The Chlamydomonas FLA10 Gene Encodes a Novel Kinesin-homologous Protein
Zenta Walther, Malini Vashishtha, and John L. Hall
The Rockefeller University, New York 10021

Abstract. Many genes on the uni linkage group of Chlamydomonas affect the basal body/flagellar apparatus. Among these are five FLA genes, whose mutations cause temperature-sensitive defects in flagellar assembly. We present the molecular analysis of a gene which maps to flaI0 and functionally rescues the flaI0 phenotype. Nucleotide sequencing revealed that the gene encodes a kinesin-homologous protein, KHP1. The 87-kD predicted KHP1 protein, like kinesin heavy chain, has an amino-terminal motor domain, a central \(\alpha\)-helical stalk, and a basic, globular carboxy-terminal tail. Comparison to other kinesin superfamily members indicated striking similarity (64% identity in motor domains) to a mouse gene, KIF3, expressed primarily in cerebellum. In synchronized cultures, the KHP1 mRNA accumulated after cell division, as did flagellar dynein mRNAs. KHP1 mRNA levels also increased following deflagellation. Polyclonal antibodies detected KHP1 protein in Western blots of purified flagella and axonemes. The protein was partially released from axonemes with ATP treatment, but not with AMP-PNP. Western blot analysis of axonemes from various motility mutants suggested that KHP1 is not a component of radial spokes, dynein arms, or the central pair complex. The quantity of KHP1 protein in axonemes of the mutant flaI0-1 was markedly reduced, although no reduction was observed in two other uni linkage group mutants, fla9 and fla11. Furthermore, flaI0-1 was rescued by transformation with KHP1 genomic DNA. These results indicate that KHP1 is the gene product of FLA10 and suggest a novel role for this kinesin-related protein in flagellar assembly and maintenance.

In recent years, it has become clear that a wide variety of intracellular movements are accomplished by proteins of the kinesin superfamily. Kinesin, originally isolated from squid axoplasm (Vale et al., 1985), is a mechanochemical enzyme that uses the energy from ATP hydrolysis to transport vesicles and organelles along microtubules (Hirokawa et al., 1991; Urrutia et al., 1991; Wright et al., 1991; Gho et al., 1992; Schnapp et al., 1992). Its force-generating motor domain is well conserved among a class of related proteins which function in organelle transport (Hall and Hedgecock, 1991; Aizawa et al., 1992), as well as diverse processes such as mitotic spindle assembly and maintenance, spindle pole separation, nuclear fusion, and meiotic chromosome segregation (for reviews see Endow and Titus, 1992; Skoufias and Scholey, 1993; Walker and Sheetz, 1993). Kinesin is tetrameric, with two force-generating heavy chains and two light chains (Kuznetsov et al., 1988). Kinesin heavy chain (KHC) has a secondary structure analogous to that of myosin: an amino-terminal motor domain has both ATP- and microtubule-binding sites, an \(\alpha\)-helical rod/stalk region forms a coiled-coil with the other KHC in the tetramer, and a small globular carboxy-terminal tail interacts with light chains and the organelle being transported (reviewed in Goldstein, 1991). Numerous variations on this structure have been found among members of the superfamily (for reviews, see Endow and Titus, 1992; Skoufias and Scholey, 1993; Walker and Sheetz, 1993). Motor domains have been found to be carboxy-terminal in some kinesin-like proteins, and internal in others. The lengths of the rod domains vary, and in some cases, there is no \(\alpha\)-helical stalk at all. A novel heterotrimeric kinesin has recently been described (Cole et al., 1992). Clearly, there are high degrees of both structural and functional complexity among kinesin-related proteins, and the full range of diversity within the kinesin superfamily is doubtless yet to be uncovered.

Eukaryotic cilia and flagella are elaborate cellular appendages which display several forms of microtubule-based motility. Their whip-like motions are well known, and the dynein-mediated microtubule sliding which underlies flagellar beating has been extensively studied (see Witman, 1992). Recently, interest has grown in the movements of particles on the flagellar surface (reviewed in Bloodgood, 1992) as well as those observed between the flagellar membrane and...
the axoneme, the internal microtubule core of the flagellum (Kozminski, et al., 1993). In addition, the generation of flagella by aflagellate cells requires a dramatic rearrangement of the microtubule cytoskeleton, and this process itself may involve microtubule-directed motility. It is not known what role(s), if any, is played by kinesin-related proteins in the various movements contributing to flagellar assembly and function.

Much of what is known about flagella has been learned through the study of *Chlamydomonas*, a unicellular biflagellate green alga. The ease with which genetic manipulations can be performed in this organism, combined with simple procedures for experimental detachment of flagella for biochemical analysis, make *Chlamydomonas* an excellent model system for the study of flagellar motility (Luck, 1984). A variety of mutants with abnormal flagellar motion are available, and in many cases, corresponding morphological and biochemical defects have been determined. The recent application of molecular techniques to the study of these mutants has led to the identification of a number of genes encoding flagellar structural components. The goal of these combined approaches is to provide a detailed mechanistic description of flagellar beating. Much less is known about the process of flagellar morphogenesis. There are *Chlamydomonas* mutants which lack flagella altogether (either constitutively or conditionally), but none has been characterized in molecular detail. Among these are the fla (flagellar assembly) mutants, which exhibit temperature-sensitive defects in flagellar synthesis and maintenance (see Harris, 1989). Genetic analysis has indicated that at least five of these FLA genes map to the uni chromosome (or uni linkage group, ULG) as do many other genes affecting the basal body/flagellar apparatus (Ramanis and Luck, 1986). Our efforts have focused on the molecular analysis of such ULG genes. We describe here the cloning and characterization of a *Chlamydomonas* kinesin-related gene whose protein product, KHP1, is found in the flagellar axoneme. We show that KHP1 is encoded by *FLA10*, a ULG gene which is required for both flagellar assembly and stability.

**Materials and Methods**

**Cell Culture, Strains, and Mutants**

Vegetative cells were cultured in Sager-Granick medium supplemented with acetate (Harris, 1989), and gametes were grown as described previously (Luck et al., 1977). For synchronisation experiments, cells were grown in minimal Sager-Granick medium (Harris, 1989), bubbled continuously with 5% CO₂ in air, at 23°C, in alternating 14 h light/10 h darkness for at least 6 d (modified from Szurzycki, 1971).

Wildtype cells were of the standard laboratory strain 137c. Mutants *fla9* and *fla1* (as well as the *nlt-305*; wild double mutant strain, were obtained from the Chlamydomonas Genetics Center, Duke University (Durham, NC). *pf28* and *pf30* were kindly donated by Dr. Gianni Piperno (Mt. Sinai School of Medicine, New York). Mutant strains of *pf4* and *pf8* are from our laboratory stock collection and were originally obtained from J. R. Warr (University of York, Heslington, UK) and P. R. Levine (Washington University, St. Louis, MO), respectively. The *fla10* strain was isolated in this laboratory and was originally designated *da-a-224* (Huang et al., 1977). Other mutant alleles of this locus were obtained in later mutageneses (Adams et al., 1982); the one referred to in this paper as *fla10-519* was initially called *519*, and has been referred to elsewhere (Lux and Dutcher, 1991) as *fla10-14*. The *pf10* and *apm1* strains used in genetic crosses (Table I) were generated in this laboratory (Ramanis and Luck, 1986; and unpublished data).

