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The *Chlamydomonas FLA10* Gene Encodes a Novel Kinesin-homologous Protein

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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 *fla10* and functionally rescues the *fla10* 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 α-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 *fla10-1* was markedly reduced, although no reduction was observed in two other *uni* linkage group mutants, *fla9* and *fla11*. Furthermore, *fla10-1* 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 a 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)[1] has a secondary structure analogous to that of myosin: an amino-terminal motor domain has both ATP- and microtubule-binding sites, an α-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 α-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

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1. *Abbreviations used in this paper*: aa, amino acid; *fla*, flagellar assembly mutants; KHC, kinesin heavy chain; nt, nucleotide; PVDF, polyvinylidene difluoride; ULG, *uni* linkage group.

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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 synchronization experiments, cells were grown in minimal Sager-Granick medium (Harris, 1989), bubbled continuously with 5% CO_2 in air, at 23°C, in alternating 14 h light/10 h darkness for at least 6 d (modified from Surzycki, 1971).

Wildtype cells were of the standard laboratory strain 137c. Mutants *fla9* and *fla11*, as well as the *nitl-305,cw15* 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 *pf14* and *pf18* 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 *fla10-1* mutant was isolated in this laboratory and was originally designated *dd-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% washed agar). 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.

Table I. Genetic Linkage Data

Markers		Cross*	PD:NPD:T‡	Map distance (cM)§
apm	*pf10*	1	2:0:15	44.1
fla10	*pf10*	1	10:0:7	20.6
fla10	*apm*	1	9:0:8	–
		2	5:0:14	30.5
p136-420‖	*pf10*	1	10:0:7	20.6
p136-420	*apm*	1	9:0:8	–
		2	5:0:14	30.5
p136-420	*fla10*	1	17:0:0	–
		2	19:0:0	0 (≤1.4 cM)¶

C. reinhardtii Crosses:
fla10-1, mt+ × *fla10-519*, mt− 153:0:0
fla10-1, mt− × *fla10-519*, mt+ 280 tetrads analyzed (undissected) no recombinants

* 1 *Chlamydomonas smithii* (mt+) × *Chlamydomonas reinhardtii* (*apm*, *fla10-1*, *pf10*, mt−);
2 *Chlamydomonas smithii* (mt+) × *Chlamydomonas reinhardtii* (*apm*, *fla10-1*, *pf29*, *ac17*, mt−)
‡ PD, parental ditype; NPD, nonparental ditype; T, tetratype.
§ cM, centiMorgans; calculated as $[(6NPD + T)/(PD + NPD + T)] \times 50$. For calculation of map distances in the intervals *apm-fla10*, *p136-420-apm*, and *p136-420-fla10*, tetrad data from (1) and (2) were combined.
‖ p136-420 is a 420-bp plasmid subclone of λ136 (cDNA nt 2002–2072 plus intron 19); it recognizes a SacI RFLP between *C. smithii* and *C. reinhardtii* DNAs, and was followed as a molecular marker.
¶ Estimate based on the calculation of map distance if an additional tetrad were to show recombination (36:0:1). *mt*, mating type; *apm*, *fla10*, and *pf10* are ULG markers.

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 ~10^7 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–Cl, pH 7.5, 40-mM EDTA, 2 mg/ml Proteinase K), and an equal volume of 10% SDS was immediately added, to give a final concentration of ~10^8 (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 DNA 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 H_2O 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 pBluescript plasmids (Stratagene, Inc., La Jolla, CA). Selected clones were used to identify RFLPs between *C. reinhardtii* and *C. smithii* genomic DNAs on

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Southern blots. Linkage to ULG was then confirmed by molecular tetrad analysis: the segregation of these RFLPs was monitored in a cross (generation F_6, described in Hall et al., 1989) in which RFLPs among the progeny arise from the segregation of *C. smithii* and *C. reinhardtii* ULG chromosomes against a common genetic background. λ136 and overlapping clones were obtained by using one of the ULG plasmid clones to screen a lambda library containing *C. reinhardtii* genomic DNA, partially digested with Sau3A.

Northern and Southern Blot Hybridizations

RNA samples were routinely treated with RNase-free DNase before Northern analysis. For RNA gel electrophoresis, we used formaldehyde denaturation, following standard methods (Setlow and Hollaender, 1982). Hybridization and wash conditions for Northern and Southern blots were as described (Hall et al., 1989).

RACE cDNA Cloning and Sequencing

All sequencing was done using the Sequenase DNA Sequencing Kit (US Biochemical Corp., Cleveland, OH) on double stranded templates cloned in pBluescript. Genomic sequence information from λ136 was used to make primers for cDNA cloning by rapid amplification of cDNA ends (RACE): 3′ partial cDNAs were obtained by the method of Frohman (Innis et al., 1990), and overlapping internal and 5′ partial cDNAs were generated using the 5′RACE System Kit (GIBCO BRL, Gaithersburg, MD). All sequence information shown for the cDNA (see Fig. 3) was obtained from both strands; most data are from both cDNA strands and at least one genomic strand, but some are from one cDNA strand and its complement in genomic DNA. In rare cases of single-base discrepancies between different cDNA clones (assumed to be PCR artifacts), genomic sequence information was used. DNA and protein sequence analysis were performed using PC/Gene (IntelliGenetics, Inc., Mountain View, CA)

Antibody Preparation and Affinity Purification

Two nonoverlapping cDNA segments encoding portions of the COOH-terminal rod and tail domains of KHP1 (residues 596–667 and 669–752) were used to make fusion proteins with glutathione-S-transferase (pGEX vectors; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Fusion proteins were recovered from bacterial lysates as described (Smith and Johnson, 1988) and frozen in aliquots on dry ice for storage at −70°C.

