

Chromosome Instability Mutants of *Saccharomyces cerevisiae* That Are Defective in Microtubule-Mediated Processes

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By using a multiply marked supernumerary chromosome III as an indicator, we isolated mutants of *Saccharomyces cerevisiae* that display increased rates of chromosome loss. In addition to mutations in the tubulin-encoding *TUB* genes, we found mutations in the *CIN1*, *CIN2*, and *CIN4* genes. These genes have been defined independently by mutations causing benomyl supersensitivity and are distinct from other known yeast genes that affect chromosome segregation. Detailed phenotypic characterization of *cin* mutants revealed several other phenotypes similar to those of *tub* mutants. Null alleles of these genes caused cold sensitivity for viability. At 11°C, *cin* mutants arrest at the mitosis stage of their cell cycle because of loss of most microtubule structure. *cin1*, *cin2*, and *cin4* mutations also cause defects in two other microtubule-mediated processes, nuclear migration and nuclear fusion (karyogamy). Overproduction of the *CIN1* gene product was found to cause the same phenotype as loss of function, supersensitivity to benomyl. Our findings suggest that the *CIN1*, *CIN2*, and *CIN4* proteins contribute to microtubule stability either by regulating the activity of a yeast microtubule component or as structural components of microtubules.

Microtubules are ubiquitous fibrous structures in eucaryotic cells that participate in numerous processes involving motility and cell structure. They are composed primarily of tubulin, a heterodimeric protein consisting of one polypeptide chain each of α -tubulin and β -tubulin (29). A number of less well-understood microtubule-associated proteins (MAPs) also participate in these structures (35). In vivo, microtubules often function as dynamic structures, rapidly assembling and disassembling in response to uncharacterized molecular signals (41). These processes are regulated both spatially and temporally with respect to the cell division cycle. The observed properties of microtubules must reflect aspects of the molecular functions of both the tubulin subunit and the nontubulin MAPs. Despite our accumulated knowledge of the in vitro assembly properties of tubulin and some MAPs, their relationships to in vivo microtubule function remain obscure.

The yeast *Saccharomyces cerevisiae* is a particularly tractable organism for the study of microtubule function (17). (i) Yeast cells contain relatively simple microtubule arrays that participate in only three well-defined and temporally distinct cellular processes: mitosis, meiosis, and nuclear fusion during conjugation (karyogamy). Two of these processes, meiosis and karyogamy, are nonessential for mitotic growth; they are expressed only as alternative developmental pathways. (ii) The yeast genome contains relatively few genes that encode tubulin polypeptides. *S. cerevisiae* contains two genes that encode 90% homologous α -tubulin polypeptides, *TUB1* and *TUB3* (43, 44). The only detectable functional difference between the two is in their levels of expression. *TUB1* is essential for mitotic viability because it provides most of the α -tubulin for the cell. *TUB3* is a nonessential gene; null alleles are viable but do cause

detectable phenotypes (see below). *S. cerevisiae* contains a single and essential β -tubulin gene, *TUB2* (34). Interestingly, the distantly related fission yeast *Schizosaccharomyces pombe* has an identical tubulin gene configuration (57).

In *S. cerevisiae*, as in other eucaryotic cell types, replicated chromosomes are segregated in mitosis by a structure that consists of an array of microtubules (the mitotic spindle). Yeast cells with impaired tubulin activity show unfaithful mitotic chromosome transmission (18, 55). These cells lose chromosomes at higher frequencies than do wild-type cells; that is, cells that have failed to inherit a particular chromosome appear more often. This is presumably due to increased rates of nondisjunction events. Three other phenotypes have been associated with impaired tubulin activity in *S. cerevisiae*. (i) Tubulin mutants are often sensitive to what are normally sublethal concentrations of the antimicrotubule agent benomyl. Indeed, benomyl supersensitivity appears to be an almost universal property of α -tubulin mutants. Of 38 conditional *tub1* mutants generated by site-specific mutagenesis, 35 had this phenotype (45). *tub3* null and missense mutants are also supersensitive to benomyl (44, 51). Resistance to high levels of benomyl appears to be conferred exclusively by alterations in *TUB2* (52). However, some *tub2* mutant alleles cause a supersensitivity phenotype (18). (ii) Impaired tubulin activity causes karyogamy failure, demonstrating the essential role performed by microtubules in the process of nuclear fusion (9, 18). (iii) Complete loss of tubulin activity caused by either drug treatment or mutation results in specific arrest of the yeast cell cycle (18, 21, 38, 56). Cells arrest at the large-budded stage with single nuclei containing replicated but nonsegregated chromosomes.

We anticipated that mutant forms of some microtubule-related proteins would cause tubulin mutantlike phenotypes. Therefore, we screened for new mutants that show increased chromosome loss and then examined these mutants for other microtubule-related defects. Using these criteria, we found new mutant alleles of the yeast tubulin genes, as well as alleles of three nontubulin genes, *CIN1*, *CIN2*, and *CIN4*. *cin* mutants appear to be distinct from previously isolated

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TABLE 1. Strains and plasmids

Yeast strain or plasmid	Relevant genotype
F122	<i>MATa/MATa leu2-1/+ his4-Δ29/his4-Δ29</i> (chromosome III disome) <i>ade2-1</i>
DBY3363	<i>MATa/MATa +/cry^r1LEU2::SUP11-1 CAN1/</i> <i>leu2</i> (chromosome III disome) <i>ade2-101</i> <i>his3-Δ200 ura3-52 can^r1</i>
DBY3365	<i>MATa/MATa +/cry^r1LEU2::SUP11-1 CAN1/</i> <i>leu2::LUKA14</i> (chromosome III disome) <i>ade2-101 lys2-801 ura3-52 can^r1</i>
DBY3382	<i>MATa his3-Δ200 leu2-3,112 ura3-52 ade2-101</i> <i>cyh² cry^r1 [rho⁰]</i>
DBY3383	<i>MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801</i>
DBY3384	Same as DBY3383 but <i>cin1::HIS3a</i>
DBY3385	Same as DBY3382 but <i>cin1::HIS3a</i>
DBY5350	<i>MATa/MATa CIN⁺/CIN⁺</i>
DBY5351	<i>MATa/MATa cin1::HIS3b/cin1::HIS3b</i>
DBY5352	<i>MATa/MATa cin2::LEU2/cin2::LEU2</i>
DBY5353	<i>MATa/MATa cin4::URA3/cin4::URA3</i>
DBY5354	<i>MATa leu2-3,112 ura3-52 his4-519 GAL⁺</i>
DBY5355	<i>MATa leu2-3,112 ura3-52 lys2-801 cin1::HIS3a</i> <i>GAL⁺</i>
DBY5356	<i>MATa his3-Δ200 leu2-3,112 lys2-801 ura3-52</i>
pRB327	<i>TUB1 LEU2</i> (2 μ m)
pRB1252	<i>LEU2 SUP11-1 CAN1</i>
pBM258	<i>P_{GALI} URA3 (CEN)</i>
pGM65	<i>P_{GALI} URA3</i> (2 μ m)
pRB1310	<i>P_{GALI}-CIN1 URA3 (CEN)</i>
pRB1311	<i>P_{GALI}-CIN1 URA3</i> (2 μ m)

