Two *Saccharomyces cerevisiae* Kinesin-related Gene Products Required for Mitotic Spindle Assembly

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Abstract. Two *Saccharomyces cerevisiae* genes, *CIN8* and *KIP1* (a.k.a. *CIN9*), were identified by their requirement for normal chromosome segregation. Both genes encode polypeptides related to the heavy chain of the microtubule-based force-generating enzyme kinesin. Cin8p was found to be required for pole separation during mitotic spindle assembly at 37°C, although overproduced Kiplp could substitute. At lower temperatures, the activity of at least one of these proteins was required for cell viability, indicating that they perform an essential but redundant function. Cin8p was observed to be a component of the mitotic spindle, colocalizing with the microtubules that lie between the poles. Taken together, these findings suggest that these proteins interact with spindle microtubules to produce an outwardly directed force acting upon the poles.

CHROMOSOMES are segregated in mitosis by the spindle, a microtubule-based structure that orchestrates a series of spatially and temporally controlled motility events. Although elegant observational studies have produced detailed descriptions of mitotic movements (reviewed in Hyams and Brinkley, 1989; Inoue, 1981; Nicklas, 1988), the mechanisms by which forces are produced by the spindle have remained uncharacterized. A molecular description of mitosis will require the identification of the force generators that produce the following motility events: separation of duplicated spindle poles to generate the bipolar spindle structure; chromosome attachment and congression to the metaphase plate; pre-anaphase chromosome oscillations; intracellular spindle positioning; and the two major anaphase movements, sister chromatid movement toward opposite poles (anaphase A), and further separation of the spindle poles (anaphase B). Observations of mitosis in numerous eukaryotic cell types suggest that many of these motility events are conserved.

It is probable that some spindle motile forces are produced through the actions of microtubule-based mechanochemical enzymes (McIntosh and Pfarr, 1991; Sawin and Scholey, 1991). These enzymes (or microtubule-based "motors") translate chemical energy into forces exerted along the length of microtubules (Vallee and Shpetner, 1990). The discovery of kinesin (Vale et al., 1985), a motor protein capable of translocating vesicles and organelles toward the plus ends of microtubules, suggested the possibility that similar types of motors may accomplish mitotic movements. Indeed, roles for kinesin-related proteins have been suggested by the localization of related epitopes to the spindle (Sawin et al., 1992) and by genetic observations (see Discussion).

An expected phenotypic consequence of mitotic spindle malfunction is a decrease in the fidelity of chromosome transmission. We have previously reported the identification of *Saccharomyces cerevisiae* mutants that lose a supernumerary chromosome III at elevated rates during mitotic growth (Hoyt et al., 1990). Among this collection of mutants was recognized a subset with defective microtubule structure. New mutant alleles of the two α-tubulin-encoding loci were identified, as well as mutant alleles of the *CIN1*, *CIN2*, and *CIN4* genes (*c*hromosome *in*stability), required for normal microtubule stability. These findings demonstrated the utility of this chromosome loss method for the identification of gene products that participate in spindle action.

In this paper, we report the genetic identification of the *CIN8* gene by its requirement for normal chromosome transmission fidelity. The *CIN9* gene was identified by its ability to suppress a *cin8* conditional-lethal phenotype. Both genes encode polypeptides related to the heavy chain of kinesin. Analysis of *CIN9* revealed that it is allelic to *KIP1*, identified in a kinesin homology-based screen and also described in the accompanying paper (Roof et al., 1992). Henceforth, we will use the *KIP1* designation. We demonstrated that the *CIN8* and *KIP1* gene products (Cin8p and Kiplp) perform overlapping and essential roles. The function of at least one of these proteins is required to separate duplicated spindle poles during the assembly of the mitotic spindle. Cin8p was observed to be a component of the mitotic spindle, colocalizing with the microtubules that lie between the poles.

Materials and Methods

Yeast Strains and Media

The yeast strains used in these experiments are derivatives of S288C and are listed in Table I. The original *cin8-1*, *-2*, and *-3* strains were derived from the collection of strains elevated for mitotic loss of a supernumerary chromosome III (Hoyt et al., 1990). Allelism was demonstrated by linkage

Table I. Yeast Strains and Plasmids

Strain or plasmid	Relevant genotype
MAY589	*a CIN8 KIP1*
MAY1558	*a cin8-1*
MAY1561	*a cin8-2*
MAY1563	*a cin8-3*
MAY2061	*a cin8::URA3*
MAY2077	*a kip1::HIS3*
MAY2123	*α::CYH2/a LEU2::CAN1-SUP11-1/leu2* (chromosome III disome) *ade2-101 his3-Δ200 ura3-52 can1^R cyh2^R*
MAY2166	same as MAY2123, but *kip1::HIS3*
MAY2169	*a cin8-3 kip1::HIS3*
MAY2177	*a cin8::URA3*
MAY2205	*a cin8::LEU2 kip1::HIS3 ura3-52* (pMA1200)
MAY2209	*a/α cin8::LEU2/cin8::LEU2 kip1::HIS3/kip1::HIS3* (pMA1200)
MAY2210	*a/α cin8::LEU2/cin8::LEU2 kip1::HIS3/kip1::HIS3* (pMA1201)
MAY2275	*a cin8::URA3 kip1::HIS3 leu2-3,112 cyh2^R* (pMA1208)
MAY2305	*a cin8::URA3 kip1::HIS3 lys2-801* (pMA1212)
MAY2551	same as MAY2123, but *cin8::URA3*
pMA1112	*CIN8 URA3*
pMA1125	*CIN8 URA3 (CEN)*
pMA1129	*KIP1 URA3 (CEN)*
pMA1200	*CIN8*-hemagglutinin tag *URA3 (CEN)*
pMA1201	*CIN8*-hemagglutinin tag *URA3* (2 μm)
pMA1208	*CIN8 CYH2 LEU2 (CEN)*
pMA1212	*CIN8-Δ99 LYS2 (CEN)*

analysis and by the failure of the three mutants to complement for the temperature-sensitive viability phenotype.

Rich (YPD), minimal (SD) and sporulation media were as described (Sherman et al., 1983). α-factor (Sigma Chemical Co., St. Louis, MO) was added to log-phase cultures in YPD (pH = 4.0) to a final concentration of 6 μg/ml. The cultures were then incubated at 26°C until >80% of the cells had assumed an unbudded morphology (3–4 h). To release from α-factor arrest, cells were centrifuged and resuspended in the same media minus the inhibitor.

Quantitative Measurement of Chromosome Loss

The chromosome loss tester strains originally described carried a supernumerary chromosome III marked with two loci that cause sensitivity to antibiotics, one on each side of the centromere (Hoyt et al., 1990). The left arm of the tester chromosome III was marked with *CAN1* and the right arm with *CRY1*. The tester strains also carried recessive resistance alleles for these two genes at their normal chromosomal loci. Therefore, loss of the marked chromosome III allowed the simultaneous phenotypic expression of the two recessive alleles causing resistance to both canavanine and cryptopleurine. The current unavailability of cryptopleurine necessitated the construction of a new tester strain (to be described elsewhere). Briefly, in strain MAY2123 (Table I), DNA encoding the *CYH2* gene was inserted near the *MAT* locus on the right arm of the marked chromosome III. This chromosome III also carries *CAN1* on the left arm, as did the original tester strains. The normal genomic *CAN1* and *CYH2* loci (chromosomes V and VII, respectively) are marked with recessive resistance alleles. Loss of this copy of chromosome III, therefore, results in the simultaneous acquisition of resistance to canavanine and cycloheximide.

MAY2123 and *cin8::URA3* and *kip1::HIS3* derivatives (MAY2551 and MAY2166, respectively) were grown into colonies from single cells at 26°C on YPD agar media. 3 d after plating, eight to ten colonies were cut from the agar surface, separately suspended in water, lightly sonicated, and titered. The frequencies reported represent the titer of cells that had lost the marked chromosome III, determined by plating on SD plus canavanine (75 μg/ml) and cycloheximide (5 μg/ml) (supplemented with adenine and uracil and all amino acids except arginine), divided by total cell number, determined by plating on YPD. The median value in each experiment is reported in Table II. A portion of each colony suspension was diluted into YPD and incubated at 26°C for 3 h followed by 12 h at 37°C. The frequency of cells that had lost chromosome III was then determined as described for the 26°C samples.

