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Heterogeneity and maintenance of centromere plasmid copy number in *Saccharomyces cerevisiae*

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Abstract. We developed a novel approach to quantitate the heterogeneity of centromere number in yeast, and the cellular capacity for excess centromeres. Small circular plasmids were constructed to contain the *CUP1* metallothionein gene, *ARS1* (autonomously replicating sequence) and a conditionally functional centromere (*GAL1-GAL10* promoter controlled centromere). The *CUP1* gene provided a gene dosage marker, and therefore a genetic determinant of plasmid copy number. Growth of cells on glucose is permissive for centromere function, while growth on galactose renders the centromere nonfunctional and the plasmids are segregated in an asymmetric fashion. We identified "lines" of cells containing increased numbers of plasmids after transformation. Cell lines containing as many as five to ten active centromeres are stably maintained in the absence of genetic selection. Thus haploid yeast cells can tolerate a 50% increase in their centromere number without affecting progression through the cell cycle. This system provides the opportunity to address issues of specific cellular controls on centromere copy number.

Introduction

An essential component of the chromosome segregational apparatus in eukaryotic organisms is the centromere. The centromere has been characterized at the DNA sequence level in the yeast *Saccharomyces cerevisiae* (Fitzgerald-Hayes et al. 1982; Heiter et al. 1986). Strategies that involve centromere replacement, conditionally functional centromeres, and directed DNA mutagenesis have delineated the sequence and structural domains to approximately 200 bp of DNA (reviewed in Resnick and Bloom 1987). Chromosomes with a wild-type centromere exhibit a frequency of malsegregation of approximately 1 loss event per every 10^5 cell divisions (Hartwell et al. 1982; Whittaker et al. 1988). Specific mutations in the centromere can result in partial to complete abolishment of orderly chromosomal segregation (McGrew et al. 1986; Panzeri et al. 1985). The addition of a centromere to autonomously replicating plasmids reduces malsegregation rates from 0.8 to 0.02 errors per cell division for small circular plasmids and to less than 0.001 errors per cell division for very large circular or linear plasmids (greater than 100 kb, Heiter et al. 1985).

Malsegregation of the small circular molecules can result in plasmid loss or gain. To examine the extent of instability elegant genetic systems have been developed based on colony color to detect the absence, or the presence of one and two copies of centromere plasmids per cell (Heiter et al. 1985; Koshland et al. 1985). While it has been shown that two copies of centromere plasmids can be stably maintained in haploids in the absence of selection, it has not been possible to evaluate the normal limitations on centromere number in the absence of selection or as a consequence of mutational change in the genome.

Several studies have demonstrated the feasibility of developing cells with many copies of centromere-containing plasmids, either by mutation, selection for high copy using a drug resistance marker (Bitoun and Zamir 1986; Tschumper and Carbon 1987), weakly complementing genetic markers (Chlebowicz-Sledziewska and Sledziewski 1985) or by complementation using centromere plasmids containing different genetic markers (Futcher and Carbon 1986). Because of the selection systems used, it has not been possible to investigate the heterogeneity of centromere number in a cell population or the consequences of altered copy number with respect to growth and meiotic development. In this report we describe the development of a plasmid system containing a centromere and the copper chelatin gene *CUP1* (Karin et al. 1984) which allows these issues to be addressed without a requirement for direct selection of cells containing high copy number. We have been able rapidly to identify lines or populations of cells containing var-

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ious numbers of plasmids and assess the degree of heterogeneity of copy number in populations of cells. Cell lines containing between one and ten functional copies of the plasmid can be isolated in the absence of selection and the average plasmid copy number in these lines remains stable. There appears to be a copy number barrier at five to ten extra copies of centromeres per haploid cell without affecting the growth of the cell.

Materials and methods

Strains. The strains used were: ABDE1, *MATa ade2 his7-2 leu2-3, 112 arg4-8 cup1::URA3 thr1-4 trp1-289 ura3-52,* in which the *CUP1* gene has been deleted, provided by the laboratory of Dr. Seymour Fogel;
BR1669-5α, *MATα ade2-1 his4 arg4-8 CUP1^S thr1-4 trp1-1*, containing a single copy of *CUP1* and derived from BR1669 (Whittaker et al. 1988);
MR104-1d, *MATα ade2-1 leu2-3, 112 arg4-8 CUP1::URA3 thr1-4 trp1-1*;
MR104-4a, *MATa ade2-1 leu2-3, 112 arg4-8 CUP1::URA3 thr1-4 trp1-1;*
MR104-10c, *MATα ade2-1 his4 arg4-8 CUP1::URA3 thr1-4 trp1-1*;
MR108-11c, *MATa ade2-1 his4 arg4-8 CUP1::URA3 thr1-4 trp1-1.*
These congenic strains were derived from a cross of ABDE1 with BR1669-5α, followed by two backcrosses to BR1669-5α; MR108-11c corresponded to three backcrosses. These were used for transformation and determination of copper resistance.

