

Mutant Kinesin-2 Motor Subunits Increase Chromosome Loss

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Submitted May 9, 2005; Revised May 26, 2005; Accepted May 31, 2005
Monitoring Editor: Ted Salmon

The *Chlamydomonas* anterograde intraflagellar transport motor, kinesin-2, is isolated as a heterotrimeric complex containing two motor subunits and a nonmotor subunit known as kinesin-associated polypeptide or KAP. One of the two motor subunits is encoded by the *FLA10* gene. The sequence of the second motor subunit was obtained by mass spectrometry and sequencing. It shows 46.9% identity with the Fla10 motor subunit and the gene maps to linkage group XII/XIII near RPL9. The temperature-sensitive flagellar assembly mutants *fla1* and *fla8* are linked to this kinesin-2 motor subunit. In each strain, a unique single point mutation gives rise to a unique single amino acid substitution within the motor domain. The *fla8* strain is named *fla8-1* and the *fla1* strain is named *fla8-2*. The *fla8* and *fla10* alleles show a chromosome loss phenotype. To analyze this chromosome loss phenotype, intragenic revertants of *fla8-1*, *fla8-2*, and *fla10-14* were generated. The analysis of the mutants and the revertants demonstrates the importance of a pocket in the amino terminus of these motor subunits for both motor activity and for a novel, dominant effect on the fidelity of chromosome segregation.

INTRODUCTION

Kinesin-2 is the motor for anterograde intraflagellar transport (IFT) that serves to assemble and to maintain cilia and flagella in eukaryotic organisms. Cilia in many of these organisms are used for both motile and sensory functions. Defects that affect motile cilia/flagella in vertebrates cause randomization of the left-right axis of symmetry, chronic respiratory defects, and male sterility (McGrath and Brueckner, 2003; Snell *et al.*, 2004). Defects in sensory cilia have been associated with polycystic kidney disease, retinal degeneration, smell and hearing loss, learning disabilities, obesity, and skeletal defects (Zhang *et al.*, 2004). Genetic analysis of flagellar/ciliary assembly in *Chlamydomonas*, *Caenorhabditis elegans*, *Drosophila*, *Tetrahymena*, and mice show that these motors and proteins involved in IFT are conserved. Large particles (IFT particles) are moved from the basal body region to the tip of the cilia/flagella by kinesin-2 and returned to the cell body by a cytoplasmic dynein (Pazour *et al.*, 1999; Porter *et al.*, 1999).

Kinesin-2 is composed of two motor subunits and a third kinesin-associated protein known as KAP. This complex was

first identified biochemically from sea urchin eggs (Cole *et al.*, 1993; Wedaman *et al.*, 1996). Its ciliary role was clarified by the identification of the gene product of the *FLA10* gene as a kinesin-2 motor subunit (Walther *et al.*, 1994), and observations that a mutant allele affected movement of particles in the flagella of *Chlamydomonas* (Kozminski *et al.*, 1995). The *fla10-1* allele is a temperature-sensitive mutation that lacks flagella at the restrictive temperature of 32°C (Huang *et al.*, 1977; Adams *et al.*, 1982; Lux and Dutcher, 1991). In this work, we show that the other motor subunit is encoded by the *FLA8* gene (see below). The KAP subunit is encoded by the *FLA3* gene (Mueller *et al.*, 2005). Mutations in *FLA8* and *FLA3* also show temperature-sensitive defects in flagellar assembly (Adams *et al.*, 1982).

Kinesin-2 also is required for ciliary assembly in *C. elegans* (Shakir *et al.*, 1993), *Tetrahymena* (Brown *et al.*, 1999), *Drosophila* (Han *et al.*, 2003; Sarpal *et al.*, 2003), sea urchins (Morris and Scholey, 1997), and mice (Yamazaki *et al.*, 1995; Yang *et al.*, 2001). In addition to roles in ciliary assembly, kinesin-2 has been implicated in other microtubule-based transport events. In *Xenopus*, kinesin-2 is involved in pigment granule dispersion (Tuma *et al.*, 1998; Deacon *et al.*, 2003); in endoplasmic reticulum-to-Golgi transport (Le Bot *et al.*, 1998); and Vg1 mRNA localization (Betley *et al.*, 2004) based on the introduction of dominant negative mutations. In *Drosophila*, neuronal transport of choline acetyltransferase (Ray *et al.*, 1999) and transport of cell fate components during oogenesis require kinesin-2 (Pflanz *et al.*, 2004). Kinesin-2 also seems to be involved in the establishment of neuronal polarity via association with the PAR-3 complex (Fan and Beck, 2004; Nishimura *et al.*, 2004).

In ciliated cells, kinesin-2 localizes to the basal body region of cells and within the cilia/flagella (Vashishtha *et al.*,

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-05-0404>) on June 8, 2005.

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1996; Cole *et al.*, 1998; Deane *et al.*, 2001). During mitosis, kinesin-2 is found near centrosomes and along the mitotic spindle (Henson *et al.*, 1995; Vashishtha *et al.*, 1996; Deane *et al.*, 2001). Recently, Morris and colleagues showed in sea urchin blastulae using a green fluorescent protein GFP-KAP construct that KAP moves into the nucleus before nuclear envelope breakdown when the cilia disassemble and moves out of the nucleus after nuclear envelope reformation (Morris *et al.*, 2004). Fan and Beck (2004) showed that KIF3B, a kinesin motor subunit, localizes to the midbody and that overexpression of the KIF3B tail results in cytokinesis defects. Previously, we had observed synthetic phenotypes in double mutants of *fla10* and *apm1* mutant alleles. The synthetic phenotypes were observed with three different alleles of *fla10* (*fla10-1*, *fla10-14*, and *fla10-15*) in combination with the mutant *apm1-122* allele (Lux and Dutcher, 1991). Mutations in the *APM1* gene confer resistance to the microtubule-destabilizing drug ami-prophos-methyl or oryzalin (James *et al.*, 1988), which suggests that the microtubules may be hyperstabilized in this mutant background. The double mutant *fla10-1*; *apm1-122* showed a cell cycle arrest at 21°C but growth at 32°C. The double mutants *fla10-14*; *apm1-122* and *fla10-15*; *apm1-122* show a slow growth phenotype at 21°C but show wild-type growth at 32°C. Given that no detectable Fla10 gene product is present at 32°C in *fla10-1* cells (Cole *et al.*, 1998), it is likely that these phenotypes arise via toxic interactions of the mutant gene products rather than by redundancy of the two genes. To ask whether the mutant gene products have other effects on the cell cycle, we have investigated the rates of chromosome loss and cytokinesis failures in several *fla10* and *fla8* alleles as well as in intragenic revertants of the *fla8-1*, *fla8-2*, and *fla10-14* alleles.

MATERIALS AND METHODS

Cell Culture and Genetics

Cells were grown as described previously (Holmes and Dutcher, 1989). Matings were performed as described previously (Dutcher, 1995a). Revertants were isolated as described previously (Preble *et al.*, 2001) but at 32°C.