Two female New Zealand white rabbits were immunized with each fusion protein. Immunizations were carried out by personnel of the Rockefeller University Laboratory Animal Research Center. For initial injections, 50 to 150 μg of thawed fusion protein was emulsified 1:1 in complete Freund's adjuvant and administered i.d. at multiple sites. Boosts were performed i.d. at ~8, 12, 16, and 20 wk with 25–100 μg fusion protein, either emulsified 1:1 in incomplete Freund's adjuvant or adsorbed onto Alu-Gel-S. Rabbits were bled 10 to 15 d after each boost.

In order to deplete our rabbit sera of anti-GST antibodies, we incubated the sera with Sepharose-4B beads (Pharmacia Biotech Inc.) to which we had coupled the GST protein fragment encoded by pGEX. Further affinity purification with fusion proteins bound to polyvinylidene difluoride (PVDF) membranes was performed as described (Rosen et al., 1990).

Preparation of Flagellar and Axonemal Proteins

Deflagellation of gametic cells was performed by pH shock (Piperno and Ramanis, 1991). After deflagellation, supernatants containing flagella were cleared of cell bodies and debris by repeated centrifugation at 500 *g* for 15 min. Flagella were then pelleted by centrifugation at 10,000 *g* for 45 min; axonemes were prepared by adding 0.1% NP-40 to flagellar supernatants before pelleting similarly. Flagellar and axonemal protein pellets were resuspended in the concentration range of 1–5 mg/ml in a buffer containing 50 mM NaCl, 4 mM $MgCl_2$, 10 mM Hepes, pH 7.2, and 0.2 mM $SrCl_2$. Aliquots were taken for protein concentration measurement by the Bradford assay (Protein Assay Kit; Bio Rad Laboratories, Hercules, CA). After addition of 2% SDS and 1.5% β-mercaptoethanol, samples were frozen on dry ice and stored at −70°C.

PAGE and Western Blotting

Protein concentrations were always checked on test gels stained with Coomassie blue R-250 and minor adjustments were made before use in Western blots. Protein samples were separated on 8% acrylamide gels and transferred to PVDF membranes in a semi-dry blotting apparatus. Transfer buffer was 48 mM Tris, 39 mM glycine, 10% methanol. For Western analysis, blots were incubated in blocking solution (150 mM NaCl, 50 mM Tris–Cl, pH 7.5, 0.1% Tween-20, 3% bovine serum albumin, 1.5% goat serum) for at least 30 min, followed by primary antibody, three changes of wash solution (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% Tween-20), and alkaline phosphatase-conjugated goat anti-rabbit IgG (Vector Labs, Burlingame, CA). Antibodies were diluted in blocking solution minus goat serum, and incubations were at room temperature for 1 h. Blots were developed with BCIP/NBT (alkaline phosphatase substrate kit IV; Vector Labs).

Transformation Rescue

To create a recipient strain for transformation, we crossed the *nitl-305,cwl5* strain with *fla10-1* and selected a triple mutant clone from among the progeny. The *fla10-1,nitl-305,cwl5* strain was cotransformed with DNA from λ136 (or overlapping clones) and a plasmid carrying the gene for nitrate reductase (pMN24; Fernandez et al., 1989). We followed the method of (Kindle, 1990), with minor modifications: cells were resuspended at a density of 0.5–2.0 × 10^8/ml and 0.3 ml were used for each transformation; vortexing with glass beads was done twice (once for 15 s, followed by a 2.5-min wait, then again for 10 s); 2 ml of medium were added immediately after vortexing, and 0.3-ml aliquots were spread on selective and nonselective plates. We used 2 μg of each DNA sample for transformation. Lambda clones were used intact in most cases; for a few of the transformations, gel-purified lambda inserts were used, resulting in only minor increases in *fla10* rescue rates.

