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Toxic Effects of Excess Cloned Centromeres

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Plasmids carrying a *Saccharomyces cerevisiae* centromere have a copy number of one or two, whereas other yeast plasmids have high copy numbers. The number of *CEN* plasmids per yeast cell was made artificially high by transforming cells simultaneously with several different *CEN* plasmids carrying different, independently selectable markers. Some host cells carried five different *CEN* plasmids and an average total of 13 extra copies of *CEN3*. Several effects were noted. (i) The copy number of each plasmid was unexpectedly high. (ii) The plasmids were mutually unstable. (iii) Cultures contained many dead cells. (iv) The viable host cells grew more slowly than control cells, even in nonselective medium. (v) There was a pause in the cell cycle at or just before mitosis. We conclude that an excess of centromeres is toxic and that the copy number of centromere plasmids is low partly because of selection against cells carrying multiple centromere plasmids. The toxicity may be caused by competition between the centromeres for some factor present in limiting quantities, e.g., centromere-binding proteins, microtubules, or space on the spindle pole body.

Functional centromeres (*CEN*s) from at least 11 different chromosomes of the yeast *Saccharomyces cerevisiae* have recently been cloned and characterized (6, 11, 12, 17, 21, 26, 27, 30). When one of these *CEN* sequences is placed on an *ARS* plasmid (31), it increases the efficiency of mitotic plasmid partitioning and so stabilizes the plasmid through mitotic divisions. In addition, a *CEN* sequence enables a plasmid to segregate in a Mendelian fashion in meiosis (6). The centromeres characterized so far have a region of 80 to 90 base pairs which is more than 90% A + T (element II) sandwiched between two conserved regions of 8 (element I) and 25 base pairs (element III) (12, 17).

A useful property of *S. cerevisiae* centromere plasmids is that they have copy numbers of just one or two per haploid genome. Evidence for this comes from Southern hybridization analysis of DNA from *CEN* plasmid transformants (3, 34) and also from tetrad analysis; *CEN* plasmids segregate 2:2 in about half the tetrads dissected, indicating that most parental diploids have one plasmid copy (6, 12). In contrast, *ARS* plasmids without centromeres have copy numbers of 50 to 100 per plasmid-bearing cell (18). Similarly, the 2μm circle plasmid of yeast has a copy number of about 60 (7), and 2μm circle derivatives have copy numbers ranging from 15 to 300 (13).

The reason for the high copy number of an *ARS* plasmid is selection for the plasmid combined with poor plasmid partition (25). Because of poor partition, the plasmids accumulate in a subpopulation of the cells, and because of selection, only these cells can grow. *CEN* plasmids might have low copy numbers simply because their efficient partition prevents them from using this mechanism to achieve high copy number. There are at least three counterarguments to this. First, it is clear that several plasmid copies are taken up in many if not most transformation events (15). In the absence of any upward or downward force on plasmid copy number, a clonal culture originating from a cell that had taken up three plasmid copies should still contain the plasmid at a copy number of three. Such cultures have not been observed. Second, a centromere does not make plasmid partition perfect; plasmid loss still occurs in up to 4% of cell divisions, and about one-fifth of these are 2-0 events, in which one daughter receives two plasmid copies (16, 20). Over a long period of culture growth in selective medium, these 2-0 events could increase average plasmid copy number. Third, Tschumper and Carbon (34) attached a centromere to a 2μm circle-based plasmid to see if the 2μm circle copy number amplification system could increase copy number. They found that plasmid copy number stayed at about one and concluded that the centromere had some positive function as a copy number control element.

To find what forces keep centromere plasmid copy number low, we tried to force copy numbers to higher levels and then observe the results.

MATERIALS AND METHODS

Strains. The genotypes of the strains used are shown in Table 1. All of the strains were constructed during the course of this work, mostly from parental strains provided by the Yeast Genetic Stock Center at Berkeley.

Media. YEPD was 1% yeast extract, 2% peptone, and 2% glucose. YNB was 0.67% Yeast Nitrogen Base (Difco Laboratories, Detroit, Mich.), 2% glucose, and 50 μg of each required base and amino acid per ml except ornithine, which was added to 500 μg/ml. The pH of YNB was adjusted to 6.5. All plates contained 2% agar. For both YEPD and YNB, glucose was autoclaved separately as a 20% stock.

YNB-leu lacked leucine, YNB-arg lacked arginine, etc.; YNB-5 lacked leucine, tryptophan, arginine, uracil, and histidine. When "selective medium" is referred to in the text, it means YNB lacking various supplements such that selective pressure is maintained for whatever plasmids are in the strain in question. In some cases noted in the text, galactose was substituted for glucose.

Yeast transformations. The lithium acetate method (19) was used with modifications. Cells were treated with lithium acetate at a cell concentration of about 5×10^8 per ml; 30 μg of sonicated calf thymus DNA was added to each transformation (5×10^7 cells); and no heat shock was used.

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TABLE 1. Yeast strains

Strain	Genotype										
BF231-4d	*MATα*	*leu2*	*ilv1*	*met8*	*ade5,7*		*his4*	*lys1*	*aro1D*	*ura4*	*CUP1?*
BF305-15d	*MATa*	*leu2*	*trp1*		*ura3*	*his3*		*ade1*	*arg5,6*	*met14*	
BF306-11b	*MATa*	*leu2*	*trp1*	*arg4*	*ura3*		*his4*	*ade1*	*arg5,6*	*ade2*	
BF306-12a	*MATα*	*leu2*	*trp1*	*arg4*	*ura3*		*his4*		*arg5,6*	*ade2*	
BF307-3	*MATa*	*leu2*	*trp1*	*arg4*	*ura3*	*his3*	*his4*	*ade1*	*arg5,6*	*met14*	
BF307-10	*MATα*	*leu2*	*trp1*	*arg4*	*ura3*	*his3*		*ade1*		*met14*	*ade6*
BF309[a]	*MATa*	*leu2*	*trp1*	*arg4*	*ura3*	*his3*	*his4*	*ade1*	*arg5,6*	*met14*	+
	MATα	*leu2*	*trp1*	*arg4*	*ura3*	*his3*	+	*ade1*	+	*met14*	*ade6*

[a] BF309 was constructed by crossing BF307-10 and BF307-3.

Plasmids. Plasmid pGT26 (Fig. 1) was obtained from G. Tschumper; pYF92 (which carries the yeast *HIS3* gene on a 1.5-kilobase (kb) *Bam*HI fragment) was obtained from R. Storms (32); pBR322-*ARG4* (which carries the yeast *ARG4* gene on a 3-kb *Hin*dIII fragment) and pBR322-*URA3* (which carries the yeast *URA3* gene in a 1-kb *Hin*dIII fragment) were obtained from S. Elliot; pYe(*CEN3*)30, pYe(*CEN11*)12, and pYe(*MET14*)2 were obtained from M. Fitzgerald-Hayes (11, 12); pYe(*CEN3*)41 was obtained from L. Clarke (6); and p*GALCEN3*, which consists of *CEN3* cloned just downstream of the *GAL1* promoter on a *URA3 ARS1* plasmid, was obtained from A. Hill and K. Bloom. The plasmid p*GALCEN*[2] was constructed by subcloning a *Bam*HI-*Eco*RI fragment of p*GALCEN3* carrying the *GAL1* promoter and *CEN3* into YCp50 (obtained from M. Rose), which carries *URA3*, *ARS1*, and *CEN4*. Plasmid p*GALCEN*[2] had previously been constructed and characterized by A. Hill and K. Bloom (personal communication). Plasmids carrying *CEN3* and either *HIS3*, *ARG4*, or *URA3* were constructed by standard methods as shown in Fig. 1. For clarity of nomenclature, pYe(*CEN3*)30, pYe(*CEN3*)41, and pYe(*MET14*)2 are referred to here as p*CEN3-TRP1*, p*CEN3-LEU2*, and p*CEN11-TRP1*, respectively.