mutants that display unstable inheritance of minichromosomes (30), chromosome fragments (50), and the *MAT* locus in chromosome III disomes (25, 27). In this report, we describe the identification of these three *CIN* genes as chromosome loss mutants and phenotypic characterization that suggests that wild-type *CIN* gene products function to increase microtubule stability. In a separate paper (T. Stearns, M. A. Hoyt, and D. Botstein, Genetics, in press), we describe the independent identification of *CIN1*, *CIN2*, and *CIN4* via mutations that result in benomyl supersensitivity, as well as genetic experiments that suggest that the *CIN1*, *CIN2*, and *CIN4* gene products act in concert to affect microtubule function.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in these experiments are listed in Table 1. The original chromosome III disomic strain, F122, was described by Shaffer et al. (47). The disomic chromosome loss tester strains were constructed from F122 by standard genetic techniques. The insertion of the *CAN1* and *SUP11-1* genes next to *LEU2* was constructed as follows. DNA containing the *SUP11-1* locus bounded by *Bam*HI sites (obtained from C. Newlon) was inserted between two *Bam*HI sites adjacent to the *CAN1* gene in YEPCAN1-1 (from K. Tatchell via G. Fink; unpublished data). A fragment of DNA containing both genes and bounded by *Xho*I and *Sal*I was then inserted into the *Xho*I site adjacent to the *LEU2* gene in YEpl3 (2) to create pRB1252. Cutting with *Pst*I liberated a 6,020-base-pair fragment that contained *SUP11-1*, *CAN1*, and *LEU2*. Transformation of the appropriate *leu2* mutant host transferred the insertion onto chromosome III.

Strains bearing chromosomal disruptions of each of the *CIN* genes were made by fragment-mediated one-step re-

placement (40) of the chromosomal sequences. The disruption constructions, except *cin2::URA3*, are described in detail elsewhere (Stearns et al., in press). *cin1::HIS3a*, *cin1::HIS3b*, *cin2::LEU2*, and *cin4::URA3* are deletion-substitution alleles constructed in vitro by replacement of the *CIN* gene containing sequences with DNA containing selectable yeast genes; the amounts of *CIN* DNA deleted in these constructs were, respectively, 1,475, 3,050, 590, and 520 base pairs. Additional *CIN1* disruptions were created by insertion of the mini-Tn10-LUK transposable element (19); the *cin1::LUK7* allele contains an insertion at the 5' end of the gene. The three *cin1* disruptions behaved similarly. *cin2::URA3* was constructed from *cin2::LEU2* by removal of part of the *LEU2*-containing DNA and replacement with a fragment carrying the *URA3* gene.

The rich (YPD) and minimal (SD) media used were as previously described (49). Nutritional supplements were added to 30 μ g/ml, except for adenine, which was added to 6 μ g/ml. Benomyl (Du Pont Co.) was added to media from a 10-mg/ml stock in dimethyl sulfoxide to 5 or 10 μ g/ml. Canavanine (Sigma Chemical Co.) was added to SD at 75 μ g/ml. Cryptopleurine (Corkwood Enterprises) was used at 3 to 5 μ g/ml, except in the karyogamy experiment, in which it was used at 0.5 μ g/ml.

Screen for mutants with increased rates of chromosome loss. DBY3363 and DBY3365 were mutagenized with ethyl methanesulfonate to 10% survival and plated onto YPD at 26°C. The colonies formed were then replica transferred to SD plus canavanine and cryptopleurine (and leucine, adenine, histidine, lysine, and uracil), as well as three YPD plates that were incubated at 14, 26, and 37°C. The cells on these plates were also subsequently replica transferred to canavanine-cryptopleurine medium. Potential *cin* mutants were recognized by their increased numbers of red papillae on one or more of the canavanine-cryptopleurine replicas.

Quantitative measurement of chromosome loss and mitotic recombination. For each strain tested, 8 to 10 colonies were cut from YPD agar surfaces after 3 days of growth at 26°C. The cells were suspended in water, lightly sonicated, and plated on SD plus canavanine and cryptopleurine (plus leucine, adenine, histidine, lysine, and uracil) to determine the titer of cells that had lost chromosome III. To determine the titer of cells that had recombined in the *CENIII-CRYI* interval, cells were plated on SD plus cryptopleurine minus leucine (plus adenine, histidine, lysine, and uracil). Total cells were determined by plating on YPD. Rates of chromosome loss were estimated by the method of the median (26). For each strain tested at 11°C, a portion of each cell suspension was diluted into YPD at 11°C and incubated for 4 days. The titers of cells with the various phenotypes were then determined as described for the 26°C samples.

Immunofluorescence staining of yeast cells. Yeast cells were treated for immunofluorescence essentially as described by Kilmartin and Adams (24), with the exceptions that the cells were fixed by addition of formaldehyde directly to the culture medium to a final concentration of 5% formaldehyde and incubated at room temperature for 2 h and that cell walls were subsequently removed by incubation at 37°C for 30 min in 50 μ g of Zymolyase 100,000 per ml in 0.1 M potassium phosphate, pH 7.5. Rat monoclonal anti-tubulin antibody YOL1/34 was obtained from Bioproducts for Science, Inc. Fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin G serum was obtained from Organon Teknica. DNAs in the fixed and digested cells were stained with 1 μ g of 4,6-diamidino-2-phenylindole (DAPI) per ml for 1 min. Preparations were viewed on a Zeiss Axioskop

equipped for epifluorescence microscopy and photographed on Kodak T-MAX 400 film. Intact cells were visualized microscopically by phase-contrast or Nomarski optics. Cell morphologies were arbitrarily assigned as follows: unbudded cells, no visible bud; small-budded cells, buds of up to one-half of the diameter of the mother cell; large-budded cells, buds of greater than one-half of the diameter of the mother cell.

Flow cytometry. Cells were fixed in ethanol and stained with propidium iodide by the method of Hutter and Eipel (20). For each sample, the DNA content of 20,000 cells was determined with a Coulter EPICS 752 flow cytometer.

Karyogamy measurement. α strains DBY3383 and DBY3384 and α strains DBY3382 and DBY3385 (see Table 1 for genotypes) were mated on filters in all four combinations as previously described (12). Matings at 26°C proceeded for 5 h; those at 11°C went on for 48 h. After mating, cells were removed from the filters by sonication into water and their titers were determined. The numbers of diploids formed were determined by plating on SD plus histidine, leucine, and uracil. The numbers of cytoductants generated which carried the α parent nucleus were determined by plating on YEP plus 3% glycerol and cycloheximide and cryptopleurine at 5 and 0.5 μ g/ml, respectively.

Analysis of tubulin levels. Cultures were grown to the late-log phase in minimal medium (SD) and harvested. Cells were disrupted by hard vortexing with glass beads for 1 min, followed by addition of sodium dodecyl sulfate-containing sample buffer and boiling. Equivalent amounts of the samples were subjected to electrophoresis through a sodium dodecyl sulfate–8% polyacrylamide gel and then transferred to nitrocellulose filters (4). The filters were stained with Ponceau S to ascertain even transfer of proteins. The filters were then probed with antisera 124, 345, and 346, which were generated against peptides from TUB2, TUB1, and TUB3, respectively. These sera were a generous gift from F. Solomon, and their generation and specificity have already been described (1, 42; P. J. Schatz, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1987). The weak cross-reactivities of antiserum 345 with TUB3 and antiserum 346 with TUB1 previously noted (Schatz, Ph.D. thesis) were judged to make insignificant contributions to the results of these experiments. To visualize the tubulin polypeptides, the filters were then probed with ¹²⁵I-labeled protein A and exposed to X-ray film.

Overproduction of CIN1. Cultures of DBY5355 (*cin1::HIS3a*) or DBY5354 (*CIN⁺*) carrying the 2 μ m plasmid pGM65 (*GAL1* promoter only) or pRB1311 (*GAL1* promoter driving the *CIN1* gene) or the *CEN* plasmid pBM258 (*GAL1* promoter only) or pRB1310 (*GAL1* promoter driving the *CIN1* gene) were grown on minimal medium containing 3% glycerol and 2% lactate as carbon sources to remove catabolite repression. Uracil was omitted to select for the plasmids. Cells from these cultures were then inoculated in spots on minimal medium (minus uracil) containing either glucose or galactose and different concentrations of benomyl. Growth of the spots was scored qualitatively.