Results

Genetic Mapping

For purposes of molecular genetic mapping of the ULG, we isolated several ULG genomic DNA clones which identify RFLPs 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, p136-420, was found to contain sequences that were closely linked to the ULG genetic marker *fla10*: in two interspecific crosses, tetrad analysis showed that the p136-420 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 p136-420 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 p136-420 to retrieve several overlapping genomic DNA fragments from a lambda phage library. A restriction map of one such clone, λ136, 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 λ136 identify a single polyadenylated mRNA of ~3.4 kb in vegetative and gametic cells (Fig. 2 *a*). Two features

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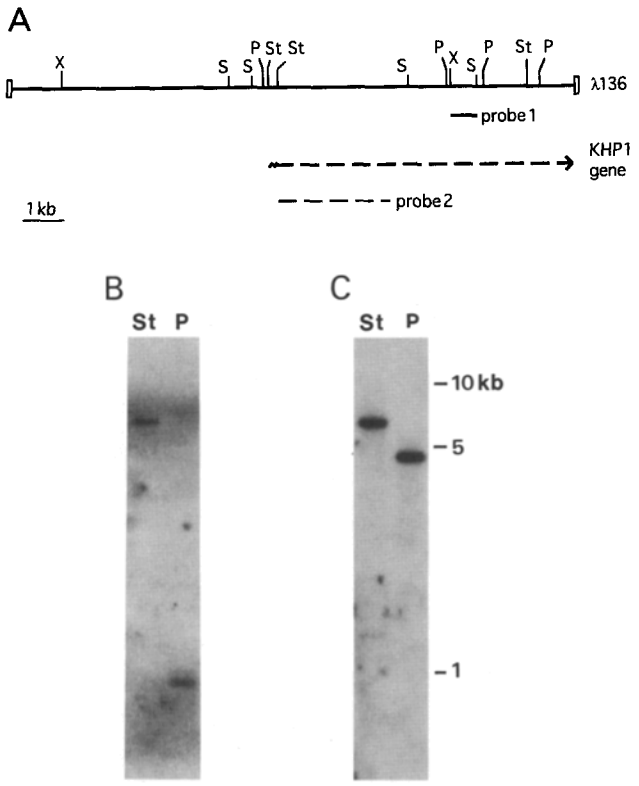

Figure 1. Genomic organization of λ136. (*A*) Partial restriction map of genomic insert in lambda clone 136. *probe1* is a genomic fragment used as a probe on Southern and Northern blots. The dashed arrow indicates an extensively spliced mRNA (see Fig. 3) encoded by the corresponding genomic region above. *probe2* represents a cDNA fragment (nucleotides −55 to 927) also used as a probe in hybridization experiments. Restriction enzymes: *X*, XhoI; *S*, SalI; *P*, PvuII; *St*, StuI. Possible additional PvuII and StuI sites upstream of KHP1 are not shown. (*B*) Southern blot hybridization of *probe1* to StuI and PvuII genomic digests. (*C*) The same Southern blot as in *B*, hybridized with cDNA *probe2*, which encodes the motor domain of KHP1. In addition to the ~6.4-kb StuI band recognized in the first lane, a faint cross-reacting band of ~1.3-kb is visible, possibly indicating the existence of another genomic locus with homology to kinesin motor domain. Bars at right show the positions of molecular masses 10, 5, and 1 kb.

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 threefold 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 Db1).

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 G_2 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 KHP1, 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 KHP1, for kinesin-homologous protein.

A striking structure feature of the KHP1 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.

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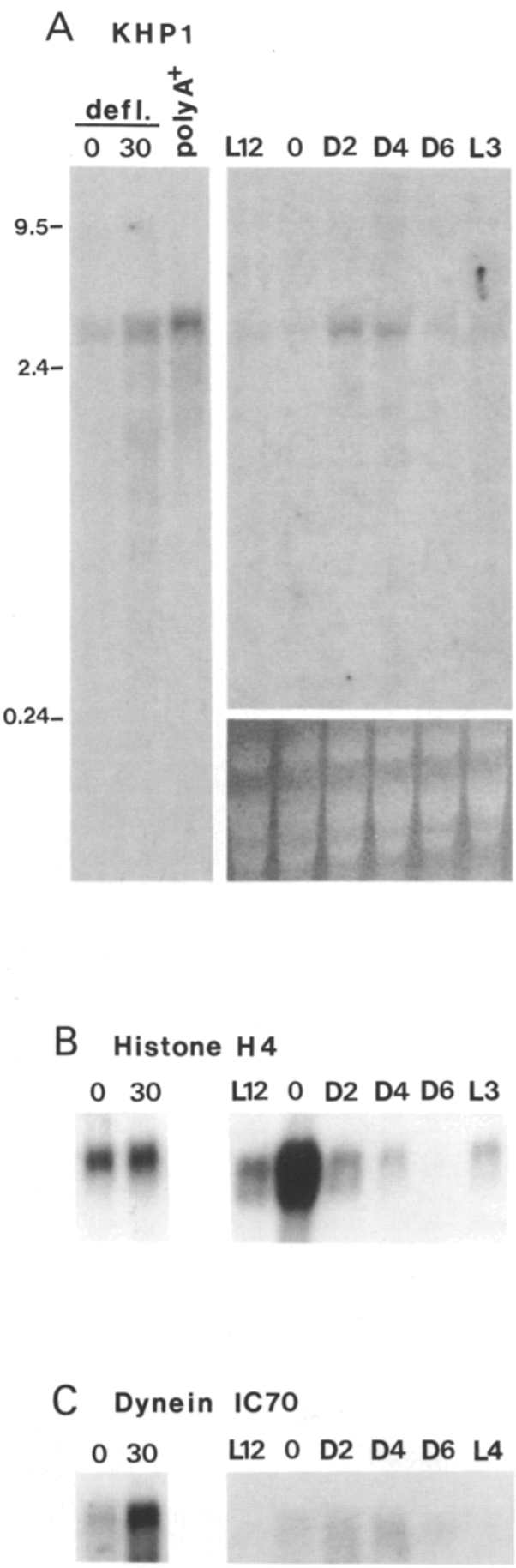

