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Genetic Dissection of Centromere Function

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A system to detect a minimal function of *Saccharomyces cerevisiae* centromeres in vivo has been developed. Centromere DNA mutants have been examined and found to be active in a plasmid copy number control assay in the absence of segregation. The experiments allow the identification of a minimal centromere unit, CDE III, independently of its ability to mediate chromosome segregation. Centromere-mediated plasmid copy number control correlates with the ability of CDE III to assemble a DNA-protein complex. Cells forced to maintain excess copies of CDE III exhibit increased loss of a nonessential artificial chromosome. Thus, segregationally impaired centromeres can have negative effects in *trans* on chromosome segregation. The use of a plasmid copy number control assay has allowed assembly steps preceding chromosome segregation to be defined.

The inheritance of genetic information depends on the faithful segregation of chromosomes during mitosis and meiosis. In eukaryotic cells, the structure that mediates the attachment of spindle microtubules to chromosomes and governs chromosome movement is the kinetochore. Kinetochores are assembled at a unique chromosomal domain, the centromere, frequently observed as a constriction in mitotic chromosomes. Centromeric DNA most likely provides sequence and structural information required for the assembly of functional kinetochores. The centromere region also mediates the complex process of homologous chromosome pairing and separation of sister chromatids during meiosis (for reviews, see references 9, 14, and 33).

In contrast to the large and repetitive centromeric domains characteristic of higher eukaryotes, centromeres of the budding yeast *Saccharomyces cerevisiae* consist of only 130 bp of DNA and interact with a single microtubule (14, 27). This small size, along with simple genetic assays for centromere function, has allowed a thorough characterization of this organism's centromere (14, 33). *S. cerevisiae* centromeric DNA consists of three centromere DNA elements, CDE I, CDE II, and CDE III. These elements are characterized by two short regions of conserved DNA (CDE I [8 bp] and CDE III [25 bp]) separated by a 78- to 86-bp A+T-rich CDE II (>90% A+T). Analysis of in vitro mutations generated in centromeric DNA has determined an essential role for specific base pairs in CDE III (17). These base pairs appear to be critically involved in the binding of CBF3, a multimeric complex of at least three polypeptides (25). Analyses of numerous deletions and insertions have determined a requirement for A+T richness in CDE II without conservation of a specific primary sequence. These results indicate that the conformation or structural deformation of this element contributes to centromere function (17, 28). A complete deletion of CDE I and CBF1, the protein that binds to CDE I (also referred to as CP1 and CPF1), has only a small effect (<10-fold) on the fidelity of chromosome segregation (3, 11, 17, 26). CDE I therefore plays a minor role in mitotic centromere function. Binding sites for CBF1 are also present at other chromosomal locations, and in some cases this protein appears to function as a regulator of transcription (8). The precise role of CBF1 at centromeres, however, is not known.

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Nuclease digestion experiments have shown that *S. cerevisiae* centromeres are packaged into a 200- to 250-bp nuclease-resistant DNA-protein complex (7, 32). Sedimentation analysis of centromere complexes excised from centromere plasmids indicates that the complex is greater than 15S (34), and functional centromere complexes have also been shown to mediate the interaction of centromere plasmids with microtubules in vitro (23). Importantly, mutations in CDE III that functionally inactivate centromeres in vivo do not show any evidence for the presence of a nuclease-resistant complex (32). These same mutations decrease the S value of excised centromere complexes (34), abolish the ability of centromeres to mediate an interaction with microtubules in vitro (23), and decrease the binding of CBF3 in vitro (25, 29). Taken together, these results indicate a direct correspondence between the ability to assemble a multimeric DNA-protein complex on centromeric DNA and the fidelity of chromosome segregation.

The results discussed above suggest that the proper assembly of a DNA-protein complex (kinetochore) on centromeric DNA is an absolute requirement for subsequent kinetochore function. The inability to visualize *S. cerevisiae* kinetochores cytologically has made the assembly process difficult to study in this organism. Footprinting of centromeres by indirect end labeling has shown that centromere DNA-protein complexes are indistinguishable throughout the cell cycle (40). Nevertheless, the finding that an assembly factor or chaperonin is required for the binding of CBF3 to CDE III in vitro (25) suggests that kinetochore assembly may be tightly controlled in vivo. Although sensitive assays for monitoring chromosome segregation in *S. cerevisiae* exist, these assays cannot distinguish defects in assembly from defects in other aspects of segregation. In this study, an assay based on the ability of exogenous centromere sequences to control plasmid copy number has been developed (16, 30). Unlike segregation assays, the plasmid copy number assay described herein can be used to measure centromere function in the absence of segregation. The results of this study suggest that a factor(s) involved in the assembly of the specialized DNA-protein complex at centromeres is required for centromere-mediated plasmid copy number control. This study also shows that the presence of extra centromere sequences which can bind components of the DNA-protein complex but are segregationally defective nonetheless can have significant negative effects in *trans* on the segregation of endogenous chromosomes.