Restriction Fragment Polymorphisms and Sequencing

DNA from *fla10-14* and *fla10-14* revertant cells was isolated and colony PCR was performed as described previously (Bowers *et al.*, 2003). The coding region of *FLA10* was amplified with overlapping primers that were designed with a melting temperature of 57°C with a spacing of 600 base pairs (sequences available upon request) and cloned into the pCR4-TOPO vector (TOPO TA cloning kit; Invitrogen, Carlsbad, CA). Transformed plasmids were prepared with Wizard Plus SV Minipreps DNA purification system (Promega, Madison, WI) and digested with *EcoRI* to verify the presence of an insert. Plasmid DNA was sequenced in conjunction with the Protein and Nucleic Acid Sequencing Laboratory (Washington University, St. Louis, MO) using M13 primers. All primers are given in the 5'-to-3' direction. The E₂₄K mutation was amplified using primers *fla10-14F* (CATCGTTGCAGCAAGCTTTA) and *fla10-14R* (ATGTAGACCGCTTCGAGTC). A 207-base pair fragment from -53 to +155 in wild-type cells is not cut by *MboII*, whereas the E₂₄K mutant is cut. The L₁₉₆F mutation was amplified using primers *fla10-3F* (GTTGGCGTGTGTGTTATG) and *fla10-3R* (GACTCATAACCGACCGCTGT) and produced a 958-base pair fragment from +1417 to +2374. *PvuMI* cuts the wild-type PCR product into four fragments (512, 252, 149, and 45 base pairs), whereas the mutant is cut into three fragments (557, 252, and 149 base pairs). Primers *fla10-5F* (TTGGAATACCGCCATCT) and *fla10-5R* (CCAATCCCGTAAGGAAACAG) amplified the G₃₃₄S mutation and produce a 894-base pair fragment from +3035 to +3928. The wild-type PCR product is cut into five fragments (270, 259, 153, 144, and 68 base pairs) with *Sau96I*, whereas the mutant is cut into four fragments (403, 270, 153, and 68 base pairs). The N₃₂₉H mutation was amplified with the primers *fla10-5F* and *fla10-5R*, cloned into the TA vector, and sequenced because it does not have a restriction polymorphism with wild-type sequence.

A 242-base pair fragment was amplified from *fla1*, *fla8*, and CC1952 DNA with primers *fla8-1F* (TATCAAGCCACGGGAGTAG) and *fla8-1R* (CGGATGTGTTACGAGTGTCTG). The *fla1* and *fla8* PCR products were cut with *RsaI* to produce fragments of 178, 37, and 27 base pairs. The CC1952 DNA was cut to produce a fragment of 205 and 37 base pairs.

Cloning of a Kinesin-2 Motor Subunit

When *Chlamydomonas* expressed sequence tags (ESTs) first became available, BLAST searches using kinesin-2 motor sequences revealed a single 578-nucleotide EST (accession no. BE337430) that encoded part of a *FLA10*-related kinesin. This partial cDNA sequence was outside of the highly conserved kinesin motor domain, so it was suitable as a probe to screen both a cDNA library and a genomic BAC library (Clemson University Genomics Institute, Clemson, SC). Three BAC clones (28P7, 02I16, and 07F13) were identified. BAC clones and genomic DNA isolated directly from *Chlamydomonas* strains were used as PCR templates to amplify 4.8- to 5.2-kb products with high fidelity KOD XL DNA polymerase (Novagen, Madison, WI) supplemented with 2 M GC Melt (BD Biosciences Clontech, Palo Alto, CA). The sets of primers used were Kin2F1 (ACCCGCTGATTACGTTTCGAGTCTGG) and Kin2R1 5'-(GAGCGGGTCTCAAGACGGGGATAG) and Kin2F2 (GCGCGCTGGAGCTGAAGACA) and Kin2R2 (GTAACGCGCAGATACCGGATG-GAATAGACA). The 5-kb PCR products were cloned into TOPO blunt vectors (Invitrogen); the resulting plasmids were purified, and *EcoRI* digests were analyzed as described above. Sequencing primers were designed with a melting temperature of ~55°C at an average spacing of ~400 base pairs (sequences available upon request). An additional EST sequence (accession no. AV640755) corresponded to a cloned cDNA plasmid (#755) that was obtained from Kazusa DNA Research Institute (Chiba, Japan) (Asamizu *et al.*, 1999; Asamizu *et al.*, 2000). Plasmid #755 contained a 2.98-kbp cDNA insert with an open reading frame of 2304 base pairs that encodes a 768-amino acid protein. Sequencing of plasmid #755 confirmed the intron-exon structure determined from earlier analysis but it was found to contain a single base pair substitution at position 1721 of the open reading frame changing an A to a G transition. This produced a conservative amino acid substitution of arginine for lysine at residue 574. The *Chlamydomonas reinhardtii* strain used to generate clone #755 was C9 mt⁻, whereas all other sources of genomic and cDNA, including cDNA and BAC libraries, were of strains that were either 137c (CC-124 or CC-125) or mutants that were derived from the 137c strains. According to Harris (1989) C9 mt⁻ and 137c arose from the same laboratory isolate but have been separated for 60 years. This length of time may account for the variation observed at amino acid residue 574.

Sequencing of Second Kinesin Motor Subunit Gene in *fla* Mutants and Revertants

Genomic DNA was isolated from *Chlamydomonas flA1*, *flA3*, *flA8*, and revertants by a modified procedure of (Koutoulis *et al.*, 1997). The *flA3* strain was chosen as a control. *Chlamydomonas* strains were plated onto solid medium and grown under continuous light for 7–9 d. Cells were removed from the plates and resuspended in 20 mM Tris, pH 7.5, 20 mM EDTA, 5% SDS, 1 mg/ml proteinase K and incubated overnight at 50°C. Phenol:chloroform (50:50) extraction was followed by chloroform extraction. Genomic DNA was precipitated with 7.5 M ammonium acetate in ice-cold ethanol followed by centrifugation for 5 min at 16,000 × g. The resulting DNA precipitate was washed two times with 80% ethanol. After drying, the DNA was resuspended in 10 mM Tris, 0.10 mM EDTA, pH 8.0, and stored at -28°C. PCR products (~5 kb) containing the second motor subunit gene were amplified from each of three *fla* strains using KOD XL DNA polymerase with the Kin2F1, Kin2F2, Kin2R1, and Kin2R2 primers described above. Products from two different PCR reactions for each of the strains were cloned into TOPO Blunt vectors and resulting plasmids were purified as described above. For *flA1*, *flA3*, and *flA8*, the entire gene was sequenced with threefold coverage for at least two different PCR products. For revertant sequencing, the focus was on the motor domain of the kinesin with the primers Kin2F3 (CTTGCGCCGAGAGCTGTACA-GATTCAG) and Kin2R3 (CAGGGGCCATCAGCATCAATACACAGTTTG). The resulting 2254 base pairs PCR products were cloned into TOPO Blunt vectors (Invitrogen) and sequenced as described above.