All introns are flanked by highly conserved splice junction sequences which conform tightly to the previously described *Chlamydomonas* consensus: 16 of the 20 intron junctions match the consensus sequence at ≥10 out of 11 positions (Zimmer et al., 1988; Schloss and Croom, 1991). Interestingly, all 20 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). A conserved ATP-binding

Figure 2. Northern blot analysis. (*A*) A radiolabeled 700-bp genomic fragment encoding part of KHP1 (*probel* 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 *O* indicates the peak of S phase (see text). (*C*) A genomic DNA clone containing the intermediate chain dynein gene IC70 (clone Da1 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.

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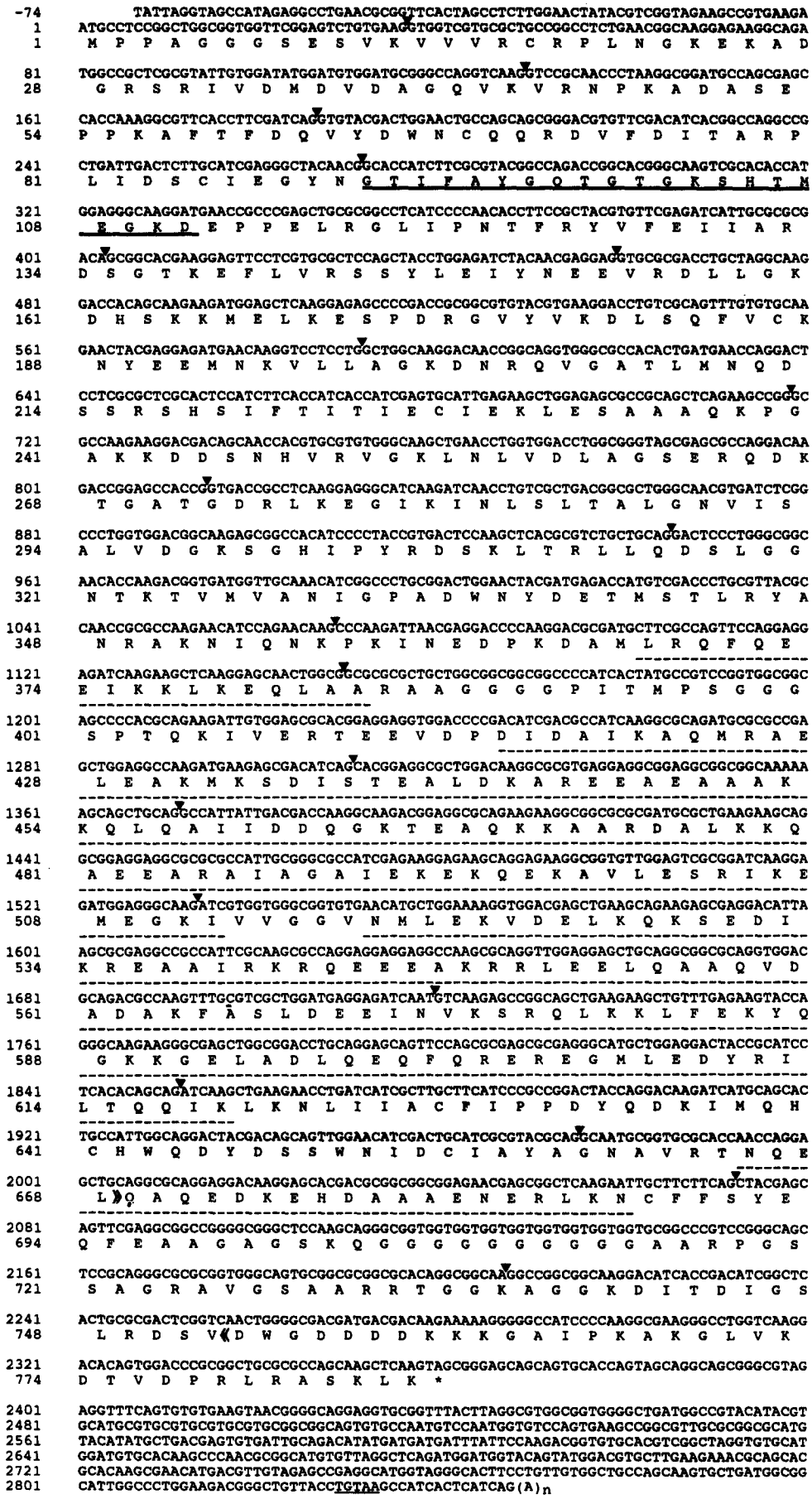

Figure 3. Nucleotide and predicted amino acid sequences of KHP1. Sequence shown was derived from several overlapping cDNA clones. Nucleotide residues are numbered from the first letter of the initiator methionine. An asterisk marks the TAG stop codon. The conserved *Chlamydomonas* poly(A) addition signal (TGTAA, 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 protein regions with the heptapeptide pseudorepeat structure characteristic of coiled-coil domains; repeat structure was analyzed using the program MATCHER, which was designed for prediction of coiled-coil domains (Fischetti et al., 1993). Double carats within the predicted protein sequence (aa 669–752) denote the fragment used to prepare the polyclonal antibody 14-02. This sequence data is available from EMBL/DDBJ under accession number L33697.