Media. Synthetic complete medium (SC) was as described by Whittaker et al. (1988) except that Gibco Phytagar was replaced by Difco Bactoagar. Copper-containing agar medium (SC + CUP) was made by adding the appropriate dilution of a 50 mM stock solution of copper sulfate to SC following the cooling of the autoclaved medium to 65° C and shortly before pouring plates. Media lacking particular components such as tryptophan (i.e., SC-TRP) were also used in genetic analysis. To induce the *GAL1-GAL10* promoter, the glucose in SC or SC-TRP was replaced by 2% D-galactose.

Liquid replica plating. In order to assure uniformity in the transfer of cells from colonies to medium containing copper, liquid replica plating was used instead of the traditional replica plating involving imprints on velvet. Colonies were inoculated to liquid growth medium (200 µl) in 96 well microtiter dishes (the dishes were not tissue culture treated) and grown overnight to stationary phase at 30° C. The wells were sampled using a 48 pin replicator (West Coast Scientific) and spotted to the appropriate medium. Approximately 1.1 to 1.3 µl was delivered per spot. Plates were incubated for 2 days at 30° C. The resistance level of an isolate corresponds to the highest copper concentration at which there is no clearly detectable reduction in growth relative to growth on a plate lacking copper. Higher concentrations lead to growth retardation as demonstrated in Figure 2.

Plasmid construction. Plasmids YCp5T and YCp5TΔA were constructed from the plasmid pBM150. Plasmid pBM150 contains a 685 bp *Eco*RI-*Bam*HI DNA fragment with the divergent *GAL1-GAL10* yeast promoters (Johnston and Davis 1984). The *Bam*HI site is located 56 nucleotides downstream from the *GAL1* transcription initiation site. A conditional centromere was constructed by isolating a 289 bp *Rsa*I-*Alu*I fragment containing *CEN3* DNA (Bloom et al. 1984). The fragment was ligated to synthetic oligonucleotide linkers encoding the *Bam*HI restriction recognition site in the presence of T4 DNA ligase. The *Bam*HI linker-*CEN3* DNA complex was subsequently ligated to *Xho*I oligonucleotide linkers in the presence of T4 DNA ligase.

The *CEN3-Bam*HI linker cassette could then be shuttled into substitution vectors at unique *Xho*I or *Sal*I restriction sites without disrupting the tandem *Bam*HI sites (Kenna et al. 1988). This fragment was introduced into pBM150 at the *Sal*I site 275 bp downstream from the *GAL1* initation site. Ligation was followed by transformation into HB101 and the presence and orientation of the insert were confirmed by restriction digestion of a miniplasmid preparation from ampicillin-resistant transformants. *CEN4* sequences were removed by digesting the resulting plasmid with *Sma*I + *Hpa*I, and blunt-end ligation of the resultant molecules. The resulting plasmid pBMBX289-C4 was digested with *Kpn*I, and a 2.2 kb *Kpn*I fragment containing the *CUP1* gene from plasmid JW6 (Karin et al. 1984) was inserted. Finally, the *TRP1-ars1* 1.4 kb *Eco*RI fragment (Tschumper and Carbon 1980) was introduced into the unique *Eco*RI site adjacent to the *GAL10* initiation site. Plasmid replication does not require this *ars1* element. The resulting plasmid YCp5T is shown in Fig. 1. The plasmid YCp5TΔA was constructed by digesting YCp5T with *Bam*HI to remove the wild-type *CEN3* sequences and substitution with a 289 bp *Bam*HI fragment containing a single point mutation in *CEN3* with a C to A transversion at the central position of dyad symmetry in CDEIII (Saunders et al. 1988). The resulting plasmid YCp5TΔA was used for transformation as indicated in the text.

Restriction endonucleases and T4 DNA ligase were used following conditions recommended by suppliers (New England Biolabs, Bethesda Research Laboratories or Boehringer Mannheim Biochemicals). Restriction enzyme linkers were purchased from P-L Biochemicals. Standard molecular cloning techniques were as described by Maniatis et al. (1982).

Transformation of yeast. The lithium acetate (LiAc) method of Ito et al. (1983) was used for transforming yeast.

Preparation and restriction digestion of yeast DNA for plasmid analysis. Transformed yeast strains were grown for 24–36 h at 32° C in 5 ml minimal medium with various amounts of copper supplemented as indicated in the text. The cells were harvested by centrifugation, washed once with water, resuspended in lysis buffer (0.1 M Tris-HCl, pH 8.0, 50 mM EDTA, 1% SDS) and transferred to Eppendorf tubes. Cells were lysed with glass beads by shaking on an Eppendorf mixer for 15 min. The supernatant was transferred to a new tube, extracted with phenol and 24:1 chloroform: isoamyl alcohol, ethanol precipitated and dissolved in 50 µl water.