Genetic tetrad analysis was carried out using standard methods (Harris, 1989). For random spore analysis, zygotes (pretreated with chloroform to kill unmated gametes) were removed from zygote plates (4% agar) and placed on germination plates (1.5% agar) for 5 days. Upon germination, a small amount of liquid medium was added to each plate, and spread such that the meiotic progeny were separated to form single colonies. Colonies were picked and propagated for phenotypic testing as well as RFLP analysis. Mating type segregation was checked and found to be independent of markers in the cross, confirming that meiotic progeny were being analyzed.

**Preparation of Chlamydomonas DNA and RNA**

DNA and RNA were prepared by similar methods. Cells grown on agar plates were resuspended in liquid media at a density of ~10⁷ cells/ml and allowed to grow flagella in bright illumination for at least 45 min. After sedimentation at 200 g, cell pellets were quickly resuspended at 50°C in prewarmed TEK (20 mM Tris pH 8.0, 40 mM EDTA, 2 mg/ml Proteinase K), and an equal volume of 10% SDS was immediately added, to give a final concentration of ~10⁷ (lysed) cells/ml. Samples were left at 50°C overnight for complete proteolysis. 1/10 vol of 3 M sodium acetate, pH 5.2, was then added, and the samples were extracted with 2:1 phenol-chloroform. For RNA isolation, this extraction was done by gentle inversion; RNA preparations were extracted vigorously. Nucleic acids were then precipitated at ~20°C with equal volumes of isopropanol. For DNA preparations, the DNA pellet was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0), digested with 10 μg/ml RNase for 30 min at 37°C, extracted again with 1:1 phenol-chloroform, and precipitated with ethanol. Final DNA pellets were allowed to dissolve in TE slowly, with gentle rotation at 4°C. For RNA preparations, pellets were resuspended in DEPC-treated water at 80°C. CsCl was added (1 g per 2.5-ml sample), and samples were layered over 6 M CsCl cushions for ultracentrifugation in a Sorvall SW55Ti rotor, 35,000 rpm, 20°C, 15-18 h. RNA pellets were resuspended in DEPC-treated H₂O and ethanol precipitated once more.

**Isolation of ULG-linked Genomic DNA Clones**

The ULG chromosome was partially purified by pulsed field gel electrophoresis (Hall et al., 1989), digested with *PstI*, and cloned into *PstI*-containing plasmids (Stratagene, Inc., La Jolla, CA). Selected clones were used to identify RFLPs between *C. reinhardtii* and *C. smithii* genomic DNA copies using Southern hybridization.
Southern blots. Linkage to ULG was then confirmed by molecular tetrad analysis: the segregation of these RFLPs was monitored in crosses between C. reinhardtii and C. smithii. These clones were obtained in our initial molecular analysis of the ULG (Hall, J. L., unpublished data). Briefly, intact Chlamydomonas chromosomes were partially resolved on agarose gels by pulsed field gel electrophoresis, and ULG-enriched fractions of DNA were recovered from these gels and cloned into pBluescript (see Materials and Methods). One clone, p36-420, was found to contain sequences that were closely linked to the ULG genetic marker fla10; in two interspecific crosses, tetrad analysis showed that the p36-420 RFLP and fla10-1 are within 1.4 cm of each other (Table I). Furthermore, in random spore analysis of another interspecific cross, the fla10 phenotype and the C. reinhardtii RFLP pattern were always found to cosegregate (n = 91), whereas all wildtype progeny analyzed had the C. smithii RFLP (n = 15). This implies a map distance of less than 0.5 cm between the p36-420 RFLP and fla10-1. According to rough estimates of physical map distances in Chlamydomonas, this corresponds to less than 50 kb (Ranum et al., 1988).

A Cell Cycle-regulated Gene on the ULG of Chlamydomonas

We used p36-420 to retrieve several overlapping genomic DNA fragments from a lambda phage library. A restriction map of one such clone, \( \lambda 36 \), is shown in figure 1 a. Although there are repetitive sequences at one end of this clone (left end as depicted in Fig. 1 a; data not shown), some regions identify unique bands on Southern blots (Fig. 1, b and c).

Northern blot hybridizations of unique DNA fragments from \( \lambda 36 \) identify a single polyadenylated mRNA of \( \sim 3.4 \) kb in vegetative and gametic cells (Fig. 2 a). Two features

Walther et al. FLA10 Encodes a Novel Kinesin-homologous Protein
of this RNA's regulation suggest a function in flagellar morphogenesis. First, we tested the response of mRNA levels to experimental deflagellation. *Chlamydomonas* cells, like many other organisms, shed their flagella when transiently exposed to various toxic conditions (extremes of pH, heat, alcohol, detergents, etc.; see Lewin et al., 1982). Flagellar regeneration follows rapidly, and is characterized by coordinate induction of many genes, several of which are known to encode flagellar components (Schloss et al., 1984). The λ136 mRNA is induced by deflagellation: there is an approximately twofold increase in mRNA level 30 min after flagellar amputation by pH shock (Fig. 2 a, left; quantitation determined by analyzing densitometric scans of autoradiograms using the NIH Image software). This increase is comparable to that observed in several other mRNA species which are up-regulated by deflagellation (see Schloss et al., 1984). By comparison, the 2.6-kb mRNA encoding the intermediate chain flagellar dynein IC70 is induced approximately three-fold in this experiment (Fig. 2 c, left) whereas histone H4 mRNA is uninduced (in Fig. 2 b, the band in the 30' lane is 1.2-fold the intensity of the band in lane 0).

Second, gene expression appears to be cell cycle regulated. We entrained cells, under a 24-h cycle of alternating light/dark, to undergo mitosis synchronously (see Materials and Methods). Cell division was monitored by hourly microscopic examination of fixed cells. Under these conditions, the peak of cell division was in the first hour of darkness: typically 50-60% of the cells had completed at least one round of division by the end of the first dark hour. On Northern analysis, we found that little of the λ136 mRNA was detected in the hours before and at the peak of cell division, but mRNA levels increased shortly thereafter (Fig. 2 a, right). The time of its expression is likely to represent the stage at which recently formed daughter cells synthesize new flagella. Flagellar dynein mRNAs exhibit a similar pattern of cell cycle regulation (Fig. 2 c; and Walther, Z., unpublished results with heavy chain dynein clone Dbl).

As a molecular marker of cell division in our synchronous cultures, we monitored the expression of *Chlamydomonas* histone H4 mRNA and found that it is also dramatically cell cycle regulated, but in a different pattern from the flagellar genes. Histone gene transcription and mRNA stability are tightly regulated in most organisms, such that histone gene expression is largely confined to S phase (reviewed in Maxson et al., 1983). In our synchronous cultures, H4 expression peaks at the light-to-dark transition, at a time when the number of cells entering mitosis, as monitored by light microscopy, is near maximal. This is consistent with the observation that in *Chlamydomonas*, cell division follows S phase immediately, without a G2 delay; in populations of light-synchronized cells, the period during which cells are undergoing DNA replication overlaps almost entirely with the time in which mitosis occurs (Coleman, 1982). Thus, H4 mRNA was a convenient marker of cell division for Northern analysis, and its expression pattern suggested that mitosis precedes the appearance of the λ136 mRNA.