MATERIALS AND METHODS

Strains. KR36-60 (*MATa ade2-1* or *ade2-101 ade8-18 his3-100 ura3-52 trp1-901 leu2::HIS3 cyh2*) (31) was used for most of the experiments reported in this study. For the sectoring assay, strain 5MO (MATa/α *ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trpΔ1/trpΔ1 ura3-52/ura3-52*) (provided by M. Resnick, National Institute of Environmental Health Sciences), which carries a *URA3 TRP1 SUP11* yeast artificial chromosome (YAC) with approximately 500 kb of mouse DNA was plated for single colonies in the presence of 5-fluoro-orotic acid. Colonies that arose on 5-fluoro-orotic acid were tested for growth in the absence of uracil. Potential *ura3* isolates were transformed with plasmid YEpFAT7 (Fig. 1) to determine whether uracil auxotrophy could be complemented by a wild-type *URA3* gene. The integrity of the YAC was determined by the presence and cosegregation of the *TRP1* and *SUP11* markers. The same *ura3* derivative of 5MO, 5MO-1, was used for all sectoring experiments.

Plasmid construction. YEpFAT7-Loc1 and YEpFAT7-Loc2 were constructed by cloning a *Sca*I fragment from plasmids pLoc1 and pLoc2 into the *Sma*I site present in the polylinker of YEpFAT7 (31) (Fig. 1). The *Sca*I fragment contains a partial fragment of the *Escherichia coli* ampicillin resistance gene, a 289-bp *CEN3* fragment, a 36-bp consensus binding site for the *lac* repressor, the *GAL1-10* promoter, the *E. coli lacI* gene, and a partial fragment of the *S. cerevisiae URA3* gene in the order given. YEpFAT7-Loc2 contains a C-to-A point mutation at position 14 of CDE III. YEpFAT7-1, YEpFAT7-6, YEpFAT7-7, YEpFAT7-16, YEpFAT7-21, YEpFAT7-31, and YEpFAT7-42 were constructed by polymerase chain reaction amplification of the centromeres from plasmids 303-1AR, 303-6AR, 303-7AR, 303-16AR, 303-21AR, 303-31AR, and 303-42AR (12, 32), using oligonucleotides 5′ to the *Bam*HI site of pBR322 (nucleotides 331 to 360) and 120 bp 3′ to the chromosomal *Bam*HI site flanking *CEN3*. YEpFAT7-150, YEpFAT7-C+Fl, and YEpFAT7-Fl were constructed by polymerase chain reaction amplification of *CEN3*, using genomic DNA from strain KR36-60 and oligonucleotides 15 bp upstream of CDE I and 41 bp downstream of CDE III (YEpFAT7-150), 15 bp upstream of CDE I and 1,186 bp downstream of CDE III (YEpFAT7-C+Fl), and 162 to 1,186 bp downstream of CDE III (YEpFAT7-Fl). A *Sph*I site was added to the end of each oligonucleotide to allow cloning into the unique *Sph*I site present in the polylinker of YEpFAT7 (31) (Fig. 1).