Preparation of DNA. The alkaline lysis method (2, 23) was used to prepare plasmid DNA from *Escherichia coli*. Total DNA was prepared from yeast as follows: mid-log phase cells were harvested and suspended in 1 M sorbitol–50 mM Tris–5 mM EDTA–0.5% 2-mercaptoethanol (pH 8). Zymolyase 100,000 (Miles Laboratories, Inc., Elkhart, Ind.) was added to about 0.1 mg/g of cells. The cells were incubated at 37°C for 15 to 60 min; spheroplasts were harvested and washed twice with 1 M sorbitol–50 mM Tris–5 mM EDTA (pH 8). The pellet was suspended in TE (10 mM Tris, 1 mM EDTA [pH 8]), and a 10 mg/ml solution of pronase E (type XIV; Sigma Chemical Co., St. Louis, Mo.) was added to a final ratio of 10 mg of pronase per gram of cells. Sodium dodecyl sulfate was added to 1%. The suspension was mixed and then incubated at 37°C for at least 1 h. Cell debris was removed by centrifugation, and one-half volume of 40% polyethylene glycol 4000–2 M LiCl was added to the supernatant. After a 30-min incubation at 0°C, nucleic acids were recovered by centrifugation. The pellet was dissolved in TE, and the solution was extracted with 1:1 phenol-chloroform. Nucleic acids were precipitated with ethanol. The pellet was dissolved in TE, and various procedures were used to remove RNA, usually precipitation in 2.5 M LiCl at −20°C for 30 min, followed by centrifugation at 30,000 × *g* for 30 min followed by precipitation of the DNA in the supernatant with ethanol and treatment of the redissolved pellet with RNase A.

Southern analysis. A modification of the technique of Southern (29) was used. To make ^{32}P-labeled probe, the *Hin*dIII-*Bam*HI fragment from pYe(*CEN3*)41 containing the centromere (Fig. 1) was subcloned into plasmid pSP64 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). A ^{32}P-labeled 2-kb transcript of the centromere-containing fragment was made by using bacteriophage SP6 polymerase. Gel analysis showed that virtually all of the incorporated radioactivity ran in a band corresponding to a fragment length of 2 kb. There were slight differences in the length of homology between the probe and the various target sequences, but since almost all of the probe was full length and since hybridizations were for 16 h (i.e., a high Cot), these differences should have made little or no difference to the degree of hybridization observed in Fig. 2.

Plasmid instability measurements. Plasmid instabilities were measured as previously described (13). Briefly, cells were pregrown in selective medium and then switched at 0 time to a YNB medium that selected for all but one of the plasmids present. Serial dilutions were made when necessary to keep the cells in exponential growth. The percentage of cells containing the unselected plasmid was assayed by plating samples daily and by replica plating the resulting colonies. Plasmid loss was generally followed for about 5 days. Instability is the rate at which plasmid$^+$ cells generate plasmid^0 cells per generation.

Viability measurements. Cells were grown in selective YNB medium to mid-log phase, harvested, suspended in water, sonicated, and counted with a hemacytometer. Then 300 plating units were spread onto each of two YEPD plates. After incubation, the number of colonies was counted. Results were normalized (i.e., control viability was set equal to 100%). In general, controls gave close to 100% viability even before normalization.

Chromosome loss. An *S. cerevisiae* cell that has no mating type information mates as if it were *MATa* (33). Therefore, haploids that have lost their chromosome III (which carries the *MAT* locus) can be rescued by mating them to *MATα* cells. This fact was used to measure the rate of loss of chromosome III from a *MATα* haploid. Strain BF307-10 (*MATα*) with or without five centromere plasmids was grown to mid-log phase, sonicated, and mixed with a twofold excess of similarly treated strain BF231-4d (*MATα*). Cells (3×10^7) from the mixture were plated onto YEPD and incubated at 30°C for 22 h to allow chromosome loss and mating to occur. They were then recovered from the YEPD plate and plated onto YNB-ade-met. Since the two strains had complementing *ade* and *met* markers, only diploids (actually, 2N-1 aneuploids) grew on the YNB-ade-met plates. These diploids were the result of the loss of chromosome III of either strain, allowing it to mate as a *MATa*. The two possibilities could be distinguished because chromosome III of strain BF231-4d was marked *his4*, whereas strain BF307-10 was *HIS4*. Therefore, the diploid formed when

chromosome III was lost from BF231-4d was *HIS4*, while the diploid formed when chromosome III was lost from BF307-10 was *his4*. Thus, the ratio of the rates of loss of chromosome III in the two strains was approximately the ratio of His^- to His^+ diploids. The ratio of His^- diploids to His^+ diploids obtained when BF307-10 carried no centromere plasmids was compared with the ratio obtained when BF307-10 carried five different centromere plasmids. A fluctuation test was not needed because haploids that lose a chromosome cannot produce viable daughter cells.

The rate of loss of chromosome III was also measured in diploid strain BF309 (*MAT*a/α). In this case, the rate at which BF309 mated as a *MAT*a cell was measured, with complementation of strain BF231-4d as an assay, in the same way as described above. Strain BF309 would mate as a *MAT*a cell if it had lost the *MAT*α homolog of chromosome III or, alternatively, if mitotic crossing over or gene conversion had replaced *MAT*α information with *MAT*a information from the other homolog. Since the chromosome III homolog carrying *MAT*a was marked with *his4* and the *MAT*α homolog was marked with *HIS4* and since BF231-4d was *his4*, the following possibilities could be distinguished. When chromosome loss was responsible for mating, the triploid (actually, a 3N-1 aneuploid) produced was His^-, and when mitotic crossing over or conversion was responsible, the triploid was His^+.

Strain BF309 was *his3* homozygous, but plasmid p*CEN3-HIS3* was present even in the control diploid. This allowed the diploid to be grown in YNB-his, which prevented the accumulation of diploids that had lost the *HIS4* homolog of chromosome III.