Although we cannot exclude the possibility that the mRNA detected by λ136 sequences plays a role late in cell division, this seems unlikely. In the experiment shown in Fig. 2 a, by the time the λ136 mRNA levels had increased (lane D2), not only had H4 expression declined significantly, but 68% of cells had already divided (as monitored by light microscopy). The observed mRNA expression pattern best correlates not with mitosis, but with postmitotic flagellar morphogenesis.

cDNA Cloning and Sequencing of a Gene Encoding *KHPI*, a Kinesin-homologous Protein

We used genomic sequence information from λ136 to generate overlapping partial cDNA clones of the transcript it encodes (by RACE; see Materials and Methods). Sequencing revealed that this cDNA encodes a predicted protein of 86.7 kD (Fig. 3) in which the first 360 amino acids bear strong homology to the kinesin motor domain; we have named this gene product KHPI, for kinesin-homologous protein.

A striking structure feature of the KHPI gene is the large number of introns: there are at least 20 intervening sequences (see Fig. 3), ranging in size from 68 to 359 bp (mean size, 220 bp). To our knowledge, this represents the largest number of introns found in a *Chlamydomonas* gene to date.
All introns are flanked by highly conserved splice junction sequences which conform tightly to the previously described *Chlamydomonas* consensus: 16 of the 20 intron junctions match the consensus sequence at ≥10 out of 11 positions (Zimmer et al., 1988; Schloss and Croom, 1991). Interestingly, all 20 intervening sequences appear within the coding region. On average, an intron interrupts the coding sequence every 118 bp, whereas none is found in the 487 bp of 3' untranslated region. Despite the large number of introns, we find no evidence, either on Northern blots or by PCR analysis, of alternative splicing products of the KHP1 gene.

The cDNA sequence shown spans 2.9 kb (Fig. 3). Since the mRNA detected on Northern blots is ~3.4 kb, and the poly A tail is not likely to be more than a few hundred nucleotides in length, our cDNA clones probably do not encompass the entire 5' untranslated region of the transcript. It is likely, however, that the entire coding region is represented. In the 75 nt upstream of the putative initiator AUG, there are seven stop codons, and three of these occur in-frame (positions -6, -15, and -39, Fig. 3). Moreover, the strong homology to murine KIF3 (see below), beginning at residue 11 of KHP1 (residue 15 of KIF3), defines a motor domain of about the same size (360 aa) as that in KIF3 (352 aa) (Aizawa et al., 1992).

The predicted KHP1 protein has a three-domain structure similar to that of kinesin heavy chain. The amino-terminal 360 amino acids (aa) display homology to all kinesin-like motor domains; for example, this region is 45% identical to the motor domain of murine kinesin heavy chain (KIF5 in Fig. 4) (Aizawa et al., 1992).

**Figure 2.** Northern blot analysis. (A) A radiolabeled 700-bp genomic fragment encoding part of KHP1 (probe 1 in Fig. 1) detects a 3.4-kb mRNA in hybridizations to total RNA (30 μg/lane) and poly(A)⁺ RNA (10 μg). Bars at left show positions of molecular mass markers (9.5, 2.4, and 0.24 kb). The first two lanes contain gamete RNA before (0) and 30 min after (30) deflagellation. The six lanes on the right contain RNA from synchronized vegetative cells at intervals through a cell division cycle. Liquid cultures were synchronized by growth in minimal medium for at least six cycles of alternating 14 h light/10 h darkness; L12 is the end of the twelfth hour of illumination, 0 is the light-to-dark transition, D2 is the end of the second hour of darkness, etc. Under these conditions, cells begin to divide during L12, and mitosis peaks in the first hour of the dark (cells were monitored microscopically). KHP1 mRNA is most abundant after cell division, at D2 and D4. The bottom right panel shows ethidium bromide staining of the synchronized-cell RNAs before transfer (size range shown: 1-2.5 kb). (B) A genomic clone of the *Chlamydomonas* histone H4 gene (Walther, Z., and J. L. Hall, manuscript in preparation) was used to probe RNA samples from the same deflagellation and cell synchronization experiments as in A, (30 μg/lane). H4 mRNA is not significantly increased after deflagellation; in synchronized cell RNAs, strong hybridization to lane 0 indicates the peak of S phase (see text). (C) A genomic DNA clone containing the intermediate chain dynein gene IC70 (clone Dal from Williams et al., 1986) was radiolabeled and hybridized to RNAs from the same deflagellation experiment shown above (0, 30), and to another series of synchronized-cell RNAs (lanes L12-L4). This 2.6-kb dynein mRNA is regulated similarly to KHP1. Mitotic synchrony was the same in this experiment as in A, as determined by microscopy and Northern analysis with the H4 DNA probe (not shown). 30 μg of total RNA were loaded in each lane.
Figure 3. Nucleotide and predicted amino acid sequences of KHPI. Sequence shown was derived from several overlapping cDNA clones. Nucleotide residues are numbered from the first letter of the initiator methionine. An asterisk marks the TAG stop codon. The conserved *Chlamydomonas* poly(A) addition signal (TGTTA, underlined) precedes the poly(A) tail by 15 nucleotides in the clone containing the 3' end. Solid triangles show the positions of 20 introns found in the corresponding genomic sequence. The putative ATP-binding domain is marked by bold underline. Dashed underlining indicates the region with the heptad repeat characteristic of coiled-coil domains; repeat units are numbered by bold underline. The predicted amino acid sequence is shown, with putative ATP-binding domain marked by bold underline. The polyclonal antiserum to KHPI was raised against a fragment comprised of the protein sequence (aa 669-752) designated L33697.

The Journal of Cell Biology, Volume 126, 1994 180
site (see Fig. 3) and all peptide motifs characteristic of kinesin motor domains are present in the appropriate positions (SSRSHSIF, aa 214-221; LVDLAGSE, aa 256-263; HIPYRDSKLTR, aa 302-312). The middle section of KHP1 (aa 367-688) is predicted to have α-helical structure (not shown) (methods of Garnier et al., 1978; Novotny and Auffray, 1984). It is likely to form coiled-coil dimers by virtue of a heptapeptide repeat pattern in which the first and fourth positions of each heptad are enriched in hydrophobic residues; this type of pseudorepeat is characteristic of coiled-coil domains in a wide variety of proteins (reviewed in Cohen and Parry, 1986) (see Fig. 3). The carboxy-terminal 100 amino acids of KHP1 are likely to form a small, globular, basic domain (pl = 10.5), with no notable similarity to other proteins in the GenBank database. There are several sites of possible flexibility in the rod and tail domains of KHP1: the α-helical middle region is interrupted by two short glycine and proline-rich segments (aa 385-415 and aa 513-517) and a longer nonhelical segment near the tail domain (aa 620-664). In addition, a stretch of 10 consecutive glycine residues (aa 705-715) may add flexibility to the C-terminal tail. The rod domains of some other kinesins are similarly interrupted (Yang et al., 1989; Meluh and Rose, 1990).

We compared the protein sequence of KHP1 to that of other kinesin family members. KHP1 is most strikingly similar to the murine kinesin relative, KIF3: these proteins are 64% identical in the motor domain (see Fig. 4), and about 30% identical in the remaining residues. The region of strongest homology extends past the motor domain (defined by similarity to Drosophila kinesin heavy chain; Yang et al., 1989) for an additional 19 amino acids, apparently into the stalk region. Furthermore, there is a section in the rod domain of 38% identity between these two proteins (KHP1 residues 493-663). Although KHP1 and KIF3 are clearly closely related, they are not as similar to each other as are kinesin heavy chain genes from different organisms. Kinesin heavy chain homologues in different species share extensive sequence identity (motors, ~80%; stalks and tails, ~60%), and the identity encompasses all three domains, whereas KHP1 and KIF3 tail domains are quite divergent (15% identity). KHP1 is more distantly related to other kinesin family members. Pairwise comparisons between KHP1 and various other kinesin-like proteins reveal 30 to 45% identity in motor domains and less similarity elsewhere.