DNA Manipulations

A *URA3-CEN* library of *S. cerevisiae* DNA fragments (Rose et al., 1987) was transformed into both MAY846 (*cin8-1*) and MAY851 (*cin8-2*). Ura$^+$ transformants were replica transferred to YPD plates and incubated at 37°C. The plasmids from 12 temperature-resistant clones were extracted and transformed into *Escherichia coli* for analysis. Restriction enzyme analysis revealed that eleven of the plasmids contained genomic DNA inserts that overlapped. A fragment from this locus was introduced into a *URA3*-containing yeast integrating vector and the resulting plasmid (pMA1112) was cut within the insert and transformed into a *ura3* strain. This resulted in a duplication at this genomic locus that is marked with *URA3*. The strain with the *URA3*-marked locus was crossed to *ura3 cin8-1, -2,* and *-3* strains and the resulting diploids were sporulated. Only parental genotypes were recovered; all asci analyzed contained two temperature-resistant, Ura$^+$ spores and two temperature-sensitive, Ura$^-$ spores. This indicated that the isolated genomic DNA contains the bona fide *CIN8* locus. The unique genomic insert was found to contain the *cin8*-suppressing *KIP1* locus (originally designated *CIN9*).

The regions encoding the *CIN8* and *KIP1* genes were determined by the subcloning of smaller genomic fragments (Sambrook et al., 1989) and by mutagenesis with a modified bacterial Tn*10* transposon (Huisman et al., 1987). DNA sequencing was accomplished by subcloning into M13-based vectors and use of the Sequenase reagent kit (United States Biochemical, Cleveland, OH). Only 600 bp of *CIN9* were sequenced as this was sufficient to reveal that this locus is equivalent to *KIP1* (Roof et al., 1992). Database searches were performed using the FASTA computer program (Pearson and Lipman, 1988). Pairwise comparisons of amino acid sequence similarity and multiple sequence alignments were performed using the GAP, PILEUP, and PRETTY computer programs (Devereux et al., 1984). Adjacent to *CIN8* was found the sequence of the end of *PRB1*, a gene located at the distal tip of the left arm of chromosome V near the *CAN1* locus (Moehle et al., 1987; Hoffmann, 1985; Mortimer et al., 1989). This finding agreed with the observed tight linkage of *CIN8* to the *CAN1* locus (58 PD: 0 NPD: 3 T) and the coincidence of restriction enzymes fragments when *CIN8* and *CAN1* DNAs were compared. The order on chromosome V is *CEN-PRB1-CIN8-CAN1*.

The 12CA5 epitope sequence was attached to the 3′-end of the *CIN8* open reading frame by oligonucleotide-directed mutagenesis with the Muta-Gene reagent kit (Bio-Rad Laboratories, Cambridge, MA). The following oligonucleotide was used:

5′-GAAAAATGTTAAAGATTGAATACCCATACGACGTCCCAGACTAC-GCTTAGTTGATATTGCCTTTC-3′

The *CIN8-Δ99* allele is a deletion of 99 amino acids from the motor domain of Cin8p that are replaced with seven amino acids from Kiplp (see Results). The following oligonucleotide was used to create *CIN8-Δ99*:

5′-GCAAATTCTGGATGTATATGGATGAATTATTGTTGTTATTAGCAA-AAATCCTCAATTTTTTC-3′

The final mutagenesis products were confirmed by DNA sequencing.

Marked deletions of *CIN8* and *KIP1* were generated by the one-step gene replacement method (Rothstein, 1983). For *cin8::LEU2*, the XhoI (site −337; see Fig. 1) to BglII (site 1,180) fragment was replaced with a fragment encoding *LEU2*. For *cin8::URA3*, the EcoRI (site 942) to EcoRI (site 2,611) fragment was replaced with a fragment encoding *URA3*. For *kip1::HIS3*, the SphI–ClaI fragment (see Roof et al., 1992) was replaced with a fragment encoding *HIS3*. Linear DNA from these constructs was used to replace the wild-type alleles in the appropriate strains.

Test for Double Deletion Viability

Crosses between *cin8* and *kip1* deletion strains yielded double mutant spores that did not germinate into viable colonies. This could reflect either mitotic inviability of the double deletion or an inability of spores of this

genotype to germinate. To rule out the germination defect possibility, strains with the double deletion genotype were constructed that are kept viable by a *CIN8*-containing plasmid (MAY2205 and MAY2275; see Table I). The plasmids also contained markers that could be selected against in the appropriate media; 5-fluoro-orotic acid selects against *URA3*, carried by the plasmid in MAY2205 and cycloheximide selects against *CYH2* carried by the plasmid in MAY2275. Cells were grown on rich media (YPD) for two to three days at temperatures ranging from 11 to 37°C and then were transferred to the appropriate test media at the same temperatures. At all tested temperatures, MAY2205 was unable to segregate 5-fluoro-orotic acid-resistant colonies and MAY2275 was unable to segregate cycloheximide-resistant colonies. This indicated that the mitotic viability of these two strains depended upon the presence of the respective *CIN8*-containing plasmid.

Microscopic Analysis of Cells

To determine the distribution of cell morphologies, culture samples were fixed in 70% ethanol and stained for DNA with 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI)[1] plus 1 mg/ml *p*-phenylene diamine. Using differential interference contrast optics, cells were scored as unbudded, small budded (bud size roughly ≤50% size of mother), or large budded (bud size roughly >50% size of mother). The number of nuclei per cell was determined by observation of chromosomal DNA masses by epifluorescent illumination. Microtubule structures were observed after formaldehyde fixation using the anti-tubulin mAb YOL1/34 (Kilmartin et al., 1982; Bioproducts for Science, Inc., Indianapolis, IN) and a fluorochrome-conjugated secondary antibody as previously described (Stearns et al., 1990). Spindle length was measured using a calibrated eyepiece reticule. For quantitative analyses, at least 200 cells from each sample were examined.

Cells were prepared for thin-section EM as described (Byers and Goetsch, 1991) with the following modifications: glutaraldehyde fixed cells were treated with 50 μg/ml zymolyase at 24°C until a majority of the cells had become spheroplasts (as assayed by loss of refractility when viewed by phase contrast optics; ~1–2 h). Spheroplasts were treated with uranyl acetate overnight at 4°C and dehydrated with successive ethanol baths of 35, 50, 65, 85, 95, and 100% twice each for 5 min.

To visualize the intracellular location of the Cin8p marked with the epitope tag from the influenza hemagglutinin protein, cells were double labeled for immunofluorescence microscopy with mAbs directed against α-tubulin [YOL1/34 from rat] and hemagglutinin [12CA5 from mouse (Wilson et al., 1984; Berkeley Antibody Co., Berkeley, CA)]. Immunofluorescent visualization of the 12CA5 epitope in this context was found to be sensitive to formaldehyde fixation. Since formaldehyde is the only suitable fixative for the visualization of yeast microtubules, relatively short fixation conditions were used that could satisfactorily maintain microtubule structure and also not obscure the hemagglutinin epitope. MAY2209 (tagged Cin8p encoded on a low-copy *CEN* plasmid) and MAY2210 (tagged Cin8p encoded on a high-copy 2-μm plasmid) were fixed with 4% formaldehyde in 50 mM KPO_4 (pH = 6.5), 0.5 mM $MgCl_2$ for 15–20 min at 24°C. The fixed cells were prepared for immunofluorescence microscopy as described (Stearns et al., 1990). Cells fixed to poly-lysine–coated slides were sequentially incubated with antibodies in the following order: (*a*) 1:30 dilution of 12CA5, pre-treated by adsorption with formaldehyde-fixed and -spheroplasted wild-type yeast cells; (*b*) 1:200 dilution of FITC-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) pre-treated in the same was as in *a* (*c*) 1:250 dilution of YOL1/34; (*d*) 1:50 dilution of rhodamine-conjugated goat anti-rat IgG (Cappel Laboratories). All antibodies were diluted into a paper-filtered solution of 50 mM KPO_4 (pH = 7.0), 150 mM NaCl containing 2.5% powdered milk. Control antibody incubations demonstrated that cross-species reactivity could not contribute to the final images when the sequential protocol was used. These control experiments also indicated that light channel spill-over made an insignificant contribution to the final images.