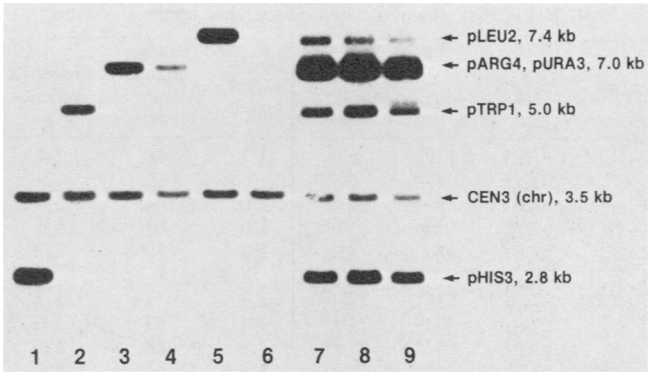

FIG. 2. Autoradiogram of filter used to measure plasmid copy number. DNA was extracted from strain BF307-10 containing the following plasmids. Lanes: 1, p*CEN3-HIS3*; 2, p*CEN3-TRP1*; 3, p*CEN3-ARG4*; 4, p*CEN3-URA3*; 5, p*CEN3-LEU2*; 6, no plasmid; 7, 8, and 9, all five plasmids (i.e., p*CEN3-HIS3*, p*CEN3-TRP1*, p*CEN3-ARG4*, p*CEN3-URA3*, and p*CEN3-LEU2*). The DNA was cut with *Eco*RI and *Hin*dIII, fractionated on an agarose gel, blotted to nitrocellulose, and hybridized to a probe complementary to *CEN3*. The chromosomal *CEN3* fragment was 3.5 kb long, and the sizes of the *CEN3*-containing fragments released from the plasmids are indicated at the right. Extra bands in lanes 4 and 7 are due to incomplete DNA digestion. Lanes 1 through 6 and 7 through 9 are from two different Southern blots, and the negatives were enlarged differently; therefore, band intensities cannot be compared between the two parts of the figure.

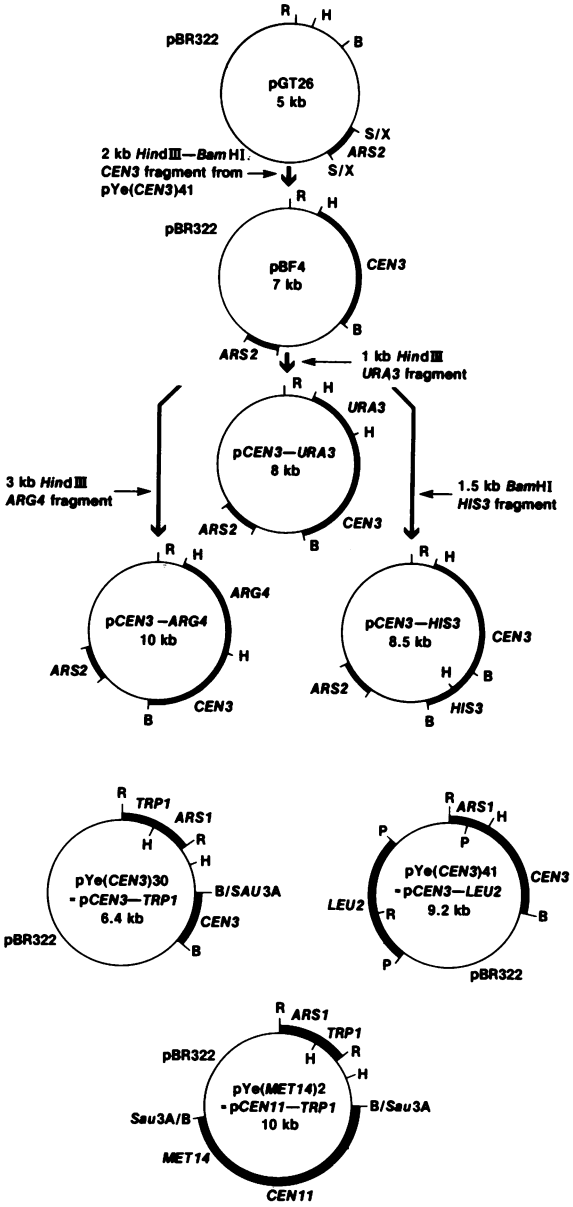

FIG. 1. Plasmids. ▬ *S. cerevisiae* DNA; —— pBR322 DNA. Restriction sites: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xho*I; S/X, junction of a *Sal*I fragment and a *Xho*I fragment; B/Sau, junction of a *Bam*HI fragment and a *Sau*3A fragment.

Giemsa staining. Cell nuclei were stained with Giemsa by a modification of the method of Bilinski and Miller (1). Briefly, a microscope slide was painted with egg white and allowed to dry. It was then painted with a suspension of log-phase cells that had been sonicated, harvested, washed, and suspended at a concentration of about 5×10^7 per ml. The slides were allowed to dry and then were fixed by passing them slowly, cells up, through the flame of a Bunsen burner three times. After the slides cooled, they were placed in a beaker of 5 N HCl (room temperature) and left for up to 10 min, depending on the strain. The slides were moved to a beaker of deionized water and left there for about 3 min. This step was repeated twice. Giemsa stain was freshly made up in a staining jar by diluting 5 ml of concentrated Giemsa Stain Solution (SO-G-28; Fisher Scientific Co., Pittsburgh, Pa.) into 35 ml of phosphate-buffered saline (PBS) (pH 6.9) (a 10× stock solution of PBS contained [per liter] 2 g of KCl, 2 g of KH_2PO_4, 80 g of NaCl, 11.4 g of Na_2HPO_4). Wet slides were immersed in the stain and rocked gently for times

TABLE 2. *CEN* plasmid copy number per haploid genome[a]

Strain	Plasmid[b]	cpm of:		Uncorrected copy no.	% prototrophic (i.e., plasmid$^+$)	Corrected copy no.
		Plasmid	Chromosome			
1-*CEN*	p*HIS*	1393	672	2.1	86	2.4
	p*TRP*	598	602	1.0	52	1.9
	p*ARG*	897	549	1.6	64	2.6
	p*URA*	586	599	1.0	61	1.6
	p*LEU*	1285	528	2.4	89	2.7
5-*CEN*[c]	p*HIS*	5161	2180	2.4	72	3.3
	p*TRP*	4049	2180	1.9	33	5.6
	p*ARG*	8525	2180	3.9	60	6.5
	p*URA*	8525	2180	3.9	61	6.5
	p*LEU*	2620	2180	1.2	76	1.6

[a] Relevant sections of the nitrocellulose filter that produced the autoradiogram shown in Fig. 2 were cut out. The amont of ^{32}P on each section was assayed with a scintillation counter. Counting errors were 3% or less. Background counts have been subtracted from the values shown.

[b] Plasmid names have been abbreviated.

[c] Results for the three 5-*CEN* transformants were very similar and so were pooled. Since the fragments derived from p*CEN3-ARG4* and p*CEN3-URA3* comigrate in the 5-*CEN* lanes, the total counts in the band (17,050) were attributed equally to each of the two plasmids.

ranging from 2 to 15 min, depending on the strain. After staining, slides were dipped in PBS and then placed in a beaker of distilled water for about 1 min. In rare cases where overstaining was a problem, slides were dipped in 0.02% acetic acid for 2 to 4 s, dipped and swirled in PBS, and placed in distilled water. After air drying, immersion oil was placed directly onto the slide, and slides were examined by light-field microscopy with a total magnification of 1,000.