Determination of copy number control values. Single colonies of KR36-60 transformed with the appropriate plasmid were inoculated into 5 ml of YALT low-copy medium (0.66% yeast nitrogen base, adenine [20 μg/ml], leucine [40 μg/ml], tryptophan [50 μg/ml], 8% glucose or galactose) and grown at 32°C until a cell density of 2×10^7 to 4×10^7 cells per ml was reached. This culture was then used to inoculate a fresh culture at a density of 5×10^4 cells per ml. The second culture was grown at 32°C until a cell density of 2×10^7 to 4×10^7 cells per ml was reached. The cells were then pelleted at 3,000 × *g* for 5 min at room temperature, resuspended in 5 ml of H_2O, and incubated at 32°C for 60 min. This starvation step eliminated the appearance of microcolonies on minus-leucine (−leucine) plates. Appropriate dilutions were then plated on YALT low-copy plates and YAT high-copy plates (0.66% yeast nitrogen base, adenine [10 μg/ml], tryptophan [50 μg/ml], 8% glucose or galactose). The decreased concentration of adenine in YAT plates allows an estimate of plasmid copy number to be made by colony color as a result of weak complementation of the *ade8-18* mutation by the *Drosophila* glycinamide ribotide transformylase gene (fly ADE8) present on YEpFAT7 (31) (Fig. 1). After 4 days at 32°C, colonies were counted and a high copy (YAT)/low copy (YALT) ratio was determined.

Mitotic stability assays. Single colonies of KR36-60 carrying each plasmid were picked from YALT low-copy selective plates and used to inoculate 5 ml of nonselective YAULT medium (0.66% yeast nitrogen base, adenine [20 μg/ml], uracil [40 mg/ml], leucine [40 μg/ml], tryptophan [50 μg/ml], 8% glucose or galactose). After 5, 10, and 20 generations of growth at 32°C, cells were plated for single colonies on YAULT plates. After 3 days at 32°C, colonies were replica plated to YALT low-copy selective plates. These plates were incubated for 24 h at 32°C, and the percentage of the population bearing the plasmid was determined. The loss rate was derived from the change in the percentage of plasmid-bearing cells over time.

DNA isolation. Approximately 10^8 cells were pelleted at 3,000 × *g* for 5 min at 4°C and resuspended in 0.5 ml of lysis buffer (0.1 M Tris [pH 8.0], 50 mM EDTA, 1% sodium dodecyl sulfate [SDS]). The cell suspension was transferred to an Eppendorf tube to which an equal volume of glass beads was added. The cells were lysed by vortexing for 20 min at 4°C. The lysed cells were spun into a fresh tube by pelleting the suspension through a hole made in the bottom of the first tube at 3,000 × *g* for 5 min at 4°C. The suspension was brought to a final concentration of 0.5 M NaCl, extracted with phenol, with phenol-chloroform, and with chloroform, and precipitated with 1 volume of isopropanol. Nucleic acid was pelleted at 15,000 × *g* for 15 min at 4°C, washed once with 70% ethanol, and dried. The pellet was resuspended in 190 μl of STE (10 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and digested with RNase A (0.5 mg/ml, final concentration) for 30 min at 37°C. After RNase A digestion, the solution was brought to a final concentration of 0.1% SDS and digested with proteinase K (0.5 mg/ml, final concentration) for 30 min at 65°C. The solution was then extracted with phenol, with phenol-chloroform, and with chloroform and precipitated with 2 volumes of ethanol. The DNA was pelleted at 15,000 × *g* for 15 min at 4°C, washed once with 70% ethanol, dried, and resuspended in 50 μl of H_2O.

Copy number quantitation. Cells were grown as described for determination of copy number control values, and appropriate dilutions were plated on duplicate YALT and YAULT plates to determine the percentage of the population bearing the plasmid in question. DNA from 10^8 cells isolated as described above was brought to a final volume of 450 μl by the addition of 400 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The DNA was denatured by boiling for 4 min followed by quick cooling on ice. DNA from 4×10^7 cells was then transferred to nitrocellulose with a Schleicher & Schuell Manifold II slot blotting device. Filters were hybridized under standard conditions with nick-translated pBR322 to detect plasmid sequences or with a 1.6-kb *Sal*I fragment from pR285 containing the *HIS4* gene. For quantitation, duplicate samples from two individual transformants with each plasmid were analyzed with the AMBIS radioanalytic imaging system version 3.02 (AMBIS Systems Inc., San Diego, Calif.). A detection time of 4 h was used. The results of the blot hybridized with the *HIS4* probe were used to correct for differences in loading. After this correction, the relative copy number of each sample was determined by dividing the counts per minute obtained for each sample from the blot hybridized with pBR322 by the

