNA (pK101) and translated in a rabbit reticulocyte lysate system. An autoradiograph of an SDS-polyacrylamide gel of the product is shown. M_r standards were β-galactosidase (*116*), phosphatase b (*97*), and BSA (*66*).

(TAG). However, it was noted that an additional in-frame 447-bp open reading frame was present immediately after the amber stop codon. Both open reading frames exhibited the strong codon bias typical of *C. reinhardtii* coding sequence (Williams et al., 1989; Mitchell and Brown, 1994). To understand this phenomenon, the ~2.4-kb clone was sequenced through this region. The sequence was identical in both clones except that the TAG codon was CAG in the ~2.4-kb clone. We conclude that the amber stop codon in the ~3.0-kb clone was the result of a point mutation that occurred during cDNA library construction. The correct codon should be CAG, resulting in a 2,247-bp open reading frame terminating in TAA (Fig. 5). Two additional in-frame stop codons (TAA and TGA) were located 24 and 66 nucleotides downstream from the first TAA, providing further evidence that the first TAA represents the actual termination codon.

The ~2.4-kb clone was not full length, but a convenient NcoI site upstream from the TAG/CAG codon was used to link portions of the ~3.0- and ~2.4-kb clones to yield a correct full-length clone (pK101). Hybridization of the full-length *ODA*3 cDNA to Southern blots of total genomic wild-type *C. reinhardtii* DNA digested with several restriction enzymes indicated that this sequence occurs once in the *C. reinhardtii* genome (Fig. 4 *B*).

The nucleotide sequence of the *ODA*3 cDNA predicts a protein of 749 amino acids (Fig. 5) with a mass of 83,376 daltons and an isoelectric point of 5.72. Based on secondary structure predicted using the NEWCOILS program (Lupas et al., 1991; Lupas, 1996*a*), the *ODA*3 gene product can be divided into three domains (Fig. 6): an NH_2-terminal domain (residues 1–85), a central domain (residues 86–462), and a COOH-terminal domain (residues 463–749). The central domain contains four predicted coiled-coil regions (A, B, C, and D) that are interrupted by three short non-coil regions (Fig. 6). Regions A, B, and D contain a total of 226 (120+70+36) amino acid residues and are very likely to form coiled-coils (probability ~1), whereas region C (28 amino acids) is slightly less likely to form a coiled-coil (probability ~0.67). An imperfect 11–amino acid tandem repeat ($_{289}$KQLERERKMRE$_{299}$ and $_{300}$KQLERERQERE$_{310}$) is located at the predicted junction of the COOH end of region B and the adjacent non-coil region. The COOH-terminal portion of the molecule contains both a highly basic region ($_{676}$KRKKGKKK$_{683}$) and a highly acidic region ($_{701}$DVEEEEPESEEETEEE$_{716}$). Overall, the protein has an unusually high percentage of glutamic acid residues (13.6%; the average in the NCBI database is 6.3%).

The predicted amino acid sequence was compared to the protein sequences in the nonredundant PDB + SwissProt + SPupdate + PIR + GenPept + Genupdate data-