RESULTS

Mutants with increased rates of chromosome loss. One of the major roles for microtubules in *S. cerevisiae* is partitioning of replicated chromosomes at mitosis. The rate of loss of individual chromosomes has been determined to be approximately one in every 10⁵ cell divisions (13, 14, 32). Impairment of tubulin activity dramatically increases this rate

TABLE 2. Measured frequencies of chromosome loss and mitotic recombination

Genotype ^a	Median frequency of:			
	Chromosome loss (10 ⁴) ^b		Mitotic recombination (10 ⁵) ^c	
	26°C	11°C	26°C	11°C
Wild type	1.3	3.5	1.5	1.8
<i>tub1-1</i>	1.9	930	2.2	5.8
<i>tub3::URA3</i>	5.0	170	1.0	1.7
<i>cin1::LUK7</i>	6.7	150	2.3	2.7
<i>cin1-4</i>	5.9	1,300	NT ^d	NT
<i>cin1-5</i>	7.8	1,100	NT	NT
<i>cin4::URA3</i>	0.85	130	0.85	4.5
Wild type ^e	1.6	4.5	2.7	3.2
<i>cin2::URA3</i> ^e	3.0	91	1.4	3.4

^a All of the strains used were isogenic to DBY3363 (wild type; see Table 1), except for the following mutations: DBY5343 (*tub1-1*); DBY5344 (*tub3::URA3*); DBY5345 (*cin1::LUK7*); DBY5346 (*cin1-4*); DBY5347 (*cin1-5*); DBY5348 (*cin4::URA3*); DBY5349 (*cin2::URA3*).

^b Median frequency at which colonies resistant to both cryptopleurine and canavanine appeared.

^c Median frequency at which colonies resistant to cryptopleurine and prototrophic for leucine appeared.

^d NT, Not tested.

^e The comparison of *cin2::URA3* with the wild type was performed on a separate occasion and is therefore reported separately.

(Table 2; 18, 55). Mutant forms of other microtubule-specific functions were anticipated to cause a similar elevation in chromosome loss frequency.

Figure 1 depicts the genotype of a yeast strain that we constructed that allows one to monitor easily the rate of chromosome loss events in a growing colony. It is a haploid strain disomic for chromosome III. One chromosome III homolog (the upper copy in Fig. 1) was marked such that if a cell failed to inherit it, four distinct phenotypic changes would result. Loss of this chromosome results in a change from sensitivity to resistance to the antibiotics canavanine and cryptopleurine. This is due to loss of the dominant wild-type alleles of the *CAN1* and *CRY1* genes carried on opposite sides of the centromere (31, 54). This copy of chromosome III also carries the wild-type *LEU2* gene and the *ade2* ochre-suppressing *SUP11-1* tRNA gene (15). Loss results in auxotrophy for leucine and adenine. The change from Ade⁺ to Ade⁻ causes a colony change from white to red due to accumulation of a metabolic pigment. The rate of loss of the marked chromosome III, determined by fluctuation analysis, was close to the rates that have been measured for chromosome loss in euploid strains (Fig. 1).

To screen for *cin* (chromosome instability) mutants, mutagenized strains carrying the disomy described above were allowed to grow into colonies on rich medium and then replica transferred to media containing both canavanine and cryptopleurine. Cells that lost the marked chromosome III appeared as rapidly growing red papillae in a white hazy background. We recognized 550 potential *cin* mutants by their higher-than-wild-type rates of papillation.

Increased sensitivity to benomyl in a subset of *cin* mutants. Since supersensitivity to benomyl is a common phenotype among tubulin mutants, we were curious to see whether any of our *cin* mutants were affected by this drug. Our wild-type haploid strains withstood benomyl concentrations in the medium of up to approximately 15 μ g/ml (at 26°C). Among 550 *cin* mutants selected by the above-described protocol, 99

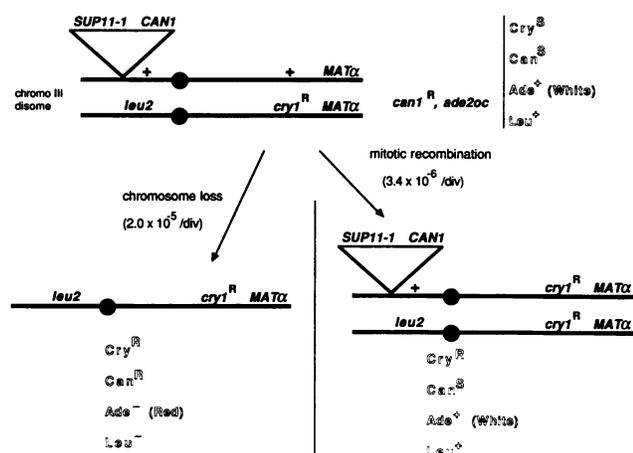


FIG. 1. Chromosome loss tester strain. The genotype of a chromosome (chromo) III disomic haploid strain used to monitor chromosome loss and mitotic recombination rates is depicted at the top. Relevant markers include the ochre-suppressing tRNA gene *SUP11-1* and the wild-type allele of the *CAN1* gene, both inserted by homology-directed transformation adjacent to the *LEU2* locus. The lower chromosome III pictured carries a recessive mutation (*cry1^R*) that confers cryptopleurine resistance; the recessive mutation (*can1^R*) that confers canavanine resistance is located at its normal locus on chromosome V. *ade2oc* indicates an ochre allele at this locus (on chromosome XV). Open letters show the phenotypes produced by the indicated genotypes. Failure to inherit the upper copy of chromosome III (chromosome loss) would produce the genotype and phenotypes indicated at the lower left. A mitotic recombination event that occurs between the centromere and the *CRY1* locus, followed by the proper pattern of segregation, produces the genotype and phenotypes indicated at the lower right. Also indicated are the measured rates at which these genotypes are produced by the disome, as determined by the method of the medium (26). div, Division.

were inhibited for growth by benomyl at 10 $\mu\text{g/ml}$. Fifteen of these mutants were extremely sensitive to benomyl, being affected by 5 μg or less per ml. Complementation and recombination testing of this set of highly benomyl-supersensitive mutants revealed that five contained mutations in the yeast α -tubulin-encoding genes: three in the *TUB1* gene and two in the *TUB3* gene. The remaining 10 benomyl-supersensitive mutants were each found to contain a mutation in one of three new genes that we termed *CIN1*, *CIN2*, and *CIN4*. Mutations in these three *CIN* genes, as well as in the three yeast tubulin genes, were isolated independently by screening mutants directly for benomyl supersensitivity (Stearns et al., in press). The genetic map positions of *CIN1*, *CIN2*, and *CIN4* do not correspond to those of the tubulin genes or any other known genes (Stearns et al., in press). Biochemical and genetic experiments have led to the conclusion that tubulin is the only intracellular target of benomyl (8, 16, 48, 52); thus, it seems likely that many of the benomyl-supersensitive *cin* mutants define genes involved with microtubule function.

Below, we describe our phenotypic characterization of *cin1*, *cin2*, and *cin4* mutants; the other *cin* mutants will be described elsewhere. In a separate report (Stearns et al., in press), we will describe the cloning, molecular characterization, and disruption of these genes. The results of these experiments demonstrate that the *CIN1*, *CIN2*, and *CIN4* genes are not essential for viability and that the null alleles have phenotypes similar to those of the ethyl methane-

sulfonate-induced mutants. For this reason, we performed much of the following phenotypic characterization with null alleles.