RESULTS

Generation of strains containing multiple *CEN* plasmids. A system was devised that required cells to maintain several different *CEN* plasmids simultaneously. Strains were constructed with multiple auxotrophies, including *leu2*, *his3*, *trp1*, *ura3*, and *arg4*. A matching series of centromere plasmids was then constructed, with each plasmid carrying a different selectable marker. These plasmids, when based on *CEN3*, were called p*CEN3-LEU2*, p*CEN3-HIS3*, p*CEN-TRP1*, p*CEN3-URA3*, and p*CEN3-ARG4*. Some analogous plasmids based on *CEN11* were also built. By transforming a strain with a mixture of the plasmids and selecting for several plasmid markers simultaneously, strains containing four or five different *CEN* plasmids were obtained. These will be referred to as 4-*CEN* and 5-*CEN* strains.

TABLE 3. Plasmid instability

Strain	Plasmid followed	No. of *CEN* plasmids in strain	Instability (% loss/ Generation)[a]	Relative instability
BF307-3	p*CEN3-LEU2*	1	0.6 (0.1)	1
	p*CEN3-LEU2*	4	2.3 (1.1)	3.8
	p*CEN11-TRP1*	1	4.3 (1.0)	1
	p*CEN11-TRP1*	4	14.4 (6.5)	3.3
BF307-10	p*CEN3-TRP1*	1	7.5 (1.6)	1
	p*CEN3-TRP1*	5	9.7 (2.5)	1.3
	p*CEN3-URA3*	1	3.8 (1.0)	1
	p*CEN3-URA3*	4	5.7 (1.0)	1.5
	p*CEN3-URA3*	5	8.1 (0.9)	2.1
BF309	p*CEN3-TRP1*	1	6.0 (0.4)	1
	p*CEN3-TRP1*	5	7.5 (1.1)	1.3
	p*CEN3-LEU2*	1	0.7 (0.1)	1
	p*CEN3-LEU2*	5	1.7 (0.4)	2.4

[a] Percentage of plasmid° cells produced per plasmid$^+$ cell per generation during continuous exponential growth without selection for the plasmid of interest. The standard deviation of the instability is given in parentheses.

TABLE 4. Normalized viability of multiple *CEN* cells

Strain[a]	Expt. no.	No. of *CEN* plasmids	Viability (%)[b]
BF307-3	1	4	57
	2	4	56
	3	4	74
	4	4	57
BF307-10	1	5	85
	2	5	76
BF309	1	5	41

[a] For strain BF307-3, the plasmids used were p*CEN3-LEU2*, p*CEN3-TRP1*, p*CEN3-ARG4*, and p*CEN3-URA3* for experiments 1, 2, and 3. For experiment 4, p*CEN11-TRP1* was substituted for p*CEN3-TRP1*.

[b] When BF307-10 or BF309 cells were held at 30°C for 24 h in YNB-5, their viability was 80 to 90%.

Several genetic tests were done to show that the transformants were haploids and that they contained multiple, independently segregating *CEN* plasmids. In a few transformed clones, markers did not segregate independently, e.g., all the His$^-$ colonies were also Trp$^-$ and vice versa. Further analysis showed that two plasmids could recombine and subsequently lose one *CEN* sequence through various types of deletions (data not shown).

Several haploid transformants displaying independent segregation of all markers were stored at −70°C in 50% glycerol. All experiments were done with samples of cells taken from these frozen stocks.

Properties of multiple *CEN* strains. (i) Plasmid copy number. To determine how many plasmid copies the 5-*CEN* strains contained, we prepared DNA from each of the five types of 1-*CEN* strains and also from four independent 5-*CEN* transformants. The DNA was digested with *Eco*RI and *Hin*dIII, fractionated on an agarose gel, blotted to nitrocellulose, and hybridized to a 2-kb single-stranded ^{32}P-labeled RNA probe complementary to *CEN3*. Autoradiograms showing the chromosomal *CEN3* band and the

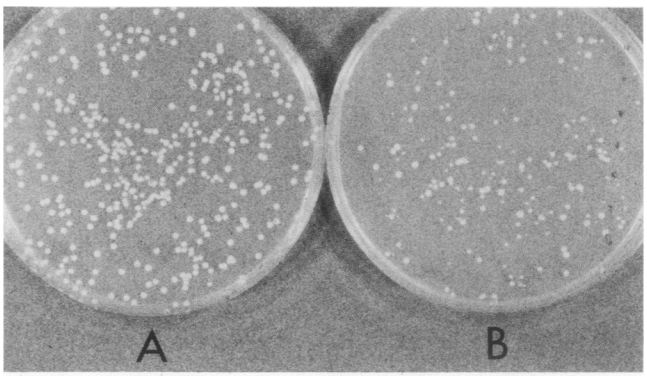

FIG. 3. Colonies formed by 5-*CEN* cells are heterogeneous in size. Control cells (A) or 5-*CEN* cells (B) were sonicated, spread on YEPD plates, and incubated for 3 days.

plasmid bands were obtained (Fig. 2). By comparing the amount of radioactivity hybridized to plasmid and to chromosomal restriction fragments, we estimated the absolute copy number of each plasmid per haploid genome. Results are shown in Table 2. In 1-*CEN* strains, copy numbers ranged from 1.6 to 2.7, with an average of 2.2 (after correcting for plasmid^0 cells; i.e., the average copy number over all cells is divided by the fraction of cells that have the phenotype conferred by the plasmid). In the 5-*CEN* transformants, copy numbers ranged from 1.6 to 6.5, with an average of 4.7. The total number of extra copies of *CEN3* carried by the 5-*CEN* strains is about 13 (the sum of the uncorrected copy numbers).

(ii) Plasmid instability. For any of the five markers, more auxotrophs were observed in 4- or 5-*CEN* strains than in the corresponding 1-*CEN* strain. This suggested that *CEN* plasmids might be unstable in each other's presence. Plasmid instability was therefore measured as described in Materials and Methods. In most cases, plasmid instability was about twofold higher in multiple *CEN* strains (Table 3). This modest increase in instability on a per cell basis may reflect a relatively large increase on a per plasmid basis, since the 5-*CEN* strains have elevated plasmid copy numbers.

The increased instability was not specific to *CEN3*; when a *CEN11*-based plasmid was examined in a strain containing three *CEN3* plasmids, the instability of the *CEN11* plasmid increased (Table 3).

There was a hierarchy of stability among the plasmids. p*CEN3-LEU2* (the second largest plasmid) was most stable, while p*CEN3-TRP1* (the smallest plasmid) was least stable. There seemed to be some selection for the *LEU2* gene of p*CEN3-LEU2*, since strain BF307-10 grew slightly faster when it contained p*CEN3-LEU2* than when it did not, even in rich medium (data not shown). Similar effects in *leu2* strains have been noted previously (9). There was no such effect for any of the other markers.

A consequence of the high instability in the 5-*CEN* transformants was that less than 10% of the cells in selective medium actually contained all five plasmids. The average cell contained only three different plasmids, but it carried about four copies of each (Table 2).