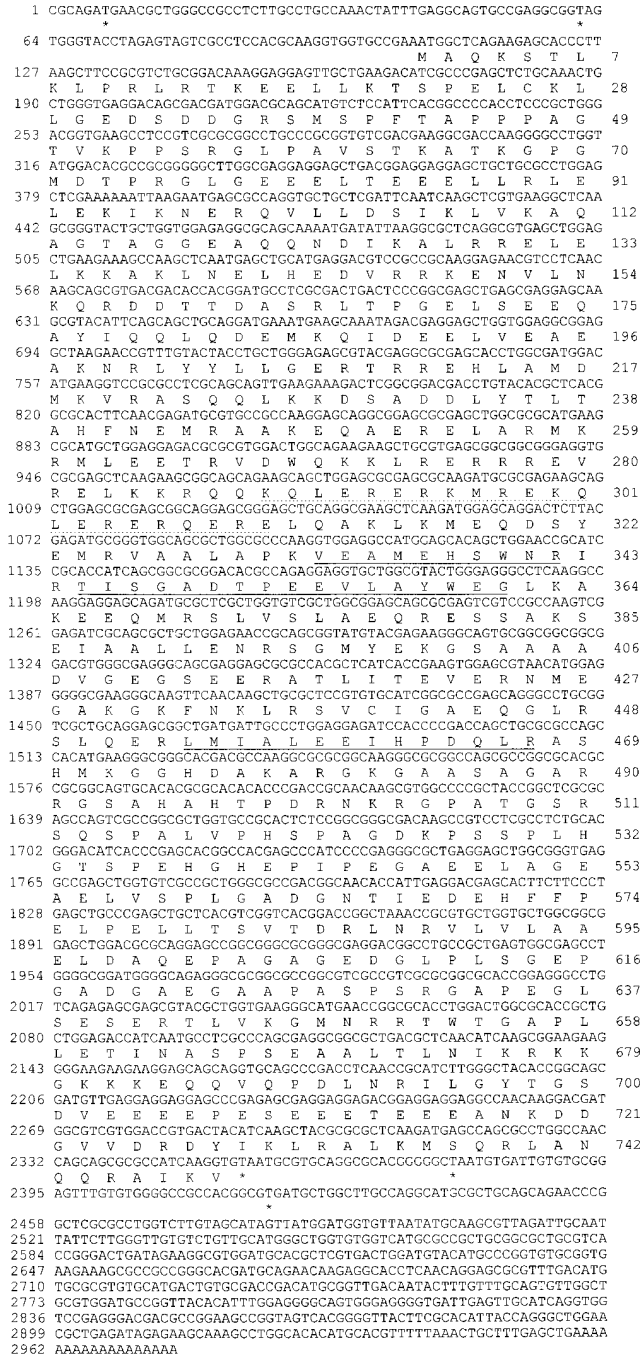


Figure 5. Nucleic acid and deduced amino acid sequence of *ODA*3. The numbers on the lefthand and righthand sides refer to nucleotides and amino acid residues, respectively. In-frame stop codons before and after the open reading frame are marked with asterisks. Underlined amino acids correspond to sequence obtained directly from tryptic fragments of the ~105-kD polypeptide of the ODA-DC. Dotted underlines mark the imperfect 11–amino acid tandem repeat. These sequence data are available from GenBank/EMBL/DDBJ under accession number AF001309.

base using the BLAST program (Altschul et al., 1990). The hypothetical coiled-coil regions of the *ODA*3 gene product were similar to the coiled-coil regions of other proteins, but no convincing similarities were found when non–coiled-coil regions were used to search the database. ODA3 therefore represents a novel protein. Interestingly, a BLAST search using only the tandem repeat of ODA3 revealed that the motif is very similar to the extensive repeats present in the mammalian intermediate filament–associated protein trichohyalin (Fietz et al., 1993):

```
ODA3 repeat                KQLERERQERE
                           :|| |  |||||
ovine trichohyalin         RKFREEEQLLQEREEQL-R-RQERE/D
consensus repeat
```

The ODA3 Gene Encodes a Component of the ODA-DC

*oda*3 mutants lack the ODA-DC (Takada and Kamiya, 1994; Fig. 1 *D*), which contains polypeptides of ~105, 62.5, and ~25 kD (Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation). A cDNA encoding the 62.5-kD protein recently has been cloned and shown to correspond to the *ODA*1 gene (Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation). To investigate the possibility that the *ODA*3 gene product corresponds to the ~105-kD protein, the latter was partially purified and four tryptic fragments were sequenced directly (see Materials and Methods). Three of these (VEAMEHSWNR, TISGADTPEEVLAYWEG, and LMIALEEIHPDQLR) matched the ODA3 sequence exactly (Fig. 5, *underlines*) (the fourth fragment did not match and presumably was from a contaminant). This provides direct evidence that the *ODA*3 gene product is the largest component of the ODA-DC.

