Chlamydomonas Inner-Arm Dynein Mutant, *ida5*, Has a Mutation in an Actin-encoding Gene

Takako Kato-Minoura,* Masafumi Hirono,‡ and Ritsu Kamiya‡§

*Department of Molecular Biology, School of Science, Nagoya University, Nagoya 464-01, Japan; ‡Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan; and §National Institute for Basic Biology, Okazaki 444, Japan

Abstract. *Chlamydomonas* flagellar inner-arm dynein consists of seven subspecies (*a*–*g*), of which all but *f* contain actin as subunits. The mutant *ida5* and a new strain, *ida5-t*, lack four subspecies (*a*, *c*, *d*, and *e*). These mutants were found to have mutations in the conventional actin gene, such that its product is totally lost; *ida5* has a single-base deletion that results in a stop codon at a position about two-thirds from the 5′ end of the coding region, and *ida5-t* lacks a large portion of the entire actin gene. Two-dimensional gel electrophoresis patterns of the axonemes and inner-arm subspecies *b* and *g* of *ida5* lacked the spot of actin (isoelectric point [*p*I] = ∼5.3) but had two novel spots with pIs of ∼5.6 and ∼5.7 instead. Western blot with different kinds of anti-actin antibodies suggested that the proteins responsible for the two novel spots and conventional actin are different but share some antigenicity. Since *Chlamydomonas* has been shown to have only a single copy of the conventional actin gene, it is likely that the novel spots in *ida5* and *ida5-t* originated from another gene(s) that codes for a novel actin-like protein(s) (NAP), which has hitherto been undetected in wild-type cells. These mutants retain the two inner-arm subspecies *b* and *g*, in addition to *f*, possibly because NAP can functionally substitute for the actin in these subspecies while they cannot in other subspecies. The net growth rate of *ida5* and *ida5-t* cells did not differ from that of wild type, but the mating efficiency was greatly reduced. This defect was apparently caused by deficient growth of the fertilization tubule. These results suggest that NAP can carry out some, but not all, functions performed by conventional actin in the cytoplasm and raise the possibility that *Chlamydomonas* can live without ordinary actin.

SINCE the first discovery by Piperno and Luck (1979) from studies on *Chlamydomonas* flagella, the inner-arm dynein of cilia and flagella in various organisms has been shown to contain actin as a subunit (Pratt, 1986; Muto et al., 1994). Immunological evidence (Pratt, 1986; Muto et al., 1994; Sugase et al., 1996) strongly suggests that the actin contained in the inner-arm dynein is a true actin, not an actin-related protein that has been shown to be associated with dynactin, an activator of cytoplasmic dynein (Lees-Miller et al., 1992; Paschal et al., 1993). A recent study has indicated that *Chlamydomonas*, like *Volvox* (Cresnar et al., 1990), has only a single gene for actin, suggesting that the same actin is used for both cytoplasmic and axonemal functions (Sugase et al., 1996). The deduced sequence of *Chlamydomonas* actin indicated that it is a typical conserved actin, sharing ∼90% homology to rabbit skeletal muscle actin.

Please address all correspondence to Ritsu Kamiya, Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, 113 Tokyo, Japan. Tel. and Fax: (81) 3-5800-6842. e-mail: kamiyar@biol.s.u-tokyo.ac.jp

The inner-arm dynein of *Chlamydomonas* consists of several distinct subspecies, each containing one or two heavy chains (Piperno et al., 1990; Kagami and Kamiya, 1992). Our study using ion-exchange chromatography has shown that the inner-arm dynein from outer arm–missing mutants can be separated into seven different subspecies, named *a*–*g*, containing eight distinct heavy chains altogether (Kagami and Kamiya, 1992). Actin is associated with all subspecies except *f.* Several inner arm–deficient mutants we isolated were found to lack distinct sets of inner-arm subspecies. For example, *ida1* lacks subspecies *f* (containing two heavy chains) and *ida4* lacks subspecies *a*, *c*, and *d* (Kagami and Kamiya, 1992; for another terminology system of inner arm subspecies, see Piperno [1995]). The mutant *ida4* has recently been shown to have a mutation in the structural gene for a 28-kD component of the subspecies *a*, *c*, and *d* (LeDizet and Piperno, 1995).

In the present study we further investigated the structural defects in the mutant *ida5*, which lacks subspecies *a*, *c*, *d*, and *e* and displays slow swimming (Kato et al., 1993). Here, we report an unexpected finding that *ida5* has a mutation in the actin-encoding gene that results in conventional actin not being expressed at all. Instead, a novel

© The Rockefeller University Press, 0021-9525/97/05/649/8 $2.00

protein, previously undetected in wild type, appears to substitute for the ordinary actin as a subunit of the inner-arm subspecies *b* and *g*.

Materials and Methods

Strains and Cell Culture

Chlamydomonas reinhardtii 137c (wild type), *nit1/cw15*, *ida5* (Kato et al., 1993), and a new *ida5* strain, *ida5-t*, were used. The mutant *ida5* was produced by mutagenesis with UV light, while *ida5-t* was produced by insertional mutagenesis (see below). *nit1/cw15* was obtained from Dr. E. Harris of the *Chlamydomonas* Genetic Center (Department of Botany, Duke University, Durham, NC). Cells were grown either in liquid Tris-acetic acid-phosphate (TAP) medium (Gorman and Levine, 1965) or on TAP/agar plates.

Isolation of an ida5 Allele by Insertional Mutagenesis

An *ida5* allele, *ida5-t*, was obtained using a gene-tagging technique as described by Tam and Lefebvre (1993). Briefly, a nitrate reductase–encoding plasmid, pMN24, was introduced into the mutant *nit1/cw15* lacking nitrate reductase by vigorous stirring of the cells and plasmid with glass beads (Kindle, 1990). Transformants were selected by growing the cells on SGII/agar plates (Sager and Granick, 1953) containing nitrate as the sole source of nitrogen. The mutant *ida5-t* was one of the mutants deficient in inner-arm dyneins obtained by screening ~6,500 transformants for slow swimming phenotypes. Southern blot analyses indicated that this mutant carries four plasmid insertions in the genome.

Isolation of Axoneme and Dynein

Flagella were detached from the cell body using dibucaine and purified by differential centrifugation (Witman, 1986). Dynein was obtained by extracting the demembranated flagellar axoneme with a buffer solution containing 0.6 M KCl and fractionated by chromatography on a Mono-Q column, as described previously (Kagami and Kamiya, 1992).