Gel electrophoresis and hybridization analysis of DNA. DNA samples were analyzed on 1.0% agarose slab gels containing 0.09 M Tris-borate, pH 8.3 and 2.5 mM EDTA. Transfer of DNA from agarose gels to nitrocellulose paper, nick translation and hybridization analysis were performed as described by Bloom and Carbon (1982). Autoradiography was performed for 24–72 h at −80° C with Kodak XAR-5 film (Eastman Kodak, Rochester, NY) and a DuPont Cronex Lightning-Plus intensifying screen (DuPont Instruments, Wilmington, Del). The film was preflashed to an A_{800} of 0.065.

Copy number determination. The copy number of YCp5T was determined by preparing genomic DNA from selected transformants. DNA was digested with *Eco*RI which linearizes the plasmid and generates a 5.1 kb genomic *CEN3* fragment, and a 9.2 kb plasmid fragment. DNA was electrophoresed in a 1% agarose gel, transferred to nictrocellulose and probed with a radiolabeled *CEN3* fragment. The genomic *CEN3* band was used as a single copy standard with which to compare the plasmid band. Dilutions of linearized YCp5T plasmid equivalent to 1, 2, 5, 20, 50, and 100 copies per cell were also electrophoresed and probed with *CEN3*. Autoradiographs were scanned using a Joyce Loebl Chromoscan 3. Peaks were analyzed by the built-in Rev 4.3 software (Resnick et al. 1987). The reproducibility of extraction was examined by isolating DNA from a single copy isolate that had been apportioned equally into three tubes. The copy number determinations revealed a range from 1–1.2 for the three samples.

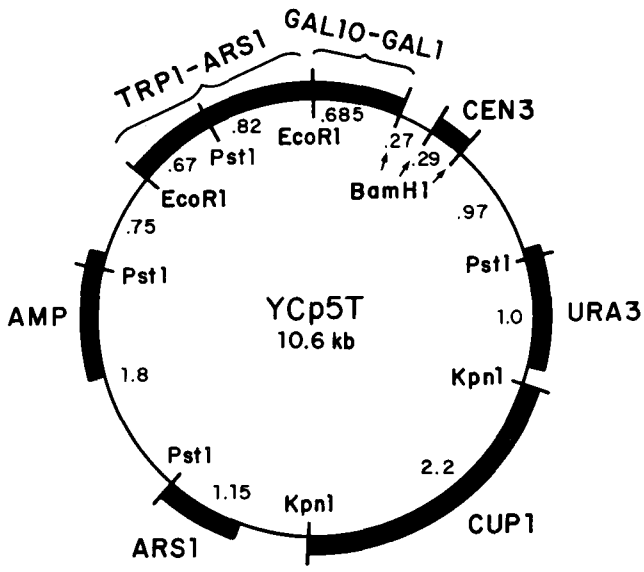

Fig. 1. The YCp5T plasmid containing a single copy of the *CUP1* gene and *CEN3*

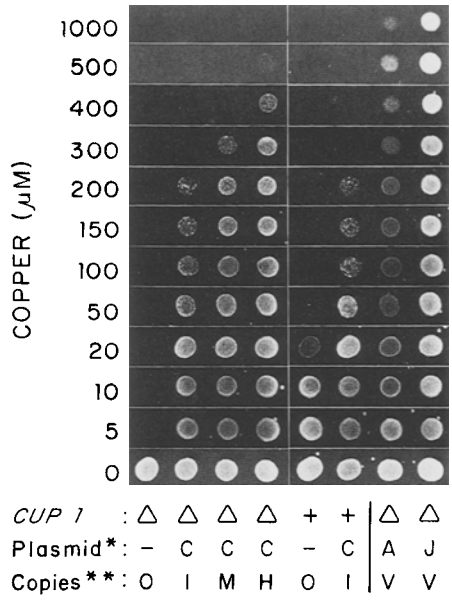

Fig. 2. Growth of haploid cells containing various copies of the *CUP1* gene on plasmids or in the genome. Cells were liquid replica plated (see Materials and methods) following growth to stationary phase in SC-TRP medium [except for the *CUP1* deletion strain lacking a plasmid, in which case tryptophan was added to the medium]. All strains were tested at the same time on each of the copper-containing plates. [For presentation purposes, the plates were photographed and groups of four strains were arranged as shown.] *CUP1*: Δ, strains with *CUP1* deleted from the genome; +, presence of a single copy of the *CUP1* gene. Plasmid*: −, no plasmid; C, presence of the YCp5T plasmid; A, mutated CEN plasmid YCp5TΔA; J, YRp(*CUP1*)$_3$ plasmid containing three copies of the *CUP1* gene and lacking a centromere. Copies**: 0, none; 1, one; M, medium copy number (see Fig. 5); H, high copy number (5 to 10; see Fig. 5); V, greater than 10–20 copies, typical of YRp plasmids and YCp5TΔA

