Yeast Mutants Sensitive to Antimicrotubule Drugs Define Three Genes That Affect Microtubule Function

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ABSTRACT

Three new genes affecting microtubule function in *Saccharomyces cerevisiae* were isolated by screening for mutants displaying supersensitivity to the antimicrotubule drug benomyl. Such mutants fall into six complementation groups: *TUB1*, *TUB2* and *TUB3*, the three tubulin genes of yeast, and three new genes, which we have named *CIN1*, *CIN2* and *CIN4*. Mutations in each of the *CIN* genes were also independently isolated by screening for mutants with increased rates of chromosome loss. Strains bearing mutations in the *CIN* genes are approximately tenfold more sensitive than wild type to both benomyl and to the related antimicrotubule drug, nocodazole. This phenotype is recessive for all alleles isolated. The *CIN1*, *CIN2* and *CIN4* genes were cloned by complementation of the benomyl-supersensitive phenotype. Null mutants of each of the genes are viable, and have phenotypes similar to those of the point mutants. Genetic evidence for the involvement of the *CIN* gene products in microtubule function comes from the observation that some tubulin mutations are suppressed by *cin* mutations, while other tubulin mutations are lethal in combination with *cin* mutations. Additional genetic experiments with *cin* mutants suggest that the three genes act together in the same pathway or structure to affect microtubule function.

A basic question in understanding the eukaryotic cytoskeleton is how the assembly and disassembly of the subunits that make up the polymeric cytoskeletal elements is temporally and spatially regulated. The answer most likely lies in both the properties of the subunits themselves, and in the proteins that the cytoskeletal elements interact with. The microtubule cytoskeleton is involved in a wide variety of cellular events, including mitosis and meiosis, cellular morphogenesis, and organelle transport, each of which is likely to be mediated by different microtubule interactions. In all of these functions, regulation of microtubule stability is likely to play a major role. Microtubules *in vitro* (MITCHISON and KIRSCHNER 1984) and certain classes of microtubules *in vivo* (SAMMAK and BORISY 1988) display dynamic instability, with growing and shrinking phases in the same population. Little is known, however, of how the dynamic nature of the microtubule cytoskeleton is harnessed *in vivo* to participate in the diverse microtubule-mediated processes. In certain cases, it may be that controlling polymerization and depolymerization is sufficient to account for a given function; it has recently been proposed that depolymerization of microtubules may itself be the driving force behind the movement of chromosomes to the pole in mitosis (KOSHLAND, MITCHISON and KIRSCHNER 1988). In an effort to understand the factors regulating microtubules we have taken a genetic approach, isolating mutants in the yeast *Saccharomyces cerevisiae* that have phenotypes that might be expected of a defect in the regulation of microtubule function.

We have used two approaches to isolate such mutants. The first, presented in this paper, was to search for mutants that are supersensitive to the antimicrotubule drug benomyl. Benomyl is a member of the benzimidazole class of compounds that, at appropriate concentration, cause the depolymerization of yeast microtubules both *in vivo* (JACOBS *et al.* 1988) and *in vitro* (KILMARTIN 1981). Treatment *in vivo* results in the failure of the microtubule-mediated processes of nuclear division, nuclear migration, and nuclear fusion (QUINLAN, POGSON and GULL 1980; DELGADO and CONDE 1984; JACOBS *et al.* 1988). This approach was prompted by the observation that many tubulin mutants have been found to be benomyl supersensitive in both *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (UMESONO *et al.* 1983; SCHATZ, SOLOMON and BOTSTEIN 1986; STEARNS and BOTSTEIN 1988; HUFFAKER, THOMAS and BOTSTEIN 1988; SCHATZ, SOLOMON and BOTSTEIN 1988; KATZ and SOLOMON 1988; MATSUZAKI, MATSUMOTO and YAHARA 1988). By specifically screening for such mutants, we reasoned that we might isolate mutations in genes, other than those encoding tubulins, that regu-

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late the stability of microtubules. Particularly inspiring were the results of UMESONO *et al.* (1983), who isolated mutations in α- and β-tubulin genes of *S. pombe* and at least two other genes by screening for supersensitivity to the related drug thiabendazole, although these other genes have not been characterized.

The second approach, described in a separate paper (HOYT, STEARNS and BOTSTEIN 1990), was to search for mutants that lose chromosomes at a higher rate than wild-type. Interestingly, the spectrum of mutants isolated by these two approaches is very similar. As anticipated, we isolated a number of tubulin mutations, in both α-tubulin and β-tubulin genes. More interestingly, we also isolated mutations in three genes that do not encode tubulins, which we have named *CIN* (*C*hromosome *IN*stability) genes. In this paper, the isolation, cloning, and genetic characterization of these genes is presented. Genetic evidence suggests that the three *CIN* gene products play a role in regulating microtubule function in yeast, and that they might act in the same pathway or structure to perform this role. Evidence from morphological studies of the *cin* mutants (HOYT, STEARNS and BOTSTEIN 1990) shows that *cin* mutations result in phenotypes much like those of mutations in the tubulin genes themselves.

MATERIALS AND METHODS

Media and strains: Media for yeast growth and sporulation were as described by SHERMAN, FINK and LAWRENCE (1974), except for 5-fluoro-orotic acid medium, prepared as described by BOEKE, LACROUTE and FINK (1984). Benomyl, 98.6% pure, was a generous gift from IAN WELLINGS of E. I. duPont de Nemours and Co., Inc.; nocodazole was purchased from Sigma. Each drug was kept as a 10 mg/ml stock in dimethyl sulfoxide at −20°, which was thawed and added to warm (65°) YPD medium immediately before the plates were poured. The yeast strains DBY1034 *MAT***a***his4-539 lys2-801 ura3-52* and DBY1399 *MATα ade2-101 ura3-52* were used as the wild-type strains in these experiments, both for mutagenesis and backcrossing. For gene disruption experiments, strains DBY1826 *MAT***a** *ade2-101 his3-Δ200 leu2-3,112 ura3-52* and DBY1827 *MATα his3-Δ200 leu2-3,112 ura3-52* were used.

Isolation of mutants: Cells were grown in YPD to stationary phase and mutagenized with ethyl methanesulfonate (EMS) according to SHERMAN, FINK and LAWRENCE (1974). Aliquots were taken at 50, 60 and 70 min and plated to determine viability; one that had between 10% and 25% viable cells was chosen and plated on YPD to give a density of approximately 150 colonies per plate. These master plates were incubated at 26°, then replica-plated to YPD plates containing 5 μg/ml benomyl, as well as to YPD without benomyl. These plates were incubated at 26° for 2 days, then examined for clones unable to grow on the benomyl plates. The putative benomyl-supersensitive strains were purified by streaking for single colonies and retested for the benomyl-supersensitive phenotype. The strains that remained benomyl supersensitive after retesting were backcrossed to wild type. The benomyl-supersensitive phenotype was tested for dominance or recessiveness, and segregation through tetrad analysis. In all cases the phenotype segregated 2 wild type:2 benomyl-supersensitive, as expected for a trait determined by a single nuclear gene.