Mass Spectrometry

Chlamydomonas kinesin-2 was purified from flagella essentially as described previously (Cole *et al.*, 1998) with the exception that gel filtration was omitted. In brief, flagella were isolated from the wild-type CC-125 *Chlamydomonas* strain by pH shock followed by differential centrifugation (Witman *et al.*, 1972) and storage as pellets at -80°C. The following steps were performed on ice or at 4°C. A 100-ml flagellar aliquot was extracted with 400 ml of 0.05% NP-40 in HMDEK buffer (10 mM HEPES, 5 mM MgSO₄, 1.0 mM dithiothreitol, 0.5 mM EDTA, 25 mM KCl, pH 7.2) supplemented with 2.0 mM adenylyl-5'-yl imidodiphosphate (AMPPNP) to keep kinesin-2 bound to the axonemal microtubules. After a 15-min centrifugation at 16,000 × g, the axonemal pellet was resuspended and extracted for 10 min with 10 mM MgATP in HMDEK buffer. After another 15-min centrifugation at 16,000 × g, the resulting supernatant was loaded onto a 13 ml of 10–25% sucrose density gradient (14 × 89-mm centrifuge tubes) and spun in an SW41-Ti rotor (Beckman Coulter, Fullerton, CA) for 12 h at 41,000 rpm. Fractions (0.5 ml) were analyzed by SDS-PAGE stained with Coomassie Blue. The heterotrimeric kinesin-2 was resolved at ~10 S as three protein bands with relative mobilities of 86, 90, and 96 kDa. Each of the protein bands was carefully excised, digested with trypsin, and analyzed by matrix-assisted laser desorption/ionization time-of-

flight (MALDI-TOF) mass spectrometry to identify peptide masses. The peptides obtained for KRP86 were TTFDNAFDWNVTQR, ASYLEIYNEEVVR, SHSIFITITITIEQTQAQPEGHIR, LNLVDLAGSER, AALEAEGGALPEGFAT-GPGGEEIIVEK, ALDASFLEQMR, DMVIEAFIPPEEVQKVMK, AHWDDER-EVWVLER, and FKSENILNLELDLPER. The peptides obtained for Fla10 were GLIPNTFR, YVFEIAR, DLSQFVCK, LNLVDLAGSER, SSYLEIYNEEVVR, and KGELADLQEQFQR. The peptides obtained for KAP were ATGMIAQLFR, LLHNLSDFDHSLR, VVFYLVLDLQDK, SNVELLILATFLK, AGQVEEL-LYQLQSR, NTPELIALAVNLTQNPR, and NTENFEVLLSHETLMQTLR.

Chromosome Loss

Diploid strains were constructed by using either complementing *arg7* alleles that had been taken through meiosis within 1 month of their use to ensure the absence of other mutations or by using complementation with *nit2* and *ac17* mutations in a trans-configuration. For chromosome loss on linkage group VI, one arm was marked with the recessive *act2* mutation that confers resistance to 18 $\mu\text{g}/\text{ml}$ cycloheximide. The other arm was marked with the recessive mating-type plus allele (*mt+*) and scored by mating or by a PCR reaction to identify the *MID* or *FUS1* genes (Werner and Miergenhagen, 1998). Chromosome loss and mitotic recombination on linkage group III was assayed by the presence of *maa7*, *nit2*, and *ac17* mutations. 5-Methylanthranilic acid or 5-aminobenzoic acid was used at 1.6 mM and maintained in yellow Lucite boxes to reduce breakdown (Palombella and Dutcher, 1998). One arm was marked by *maa7* and *nit2*. The other arm is marked by *ac17*. Diploid cells were plated for single colonies on Sager and Granick rich medium with acetate and allowed to grow 5 d in the constant light at 21° or 32°C. Individual colonies were then transferred to liquid medium and grown for 24–48 h to a cell density of 10^6 cells/ml at 21°C to avoid clumps of cells that had been released from the mother cell wall. For experiments with *fla10-2*, the cells were treated with autolysin (Dutcher, 1995b) to release the cells from the mother cell to allow the plating of individual cells rather than groups of cells. Approximately 10^5 cells were inoculated onto cycloheximide or 5-methyl anthranilic acid (5-MAA) plates from each individual colony in four replicates. Approximately 3×10^2 cells were inoculated onto nonselective plates to determine cell number more precisely. After 10–14 d, the number of resistant colonies and the total number of viable cells on nonselective medium were determined. Because resistance could occur via spontaneous mutations, the rate of cycloheximide-resistant colonies in a haploid strain was determined. It occurred at $<1 \times 10^{-8}$ events/cell cycle. For linkage group VI, the mating type was determined and for linkage group III, the ability to grow on medium lacking acetate was determined.

Fluctuation analyses were performed on cultures, each derived from a single colony (Luria and Delbruck, 1943). For each fluctuation test, nine or 10 cultures were examined in parallel. The mean number of colonies was determined from the four replicates from each individual culture on selective medium and on nonselective medium. The number of distinct events rather than the number of resistant colonies was calculated through application of the “method of the median” (Lea and Coulson, 1949).

Modeling of Kinesin-2 onto KIF1A

The program Insight (Accelrys, Cambridge, MA) was used for modeling on a SGI workstation.

RESULTS

Identifying the Second Motor Subunit of Kinesin-2

Chlamydomonas kinesin-2 was previously isolated from flagella and shown to be a heterotrimeric complex with two distinct motor subunits, one encoded by the *FLA10* gene and a second motor subunit of unknown origin (Cole *et al.*, 1998). As *Chlamydomonas* expressed sequence tags became available, a sequence closely related to the *FLA10* gene was identified and used as a probe to screen libraries to identify both cDNA and genomic BAC clones for subsequent sequencing. cDNA and genomic DNA also were used directly as PCR template to amplify the gene for the purpose of sequencing. This kinesin-2 gene (KRP86) contains 12 exons with 11 introns (Figure 1A). The cDNA was determined to be 2926 base pairs with a 5'-untranslated region (UTR) of 141 base pairs and a 3'-UTR of 478 base pairs. The 2304-base pair open reading frame of KRP86 encodes a 768-amino acid kinesin with a predicted molecular mass of 86,056 Da and a pI of 6.59. The predicted protein was surprisingly similar to Fla10, displaying 46.9% identity throughout the protein and 71.7% identity within the NH₂-terminal 360 amino acid motor domain (Figure 1B).

To verify that the KRP86 gene encoded the second motor subunit, kinesin-2 was removed from *Chlamydomonas* flagella by AMPPNP extraction followed by ATP release of the kinesin. The kinesin-2 complex was purified on a 10–25% sucrose gradient, and the three subunits were fractionated by SDS-PAGE as described previously (Figure 1C; Cole *et al.*, 1998). MALDI-TOF mass spectrometry analysis of tryptic peptides of the 86-kDa kinesin-2 subunit generated 10 masses that matched nine predicted tryptic peptides for this kinesin (Figure 1D); one mass was unmatched. One of the nine sequences, LNLVDLAGSER, was not unique in that both Fla10 and the KRP86 protein both contain this conserved sequence. The kinesin-2 subunit with relative mobility of 90 kDa produced nine tryptic masses that matched exactly to five predicted Fla10 tryptic peptides (Figure 1D); four masses were unmatched. The 96-kDa protein generated 11 masses matched exactly eight predicted KAP tryptic peptides; three masses were unmatched (see *Materials and Methods*).