Results

Genetic Identification of CIN8 and KIP1

Three *cin8* mutant strains were identified among the previously described collection of *S. cerevisiae* mutants that segregate a supernumerary chromosome III with reduced fidelity (Hoyt et al., 1990). In growing cultures, a *cin8* deletion allele (*cin8-Δ*; see below) caused cells that have lost a marked chromosome III to appear at a 16-fold higher frequency than wild-type at 26°C (Table II). After incubation at 37°C for 12 h, this difference increased to approximately 90-fold. All three original *cin8* mutants were recessively temperature sensitive for viability (at 37°C) and failed to complement with each other for this defect. After a shift to the non-permissive temperature, all three strains exhibited a phenotype characteristic of a block at or in mitosis. Cells were predominantly large budded and contained a single nucleus (see below). The arrested cells contained replicated chromosomes, as determined by flow cytometric analysis of DNA content (data not shown).

The *CIN8* locus was cloned by complementation of the temperature-sensitive viability defect. 12 rescuing plasmids were isolated from a library of *S. cerevisiae* genomic fragments constructed in a low-copy centromere-containing yeast vector (Rose et al., 1987). 11 of the plasmids contained overlapping DNA inserts that were demonstrated by a linkage experiment to contain the bona fide *CIN8* locus (see Materials and Methods). The *CIN8* locus was determined to reside between the *PRB1* and *CAN1* genes at the distal tip of the left arm of chromosome V (see Materials and Methods). The last centromere plasmid contained a locus distinct from *CIN8* that could suppress the temperature sensitivity of *cin8* mutant strains when present in extra copy. The temperature sensitivity of all three original *cin8* alleles, as well as insertion and deletion alleles were suppressed by this plasmid. Limited DNA sequence analysis revealed that the suppressing locus was equivalent to *KIP1* (Roof et al., 1992). We have not determined the number of excess *KIP1* copies required to suppress the temperature sensitivity of *cin8* mutants, however, centromere plasmids are typically maintained at near one or two copies per haploid genome; higher levels cause deleterious effects (Futcher and Carbon, 1986).

cin8 and *kip1* mutant alleles were generated by in vitro deletion of coding sequence and by insertion of a modified bacterial Tn*10* transposon (see Materials and Methods). When transferred back to the genomic locus, all *cin8-Δ* and insertion alleles produced phenotypes similar to the original alleles (elevated chromosome loss, Table II, and temperature-sensitivity for viability at 37°C). In contrast, *kip1* mutant

Table II. Measured Frequencies of Chromosome Loss

Strain	Genotype	Median frequency of chromosome loss ($\times 10^4$) 26°C	37°C
MAY2123	*CIN8 KIP1*	1.2	0.83
MAY2551	*cin8-Δ KIP1*	19	77
MAY2123	*CIN8 KIP1*	0.93	1.4
MAY2166	*CIN8 kip1-Δ*	0.80	1.5

Mitotic loss of a marked copy of cromosome III from disomic haploid strains was measured (see Materials and Methods for details). The values reported represent the frequency of cells resistant to both cycloheximide and canavanine relative to total cells. Frequencies were determined after three days of growth at 26°C from single cells and then after a shift to 37°C for 12 h. All strains were isogenic except for the indicated genotype. The two comparisons were performed on separate occasions and therefore are reported separately.

1. *Abbreviations used in this paper*: DAPI, 4,6-diamidino-2-phenylindole; SPB, spindle pole body.

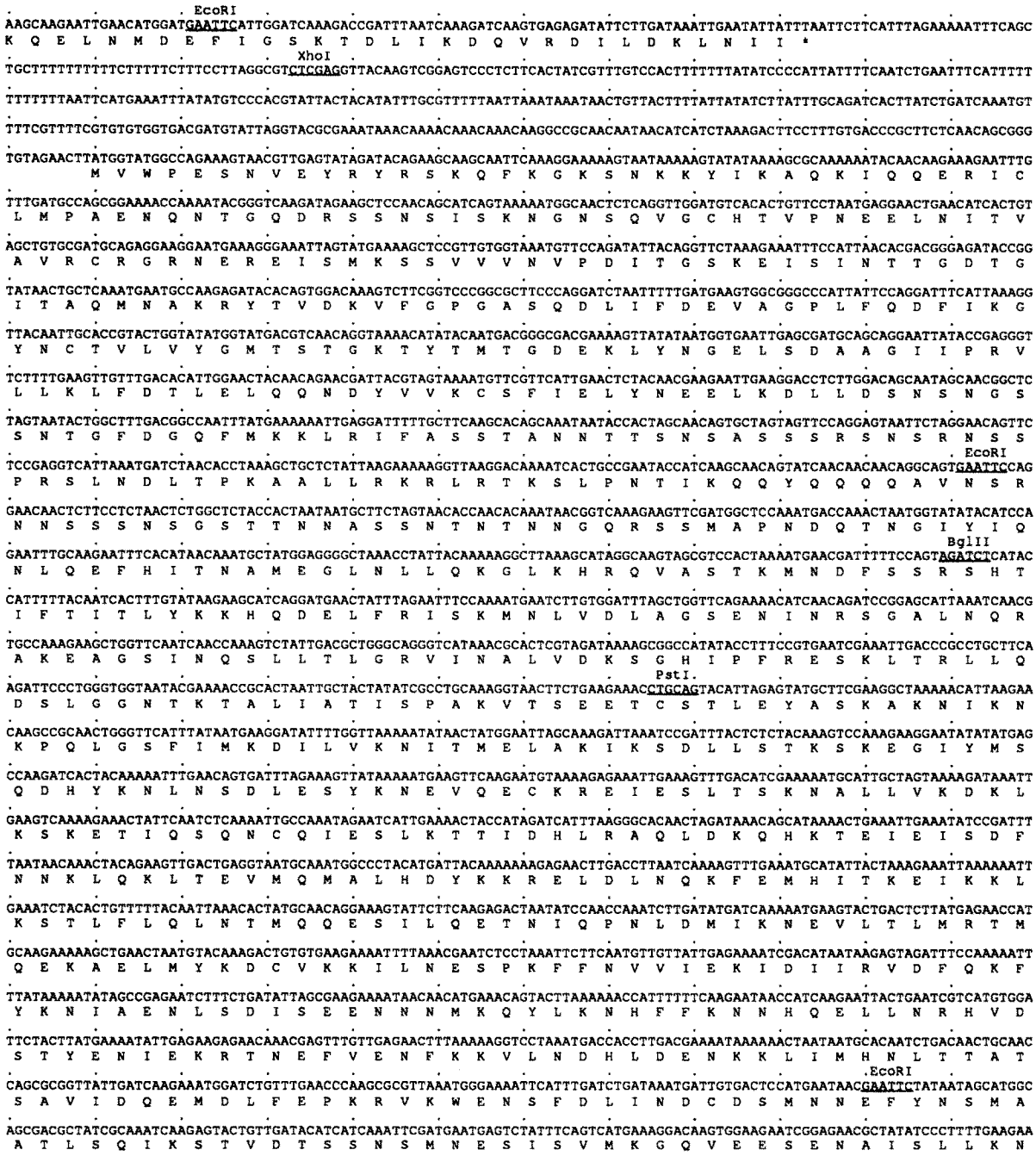


Figure 1. DNA and predicted polypeptide sequence of *CIN8*. The DNA sequence is numbered on the left with position 1 equivalent to the A in the predicted initiation codon. The polypeptide sequence is numbered on the right beginning with the initiator methionine. The first 96 nucleotides encode the 3′-end of the *PRB1* open reading frame (Moehle et al., 1987). Relevant restriction enzyme cut sites are underlined. These sequence data are available from EMBL under accession number Z11859 and GenBank under accession number M90522.

strains displayed no detectable mutant phenotype; measured chromosome loss rates were no higher than wild-type (Table II). Strains were constructed that are deleted for both chromosomal *CIN8* and *KIP1* loci, but carry *CIN8* on a plasmid (see Materials and Methods). At temperatures ranging from 11 to 37°C, these strains were unable to remain viable following loss of the *CIN8* plasmid. This demonstrated that a functional allele of either *CIN8* or *KIP1* is required for viability.