Genetic techniques and transformation: Yeast mating, sporulation, and tetrad analysis were performed as described by SHERMAN, FINK and LAWRENCE (1974). Growth on plates was assessed by spotting suspensions of cells in sterile water onto plates using a 32- or 48-point inoculator. Yeast cells were transformed with DNA by the lithium acetate method of ITO *et al.* (1983) as modified by KUO and CAMPBELL (1983) with sonicated salmon sperm DNA as carrier. Transformants were plated on synthetic complete (SC) medium lacking the appropriate nutrient to select cells carrying the plasmid. When Ura$^+$ was to be selected, 0.3% clarified casamino acids was added to the medium after autoclaving. Transformants were purified by streaking on selective medium. The *CIN* genes were cloned by transforming *cin* mutants with DNA from either a YCp50-based yeast DNA library (ROSE *et al.* 1987) or a YEp24-based yeast DNA library (CARLSON and BOTSTEIN 1982). In each case approximately 3,000 transformants were screened for complementation of the benomyl-supersensitive phenotype by replica-plating to medium containing 10 μg/ml benomyl.

Insertional mutagenesis of *CIN1* with mini-Tn*10*-LUK: A yeast centromere plasmid containing the *CIN1* gene was used as the target for mini-Tn*10*-LUK transposon hops as previously described (HUISMAN *et al.* 1987). The transposon provides a *URA3* gene for selection of Ura$^+$ in yeast. Bacterial colonies carrying plasmids that had suffered insertions were pooled and plasmid DNA extracted. This DNA was transformed into DBY3402 and Ura$^+$ transformants were tested for benomyl resistance. The plasmid DNA from transformants that remained supersensitive was extracted and introduced into *Escherichia coli* for analysis. Restriction digestion revealed the location of insertions that disrupted the function of the *CIN1* gene.

Immunofluorescence staining of yeast cells: Yeast cells were treated for immunofluorescence essentially as described by KILMARTIN and ADAMS (1984), with the exceptions that the cells were fixed by adding formaldehyde directly to the culture medium to a final concentration of 5%, and incubating at room temperature for 2 hr, and that cell walls were subsequently removed by incubating at 37° for 30 min in 50 μg/ml Zymolyase 100,000 in 0.1 M potassium phosphate (pH 7.5). Rat monoclonal antitubilin antibody, YOL 1/34, was obtained from Bioproducts for Science, Inc. FITC-conjugated goat anti-rat IgG antiserum was obtained from Cappel, Inc. DNA in the fixed and digested cells was stained with 1 μg/ml DAPI for 1 min. Preparations were viewed on a Zeiss Axioskop equipped for epi-fluorescence microscopy, and photographed on Kodak T-MAX 400 film.

Plasmid manipulations: The *E. coli* strain HB101 was the host for all plasmid manipulations. Restriction digests were performed according to the suggestions of the manufacturer (New England Biolabs). All plasmid constructions were made by purification of the desired DNA fragments on low melting point agarose gels, followed by ligation in the presence of the agarose, essentially as described by STRUHL (1983). Gel slices containing the fragments (usually about 50 μl of gel) were melted at 65° for 7 min, and then combined so that the total volume of agarose was 10 μl. After this mixture had cooled to 37°, 10 μl of 2 × T4 ligase buffer containing T4 DNA ligase was added, mixed, and incubated at 14° for approximately 18 h. To transform *E. coli* the reaction mixture was remelted at 65°, and diluted tenfold with 100 mM $CaCl_2$ 10 mM Tris (pH 8.0). Approximately one-fifth of this was then used in the transformation.

Gel-transfer hybridizations: DNA from agarose gels was transferred to Zetapor membrane as described by SOUTH-

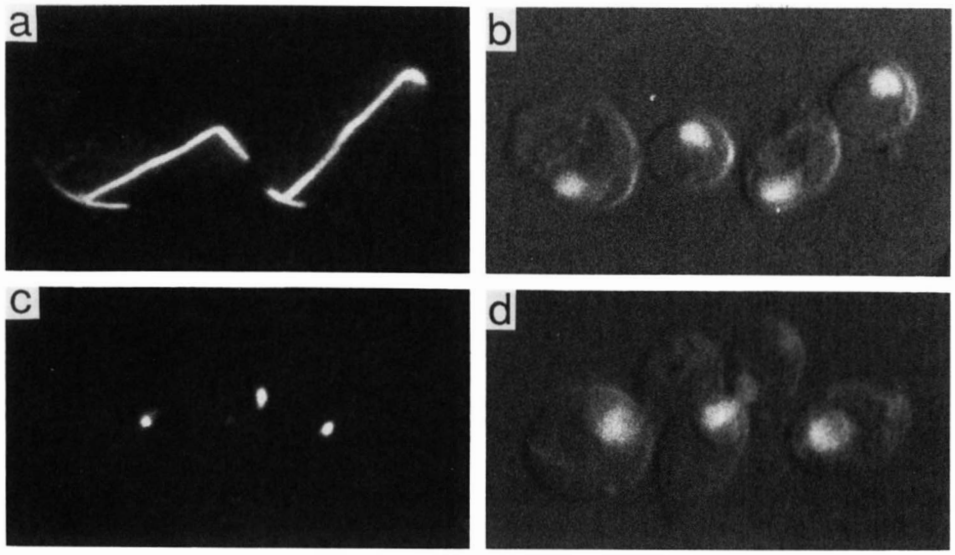


FIGURE 1.—Effect of benomyl on the microtubule cytoskeleton of yeast. The diploid yeast strain DBY5309 was grown in YPD at 26°. This culture was split, and to one portion benomyl was added to 30 μg/ml, the other received no additions. Incubation was continued for 2 hr, when the cells were fixed and prepared for immunofluorescence. Top panels (a and b), no benomyl; bottom panels (c and d), 30 μg/ml benomyl. Left (a and c), cells stained with antitubulin primary antibody (YOL1/34) and fluorescent secondary antibody; right (b and d), same cells stained with DAPI.

ERN (1975). OFAGE gels carrying chromosome sized DNA pieces (CARLE and OLSON 1984) were first depurinated in 0.25 N HCl for 20 min. Hybridizations with ^{32}P-labeled probes were performed under conditions described by WAHL, STERN and STARK (1979); washes were carried out in 2×SSPE at 65°. For low stringency hybridization the washes were carried out in 2×SSPE at 37°. ^{32}P-labeled probes were prepared by random hexamer labeling of restriction fragments in low melting point agarose (FEINBERG and VOGELSTEIN 1983).

RESULTS

Effect of benomyl on the yeast microtubule cytoskeleton: The effect of benomyl on the growth of *S. cerevisiae* has been extensively characterized (THOMAS 1984; SCHATZ, SOLOMON and BOTSTEIN 1986). We wished to determine the effect of high and low concentrations of benomyl on the yeast microtubule cytoskeleton directly, using antitubulin immunofluorescence. An exponentially growing culture of a wild-type diploid strain, DBY5309, was split into three cultures. Benomyl was added to two of the cultures (2 μg/ml or 30 μg/ml), and incubation was continued for 2 hr. Benomyl at 30 μg/ml completely inhibits the growth of diploid cells on solid medium, while at 2 μg/ml, benomyl has little effect under the same conditions. Cells from these cultures were then fixed, and stained with an anti-α-tubulin primary antibody and a fluorescent secondary antibody to visualize microtubules, and with DAPI to stain the DNA (Figure 1). Cells from the culture that did not receive benomyl show characteristic microtubule staining; the two cells shown in Figure 1, a and c, are in the large-budded stage of the cell cycle, and have a long spindle connecting the divided nuclear DNA. In contrast, the only microtubule staining present in cells that were incubated in 30 μg/ml benomyl is localized to one small dot on the periphery of the nucleus in each cell, probably corresponding to the spindle pole body(s) (JACOBS *et al.* 1988). This residual staining may represent the presence of a class of microtubules in yeast that are resistant to benomyl, or it may be that tubulin within the spindle pole body is inaccessible to the drug. The latter seems unlikely as similar treatment with nocodazole is able to cause the complete loss of stainable material (JACOBS *et al.* 1988). The microtubule structures of cells treated with 2 μg/ml benomyl appeared to be normal.