Cell Body Extract

About 10^9 cells in 100 ml of TAP medium were deflagellated as above. The cell bodies were collected by centrifugation, resuspended in 5 ml of a solution containing 0.5 mM ATP, 0.1 mM $CaCl_2$, 10 mM imidazole, 0.01% β-mercaptoethanol, and 1 mM PMSF (Hirono et al., 1989), and sonicated with a model US50 ultrasonic generator (Nihon Seiki Ltd., Tokyo, Japan) at 0°C for 2 min. The sonicated material was centrifuged at 200,000 *g* for 1 h, and the supernatant was used as the cell body extract. The final protein concentration was ~1 mg/ml.

Electrophoresis

One-dimensional SDS-PAGE was performed by the method of Laemmli (1970). Two-dimensional gel electrophoresis was performed using Immobiline DryStrip gel (Pharmacia LKB Biotechnology, Uppsala, Sweden), which covered a pH range of 4.0–7.0. Isoelectric focusing was performed at 2,300 V for 16 h, in the presence of 5 M urea and 2 M thiourea (Nakamura et al., 1989), followed by SDS-PAGE in the second direction. Gels were stained with silver or colloidal gold (AuroDye; Amersham Intl., Amersham, UK) or used for immunoblot analysis.

Immunoblotting

Western blotting of axonemes and cell body extracts was performed by the method of Towbin et al. (1979). Samples were electrophoresed, transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA), and probed with antibodies. Antibodies used were a polyclonal antiserum raised against an 11–amino acid peptide corresponding to the NH_2-terminal sequence of *Chlamydomonas* actin (Sugase et al., 1996); an anti-*Physarum* actin polyclonal antibody (IgG fraction obtained by ammonium sulfate fractionation; a gift from Dr. K. Owaribe [Nagoya University, Japan]); and two commercially available anti–chicken gizzard actin mAbs, C4 (Lessard, 1988; ICN Biomedicals, Costa Mesa, CA) and N350 (Lin, 1981; Amersham Intl.). Immunoreactive spots were visualized with an alkaline phosphatase–conjugated secondary antibody (Cappel Research Products, Durham, NC) with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) as the chromogen.

Isolation and Sequence Analysis of ida5 Actin cDNA

Total RNA of *ida5* and *ida5-t* was prepared by acid guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). We used cells that were in the process of flagellar regeneration after being deflagellated by pH shock (Luck et al., 1977), since we speculated that the actin gene transcription may be activated during flagellar growth (see Sugase et al., 1996). Poly$(A)^+$ RNA was purified using oligotex-dT30 (Nihon Gousei Gum Co., Tokyo, Japan). 5 μg of poly$(A)^+$ RNA was used for the construction of Uni-ZAP XR cDNA library (Stratagene, La Jolla, CA). For screening for actin cDNA, a wild-type actin cDNA (Sugase et al., 1996) was used as a probe. One of the two positive clones obtained from the *ida5* library was sequenced over its entire length. The sequence difference observed between the mutant and wild type was verified by sequencing a segment of cDNA containing the possible mutation site. For this purpose, a 200-bp sequence starting at nucleotide 730 (in the cDNA sequence registered in EMBL/DDBJ/GenBank under accession number D50839) was amplified by reverse transcription (RT)[1]–PCR from both wild-type and *ida5* total RNA using 5′-GGCCACCGCGCTGTCCAGT-3′ and 5′-TACTGGCGCACTCAAAAGCG-3′ as primers. A first-strand cDNA synthesis kit (Clontech, Palo Alto, CA) was used. The fragments were cloned into pBluescript vector (Stratagene) and sequenced for both strands.

DNA and RNA Blotting

DNA was isolated by the method of Weeks et al. (1986). For Southern blot analysis, DNA was digested with restriction enzymes, loaded on a 1% agarose gel, transferred to a Hybond-N+ membrane (Amersham Intl.), and probed with various fragments from genomic and cDNA clones of wild-type actin. As a control, the same samples were probed with a fragment of α-1 tubulin gene (Brunke et al., 1984) (obtained from the *Chlamydomonas* Genetics Center). The probes were labeled using a DIG DNA labeling kit (Boehringer Mannheim GmBH, Mannheim, Germany). The membranes were hybridized at 67°C overnight in 5 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 0.2% SDS, 5 × Denhardt's reagent (1 × Denhardt's reagent is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), and 0.5 mg/ml salmon sperm DNA, and then washed with 0.1 × SSPE containing 0.1% SDS at 67°C.

For Northern blot analysis, total RNA, prepared as above, was separated on a 1.5% agarose/formaldehyde gel and transferred to a Hybond-N+ membrane. Hybridization was performed at 65°C overnight in 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 7% SDS, 2% blocking reagent (Boehringer Mannheim GmBH), and 0.5 mg/ml salmon sperm DNA, and the membranes were washed with 0.2 × SSC (1 × SSC is 150 mM NaCl and 15 mM Na_3 citrate, pH 7.0) containing 0.5% SDS at 65°C.

Mating Efficiency

Gametes were produced by incubating cells for 4–5 h in the nitrogen-free medium (see Harris, 1989). For the determination of mating efficiency, equal numbers of plus mating type (mt^+) and minus mating type (mt^-) gametes (density: 1×10^7 cells per ml) were mixed together, and the numbers of biflagellate cells (B) and quadriflagellate cells (Q) were counted after fixing the cells with 0.5% formaldehyde at different times. The percentage of the gametes that underwent cell fusion was calculated as $100 \times 2Q/(2Q + B)$.

Observation of Fertilization Tubules

Gametes (mt^+) of wild type or *ida5* were incubated in the presence of 10 mM dibutyryl-cAMP and 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 1 h to induce fertilization tubule growth (Pasquale and Goodenough, 1987). For optical microscopy, gametes were fixed with 3.6% formaldehyde and observed using differential-contrast optics. For EM, gametes were fixed with 1% glutaraldehyde and processed after the method of Detmers et al. (1983). A 100CX microscope (Jeol Co., Tokyo, Japan) was used for the observation.

1. *Abbreviations used in this paper*: IBMX, 3-isobutyl-1-methylxanthine; mt^+, mating type plus; mt^-, mating type minus; NAP, novel actin-like protein; RT, reverse transcription; TAP, Tris-acetic acid-phosphate.