Identifying the FLA8 Locus

A restriction fragment length polymorphism (RFLP) for the kinesin-2 motor subunit was found previously and mapped to linkage group XII/XIII using meiotic progeny from a cross of a wild-type strain (137 or CC124) with a highly polymorphic strain (CC1952) (Gross *et al.*, 1988). It is tightly linked to RPL9 in 40 progeny (Bowers *et al.*, 2003). No known flagellar mutants mapped to this region. Although Adams *et al.* (1982) mapped many temperature-sensitive flagellar assembly mutants in *Chlamydomonas*, many of these were mapped incorrectly. Therefore, the mutant strains *fla1* and *fla8* were chosen to examine because the antero-grade-to-retrograde particle frequency ratio in these mutants was similar to the ratio for particles in the *fla10-1* mutant flagella (Iomini *et al.*, 2001). In addition, the flagellar IFT content of these mutants was biochemically similar to the patterns observed in the *fla10-1* mutant (Cole *et al.*, 1998). Thirty-nine progeny from a cross of *fla1* by CC1952 and 45 progeny from a cross of *fla8* by CC1952 were examined using the polymorphism used for mapping (see *Materials and Methods*). There was complete linkage between the temperature-sensitive flagellar assembly defect and the RFLP. As a control, no linkage was observed with the *fla2* mutation in 25 progeny. The gene was sequenced from the *fla1* strain and a single change was identified. The phenylalanine at position 55 is changed to a serine (F₅₅ to S₅₅) via a T-to-C transition. The gene from the *fla8* strain was sequenced and a single change was identified. The glutamic acid at position 21 is changed to a lysine (E₂₁ to K₂₁) via a G-to-A transition. Therefore, we will refer to the KRP86 locus as *FLA8*; the *fla8* allele (CC-1396) will be referred to as *fla8-1*. The *fla1* allele (CC-1389) will be referred to as *fla8-2*.

Identifying the *fla10-14* Mutation

Previously, we have shown that two of these mutations identified as temperature-sensitive flagellar assembly mutants in *Chlamydomonas* by Adams *et al.* (1982) were new alleles of *fla10* (Lux and Dutcher, 1991). We sequenced the *fla10-14* allele and found two changes between it and the published sequence (Walther *et al.*, 1994). The glutamic acid at position 24 is changed to a lysine (E₂₄ to K₂₄) via a G-to-A transition and the leucine at position 196 is changed to a phenylalanine (L₁₉₆-to-F₁₉₆) via a C-to-T transition. This glutamic acid is in a similar position to the glutamic acid that is changed in the *fla8-1* allele (Figure 1C).

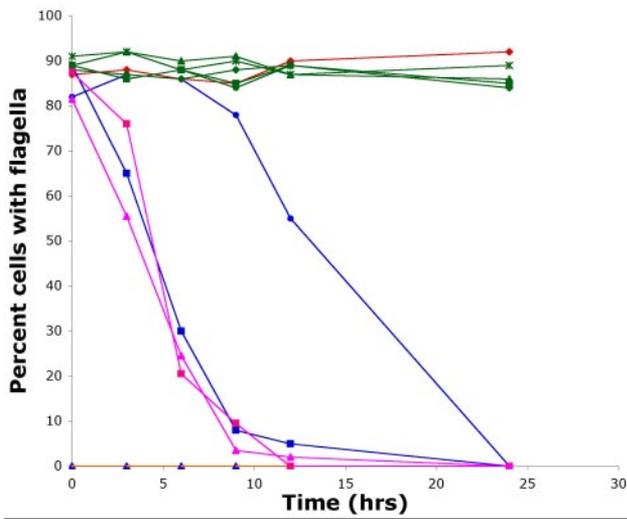


Figure 2. Flagellar loss of *fla8* and *fla10* mutant alleles when shifted to the restrictive temperature. Compared with wild-type cells (red diamond), the *fla10-1* (blue square), *fla10-14* (blue circle), *fla8-1* (pink square), and *fla8-2* (pink triangle) alleles begin to lose their flagella after 3 h at 32°C, whereas *fla10-2* (blue triangle) and *fla10-14; fla3* (orange triangle) are aflagellate at all temperatures. The *fla10-14* intragenic revertants, R16 (green square), R27 (green diamond), R10 (green circle), R8 (green asterisk), and R13 (green triangle) are identical to wild-type cells at the permissive and restrictive temperature.

gests that the L₁₉₆F lesion has no detectable phenotypic effect on flagellar assembly. It is likely that the aflagellate phenotype arises from the change at glutamic acid to lysine at amino acid 24. The L₁₉₆F change is likely to have been introduced by mutagenesis, because it is not present in the 137c parent.

Three of the remaining 17 revertants were picked at random and 7793 base pairs were sequenced to identify other reversion events. Three additional changes were found. Revertant R8 changes N₃₂₉ to H₃₂₉ via a transversion of an A to a C, R10 changes G₃₃₁ to S₃₃₁ via a transition of a G to an A, and R13 changes N₃₂₉ to T₃₂₉ via a transversion of an A to a C. An RFLP in an 894-base pair PCR product is observed with *Sau96I* in DNA from the G₃₃₁ to S₃₃₁ revertant. It cleaves the wild-type product into five bands and the mu-

tant product into four bands. Using this polymorphism, three additional revertants were demonstrated to have the G₃₃₁-to-S₃₃₁ event. The remaining 11 revertants have not been sequenced at this time, and the change(s) is not known in these revertants.

Fifteen revertants of *fla8-1* and 35 revertants of *fla8-2* were obtained. Each was crossed by wild-type cells, and 20–32 tetrads were examined for each revertant. The reversion event in each of the strains was linked to the original mutation and no temperature-sensitive progeny were recovered, which suggests that these are intragenic revertants. The flagellar phenotypes were determined by counting 200 cells at 21°C and at several time points for up to 24 h after shifting the cultures to 32°C. All showed wild-type frequencies of flagella with wild-type lengths (our unpublished data).

The motor domain of the *FLA8* gene from each revertant strain was sequenced (Table 2). For *fla8-1*, six true revertants that changed the serine back to phenylalanine were found. Nine other single or double mutants were found. The most frequent class (n = 6) changes M₃₁₃ to an isoleucine. For *fla8-2*, three true revertants were found that changed K₂₁ back to a glutamic acid. Thirty-two of the 35 revertants have a second-site alteration. Twenty-one of the strains change M₃₁₃. Four change the methionine to a valine and the remaining ones change it to an isoleucine. Eight of the revertants change G₃₁₄ to a serine, which was altered in three of the *fla8-1* revertants and in four of the *fla10-14* revertants.

Measuring Chromosome Loss and Mitotic Recombination in *Chlamydomonas*

The *apm1-122* allele confers resistance to the microtubule depolymerizing agent, oryzalin, which binds to α -tubulin (Morrisette *et al.*, 2004). The synthetic lethality of *apm1-122* with the *fla10* alleles and the dominance of this phenotype suggest that these alleles may interact with microtubules of the mitotic spindle in an aberrant way. We developed an assay for monitoring chromosome loss in *Chlamydomonas*.