It should be noted that diploid yeast cells are more sensitive to benomyl than are haploid cells; typically half as much benomyl is required to achieve a given effect in diploid cells in comparison to haploid cells (STEARNS and BOTSTEIN 1988). Equally important is that the level of benomyl sensitivity is temperature dependent; sensitivity decreases as the incubation temperature is increased (THOMAS 1984). For this reason, all experiments in which benomyl sensitivity was assessed were performed at 26°.

Isolation of benomyl-supersensitive mutants: Wild-type haploid cells are able to grow on solid medium containing up to 15–20 μg/ml benomyl. We sought mutants that are unable to grow in the presence of 5 μg/ml benomyl. Wild-type haploid cells appear unaffected by this concentration of benomyl, similar to diploid cells treated with 2 μg/ml above. A wild-type haploid yeast strain was mutagenized with ethyl methane sulfonate (EMS) and individual mutagenized colonies were screened by replica-plating for inability to grow on medium containing 5 μg/ml benomyl. Of approximately 20,000 colonies screened, 29 were reproducibly supersensitive to benomyl. The mutants were backcrossed to wild-type and those recessive for benomyl-supersensitivity were assigned to complementation groups. The identity of individual genes was confirmed by recombination analysis.

Six different genes are represented by the mutants. Three of these are the tubulin genes of yeast: *TUB1*, *TUB2* and *TUB3*. Eight alleles of *TUB1* and one allele

TABLE 1

Strain list

Name	Genotype	Source
DBY1034	*MAT***a** *his4-539 lys2-801 ura3-52*	This laboratory
DBY1399	*MATα ade2-101 ura3-52*	This laboratory
DBY1826	*MAT***a** *ade2-101 his3-Δ200 leu2-3,112 ura3-52*	This laboratory
DBY1827	*MATα his3-Δ200 leu2-3,112 ura3-52*	This laboratory
DBY5309	*MAT***a**/*MATα ade2-101/ADE2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52*	This laboratory
DBY3393	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 cin1::HIS3*	This study
DBY3391	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 cin2::LEU2*	This study
DBY3444	*MATα his3-Δ200 leu2-3,112 ura3-52 cin4::URA3*	This study
DBY3397	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 cin1::HIS3 cin2::LEU2*	This study
DBY3399	*MATα his3-Δ200 leu2-3,112 ura3-52 cinl::HIS3 cin4::URA3*	This study
DBY3396	*MATα his3-Δ200 leu2-3,112 ura3-52 cin2::LEU2 cin4::URA3*	This study
DBY3390	*MATα his3-Δ200 leu2-3,112 ura3-52 cin1::HIS3 cin2::LEU2 cin4::URA3*	This study
DBY5340	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 tub1-1 cin1::HIS3* (pRB316)	This study
DBY5341	*MATα his3-Δ200 leu2-3,112 trp1-1 ura3-52 tub1-1 cin2::LEU2* (pRB316)	This study
DBY5342	*MAT***a** *ade2-101 lys2-801 ura3-52 tub1-1 cin4-7* (pRB316)	This study
DBY5280	*MAT***a** *his4-539 ura3-52 tub2-504*	This study
DBY5281	*MAT***a** *ade2-101 his4-539 ura3-52 cin4-4 tub2-504*	This study
DBY5282	*MAT***a** *his4-539 ura3-52*	This study
DBY5283	*MATα ura3-52 cin4-4 tub2-150*	This study
DBY5284	*MATα ade2-101 his4-539 ura3-52 cin4-4*	This study
DBY5285	*MAT***a** *ade2-101 ura3-52 tub2-150*	This study
DBY1662	*MATα ade1 leu1 cdc60-1*	P. JOYCE
DBY1831	*MATα ade1 his1 lys1 ura1 rnal-1*	G. FINK
DBY1833	*MAT***a** *leu2-3 ade3 arg1 lys7 met6 pet14*	G. FINK
DBY5365	*MATα his4-539 trp1-1 ilv2-1 cin4-4*	This study
DBY5366	*MAT***a**/*MATα ade2-101/ADE2 his6/HIS6 leu1/LEU1 lys2/LYS2 ura1/URA1 cdc64-1/CDC64 cin1-1/CIN1*	This study
DBY5367	*MAT***a**/*MATα his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/LYS2 ura3-52/ura3-52 pep4::HIS3/PEP4 gal4::LEU2/GAL4 ipl1-2/IPL1 cin2-7/CIN2*	This study
	Benomyl supersensitive mutants isolated in this study:	
DBY3401	*MATα ade2-101 lys2-801 ura3-52 cin1-1*	
DBY3402	*MAT***a** *ura3-52 cin1-1*	
DBY3403	*MATα ade2-101 lys2-801 ura3-52 cin1-2*	
DBY3404	*MAT***a** *ura3-52 cin1-2*	
DBY3405	*MATα ade2-101 his4-539 lys2-801 ura3-52 cin1-3*	
DBY3406	*MAT***a** *ura3-52 cin1-3*	
DBY3407	*MATα ade2-101 ura3-52 cin1-11*	
DBY3408	*MATα ade2-101 ura3-52 cin1-12*	
DBY3409	*MATα ade2-101 ura3-52 cin1-13*	
DBY3410	*MATα ade2-101 ura3-52 cin1-14*	
DBY3423	*MATα ade2-101 ura3-52 cin2-1*	
DBY3424	*MAT***a** *his4-539 ura3-52 cin2-1*	
DBY3425	*MATα ade2-101 ura3-52 cin2-2*	
DBY3426	*MAT***a** *his4-539 lys2-801 ura3-52 cin2-2*	
DBY3427	*MATα ade2-101 ura3-52 cin2-5*	
DBY3428	*MATα ade2-101 ura3-52 cin2-6*	
DBY3429	*MATα ade2-101 his4-539 ura3-52 cin2-7*	
DBY3430	*MAT***a** *ura3-52 cin2-7*	
DBY3434	*MATα ade2-101 his4-539 lys2-801 ura3-52 cin4-1*	
DBY3435	*MAT***a** *ura3-52 cin4-1*	
DBY3436	*MATα ade2-101 his4-539 ura3-52 cin4-2*	
DBY3437	*MAT***a** *his4-539 lys2-801 ura3-52 cin4-2*	
DBY3438	*MATα ade2-101 ura3-52 cin4-3*	
DBY3439	*MATα ade2-101 ura3-52 cin4-4*	
DBY3440	*MAT***a** *his4-539 ura3-52 cin4-4*	
DBY3441	*MATα ade2-101 lys2-801 ura3-52 cin4-5*	
DBY3442	*MATα made2-101 his4-539 lys2-801 ura3-52 cin4-7*	
DBY3443	*MAT***a** *his4-539 ura3-52 cin4-7*	
DBY3452	*MATα ade2-101 ura3-52 cin4-8*	
DBY3463	*MATα ade2-101 ura3-52 tub1-2*	

each of *TUB2* and *TUB3* were isolated (the *TUB2* and *TUB3* mutants were isolated concurrently as unlinked noncomplementers of a *tub1-1* mutant, and have been described; STEARNS and BOTSTEIN 1988). Some of the