Results

Altered Actin in ida5

Fractionation by chromatography of the high salt extract of *ida5* axonemes showed that its axonemes lack four (*a*, *c*, *d*, and *e*) of the seven (*a*–*g*) inner-arm dynein subspecies normally present in the wild-type axoneme (Kato et al., 1993). SDS-PAGE patterns of the dynein fractions indicated that its subspecies *b* and *g* had a 43-kD band at a position similar to that of the actin band in the wild-type counterparts (Kato et al., 1993). However, two-dimensional gel electrophoresis of the axoneme revealed a striking difference (Fig. 1, *A* and *B*); the *ida5* axoneme lacked the spot corresponding to that of actin (isoelectric point [pI] = ~5.3) but had two novel spots at apparent pIs of ~5.6 and ~5.7. The same change in the actin spot was observed with the dynein fractions *b* (data not shown) and *g* (Fig. 1 *B*). In one-dimensional SDS-PAGE, the 43-kD band in *ida5* had a slightly larger mobility than that in wild type (Fig. 1 *C*).

Sequence Analysis of ida5 Actin cDNA

The above finding, together with the recent mapping of the actin gene to a locus near that of *ida5* (right arm of linkage group XIV) by C. Silflow and P. Lefebvre (personal communication), suggested that the mutant *ida5* might have a mutation in the actin-encoding gene itself. We thus analyzed its actin gene. Actin cDNA was isolated from an *ida5* cDNA library using the wild-type actin cDNA as the probe. One of the two clones obtained was sequenced over its entire length. The sequence indicated that a base (C) had been deleted from the CCCC sequence starting at nucleotide 838 (in the cDNA nucleotide sequence registered in EMBL/DDBJ/GenBank). The same deletion was found in a cDNA fragment obtained by RT-PCR from total *ida5* RNA (see Materials and Methods). This deletion should cause a frame shift and produce a stop codon at the amino acid position 268, i.e., at about two-thirds of the total of 377 amino acids (Fig. 2). Hence, no functional actin should be produced in this mutant since the COOH-terminal 110–amino acid sequence is essential to the actin assembly and function (Kabsch et al., 1990). However, although Northern blotting detected the presence of full-length actin mRNA (data not shown), Western blot analysis using an antibody against NH_2-terminal 11–amino acid sequence did not detect any truncated actin (see Fig. 4). Truncated products, if any, may be degraded quickly in the cytoplasm.

Loss of the Actin Gene in an ida5 Allele Produced by Insertional Mutagenesis

We isolated a mutant similar to *ida5* by screening a population of cells mutagenized by plasmid insertion. Its axoneme lacked the same inner-arm subspecies as those

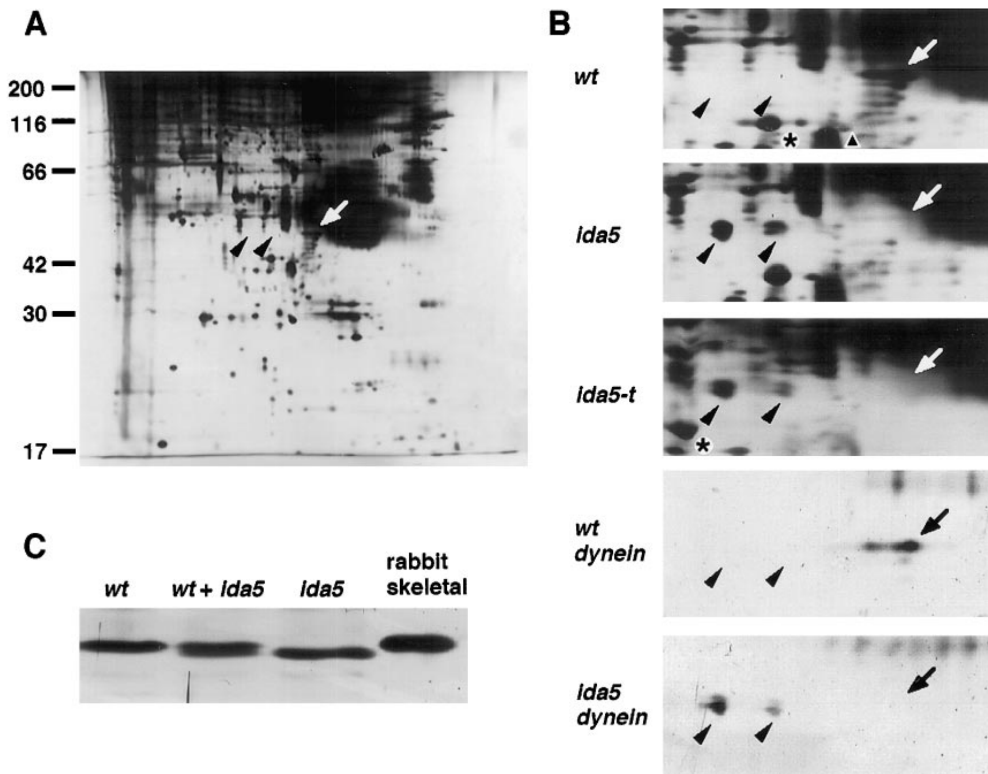

Figure 1. Electrophoresis patterns of wild-type and mutant axonemes. (*A*) Two-dimensional SDS-PAGE pattern of wild-type axonemes. pH range: 4.0–7.0. (*Left*) Basic polypeptides. (*Bars with numbers*) Positions of molecular mass standards shown in $M_r \times 10^{-3}$. (*B*) Portions of two-dimensional electrophoresis showing spots of actin and NAP appearing in the mutants. (*wt, ida5*, and *ida5-t*) Axonemes of wild type and mutants. (*wt dynein* and *ida5 dynein*) Inner-arm subspecies *g* separated by chromatography. Arrows in *A* and *B* indicate the position of actin; arrowheads indicate those of NAP. In *ida5-t*, two spots of unidentified origins were shifted by ~0.2 pH unit to more alkaline positions (*). This shift may be caused by another gene disruption event in this mutant. The smear (*triangle*) is an artifact of silver staining. The faint spots seen in the dynein patterns (shown in a series) are those of tubulins with various degrees of posttranslational modification. (*C*) One-dimensional SDS-PAGE of subspecies *g*. Samples from wild type, *ida5*, and their mixture were loaded on the same 11% polyacrylamide gel. (Lane *rabbit skeletal*) Rabbit skeletal muscle actin. Only a portion near the actin band is shown. All gels were stained with silver.