Codon usage in KHP1 is biased in the manner typical of Chlamydomonas nuclear genes. This codon usage bias exists in genes on the ULG as well as the other chromosomes (Schloss and Croom, 1991; and ULG histone genes, Walther, Z., and J. L. Hall, manuscript in preparation), and is distinct from the codon usage patterns found in chloroplast and mitochondrial genomes (Dronet al., 1982; Boer and Charbonnel, 1988). Different Chlamydomonas nuclear genes exhibit the characteristic codon usage bias to differing degrees. A good measure of the strength of the codon bias in a particular gene is the percentage of codons ending in A: in the strongly biased tubulin genes, only 0.1% of codons have A in the third position (Youngblom et al., 1984; Silflow et al., 1985), whereas in genes with the weakest codon preferences, this number is 8-9% (deHostos et al., 1989; Schloss and Croom, 1991). KHP1, by comparison, has 2.8% codons with A in the third position, indicating moderately strong codon usage preferences.

**KHP1 Protein Is Found in Flagella**

Polyclonal rabbit antibodies were raised against two fusion proteins containing nonoverlapping carboxy-terminal fragments of KHP1. Two rabbits were immunized with each fusion protein. All four sera recognized a common band of...
The Journal of Cell Biology, Volume 126, 1994

Figure 5. Identification of KHPI protein by Western analysis. (A) Affinity-purified antisera were incubated with Western blots containing Chlamydomonas total protein (TP, ~10^9 cells/lane were lysed in protein sample buffer and loaded directly onto the gel), flagella (Fl, ~15 µg/lane), and axonemes (Ax, ~15 µg/lane). Molecular mass markers are indicated at left; the calculated molecular mass of the immunoreactive band is 91 kD. Drosophila kinesin heavy chain protein is predicted to be 110 kD and its apparent molecular mass on electrophoresis is 120 kD (Yang et al., 1989); a number of other proteins with long α-helical domains also appear larger on SDS-PAGE than their deduced protein sequences predict (e.g., yeast tropomyosin; Liu and Bretscher, 1989).

In order to determine whether the 91-kD protein is a flagellar component, we analyzed proteins of isolated flagella and axonemes. These preparations were obtained after pH shock deflagellation by a series of differential centrifugations (see Materials and Methods). They have been shown, by electron microscopy and iodination studies, to be highly purified, with virtually no contaminating cell body material (Piperno et al., 1977). The 91-kD protein was found in purified flagella by Western analysis (Fig. 5a, lane 2). As expected for a microtubule-binding protein, it remained associated with the microtubule-based flagellar core, the axoneme, upon removal of the flagellar membrane with a non-ionic detergent (Fig. 5a, lane 3). Furthermore, in Western analysis of equal amounts of protein from isolated axonemes and from deflagellated cell bodies, the 91-kD protein, though detectable in both fractions, showed clear enrichment in the axoneme fraction (data not shown).

When flagella and axonemes were analyzed in adjacent lanes, we observed a small but reproducible difference in the mobility of the immunoreactive protein. The slight increase of its mobility in axonemes could result from the enzymatic removal of a covalent modification of the protein (e.g., dephosphorylation, deglycosylation, etc.), upon disruption of the flagellar membrane with NP-40. It is interesting to note that KHC is known to be phosphorylated in vivo (Hollenbeck, 1993). These observations will require further investigation.

A distinguishing characteristic of kinesins is that their microtubule binding is nucleotide dependent: they bind microtubules when ATP has been depleted or when in the presence of nonhydrolyzable ATP analogues, and they are released from microtubules upon binding ATP (Vale et al., 1985). Chlamydomonas axonemes have high ATPase activity (Piperno and Luck, 1979), and ATP is quickly depleted in flagella during isolation. We treated axonemes with exogenous ATP and found that some of the 91-kD protein was liberated and could be recovered from the supernatant (Fig. 5b). Similar treatment with the nonhydrolyzable ATP analogue AMP-PNP failed to release the protein from axonemes. Thus, the 91-kD axonemal species responds to nucleotides in the manner expected of a kinesin-related protein. We have not optimized this procedure for maximum ATP-dependent release of the 91-kD species from axonemes.

The depletion of ATP by dynein and other axonemal ATPases, as well as the high concentration of microtubules to which proteins can rebind after ATP hydrolysis, may account for the limited degree to which the 91-kD species was recovered by treatment with ATP.

KHPI in Mutants with Axonemal Defects

To determine the axonemal location of KHPI, we performed Western analysis of several mutants (Fig. 6). Many Chlamydomonas mutants which display abnormal motility have flagella that lack certain axonemal substructures. Flagella of pfl4 and pf28 are completely deficient in radial spokes and outer dynein arms, respectively; this has been determined by both electron microscopy and biochemical studies (Witman et al., 1978; Piperno et al., 1981; Mitchell and Rosenbaum, 1985). In these mutants, a single gene mutation leads to the failure of a multimolecular complex to be assembled into the axoneme. Consequently, the axonemes of these mutants each lack a particular set of polypeptides on two-dimensional SDS-protein gels. The clear presence of the 91-kD species in axonemes isolated from either pfl4 or pf28 suggests that KHPI is not a component of radial spokes or outer dynein arms. pf30 has a more subtle defect, in which only one of...
three different types of inner dynein arm complex, II, is missing (Piperno et al., 1990). Since KHP1 is abundant in pfl30 axonemes, it is not likely to be a component of II; there is no known mutant lacking all inner dynein arm structures.

In flagella of the pfl8 mutant, the central pair microtubule complex is reduced to an amorphous, dense material (visible by EM) (Adams et al., 1981), which is completely lost during the preparation of axonemes. Therefore, pfl8 axonemes are analogous to those of pfl4 and pfl28 in that they are totally lacking a specific set of 18 microtubule-associated proteins; they also have less tubulin than wildtype axonemes, due to the absence of the central pair. Western analysis of pfl8 axonemes (Fig. 6) reveals the presence of the 91-kD species, indicating that KHP1 is not among those 18 central pair-associated proteins described. However, although approximately equal amounts of wildtype and pfl8 axonemes were analyzed in Fig. 6, the amount of KHP1 protein detected in pfl8 seems somewhat reduced; this raises the possibility that some of the protein might normally be associated with the central pair microtubules. In this technique, protein quantitation is not precise enough to allow conclusive interpretation of such subtle differences in signal intensity.

The fla10 Mutant Is Deficient in KHP1 Protein

fla10 is a temperature-sensitive mutant defective in flagellar assembly: at restrictive temperature (32°C), cells slowly resorb their flagella (complete in 4–12 h), and nonflagellated cells do not assemble flagella; in addition, experimentally deflagellated cells are unable to regenerate flagella at 32°C (Huang et al., 1977; Adams et al., 1982). We prepared axonemes from two fla10 mutants that had been shifted to restrictive temperature for one hour. This incubation was not long enough to cause significant flagellar loss, but was intended to reveal any molecular defect that might precede flagellar resorption at restrictive temperature. As seen in Fig. 7a, fla10-1, but not its allele fla10-519, has significantly reduced levels of KHP1 protein in axonemes; a faint band of KHP1 reactivity is seen in overdeveloped blots (not shown). KHP1 is also reduced in axonemes of fla10-1 grown at permissive temperature (Fig. 7b). This reduction is observed in Western blots of fla10-1 total cell protein (after 1 hour at 32°C; data not shown), indicating that KHP1 protein is indeed deficient in fla10-1, and is not merely redistributed from flagella to the cell body.