Name	Genotype	Source
DBY3464	*MAT***a** *his4-539 ura3-52 tub1-2*	
DBY3467	*MAT***a** *lys2-801 ura3-52 tub1-3*	
DBY3468	*MATα ade2-101 ura3-52 tub1-3*	
DBY3469	*MATα ade2-101 lys2-801 ura3-52 tub1-4*	
DBY3470	*MAT***a** *his4-539 lys2-801 ura3-52 tub1-4*	
DBY3473	*MAT***a** *his4-539 lys2-801 ura3-52 tub1-8*	
DBY3474	*MATα ade2-101 lys2-801 ura3-52 tub1-8*	
DBY3475	*MATα ade2-101 ura3-52 tub1-9*	
DBY3476	*MAT***a** *his4-539 ura3-52 tub1-9*	
DBY3479	*MAT***a** *his4-539 lys2-801 ura3-52 tub1-10*	
DBY3480	*MATα lys2-801 ura3-52 tub1-10*	
DBY3481	*MATα ade2-101 lys2-801 ura3-52 tub1-11*	
DBY3482	*MAT***a** *his4-539 lys2-801 ura3-52 tub1-11*	
DBY3483	*MAT***a** *ura3-52 tub1-12*	
DBY3485	*MATα ade2-101 his4-539 lys2-801 ura3-52 tub1-12*	
DBY2510	*MAT***a** *his4-539 ura3-52 tub2-501*[a]	
DBY2511	*MATα ade2-101 ura3-52 tub2-501*[a]	
DBY2505	*MAT***a** *his4-539 lys2-801 ura3-52 tub3-1*[a]	

[a] Isolated concurrently as unlinked noncomplementers of *tub1-1* (STEARNS and BOTSTEIN 1988).

new *tub1* alleles exhibit cold sensitivity as well as benomyl supersensitivity, appearing to be similar to the original *tub1-1* mutant in this respect. The other three genes are unlinked to the tubulin loci and to each other. Because mutations in these three genes were isolated independently by screening for mutants with an increased frequency of chromosome loss (HOYT, STEARNS and BOTSTEIN 1990), we have designated them *CIN* genes (*C*hromosome *IN*stability). In total, seven alleles of *CIN1*, five alleles of *CIN2*, and seven alleles of *CIN4* were found by screening for benomyl supersensitivity, and an additional seven alleles of *CIN1*, two alleles of *CIN2*, and one allele of *CIN4* were found by screening for chromosome loss mutants. A complete list of the *tub* and *cin* mutant alleles isolated as benomyl-supersensitive mutants is presented in Table 1. All of the *cin* alleles are recessive to wild type for benomyl supersensitivity. The benomyl supersensitivity of the *cin2-7* mutation is suppressible by the *SUP11* ochre suppressor, thus this allele is presumably an ochre mutation in *CIN2*.

Cloning *CIN1*, *CIN2* and *CIN4*: The three *CIN* genes were cloned from yeast genomic DNA plasmid libraries by complementation of the recessive benomyl supersensitive phenotype that each displays. A representative mutant from each complementation group was transformed with plasmid library DNA and transformants able to grow on 10 μg/ml benomyl were isolated by replica plating. Plasmid DNA was recovered from these strains and examined after transformation into *E. coli*. *CIN1* was cloned from a YCp50 plasmid library (ROSE *et al.* 1987), *CIN2* and *CIN4* were cloned from both the YCp50 library and a YEp24 plasmid library (CARLSON and BOTSTEIN 1982). For each *CIN* gene, all recovered clones shared common restriction fragments, and the cloned sequences were shown to be capable of directing homologous recombination to the proper locus, demonstrating that the clones carry the authentic genes, and not unlinked suppressors.

The *CIN1* gene was localized on the cloned DNA fragment using the mini-Tn*10*-LUK bacterial transposon system (Figure 2). Insertions at four different sites, distributed over 3 kb, eliminated the ability of the cloned gene to complement the benomyl supersensitivity of a *cin1* mutant. The mini-Tn*10*-LUK element contains a *lacZ* gene without transcriptional and translational start signals; it can be activated only by appropriate insertion into an expressed gene. Mini-Tn*10*-LUK insertion number eight disrupts *CIN1* complementing activity and causes a blue color when yeast cells carrying the plasmid are grown on media containing X-gal. This indicates that the orientation of the *CIN1* gene is from left to right as depicted in the map in Figure 2. The *CIN2* (Figure 3A) and *CIN4* (Figure 3B) genes were localized by subcloning of the original inserts, and scoring the ability of the subclones to complement the benomyl supersensitivity of representative mutants. The smallest *cin2* complementing clone is a 1.1-kb *Mlu*I-*Sph*I fragment. The smallest *cin4* complementing clone is a 0.95-kb fragment that extends from the *Bgl*II site in the insert to the junction of insert and vector.

Genetic Mapping of *CIN1*, *CIN2* and *CIN4*: We used the cloned *CIN* genes to aid in determining the location of the genes on the yeast genetic map. For each of the three genes a radioactive probe was prepared from a restriction fragment internal to the

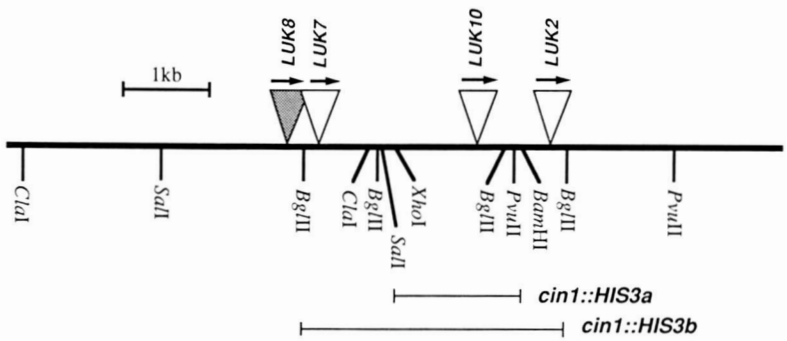
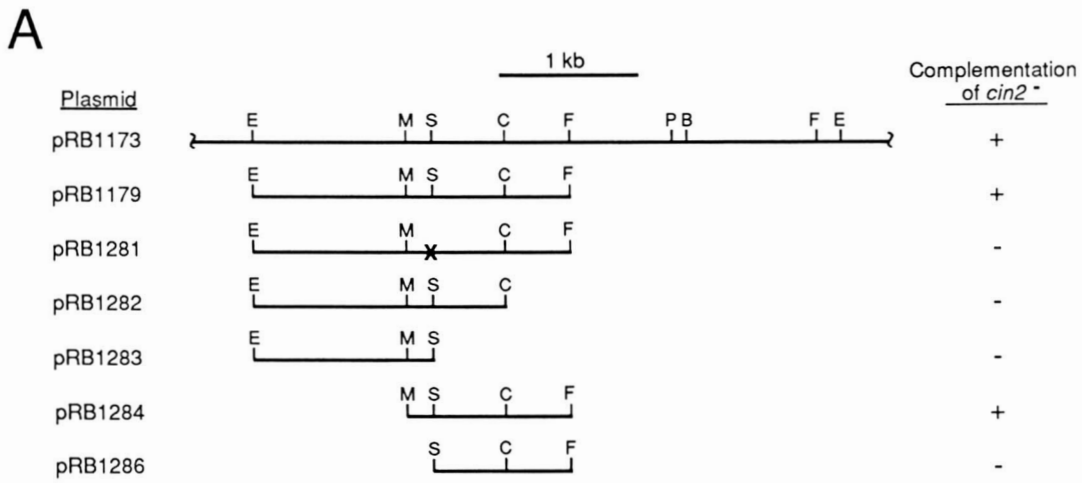


FIGURE 2.—Localization of the *CIN1* gene. A restriction enzyme map of the DNA in the vicinity of the *CIN1* locus is depicted. The triangles indicate the mapped positions of four mini-Tn*10*-LUK insertions that disrupt *CIN1* complementing activity. mini-Tn*10*-LUK insertion 8 is shaded to indicate that this insertion causes a blue color to develop on X-gal plates. The arrows above each insertion indicate the orientation of the element with respect to the translational orientation of the *lacZ* gene. The positions of two *in vitro* generated deletions of the *CIN1* gene are also shown. For *cin1*::*HIS3*a and *cin1*::*HIS3b*, the lines indicate the DNA that was removed and replaced with the yeast *HIS3* gene. *cin1*::*HIS3*a removes the DNA between the *Sal*I and *Bam*HI sites; *cin1*::*HIS3b* removes the DNA between the outside *Bgl*II sites.