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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 (pI = 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 (Dron et al., 1982; Boer and Gray, 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


```
Cr KHP1  MPPAGG----GSESVKVVVRCRPLNGKEKADGRSRIVDMDVDAGQVKVRNPKADASEPPKAFTFDQVYD  65
Mm KIF3  MPINKSEKPESCDNVKVVVRCRPLNEREKSMCYRQAVSVDEMRGTITVHKTDS-SNEPPKTFTFDTVFG  68
Mm KIF5  MA-----DPAEC-SIKVMCRFRPLNEAE---------ILRGDKFIPKFKGEETVVIGQGKPYVFDRVLP  54

Cr KHP1  WNCQQRDVFDITARPLIDSCIEGYNGTIFAYGQTGTGKSHTMEGKDEPPELRGLIPNTFRYVFEIIARD 134
Mm KIF3  PESKQLDVYNLTARPIIDSVLEGYNGTIFAYGQTGTGKTFTMEGVRAVPGLRGVIPNSFAHIFGHIAKA 137
Mm KIF5  PNTTQEQVYNACAKQIVKDVLEGYNGTIFAYGQTSSGKTHTMEGKLHDPQLMGIIPRIAHDIFDHIYSM 123

Cr KHP1  SGTKEFLVRSSYLEIYNEEVRDLLGKDHSKKMELKESPDRGVYVKDLSQFVCKNYEEMNKVLLAGKDNR 203
Mm KIF3  EGDTRFLVRVSYLEIYNEEVRDLLGKDQTQRLEVKERPDVGVYIKDLSAYVVNNADDMDRIMTLGHKNR 206
Mm KIF5  DENLEFHIKVSYFEIYLDKIRDLLDVSKTN-LAVHEDKNRVPYVKGCTERFVSSPEEVMDVIDEGKANR 191

Cr KHP1  QVGATLMNQDSSRSHSIFTITIECIEKLESAAAQKPGAKKDDSNHVRVGKLNLVDLAGSERQDKTGATG 272
Mm KIF3  SVGATNMNEHSSRSHAIFTITIECSEKGV-----------DGNMHVRMGKLHLVDLAGSERQAKTGATG 264
Mm KIF5  HVAVTNMNEHSSRSHSIFLINI----KQENVETEK----------KLSGKLYLVDLAGSEKVSKTGAEG 246

Cr KHP1  DRLKEGIKINLSLTALGNVISALVDGKSGHIPYRDSKLTRLLQDSLGGNTKTVMVANIGPADWNYDETM 341
Mm KIF3  QRLKEATKINLSLSTLGNVISALVDGKSTHVPYRNSKLTRLLQDSLGGNSKTMMCANIGPADYNYDETI 333
Mm KIF5  AVLDEAKNINKSLSALGNVISALAEGTKTHVPYRDSKMTRILQDSLGGNCRSRMFICCSPSSYNDAETK 315

Cr KHP1  STLRYANRAKNIQNKPKINEDPKDAMLRQFQEEIKKLK                                379
Mm KIF3  STLRYANRAKNIKNKARINEDPKDALLRQFQKEIEELK                                371
Mm KIF5  STLMFGQRAKTIKNTASVN                                                    334
```

Figure 4. Comparison of motor domains of *Chlamydomonas* KHP1 and murine kinesin-family genes. Protein alignments were performed by the method of (Myers and Miller, 1988). The motor domain of KHP1 is 360 aa in length, as defined by homology to murine kinesin heavy chain KIF5 (shown at bottom); homology to murine KIF3 extends 19 residues further, as shown. Bars indicate identity between residues of KHP1 and murine KIF3. Motor domains of these proteins are 64% identical in the first 360 aa of KHP1 and 65% identical if the following 19 residues are included. Amino acids conserved in all three genes are underlined. Residue numbers for each partial sequence are shown at right.

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~91 kD molecular mass on Western blots of total *Chlamydomonas* cell protein (not shown). All data presented here were obtained with one serum, called 14-02, which was raised against a fusion protein bearing residues 669–752 of the KHP1 tail domain (see Fig. 3). Upon affinity purification, the 14-02 antiserum showed specificity for the 91-kD species on Western blots of total *Chlamydomonas* protein (Fig. 5 *a*, lane *1*). This apparent molecular mass is in reasonably good agreement with the predicted KHP1 size of 87 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. 5 *a*, 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 nonionic detergent (Fig. 5 *a*, 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. 5 *b*). 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.