Although fla10-1 and fla10-519 are allelic (Table I; Lux and Dutcher, 1991), they are unlikely to contain exactly the same molecular mutation. They were generated with different mutagens and isolated in separate screens (see Materials and Methods); moreover, their phenotypes are subtly different. fla10-1 cells lose flagella more quickly at restrictive temperature than fla10-519. In one experiment, after 9 h at 32°C, 100% of fla10-1 cells were aflagellate, whereas only 60% of fla10-519 cells had lost their flagella. This phenotypic difference has also been documented by others (Lux and Dutcher, 1991).

In order to investigate whether selective loss of KHP1 is a common phenomenon in the flagellar assembly mutants of *Chlamydomonas*, we examined the axonemes of two such mutants, fla9 and fla11 (Fig. 8). Both of these mutants, like fla10, have been mapped to the ULG (Ramanis and Luck, 1986). In both mutants, flagella are gradually resorbed at 32°C (complete in 12–24 h). Both mutants are also unable to synthesize flagella at restrictive temperature after deflagellation; in addition, fla11 has a slight defect in the kinetics of flagellar regeneration at permissive temperature (Ramanis and Luck, 1986). The axonemes of both these mutants were found to contain wildtype levels of KHP1 after

![Figure 6. Western analysis of mutant axonemes. (A) Approximately equal amounts of axonemal protein from wildtype (wt) and various mutant strains were examined (~20 μg/lane). pfl8 axonemes lack the central pair microtubules; pfl28 is missing the outer dynein arms; pfl30 is deficient in inner dynein arm II; pfl4 lacks radial spokes. (B) Diagram of axoneme in cross-section. Major structures are labeled, and mutants in which these structures are absent are indicated.](image-url)
fla/0 phenotype: they had flagella and were swimming nor-
KHP1 Genomic DNA
Rescue of the fla/0 Phenotype by Transformation with
transformants with restored motility at 32°C. Since M36II-2
really after an overnight incubation at 32°C. Similar cotrans-
duce reversion of the fla/0 phenotype at detectable rates in
our recipient strain with pMN24, a plasmid containing the
reductase structural gene (Fernandez and Matagne, 1984).
ent strain for transformation. The
mants which were able to utilize nitrate
5' end of the KHP1 and the proximal end of M36II-2 shows
extends to within 1.6 kb of the 5' end of the KHP1 and yet
no detectable transcript when used as a probe on Northern
blots of poly (A)÷ RNA (data not shown). Therefore, these
results strongly argue that it is the presence of the KHP1 tran-
1 h at 32°C, indicating that early loss of this protein is not
a shared feature of the temperature-sensitive fla phenotype.

Rescue of the fla/0 Phenotype by Transformation with
KHP1 Genomic DNA
The genetic proximity of $\lambda$36 sequences to the $FLA10$ locus
made KHP1 a candidate gene product for $FLA10$. To demon-
strate the identity of the KHP1 gene and $FLA10$ directly, we
showed that the mutant phenotype of $fla10$ cells is rescued by transformation with KHP1 genomic DNA.
We constructed a $fla10$-1, nit-305, cw15 triple mutant recipient
strain for transformation. The $cwl5$ mutation causes a cell wall deficiency, and $nit-305$ is a mutation in the nitrate
reductase structural gene (Fernandez and Matagne, 1984).
Cells without nitrate reductase activity are unable to grow when nitrate is the sole nitrogen source. Transformation of our recipient strain with pMN24, a plasmid containing the
$NIT1$ gene (Fernandez et al., 1989), yielded many transfor-
mants which were used for transformation. The complete KHP1 transcription unit (black arrows) is present in clones $\lambda$36 and $\lambda$36I-1. Insert sizes are: $\lambda$36 and $\lambda$36I-1, 14.3 kb; $\lambda$36II-2, 13.5 kb. (B) $fla10$-1, nit-305, cw15 triple mutant cells were transformed with the nitrate reductase gene (pMN24), either alone or in combination with DNA from one of the lambda clones shown above. Cells were plated on nitrate-containing medium, and after several days the number of colonies was recorded (nit÷). These colonies were picked and tested for the $fla10$ phenotype: after $\geq$12 h at 32°C, liq-
uid cultures were examined for flagellated, swimming cells. The number of nit+ transformants which are motile at 32°C is indicated (swim @ 32°C).

Discussion
This paper describes the cloning and characterization of a
kinesin-related gene in $Chlamydomonas reinhardtii$. We
have identified the gene product, KHP1, in flagella of wild-
type cells, and we have investigated its localization within the
flagellum using mutants which lack certain axonemal sub-
structures. Furthermore, we show that KHP1 is the product of the $FLA10$ gene, which is required for both flagellar assembly and flagellar maintenance.
Homology of KHPI to Kinesins

Sequence analysis of KHPI revealed that the protein has a three-domain structure analogous to that of kinesin heavy chains. There is an NH₂-terminal globular motor domain of 360 aa, a middle α-helical rod domain of ~330 aa, and a 100-residue globular COOH-terminal tail. The motor domain has the conserved ATP-binding site and characteristic peptide motifs found in all kinesins. Moreover, this region is 45% identical to murine KHC (KIF5; Aizawa et al., 1992) at the amino acid level. The middle rod/stalk region has strong α-helical character and heptapeptide repeats characteristic of proteins which form coiled-coil dimers. The small tail domain is basic (pI = 10.5). All of these features are shared by kinesin heavy chains and suggest that KHPI exists as a dimer; whether KHPI associates with light chains through its tail domain remains to be determined.

KHPI has particularly strong sequence homology with a recently discovered member of the kinesin superfamily, murine KIF3, which was isolated in a PCR screen for kinesin-related transcripts in murine CNS (Aizawa et al., 1992). KHPI and KIF3 are 64% identical in their motor domains, and 38% identical overall of the stalk; the tail domains are not well conserved (15% identity). This high degree of sequence similarity between the NH₂-termini is striking; however, the functional implications of the COOH-terminal divergence are unclear. Kinesin heavy chains from different organisms generally show even higher degrees of conservation, and the similarity extends to all three domains (Navone et al., 1992). On the other hand, it has been shown that some kinesin-related proteins with divergent tail domains can nevertheless functionally substitute for one another (O'Connell et al., 1993).

The extent of similarity between KHPI and KIF3 is reminiscent of that between members of the bimC/cul7/Eg5 subfamily of kinesin-like proteins (Hoyt et al., 1992; Roof et al., 1992; Heck et al., 1993). Proteins of the latter group are more similar to each other than any one of them is to kinesin heavy chain; in addition, they are all likely to perform functions in mitosis. The expression of murine KIF3 is restricted to adult cerebellum, where the protein is presumed to act as a transporter in neurites. As will be argued below, the Chlamydomonas KHPI protein is likely to act as a transporter in flagella. As more members of the kinesin superfamily are characterized and compared to these genes, it will perhaps become clear whether KHPI and KIF3 define a distinct subgroup of structurally and functionally related proteins.