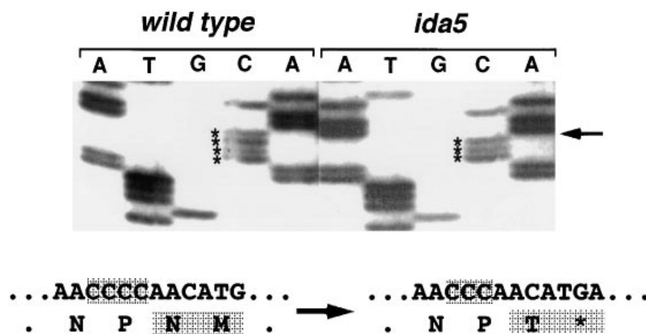

Figure 2. Sequence analysis of the *ida5* mutation. Sequencing patterns of the partial cDNA clones obtained from wild type and *ida5* by RT-PCR were shown side by side. (*Arrow, right*) Position of a base (*C*) deletion (*asterisks*). The altered cDNA and amino acid sequences are shown at the bottom. The leftmost asparagine is at amino acid 265. The deletion in *ida5* results in a stop codon TGA at position 268.

missing from *ida5* and had the same two novel spots in two-dimensional gels as observed with *ida5* (Fig. 1 *B*). Sexual crossing of this mutant with *ida5* did not produce cells with a wild-type phenotype in 40 tetrads. In addition, temporary dikaryons between them were not rescued. From these observations, we concluded that this mutation is an *ida5* allele. We named it *ida5-t.*

Unexpectedly, Southern blot analyses of the *ida5-t* genome using four different fragments from the wild-type actin genomic clone as probes did not detect bands that hybridized with any of the probes. With the parent strain *nit1/cw15*, on the other hand, the same four probes clearly detected bands at positions exactly as predicted from the sequence (Fig. 3 *A*). These findings indicate that a large portion of the actin-encoding region is missing from *ida5-t.* Such loss of a gene upon insertional mutagenesis has often been observed before, although its exact mechanism is unknown (Tam and Lefebvre, 1993).

In agreement with the major loss of the actin gene, Northern analysis detected no actin mRNA in *ida5-t*, while the same analysis detected actin mRNA in the parent strain *nit1/cw15* (Fig. 3 *B*). Thus, the findings with *ida5* and *ida5-t* both indicate that these mutants do not express conventional actin. It is likely that the two novel spots in the two-dimensional gel are products of gene(s) other than the known actin-encoding gene. The proteins responsible for these two spots will be tentatively called novel actin-like proteins (NAPs). (It is not certain whether NAP is a single protein or two, because a single protein frequently appears as two or three spots in our two-dimensional gel system for unknown reasons.)

Western Blot Analysis of NAP

Although the above findings suggest that NAP is not the product of the conventional actin gene, we speculated that it is somehow related to actin. This is because NAP has an apparent molecular weight similar to that of actin and can substitute for actin as the subunit of inner-arm dynein subspecies *b* and *g.* To assess the similarity of NAP to conventional actin, its cross-reactivity with four different anti-actin antibodies was examined by Western blotting (Fig. 4 *A*). All these antibodies were found to react with the actin in the SDS-PAGE pattern of wild-type axonemes, although most of them also reacted with a few other bands of unidentified origins. In *ida5* axonemes, the polyclonal antibody against the NH_2-terminal 11–amino acid sequence of *Chlamydomonas* actin did not react with NAP as stated before, and neither did a monoclonal anti–smooth muscle actin antibody (C4). However, a polyclonal antibody against *Physarum* actin and another monoclonal anti–smooth muscle actin antibody, N350, did react with it, although less intensely than with actin in the wild-type axoneme. In two-dimensional electrophoresis patterns of the axoneme, the *Physarum* antibody reacted with the actin spot in wild type and both of the two spots of NAP in *ida5* and *ida5-t*, while the *Chlamydomonas* antibody reacted only with the actin in wild type (Fig. 4 *B*).

To detect actin and NAP in the cell body, extracts from deflagellated cells of wild type and *ida5* were subjected to two-dimensional electrophoresis and Western analysis. Both of the *Chlamydomonas* and *Physarum* antibodies used above revealed the presence of actin in wild type but not in *ida5* (Fig. 4 *C*). Rather unexpectedly, NAP spots were not detected with the *Physarum* antibody in the *ida5* cell body. This suggests that NAP may be present in the cell body of this mutant in a much smaller amount than actin in the wild-type cell body.

Effects of Actin Mutation in Cell Growth and Fertilization

We expected that the actin mutation in *ida5* would cause some defects in its growth or cell division. However, we found no difference in the gross growth rate between the mutants and wild type (Fig. 5). Time-lapse observations of the cytokinesis process revealed that the mutants form cleavage furrows similar to those observed during the cell division in the wild-type cells (data not shown).

During the sexual conjugation of *Chlamydomonas*, mt^+ gametes produce a process of a few micrometers in length at the apical end of the cell body and thereby facilitate the cell fusion process (Goodenough and Weiss, 1975). This process, called the fertilization tubule, has been shown to contain an F-actin bundle (Detmers et al., 1983). We speculated that *ida5* gametes may be deficient in fertilization because they cannot produce the fertilization tubule normally. Just as we expected, the efficiency of cell conjugation greatly decreased when mt^+ *ida5* was mated with mt^- wild type or *ida5.* Interestingly, conjugation efficiency did not decrease when mt^- *ida5* was mated with mt^+ wild type (Fig. 6). These results suggest that the gamete of mt^+ *ida5* is deficient in fertilization tubule growth.

To confirm the above prediction, we induced fertilization tubule growth by treating gametes with dibutyryl-cAMP and IBMX (Pasquale and Goodenough, 1987) and observed them with a differential-interference microscope. After about an hour of treatment, fertilization tubules were clearly seen in $>70\%$ of the mt^+ wild-type gametes, whereas no fertilization tubules were found in mt^+ *ida5* gametes. EM indicated that a small fraction of *ida5* gametes in fact produced fertilization tubules of abnormal shapes (Fig. 7). In contrast with the slender fertilization tubules of