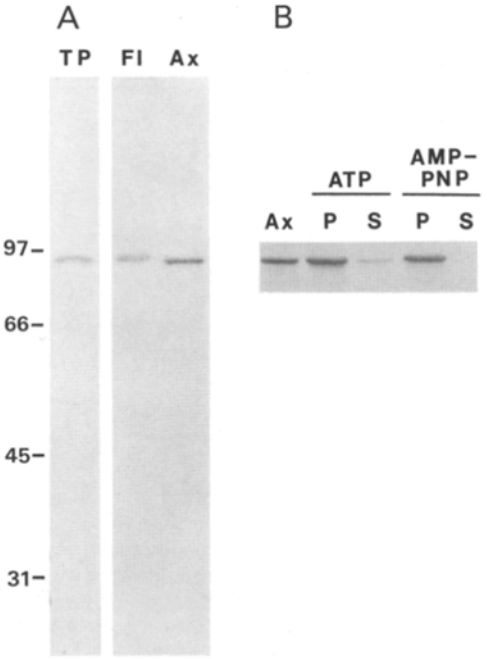

Figure 5. Identification of KHP1 protein by Western analysis. (*A*) Affinity-purified antisera were incubated with Western blots containing *Chlamydomonas* total protein (*TP*, ~10^6 cells/lane were lysed in protein sample buffer and loaded directly onto the gel), flagella (*Fl*, ~15 μg/lane), and axonemes (*Ax*, ~15 μg/lane). Molecular mass markers are indicated at left; the calculated molecular mass of the immunoreactive band is 91 kD. (*B*) Western blot analysis of axonemal proteins in untreated preparations (*Ax*) and in pellets (*P*) or supernatants (*S*) of axoneme samples that had been treated with either ATP or its nonhydrolyzable analogue AMP-PNP. Axoneme pellets were resuspended in small amounts of buffer as usual, diluted 11-fold with freshly prepared nucleotide buffer (6 mM ATP or AMP-PNP, 10 mM Hepes, 12 mM $MgCl_2$, 50 mM NaCl, pH 7.2), incubated at room temperature for 30 min, and centrifuged. Supernatants were concentrated in Centricon-10 spin-filters (Amicon, Inc., Beverly, MA). Equal fractions of pellet and supernatant were loaded (~25 μg axonemal protein/lane).

KHP1 in Mutants with Axonemal Defects

To determine the axonemal location of KHP1, 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 *pf14* 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 *pf14* or *pf28* suggests that KHP1 is not a component of radial spokes or outer dynein arms. *pf30* has a more subtle defect, in which only one of

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three different types of inner dynein arm complex, I1, is missing (Piperno et al., 1990). Since KHP1 is abundant in *pf30* axonemes, it is not likely to be a component of I1; there is no known mutant lacking all inner dynein arm structures.

In flagella of the *pf18* 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, *pf18* axonemes are analogous to those of *pf14* and *pf28* 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 *pf18* 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 *pf18* axonemes were analyzed in Fig. 6, the amount of KHP1 protein detected in *pf18* 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. 7 *a*, *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. 7 *b*). 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.

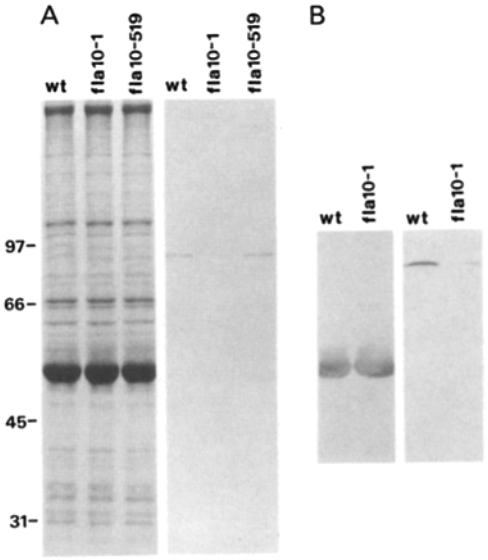

Figure 7. KHP1 in *fla10* mutant axonemes. (*A*) Coomassie stained polyacrylamide gel and Western blot of wildtype (*wt*, *fla10-1*, and *fla10-519* axonemes (~25 μg/lane) incubated with the affinity-purified anti-KHP1 antibody, 14-02. Cells were resuspended in liquid medium at 21°C and allowed to grow flagella (>1 h); cultures were then transferred to 32°C for 1 h before isolation of axonemes. (*B*) Western blots of wildtype (*wt*) and *fla10-1* axonemes, (30 μg/lane), from cells grown at permissive temperature (21°C). Filter on the left was incubated with the anti-acetylated tubulin monoclonal antibody, 6-11B-1 (Piperno and Fuller, 1985), and shows that approximately equal amounts of protein were loaded in each lane. Filter on the right was incubated with the affinity-purified anti-KHP1 antibody.