To determine the rate of chromosome loss in *Chlamydomonas*, we constructed diploid strains that are heterozygous for markers on either linkage groups VI or III. These marked strains allow the detection of chromosome loss compared with mitotic chromosome recombination by using recessive phenotypic markers on both arms of a linkage group. On linkage group VI, one arm was marked with the recessive

Table 1. Analysis of intragenic revertants of *fla10-14*

Strain name	Amino acids of interest	Restriction fragment length polymorphism			Explanation
		E ₂₄ K ₂₅	L ₁₉₆ F	G ₃₃₁ S	
Wild-type	E ₂₄ K ₂₅ L ₁₉₆ N ₃₂₉	GGAGA	AGGTCCT	GGCCC	
<i>fla10-14</i>	G ₃₃₁ E ₂₄ K; F ₁₉₆	Not cut by <i>MboII</i>	Cut by <i>PpuMI</i>	Cut by <i>Sau96I</i>	Original mutations
R27	K ₂₄ E; F ₁₉₆	GGAGA	AGGTCCT	GGCCC	True revertant at position 24
R16	E ₂₄ K; K ₂₅ E; F ₁₉₆	Not cut by <i>MboII</i>	Not cut by <i>PpuMI</i>	Cut by <i>Sau96I</i>	Pseudorevertant
R10,R35 R36, R43	E ₂₄ K; F ₁₉₆ ; G ₃₃₁ S	GAAGG	AGGTCCT	GGCCC	Pseudorevertant
R8	E ₂₄ K; F ₁₉₆ ; N ₃₂₉ H	Not cut by <i>MboII</i>	Not cut by <i>PpuMI</i>	Cut by <i>Sau96I</i>	Pseudorevertant
R13	E ₂₄ K; F ₁₉₆ ; N ₃₂₉ T	GAAGA	AGGTCCT	GGCCC	Pseudorevertant
		Cut by <i>MboII</i>	Not cut by <i>PpuMI</i>	Cut by <i>Sau96I</i>	

Table 2. Intragenic revertants of *fla8-1* and *fla8-2*

Strain	Amino acid change	Codon	Explanation
Wild-type	F ₅₅	TTC to TCC	Original mutation
<i>fla8-1</i>	F ₅₅ S	TCC to TTC	True revertant
R1, R5, R8, R19, R22, R27	S ₅₅ F	TCT to TAT	Pseudorevertant
R9	F ₅₅ S; N ₅₇ I	AAT to ATT	Pseudorevertant
R17	F ₅₅ S; N ₄₂ H	AAC to CAC	Pseudorevertant
R29	F ₅₅ S; M ₃₁₃ I	ATG to ATA	Pseudorevertant
R4, R7, R8, R18	F ₅₅ S; M ₃₁₃ I; G ₃₁₄ S	ATG GGC to ATA AGC	Pseudorevertant
R21, R24	E ₂₁ K	GAG to AAG	Original mutation
<i>fla8-2</i>	K ₂₁ E	AAG to GAG	True revertant
R17, R23, R36a	E ₂₁ K; N ₅₇ Y	AAT to TAT	Pseudorevertant
R10	E ₂₁ K; H ₁₀₀ Y	CAT to TAT	Pseudorevertant
R4, R8d	E ₂₁ K; N ₃₁₂ T	AAC to ACC	Pseudorevertant
R29	E ₂₁ K; M ₃₁₃ V	ATG to GTG	Pseudorevertant
R2, R19, R25, R26	E ₂₁ K; M ₃₁₃ I	ATG to ATA	Pseudorevertant
R8a, R9, R10, R11, R13, R18, R20, R21, R28	E ₂₁ K; M ₃₁₃ I	ATG to ATT	Pseudorevertant
R14, R15, R22, R24, R30, R33, R35	E ₂₁ K; M ₃₁₃ I; G ₃₁₄ S	ATG GGC to ATA AGC	Pseudorevertant
R16	E ₂₁ K; G ₃₁₄ S	GGC to AGC	Pseudorevertant
R5, R6, R25, R27, R31, R32, R36b			

act2 allele that confers resistance to cycloheximide and the other arm was marked by the mating-type locus. Cells heterozygous for the plus and minus mating-type alleles show a minus phenotype and cells that have lost the minus alleles show the plus mating-type phenotype. Chromosome loss is detected by resistance to cycloheximide and a mating-type plus phenotype. Mitotic recombination produces cycloheximide resistance and a mating-type minus phenotype. On linkage group III, one arm is marked with the *NIT2* and *maa7* alleles and the other arm is marked with the *ac17* allele. The other homolog has the wild-type dominant alleles for *MAA7* and *AC17* and the recessive allele, *nit2* (Figure 3). Loss of one homolog can be detected by cells that show resistance to 5-MAA, a suicide substrate for tryptophan synthetase, which is the product of the *MAA7* gene (Palombella and

Dutcher, 1998). These cells are able to grow on nitrite medium, but they require acetate. Mitotic recombination on this arm would result in cells that are resistant to 5-MAA but do not require acetate.

To verify that we could detect chromosome loss and mitotic recombination, we assayed untreated cells as well as cells treated with various agents. These agents include gamma irradiation, UV light, and addition of colchicine, a microtubule-destabilizing agent. Gamma irradiation at 500 mrem for 20 min increased the number of cycloheximide-resistant colonies from 10 per 10⁵ to 45 per 10⁵ cells (Figure 4). At the 20-min time point, 90% of the cycloheximide-resistant colonies showed a mating-type plus phenotype, which is consistent with chromosome loss. Given the starting frequency of chromosome loss events, it is likely that the increase in cells resistant to cycloheximide arises primarily from chromosome loss rather than mitotic recombination.

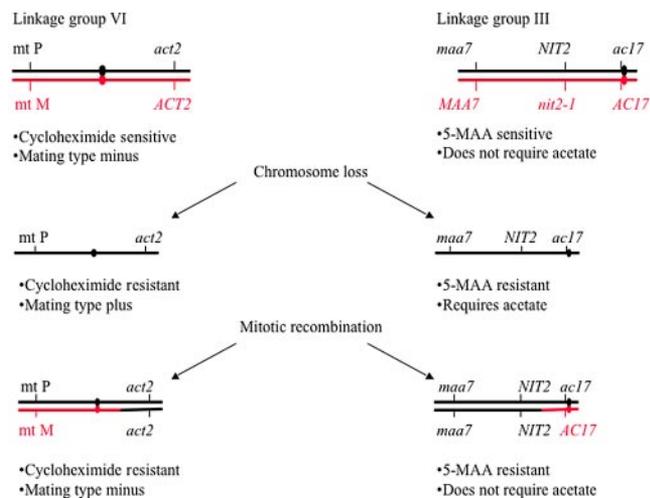


Figure 3. Determining chromosome loss using markers on linkage group III and VI. An increased frequency of chromosome loss results in cells that are cycloheximide-resistant or mating-type plus and 5-MAA-resistant and requires acetate for growth. Cells that have undergone mitotic recombination will be cycloheximide resistant and mating-type minus and 5-MAA resistant and do not require acetate for growth.

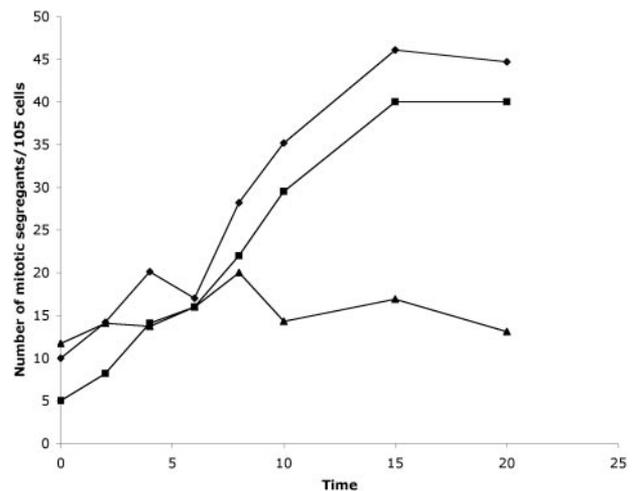


Figure 4. Effect of UV irradiation on mitotic segregation of linkage group VI after various exposure times (in minutes) to UV irradiation. Mitotic recombination and chromosome loss of linkage group VI (diamond), chromosome loss of linkage group VI (square), and mitotic recombination of the left arm of linkage group VI (triangle).