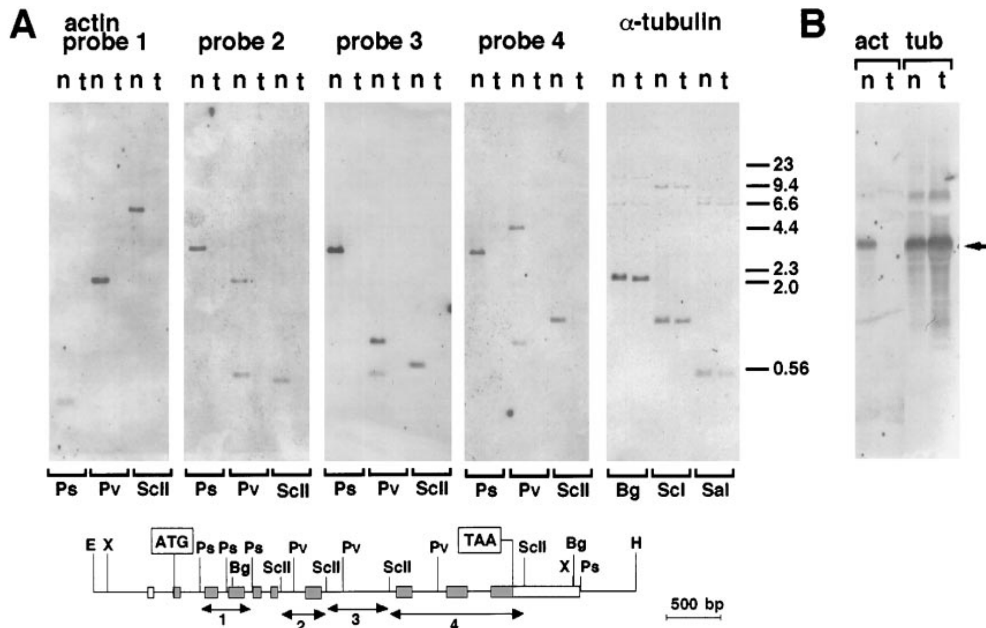

Figure 3. Southern (*A*) and Northern (*B*) hybridization analyses of *ida5-t.* Along with the actin gene, tubulin gene and message were analyzed as controls. (*A*) Genomic DNAs from *nit1/cw15* (*n*) and *ida5-t* (*t*) were digested with a restriction enzyme shown at the bottom and probed with the probes indicated in the restriction map (Sugase et al., 1996). The solid and open boxes indicate coding exons and transcribed UTR. *Pv*, PvuII; *ScII*, SacII; *Ps*, PstI; *Bg*, BglII; *ScI*, SacI; *Sal*, SalI; *E*, EcoRI; *X*, XhoI; *H*, HindIII. The membranes were hybridized at 67°C overnight. (*Bars with numbers*) Positions of the λHindIII markers shown in bp × 10^{-3}. (*B*) Total RNA was probed with either wild-type actin cDNA (*act*) or TubA1 fragment (*tub*). Hybridization was performed at 65°C. (*Arrow*) Bands of actin and α-tubulin mRNAs (almost the same size).

wild type, the *ida5* fertilization tubules were round and stubby. Appendices of round appearance were also observed in mt^- *ida5* gametes treated with dibutyryl-cAMP and IBMX, as well as in the mt^- wild-type gametes thus treated (Pasquale and Goodenough, 1987).

Southern Blot Analyses at Different Stringencies

As stated, our previous study indicated that *Chlamydomonas* has only a single copy of actin-encoding gene (Sugase et al., 1996). However, the above finding that the mutants *ida5* and *ida5-t* with serious deletions in the actin gene grow normally has raised the possibility that another protein(s), such as NAP, substitutes for actin in important functions in these mutants, because actin has been believed to be essential for cellular function. The substituting protein, if any, may be structurally related to actin. Thus, we examined if *Chlamydomonas* has other genes that become hybridized with a fragment of the conventional actin gene at low stringency. Fig. 8 shows the results of such an experiment. Under the conditions shown in Fig. 3, probe 2 did not hybridize with any band in *ida5-t*, whereas in the parent (*nit1/cw15*) it hybridized with one or two bands, at positions exactly as expected from the restriction map of the known actin gene. When the stringency was slightly decreased by lowering the temperature from 67° to 65°C, an additional band appeared in each sample of the DNA fragments of both strains. This band cannot be explained from the sequence of the conventional actin gene. With additional lowering of stringency, it was obscured by many other nonspecific bands.

Discussion

We have shown that the *Chlamydomonas* mutants *ida5* (Kato et al., 1993) and *ida5-t*, lacking four subspecies of inner-arm dynein, have mutations in an actin-encoding gene such that its gene product is totally lost. The axoneme of these mutants contain NAP instead of conventional actin, as evidenced by the two novel spots in two-dimensional electrophoresis patterns. These findings are unexpected since *Chlamydomonas* and a related species, *Volvox*, have been shown to have a single gene for actin, which is ~90% homologous to rabbit skeletal muscle actin (Cresnar et al., 1990; Sugase et al., 1996). The fact that *ida5* and *ida5-t* grow normally suggests either that NAP or other proteins may be able to substitute for conventional actin in important functions, or that actin is not essential for the growth of *Chlamydomonas.*

The molecular identity of NAP remains to be studied, but it appears to be somehow related to actin. This is because NAP and actin have similar molecular weights, can serve as the subunit of some inner-arm dynein, and share some antigenicity; although NAP did not cross-react with the antibody against the NH_2-terminal sequence of *Chlamydomonas* actin or mAb C4, it weakly cross-reacted with two other anti-actin antibodies. NAP may be another actin that is only moderately homologous to conventional actin and has hitherto been undetected. The Southern analysis in the present study under low stringency conditions revealed, in addition to the band expected from the known actin gene sequence, a weakly hybridizing band that has not been detected in our previous study (Sugase et al., 1996). This additional band cannot be explained by the