(iii) Cell inviability. Viability was measured as described in Materials and Methods. From 15 to 59% of the cells in 5-*CEN* cultures were inviable (Table 4).

(iv) Slow growth rates. We noticed that 5-*CEN* strains seemed to grow rather slowly. Two experiments were done to confirm and quantify the effect. First, sonicated cells were spread on YEPD plates and incubated for 3 days. Strains containing 5-*CEN* plasmids produced colonies of heterogeneous size that were generally smaller than those produced by the control (Fig. 3). Sizes of the control colonies were quite homogeneous. Second, control and 5-*CEN* strains were switched from selective medium into YEPD, and growth of the cultures was monitored carefully with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). Several independent experiments were done, each of which gave similar results (e.g., Fig. 4). The control strain had a doubling time of 94 ± 3 min, whereas the initial doubling time of the 5-*CEN* strains was about 200 min; after 20 h in YEPD, the average doubling time was still about 119 min, 25 min longer than that of the control strain.

Similar results were obtained with diploid strain BF309. The doubling time of the control strain in YEPD was about 96 min, whereas the doubling time of the 5-*CEN* version in the interval between 5 and 12 population doublings was about 113 min.

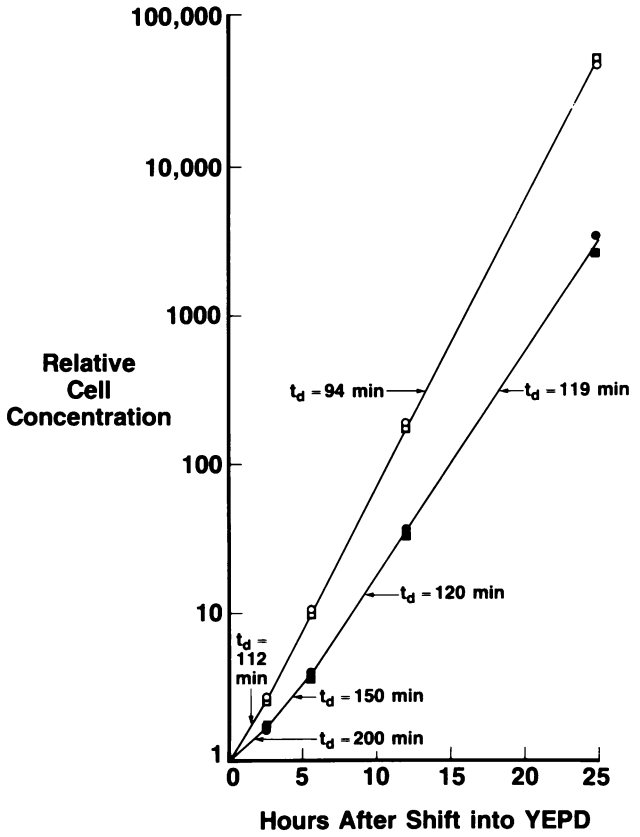

FIG. 4. 5-*CEN* cells grow slowly even in nonselective medium. Exponentially growing control cells (open symbols, two experiments) or 5-*CEN* cells (solid symbols, two experiments) were diluted from selective medium into prewarmed (30°C) YEPD medium at zero time. The initial cell concentrations were about 10^6/ml. Increases in cell concentration were monitored with a Coulter Counter. A serial dilution into prewarmed YEPD was made just after the 12-h point. The population doubling time (t_d) for various intervals is shown.

(v) Cell cycle abnormalities. Microscopic examinations of 5-*CEN* cultures revealed more budded cells than in the control strain. Many of these were doublets with two equal, exceptionally large cells. Numerous other abnormalities were apparent. 5-*CEN* cells were in general significantly larger than the controls; cells were misshapen; there were strings or complexes of three, four, or five cells which could not be separated by sonication; and many of the cells, particularly the large ones, could be broken by sonication. No such abnormalities appeared when control cells were shifted to YNB-5 medium to starve them for amino acids. Examples of 5-*CEN* cells are shown in Fig. 5.

To see if cells were having difficulty completing some particular phase of cell division, we grew control and 5-*CEN* cells in complete or selective YNB medium, respectively, switched the cells into prewarmed YEPD medium for 12 h, and then stained cell nuclei with Giemsa. As shown in Table 5, there were about twice as many 5-*CEN* cells as control cells in nuclear divison and in the stage just prior to nuclear division. The effect may have been more than twofold, because the largest and most aberrant cells were often broken during the staining procedure, so their nuclear morphologies could not always be seen. Thus, an excess of