Elevated rates of chromosome loss but not mitotic recombination caused by *cin1*, *cin2*, and *cin4* mutations. Quantitative measurements of chromosome III loss rates were performed with wild-type, tubulin mutant, and *cin* mutant strains (Table 2). In each case, multiple cultures were analyzed. For simplicity, only median loss frequencies are reported. *tub1-1* is a mutant allele of the essential α -tubulin gene that causes cold sensitivity for viability (51). Incubation of a *tub1-1* chromosome III disomic tester strain at 11°C revealed that this mutation also greatly increases chromosome loss rates. A null allele of the nonessential α -tubulin gene, *tub3::URA3*, produces no observable effect on mitotic viability (44), but we found that it caused increased chromosome loss, especially at 11°C.

The two *cin1* missense alleles and a *cin1* insertion allele (*cin1::LUK7*) caused small but reproducible elevations in chromosome III loss at 26°C. Like the two α -tubulin mutants analyzed, the *cin1* mutants caused striking elevations in chromosome III loss at 11°C. Interestingly, the two missense alleles examined reproducibly caused a higher frequency of loss than the insertion allele. *cin2* and *cin4* null alleles also caused greatly increased rates of chromosome loss at 11°C.

Nondisjunction is just one of the possible causes of chromosome loss. Mutants that fail to properly replicate or repair DNA may also display increased chromosome loss. It is expected that some mutants in this latter class leave recombinogenic lesions in their chromosomal DNAs (14). Therefore, we also compared the rates of mitotic recombination events in our chromosome III disomic tester strains. A possible mitotic recombination event involving the chromosome III homologs that occurs between the centromere and the *CRY1* locus can allow segregation of two *cry1^R* alleles into a daughter cell (Fig. 1). Such a cryptopleurine-resistant progeny cell can be distinguished from a cryptopleurine-resistant cell caused by chromosome loss by the presence of the *LEU2⁺* (and *SUP11-1*) information on the other side of the centromere. The frequency of appearance of cryptopleurine-resistant *Leu⁺* cells is therefore a measure of recombination in the *CENIII-CRY1* interval. Like α -tubulin mutants, *cin* mutants displayed no significant change in the frequency of mitotic recombination in this interval (Table 2). Additional evidence that *cin* mutants are not affected for DNA replication was provided by the flow cytometry experiment described below.

Arrest of *cin1*, *cin2*, and *cin4* mutants at mitosis at cold temperatures. *cin1*, *cin2*, and *cin4* mutants caused greatly increased rates of chromosome loss at 11°C (see above). In addition, at 11°C, the *cin* mutant alleles caused greatly reduced growth rates and plating efficiencies relative to those of the wild type. Below, we present evidence that this cold sensitivity is due to a defect in traversal of the mitotic section of the cell cycle.

Shifting a culture of *S. cerevisiae* to conditions that promote microtubule depolymerization causes a block in the cell cycle at mitosis (18, 21, 38, 56). The cells arrest growth almost homogeneously as large-budded mononucleate cells. When exponentially growing cultures of *cin* mutants grown at 26°C were shifted to 11°C for 24 h (approximately two generations) and then observed by light microscopy, a marked increase in the proportion of large-budded cells was observed (Fig. 2A). For *cin1*, 74% of the culture consisted of large-budded cells that appeared much more swollen in size than wild-type large-budded cells. This is in contrast to the

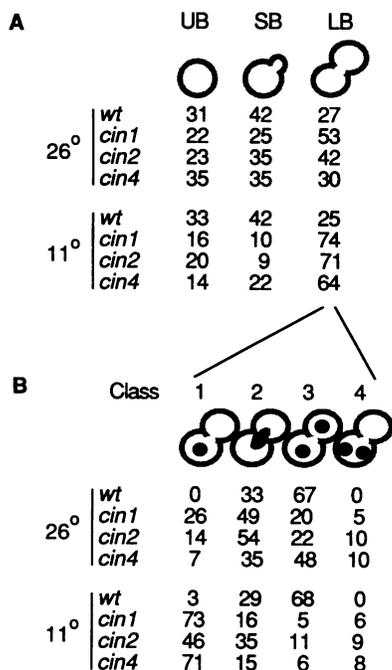


FIG. 2. Cell cycle arrest morphology caused by *cin* mutations. Log-phase cultures of wild-type (wt) and *cin* strains at 26°C were shifted to 11°C for 24 h. Pre- and postshift cell samples were fixed, stained with DAPI, and observed by light microscopy and fluorescence microscopy. (A) Percentages of morphological subtypes observed. (B) Numbers and positions of nuclear DNA staining regions in the large-budded cell class (percentage of total). Strains used: DBY5350 (wild type); DBY5351 (*cin1::HIS3b*); DBY5352 (*cin2::LEU2*); DBY5353 (*cin4::URA3*).

wild-type distribution of approximately one-third of each morphological class, unbudded, small budded, and large budded. *cin1* mutants were also consistently slightly elevated for large-budded cells, even at the permissive temperature of 26°C. *cin2* and *cin4* mutants were similarly affected by growth at 11°C, although the *Cin4* phenotype was less severe. This is consistent with the observation that *cin4* null mutants are less sensitive to benomyl than are *cin1* and *cin2* null mutants (Stearns et al., in press).

In *S. cerevisiae*, microtubules are required for separation of chromosomes during mitosis. In addition, they are required for the observed migration of the dividing nucleus to the neck between mother and daughter cell bodies (18, 21). Cytological observations and the phenotypes of certain β -tubulin mutants suggest that nuclear migration is a function of the microtubules that extend outward from the yeast microtubule-organizing center (the spindle pole body) into the cytoplasm. Intranuclear microtubules are presumed to play a more direct role in the segregation of chromosomes.

The numbers and positions of nuclear chromosomal DNA masses in the large-budded cells in our cultures were determined by staining with the DNA-specific fluorescent dye DAPI. Comparison of *cin* mutants with the wild type yielded striking differences (Fig. 2B). At both 26 and 11°C, two-thirds of the wild-type large-budded cells had divided and segregated their chromosomes into the two separate cell bodies (class 3). Most (at 11°C) or all (at 26°C) of the remaining large-budded cells had their DNA at the neck between mother and daughter, presumably in division (class 2). After incubation at 11°C, only 5 to 11% of the *cin* mutant

large-budded cells had segregated divided chromosomes properly (class 3); even at 26°C, they were significantly reduced relative to the wild type. At 11°C, most *cin* large-budded cells were mononucleate, with the DNA usually located at a position away from the neck (class 1).

In addition, the *cin* mutants showed high percentages of cells with two nuclei in one cell body (class 4), a morphology never observed in a wild-type culture. These cells were observed at high frequencies, even at 26°C, the permissive temperature for growth. In many *cin* cells, nuclei aberrantly dividing in one cell body were observed. Anti-tubulin immunofluorescence microscopy revealed misoriented mitotic spindles in these cells (data not shown). We suggest that the nuclear positioning phenotypes that we observed are all related; they arose from failure of the cytoplasmic microtubules to draw the dividing nucleus into the neck. Cultures of *cin* cells at 26°C also contained significant numbers of other aberrant morphological types. Between 1 and 3% of the cells were multinucleate (unbudded or small-budded cells containing two or more nuclei or large-budded cells containing three or more nuclei). An equal fraction was aploid (no nuclear DNA staining visible but mitochondrial DNA staining apparent). These two classes presumably arose from cytokinesis of class 4 large-budded cells.