Unlike the *cin8* deletions, two of the three original *cin8* mutant alleles (*cin8-1* and *cin8-3*) could be combined with *kip1-Δ* to yield viable strains. These double mutant strains grew well at 26°C, but were inviable at 33°C and above. This indicated that the *cin8-1* and *cin8-3* gene products retain some activity at lower temperature.

CIN8 and KIP1 Encode Kinesin-related Proteins

The observations that the *cin8-Δ kip1-Δ* genotype is inviable and that extra *KIP1* copies suppress the *cin8* temperature-sensitive mitotic block suggest a simple hypothesis. Cin8p and Kiplp may perform a redundant but essential function in mitosis. This hypothesis is supported by the finding that the two genes encode related products. *CIN8* encodes a 1,038 amino acid polypeptide (118 kD) containing a region (amino acids 67 through 522) with strong similarity to the heavy chain of the microtubule-based mechanochemical enzyme kinesin (Figs. 1 and 2). The sequence of the kinesin-related *KIP1* gene is reported in the accompanying paper (Roof et al., 1992).

Genetic and molecular studies of numerous eukaryotic organisms have identified a superfamily of proteins related to the kinesin heavy chain by primary amino acid sequence (reviewed in Endow, 1991; Goldstein, 1991; Rose, 1991). For kinesin, it has been demonstrated that a 340 amino acid NH_2-terminal fragment of the heavy chain is sufficient for generating microtubule-based motile force (Yang et al., 1990; Goldstein, 1991). The region of sequence similarity shared by all members of the kinesin superfamily, including Cin8p and Kiplp, corresponds to this 340 amino acid "motor domain" (Fig. 2). The putative motor domains of Cin8p and Kiplp are more closely related to bimC, cut7, and Eg5 than other members of the kinesin superfamily. In contrast, outside of the motor domains, kinesin superfamily members share little or no sequence similarity to kinesin or to each other. This apparently is also the case for Cin8p and Kiplp; outside of the motor domain, Cin8p and Kiplp show only limited sequence conservation (15% identity for the region prior to the motor and 22% for the "tail" region after the motor, as compared with 56% for the motor domain).

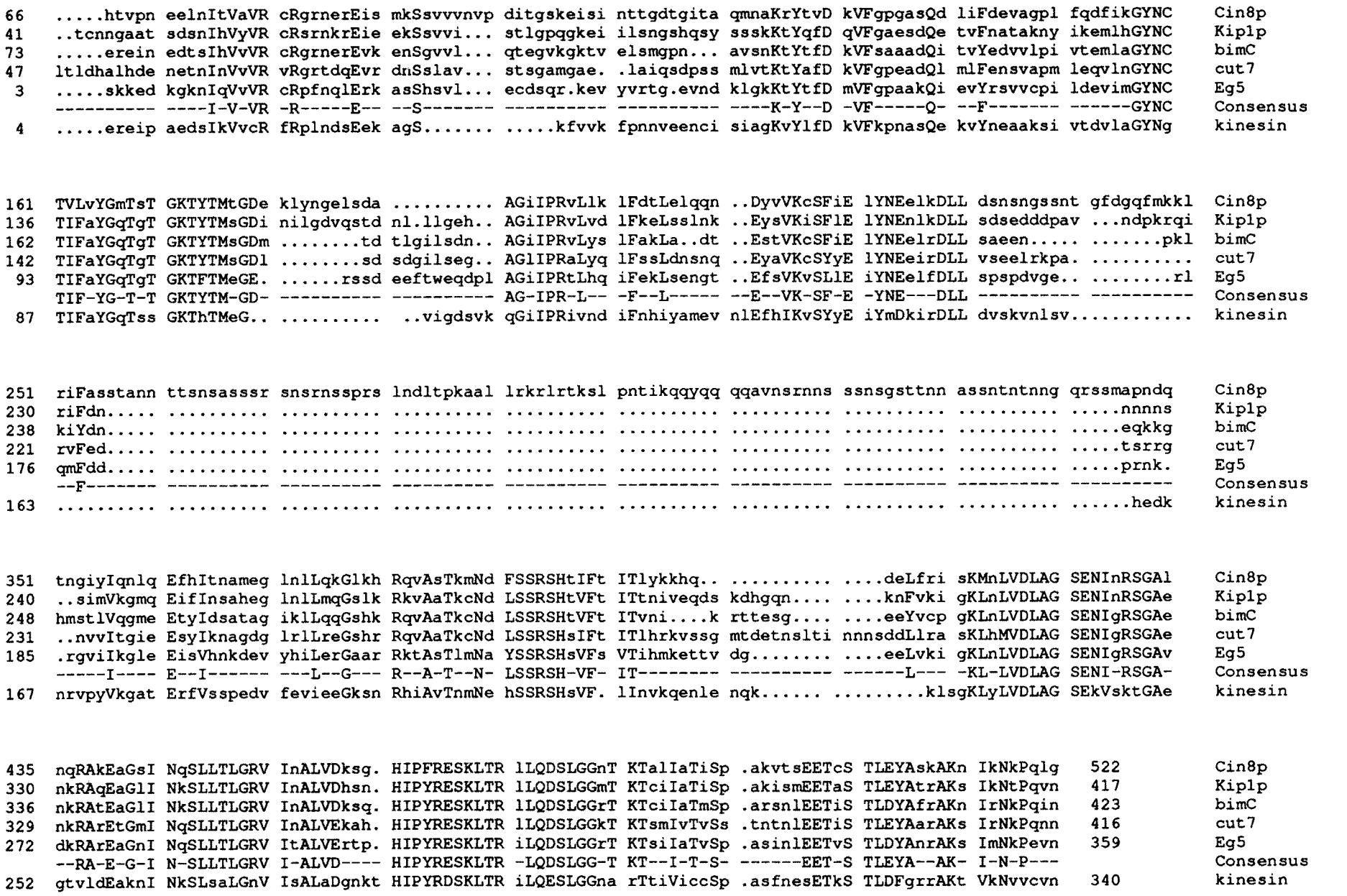
The Cin8p motor domain contains a segment of ~100 amino acids that is not present in other characterized kinesin-related proteins (residues 255–353; Fig. 2). It appears in a region of the motor domain sequence that is poorly conserved between members of the superfamily. The function of this segment is not known but is apparently not essential for Cin8p activity. A low-copy plasmid was constructed (pMA1212) that contained a *CIN8* gene in which the DNA encoding these amino acids was removed and replaced with a sequence encoding seven amino acids from the corresponding region of Kiplp (amino acids 234–240 = NNNNNSS; see Fig. 2). A strain was then constructed (MAY2305) that carried this plasmid and additionally was deleted for both chromosomal *CIN8* and *KIP1* loci. This strain was viable and had no obvious defect in growth rate at temperatures ranging from 11 to 37°C.