Results

Plasmid detection system for gene dosage

We developed a plasmid system (YCp5T, Fig. 1) to determine the cellular capacity for centromeres and the degree of copy number heterogeneity in the population. The plasmid contains an origin of replication (*ARS1*), a centromere (*CEN3*), and a reporter gene *CUP1* (copper chelatin) that allows the in vivo monitoring of copy number within individual colonies based on resistance to exogenous levels of copper (Fig. 1). It has been demonstrated that a linear relationship exists between the number of copies of the *CUP1* gene on chromosome VIII and the resistance of cells to the concentration of exogenous copper (Fogel et al. 1983). This strict relationship has been used in the detection and analysis of chromosomal aneuploidy (Whittaker et al. 1988). The plasmid YCp5T, also contains the *GAL1-10* promoter (Johnston and Davis 1984) juxtaposed to the centromere as shown in Fig. 1. This feature allows the centromere to be conditionally inactivated, which in turn leads to plasmid malsegregation and elevated copy number in the plasmid-bearing cells (Hill and Bloom 1987; Snyder et al. 1988). Reactivation of the centromere in this plasmid provides the opportunity to examine biological consequences in the distribution of high copy number centromeres.

Copper sensitivity can be measured by liquid replicaplating on plates containing increasing concentrations of copper (see Materials and methods) or, alternatively, by plating directly onto copper-containing medium and determining plating efficiency. Examples of both methods are presented in Figs. 2 and 3, respectively. Since survival is based on growth capability of individual cells and the replica plating method is based on clonal growth, the copper concentrations at which significant differences are detectable in the two methods are not strictly comparable.

Strains with a single chromosomal copy of the copper gene grow at concentrations below 20 μM copper (*CUP1*$^+$, no plasmid, Fig. 2). Deletion of the *CUP1* gene results in a considerable reduction in growth at concentrations above 2 μM (*CUP1*Δ, no plasmid, Fig. 2). Correspondingly, there is less than 1% survival of *CUP1* deletion cells on plates containing 10 μM copper compared with 100% survival for the strain containing a single copy of *CUP1* in the genome (open circles versus open triangles, Fig. 3). The ability to tolerate copper by increasing the number of copies of the *CUP1* gene was examined by transforming the cells with a *CUP1*-containing plasmid (YRp(*CUP1*)$_3$) lacking a centromere, and containing three copies of the *CUP1* gene (identical to JW6, Karin et al. 1984). The autonomously replicating plasmid (YRp) exhibits an elevated frequency of malsegregation, thereby generating a subpopulation of cells (*TRP*$^+$) with high copy number. Cultures of cells containing this plasmid in the genomic deletion *CUP1* strain, are greater than 300 times more resistant than when the plasmid is absent (far right column in Fig. 2, and closed triangles, Fig. 3). Thus there is a considerable range over which copper tolerance can be quantitated.

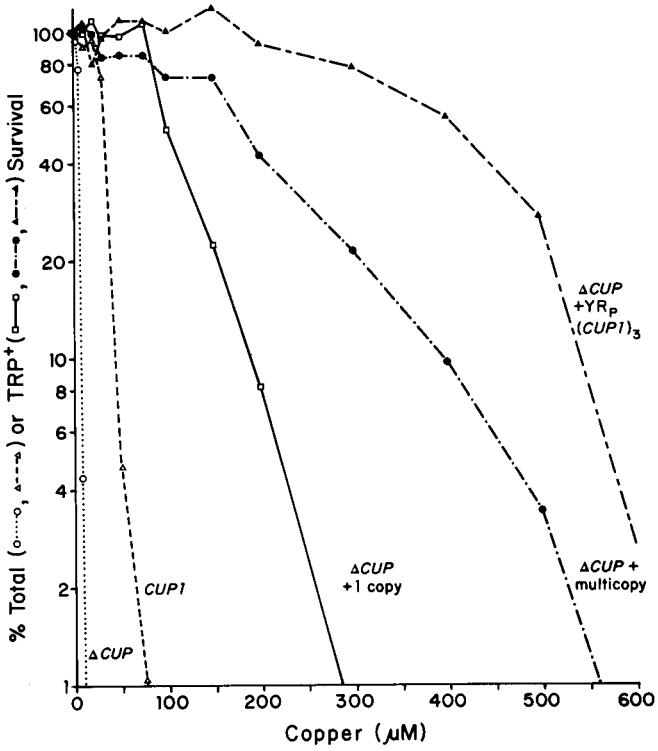

Fig. 3. Survival of strains containing different copies of the *CUP1* gene on plasmids and having one (*CUP1*) or no copies (CUP1Δ) in the genome. No plasmid, *CUP1Δ open circles*; one genomic copy, *CUP1 open triangles*; single copy plasmid, *CUP1Δ open squares*; high copy plasmid isolate, *CUP1Δ closed circles*; high copy acentric plasmid, *CUP1Δ closed triangles*

Transformation of cells lacking the CUP1 gene with centromere plasmid YCp5T results in increased copper resistance

Following transformation of the *CUP1* deletion strains with YCp5T the *TRP*$^+$ isolates exhibited resistance to greater than 50 μM copper (lane 2 from the left, Fig. 2). A small percentage of the isolates exhibited significantly greater resistance to copper. Two of the isolates that were resistant to 50 μM copper and presumed to have a low plasmid copy number per cell, were mated to an appropriate haploid lacking the plasmid. The diploids were induced to sporulate to assess the segregation fidelity of the YCp5T plasmid in meiosis. As shown in Table 1, the plasmid generally segregates in a $2^+:2^-$ fashion demonstrating that the level of resistance observed in the bulk of the transformants was due to the presence of a single plasmid bearing the *CUP1* gene. Based on these results we can also conclude that presence of the *GAL1-10* promoter does not disrupt centromere function under normal mitotic and meiotic growth conditions.