In *S. cerevisiae*, morphological tests alone cannot easily distinguish between a defect in chromosome segregation and a defect in DNA synthesis; both defects cause arrest as mononucleate large-budded cells. The point of accumulation in the cell cycle can be more accurately determined by flow cytometric analysis of the DNA content of the arrested cells. As demonstrated by the wild-type strain in Fig. 3, exponentially growing cells are divided by flow cytometry into two almost equal classes: those with G_1 DNA content ($2n$ for diploid cells) and those with G_2 DNA content ($4n$ for diploids). Addition of the DNA synthesis inhibitor hydroxyurea caused accumulation of most of these diploid cells in the $2n$ peak, while the anti-microtubule agent nocodazole shifted most of the cells into the $4n$ peak (data not shown). Samples of the cultures used in the above-described morphological analysis were fixed, stained, and tested in a flow cytometer (Fig. 3). The wild-type cells gave approximately equal $2n$ and $4n$ peaks at both 26 and 11°C. For all three *cin* mutants, two peaks were observed at 26°C, but the proportions of $4n$ cells had increased. For *cin1* and *cin2*, incubation at 11°C resulted in appearance of almost all of the cells in the $4n$ peak. The *cin4* culture showed a similar but less severe phenotype. In addition, the *cin* mutants accumulated significant numbers of cells containing higher-than- $4n$ amounts of DNA. Some or all of these polyploid cells were probably the multinucleate cells described above. We conclude that the *cin1*, *cin2*, and *cin4* mutations cause a block in the cell cycle after DNA synthesis.

Cold-sensitive microtubule structures of *cin1*, *cin2*, and *cin4* mutants. Following a shift to 11°C for 24 h, samples of wild-type and *cin* cells were prepared for anti-tubulin immunofluorescence microscopy. Figures 4A and B show typical cells from the wild-type culture. Note the two large-budded cells containing elongated late mitotic spindles (Fig. 4A) and segregated progeny nuclear DNA (Fig. 4B). Figures 4C and D show two typical large-budded *cin1* cells after the same treatment. A striking reduction in microtubule structure is evident in almost every cell in the culture (Fig. 4C). Most cells have tubulin-specific staining in only one small region, presumably from some residual structure associated with unseparated spindle pole bodies or a very short spindle. As described above, most large-budded cells contained only

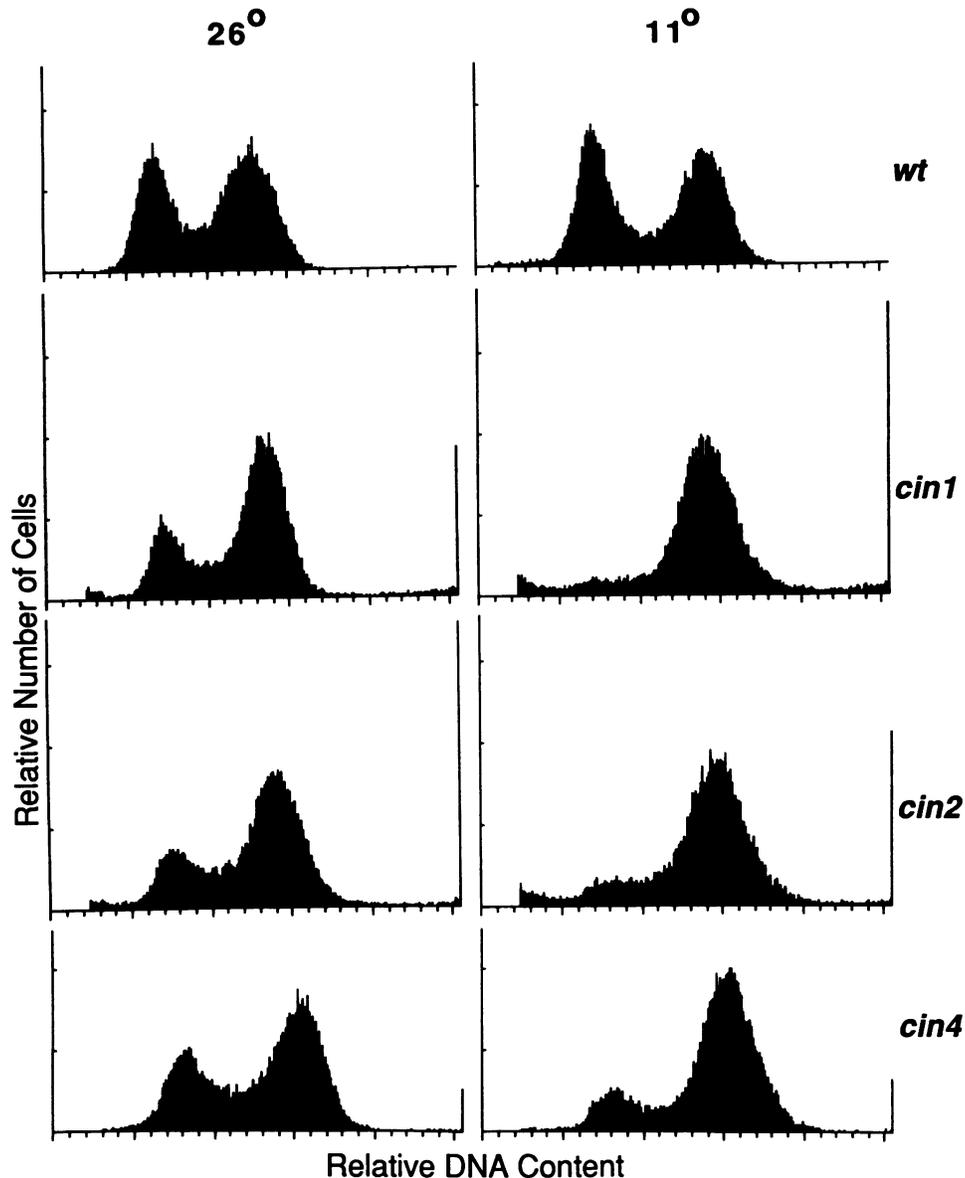


FIG. 3. Flow cytometric analysis of *cin* cell cycle defects. Samples of the cultures described in the legend to Fig. 2 were fixed, stained with propidium iodide, and examined by flow cytometry. We analyzed 20,000 cells of each sample. The bars at the right of some of the panels represent the relative numbers of cells with DNA staining that was higher than the end of the scale. wt, Wild type.

single nuclei (Fig. 4D). The *cin2* mutant showed an identical phenotype (Fig. 4E and F). The *cin4* mutant (Fig. 4G and H) behaved similarly but appeared slightly less affected. Slightly more residual microtubule structure could be detected in cultures of this strain.

Karyogamy defect of *cin1* mutants. Karyogamy is the process by which nuclei fuse during conjugation to form a cell of doubled ploidy. Cytological, drug inhibitor, and tubulin mutant studies have revealed that microtubules are essential for this process (5, 9, 18). In a newly formed zygote, the two parental nuclei are drawn together by a specialized structure composed of cytoplasmic microtubules. Nuclear fusion initiates when the two parental spindle poles meet. Rare failures in karyogamy result in production of cytoductants, cells with the haploid nuclear genotype of one of the parents but mixed cytoplasm contributed by both parents. Rare cytoductants can be detected by using prop-

erly marked strains. We found that mating *cin1* mutants resulted in 7- and 23-fold higher frequencies of cytoductants than isogenic *CIN⁺* matings at 26 and 11°C, respectively (Table 3). A slight increase in production of cytoductants was also detectable at 11°C when one mating parent carried *cin1* and the other carried *CIN⁺*.