Loss of Cin8p and Kiplp Function Prevents Spindle Pole Separation

We examined the requirement for Cin8p and Kiplp function during assembly of the pre-anaphase short spindle. The α-factor mating pheromone arrests cells in the G1 phase of the cell cycle, before spindle pole body (SPB) duplication, and subsequent spindle assembly (Byers, 1981). Cell cultures of various genotypes were synchronized with α-factor at 26°C and released from this block into fresh media at 37°C. Observation of the cultures revealed that most *cin8-3*, *cin8-Δ*, and *cin8-3 kip1-Δ* double mutants had not passed through mitosis. After 2 h at 37°C, most cells had arrested growth with a mononucleate, large-budded morphology (Figs. 3 and 4). When α-factor synchronized cells were released onto agar surfaces at 37°C and observed, they arrested growth as single large-budded cells. This indicated that the block to mitosis occurred in the first cell cycle after release. In contrast, following α-factor synchronization and release to 37°C, wild-type and *kip1-Δ* cells were able to undergo mitosis; mononucleate large-budded cells did not accumulate in these cultures (Fig. 3). Cells of all genotypes were able to pass through mitosis after release from the α-factor block into media at 26°C. Therefore, Cin8p, but not Kiplp, is required to progress from the α-factor arrest point through mitosis at 37°C.

We examined the morphology of the spindles in temperature-arrested mutant cells by anti-tubulin immunofluorescence microscopy (Fig. 4). Wild-type cells formed short (~1.5–2 μm) bipolar spindles before mitosis. These appeared as a brightly stained bar, corresponding to the nuclear microtubules extending between the SPBs, with fainter staining cytoplasmic microtubules attached to each end (Fig. 4 *A*). After α-factor synchronization and subsequent incubation at 37°C, short bipolar spindles were not observed in the *cin8* single and *cin8 kip1* double-mutant cells (Fig. 4, *C–H*). Typically, elaborate cytoplasmic microtubules extended from a single region in each large-budded cell. In arrested cells, virtually all nuclei appeared to be located adjacent to or in the neck dividing the mother and the bud cell bodies. Nuclear migration to the neck, a microtubule-dependent motility event (Huffaker et al., 1988; Jacobs et al., 1988), was therefore not noticeably affected by the *cin8* and *kip1* mutations.

EM of these cells revealed the nature of the *cin8* spindle defect (Fig. 5). When released from the α-factor block into media at 26°C, mutant cells assembled normal-appearing spindles with poles separated to opposite sides of the nucleus (Fig. 5 *A*). In contrast, when incubated at 37°C, spindle pole separation apparently did not occur in either the *cin8* single mutant or *cin8 kip1* double mutant cells (Fig. 5, *B* and *C*). SPBs were found adjacent to each other in a side-by-side orientation. In a wild-type cell cycle, before separation, duplicated SPBs are connected by a "bridge" structure (Byers, 1981). In preparations of *cin8-3 kip1-Δ* cells, we usually observed the presence of a bridge connecting the duplicated SPBs (Fig. 5 *C*). This observation further supports the conclusion that spindle poles had not separated.


66 .....htvpn eelnItVaVR cRgrnerEis mkSsvvvnvp ditgskeisi nttgdtgita qmnaKrYtvD kVFgpgasQd liFdevagpl fqdfikGYNC Cin8p
41 ..tcnngaat sdsnIhVyVR cRsrnkrEie ekSsvvi... stlgpqgkei ilsngshqsy ssskKtYqfD qVFgaesdQe tvFnatakny ikemlhGYNC Kip1p
73 .....erein edtsIhVvVR cRgrnerEvk enSgvvl... qtegvkgktv elsmgpn... avsnKtYtfD kVFsaaadQi tvYedvvlpi vtemlaGYNC bimC
47 ltldhalhde netnInVvVR vRgrtdqEvr dnSslav... stsgamgae. .laiqsdpss mlvtKtYafD kVFgpeadQl mlFensvapm leqvlnGYNC cut7
3 .....skked kgknIqVvVR cRpfnqlErk asShsvl... ecdsqr.kev yvrtg.evnd klgkKtYtfD mVFgpaakQi evYrsvvcpi ldevimGYNC Eg5
---------- ----I-V-VR -R-----E-- --S------- ---------- ---------- ----K-Y--D -VF-----Q- --F------- ------GYNC Consensus
4 .....ereip aedsIkVvcR fRplndsEek agS..... .....kfvvk fpnnveenci siagKvYlfD kVFkpnasQe kvYneaaksi vtdvlaGYNg kinesin
161 TVLvYGmTsT GKTYTMtGDe klyngelsda ..... AGiIPRvLlk lFdtLelqqn ..DyvVKcSFiE lYNEelkDLL dsnsngssnt gfdgqfmkkl Cin8p
136 TIFaYGqTgT GKTYTMsGDi nilgdvqstd nl.llgeh.. AGiIPRvLvd lFkeLsslnk ..EysVKiSFlE lYNEnlkDLL sdsedddpav ...ndpkrqi Kip1p
162 TIFaYGqTgT GKTYTMsGDm ....td tlgilsdn.. AGiIPRvLys lFakLa..dt ..EstVKcSFiE lYNEelrDLL saeen..... ....pkl bimC
142 TIFaYGqTgT GKTYTMsGDl ....sd sdgilseg.. AGlIPRaLyq lFssLdnsnq ..EyaVKcSYyE lYNEeirDLL vseelrkpa. cut7
93 TIFaYGqTgT GKTFTMeGE. ......rssd eeftweqdpl AGiIPRtLhq iFekLsengt ..EfsVKvSLlE iYNEelfDLL spspdvge.. ....rl Eg5
TIF-YG-T-T GKTYTM-GD- ---------- ---------- AG-IPR-L-- -F--L----- --E--VK-SF-E -YNE---DLL ---------- ---------- Consensus
87 TIFaYGqTss GKThTMeG.. ....vigdsvk qGiIPRivnd iFnhiyamev nlEfhIKvSYyE iYmDkirDLL dvskvnlsv..... kinesin
251 riFasstann ttsnsasssr snsrnssprs lndltpkaal lrkrlrtksl pntikqqyqq qqavnsrnns ssnsgsttnn assntntnng qrssmapndq Cin8p
230 riFdn..... ....nnnns Kip1p
238 kiYdn..... ....eqkkg bimC
221 rvFed..... ....tsrrg cut7
176 qmFdd..... ....prnk. Eg5
--F------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- Consensus
163 ....hedk kinesin
351 tngiyIqnlq EfhItnameg lnlLqkGlkh RqvAsTkmNd FSSRSHtIFt ITlykkhq.. ....deLfri sKMnLVDLAG SENInRSGAl Cin8p
240 ..simVkgmq EifInsaheg lnlLmqGslk RkvAaTkcNd LSSRSHtVFt ITtniveqds kdhgqn.... ....knFvki gKLnLVDLAG SENInRSGAe Kip1p
248 hmstlVqgme EtyIdsatag iklLqqGshk RqvAaTkcNd LSSRSHtVFt ITvni....k rttesg.... ....eeYvcp gKLnLVDLAG SENIgRSGAe bimC
231 ..nvvItgie EsyIknagdg lrlLreGshr RqvAaTkcNd LSSRSHsIFt ITlhrkvssg mtdetnslti nnnsddLlra sKLhMVDLAG SENIgRSGAe cut7
185 .rgviIkgle EisVhnkdev yhiLerGaar RktAsTlmNa YSSRSHsVFs VTihmkettv dg........ ....eeLvki gKLnLVDLAG SENIgRSGAv Eg5
-----I---- E--I------ ---L--G--- R--A-T--N- LSSRSH-VF- IT-------- ---------- ------L--- -KL-LVDLAG SENI-RSGA- Consensus
167 nrvpyVkgat ErfVsspedv fevieeGksn RhiAvTnmNe hSSRSHsVF. lInvkqenle nqk..... ....klsgKLyLVDLAG SEkVsktGAe kinesin
435 nqRAkEaGsI NqSLLTLGRV InALVDksg. HIPFRESKLTR lLQDSLGGnT KTalIaTiSp .akvtsEETcS TLEYAskAKn IkNkPqlg 522 Cin8p
330 nkRAqEaGlI NkSLLTLGRV InALVDhsn. HIPYRESKLTR lLQDSLGGmT KTciIaTiSp .akismEETaS TLEYAtrAKs IkNtPqvn 417 Kip1p
336 nkRAtEaGlI NkSLLTLGRV InALVDksq. HIPYRESKLTR lLQDSLGGrT KTciIaTmSp .arsnlEETiS TLDYAfrAKn IrNkPqin 423 bimC
329 nkRArEtGmI NqSLLTLGRV InALVEkah. HIPYRESKLTR lLQDSLGGkT KTsmIvTvSs .tntnlEETiS TLEYAarAKs IrNkPqnn 416 cut7
272 dkRArEaGnI NqSLLTLGRV ItALVErtp. HIPYRESKLTR iLQDSLGGrT KTsiIaTvSp .asinlEETvS TLDYAnrAKs ImNkPevn 359 Eg5
--RA-E-G-I N-SLLTLGRV I-ALVD---- HIPYRESKLTR -LQDSLGG-T KT--I-T-S- ------EET-S TLEYA--AK- I-N-P--- Consensus
252 gtvldEaknI NkSLsaLGnV IsALaDgnkt HIPYRDSKLTR iLQESLGGna rTtiViccSp .asfnesETkS TLDFgrrAKt VkNvvcvn 340 kinesin