For one of the two strains examined, there were 7 among 79 tetrads that were 1:3 TRP$^+$:trp$^-$. This class of segregants could represent two plasmids that segregated to the same spore. Evidence to support this idea is provided by the increased resistance of the single plasmid-containing spore in 5 of the $1^+:3^-$ tetrads (see Table 1 and spores 2c and 11c in Fig. 4). Plasmid-bearing spores generated from $2^+:2^-$ events were sensitive to 250 μM copper (Fig. 4), while plasmid-bearing spores from $1^+:3^-$ events were considerably more resistant to 250 μM copper. As shown below, the increased copper resistance reflects increased plasmid copy number. Thus with this system we can examine malsegregation events in meiosis that lead to plasmid gain.

Table 1. Tetrad analysis and copper resistance of spore colonies from a strain presumed to contain a single copy of YCp5T

Strain[a]	*TRP*$^+$: *trp*$^-$ asci					
	$0^+:4^-$	$1^+:3^-$	$2^+:2^-$		$3^+:1^-$	4^+0^-
			NPD[b] PD	TET[b]		
MR107	5	7	54	5	1	7
	—	(5/7)[c]	(3/108)[c,d]	(0/10)[c]	(1/3)[c]	(1/28)[c]
MR109	3	0	52	4	1	10
	—	—	(0/104)[c]	(0/8)[c]	(3/3)[c]	(3/40)[c]

[a] Strains MR107, MR104-4a/MR104-10c; MR109, MR104-1d/MR108-11c
[b] NPD, nonparental ditype; PD, parental ditype, TET, tetratype
[c] In parentheses are the spores containing multiple plasmids, based on copper resistance, divided by the total plasmid-containing spores. All spores that are *TRP*$^+$ are more resistant to copper (10–20 μM) than the *trp*$^-$ spores (5 μM). The presumed higher copy number spores are resistant to 20–30 μM copper
[d] From three different asci. These may have arisen during germination

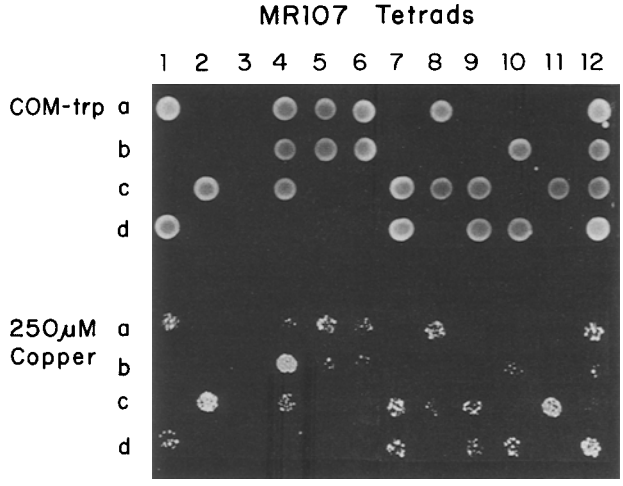

Fig. 4. Tetrad analysis of strains containing the YCp5T plasmid presumed to be present as a single copy. Spore isolates from four spore tetrads that contain the plasmid exhibited good growth on 10 to 20 μM copper while those lacking the plasmid *trp*$^-$ spores) exhibited reduced growth at 5 μM. For purposes of illustration only growth on a high copper concentration (250 μM) is presented. Only the plasmid-containing spore isolates yielded papillae at 250 μM. Note that for tetrads 2 and 11 ($1^+:3^-$) and 4 ($3^+:1^-$) one of the spores appears to be more highly resistant, indicating the presence of an additional plasmid (see Table 1)

Isolation of cell lines with increased copper resistance

Plasmid replication or segregation errors occur in about 2%–3% of mitotic cell divisions (Fitzgerald-Hayes et al. 1982). In cells with a single replicated copy of the YCp5T plasmid segregational errors would be expected to give rise to daughter cells with either no plasmid or two plasmids. Cells with two plasmids would in turn be expected to give rise to cells with three plasmids, and so on, provided cells can tolerate increased extrachromosomal plasmids.