Table 3. Rates of mitotic recombination and chromosome loss in wild-type, *fla8*, and *fla10* diploid strains

Genotype	No. trials		21°C		32°C	
	21°	32°	MR	CL	MR	CL
<i>FLA10+ / FLA10+; FLA8+ / FLA8+</i>	12	10	5.3	3.9	4.8	6.3
<i>FLA10/fla10-1</i>	4	4	7.8	11.5	8.3	12.7
<i>fla10-1/fla10-1</i>	2	8	4.9	19.2	7.8	21.5
<i>FLA10/fla10-14</i>	3	3	6.1	13.8	5.7	15.9
<i>fla10-14/fla10-14</i>	3	3	5.3	27.9	6.0	33.1
<i>FLA10/fla10-2</i>	2	2	5.6	4.5	6.7	6.2
<i>fla10-2/fla10-2</i>	2	2	5.9	6.3	6.3	5.9
<i>fla10-2/fla10-14</i>	3	3	5.9	12.9	5.4	14.7
<i>fla10-14R16/fla10-14R16</i>	2	2	4.9	4.6	4.3	5.7
<i>fla10-14R27/fla10-14R27</i>	2	2	3.7	4.6	4.6	6.2
<i>fla10-14R10/fla10-14R10</i>	2	2	4.6	5.1	3.6	4.9
<i>fla10-14R8/fla10-14R8</i>	2	2	5.4	5.2	6.1	5.7
<i>fla10-14R13/fla10-14R13</i>	2	2	5.2	5.9	6.3	6.0
<i>fla10-14/fla10-14; fla3-1/fla3-1</i>	2	2	5.2	9.3	5.7	5.4
<i>fla10-14R16/fla10-14R16; fla3-1/fla3-1</i>	2	2	4.9	5.5	5.3	4.6
<i>fla8-1/fla8-1</i>	2	2	4.3	20.1	5.2	22.6
<i>fla8-2/fla8-2</i>	2	2	4.7	17.5	3.9	18.4
<i>fla8-1/fla8-2</i>	2	2	3.6	14.4	4.3	13.6
<i>fla8-1/FLA8</i>	2	2	5.3	11.8	5.2	14.7
<i>fla8-2/FLA8</i>	2	2	5.7	13.6	4.2	15.9
<i>fla8-1R17/fla8-1R17</i>	2	2	4.5	4.9	5.0	4.9
<i>fla8-1R29/fla8-2R29</i>	2	2	4.7	5.3	4.5	4.1
<i>fla8-2R4/fla8-2R4</i>	2	2	5.6	5.9	4.5	7.1
<i>fla8-2R14/fla8R14</i>	2	2	3.3	6.0	3.7	6.9
<i>fla8-2R16/fla8R16</i>	2	2	3.9	4.8	4.3	5.7

CL, chromosome loss; MR, mitotic recombination.

UV irradiation had little effect on the rates of either mitotic recombination or chromosome loss. Treatment with colchicine resulted in only a twofold increase in cycloheximide resistance after treatment at 5 mM for 24 h, but the viability of the cultures decreased to <10% of the initial viability (our unpublished data). Wild-type cells show a similar rate of loss using linkage group III markers. Chromosome loss for linkage group III occurred at a rate of 6.9 per 10⁵ cells (n = 6).

Mitotic Segregation in *fla10* Alleles

The rate of mitotic segregation in three *fla10* alleles that fail to assemble flagella as well as four of the intragenic revertant strains was determined using linkage group VI for the assay (Table 3). In wild-type diploid strains, mitotic recombination occurs with a rate of 5.3 events per 10⁵ cells at 21°C and with a rate of 4.8 events per 10⁵ cells at 32°C. Chromosome loss is slightly increased by temperature; the rates are 3.9 and 6.3 events per 10⁵ cells at 21 and 32°C, respectively. Mitotic segregation in the two temperature-sensitive alleles, *fla10-1* and *fla10-14*, was monitored both in heterozygous and homozygous diploid strains. Both alleles show an increased rate of loss compared with the wild-type cells in both heterozygous and homozygous strains at both temperatures. Thus, the chromosome loss phenotype is dominant. It is also dosage sensitive for both alleles as the heterozygous strains show greater rate increases than the heterozygous strains (Table 3). The incubation at 32°C increases the loss for both alleles.

To ask whether the dominance results from a decrease in Fla10 gene product, the behavior of the *fla10-2* strain, which has a null allele (Matsuura *et al.*, 2002), was examined in both heterozygous and homozygous diploid strains. This allele

shows no increase in chromosome loss at either temperature (Table 3). Furthermore, a diploid strain that is heteroallelic for the *fla10-2* and *fla10-14* alleles is similar to the *fla10-14/FLA10* strain. Thus, the increased chromosome loss arises from an acquired feature of the mutant alleles.

Five different intragenic revertants were analyzed in homozygous diploid strains. None of the revertants show the loss phenotype (Table 3). Thus, both the flagellar assembly and the chromosome loss phenotypes were reverted by the second-site events. In addition, we monitored the rate of chromosome loss in the *fla3-1* allele, which encodes KAP. This allele has no effect on chromosome segregation or mitotic recombination (Table 3).

Double mutants between *fla10-14* and mutations in other kinesin-2 subunits were constructed. The *fla10-14; fla8-1*, *fla10-14; fla8-2*, and *fla10-14; fla3-1* double mutants were aflagellate at 21 and 32°C (Figure 2). The double mutant *fla10-14; fla3-1*, shows no increase in chromosome loss compared with the wild-type strains (Table 3).

Mitotic Segregation in *fla8* Alleles

We monitored chromosome loss and mitotic recombination in the two *fla8* alleles to ask whether they resulted in increased rates of chromosome loss similar to the *fla10* alleles. Both alleles show increased rates of loss (Table 3). We examined three intragenic revertants for both alleles and found they restored wild-type levels of chromosome loss (Table 3).

Monitoring Cytokinesis in *fla8* and *fla10* Cells

We monitored the fidelity of cytokinesis in wild-type, *fla8-1*, *fla8-2*, and *fla10-14* cells by examining 21 and 32°C grown cells for the presence of binucleated cells, as determined by

staining with the DNA-specific dye, 4,6-diamidino-2-phenylindole. No increase was observed ($n = 200$).

DISCUSSION

Identification of a Temperature-sensitive Flagellar Assembly Mutation in the Second Motor Subunit of Kinesin-2

The *FLA10* gene encodes one of the two motor subunits that comprise the anterograde motor kinesin-2 (Walther *et al.*, 1994; Kozminski *et al.*, 1995). We have identified the second motor subunit by its homology to the Fla10 subunit among expressed sequence tags and then by sequencing cDNA and genomic DNA. Mass spectrometry of isolated kinesin-2 confirmed the identification. This subunit is encoded by the *FLA8* gene in *Chlamydomonas* and sequencing of DNA from the *fla8-1* and *fla1* (renamed *fla8-2*) mutant strains revealed single amino acid changes in each. A phenylalanine is mutated to a serine (F₅₅S) in *fla8-1* and a glutamic acid is changed to a lysine (E₂₁K) in *fla8-2*. These results agree with the suggestion of Iomini *et al.* (2001) that the *fla8* and *fla1* mutants are defective in assembly and transport of anterograde particles but that their retrograde particle assembly and transport are comparable with wild-type cells. Six additional temperature-sensitive alleles have been identified at the *FLA8* locus, but the mutational changes have not been identified at this time (originally designated as *fla1-2* to *fla1-7*) (Iomini *et al.*, 2001). Unlike most of the loci identified with temperature-sensitive flagellar assembly defects (Adams *et al.*, 1982; Iomini *et al.*, 2001), multiple alleles are available for *FLA8* (8 alleles) and for *FLA10* (4 alleles). With the recent finding that the *FLA3* locus encodes the KAP subunit (Mueller *et al.*, 2005), conditional alleles are now available for the three subunits of kinesin-2 in *Chlamydomonas*. Double mutants between different temperature-sensitive alleles (*fla10*; *fla8*, *fla10*; *fla3*, and *fla8*; *fla3*) are unable to assemble flagella at any temperature, which suggests that the partial function of any two subunits result in the inability to assemble flagella.