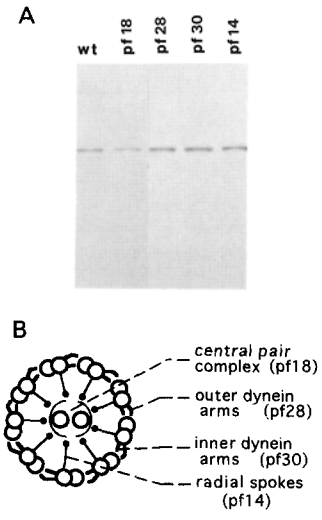

Figure 6. Western analysis of mutant axonemes. (*A*) Approximately equal amounts of axonemal protein from wildtype (*wt*) and various mutant strains were examined (~20 μg/lane). *pf18* axonemes lack the central pair microtubules; *pf28* is missing the outer dynein arms; *pf30* is deficient in inner dynein arm I1; *pf14* lacks radial spokes. (*B*) Diagram of axoneme in cross-section. Major structures are labeled, and mutants in which these structures are absent are indicated.

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 other 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

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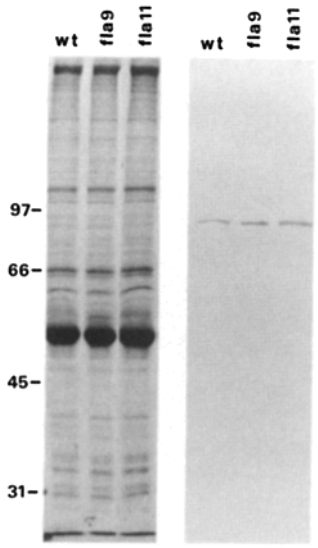


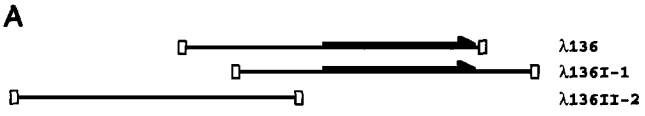
Figure 8. KHP1 is present in axonemes of other flagellar assembly mutants. Approximately 25 μg axonemal protein were analyzed from wild-type (*wt*), *fla9*, and *fla11* cells that had been grown at permissive temperature and shifted to 32°C for 1 h before axoneme isolation. Coomassie stained gel is shown at left; Western blot filter at right was made from an identical gel and incubated with the affinity-purified anti-KHP1 antibody.

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 fla10 Phenotype by Transformation with KHP1 Genomic DNA

The genetic proximity of λ136 sequences to the *FLA10* locus made KHP1 a candidate gene product for *FLA10*. To demonstrate 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 *cw15* mutation causes a cell wall deficiency, and *nitl-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 transformants which were able to utilize nitrate (*nit+ colonies*, Fig. 9 *b*). None of these transformants were motile after 12 h at 32°C (n = 218), indicating that transformation did not induce reversion of the *fla10* phenotype at detectable rates in these experiments. We cotransformed the same strain with pMN24 and either λ136 or an overlapping lambda clone also encompassing the KHP1 gene, λ136I-1 (Fig. 9 *a*). Approximately 5–10% of nit+ cotransformants were rescued for the *fla10* phenotype: they had flagella and were swimming normally after an overnight incubation at 32°C. Similar cotransformation with another overlapping lambda clone which does not cover the KHP1 gene, λ136II-2, yielded no nit+ transformants with restored motility at 32°C. Since λ136II-2 extends to within 1.6 kb of the 5′ end of the KHP1 and yet is unable to rescue *fla10*, it is extremely unlikely that the critical sequence for *fla10* rescue lies upstream of KHP1. Furthermore, a 0.6-kb Sal fragment located midway between the 5′ end of the KHP1 and the proximal end of λ136II-2 shows 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 transcription unit which accounts for the ability of both λ136 and λ136I-1 to rescue *fla10*.



B

DNAs used for Transformation	nit+ (#colonies)	swim@32°C
pMN24	218	0
pMN24, λ136	133	13
pMN24, λ136I-1	129	6
pMN24, λ136II-2	150	0

Figure 9. Transformation rescue of *fla10-1*. (*A*) Diagram of genomic DNA inserts in λ136 and two overlapping lambda phage clones which were used for transformation. The complete KHP1 transcription unit (*black arrows*) is present in clones λ136 and λ136I-1. Insert sizes are: λ136 and λ136I-1, 14.3 kb; λ136II-2, 13.5 kb. (*B*) *fla10-1, nitl-305, cw-15* 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 ≥12 h at 32°C, liquid cultures were examined for flagellated, swimming cells. The number of nit+ transformants which are motile at 32°C is indicated (*swim @ 32°C*).

In Southern analysis of the λ136 transformants, extra copies of the KHP1 sequence were detected (Fig. 10 *a*). In most cases, part of the lambda arm adjacent to KHP1 in λ136 had also integrated, accounting for the common size of the extra band in eight out of nine transformants (Fig. 10 *a*, see legend); this has been confirmed by hybridization of the same blot with a lambda DNA fragment (not shown). When these transformants were crossed to the wildtype strain 137, the *fla10* phenotype reappeared among the progeny. This indicated that the rescued strains are not revertants, and that the transforming DNA did not undergo homologous recombination at the *FLA10* locus in these cells. We followed the segregation of the extra KHP1 sequences at the molecular level in two such transformant crosses to wildtype. As illustrated in Fig. 10 *b*, these sequences segregated 2:2; furthermore, they were absent from those progeny in which the *fla10* phenotype had reappeared.