The segregation frequency of the YCp5T plasmid was examined in cells that had a single copy of the plasmid during outgrowth of cells on nonselective medium. The frequency of malsegregation was comparable to that for other centromere plasmids (approximately 2% loss per generation). Based on this frequency of malsegregation, cells with more than one copy of the plasmid should exist within the population (demonstrated by Larionov et al. 1989). The relation between the number of *CUP1* genes and copper resistance provides a convenient strategy to identify colonies (i.e., lines) with elevated plasmid copy number. Cells with a single plasmid were grown in liquid medium lacking tryptophan (selective growth for the plasmid) and subsequently plated to this medium. Individual colonies were then inoculated to microtiter wells containing selective medium (SC-Trp) and then liquid replica plated to solid medium containing increasing concentrations of copper. Most of the colonies (37/44) that arose after outgrowth from the single copy strain exhibited the copper sensitivity of the transformed parent. However, lines of cells that were more resistant (capable of normal growth above 50 μM copper, see Fig. 2, *CUP1*Δ, M and H) were also observed. The selection procedure was repeated with cells from lines exhibiting increased resistance to copper.

Following three successive rounds of screening for cell lines that exhibit higher copper resistance levels, a limit on the level of copper that could be tolerated without affecting growth was reached. An additional two rounds of screening did not yield lines with increased copper resistance. The maximum clonal resistance was less than that for cells containing a YRp plasmid and *CUP1* deletion [*CUP1* deletion strains transformed with YRp(*CUP1*)$_3$, Figs. 2 and 3]. The maximum resistance was also lower than that observed for a *CUP1* plasmid containing a functionally mutated centromere. The mutation in YCp5TΔA was due to a C to A transversion in the central nucleotide of the conserved element III (Saunders et al. 1988). Thus, the copper tolerance that can be achieved is limited in cells with functional centromere plasmids. This level is significantly lower than that observed in congenic cells containing *CUP1* plasmids that are acentric, or contain altered (nonfunctional) centromeres.

CEN plasmid copy number and stability

Copper resistance is correlated with the number of chromosomal copies of *CUP1* (Fogel et al. 1983). To determine if this correlation extends to the number of plasmid-borne *CUP1* genes, cultures of stationary cells grown in the absence of copper selection were harvested and total DNA was extracted. The DNA was either electrophoresed directly on neutral agarose gels or digested with *Eco*RI, which linearizes the plasmid, and then analyzed by gel electrophoresis. After transferring the DNA to nitrocellulose filters, the DNA was hybridized with a DNA probe specific to *CEN3*. As shown in Fig. 5, there is a correlation between resistance of a population of cells and plasmid number (based on comparisons at the 10% survival level). The relative plasmid number is based on densitometry tracings of plasmid and chromosomal centromere regions of gels as single copy standards. The maximum resistance corresponds to approximately five to ten centromere plasmids per cell.

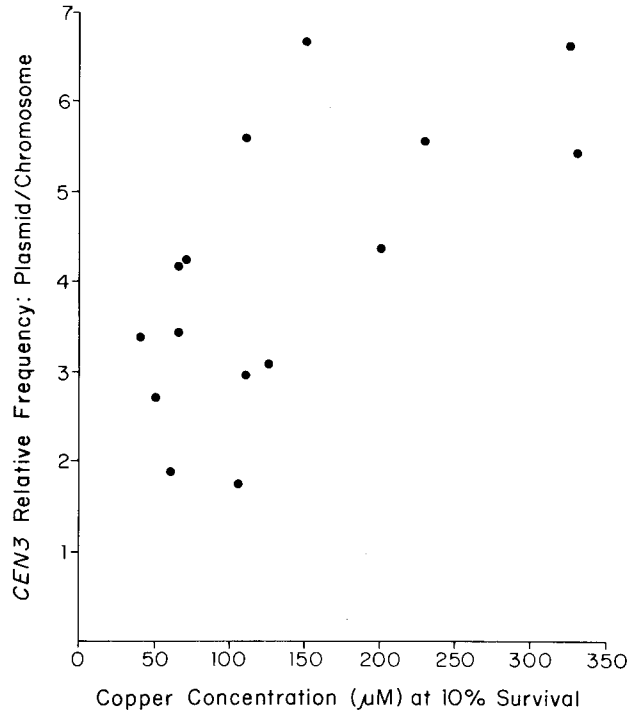

Fig. 5. Correlation between copper resistance and *CUP1* plasmid number (see Materials and methods, and Results). The ratio of *CEN3* on plasmids to the chromosomal *CEN3* is presented. These can be distinguished by their different size after *Eco*RI digestion. Resistance corresponds to level of copper at which there is 10% survival

The ability to identify cells with different levels of copper resistance, in the absence of copper selection, and therefore different copy numbers of the YCp5T plasmid, suggested that the average plasmid number per cell was stable. To test the fidelity of plasmid copy number maintenance, cell lines exhibiting various levels of resistance were outgrown without copper selection for up to 40 generations. As shown in Fig. 6, the resistance of the population did not change appreciably. Thus with each cell line, faithful segregation of centromere plasmids is evident even as the plasmid number approaches the maximum tolerated by the cell. It should be emphasized that these observations relate to the population. Individual cells may have slightly more or fewer