*Characterization of Intragenic Revertants of the *fla8* and *fla10* Mutants Reveals an Important Domain*

In addition to sequencing the *fla8* alleles, we identified two mutations in a second temperature-sensitive *fla10* allele (*fla10-14*); a glutamic acid at position 24 is changed to a lysine (E₂₄K) and a leucine at position 196 is changed to a phenylalanine (L₁₉₆F). Both *fla8-2* and *fla10-14* have the same glutamic acid-to-lysine change, which suggests that this residue has an important function. This hypothesis is strengthened by characterization of the intragenic revertants of the three mutant alleles *fla10-14*, *fla8-1*, and *fla8-2*. The sequencing of independently isolated intragenic revertants of the *fla10-14* allele revealed changes in amino acids 329 and 331. Because of a short insertion in *FLA10* (Figure 1D), the amino acid sequences of *FLA10* and *FLA8* are offset; these amino acids of Fla10 correspond to amino acids 312 and 314 in Fla8. In the *fla8-1* and *fla8-2* intragenic revertants, the same or similar mutations are observed in the revertants. At amino acid 312, one *fla8-2* revertant is changed from an asparagine to a threonine. Many *fla8-1* and *fla8-2* revertants change amino acid 313 from a methionine to either an isoleucine or a valine. Seven *fla8-2* revertants have a glycine-to-serine change at amino acid 314. Three revertants have a dinucleotide change (GG to AA) that change positions 313 and 314; the methionine is mutated to an isoleucine and the glycine is changed to a serine. The *fla10-1* mutation changes an aspar-

agine residue to a lysine (N₃₂₉K) (Walther *et al.*, 1994), whereas changes to another polar amino acid revert the defects in two of the *fla10-14* intragenic revertants (N₃₂₉H) and in one of the *fla8-2* intragenic revertants (N₃₁₂T).

To ask whether the amino acids altered in the revertants lie near the original mutations, the Fla8 and Fla10 motor domain amino acid sequences were aligned with the KIF1A motor to allow the identification of amino acids that corresponded to the sites of interest in the *fla10-14*, *fla8-1*, and *fla8-2* mutant and revertant proteins (Figure 5G). When the mutations of the intragenic revertants are positioned onto the structure (Nitta *et al.*, 2004), most are found in proximity to the original mutations (Figure 5, A–F). One notable exception is the *fla10-14* mutation, L₁₉₆F, which corresponds to M₁₉₆ in KIF1A. (Figure 5, A and B). Because reversion of the other mutation (E₂₄K₂₄E₂₄) in the *fla10-14* strain results in wild-type behavior, it seems that the L₁₉₆F mutation does not affect activity of Fla10. These results are consistent with the conclusion that the L₁₉₆F change may be a silent polymorphism.

Although KIF5 has the highest similarity with kinesin-2, KIF1A was chosen because it was crystallized with AMP-PNP and the positions of the nucleotide relative to the reversion events could be examined. The kinesin-2 mutations are not in the regions that change the interaction with the microtubule, but they are very close to the bound nucleotide and the strongly conserved P-loop (Figure 5, A–F). This suggests that reversion may result from a stabilization of the kinesin ATPase. The idea that these mutant kinesins may have reduced activity is supported by the observation that, even at the permissive temperature, the rate of anterograde IFT in *fla8-2* strains is slower than the rate of anterograde IFT observed in wild-type strains (Iomini *et al.*, 2001). Because the *fla10-14* strain carries the same mutation as in *fla8-2*, we predict that anterograde IFT will move more slowly than in wild-type flagella. The anterograde IFT rate in the *fla8-1* strain is close to wild type at the permissive temperature (Iomini *et al.*, 2001). It is possible, however, that the rates of IFT in *fla8-1*, *fla8-2*, and *fla10-14* could all dramatically decrease upon shifting to the restrictive temperature of 32°C.

Interestingly, the aromatic ring of Y₁₀₅ of KIF1A is aligned with the nucleotide base so that these two are considered to be stacked (Figure 5, D and F). This is notable, in part, because two revertants of *fla8-2*, R4 and R8d, were rescued when a C-to-T transition changed the corresponding histidine at position 100 (H₁₀₀) to a tyrosine. The N (M/I) G motif (A L S in KIF1A) is particularly close to the P-loop that wraps around the phosphate groups of the bound nucleotide (Figure 5). Without solving the motor domain structure of Fla8 or Fla10, we cannot be certain how the original and mutated amino acids might interact with the P-loop. However, it is tempting to speculate that this motif may be important for motor activity.

To determine whether these mutated residues are conserved in various orthologues or whether they are specific to the kinesin-2 subunits, BLAST searches were performed. The glutamic acid mutated to lysine in both the *fla10-14* and *fla8-2* mutants is conserved in all kinesin-2 subunits ($n = 20$). The phenylalanine, which is mutated in the *fla8-1* mutant, is conserved only in kinesin-2 subunits with one conservative change to tyrosine in chicken. The N (M/I) G motif in *FLA8* and *FLA10* is not as well conserved among kinesins. Although the majority of the residues are identical in the kinesin-2 subunits, there are a few exceptions including *Tetrahymena*, which has an alanine, isoleucine, and serine at these positions. There is little sequence conservation of these