KHPI Protein in Flagella

The expression pattern of KHPI mRNA is consistent with a proposed role in flagellar morphogenesis. In the Chlamydomonas cell cycle, flagella are resorbed prior to mitosis and resynthesized by each new daughter cell upon completion of cell division. In synchronized cultures, we found that KHPI mRNA was expressed after mitosis, at the time of flagellar outgrowth; flagellar dynein mRNAs exhibited a similar cell cycle regulation. Another indication of likely flagellar function was the response of the KHPI mRNA to experimental deflagellation. The doubling of KHPI mRNA level at 30 min after deflagellation suggested that its gene product plays a role in flagellar synthesis.

Polyclonal antibodies recognizing the COOH-terminal tail of KHPI identified the KHPI protein in whole cells and in highly purified flagellar fractions on Western blots. Upon removal of the flagellar membrane/matrix fraction with a nonionic detergent, KHPI protein remained associated with axonemes, the microtubule-based structures which generate flagellar beating.

There are several different functions which might be performed by kinesin-like motor proteins in axonemes. A different class of mechanochemical enzymes, dyneins, are known to generate the sliding of axonemal outer doublet microtubules against each other which gives rise to flagellar bending (Summers and Gibbons, 1971). However, much remains to be learned about the molecular nature of the complex regulatory mechanisms which must operate to convert this sliding motion into a coordinated flagellar beat (for review see Witman, 1992). Kinesin-related proteins might contribute to this modulation of dynein activity. Interestingly, the central pair microtubules are thought to rotate as a unit within the cylinder of outer doublet microtubules, a phenomenon for which the motive force is unknown (Omoto and Witman, 1981). In addition to axonemal bending, several other forms of motility have been observed in flagella. Directed movements of glycoproteins in the flagellar membrane give rise to gliding motility along solid substrates as well as aggregation of specialized agglutinin proteins at the tips of flagella during the mating reaction (reviewed in Bloodgood, 1992). Fundamental to all these forms of motility in Chlamydomonas is the ability to properly assemble and maintain
flagella. There might be a role played by kinesin-like proteins in the directed transport of axonemal components to their incorporation sites during flagellar assembly.

We began investigating the function of KHP1 by examining the axonemes of various motility-defective mutants. We were particularly interested in those mutants whose axonemes lack a particular structure morphologically, and show the corresponding absence of a specific set of polypeptides on molecular analysis. Such mutants have been used to identify the components of multimolecular structures such as inner and outer dynein arms, radial spokes, and the central pair microtubule complex. Since KHP1 was detected in the axonemes of mutants lacking each of these structures, we concluded that the KHP1 protein is not one of the ~50 polypeptides deficient in these mutants, and is probably not a component of dynein arms, radial spokes, or the central pair complex (we were unable to examine all inner arms and therefore cannot draw conclusions about complexes I2 and I3). We note that this analysis would not identify the location of KHP1 if it were present in more than one axonemal substructure. Furthermore, this analysis leaves open the possibility that KHP1 is associated with outer doublet microtubules on the external surface of the axoneme, since all flagellar mutants examined retain these outer doublets. Interpretation of the apparent slight reduction of KHP1 in pfl8 axonemes (central pair-less) awaits further investigation by a more precise quantitative technique.

**KHP1 and Flagellar Assembly**

*fla* mutants display normal motility but have temperature-sensitive defects in the maintenance and/or regeneration of flagella: at restrictive temperature, the flagella of swimming cells are slowly resorbed, and cells that have been experimentally deflagellated are unable to assemble new flagella (Huang et al., 1977; Adams et al., 1982). We examined two allelic *fla* mutants with subtly different phenotypes, *fla10-1* has the more severe phenotype: flagellar resorption at restrictive temperature occurs more quickly, by several hours, than in *fla10-519* (this paper and Lux and Dutcher, 1991). Interestingly, we found a marked deficiency of KHP1 protein in axonemes of the *fla10-1* mutant, whereas *fla10-519* axonemes appeared normal with respect to KHP1 protein level. Furthermore, there was no KHP1 deficiency in two other mutants, *fla9* and *fla11*, with phenotypes similar to *fla10*. Thus, reduction in KHP1 protein is not an indirect result of the flagellar resorption phenotype at restrictive temperature; in fact, we observed the KHP1 deficiency in *fla10-1* grown at both restrictive and permissive temperatures.

RFLP mapping of the KHP1 gene indicated that it lies within 0.1 cM of the *FLA10* locus. To test whether KHP1 and *FLA10* might in fact be the same gene, we transformed *fla10-1* cells with genomic DNA from the KHP1 locus. Two genomic clones encompassing the KHP1 gene were able to rescue the *fla10-1* phenotype, whereas an overlapping clone devoid of KHP1 sequences failed to rescue. Additional copies of KHP1 DNA were detected in the genomes of "rescued" transformants, and these additional copies segregated with the rescued phenotype in subsequent crosses. These experiments demonstrated the identity of the KHP1 gene and *FLA10*.

The flagellar assembly process in which *fla10* is defective is an ordered reaction. It has been shown that tubulin is incorporated into outer doublet and central pair microtubules only at the distal tips of growing flagella; radial spoke proteins, when supplied to spokeless mutant flagella, also assemble into the axonemal tips first (Johnson and Rosenbaum, 1992). The authors of these studies have suggested that there is an active transport process, perhaps involving molecular motors, that brings tubulin and other axonemal components to the tips of elongating flagella for assembly. KHP1, a flagellar kinesin-like protein, may serve just this function. In this model, *fla10* mutants, defective in KHP1 protein, are unable to assemble flagella because they cannot transport some essential axonemal component(s) to the assembly site. Since axonemal microtubules are polar, with minus-ends located at the basal bodies and plus-ends pointed distally, this is consistent with the idea that KHP1, like most kinesins, is a plus-directed motor.

The reason for this resorption of already-assembled flagella of *fla10* (at elevated temperature) is less clear. Protein turnover in flagella may require that some essential component(s) be continually replaced. There is already considerable evidence that a transport mechanism continues to operate within mature flagella after assembly is complete. This mechanism is revealed by dikaryon rescue experiments (Luck et al., 1977; Huang et al., 1981; Dutcher et al., 1984). When mutant cells with flagella that are missing certain sets of axonemal components are fused to wildtype cells, wildtype components can be transported and assembled into the mutant axonemes such that dikaryon cells display four functionally wildtype flagella. Thus, intraflagellar transport may be a constitutive function in *Chlamydomonas*. Perhaps related to this transport are the bidirectional movements of granules beneath the flagellar membrane that have been observed in living cells by differential interference-contrast microscopy (Kozmynski et al., 1993).

One cannot attribute the temperature-sensitive flagellar loss phenotype of *fla10-1* exclusively to the reduction in KHP1 protein levels: the reduction is observed at all temperatures, but flagellar resorption occurs only at restrictive temperature. However, several models can be envisioned. A simple explanation would be that in *fla10-1*, the mutant KHP1 protein is somewhat unstable at all temperatures, and that this instability is accentuated to some critical degree at elevated temperature such that the transport function is abolished. In *fla10-519*, there is likely to be a different KHP1 mutation, one in which the function of the KHP1 mechanoenzyme is impaired at 32°C, but in which protein stability is unaffected. It may be that *fla10-1* cells lose their flagella at restrictive temperature more quickly than *fla10-519* cells because they have less KHP1 to begin with, and therefore the functional loss is felt earlier.