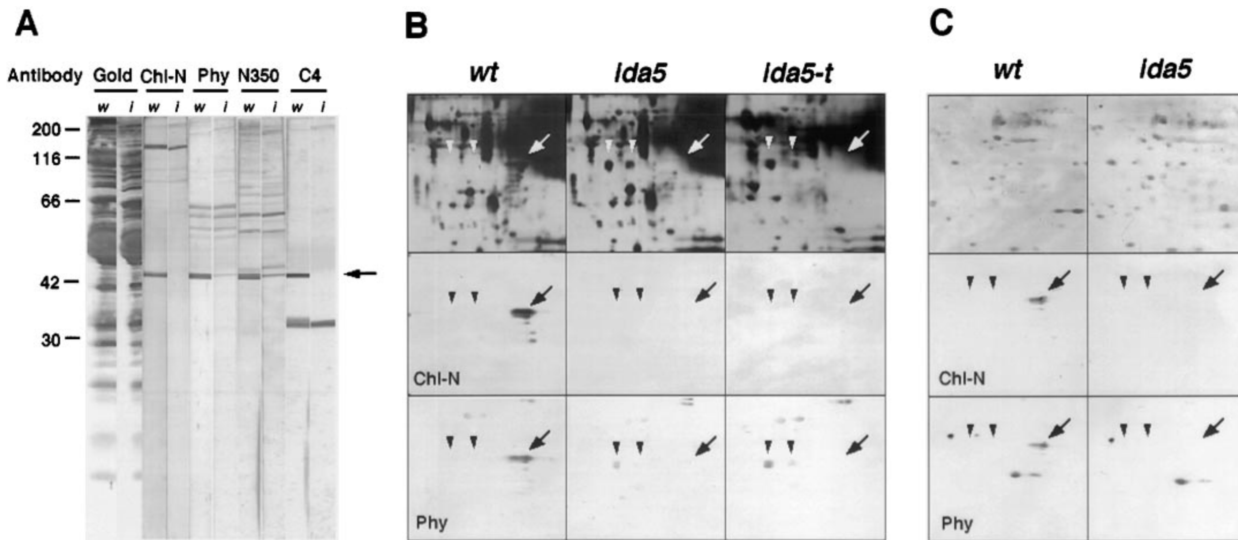

Figure 4. Western blot analyses. (*A*) Axoneme of wild type (*w*) and *ida5* (*i*) was loaded on 11% polyacrylamide gel, transferred, and stained with colloidal gold (*Gold*), or probed with anti–*Chlamydomonas* actin (*Chl-N*), anti–*Physarum* actin (*Phy*), N350, or C4 antibodies. (*Arrow*) The 43-kD band of actin or NAP. (*B*) Two-dimensional gel electrophoresis patterns and Western blot of wild-type (*wt*) and mutant axonemes. Only a region of M_r 30–66 kD and pH 5.1–5.9 is shown. (*Arrows*) Positions of actin. (*Arrowheads*) Positions of NAP. (*Top rows*) Silver-stained gel patterns; (*middle and bottom rows*) blot with anti–*Chlamydomonas* actin (*Chl-N*) and anti–*Physarum* actin (*Phy*) antibodies. (*C*) Two-dimensional gel electrophoresis patterns of cell body extracts. Cell bodies from wild type (*wt*) and *ida5* were collected immediately after deflagellation. Apparent M_r range: 24–66 kD. pH range: 5.1–5.9. (*Top row*) Stained with colloidal gold; (*middle and bottom rows*) Western blot with anti–*Chlamydomonas* actin (*Chl-N*) and anti–*Physarum* actin (*Phy*) antibodies.

known actin sequence and may well have originated from the gene of NAP. Whatever the gene is, it may not be highly homologous to conventional actin, because it cannot be detected at higher stringencies; if the two genes were members of a gene family of conventional actin, like the multiple actin genes in other organisms such as *Dictyostelium* (Romans and Firtel, 1985) and mouse (Minty et al., 1983), the two sets of bands might well have appeared even under high stringency conditions. It is likely that conventional actin is totally missing in the *ida5* mutants.

The association of actin with axonemal inner-arm dynein (Piperno and Luck, 1979; Piperno et al., 1990; Muto et al., 1994; Sugase et al., 1996) and that of an actin-like protein (dynactin, Arp1) with an activator of cytoplasmic dynein, dynactin complex, have been established (Lees-Miller et al., 1992; Paschal et al., 1993; Schafer et al., 1994). The dynactin complex probably serves to bind dynein to intracellular membranes to facilitate their movement on microtubules (see Schroer, 1994). By analogy, the actin in inner-arm dynein may function to facilitate binding of dynein to the A-tubule of the outer doublet. This idea is supported by the present finding that the actin mutation in

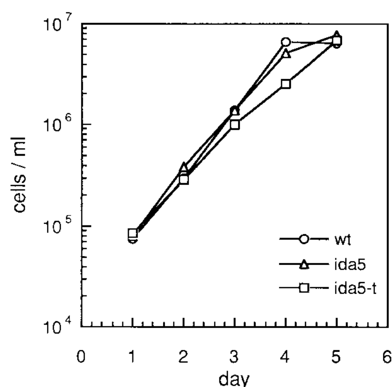

Figure 5. Proliferation rate of wild type and mutants. Cells cultured in the liquid TAP medium were sampled every 24 h and their numbers were counted.

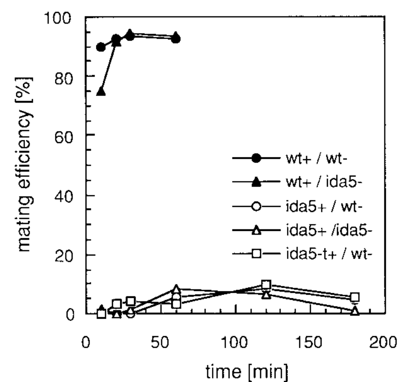

Figure 6. Mating efficiency. (*Ordinate*) Percentage of cells that underwent cell fusion and became quadriflagellate. (*Abscissa*) Time after the onset of mating. The combinations of mating pairs are shown with a slash. + and − denote the mating type of the gametes used. Since the flagella of zygotes were gradually resorbed with time, the apparent mating efficiency decreased after ∼2 h of mating.

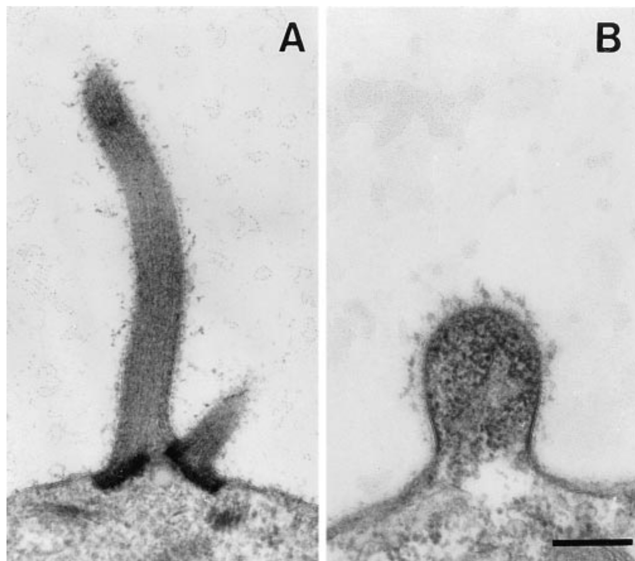

Figure 7. Fertilization tubules in wild-type (*A*) and *ida5* (*B*) mt^+ gametes produced in response to a 1-h exposure to 10 mM dibutyryl-cAMP and 1 mM IBMX. Bar, 0.3 μm.