Reduced microtubule stability in *cin1* mutants not due to reduced tubulin levels. The observed reduced microtubule stability in *cin1* strains implies that the *CIN1* gene product regulates the activity of a yeast microtubule component or is itself a component of microtubules (see Discussion). With respect to the former model, we considered the possibility that the *CIN1* gene product regulates the intracellular levels of tubulin. To test this, rapid-lysis protein extracts from an isogenic strain set were subjected to gel electrophoresis, transferred to nitrocellulose, and probed with antibodies directed against α - or β -tubulin. No differences in total

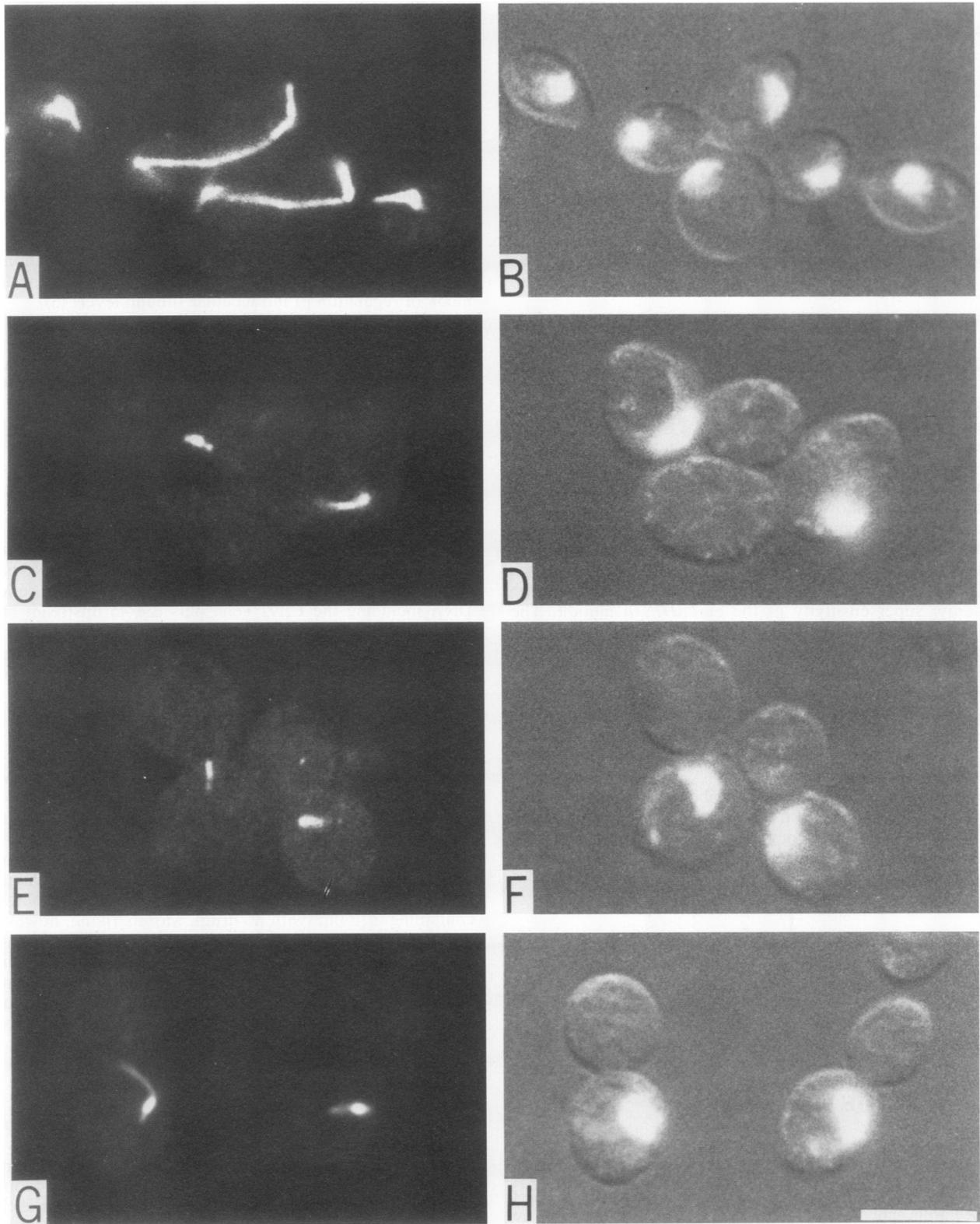


FIG. 4. Tubulin staining of *cin* mutants. Diploid cells from cultures (described in the legend to Fig. 2), after a shift to 11°C for 24 h, were fixed and stained with anti-tubulin antibodies (A, C, E, and G) and DAPI (B, D, F, and H) as described in Materials and Methods. Genotypes: A and B, wild type; C and D, *cin1::HIS3b*; E and F, *cin2::LEU2*; G and H, *cin4::URA3*. Bar, 6 μ m.

TABLE 3. Effect of *cin1* mutation on karyogamy

Genotype of:		Cytoductant/diploid ratio at ^a :	
Parent 1 (<i>[rho</i> ⁺] <i>CYH</i> ⁺ <i>CRY</i> ⁺)	Parent 2 (<i>[rho</i> ⁰] <i>cyh2 cry1</i>)	26°C (10 ⁵)	11°C (10 ⁴)
<i>CIN</i> ⁺	<i>CIN</i> ⁺	1.7	3.9
<i>CIN</i> ⁺	<i>cin1</i>	1.9	13.2
<i>cin1</i>	<i>CIN</i> ⁺	2.7	17.9
<i>cin1</i>	<i>cin1</i>	12.0	87.6

^a Titers of cytoductants that had inherited nuclei from only parent 2 were determined by plating on medium containing glycerol as the carbon source (selects for the presence of mitochondrial DNA or *rho*⁺) and cycloheximide and cryptopleurine (selects against the dominant *CYH*⁺ and *CRY*⁺ alleles). Diploid titer was determined by complementation of auxotrophies.

β -tubulin levels between wild-type and *cin1* cells were detected (data not shown). Under the same conditions, antibody 345, a serum with high specificity for the α -tubulin encoded by *TUB1* but not that encoded by *TUB3*, detected a reduction in total intracellular TUB1 in the *cin1::HIS3a* strain relative to the wild type (top of Fig. 5, lanes 2 and 3). The level of TUB1 appeared to be reduced approximately 45% (by densitometer tracing). This observed reduction was reproducible and was also seen in *cin1::LUK7* and *cin1-1* strains (data not shown). Transformation of the *cin1::HIS3a* strain with a 2 μ m multicopy plasmid that carries the *TUB1* gene restored TUB1 levels to a higher-than-wild-type level (although not quite to the wild-type-plus-plasmid level; lanes 1 and 4). However, *TUB1* in multicopy did not suppress the benomyl supersensitivity of the *cin1::HIS3a* mutant. The additional *TUB1* gene product did confer a slight increase in benomyl resistance to the *cin1::HIS3a* strain, allowing growth on 1 μ g/ml but no higher. This strain was still much more sensitive than the wild type, which can withstand up to 15 μ g/ml. The slight increase in benomyl resistance of the *cin1::HIS3a* strain is probably analogous to the increase conferred upon the wild type by multiple copies of α -tubulin genes (44).