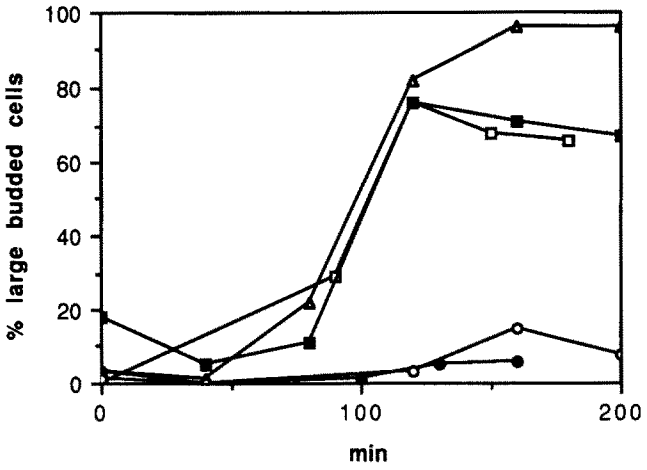


Figure 3. Percent large-budded mononucleate cells after release from α-factor to non-permissive temperature. Cells of various genotypes were synchronized with α-factor and released into media at 37°C. The percent of total cells with a large-budded mononucleate morphology was determined as a function of time at 37°C. (●) Wild-type (MAY589); (○) *kipl-Δ* (MAY2077); (□) *cin8-3* (MAY-1563); (■) *cin8-Δ* (MAY2177); (△) *cin8-3 kipl-Δ* (MAY2169).

Cin8p Colocalizes with Nuclear Microtubules

The Cin8p sequence similarity to kinesin and the phenotypes of *cin8* mutants suggested that this protein interacts with microtubules. We examined this possibility by tagging Cin8p with an epitope from the influenza hemagglutinin protein that is recognized by a mAb (Wilson et al., 1984; Kolodziej and Young, 1991). An oligonucleotide encoding the nine amino acid epitope was fused to the 3'-end of the *CIN8* reading frame (see Materials and Methods). The fusion protein thus formed was fully functional for Cin8p activity. Strains were constructed that produced no wild-type Cin8p and Kiplp but were kept viable by the tagged *CIN8* gene carried on a plasmid. These cells were fixed and stained with two mAbs, one directed against tubulin and the other against the hemagglutinin epitope. The antibodies were from two different species, allowing simultaneous labeling of both epitopes with different fluorophores (see Materials and Methods).

When the tagged Cin8p was expressed from a low-copy centromere-containing plasmid, faint anti-hemagglutinin staining colocalized with the spindle microtubules that lie between the poles (Fig. 6, *A–E*). This staining was only observed on short spindles, however, it is likely that if an equal amount of antigen was present on long spindles, it would be so dilute as to preclude visualization. In no case were anti-hemagglutinin antibodies observed to decorate cytoplasmic microtubules. When the gene encoding the tagged Cin8p was carried by a high-copy 2 μm-based plasmid, the same pattern was observed, but the intensity of nuclear microtubule staining by anti-hemagglutinin was considerably higher (Fig. 6, *F–K*). In contrast to the low-copy plasmid experiment, staining of long spindles was also observed, although this was a relatively rare occurrence. Again, staining of cytoplasmic microtubules by anti-hemagglutinin was never observed.

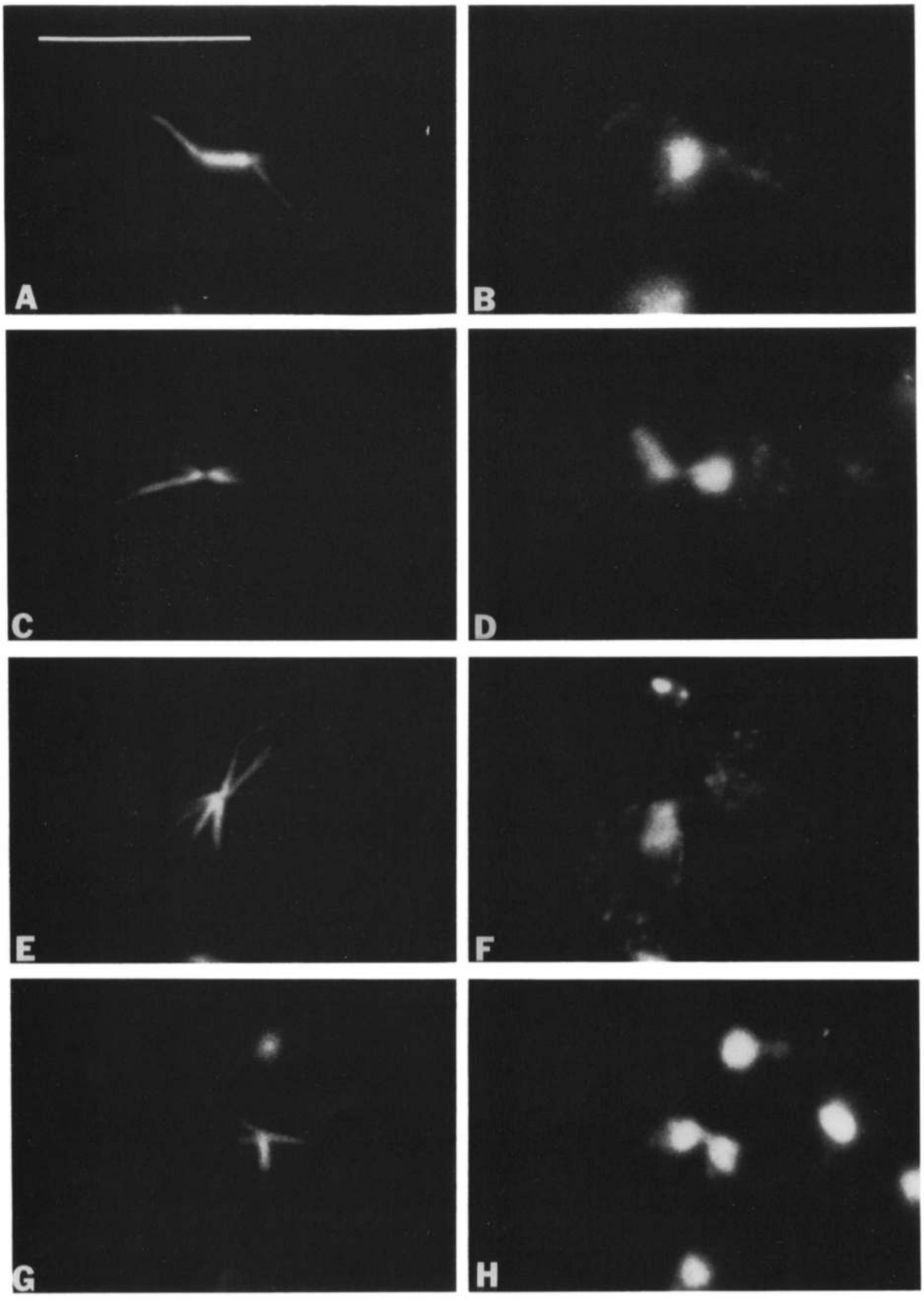
The antibody staining of the epitope-tagged Cin8p was often observed to be more intense in regions of nuclear microtubules closer to the spindle poles. The related fission yeast protein cut7 was also recently reported to be localized to nuclear microtubules and is more concentrated near the poles of spindles (Hagan and Yanagida, 1992). In our observations of doubly labeled cells, the staining by both anti-hemagglutinin and anti-tubulin was reduced for intensity in the middle of spindles (i.e., compare Fig. 6, *F* and *G*). Therefore, the asymmetric distribution of Cin8p between the poles probably reflects the distribution of nuclear microtubules.