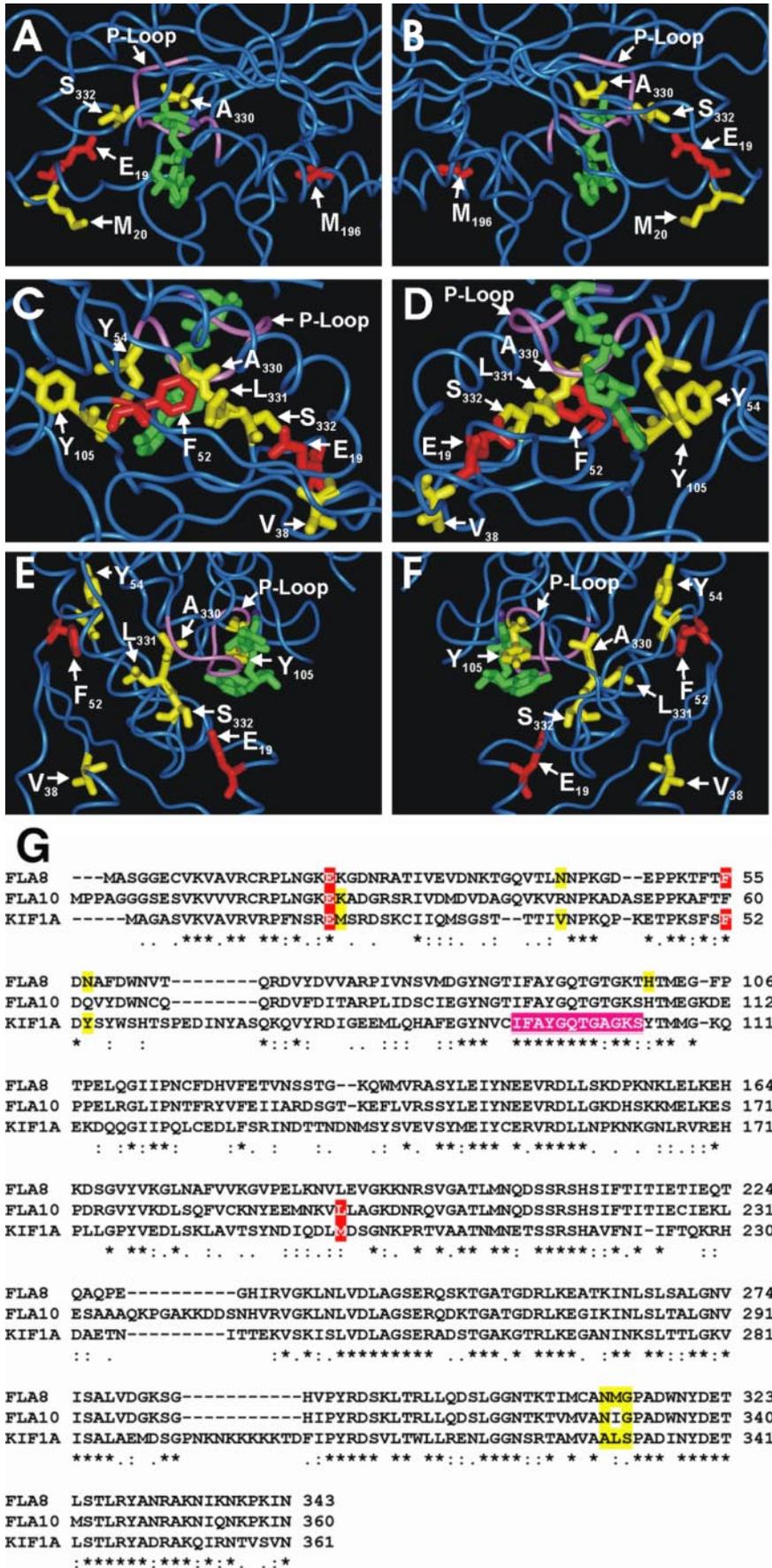


Figure 5. Location of amino acids altered in the *fla8* and *fla10* mutant and revertant alleles as modeled onto the crystal structure of KIF1A with AMPPNP (green) bound (PDB 1VFV). The P-loop is shown in purple. (A and B) Fla10 E₂₄ and L₁₉₆ correspond to KIF1A E₁₉ and M₁₉₆, respectively, and are shown in red. Revertant *fla10-14* amino acids K₂₅, N₃₂₉, and G₃₃₁ correspond to KIF1A M₂₀, A₃₃₀, and S₃₃₂, respectively, and are shown in yellow. (C–F) Fla8 amino acids E₂₁ and F₅₅ correspond to KIF1A E₁₉ and F₅₂, respectively, and are shown in red. Revertant *fla8-1* and *fla8-2* amino acids N₄₂, N₅₇, H₁₀₀, N₃₁₂, M₃₁₃, and G₃₁₄ correspond to KIF1A V₃₈, Y₅₄, Y₁₀₅, A₃₃₀, L₃₃₁, and S₃₃₂, respectively, and are shown in yellow. (G) Alignment of Fla8, Fla10, and KIF1A in the regions of interest. Red shading indicates mutations in one of the kinesin-2 motor subunits, yellow shading indicates amino acids that are altered in the revertants, and purple shading indicates the P-loop residues.

residues in other kinesin family members. As such, it seems as though the mutated residues are specific to the kinesin-2 subunits and reinforce the hypothesis that they are critical to its proper function.

The *fla8* and *fla10* Mutant Alleles Produce Proteins with Aberrant Function

It was recently shown that overexpression of the KIF3B tail results in cytokinesis defects in mammalian cells (Fan and Beck, 2004). To determine whether the *fla8* and *fla10* alleles showed this phenotype, we assayed for the presence of binucleated cells. There is no cytokinesis defect observed in the *fla10-14*, *fla8-1*, and *fla8-2* alleles; however, there is a small, but significant, increased frequency of chromosome loss in both homozygous and heterozygous diploids, which also was observed in the *fla10-1* allele. These results indicate that the chromosome loss phenotype is dominant unlike the flagellar assembly defect, which is recessive. This phenotype was not observed in the *fla10-2* allele, which is a null allele (Matsuura *et al.*, 2002). Diploid strains heteroallelic for the *fla10-2* and *fla10-14* alleles also show an increased frequency of chromosome loss. Together, these data suggest that the *fla10-1*, *fla10-14*, *fla8-1* and *fla8-2* alleles show a gain of function phenotype and the mutant proteins have acquired an aberrant or toxic function(s). Previous studies showed that the Fla10 protein is found at the mitotic spindle poles as well as at the basal bodies during interphase in *Chlamydomonas* (Vashishtha *et al.*, 1996; Deane *et al.*, 2001). We hypothesize that these mutant kinesins could act at the spindle poles to alter centrosome separation or attachment. Alternatively, it is possible that one of the IFT polypeptides is required for chromosome segregation and the IFT polypeptide is mislocalized by the mutant kinesin-2.

Intriguingly, diploids homozygous for both *fla10-14* and *fla3-1* do not have a chromosome loss phenotype, which suggests that the phenotype relies on a functional Fla3 protein. Mueller and colleagues (2005) showed that the Fla10 protein does not localize to the basal body region properly in *fla3* mutant cells. In sea urchins, it has been reported that the KAP subunit translocates into the nucleus before mitosis (Morris *et al.*, 2004). In *Chlamydomonas*, the KAP subunit localizes to the basal body region in *Chlamydomonas* in wild-type interphase and mitotic cells, but it was not reported to be in the nucleus (Mueller *et al.*, 2005). Regardless of its localization, the mutant motor subunits require wild-type Fla3 to affect chromosome segregation. The chromosome loss phenotype was not present in the *fla10-14*, *fla8-1*, or *fla8-2* intragenic revertants, which suggests that these changes removed the toxic or aberrant interactions caused by the original mutations in the *fla10* and *fla8* alleles.

This aberrant function of mutant Fla10 protein could explain the genetic interaction between *apm1-122* and various *fla10* alleles that causes a synthetic cold-sensitive cell division phenotype (*fla10-1*) or a slow growth phenotype (*fla10-14*) at 21°C (Lux and Dutcher, 1991). Although the product of the *APM1* gene is unknown, it may encode a microtubule-associated protein needed for spindle function.

ACKNOWLEDGMENTS

We thank Dr. Gary Daughdrill for assistance with the modeling. Alex Edwards and Rona Robinson-Hill helped with the sequencing of the *fla10-14* allele. They were supported by funds from Washington University and the Howard Hughes Medical Institute Award to Dr. Sarah Elgin. We thank Samantha Reed and Julie Sahalie for technical assistance cloning the *FLA8* gene. They were supported by funds from the University of Idaho. This work was supported by National Institutes of Health Grants GM-32843 (to S.K.D.) and GM-61920 (to D.G.C.).

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