The size of this protein previously was estimated to be ~105 kD by SDS-PAGE (Takada and Kamiya, 1994). To understand the discrepancy between this value and the mass of 83.4 kD predicted from the sequence, we produced synthetic mRNA from pK101 and translated it in an in vitro reticulocyte lysate translation system. The major translational product migrated with an apparent mass of ~105 kD (Fig. 4 *C*). Therefore, the *ODA*3 gene product either runs anomolously in SDS-PAGE, or undergoes significant posttranslational modifications that also occur in the reticulocyte lysate system (see Discussion).

Discussion

Utility of Insertional Mutagenesis in Chlamydomonas

Outer dynein arm mutants (*oda*1-*oda*11) of *C. reinhardtii* are defective in outer dynein arm assembly, and all but *oda*11 swim with a jerky motion about one-third the speed of wild-type (for reviews see Kamiya, 1988, 1995). Previously, five of the 11 known *ODA* genes (*ODA*2, *ODA*4, *ODA*6, *ODA*9, and *ODA*11) had been cloned (Table I; Mitchell and Kang, 1991; Mitchell and Brown, 1994; Wilkerson et al., 1994, 1995); these encode outer dynein arm heavy or intermediate chains. Simultaneously with the work reported here, a sixth *ODA* gene (*ODA*1) has been cloned and shown to encode a polypeptide of the ODA-DC (Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation). The gene products of the remaining *ODA* genes are unknown. By using a gene disruption-tagging technique based on insertional mu-

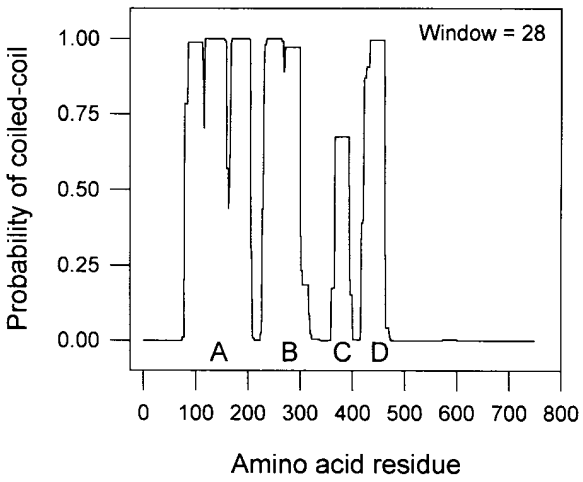


Figure 6. Graphical representation of the probability of coiled-coil regions in the *ODA*3 gene product as determined by the NEWCOILS program (Matrix = MTDIX; with a 2.5-fold weighting of positions *a* and *d*).

tagenesis (Tam and Lefebvre, 1993; Gumpel and Purton, 1994; Pazour et al., 1995), we have begun to identify insertional mutants defective in *ODA* genes that are still uncloned. Since the defective genes in these insertional mutants are likely to be tagged by exogenous DNA, it should be relatively straightforward to clone and characterize the genes that are affected.

In two separate transformations, 39 *oda* mutants were identified in a total of 9,881 transformants screened. 32 of these represent "complete" *oda* mutants that totally lack outer dynein arms as assayed by thin-section EM, whereas seven are "partial" *oda* mutants that have decreased numbers of outer arms. By combining RFLP analysis and complementation studies in stable diploids, we determined that 13 of the 32 complete *oda* mutants are alleles of *ODA*4 (cloned), 15 are alleles of *ODA*9 (cloned), two are alleles of *ODA*3 (cloning reported here), one is an allele of *ODA*10 (uncloned), and one represents an allele of a novel *ODA* gene (assigned *oda*12, uncloned) (Table II). The seven partial *oda* mutants have yet to be completely characterized and may represent additional novel *ODA* genes.