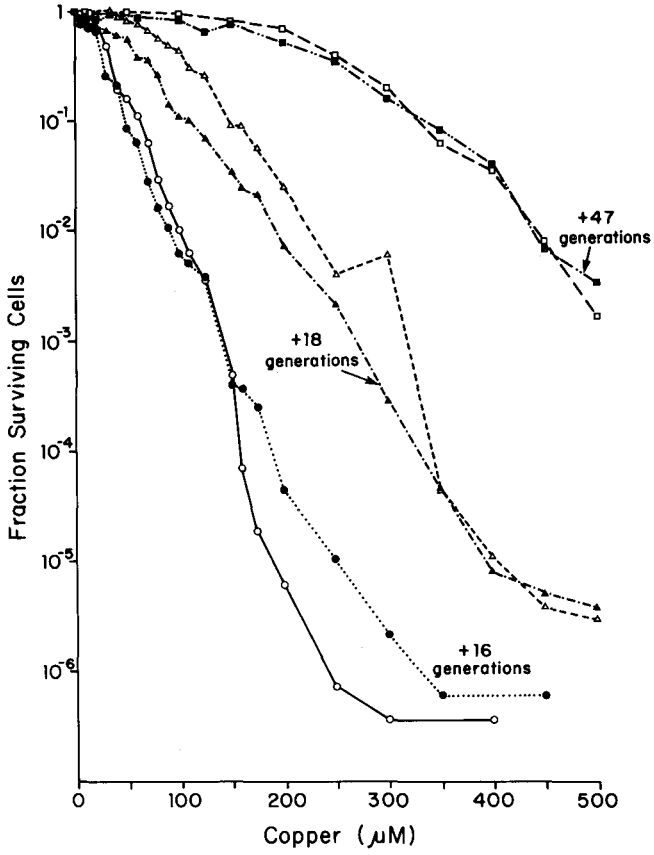

Fig. 6. Stability of copper resistance in cell lines. Clonal isolates exhibiting low (*circles*), medium (*triangles*) and high (*squares*) copper resistance were grown in SC-TRP. Survival on copper-containing plates was determined before (*open data points*) and after 16, 18 and 47 generations (*closed data points*), respectively

plasmid molecules. For example, single colony isolates from the resistant line exhibited a variety of levels of copper resistance, although most of the sublines were highly copper resistant (Fig. 7).

We attempted to surpass the centromere barrier in cells harboring functional centromeres by conditionally inactivating the centromere with the *GAL1-10* promoter, thereby increasing the rate of malsegregation. As previously shown (Hill and Bloom 1987), activating the *GAL1-10* promoter adjacent to *CEN3* results in loss of centromere function; plasmids exhibit biased patterns of segregation and accumulate to high copy number in less than 20% of the population. Returning cells to glucose medium results in repression of the promoter and reactivation of centromere function. This procedure was followed with cells containing the YCp5T plasmid; they were grown on galactose medium lacking tryptophan, and then plated to glucose medium lacking tryptophan. Colonies that arose after the centromere was returned to function were subsequently examined for levels of copper resistance. Considerable variation in resistance was obtained, but no colonies exhibited normal growth at levels above 300 μM copper (Fig. 7). Comparable results were also obtained if lines of cells with high copper resistance were subjected to the same growth regime (middle panel, Fig. 7). Since some of the colonies exhibited low resistance, we conclude that the high level of copper resistance (prior to induction by galactose) was not due to chromosomal mutation(s) rendering cells more copper resistant. None of the colonies arising after galactose induction exhibited tolerance to the levels afforded by YRp plasmids (greater than 500 μM copper). Thus the distribution of plasmid copy number can be dramatically altered in the population. Low copy isolates resistant to less than 200 μM copper (Fig. 2) when shifted to galactose give rise to many (>25%) colonies exhibiting resistance to 300 μM copper (left panel, Fig. 7). High copy isolates give rise to nearly 50% resistant colonies (right panel, Fig. 7). Only through analysis of individual colonies by such an in vivo monitoring system can the heterogeneity in the distribution of plasmid copy number be distinguished in plasmid-bearing cells.