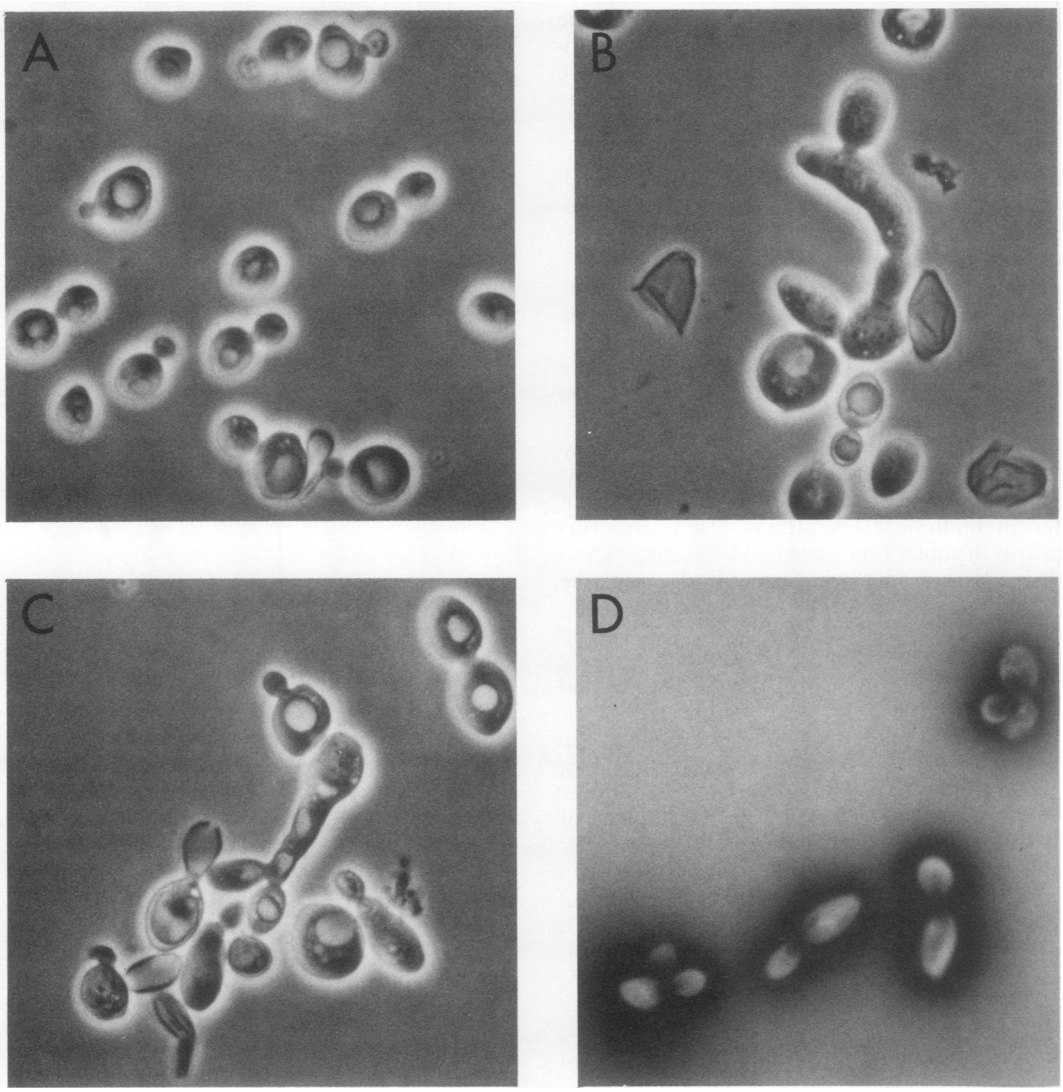

FIG. 5. 5-*CEN* cells have aberrant morphologies. Phase micrographs of sonicated BF309 control cells (A) or 5-*CEN* cells (B and C) growing exponentially in selective YNB medium. The fields shown in panels B and C are typical. (D) Four Giemsa-stained 5-*CEN* BF309 cells in mitosis.

centromeres may cause cells to pause in their cell cycles at or just before nuclear division.

When the aberrant cell complexes were not lysed it was generally found that several nuclei were present, i.e., syncytia were being formed. However, there were often fewer nuclei than cell bodies. The syncytia resembled those formed by *cdc3*, *cdc10*, *cdc11*, and *cdc12* mutants (14).

The measurement of relative cell volumes with a Coulter Channelizer proved to be a sensitive and reproducible assay for the effects of *CEN* plasmids on cell morphology. Cells were pregrown in selective YNB and then switched to YEPD and grown for various times. Their volumes were then measured (Fig. 6 and 7) and found to be aberrantly large.

Diploid strain BF309 showed a similar but somewhat less extreme increase in volume when it contained five *CEN* plasmids (data not shown).

We studied the effect of various numbers of *CEN* plasmids on cell size in several different strains, and for some strains, even a single *CEN* plasmid caused a small but reproducible increase in cell size. Three or four different plasmids (in various combinations) produced significant and increasingly large effects in all of the strains assayed (Fig. 7).

(vi) Chromosome loss. Our plasmid stability studies suggested that plasmid centromeres functioned inefficiently in each other's presence. If chromosomal centromeres were also functioning inefficiently in the 5-*CEN* strains, resulting in high levels of chromosome loss, this might explain the various toxic effects observed. Therefore, to see if the presence of 13 extra centromeres affected the efficiency of chromosomal centromere function, we measured relative rates of chromosome III loss as described in Materials and Methods. We chose chromosome III because (i) the rate of its loss could be measured in a haploid and (ii) since the plasmids carried *CEN3*, it was possible that chromosome III was more severely affected than other chromosomes (i.e., the toxicity might be caused by chromosome III loss specifically). Results are shown in Table 6; the frequency of chromosome III loss from BF307-10 increased two- to sixfold when five *CEN* plasmids were present. For diploid strain BF309, the increase due to five *CEN* plasmids was three- to fivefold. In both cases, the increase in frequency

somewhat underestimates the increase in rate, because the 5-*CEN* cells would have gone through fewer generations in the 22-h test period than the controls.

For the control strain BF307-10 the absolute rate of loss of chromosome III was very roughly 10^{-5} per cell per generation (data not shown).

Thus, a slightly increased rate of loss was observed, but on the other hand, the rate of loss was only about 10^{-4}, and even supposing that all other chromosomes were lost at the same rate, this was not enough chromosome loss to explain the inviability or other toxic effects observed.

We are not sure what significance to attach to the slight increase in chromosome loss. It could have been due to integration of a *CEN* plasmid into chromosome III, causing a dicentric, or it could have been due to competition between chromosomal and plasmid centromeres for some mitotic factor present in limiting quantities. Furthermore, since events that cause a pause at mitosis often cause some chromosome loss (G. Kawasaki, Ph.D. thesis, University of Washington, Seattle, 1979, quoted in reference 28), it is possible that the extra loss was a secondary effect of the mitotic pause described in section v above.

Effects of conditional centromeres. We were concerned that the toxicity observed might have been caused or influenced by our method of making multiple *CEN* strains, i.e., simultaneously selecting for five plasmid markers. We therefore tried another approach to obtaining multiple *CEN* strains.

It has recently been shown that transcription toward a centromere from a strong promoter can inhibit yeast centromere function (5; I. Groth-Clausen and P. Philipsen, personal communication; A. Hill and K. S. Bloom, J. Cell. Biochem., Suppl. 9C, p. 134, 1985). For instance, plasmids that have a centromere cloned downstream of the yeast *GAL1* gene behave as *CEN* plasmids when they are in a cell utilizing glucose but behave as *ARS* plasmids when they are in a cell utilizing galactose, because the centromere function is inhibited by transcription from the *GAL1* promoter (K. Bloom, personal communication). When cells containing such a plasmid are grown in galactose medium and then switched into glucose medium, the cells that happened to have a high copy number of the *ARS* plasmid will now have a high copy number of the *CEN* plasmid and so may show toxic effects.

To test this possibility, we obtained the plasmid p*GALCEN3* from A. Hill and K. Bloom. This plasmid carries *URA3*, and has *CEN3* cloned downstream of the *GAL1* promoter. Transformed cells of strain BF503-15d were grown in selective galactose medium, sonicated, and then plated onto YEPD or YEP galactose. The resulting colonies were examined to see if some were smaller than others. On the galactose plates there was very little size heterogeneity, but on the glucose plates the p*GALCEN3* culture (but not a control culture) yielded an obvious class of small colonies. The positions of all small colonies were marked, and then the colonies were replicated onto YNB-ura plates. After incubation, colonies were scored as Ura^+ or Ura^-. The replicas were then compared with the marked originals to see if there was any correlation between presence of p*GALCEN3* and colony size. Results are shown in Table 7. For the colonies grown on galactose plates, there was no correlation. For the p*GALCEN3* colonies grown on glucose plates, nearly all of the small colonies were derived from Ura^+ (i.e., p*GALCEN3*$^+$) cells. The reverse was not true; about two-thirds of the Ura^+ cells gave rise to average-sized colonies. This suggests that some of the plasmid$^+$ cells had the *ARS* plasmid in high copy number, and some had it in low copy number.