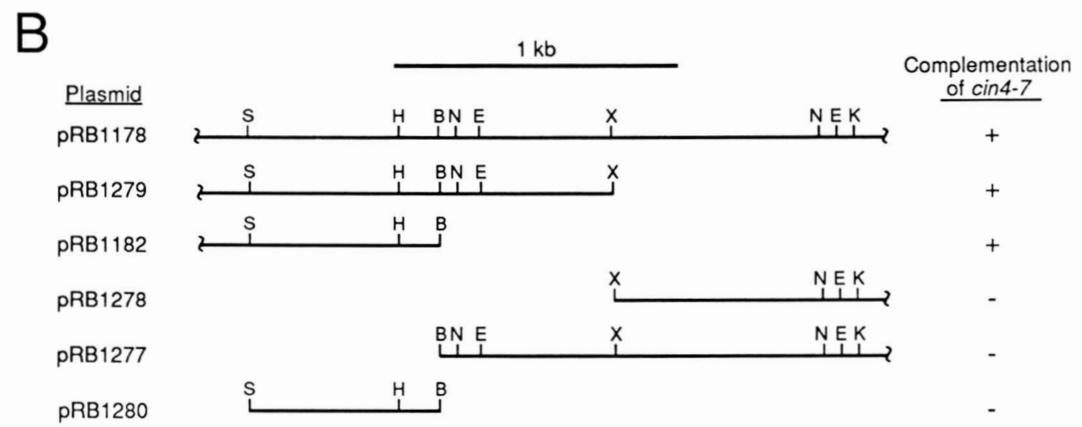


FIGURE 3.—Localization of the *CIN2* and *CIN4* genes. (A) pRB1173 was the smallest *cin2* complementing insert isolated from the YCp50 yeast DNA library. The two ends of this insert are *Bam*HI-*Sau*3A junctions. All other plasmids were constructed by inserting fragments of pRB1173 into YCp50, YCp412 (STEARNS, MA and BOTSTEIN 1990), or YEp420. In pRB1281 the *Sac*I site was destroyed by removing the 3′ overhanging ends with Klenow fragment and religating, creating a frameshift mutation. (B) pRB1178 was the smallest *cin4* complementing insert isolated from the YEp24 yeast DNA library. The two ends of this insert are *Bam*HI-*Sau*3A junctions. All other plasmids were constructed by inserting fragments of pRB1178 into YEp24 or YCp412. Restriction enzyme abbreviations are as follows: S, *Sac*I, H, *Hin*dIII; B, *Bgl*II; N, *Nhe*I; E, *Eco*RI; X, *Xba*I; K, *Kpn*I; M, *Mlu*I; C, *Cla*I; and F, *Sph*I.

complementing region and hybridized to a Southern blot containing yeast chromosomes separated by OFAGE electrophoresis (CARLE and OLSON 1985). In each case this provided an unambiguous chromosome assignment; *CIN1* on chromosome *XV*, *CIN2* on chromosome *XVI*, and *CIN4* on chromosome *XIII* (data

TABLE 2

Genetic mapping of *CIN* genes

Interval	PD:NPD:TT	Distance (cM)
cin1-cdc63	74:0:11	6.5
cin1-cdc64	48:0:34	20.7
cdc63-cdc64	23:0:23	25.0
cin2-gal4	184:0:26	6.2
cin2-pep4	11:1:36	43.8
cin2-cdc60	18:2:25	41.1
cin2-ipl1	74:0:64	23.2
ipl1-pep4	22:0:26	27.1
gal4-ipl1	37:2:52	35.2
gal4-pep4	11:3:34	54.2
cin4-ilv2	42:0:24	18.2
cin4-lys7	1:0:22	—
cin4-rna1	2:0:24	—

PD = parental ditype, NPD = nonparental ditype, TT = tetratype. Map distances were calculated according to the formula 100 × (1/2TT + 3NPD)/total number of tetrads (MORTIMER and SCHILD 1981). *ipl1-2* is a temperature sensitive mutation (C. CHAN, personal communication).

not shown). We then proceeded with genetic mapping by crossing *cin* mutant strains to strains bearing markers on the chromosomes of interest. The results of these mapping experiments are shown in Table 2. *CIN1* and *CIN2* were ordered with respect to neighboring genes in multifactor crosses. The determined orders, listed centromere proximal to centromere distal, are: *cdc64-cin1-cdc63*; *pep4-ipl1-cin2-gal4*. The map positions of the three *CIN* genes do not correspond to any known loci (MORTIMER and SCHILD 1985); we conclude that *CIN1*, *CIN2* and *CIN4* are newly identified genes.

Gene Disruptions of *CIN1*, *CIN2* and *CIN4*: The complementing region of each of the *CIN* genes was disrupted by deleting a portion of the coding sequence and inserting a yeast selectable marker. In each case the disruption construction removes sequences shown to be required for function in the gene localization experiments and would be expected to result in a null mutation. *CIN1* was disrupted by replacing the sequences between the *Bam*HI and *Xho*I sites with a 1.2-kb fragment containing the yeast *HIS3* gene, to make *cin1::HIS3a* (Figure 2). A more extensive *CIN1* disruption (*cin1::HIS3b*) was created by replacing the sequences between the *Bgl*II sites with a 1.6-kb fragment containing *HIS3*. These two disruptions behave identically, and *cin1::HIS3a* was used in the experiments described here. We also used *CIN1* disruptions created by insertion of the mini-Tn*10*-LUK transposable element; this transposon contains a *URA3* gene, allowing for selection of transplacement into the chromosome. *CIN2* was disrupted by replacing the sequences between the *Sac*I and *Cla*I sites with a fragment containing the yeast *LEU2* gene to make *cin2::LEU2*. *CIN4* was disrupted by replacing the sequences between the *Sac*I and *Hin*dIII sites and inserting *URA3* to make *cin4::URA3*. In each case a linear DNA fragment containing the disruption construction was transformed into a wild-type diploid strain such that it replaced one of the two chromosomal loci (ROTHSTEIN 1983), resulting in strains heterozygous for the disruption mutations. These strains were then sporulated and tetrads were dissected. For each of the *CIN* genes, all four spores were viable in the majority of tetrads, and the disruption marker segregated 2:2. Southern analysis confirmed that strains bearing the disruption marker lacked a wild-type copy of the *CIN* gene in question (not shown). Low stringency hybridization experiments failed to reveal the presence of any homologous loci in the yeast genome. These results indicate that *CIN1*, *CIN2* and *CIN4* are single-copy genes that are not required for viability under standard growth conditions.