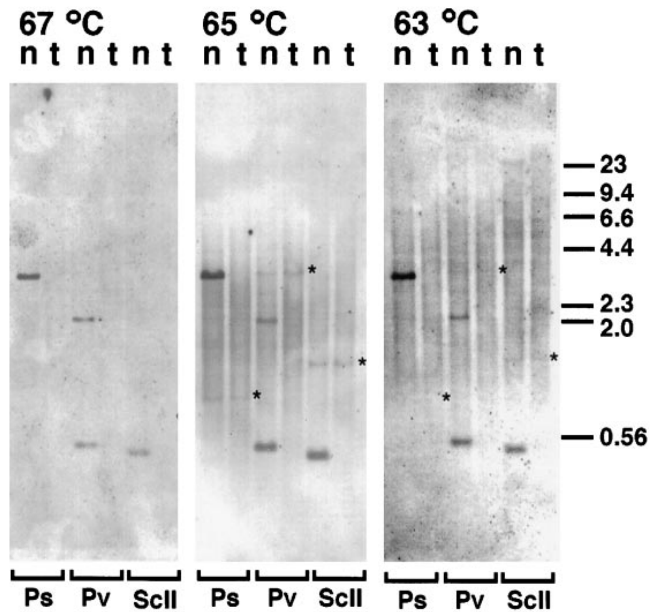

Figure 8. Southern hybridization analyses at different stringencies. Probe 2 in Fig. 3 was used. *n*, restriction fragments from *nit1/cw15* (parent strain); *t*, fragments from *ida5-t.* For abbreviations of restriction enzymes, see Fig. 3 legend. Experimental procedure was the same as in Fig. 3 *A* except for the temperature at which hybridization and wash were performed. At lower stringencies, an additional band (*) appears in each lane of both samples.

ida5 results in the loss of four inner-arm subspecies. The fact that the mutant axoneme has NAP in place of actin and retains inner-arm subspecies *b* and *g* suggests that NAP can functionally replace actin in these subspecies. However, it is not understood why NAP is absent from the wild-type axoneme (Fig. 1 *B*). It may be that, in wild-type cells, the association of NAP with the inner-arm dynein is prevented by the abundance of conventional actin. Alternatively, the cell may regulate the expression of NAP so that it is produced in a significant quantity only when the expression of conventional actin is blocked. Whether the wild-type cell also expresses NAP will be made clear when its specific antibody or DNA clone is obtained.

We have found that *ida5* and *ida5-t* have a serious defect in the fertilization tubule growth. Our observation is in good agreement with that of Detmers et al. (1983) who showed that the mating efficiency is greatly reduced if mt^+, but not mt^- , gametes have been preincubated with cytochalasin D. They showed that cytochalasin-treated mt^+ gametes produce short fertilization tubules that can make contact with mt^- gametes, permitting the gametes to fuse with low efficiency. Thus, our observation that mt^+ *ida5* gametes with stubby round fertilization tubules can undergo cell fusion at low efficiency is compatible with the idea that the gamete does not express polymerizable actin at all.

Harper et al. (1992) have examined the actin distribution during the cell cycle of *Chlamydomonas* using mAbs C4 and N350, which we also used in this study. They found that actin surrounds the nucleus in interphase cells and undergoes dynamic reorganization during mitosis and cytokinesis, including relocalization to the cleavage furrow during cytokinesis. From these observations they suggested that actin may play a role in the movement of basal bodies and cytokinesis. It is important to note, however, that the actin in the cytoplasm could not be visualized by staining with FITC-phalloidin, nor could the cytokinesis be blocked with cytochalasin B or cytochalasin D. Hence, they also suggested the possibility that the change they observed may be a change in the distribution of G-actin and not that of F-actin (Harper et al., 1992). It is possible that F-actin is present in wild-type *Chlamydomonas* only in the fertilization tubule, which has been demonstrated to be clearly stained with fluorescence-labeled phallacidin (Detmers et al., 1985).

Although a large number of actin mutants have been isolated in other organisms (for review see Sheterline and Sparrow, 1994), all the null mutants, including those of yeast (Shortle et al., 1982), have been found to be lethal. The fact that the *ida5* mutations are null alleles yet nonlethal opens the way for experiments in which functions of conventional actin are investigated by transforming the mutants with modified constructs of the gene. In fact, we have succeeded in rescuing the *ida5* phenotype by transforming it with a wild-type actin gene clone (Ohara, A., T. Kato-Minoura, R. Kamiya, and M. Hirono, unpublished result). Initial experiments aimed at elucidation of the functional domains of actin that are important for the assembly of dynein are underway.

We thank Ms. Yasuko Sugase (University of Tokyo) for her technical advice, Mr. Akinori Yoda (University of Tokyo) for his help in mutant isolation, Dr. Katsushi Owaribe (Nagoya University) for the *Physarum* actin antibody, and Dr. Hirokazu Hotani (Nagoya University) for his encouragement.

This work was supported by grants (06304006, 07408034, and 06275101) from the Ministry of Education, Science, Sports, and Culture of Japan.

Received for publication 2 July 1996 and in revised form 22 January 1997.

References

Brunke, K.J., J.G. Anthony, E.J. Sternberg, and D.P. Weeks. 1984. Repeated consensus sequence and pseudopromoters in the four coordinately regulated tubulin genes of *Chlamydomonas reinhardtii. Mol. Cell. Biol.* 4:1115–1124.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

Cresnar, B., W. Mages, K. Muller, J.M. Salbaum, and R. Schmitt. 1990. Structure and expression of a single actin gene in *Volvox carteri. Curr. Genet.* 18: 337–346.

Detmers, P.A., U.W. Goodenough, and J. Condeelis. 1983. Elongation of the fertilization tubule in *Chlamydomonas*: new observations on the core microfilaments and the effect of transient intracellular signals on their structural integrity. *J. Cell Biol.* 97:522–532.

Detmers, P.A., J.M. Carboni, and J. Condeelis. 1985. Localization of actin in *Chlamydomonas* using antiactin and NBD-phallacidin. *Cell Motil.* 5:415–430.

Goodenough, U.W., and R.L. Weiss. 1975. Gametic differentiation in *Chlamydomonas reinhardtii.* III. Cell wall lysis and microfilament-associated mating structure activation in wild-type and mutant strains. *J. Cell Biol.* 67:623–637.

Gorman, D.S., and R.P. Levine. 1965. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA.* 54:1665–1669.