Discussion

In the experiments reported herein, the requirement for Cin8p and Kiplp for normal mitotic chromosome segregation was demonstrated both genetically and morphologically. Our characterization of mutants revealed that the action of these proteins is required for the assembly of the pre-anaphase mitotic spindle. In the absence of both Cin8p and Kiplp functions, duplicated spindle poles did not separate and cells were inviable. No phenotypic consequence was observed for loss of Kiplp function alone and Cin8p function was required for viability only at 37°C. At this temperature, Kiplp must be capable of performing the Cin8p-requiring step since *KIP1* in extra copy could substitute for *CIN8*. These findings taken together are most simply explained by a model in which Cin8p and Kiplp redundantly perform an essential mitotic function.

Cin8p and Kiplp were found to be members of the superfamily of proteins related to the microtubule-based mechanochemical enzyme kinesin. Mitotic roles for kinesin-related proteins have been suggested by the localization of related epitopes to the spindle (Sawin et al., 1992) and by numerous genetic observations. Although Drosophila kinesin heavy chain gene mutants appear to be unaffected for mitotic function (Saxton et al., 1991), mutant forms of many kinesin-related genes cause mitotic and/or meiotic defects (Davis, 1969; Sequeira et al., 1989; Endow et al., 1990; McDonald and Goldstein, 1990; Carpenter, 1973; Zhang et al., 1990; Meluh and Rose, 1990). Of particular relevance to the experiments reported here, *Aspergillus nidulans bimC* mutants and *Schizosaccharomyces pombe cut7* mutants are defective for mitotic spindle formation at non-permissive temperatures (Enos and Morris, 1990; Hagan and Yanagida, 1990). Strikingly similar to loss of Cin8p/Kiplp function, both *bimC* and *cut7* mutations appear to block the separation of duplicated spindle poles. Of the previously characterized

Figure 2. Comparison of kinesin-related polypeptides. The motor domain sequences of Drosophila kinesin, Cin8p, and four bimC-related polypeptides were aligned with the PILEUP computer program (Devereux et al., 1984). By excluding the kinesin sequence, a bimC family consensus was derived with the PRETTY program. Conservative substitutions were allowed (Threshold value ≥ 1.00), but the derived consensus required agreement in all five bimC-like sequences (Plurality = 5). Agreements to the consensus, within the acceptable substitution range, are indicated by upper case letters. Kiplp from *S. cerevisiae* (Roof et al., 1992); bimC from *A. nidulans* (Enos and Morris, 1990); cut7 from *S. pombe* (Hagan and Yanagida, 1990); Eg5 from *Xenopus laevis* (Le Guellec et al., 1991); kinesin heavy chain from *Drosophila melanogaster* (Yang et al., 1989).


A
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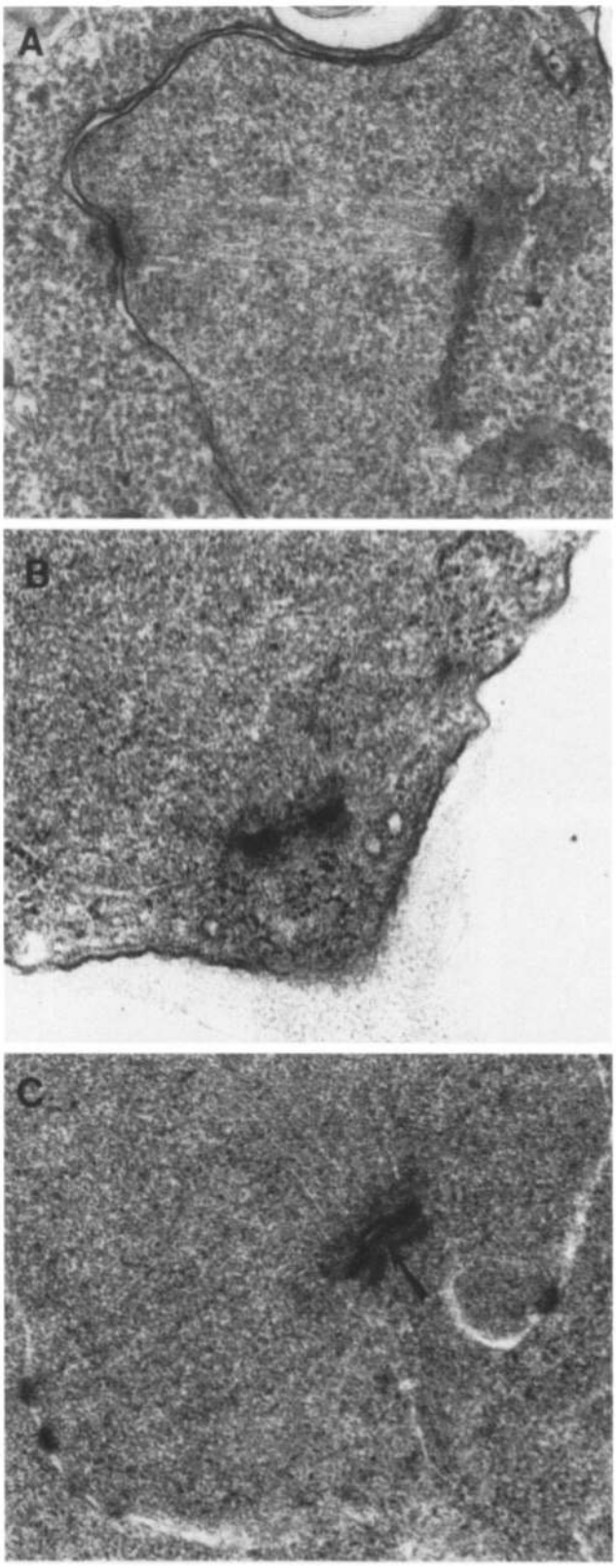