Phenotypes of *cin1*, *cin2* and *cin4* mutants: The disruption alleles were used to examine the drug sensitivity of the *cin* mutants in more detail. This was done by spotting suspensions of cells on plates containing different concentrations of either benomyl or the related antimicrotubule drug nocodazole; the results are presented in Table 3. Wild-type haploid strains are able to grow well on up to 10 μg/ml benomyl, while *cin1* and *cin2* disruption mutants are inhibited for growth on plates by 0.5 μg/ml benomyl. Interestingly, *cin4* disruption mutants are not as sensitive to benomyl; they are inhibited by 2.5 μg/ml, but are able to grow on 1 μg/ml. The same relative drug sensitivities are seen on nocodazole-containing medium, although a lower concentration of drug is effective.

For *CIN1* and *CIN2* the level of sensitivity displayed by the null mutants and the EMS-induced mutants is identical (not shown). For *CIN4* however, certain of the EMS-induced mutants are more sensitive to benomyl than the disruption mutant. The nine *cin4* alleles (eight induced by EMS, one constructed *in vitro*) can be separated into two distinct groups by the degree of benomyl supersensitivity of strains bearing them. The null mutant *cin4::URA3*, and three EMS-induced mutants, *cin4-1*, *-2*, and *-3* are able to grow on medium containing 1 μg/ml benomyl, while *cin4-4*, *-5*, *-6*, *-7* and *-8* are not able to grow on 1 μg/ml benomyl. Representative members of these groups are shown in Figure 4.

The benomyl-supersensitive phenotype of all of the *cin4* mutants is recessive, thus it is particularly interesting that the disruption mutant is less sensitive to benomyl than a number of the point mutants. This is unlikely to be due to residual function of the disrupted protein, as preliminary sequence information indicates that only 80 bp of carboxy-terminal coding sequence remain in the *cin4::URA3* construction. In addition, two other different disruption constructions result in exactly the same phenotype (not shown). To exclude the possibility that the disruption mutant is

not truly null in phenotype, we constructed diploid strains in all possible combinations between the wild-type allele, *CIN4*, the most sensitive allele, *cin4-4*, and the disruption allele, *cin4::URA3*. The heterozygous strains *CIN4/cin4-4* and *CIN4/cin4::URA3* are both identical to *CIN4/CIN4* in benomyl-supersensitivity, establishing that each of the mutations is recessive. The homozygous mutant strains *cin4-4/cin4-4* and *cin4::URA3/cin4::URA3* display the same relative sensitivities as the haploid strains described above, with the disruption strain being less sensitive than the point mutant. The heteroallelic *cin4-4/cin4::URA3* strain displays the same level of sensitivity as the *cin4-4/cin4-4* strain. This experiment shows that *cin4::URA3* is a true null allele, and that the protein encoded by the *cin4-4* allele has some activity that is expressed in a null mutant background, but is recessive to wild type.

Because it is possible that a defect in permeability of the cell might result in benomyl supersensitivity, we examined the degree of sensitivity of representative members of each of the *CIN* complementation groups to drugs which are not known to affect microtubules. The sensitivity of these strains to canavanine, cyclohexamide, cryptopleurine, and ethidium bromide was identical to that of wild type, while the sensitivity to benomyl was manyfold greater than that of wild type.

Genetic interactions between *cin* and *tub* mutations: We have performed a variety of genetic tests to begin to understand the functional relationship between the *CIN* gene products and the microtubule cytoskeleton. First, while crossing the benomyl-supersensitive mutants to each other and to known tubulin mutants, it was noted that any cross between a *cin1, 2* or *4* mutant and a strain bearing a mutation in the α-tubulin gene *TUB1* resulted in a regular pattern of spore lethality. In all cases the inviable spores could be deduced to be *cin tub1* double mutants, and no double mutants could be recovered from the cross, despite the fact that the temperature at which the spores were germinated (26°) is permissive for each of the mutations. This suggested that the combination of a mutation in either *CIN1*, *CIN2* or *CIN4* with a mutation in *TUB1* is a lethal event in a haploid cell.

It has been reported that certain α-tubulin mutations result in poor spore viability, though they do not affect mitotic viability (SCHATZ, SOLOMON and BOTSTEIN 1986). We endeavored to determine whether the inviability of *cin tub1* double mutants is due to an actual mitotic growth defect, or to an inability to germinate. Double mutants were constructed between *tub1-1*, which causes a cold-sensitive cell cycle arrest and supersensitivity to benomyl (STEARNS and BOTSTEIN 1988), and alleles of each of the *CIN* genes, *cin1::HIS3*, *cin2::LEU2*, and *cin4-7*, in the presence of plasmid pRB316. This plasmid carried the wild-type α-tubulin gene *TUB3*, the *URA3* gene as a selectable marker, and the 2μ replication origin. The *TUB3* gene on this plasmid completely suppresses the defects of *tub1-1* (STEARNS and BOTSTEIN 1988), and the presence of the plasmid allowed the construction of the desired double mutants (DBY5340, 5341 and 5342). The double mutant strains were then assayed for the ability to grow without pBR316 by selecting against *URA3* function with 5-fluoro-orotic acid (BOEKE, LACROUTE and FINK 1984). In each case, no segregants that had lost the plasmid could be recovered, demonstrating that the combination of a mutation in any of the *CIN* loci with the *tub1-1* mutation results in a strain that is inviable for mitotic growth at a temperature at which each of the single mutants is viable.

Another genetic interaction between *cin* mutations and tubulin mutations was observed when we analyzed suppressors of the benomyl-supersensitive phenotype of *cin4-4*. Among the suppressors was one that confers benomyl resistance, and is tightly linked to the *TUB2* locus (38 PD:0 NPD:0 TT), indicating that the suppressor is a mutation in *TUB2*, the single yeast β-tubulin gene. We have named this allele *tub2-504*. Interestingly, both *tub2-504* and *cin4-4* are able to grow at 16°, yet the double mutant *cin4-4 tub2-504* is completely inhibited for growth at this temperature (Figure 5A). These two mutations then define a conditional-lethal synthetic interaction between the *CIN4* gene product and β-tubulin, the *TUB2* gene product. An identical interaction was observed between *cin1-3* and *tub2-505*; the *tub2* mutation was isolated as a suppressor of the benomyl supersensitivity of *cin1-3*, and the double mutant is cold sensitive at a temperature at which each of the single mutants is viable.

The benomyl-supersensitive phenotype of the *cin* mutants, as well as the range of other phenotypes observed (HOYT, STEARNS and BOTSTEIN 1990), suggested that a lack of *CIN* function results in microtubules that are less stable than those in wild-type cells. One prediction from this hypothesis is that a *cin* mutation might be able to suppress the phenotypic defects of a tubulin mutation that causes microtubules to be too stable. The β-tubulin mutation *tub2-150* (THOMAS 1984) can be thought of as such a mutation; it was isolated as a benomyl resistant allele of β-tubulin and was then found to require benomyl for mitotic growth at 34°. This benomyl requirement can be suppressed by growing the cells at a lower temperature, 20°. As both benomyl and low temperature can destabilize yeast microtubules (KILMARTIN 1981), the phenotype of *tub2-150* is consistent with the hypothesis that *tub2-150* causes microtubules to be too stable, thus requiring a destabilizing agent or condition to function properly. If a *cin* mutation can also destabilize microtubules, then it might suppress the *tub2-150* benomyl requirement. To test this prediction, we crossed a *cin4-4* strain to a *tub2-150* strain and ana-

TABLE 3

Antimicrotubule drug sensitivity of *cin* null mutants

Genotype	Benomyl (μg/ml)						Nocodazole (μg/ml)				
	0	0.25	0.5	1.0	2.5	5	0.25	0.5	1.0	2	4
Wild type	+	+	+	+	+	+	+	+	+	+	+
cin1::HIS3	+	+	−/+	−	−	−	+/−	−	−	−	−
cin2::LEU2	+	+	−/+	−	−	−	+/−	−	−	−	−
cin4::URA3	+	+	+	+	−/+	−	+	+	+/−	−	−

Cells were suspended in water and transferred to YEPD plates containing the indicated concentrations of drug with a multipronged inoculating device. Growth was scored as follows: +, uniform rapid growth; +/−, uniform slow growth; −/+, barely detectable growth; −, no growth.