It remains to be determined what cargo is carried by KHP1. Few clues are offered by the protein sequence of its tail domain, since no compelling homologies were detected through GenBank database searches. It is unlikely that the tail domain binds membraneous vesicles, because vesicles are not observed in electron micrographs of flagella (Ringo, 1967). However, nonvesicular complexes have been observed between the outer doublet microtubules and the flagellar membrane in longitudinal flagellar thin sections (Kozmynski et al., 1993). It will be interesting to determine whether the KHP1 protein is associated with these structures.
Other Possible Functions of KHPl

Given the extensive evidence for involvement of kinesin-like proteins in mitosis in other organisms, it is important to consider the possibility that KHPl plays a role in cell division. Nothing in our present study points to such a function for KHPl: the mRNA is not induced during cell division, and the closest relative to KHPl, murine KIF3, is expressed predominantly in adult mouse cerebellum, a mitotically quiescent tissue. However, nothing in our present study precludes this possibility, either. Although KHPl mRNA is not induced at the onset of mitosis, we have no information about the longevity of the KHPl protein. Since flagella are resorbed before cell division begins, some flagellar proteins could conceivably be returned to the cell body to play different roles in mitosis.

No mitotic deficits have been demonstrated in fla10, the KHPl mutant. However, the lack of a cell division phenotype in fla10 could be explained by functional redundancy of the mitotic machinery (reviewed in Goldstein, 1993). An analysis of genetic interactions involving FLA10 has led some investigators to postulate that the fla10 gene product plays a dual role in flagellar assembly and cell division (Lux and Dutcher, 1991). Synthetic phenotypes affecting mitosis have been observed in double mutants containing certain combinations of fla10 and apml alleles (apml is a ULG-linked mutation conferring resistance to several β-tubulin-binding herbicides; James et al., 1988). Whether this implies that both wildtype gene products normally function in mitosis is unclear. Synthetic phenotypes in cell division could conceivably be explained by functional redundancy of the central pair microtubules of the flagella of Chlamydomonas reinhardtii (Chlorophyceae).

Note Added in Proof

We thank David Luck for support and helpful discussion. We are grateful to Gianni Piperno for generous advice regarding axoneme preparation and analysis. We also thank Greta Segi for her excellent technical assistance with genetic crosses and transformation rescue experiments. This work was supported by National Institutes of Health grant GM17152.

Received for publication 9 December 1993 and in revised form 20 April 1994.

References

Adams, G. M. W., B. Huang, G. Piperno, and D. J. L. Luck. 1981. Central-pair microtubular complex of Chlamydomonas: polypeptide composition as revealed by analysis of mutants. J. Cell Biol. 91:59-76.

Adams, G. M. W., B. Huang, and D. J. L. Luck. 1982. Temperature-sensitive, assembly-defective flagella mutants of Chlamydomonas reinhardtii. Genetics. 100:379-586.

Alzawa, H., Y. Sekine, R. Takanura, Z. Zhang, M. Nangaku, and N. Hirokawa. 1992. Kinesin family in marine central nervous system. J. Cell Biol. 119:1287-1296.

Bloodgood, R. A. 1992. Directed movements of ciliary and flagellar membrane components: a review. Bioessays. 14:153-160.

Boer, P. H., and M. W. Gray. 1988. Transfer RNA genes and the genetic code in Chlamydomonas reinhardtii. Biochemistry. 37:583-590.

Cole, D. G., W. Z. Cande, R. J. Baskin, D. A. Skoufies, C. J. Hogan, and J. M. Scholey. 1992. Isolation of a sea urchin egg kinesin-related protein using peptide antibodies. J. Cell Sci. 101:291-301.

Coleman, A. W. 1982. The mitotic cell cycle in Chlamydomonas (Chlorophyceae). J. Physiol. 198:192-195.

delHostos, E. L., J. Schilling, and A. R. Grossman. 1989. Structure and expression of the gene encoding the periplasmic arylsulfatase of Chlamydomonas reinhardtii. Mol. Gen. Genet. 218:229-239.

Dron, M., M. RHaire, and J.-D. Rochaix. 1992. Sequence of the chloroplast DNA region of Chlamydomonas reinhardtii containing the gene of the large subunit of ribulose bisphosphate carboxylase and parts of its flanking genes. J. Mol. Biol. 162:775-793.

Flame, S. K., B. Huang, and D. J. L. Luck. 1984. Genetic dissection of the central pair microtubules of the flagella of Chlamydomonas reinhardtii. J. Cell Biol. 98:229-236.

Fischetti, F. A., G. M. Landau, P. H. Sellers, and J. P. Schmidt. 1993. Identifying periodic occurrences of a template with applications to protein structure. Bioinformatics. 45:11-18.

Fox, L. A., Sawin, K. E., and W. S. Sale. 1994. Kinesin-related proteins in Chlamydomonas: biochemistry, flagellar transport, and function. J. Cell Sci. 109:189-202.

Gamier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy of DNA replication in Chlamydomonas. J. Mol. Biol. 120:97-120.

Goto, M., K. McDonald, B. Giaume, and W. M. Saxton. 1992. Effects of kinesin mutations on neuronal functions. Science (Wash. DC). 258:313-316.

Goldstein, L. S. B. 1991. The kinesin superfamily: tails of functional redundancy. Trends Cell Biol. 1:93-98.

Goldstein, L. S. B. 1993. Functional redundancy in mitotic force generation. J. Cell Biol. 120:1-3.

Hall, D. H., and E. M. Hedgecock. 1991. Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in C. elegans. Cell. 65:837-847.

Hall, J. L., Z. Ramanis, and D. L. Luck. 1989. Basal body/cenrole and function in Chlamydomonas. J. Cell Biol. 120:1-3.

Harris, E. H. 1989. The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Harcourt Brace Jovanovich, San Diego, CA.

Hirokawa, N., K. A. Johnson, P. Forscher, and J. L. Rosenbaum. 1993. Kinesin family in murine central nervous system. J. Cell Biol. 162:775-793.

Hirokawa, N., R. Sato-Yoshitake, N. Kobayashi, K. K. Pfister, G. S. Bloom, and S. T. Brady. 1991. Kinesin associates with anterogradely transported membranous organelles in vivo. J. Cell Biol. 119:295-302.

Hollenbeck, P. J. 1993. Phosphorylation of neuronal kinesin heavy and light chains in vivo. J. Neurochem. 60:2265-2275.

Hoyt, M. A., L. He, K. K. Loo, and W. S. Saunders. 1992. Two Saccharomyces cerevisae kinesin-related gene products required for mitotic spindle assembly. J. Cell Biol. 119:1059-1071.

Huang, B., G. Piperno, Z. Ramanis, and D. J. L. Luck. 1981. Radiolabeled spokes of Chlamydomonas flagella: genetic analysis of assembly and function. J. Cell Biol. 88:80-88.

Huang, B., M. R. Rinkin, and D. J. L. Luck. 1977. Temperature-sensitive mutations affecting flagellar assembly and function in Chlamydomonas reinhardtii. J. Cell Biol. 72:67-85.

Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White, editors. 1990. PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc.

Johnson, K. A., and J. L. Rosenbaum. 1992. Polarized flagellar assembly in Chlamydomonas. J. Cell Biol. 119:1605-1611.

Johnson, K. A., and J. L. Rosenbaum. 1993. High-frequency nuclear transformation of Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA. 87:1228-1232.