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 substructures. Furthermore, we show that KHP1 is the product of the *FLA10* gene, which is required for both flagellar assembly and flagellar maintenance.

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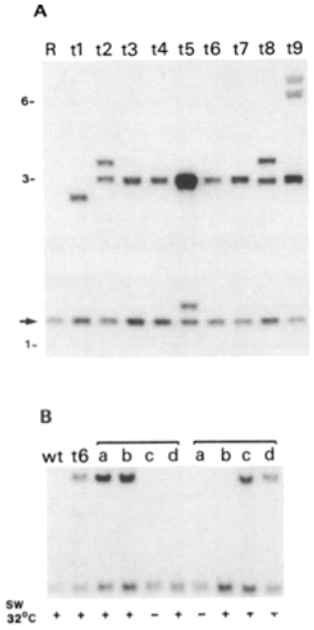

Figure 10. Southern analysis of transformants. (*A*) Southern blot of PvuII-digested genomic DNAs from the recipient strain, R (*fla10-1, nit-305, cw15*), and rescued transformant clones t1-t9, hybridized with a probe from the 3′ end of the KHP1 cDNA (nt. 2258–2472). In addition to the endogenous KHP1 gene fragment recognized in all lanes (*arrow*), all rescued clones have one or more extra copies of the KHP1 sequence. In most clones, the entire λ136 clone has been integrated, and a 3-kb fragment extending from the 3′ end of KHP1 to a PvuII site in the adjacent lambda arm is detected in the genome. Molecular masses in kb are indicated at left. (*B*) Reappearance of the *fla10* phenotype in a transformant cross to wildtype. The transformant clone t6 was crossed to wildtype (*wt*; strain 137); tetrads were dissected and analyzed for the *fla10* phenotype at 32°C. Genomic DNAs from the parents (*wt*; *t6*) and two tetrads in this cross (*a–d*) were probed with a 3′ KHP1 cDNA fragment (see *A*); each of the progeny that has inherited an extra 3-kb band is phenotypically wildtype (*sw 32°C+*). The mutant phenotype reappears in one of the progeny in each tetrad, indicating that the t6 parent is not a revertant.

Homology of KHP1 to Kinesins

Sequence analysis of KHP1 revealed that the protein has a three-domain structure analogous to that of kinesin heavy chains. There is an NH_2-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 KHP1 exists as a dimer; whether KHP1 associates with light chains through its tail domain remains to be determined.

KHP1 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). KHP1 and KIF3 are 64% identical in their motor domains, and 38% identical over half of the stalk; the tail domains are not well conserved (15% identity). This high degree of sequence similarity between the NH_2-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 KHP1 and KIF3 is reminiscent of that between members of the bimC/cut7/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* KHP1 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 KHP1 and KIF3 define a distinct subgroup of structurally and functionally related proteins.

KHP1 Protein in Flagella

The expression pattern of KHP1 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 KHP1 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 KHP1 mRNA to experimental deflagellation. The doubling of KHP1 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 KHP1 identified the KHP1 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, KHP1 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

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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 *pf18* 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 *fla10* 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* 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 (Kozminski 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 membranous 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 (Kozminski et al., 1993). It will be interesting to determine whether the KHP1 protein is associated with these structures.

Other Possible Functions of KHP1

Given the extensive evidence for involvement of kinesin-like proteins in mitosis in other organisms, it is important to consider the possibility that KHP1 plays a role in cell division. Nothing in our present study points to such a function for KHP1: the mRNA is not induced during cell division, and the closest relative to KHP1, murine KIF3, is expressed predominantly in adult mouse cerebellum, a mitotically quiescent tissue. However, nothing in our present study precludes this possibility, either. Although KHP1 mRNA is not induced at the onset of mitosis, we have no information about the longevity of the KHP1 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 KHP1 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 *apm1* alleles (*apm1* 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 result from an inappropriate interaction of mutant flagellar protein(s) with spindle microtubules and their associated proteins. To address these issues, it will be interesting to determine the distribution of KHP1 protein during mitosis in normal and mutant cells.

Clearly, there are many aspects of KHP1 function that can now be explored. In addition, there have been recent reports of multiple kinesin-related proteins in *Chlamydomonas* flagella (Fox et al., 1994; Johnson, K. A. and J. L. Rosenbaum. 1993. *Mol. Biol. Cell.* 4S:1574). A more extensive knowledge of the kinesin gene family in this organism is crucial to the understanding of flagellar function, and may shed light on the evolutionary relationships between different forms of cellular motility.

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Note Added in Proof. It has come to our attention that another kinesin-family gene with notable sequence similarity to KHP1 (66% identity of motor domains) has recently been cloned from sea urchin (Cole, D. G., S. W. Chinn, K. P. Wedaman, K. Hall, T. Vuong, and J. M. Scholey. 1993. Novel heterotrimeric kinesin-related protein purified from sea urchin eggs. *Nature (Lond.).* 366:268–270).

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