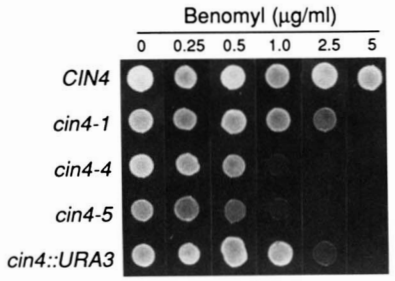


FIGURE 4.—Benomyl-supersensitivity of *cin4* mutants. Suspensions of cells in sterile water were spotted onto YPD plates containing the indicated amount of benomyl. From top to bottom, the strains used are DBY1034, DBY3435, DBY3440, DBY3441 and DBY3444.

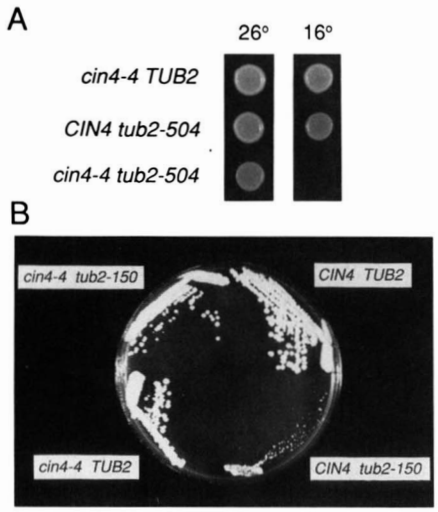


FIGURE 5.—Genetic interactions between *CIN4* and the β-tubulin gene *TUB2*. (A) A *cin4 tub2-504* double mutant is unable to grow at 16° while both of the single mutants are able to grow at that temperature. Suspensions of strains DBY3440, DBY5280 and DBY5281 in sterile water were spotted onto YPD plates and incubated at the indicated temperature. (B) The *cin4-4* mutation is able to suppress the benomyl dependence of the *tub2-150* mutation. Strains DBY5282–5285 are from a single tetratype tetrad of a cross between *cin4-4* and *tub2-150*, dissected at 20° on YPD to allow all the segregants to grow. DBY5282–5285 were then streaked on a YPD plate without benomyl and incubated at 34°.

lyzed the resulting double mutant. The segregants from a tetratype tetrad of this cross are shown in Figure 5B. The *cin4-4 tub2-150* double mutant strain is able to grow as single colonies on medium without benomyl at 34°, while the *tub2-150* mutant alone is not (the small colonies visible are revertant papillae). Thus *cin4-4* is able to suppress the benomyl dependence of the *tub2-150* allele. This result is consistent with the *CIN4* gene product playing a role in controlling microtubule stability. This experiment was also carried out with *cin1::LUK7*, a null allele of *CIN1*, and *tub2-150* with an identical result; the *cin1* mutation suppresses the benomyl dependence of the *tub2-150* mutation.

Analysis of double and triple *cin* null mutants: The three *CIN* genes isolated in the screening for benomyl-supersensitive mutants all have essentially identical mutant phenotypes (also see HOYT, STEARNS and BOTSTEIN 1990), with the exception noted above of the differences in benomyl-supersensitivity. There are a number of possible explanations for this phenotypic similarity: it could be due to redundancy of function, where all the *CIN* gene products perform the same function and loss of one of the three results in the observed phenotype; to function in a number of different pathways, such that each *CIN* gene product acts independently and a mutation disrupting any of the pathways results in the observed phenotype; or to concerted function, where the three *CIN* gene products act as part of the same pathway or structure. The first two models predict that a triple mutant, if viable, would have a more severe phenotype than single mutants, while the last predicts that the phenotype of the triple mutant would be no more severe than the single mutants. To determine the relationship between *CIN1*, *CIN2* and *CIN4*, we examined the benomyl sensitivity of single and triple *cin* mutants. The triple mutant, as well as all double mutant combinations, was isolated by tetrad dissection of a diploid strain heterozygous for disruptions of each of the *CIN* loci. Characterization of the resulting haploid strains is shown in Figure 6. Interestingly, all mutant combinations, including the triple null, are viable and grow well at 26°. In addition, the *cin1 cin2 cin4* triple null mutant is no more sensitive to benomyl than the single mutant *cin1::HIS3* (Figure 6A). That there is no additive effect in the triple null mutant suggests that the concerted function model is correct and that the *CIN* gene products act together to allow wild-type microtubule function.

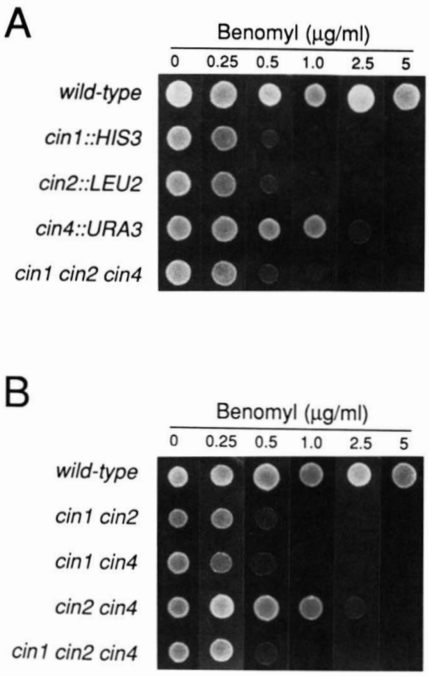


FIGURE 6.—Genetic interactions between the *CIN* genes. Suspensions of cells in sterile water were spotted onto YPD plates containing the indicated amount of benomyl. (A) The phenotypes of *cin* single mutants are compared to the phenotype of a *cin* triple mutant. The strains shown are, from top to bottom: DBY1827, DBY3393, DBY3391, DBY3444 and DBY3390. (B) The phenotypes of *cin* double and triple mutants are compared. The strains shown are, from top to bottom: DBY1827, DBY3397, DBY3399, DBY3396 and DBY3390.

The *CIN4* null mutant, *cin4::URA3*, is less sensitive to benomyl than null mutations in *CIN1* and *CIN2*. We took advantage of this difference to further examine the relationship between the *CIN* genes by performing tests of epistasis. If the genes act in the same pathway or structure, then they might show some epistasis relationship; that is, in a double or triple mutant, the phenotype of only one of the mutations would be observed. The benomyl sensitivity of the various double and triple mutants does reveal such an epistasis interaction (Figure 6B). The double mutant *cin2 cin4* is able to grow on 1 μg/ml benomyl, while all other double mutant combinations, as well as the triple mutant, are unable to grow on this concentration. Thus, the *cin4* mutant phenotype is epistatic to that of *cin2* in the *cin2 cin4* double mutant. This result is a surprise because *a priori* one might expect that the most benomyl-supersensitive phenotype would be observed when combining mutations of varying sensitivity. Similarly, the *cin1* mutant phenotype is epistatic to that of *cin4*; the *cin1 cin4* double mutant has the *cin1* phenotype. The relationship then is that *cin1* is epistatic to *cin4* and *cin4* is epistatic to *cin2*.