When the protein extracts described above were probed with antibody 346, a serum with specificity for the α -tubulin encoded by *TUB3* but not that encoded by *TUB1*, two interesting effects became clear (bottom of Fig. 5). (i) Like

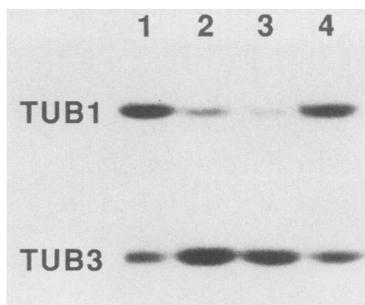


FIG. 5. Tubulin levels in *cin1* mutants. Rapid-lysis extracts of various cultures were electrophoresed through a sodium dodecyl sulfate–8% polyacrylamide gel, transferred to nitrocellulose, and probed with antibodies to TUB1 or TUB3. All four strains are derived from DBY5356 (lane 2) by transformation and are therefore isogenic, except for the *CIN1* allele and the presence or absence of the *TUB1* overproduction plasmid (pRB327). Lanes: 1, *CIN*⁺—pRB327; 2, *CIN*⁺—no plasmid; 3, *cin1::HIS3a*—no plasmid; 4, *cin1::HIS3a*—pRB327.

TUB2 but unlike TUB1, total TUB3 levels did not appear to be significantly affected by the *CIN1* genotype. (ii) The presence of the *TUB1* overproduction plasmid reduced total TUB3 levels about twofold, irrespective of the *CIN1* genotype. This implies the presence of a specific feedback regulatory system that operates on TUB3 levels. More investigation into the nature of this regulation is required.

In summary, the results presented in this section suggest that the microtubule defect of *cin1* mutants does not reflect simple reduction of total intracellular α - or β -tubulin levels. However, undetected qualitative differences in tubulin pools cannot be ruled out. The observed reduction in TUB1 levels in *cin1*-carrying strains could reflect a decreased stability of this α -tubulin polypeptide that is a secondary consequence of reduced microtubule structure. Selective degradation of unassembled α -tubulin has been proposed to be an important mode of regulation of intracellular tubulin levels (3).

***cin1* mutant phenotype produced by overproduction of CIN1.** Producing higher-than-normal intracellular levels of a number of yeast cytoskeleton-related proteins has been observed to cause deleterious phenotypes (3, 10, 39). It is believed that the deleterious effects reflect the participation of these gene products in higher-order structures with other polypeptides. Increased levels of one component of a structure may sequester a second essential component into non-functional assemblies.

We investigated the effect of overproduction of the *CIN1* gene product by removing its normal promoter and replacing it with the regulatable *GAL1* promoter (22). Preliminary DNA sequence analysis revealed an *SspI* site located 45 base pairs upstream from the *CIN1* translation initiator codon. DNA including the *CIN1* gene plus the 45 upstream base pairs was placed downstream from the *GAL1* promoter in both a low-copy-number *CEN* plasmid and a multicopy 2 μ m plasmid. These constructs were transformed into the appropriate *GAL*⁺ strains.

When transformed into a *cin1* strain, the *P_{GAL}-CIN1* (*CEN*) plasmid, but not the *P_{GAL}* vector alone, caused galactose-specific complementation of the benomyl supersensitivity phenotype (Table 4). *P_{GAL}-CIN1* (2 μ m) behaved similarly but also partially complemented on glucose-containing media. This may be due to low-level expression from remaining *CIN1* promoter elements in the construction or inefficient repression of the *GAL1* promoter in high copy number. The *P_{GAL}-CIN1* (2 μ m) plasmid slightly inhibited growth of both wild-type and *cin1* strains on galactose-containing medium (with no drug). More striking, however, was the galactose-specific benomyl supersensitivity caused by this plasmid (Table 4; Fig. 6). On galactose-containing medium, *P_{GAL}-CIN1* (2 μ m) rendered the *CIN*⁺ host sensitive to benomyl at 10 μ g/ml. This sensitivity is multicopy specific; however, the *P_{GAL}-CIN1* (*CEN*) plasmid caused only a slight increase in sensitivity.

DISCUSSION

We screened for new yeast mutants that display tubulin mutantlike phenotypes. Specifically, we isolated strains with increased rates of mitotic chromosome loss. Among our mutants, we found new alleles of the two yeast α -tubulin genes. In addition to the new tubulin alleles, mutant alleles of three previously unidentified genes were recovered. *cin1*, *cin2*, and *cin4* mutants displayed extreme sensitivity to benomyl, as well as cold sensitivity for growth, arrest in mitosis at the nonpermissive temperature, and cold-sensitive microtubule structures. Multiple alleles of the *CIN1*, *CIN2*,

TABLE 4. Effect of overproduction of CIN1 on sensitivity to benomyl

Host genotype	Plasmid	Relative growth at the indicated benomyl concn ($\mu\text{g/ml}$) ^a :									
		Glucose					Galactose				
		0	5	10	15	25	0	5	10	15	25
<i>cin1</i>	P_{GAL}^b	++	-	-	-	-	++	-	-	-	-
<i>cin1</i>	P_{GAL} - <i>CIN1</i> (<i>CEN</i>)	++	-	-	-	-	++	++	+	±	-
<i>cin1</i>	P_{GAL} - <i>CIN1</i> (2 μm)	++	+	±	-	-	+	+	-	-	-
<i>CIN</i> ⁺	P_{GAL}^b	++	++	++	++	-	++	++	++	++	-
<i>CIN</i> ⁺	P_{GAL} - <i>CIN1</i> (<i>CEN</i>)	++	++	++	++	-	++	++	+	+	-
<i>CIN</i> ⁺	P_{GAL} - <i>CIN1</i> (2 μm)	++	++	++	++	-	+	+	-	-	-

^a Assessed qualitatively. See Fig. 6. Symbols: ++, growth into a thick uniform patch of cells after 3 days; +, growth into a thick uniform patch of cells after 4 to 5 days; ±, slow, nonuniform growth; -, no uniform growth, but light papillation was usually visible.

^b Strains carrying either the *CEN* or 2 μm galactose promoter vector behaved identically and therefore are reported together (as P_{GAL}) for simplicity.

and *CIN4* genes were isolated independently as benomyl-supersensitive mutants (Stearns et al., in press).

Disruption of the *CIN1*, *CIN2*, and *CIN4* genes revealed that their products were required for mitotic viability only at lower growth temperatures. At 26°C, *cin* null strains grew at the same rate as the wild type and could be distinguished only by their increased chromosome loss rate and sensitivity to benomyl. At 11°C, *cin* null strains arrested their mitotic cell cycle, although the block was incomplete (74% large-

budded cells for *cin1*). The arrest looked extremely similar to that caused by conditions that disrupt microtubules in *S. cerevisiae*. Most of the cells adopted a swollen mononucleate, large-budded morphology characteristic of arrest in the late G₂ or early M phase. Immunofluorescence microscopy revealed a striking loss of microtubule structure in these cells. Other microtubule functions, nuclear migration and karyogamy, appeared to be defective in the *cin* strains as well. Finally, *cin*-tubulin double mutants displayed pheno-