Mutant alleles of the cloned *ODA*11 gene were not found in this study. This result was expected as *oda*11 mutants swim much faster than the other *oda* mutants (Sakakibara et al., 1991), and therefore probably would not have been picked in our screen for slow, jerky swimming mutants. More surprising is that we did not find any mutant alleles of *ODA*1, *ODA*2, *ODA*5, *ODA*6, *ODA*7, or *ODA*8, as mutations at these loci cause loss of the outer arm and slow, jerky swimming. In contrast, 28 of the 39 *oda* insertional mutants represented alleles of *ODA*4 and *ODA*9. Taken together, these results indicate that the insertion of DNA into the *C. reinhardtii* genome in our transformations was not a random event, and that there may be hot spots for the incorporation of foreign DNA. On the other hand, Mitchell and colleagues, using a similar mutagenesis and screening procedure, found alleles of *ODA*1, *ODA*2, *ODA*3, *ODA*4, *ODA*8, and *ODA*9 among 30 new Oda− insertional mutants (Mitchell, D., personal communication). Therefore, the distribution of insertionally mutated sites may depend on some still undetermined factor such as the cell line used for transformation, the state of the cells during transformation, or the presence in the transforming DNA of unidentified sequence that is homologous to sequence at the apparent hot spots.

In addition to the *oda* mutants, insertional mutants with a number of other motility phenotypes were obtained using our screening procedure. These mutants should be very useful for the identification and characterization of the genes involved in processes such as inner dynein arm assembly (slow smooth swimming mutants [Kamiya et al., 1991]), flagellar length control (long flagella mutants), and basal body maturation (uniflagellated mutants). The breadth of phenotypes obtained confirms the utility of insertional mutagenesis for studying motility phenotypes in *C. reinhardtii* (Tam and Lefebvre, 1993; Pazour et al., 1995; Smith and Lefebvre, 1996).

The ODA3 Gene Encodes the ~105-kD Protein of the ODA-DC

Beginning with the pUC119 sequence inserted into *oda*3-4 as a gene tag, we isolated a wild-type genomic cosmid clone and from that a 10-kb wild-type genomic fragment that could rescue the Oda− phenotype of both the *oda*3-4 insertional mutant and the original *oda*3-1 mutant. A 1-kb subfragment from a part of the 10-kb fragment that appeared to contain the *ODA*3 gene hybridized in Northern blots to a 3.1-kb mRNA that was induced by deflagellation. The 1-kb fragment was used to isolate *ODA*3 cDNA clones. Sequence analysis of the cDNA clones revealed that the *ODA*3 gene encodes a 749–amino acid protein with a predicted molecular mass of 83.4 kD. Southern blot analysis indicated that the *C. reinhardtii* genome contains a single copy of the *ODA*3 gene.

*oda*1 and *oda*3 mutants differ from *oda*2, *oda*4, *oda*5, *oda*6, and *oda*9 mutants in that they lack the ODA-DC in addition to the outer dynein arms (Takada and Kamiya, 1994; Fig. 1, *C* and *D*). The ODA-DC is composed of proteins of ~105 kD, 62.5 kD and ~25 kD (Takada and Kamiya, 1994; Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation). During the course of this work, a full-length cDNA encoding the 62.5-kD protein was cloned and found to correspond to the *ODA*1 gene (Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation). This raised the possibility that the *ODA*3 gene might encode the ~105-kD protein, notwithstanding the difference in size between the predicted mass of the *ODA*3 gene product and the apparent mass of the ~105-kD protein as estimated by SDS-PAGE. To investigate this possibility, the ~105-kD protein was isolated and four tryptic fragments were sequenced. The amino acid sequence of three out of four of the fragments exactly matched sequence predicted for the *ODA*3 gene product. Therefore, *ODA*3 does encode the largest polypeptide of the ODA-DC.