Kozlowski, K. G., K. A. Johnson, P. Forscher, and J. L. Rosenbaum. 1993.

Walther et al. FLA10 Encodes a Novel Kinesin-homologous Protein
A motility in the eukaryotic flagellum unrelated to flagellar beating. Proc. Natl. Acad. Sci. USA. 90:5519–5523.

Kuznetsov, S. A., E. A. Vaisberg, N. A. Shania, N. M. Magretova, V. Y. Chernyak, and V. I. Gelfand. 1988. The quaternary structure of bovine brain kinetin. EMBO (Eur. Mol. Biol. Organ.) J. 7:353–356.

Lewin, B. A., T. Lee, and L. Fang. 1987. Effects of various agents on flagellar activity, flagellar autonomy, and cell viability in four species of Chlamydomonas. In Prokaryotic and Eukaryotic Flagella. Society for Experimental Biology Symposium No. 55. W. B. Amos, and J. G. Duckett, editors. Cambridge University Press, London. 421–437.

Liu, H. and A. Bretscher. 1989. Disruption of the single tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. Cell. 57:233–242.

Luck, D. J. L. 1984. Genetic and biochemical dissection of the eukaryotic flagellum. J. Cell Biol. 87:789–794.

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

Liu, H. and A. Bretscher. 1989. Disruption of the single tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. Cell. 57:233–242.

Lux, F. G., and S. K. Dutcher. 1991. Genetic interactions at the mitotic spindle. J. Cell Biol. 117:239–277.

Melul, P. B., and M. D. Rose. 1990. KAR3, a kinetin-related gene required for yeast nuclear fusion. Cell. 60:1029–1041.

Mitchell, D. R., and J. L. Rosenbaum. 1985. A motile Chlamydomonas flagellar mutant that lacks outer dynein arms. J. Cell Biol. 100:1228–1234.

Myers, E. W., and W. Miller. 1988. Optimal alignments in linear space. CABIOS 4:11–17.

Navone, F., J. Niclas, N. Hom-Booher, L. Sparks, H. D. Bernstein, G. McCaffrey, and R. D. Vale. 1992. Cloning and expression of a human kinesin heavy chain: interaction of the COOH-terminal domain with cytoplasmic microtubules in transfected CV-1 cells. J. Cell Biol. 117:1263–1275.

Novotny, J., and C. Auffray. 1984. A program for prediction of protein secondary structure from nucleotide sequence data: application to histocompatibility antigens. Ann. Rev. Genet. 17:239–277.

Novotny, J., and C. Auffray. 1984. A program for prediction of protein secondary structure from nucleotide sequence data: application to histocompatibility antigens. Nucl. Acids Res. 12:243–255.

O’Connell, M. J., P. B. Meluh, M. D. Rose, and N. R. Morris. 1993. Suppression of the bimCa mitotic spindle defect by deletion of klpA, a gene encoding a KAR3-related kinetin-like protein in Aspergillus nidulans. J. Cell Biol. 120:153–162.

Omonto, C. K., and G. B. Witman. 1981. Functionally significant central-pair rotation in a primitive eukaryotic flagellum. Nature (Lond.) 290:708–710.

Perkins, D. D. 1949. Biochemical mutants in the smut fungus Ustilago maydis. Genetics. 34:607–626.

Piperuo, G., and Z. Ramanis. 1991. The proximal portion of Chlamydomonas flagella contains a distinct set of inner dynein arms. J. Cell Biol. 112:701–709.

Piperuo, 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.

Ringo, D. L. 1967. Flagellar motion and fine structure of the flagellar apparatus in Chlamydomonas. J. Cell Biol. 33:543–571.

Roof, D. M., P. B. Meluh, and M. D. Rose. 1992. Kinesin-related proteins required for assembly of the mitotic spindle. J. Cell Biol. 118:95–101.

Rosen, A., K. F. Koonen, M. Thelan, A. C. Nairn, and A. A. Aderem. 1990. Activation of protein kinase C results in the displacement of its myristoylated, alanine-rich substrate from punctate structures in macrophage fibroblasts. J. Exp. Med. 172:1211–1215.

Schloss, J. A., and H. B. Croom. 1991. Normal Chlamydomonas nuclear gene structure on linkage group XIX. J. Cell Sci. 100:877–881.

Schloss, J. A., C. D. Sillifo, and J. L. Rosenbaum. 1984. mRNA abundance changes during flagellar regeneration in Chlamydomonas reinhardtii. Mol. Cell. Biol. 4:424–434.

Seelow, J. K., and A. Hollaender, editors. 1982. Genetic Engineering: Principles and Methods. Vol. 4. Plenum Press, New York. 98–99.

Sillifo, C. D., R. L. Chisholm, T. W. Conner, and L. P. W. Ramun. 1985. The two a-tubulin genes of Chlamydomonas reinhardtii code for slightly different proteins. Mol. Cell. Biol. 5:2389–2398.

Skoufas, D. A., and J. M. Scholey. 1993. Cytoplasmic microtubule-based motor proteins. Curr. Opin. Cell Biol. 5:95–104.

Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusion proteins with glutathione S-transferase. Gene (Amst.). 67:31–40.

Sumners, K. E., and I. R. Gibbons. 1971. Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea urchin sperm. Proc. Natl. Acad. Sci. USA. 68:3092–3096.

Surzycki, S. 1971. Synchronously grown cultures of Chlamydomonas reinhardtii. Methods Enzymol. 23:67–73.

Urrutia, R., M. A. McNiven, J. P. Albanese, D. B. Murphy, and B. Kachar. 1991. Purified kinesin promotes vesicle motility and induces active sliding between microtubules in vitro. Proc. Natl. Acad. Sci. USA. 88:6701–6705.

Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. Cell. 42:39–50.

Walker, R. A., and M. P. Sheetz. 1993. Cytoplasmic microtubule-associated motors. Annu. Rev. Biochem. 62:429–451.

Williams, B. D., D. R. Mitchell, and J. L. Rosenbaum. 1986. Molecular cloning and expression of flagellar radial spoke and dynein genes of Chlamydomonas. J. Cell Biol. 103:1–11.

Witman, G. B. 1992. Axonemal dyneins. Curr. Opin. Cell Biol. 4:70–74.

Witman, G. B., J. Blumberg, and G. Sander. 1978. Chlamydomonas flagellar mutants lacking radial spokes and central tubules: structure, composition, and function of specific axonemal components. J. Cell Biol. 76:729–747.

Wright, B. D., J. H. Henson, K. P. Wedaman, P. J. Willy, J. N. Morand, and J. M. Scholey. 1991. Subcellular localization and sequence of sea urchin kinesin heavy chain: evidence for its association with membranes in the mitotic apparatus and interphase cytoplasm. J. Cell Biol. 113:817–833.

Yang, J. T., R. A. Laymon, and L. S. B. Goldstein. 1989. A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analyses. Cell. 56:879–889.

Youngblom, J. A., J. A. Schloss, and C. D. Sillifo. 1984. The two b-tubulin genes of Chlamydomonas reinhardtii code for identical proteins. Mol. Cell. Biol. 4:2586–2596.

Zimmer, W. E., J. A. Schloss, C. D. Sillifo, J. Youngblom, and D. M. Waterson. 1988. Structural organization, DNA sequence, and expression of the calmodulin gene. J. Biol. Chem. 263:19370–19383.