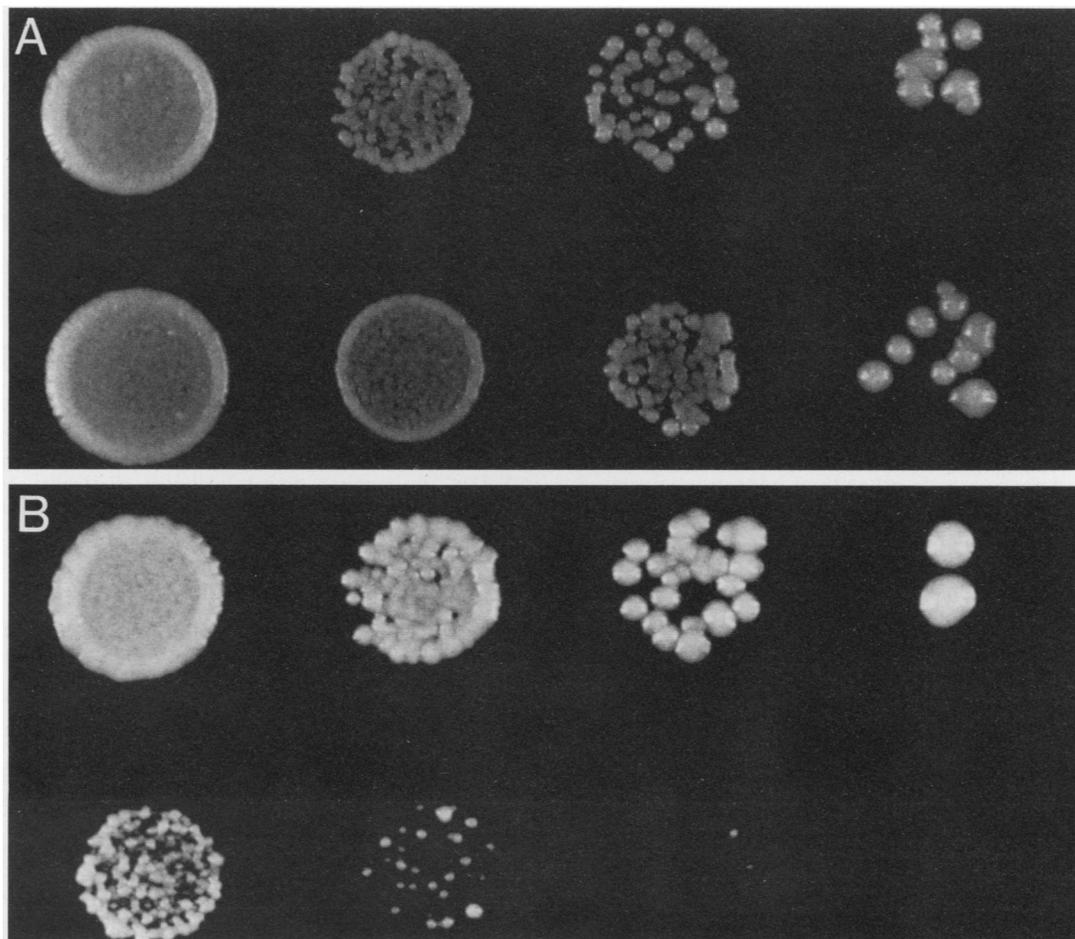


FIG. 6. Benomyl sensitivity caused by overproduction of CIN1. DBY5354 (*CIN*⁺) cells carrying pGM65 (P_{GAL}) (top row of spots in each panel) or pRB1311 (P_{GAL} -*CIN1*) (bottom row of spots in each panel) were inoculated into spots on media containing benomyl at 10 $\mu\text{g/ml}$ and either glucose (A) or galactose (B). Each row is a dilution series of the same cells, with the leftmost spot being the most concentrated.

types that suggest a functional relationship between their gene products (Stearns et al., in press).

We propose that the *CIN1*, *CIN2*, and *CIN4* gene products function to provide stability to yeast microtubules. Their absence becomes significant only under conditions that favor microtubule depolymerization, i.e., in the presence of benomyl or at low temperatures. Benomyl and other related benzimidazole compounds are believed to function in a manner similar to that of colchicine; by binding to tubulin, they shift the equilibrium of assembly toward the monomer form (7). We think it unlikely that the supersensitivity to benomyl is due to increased cell envelope permeability or altered metabolism of the drug for two reasons. Sensitivity to other antibiotics was not detected (Stearns et al., in press), but more importantly, the other microtubule-related phenotypes were observed when no drug was present.

The cold sensitivity observed in the absence of the *CIN1*, *CIN2*, or *CIN4* gene product probably reflects the intrinsic cold sensitivity of microtubule structures. Vertebrate microtubules are often observed to be cold-sensitive structures, both in vivo and in vitro (53; reviewed in reference 11). Microtubules polymerized in vitro from purified yeast tubulin have also been observed to be cold sensitive, although much less so than similarly formed vertebrate microtubules (23). Since *S. cerevisiae* can successfully divide at temperatures as low as 4°C, we propose that a mechanism(s) exists specifically to stabilize yeast microtubules against cold. The evolutionary advantage of cold-stable microtubules for fungi is self-evident.

The observed requirement of *CIN1*, *CIN2*, and *CIN4* for maximal microtubule stability suggests that their gene products are themselves structural components of yeast microtubules or that they regulate the activity of a microtubule component (or both). It appears that the major function of some MAPs is to increase the stability of microtubules; in vitro, some MAPs reduce the critical concentration of tubulin required for polymerization (6, 33). *CIN* gene products could serve an analogous role for yeast microtubules. However, demonstration of a MAP-like association of *CIN1*, *CIN2*, or *CIN4* with microtubules will require physical evidence, such as localization with specific antibodies or copolymerization with tubulin in vitro.

If *CIN1*, *CIN2*, and *CIN4* exert their effects as regulators of a microtubule component, a number of possible mechanisms can be suggested. As no obvious phenotypes could be found for *cin*-defective cells other than microtubule-related defects, we think it unlikely that *CIN* proteins affect microtubules in an indirect manner. For example, since microtubule structures are sensitive to the Ca^{2+} ion concentration (53), it can be proposed that *CIN* gene products regulate intracellular levels of this ion. We do not favor this possibility, as a defect in such an important function would be expected to have more severe consequences. However, *CIN* proteins may specifically regulate the ionic environment within a microtubule-related specialized structure, such as the spindle pole body.

Our experiments indicate that *CIN1* does not function by regulating total intracellular tubulin levels. However, regulation of the activity of specific intracellular pools of tubulin remains a strong possibility. Tubulins that differ in either posttranslational modification or intracellular location may play pivotal roles in the dynamics of microtubule function. Little is known of the intracellular distribution of tubulin subunits, its regulation, or its relevance to microtubule dynamics in any cell type. The problem of intracellular tubulin localization may be especially significant for fungi,

because their nuclear envelopes do not break down during mitosis. In *S. cerevisiae*, tubulin functions on both sides of the nuclear envelope simultaneously. This implies that a system for compartmentalizing tubulin exists, and at least two tubulin pools should be considered. *CIN1*, *CIN2*, and *CIN4* may play a role in this compartmentalization process. Although no posttranslational modifications for yeast tubulins have been described, they have been observed in many other cell types. In some cases, these modifications have been correlated with differences in microtubule stability (37, 46). Another possible role for *CIN* proteins is their participation in the covalent modification of tubulin subunits.

Overproduction of the *CIN1* gene product was found to cause benomyl supersensitivity, a phenotype also caused by *cin1* loss-of-function alleles. The most reasonable explanation for this effect is that *CIN1* participates in a multimeric complex whose proper function is sensitive to the level of *CIN1* relative to other components. High levels of *CIN1* may sequester another essential component(s) into nonfunctional assemblies. In a separate paper (Stearns et al., in press), we will present evidence that indicates that the products of the *CIN2* and *CIN4* genes act in the same pathway as *CIN1*, leading to microtubule stability. High levels of *CIN1*, therefore, may inhibit the action of *CIN2*, *CIN4*, or both.

It is not unreasonable to expect that the complex yeast mitotic spindle structure requires a large number of different interacting components to operate properly. By analogy, the flagellar axoneme of *Chlamydomonas* sp., another microtubular structure, is assembled from tubulin and over 250 other polypeptide components (28). Only a few yeast microtubule-related genes and proteins have been identified, and little is known of their functional roles (17, 36). It is anticipated that the collection of strains that show increased rates of chromosome loss (*cin*) will constitute a rich source of mutants in mitotic spindle function. These mutants should prove valuable for analysis of spindle structure and function.

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