To understand the difference between the predicted and estimated masses for the *ODA*3 gene product, we generated synthetic mRNA from an *ODA*3 cDNA clone and translated it in vitro in a reticulocyte lysate system. The

major translational product migrated in SDS-PAGE with an M_r of ~105,000. This provided additional evidence that the cDNA clone was full length, and indicated that the protein either runs anomalously in SDS-PAGE, or undergoes significant posttranslational modification that also can occur in the in vitro translation system. A ProSite search (Bairoch et al., 1995) revealed that the *ODA*3 gene product contains 35 possible phosphorylation sites, which is in agreement with the observation that the protein is heavily phosphorylated in vivo (King, S.M., personal communication). Phosphorylation can occur in reticulocyte lysates (Hiremath et al., 1989; Schubert et al., 1994), and the presence of phosphate groups can cause a shift in the relative mobility of a protein in SDS-PAGE far in excess of the added mass (Tang et al., 1993). However, in preliminary experiments, treatment of the in vitro translated protein with alkaline phosphatase caused its relative mobility to shift only slightly. Therefore, the basis for the unexpectedly slow mobility of the *ODA*3 gene product remains unknown. A similar behavior has been reported for another microtubule-associated protein, E-MAP-115, which has a predicted mass of 84,051 daltons but migrates with an M_r = 115,000 in SDS-PAGE (Masson and Kreis, 1993).

Coiled-Coil Domains

The secondary structure of the *ODA*3 gene product is predicted to contain regions of extended α helix with a strong propensity to form coiled-coil structures. Coiled-coils have a variety of functions in proteins (Adamson et al., 1993; Lupas, 1996*b*). Most commonly, they mediate homodimer and heterodimer formation. Therefore, it may be relevant that the *ODA*1 gene product, with which the *ODA*3 gene product is associated in vivo, also is predicted to contain extensive α-helical domains that have a very high probability of forming a coiled-coil. ODA3 contains three regions (~120, ~70, and ~36 residues) with a total of ~226 residues that are highly likely to form coiled-coil structures; similarly, ODA1 has three regions (~96, ~48, and ~34 residues) with a total of ~178 residues that are strongly predicted to form coiled-coils (Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation). Thus, these two proteins may interact via one or more coiled-coils to form a heterodimer. In this case, the dynein docking function of the ODA-DC might be fulfilled if the residues on one side of the exterior of the coiled-coil interacted with tubulin or some other protein on the doublet microtubule, while residues on the other side of the coiled-coil interacted with one or more outer dynein arm polypeptides, much as the coiled-coil of vertebrate striated muscle tropomyosin interacts with actin and troponin (Phillips et al., 1986).

It also is possible that the coiled-coil domains of the ODA-DC serve as a molecular ruler, either longitudinally along or circumferentially around the doublet microtubule. Although the ODA-DC is important for outer dynein arm binding to the A-tubule, it is unlikely that it is solely responsible for establishing the 24-nm repeat of the arms, because purified sea urchin sperm outer arm dynein has been shown to assemble onto brain microtubules with a 24-nm periodicity in the apparent absence of the ODA-DC (Moss et al., 1992). However, inasmuch as the ODA-DC can assemble onto the doublet microtubule in the absence of the outer dynein arm (Takada and Kamiya, 1994), it may establish its own periodicity, perhaps by binding end-to-end along the microtubule. The length of a coiled-coil region can be estimated by assuming 1.5 Å per residue (Fraser and MacRae, 1973). If all residues in the extended α-helical regions of ODA1 formed a heterodimeric coiled-coil with an equivalent number of residues in ODA3, this would form a structure ~26.7 nm long. Interestingly, if only regions A (120 residues) and D (36 residues) of ODA3 formed a coiled-coil with ODA1, the resulting structure would be ~23.4 nm long, almost exactly the same as the outer dynein arm repeat. Alternatively, the ODA-DC, or portions of it, may interact with inner dynein arm polypeptides, or with some other topographical feature of the doublet microtubule, to place the outer dynein arm the correct distance from, and in the correct phase relative to, the inner dynein arm. In either case, the nonhelical portions between coiled-coil regions probably would form discontinuities that extended from the coil, as has been proposed for intermediate filament proteins (Steinert et al., 1983). These and other non–coiled-coil regions could form linkages to the 25-kD ODA-DC polypeptide or to proteins outside of the ODA-DC (see below).