Harper, J.D., D.W. McCurdy, M.A. Sanders, J.L. Salisbury, and P.C. John. 1992. Actin dynamics during the cell cycle in *Chlamydomonas reinhardtii. Cell Motil. Cytoskeleton.* 22:117–126.

Harris, E.H. 1989. The *Chlamydomonas* Sourcebook. Academic Press, San Diego. 780 pp.

Hirono, M., Y. Kumagai, O. Numata, and Y. Watanabe. 1989. Purification of *Tetrahymena* actin reveals some unusual properties. *Proc. Natl. Acad. Sci. USA.* 86:75–79.

Kabsch, W., H.G. Mannherz, D. Suck, E.F. Pai, and K.C. Holmes. 1990. Atomic structure of the actin: DNase I complex. *Nature (Lond.).* 347:37–44.

Kagami, O., and R. Kamiya. 1992. Translocation and rotation of microtubules caused by multiple species of *Chlamydomonas* inner–arm dynein. *J. Cell Sci.* 103:653–664.

Kato, T., O. Kagami, T. Yagi, and R. Kamiya. 1993. Isolation of two species of *Chlamydomonas reinhardtii* flagellar mutants, *ida5* and *ida6*, that lack a newly identified heavy chain of the inner dynein arm. *Cell Struct. Funct.* 18: 371–377.

Kindle, K.L. 1990. High-frequency nuclear transformation of *Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA.* 87:1228–1232.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

LeDizet, M., and G. Piperno. 1995. *ida4-1*, *ida4-2*, and *ida4-3* are intron splicing mutations affecting the locus encoding p28, a light chain of *Chlamydomonas* axonemal inner dynein arms. *Mol. Biol. Cell.* 6:713–723.

Lees-Miller, J.P., D.M. Helfman, and T.A. Schroer. 1992. A vertebrate actin-related protein is a component of a multisubunit complex involved in microtubule-based vesicle motility. *Nature (Lond.).* 359:244–246.

Lessard, J.L. 1988. Two monoclonal antibodies to actin: one muscle selective and one generally reactive. *Cell Motil. Cytoskeleton.* 10:349–362.

Lin, J.J. 1981. Monoclonal antibodies against myofibrillar components of rat skeletal muscle decorate the intermediate filaments of cultured cells. *Proc. Natl. Acad. Sci. USA.* 78:2335–2339.

Luck, D.J.L., G. Piperno, Z. Ramanis, and B. Huang. 1977. Flagellar mutants of *Chlamydomonas*: study of radial spoke-defective strains by dikaryon and revertant analysis. *Proc. Natl. Acad. Sci. USA.* 74:3456–3460.

Minty, A.J., S. Alonso, J.L. Guenet, and M.E. Buckingham. 1983. Number and organization of actin-related sequences in the mouse genome. *J. Mol. Biol.* 167:77–101.

Muto, E., M. Edamatsu, M. Hirono, and R. Kamiya. 1994. Immunological detection of actin in the 14S ciliary dynein of *Tetrahymena. FEBS Lett.* 343: 173–177.

Nakamura, M., H. Imai, and T. Hirabayashi. 1989. Coordinate accumulation of troponin subunits in chicken breast muscle. *Dev. Biol.* 132:389–397.

Paschal, B.M., E.L. Holzbaur, K.K. Pfister, S. Clark, D.I. Meyer, and R.B. Vallee. 1993. Characterization of a 50-kDa polypeptide in cytoplasmic dynein preparations reveals a complex with $p150^{GLUED}$ and a novel actin. *J. Biol. Chem.* 268:15318–15323.

Pasquale, S.M., and U.W. Goodenough. 1987. Cyclic AMP functions as a primary sexual signal in gametes of *Chlamydomonas reinhardtii. J. Cell Biol.* 105:2279–2292.

Piperno, G., 1995. Regulation of dynein activity within *Chlamydomonas* flagella. *Cell Motil. Cytoskeleton.* 32: 103–105.

Piperno, G. and D.J.L. Luck. 1979. An actin-like protein is a component of axonemes from *Chlamydomonas* flagella. *J. Biol. Chem.* 254:2187–2190.

Piperno, G., Z. Ramanis, E.F. Smith, and W.S. Sale. 1990. Three distinct inner dynein arms in *Chlamydomonas* flagella: molecular composition and location in the axoneme. *J. Cell Biol.* 110:379–389.

Pratt, M.M. 1986. Homology of egg and flagellar dynein. Comparison of ATP-binding sites and primary structure. *J. Biol. Chem.* 261:956–964.

Romans, P., and R.A. Firtel. 1985. Organization of the actin multigene family of *Dictyostelium discoideum* and analysis of variability in the protein coding regions. *J. Mol. Biol.* 186:321–335.

Sager, R., and S. Granick. 1953. Nutritional studies with *Chlamydomonas reinhardtii. Ann. NY Acad. Sci.* 466:18–30.

Schafer, D.A., S.R. Gill, J.A. Cooper, J.E. Heuser, and T.A. Schroer. 1994. Ultrastructural analysis of the dynactin complex: an actin-related protein is a component of a filament that resembles F-actin. *J. Cell Biol.* 126:403–412.

Schroer, T.A. 1994. New insights into the interaction of cytoplasmic dynein with the actin-related protein, Arp1. *J. Cell Biol.* 127:1–4.

Sheterline, P., and J.C. Sparrow. 1994. Actin. *Protein Profile.* 1:1–121.

Shortle, D., J.E. Haber, and D. Botstein. 1982. Lethal disruption of the yeast actin gene by integrative DNA transformation. *Science (Wash. DC).* 217:371–373.

Sugase, Y., M. Hirono, K.L. Kindle, and R. Kamiya. 1996. Cloning and characterization of actin-encoding gene of *Chlamydomonas reinhardtii. Gene (Amst.).* 168:117–121.

Tam, L.W., and P.A. Lefebvre. 1993. Cloning of flagellar genes in *Chlamydomonas reinhardtii* by DNA insertional mutagenesis. *Genetics.* 135:375–384.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Weeks, D.P., N. Beerman, and O.M. Griffith. 1986. A small-scale five-hour procedure for isolating multiple samples of CsCl-purified DNA: application to isolations from mammalian, insect, higher plant, algal, yeast, and bacterial sources. *Anal. Biochem.* 152:376–385.

Witman, G.B. 1986. Isolation of *Chlamydomonas* flagella and flagellar axonemes. *Methods Enzymol.* 134:280–290.