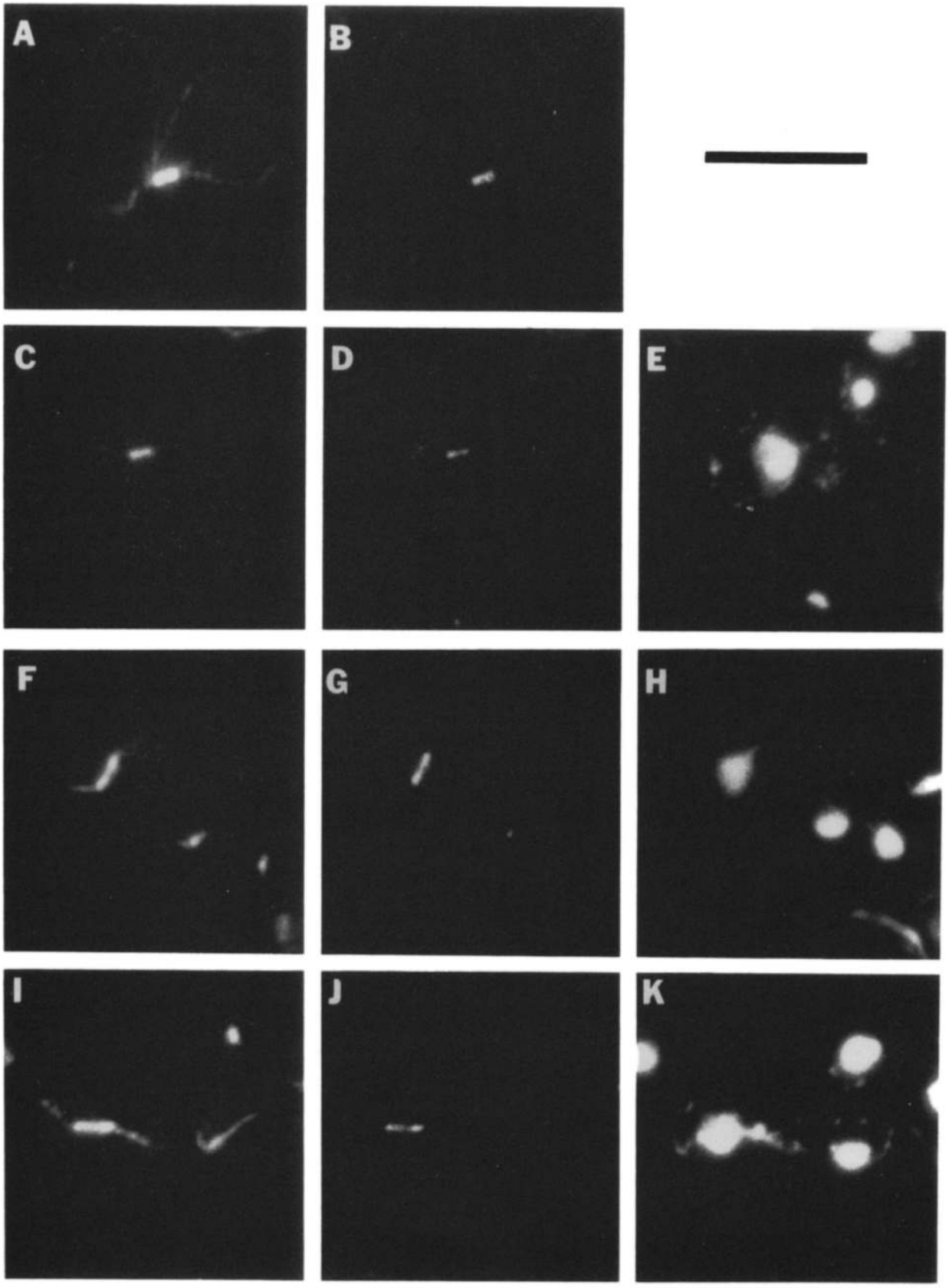
members of the kinesin-related superfamily, the motor domains of bimC, cut7, Cin8p, Kiplp, and Eg5 from *Xenopus laevis* (Le Guellec et al., 1991) are most closely related in primary sequence (Fig. 2). Recently, another member of this bimC-like family has been identified in Drosophila (L. Goldstein, personal communication). It therefore seems possible that this class of kinesin-related proteins is conserved in sequence and function across the different eukaryotic phyla. From the in vitro motility properties of kinesin (Vale et al., 1985) and the related ncd protein (McDonald et al., 1990; Walker et al., 1990), it is reasonable to presume that Cin8p, Kiplp, and relatives are directly involved in the generation of the force required to separate spindle poles.

For all members of the kinesin superfamily described to date, little primary sequence conservation is detectable in comparisons of regions outside of the motor domain. It has been suggested that the different tails affixed to the motor domain may allow diverse "cargos" to be translocated along microtubules (Goldstein, 1991). Despite their functional overlap, Cin8p and Kiplp also share extremely limited sequence similarity outside of the motor. Therefore, it can be concluded that tail domains of different primary sequence may accomplish a similar or a substituting function. Although our findings suggest functional interchangeability, we imagine that Cin8p and Kiplp may contribute to pole separation in somewhat different ways.

The consequences of loss of Cin8p and Kiplp function and their localization to nuclear microtubules (see Roof et al., 1992 for Kiplp) suggest their direct involvement in spindle assembly. In the simplest formulation, Cin8p and Kiplp cross-link interdigitated nuclear microtubules from each half spindle and slide them past one another to generate an outward force acting on the poles. Hypothetically, pole separation could be accomplished by a motor acting either between the poles or on the cytoplasmic (or astral) microtubules located outside of the poles. The epitope-tagged Cin8p was observed only on the spindle fibers lying between the poles, although visually undetectable amounts located on the cytoplasmic microtubules cannot be ruled out. Therefore, Cin8p most likely generates the pole-separating force from within the spindle, not from outside of the poles. Experimental observations by others have suggested that forces that separate spindle poles are produced by interactions between the

Figure 5. Electron microscopic analysis of mutant cells. Cells were synchronized with α-factor, released into growth media for ~2 h, fixed, and examined by thin section EM. (*A*) A *cin8-3 kipl-Δ* cell (MAY2169) released into media at 26°C for 2 h. Note the normal-appearing short mitotic spindle with spindle poles separated to opposite sides of the nucleus; (*B*) A *cin8-Δ* cell (MAY2061) released into media at 37°C; (*C*) A *cin8-3 kipl-Δ* cell (MAY2169) treated as in *B*. The arrow points to the bridge structure connecting the two SPBs.

Figure 4. Anti-tubulin immunofluorescence microscopy. Cells were synchronized with α-factor, released into media at 37°C for ~2 h, fixed, and examined by immunofluorescence microscopy. *A*, *C*, *E*, and *G* show anti-tubulin staining and *B*, *D*, *F*, and *H* show the same cells stained with the DNA-specific dye, DAPI. (*A* and *B*) wild-type (MAY589); (*C* and *D*) *cin8-3* (MAY1563); (*E* and *F*) *cin8-Δ* (MAY2061); (*G* and *H*) *cin8-3 kipl-Δ* (MAY2169). Bar, 10 μm.


A
B
C
D
E
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G
H
I
J
K
anti-
tubulin
anti-HA
DAPI

anti-parallel midzone microtubules (reviewed in Hogan and Cande, 1990).

For *S. cerevisiae*, the cytoplasmic microtubules appear to be used primarily for positioning of the dividing nucleus and its associated spindle within the cell (Huffaker et al., 1988; T. Huffaker and D. Koshland, personal communications). At the non-permissive temperature, the nuclei in our mutant cells were able to migrate efficiently to the neck dividing the mother and bud cell bodies. Therefore, consistent with the nuclear location of Cin8p, the motility functions provided by cytoplasmic microtubules apparently were unaffected. It is possible, however, that the action of an as yet unidentified cytoplasmic microtubule-based activity can contribute to pole separation in some phase of mitosis (Aist et al., 1991).

We have demonstrated a requirement for Cin8p/Kiplp function for pole separation during spindle assembly. Although not addressed in these experiments, pole separating forces are also required in subsequent mitotic stages. Indeed, we have found that the action of Cin8p or Kiplp is required following the spindle assembly step to oppose a force that draws separated poles back together (W. S. Saunders and M. A. Hoyt, manuscript in preparation). It is possible that these putative motors may function throughout mitosis whenever an outwardly directed force acting upon the spindle poles is required.

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Figure 6. Cin8p colocalizes with nuclear microtubules. Cells deleted for wild-type *CIN8* and *KIP1*, but carrying the hemagglutinin epitope-tagged *CIN8* on plasmids were labeled with anti-tubulin (*A*, *C*, *F*, and *I*), anti-hemagglutinin (*B*, *D*, *G*, and *J*) and DAPI (*E*, *H*, and *K*) as described in Materials and Methods. The cells depicted in *A*, *B*, and *C–F* (MAY2209) carried the tagged *CIN8* gene on a centromere-containing plasmid, while the cells depicted in *F–H* and *I–K* (MAY2210) carried the gene on a 2 μm plasmid. Exposure times were optimized for the best image and are not equal. Bar, 10 μm.

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