DISCUSSION

We used the phenotype of benomyl supersensitivity as an indicator for interesting mutations affecting the function of microtubules. By searching for mutants with this phenotype, we have identified three new genes, *CIN1*, *CIN2* and *CIN4*. In addition to the drug sensitivity, *cin* mutations display genetic interactions with tubulin mutations, and result in other phenotypes, including increased rates of chromosome loss and cold sensitivity (HOYT, STEARNS and BOTSTEIN 1989), that suggest an involvement of the *CIN* proteins in microtubule function.

Benomyl is a member of a large class of compounds based on the benzimidazole core that are widely used antimicrotubule drugs. This class also includes nocodazole, first introduced as an antitumor drug (DE BRABANDER *et al.* 1976), carbendazim, thiabendazole, and methyl-benzamidizol-carbamate. These drugs are believed to act by binding to free tubulin subunit (DAVIDSE and FLACH 1977), shifting the equilibrium between tubulin subunits and microtubules toward the free subunits. Benomyl has been used previously in *S. cerevisiae* to isolate mutations in *TUB2*, the single gene encoding β-tubulin, by selecting for benomyl resistance (THOMAS, NEFF and BOTSTEIN 1985); indeed *tub2* mutants are the sole class recovered when selecting for high levels of resistance. As the *cin* mutations only affect sensitivity to antimicrotubule drugs, and not various other drugs, it is likely that the benomyl-supersensitive phenotype reflects a defect in the function of microtubules, rather than a general permeability defect. A simple interpretation is that the products of the *CIN* genes normally act to increase the stability of the microtubule polymer, or alter the tubulin subunits in a way that favors their polymerization. We have obtained more compelling evidence for this interpretation by studying other phenotypes of *cin* mutants in detail (HOYT, STEARNS and BOTSTEIN 1990). In these experiments we found that *cin* mutants are cold sensitive, and that all functions of microtubules in yeast cells are compromised at low temperatures. Immunofluorescence staining of the microtubules in *cin* mutants at the nonpermissive temperature showed greatly reduced microtubule structures. It has long been known that microtubules are inherently cold-sensitive structures (DUSTIN 1984), and it seems reasonable that the same microtubule defect is responsible for both benomyl supersensitivity and cold sensitivity.

By limiting our search to those mutants sensitive to 5 μg/ml of benomyl, we have isolated mutations in only six complementation groups, the three tubulin genes and the three *CIN* genes. There are, however, other yeast loci that can mutate to benomyl supersensitivity (for example, a number of *ctf* mutants, SPENCER *et al.* 1988). Mutations at these other loci have not been observed to result in the same degree of benomyl supersensitivity as the *cin* mutants, and probably would not have been isolated by the relatively insensitive replica-plating method that was used in this study. This bias is reflected in the distribution of α-

tubulin mutants that we isolated; eight alleles of *TUB1*, and one allele of *TUB3* were isolated. This is probably due to failure to detect most *tub3* mutants in our screen, as even null mutations in *TUB3* result in only moderate levels of benomyl supersensitivity (SCHATZ, SOLOMON and BOTSTEIN, 1986) while mutations in the essential *TUB1* gene often result in greater supersensitivity (STEARNS and BOTSTEIN 1988; SCHATZ, SOLOMON and BOTSTEIN 1988).

The three *CIN* genes are not essential for viability of the yeast cell; at 26° the spore colonies carrying null mutations of the *cin* loci are indistinguishable from the wild-type spore colonies. The null mutants do, however, have phenotypes very similar to those of the mutations isolated by EMS mutagenesis of whole cells. In general, it is likely that many interesting genes will be found to be nonessential, even though the processes that they are involved in are essential. These nonessential genes may encode proteins that are important not for function itself, but for fine tuning the mechanism. While the cell can function without such fine tuning or regulation, it cannot function well, or cannot function under extreme conditions. A particularly relevant example of such a case was reported by HUANG, RAMANIS and LUCK (1982); a Chlamydomonas mutant lacking a number of components of the flagellum is able to swim, though not as efficiently as wild type. Further analysis showed that the missing components most likely play a role in generating the smooth swimming stroke characteristic of wild-type Chlamydomonas flagella (BROKAW, LUCK and HUANG 1982).

The genetic tests that we have carried out lend support to the hypothesis that the *CIN* gene products act together to regulate microtubule function in some way. These tests involved combining *cin* mutations with a number of different tubulin mutations and with each other. First, we found that the combination of either *cin1*::*HIS3*, *cin2*::*LEU2*, or *cin4-7* with *tub1-1*, a mutation in the major α-tubulin gene, is inviable for mitotic growth, even at 26°, where each single mutant is healthy. Second, for both *cin1* and *cin4* alleles we isolated suppressors of the benomyl supersensitivity that map to the β-tubulin gene *TUB2*. The *cin tub2* double mutants are strongly cold sensitive; growth is completely arrested at 16°, while each single mutant is capable of growing at 16°. An interpretation of these results is that these particular *tub* and *cin* mutations compromise microtubule function in a similar way, and that the additive effect results in the synthetic lethality. Third, and possibly most importantly for understanding the role these genes play in microtubule function, we found that either *cin4-4* or *cin1*::*LUK7* can suppress the phenotypic defect of *tub2-150*. This allele of *TUB2* requires either benomyl or low temperature (20°) to grow on YPD medium (THOMAS 1984). This suggests that microtubules in a *tub2-150* strain are hyperstable and need a destabilizing effect, *i.e.*, benomyl or low temperature, to function properly; the suppression implies that a *cin* mutation causes such a destabilizing effect. We stress, however, that this interpretation of the *tub2-150* phenotype has not yet been tested by examining the *in vitro* properties of microtubules made with this mutant β-tubulin.

The last set of genetic experiments involved combining null alleles of *CIN1*, *CIN2* and *CIN4* to generate all possible double and triple mutants. The following results were obtained from analysis of these strains: (1) the triple null mutant is viable and is phenotypically identical to *cin1* and *cin2* null mutants, (2) the *cin1* null mutant phenotype is epistatic to the *cin4* null mutant phenotype; the *cin4* null mutant phenotype is epistatic to the *cin2* null mutant phenotype. A simple explanation of these results is that the *CIN1*, *CIN2* and *CIN4* genes function in the same pathway or structure. There is still not enough information about the details of the *CIN* genes' function(s) to be very specific about this pathway or structure, although our results impose constraints on any future model. In addition to the striking epistasis results, these constraints include: the commonality of phenotype displayed by *cin1 cin2* and *cin4* mutants; the increased drug sensitivity of some point mutants relative to null mutants in the same gene; the lesser sensitivity of *cin4*-null mutants relative to *cin1* and *cin2* null mutants; and the similarity of the consequences of *cin1* overexpression to the phenotype of *cin1* loss-of-function mutations (HOYT, STEARNS and BOTSTEIN 1990). The possibility that the *CIN* gene products each separately perform the same or highly related function, for example, modification of tubulin, has not entirely been excluded. It is our hope to use the yeast system to determine how the *CIN* proteins interact with each other and the microtubule cytoskeleton to produce a fully functional cytoskeleton.

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