A sample of cells from the original galactose culture was grown in nonselective galactose medium for about 20 generations. At the end of that time, nearly all of the cells (>99.9%) were *Ura*$^-$ (as assayed on YNB galactose-ura plates). Thus, there were few if any integrated plasmids in the population, and plasmid integration could not have had

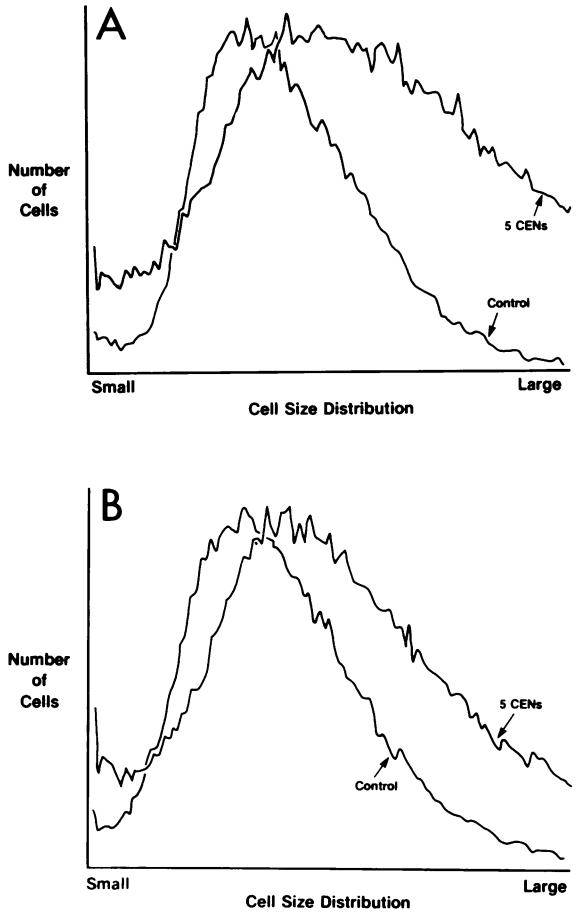

FIG. 6. 5-*CEN* cells are larger than control cells. BF307-10 control cells and 5-*CEN* cells growing exponentially in selective medium were switched to prewarmed (30°C) YEPD. After 8 (A) or 22 (B) h of growth, the cells were sonicated and their volumes were measured with a Coulter Channelizer.

TABLE 5. Cell cycle distribution of 5-*CEN* strains[a]

Strain	Medium	% Cells in Each Stage of the Cycle					No. of Cells Counted
		⊙	◔	◑	◕	●	
BF307-10 [p0]	YNB	60	22	8	5	5	509
BF307-10 [p5]	YNB-5	50	18	18	11	3	1075
BF307-10 [p0]	YEPD	45	37	6	4	8	1100
BF307-10 [p5]	YEPD	37	35	11	10	7	867
BF309 [p0]	YNB	40	30	10	9	10	862
BF309 [p5]	YNB-5	40	23	16	12	9	662

[a] [p0] and [p5] signify strains carrying 0- and 5-*CEN* plasmids.

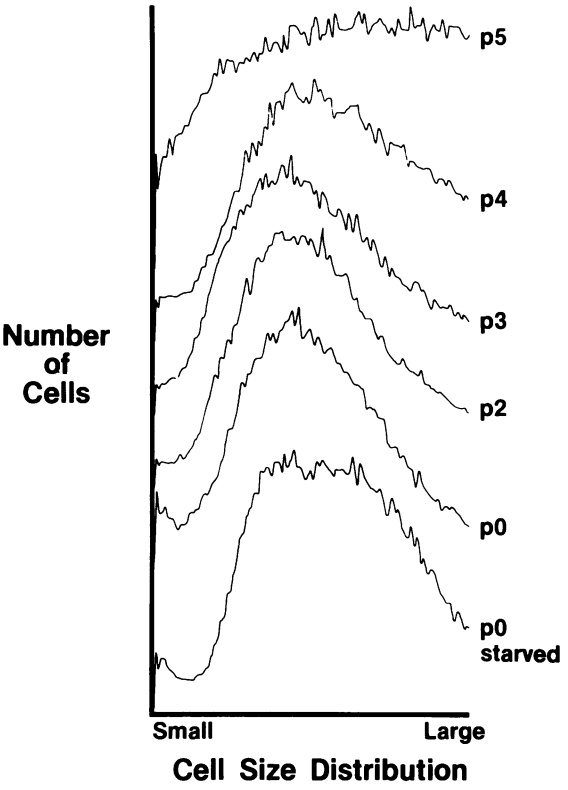

FIG. 7. Cell volume increases with the number of *CEN* plasmids. BF307-10 cells with 0-, 2-, 3-, 4-, or 5-*CEN* plasmids (p0, p2, p3, p4, and p5, respectively) were grown to mid-log phase in selective YNB, sonicated and measured with a Coulter Channelizer. The p0 starved cells were held at 30°C in YNB-5 medium for 16 h before the measurement.

anything to do with the small colonies produced on glucose plates.

Effects of a conditional dicentric. It was pointed out to us by P. Philippsen that recombination between the various plasmids in the 5-*CEN* strains could create dicentrics and that these might interfere with mitosis. To test this, a conditional dicentric was constructed. An *Eco*RI-*Bam*HI fragment carrying the *GAL1* promoter and the downstream *CEN3* was excised from p*GALCEN3* and cloned into YCp50, which carries *URA3* and *CEN4*. This new plasmid, p*GALCEN*^{2}, was functionally monocentric in galactose medium, with a working *CEN4* and a nonfunctional *CEN3*, but was dicentric in glucose medium.

Strain BF307-10 transformed with p*GALCEN*^{2} was grown to mid-log phase in YNB-ura galactose medium and then shifted to various liquid media including YNB-ura galactose, YNB-ura glucose, YNB + ura glucose, YEP galactose, and YEPD (i.e., glucose). The cultures were incubated with shaking at 30°C, and samples were taken every 2 h for 12 h. Growth rate, cell size, and cell morphology were carefully examined. No significant difference was observed between plasmid-containing cells and control cells in any medium.

To confirm that the plasmids were dicentrics in glucose medium, we grew the YEP galactose and YEPD cultures to stationary phase and then plated cells onto YEP galactose or YEPD, respectively. After colonies formed, they were replica-plated onto YNB-ura. Of cells grown in galactose, 260 of 569 (46%) produced Ura$^+$ colonies, but of the cells grown in glucose, only 5 of 835 (0.7%) produced Ura$^+$ colonies. The extraordinarily high rate of plasmid loss in glucose medium is consistent with the idea that in glucose, the plasmid is dicentric and is often broken and destroyed at mitosis.

Thus, the presence of a dicentric plasmid has little or no effect on the cells.

DISCUSSION

When a cell containing a small *CEN* plasmid divides, there is about a 1% chance that one of the daughters will not receive a plasmid. If the other daughter always received two copies (2-0 segregation), then this, combined with selection for cells containing plasmids, would tend to increase plasmid copy number. However, it has been shown that in about 80% of the cases where one daughter is plasmid^0, the other daughter contains only one copy (1-0 segregation) (16, 20). If 1-0 segregation were always more frequent than 2-0 segregation, this alone would keep *CEN* plasmid copy number low under normal circumstances.