Charged Domains

The central portion of ODA3 contains two short, highly charged repeats that are nearly identical to a portion of a sequence repeated many times in mammalian trichohyalin, an intermediate filament–associated protein of the hair follicle. In trichohyalin, the repeats are predicted to form an elongated, single-stranded, α-helical rod structure (Fietz et al., 1993). Trichohyalin is a substrate for transglutaminase, which cross-links glutamine and lysine residues, and for peptidylarginine deiminase, which converts arginine to citrulline; it has been proposed that the repeats provide an ordered array of glutamine, lysine, and arginine for catalysis by these enzymes. Further investigation will be necessary to determine if the repeats of ODA3 are similarly modified posttranslationally. In any case, the presence of these repeats in *Chlamydomonas* raises the possibility that the motif arose early in evolution and is now widespread.

The COOH-terminal end of ODA3 contains one region that is highly basic ($_{676}$KRKKGKKK$_{683}$) and another that is very acidic ($_{701}$DVEEEEPESEEETEEE$_{716}$); these regions may be important for interactions between the ODA-DC and the proteins to which it binds. The outer dynein arm together with the ODA-DC are released from the axoneme by treatment with high salt (0.6 M KCl) (Takada and Kamiya, 1994; Takada, S., C.G. Wilkerson, R. Kamiya, and G.B. Witman, manuscript in preparation), indicating that the interaction between the axonemal microtubule and the outer dynein arm/ODA-DC is ionic in nature. Both α and β tubulins of *C. reinhardtii* have highly acidic COOH termini (Silflow et al., 1985; Youngblom et al., 1984), which may interact with the basic domain of ODA3 to create the salt-sensitive bond. The COOH-terminal region of tubulin is exposed on the surface of the microtubule and has been implicated in binding of other microtu-

bule-associated proteins (Littauer et al., 1986; Paschal et al., 1989).

The outer dynein arm polypeptides that bind to the ODA-DC have not yet been identified, but IC78, which is located at the base of the dynein (King and Witman, 1990), has both acidic and basic domains (King et al., 1995). These could interact with oppositely charged domains on ODA3 to anchor the outer arm to the ODA-DC. The availability of the *ODA*3 gene and sequence will greatly facilitate identification of the polypeptides that are in direct contact with ODA3, and will permit analysis of the functional domains responsible for the interactions between ODA3 and its binding partners.

The ODA-DC as a Paradigm for Dynein Targeting

The results presented here provide definitive evidence that loss of ODA3 results in loss of the ODA-DC, with concomitant failure of the outer dynein arm to assemble onto the doublet microtubule. Therefore, ODA3 is essential for assembly of the ODA-DC, and the ODA-DC is necessary for attachment of the outer dynein arm to the doublet microtubule. These findings suggest that the ODA-DC is responsible for the specific targeting of the outer dynein arm to its correct binding site on the flagellar axoneme. Inasmuch as the inner dynein arms also are targeted to specific binding sites on the doublet microtubules (Smith and Sale, 1992), it is likely that inner dynein arm docking proteins also exist. The insertional mutagenesis technique used here should be helpful in identifying and characterizing such polypeptides. Indeed, because slow, smooth swimming is characteristic of inner armless mutants (Kamiya et al., 1991), some of the slow-smooth swimming insertional mutants that we have isolated may be defective in the protein(s) responsible for inner arm docking.

It has been proposed that the ~20S dynactin complex (Gill et al., 1991) may anchor cytoplasmic dynein to the kinetochore and various membranous structures (Vallee and Sheetz, 1996). If so, dynactin and the ODA-DC would have analogous roles in targeting of cytoplasmic dynein and outer arm dynein to their respective organelles. Although the sequences of all the polypeptides of both complexes have not been reported, the data to date do not reveal any obvious homology between dynactin and the ODA-DC. Therefore, despite their close evolutionary relationship, these two dyneins appear to bind to distinctly different docking complexes. This may turn out to be the most biologically significant difference between these dyneins. On the other hand, cytoplasmic dynein interacts with several different cell organelles at different times in the cell cycle, and it is possible that a cytoplasmic homolog of the ODA-DC will yet be found to mediate binding of cytoplasmic dynein to a specific subset of cellular sites.

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