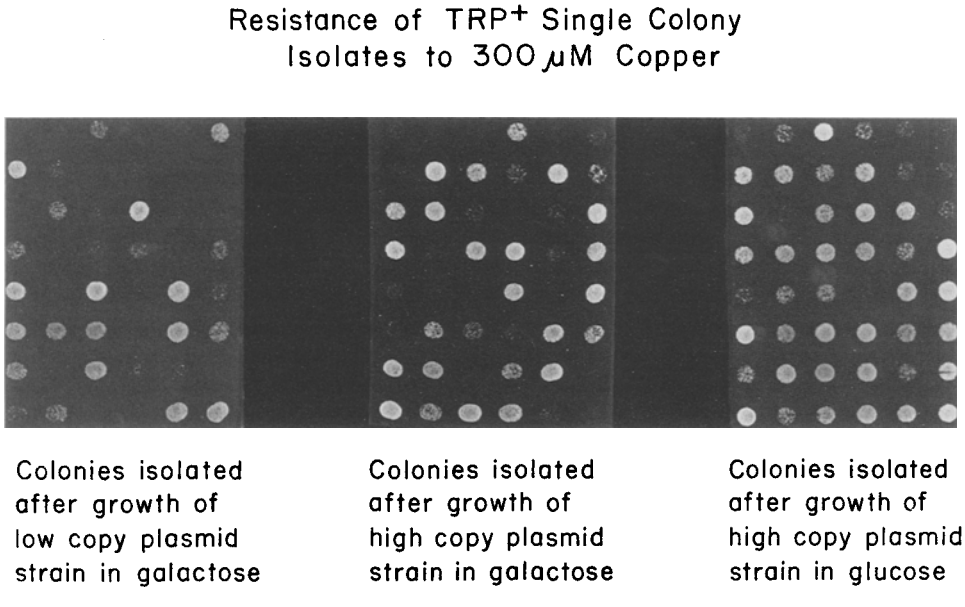

Fig. 7. Copper resistance of cell lines obtained after growth under conditions of *CEN3* inactivation. A high copy and a low copy YCp5T strain were grown in galactose medium (lacking tryptophan) to inactivate the centromere. Cells were plated to glucose medium (lacking tryptophan) and the resulting colonies were tested for copper resistance. Presented are the responses of the clonal isolates to 300 μM copper. The panel to the right corresponds to a high copy strain grown in glucose. Note the high proportion that were highly resistant (i.e., grew well on 300 μM copper) in comparison with the other two panels

Discussion

The integrity of chromosomal segregation during mitotic and meiotic cell divisions of the yeast *S. cerevisiae* is a consequence of coordinating the function of DNA-protein complexes at the centromere, of microtubule dynamics within the mitotic spindle, and of cellular feedback controls that monitor the completion of cell cycle events (for reviews see Murray and Kirschner 1989; Hartwell and Weinert 1989). We have developed a genetic system that addresses limitations on the centromere component of the segregational apparatus and the heterogeneity in copy number distribution.

The system provides the opportunity for "counting" copies of a centromere plasmid using the *CUP1* gene as a dosage marker. Starting with a population of haploid cells containing only one copy of the plasmid, it is possible to identify a small number of single colony isolates that contain more than one copy. Through successive rounds of identification of colonies with higher capabilities for growth on copper medium, lines of cells can be developed that have different numbers of plasmids per cell. Surprisingly, the cells can tolerate and stably maintain up to five to ten plasmids per cell without any apparent growth consequences. This corresponds to a 50% increase in the normal number of centromeres in a cell. All the plasmid centromeres appear to be functional based on the observation that the *CEN3* DNA of isolated nuclei is protected from nucleolytic attack (see Fig. 5 in Kenna et al. 1988, which includes cells with high copy *CEN* plasmids) as are chromosomal *CEN* DNA complexes (Saunders et al. 1988).

Previous reports have indicated that haploid yeast can tolerate an approximately 50% to 75% increase in centromere content (Futcher and Carbon 1986). The increases were accomplished either by selection and maintenance of selection for high copy (Bitoun and Zamir 1986), mutations that allowed higher levels (Tschumper and Carbon 1987), or through the use of several plasmids containing complementing markers (Chlebowicz-Sledziewska and Sledzwiewski 1985; Futcher and Carbon 1986). The latter established that high copy number could be maintained; however, the requirement for the presence of all the complementing plasmids and the occasional malsegregation of one or more of the plasmids meant that only about 10% of the cells had the entire complement and the population as a whole grew poorly. Because the present system does not require any selection for high copy numbers of plasmids (i.e. genetically neutral), it was possible to establish a limit to the number of copies without selective pressure.

It has been possible to investigate the consequences of inactivating and subsequently activating the centromere in terms of plasmid distribution in the resulting population. Hill and Bloom (1987) demonstrated that centromere inactivation results in malsegregation and the development of a small number of cells with elevated copy number. While subsequent activation of the centromere resulted in a return to low plasmid number per cell, it was not possible to determine whether the initial and final plasmid distributions were the same. The system which we have described has demonstrated that the activation/inactivation/activation cycle markedly changes the plasmid distribution in the cell population. Prior to inactivation most of the cells have one plasmid per cell. Following inactivation and activation, a large portion of the cells have up to the natural limit of approximately five to ten plasmids (the population is skewed toward the low number, Fig. 7). Approaches involving the physical determination of plasmid number do not discriminate such heterogeneity in single cells.

Several interesting questions can be addressed with a system that provides the opportunity to develop and maintain cell lines with different numbers of functioning centromere-containing plasmids. The system provides a means for obtaining more material for studies of centromere organization and function, and will allow a more accurate characterization of DNA damage and repair within the centromere (Resnick and Bloom 1987). The availability of cell lines with different copy numbers will provide the opportunity for studying gene dosage consequences of various isolated genes. The consequences of increased numbers of centromeres in meiosis and chromosomal segregation can also be investigated. Of particular importance is the potential for eventually examining the relationship between expression of genes involved in the segregational apparatus and number of functioning centromeres.

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