The difficulty encountered in forcing *CEN* plasmids to higher copy numbers (34; this paper) indicates additional complications, however. We found that an excess of centromere plasmids had a deleterious effect on cells containing them. Cultures of 5-*CEN* cells grew slowly; this was partly because viable 5-*CEN* cells grew slowly and partly because many 5-*CEN* cells were not viable. Microscopic examination showed that 5-*CEN* cells behaved aberrantly and, together with Giemsa staining, suggested that they had

TABLE 6. Loss of chromosome III[a]

Expt no.	Strain	No of colonies with indicated genotype				Ratio of *his4*/*HIS4*	Relative rate of chromosome loss
		his4 2N-1	*his4* 3N-1	*HIS4* 2N-1	*HIS4* 3N		
1a (Loss from 5-*CEN* haploids)	BF307-10 (p0) × BF231-4d	35		111		0.32	1
	BF307-10 (p5) × BF231-4d	81		50		1.6	5.1
1b	BF307-10 (p0) × BF231-4d	1278		1575		0.81	1
	BF307-10 (p5) × BF231-4d	568		377		1.5	1.9
2 (Loss from 5-*CEN* diploids)	BF309 (p1) × BF231-4d		51		185	0.28	1
	BF309 (p5) × BF231-4d		39		28	1.4	5.0
	BF309 (p1) × BF306-12a		25		35	0.71	1
	BF309 (p5) × BF306-12a		83		36	2.3	3.2

[a] (p0), (p1), or (p5) indicates a strain containing 0-, 1-, or 5-*CEN* plasmids, respectively. 2N-1, 3N-1, and 3N signify ploidy. In these experiments, loss of chromosome III from the 5-*CEN* parent generates *his4* aneuploids exclusively, whereas *HIS4* aneuploids or triploids are generated by loss of chromosome III from the control parent, in experiment 1, or by mitotic crossing over or mitotic gene conversion, in experiment 2.

difficulty with mitosis. The rate of centromere plasmid loss and possibly chromosome loss increased, again suggesting defective mitosis. Some of these effects are similar to those observed with multiply disomic strains (4).

For two reasons, we believe that the effects observed were due to high *CEN* plasmid copy number and not to the method by which high copy number was achieved. First, all of the effects were visible even in cells grown in YEPD (i.e., nonselective) medium. Second, slow growth was seen even when high copy number was achieved by switching on a conditional centromere that was already present at moderately high copy number.

One interpretation of our results would be that there was a general, partial loss of centromere function in 5-*CEN* strains. Although the increase in the rate of generation of plasmid^0 cells was small, it should be remembered that in 5-*CEN* strains plasmid copy number was relatively high. Therefore, on a per plasmid basis, rates of plasmid loss were significantly greater than those in control strains. Furthermore, most *CEN* plasmid loss is normally due to 1-0 segregation; only about 20% is due to 2-0 segregation (16). If the increase in plasmid loss in 5-*CEN* strains was entirely due to an increase in 2-0 segregation, then a 5- to 10-fold increase in 2-0 segregation would be required. This hypothetical increase in 2-0 segregation could help explain the relatively high copy numbers of each of the plasmids in the 5-*CEN* strains.

Chlebowicz-Sledziewska and Sledziewski (5) recently studied conditional centromere plasmids. They found indications that *CEN* plasmids at high copy number did not have full centromere activity, a result for which we also have evidence. However, these authors did not observe any deleterious effects of the high-copy-number centromeres. We have no good explanation for this discrepancy between their results and ours, except to point out that with conditional centromeres, the effects we observed were subtle and might not have been seen except in experiments specifically designed to look for them. This comment also applies to any other cases where moderately high-copy-number centromere sequences may have been obtained (e.g., reference 24).

Tschumper and Carbon (34) studied the behavior of 2μm circle-based plasmids carrying centromeres. Despite the fact that the 2μm circle apparently has the ability to amplify its copy number to high levels, these *CEN*-2μm circle-composite plasmids have copy numbers of about one and do not have any obvious toxic effects on the host cells. Perhaps the effects are too small to be noticed; alternatively, the *CEN* may prevent 2μm circle amplification directly, possibly by blocking replication through the centromere until late in mitosis (34). A replication block at the centromere is one way of explaining the observation that sister chromatids are held together at the centromere until anaphase (8).

We believe that the various effects noted stem from defective mitosis, but we do not know why an excess of centromere plasmids interferes with mitosis. One hypothesis is that recombination between the plasmids generates dicentric plasmids and that these inhibit the completion of mitosis. We think this unlikely for two reasons. First, if the rate of mitotic recombination between plasmids is similar to that between chromosomal markers (10, 22), dicentrics would form in the 5-*CEN* strains at a rate of very roughly 10^{-5} per generation, which seems too infrequent to explain the observed effects (however, mitotic recombination between plasmids could be more frequent than that between chromosomes). Second, a culture in which almost every cell contained a conditional dicentric plasmid showed no adverse effects when the conditional centromere was turned on.

TABLE 7. Effect of a conditional centromere[a]

Strain	Carbon source	No. of colonies	% Ura$^+$	No. of small colonies	
				Ura$^-$	Ura$^+$
307-10 (p0)	Galactose	440	0	2	0
307-10 (p0)	Glucose	503	0	1	0
307-10 (p*GALCEN3*)	Galactose	1,144	24	6	1
307-10 (p*GALCEN3*)	Glucose	1,498	24	10	128

[a] Colonies were examined at a density of about 200 per plate. More glucose plates than galactose plates were examined in the p*GALCEN3* experiment. Almost all of the small, Ura$^-$ colonies proved to be due to petite mutations.

A second hypothesis is that *CEN* plasmids integrate into genomic DNA, creating dicentric chromosomes, which would be lost. This also seems unlikely, for three reasons. First, it would require very high recombination rates. Second, chromosome III, the chromosome most likely to suffer this kind of event in our transformants, is lost at only slightly elevated rates from 5-*CEN* haploids and diploids. Third, a plasmid with a conditional centromere was found to cause toxic effects in a significant number of cells when the centromere was turned on, even though no integrated plasmid copies were found in this particular culture.

A third hypothesis, which we find more attractive, is that some factor required for centromere function is present in limiting quantities and that 13 extra centromeres are enough to titrate out this factor. Mitosis might then be aberrant. Possible limiting factors are centromere-binding proteins, tubulin, and space on the spindle pole body. It may be that competition for this limiting factor between plasmid centromeres results in plasmid instability and that competition between plasmid centromeres and chromosomal centromeres results in some chromosomal instability. The pause at nuclear division could be explained if mitosis was delayed until attachment of all centromeres to the spindle pole body was completed. In the presence of 13 extra centromeres, extra time would be required, particularly if some essential component of the mitotic apparatus was in short supply. If the pause was long enough, the slow growth of 5-*CEN* cells would be explained.

In summary, we believe that *CEN* plasmids have low copy numbers for several reasons. First, by minimizing nondisjunction, they minimize the accumulation of plasmid copies in a few cells. Second, when such accumulation does occur, the cells involved grow slowly and are diluted out of the population because of the toxicity of the excess centromeres. Third, in special cases such as 2μm circle-derived plasmids, centromeres may prevent multiple rounds of replication directly.

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