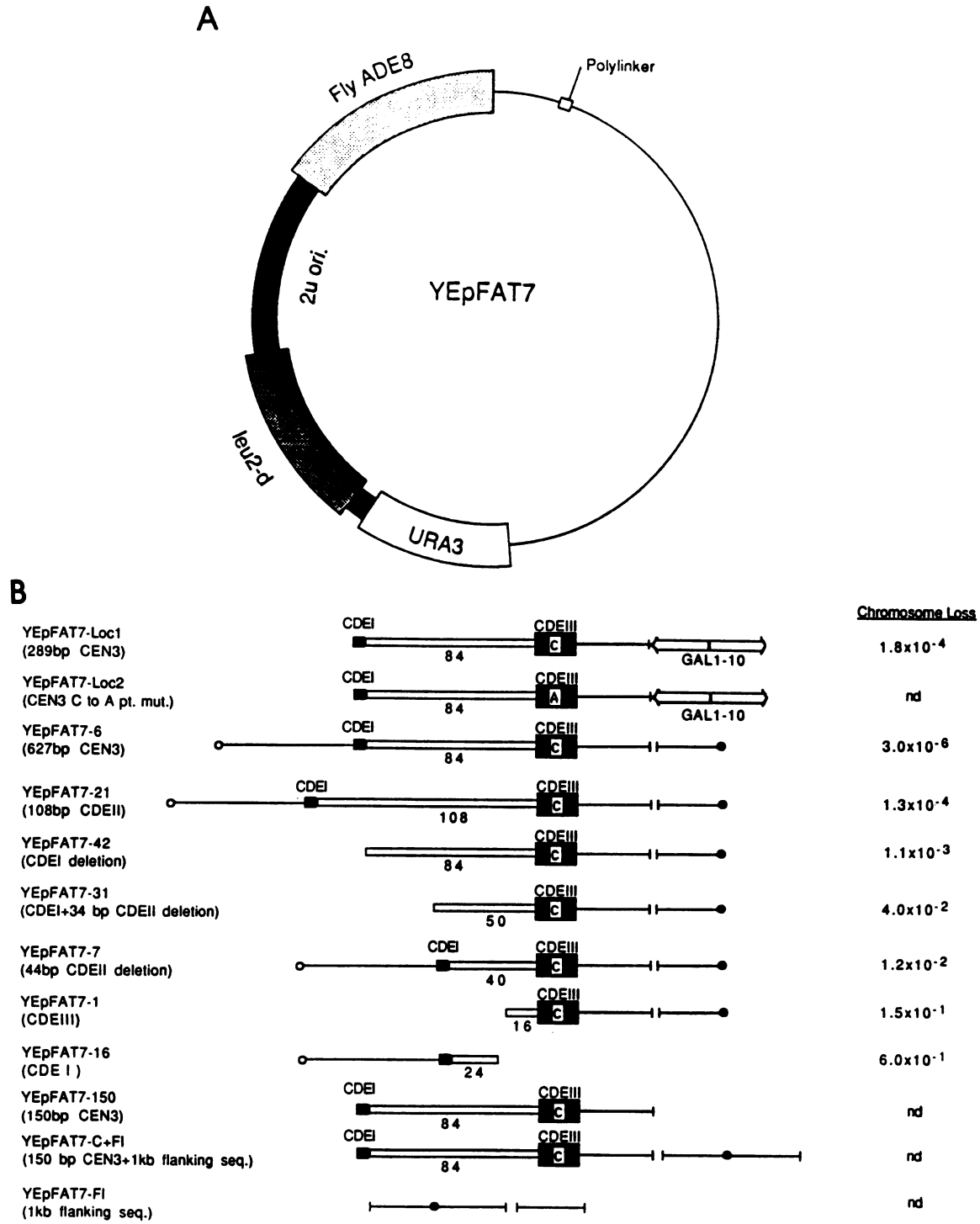

FIG. 1. Plasmid constructions. (A) Partial map of YEpFAT7. (B) YEpFAT7 derivatives. Centromere sequences were cloned into the *Sma*I or *Sph*I site of YEpFAT7 as described in Materials and Methods. The letter within CDE III represents the nucleotide at position 14, the central residue of the dyad symmetrical core. The number between CDE I and CDE III represents the length (in base pairs) of CDE II. Thin black lines, chromosomal DNA sequence flanking *CEN3*; open circle, chromosomal *Sau*3A site; closed circle, chromosomal *Bam*HI site. Chromosome loss rates were taken from Carbon and Clarke (12), Gaudet and Fitzgerald-Hayes (17), and Hill and Bloom (20). nd, not determined. The centromere containing the C-to-A point mutant does not function when placed in a chromosome (17, 19).

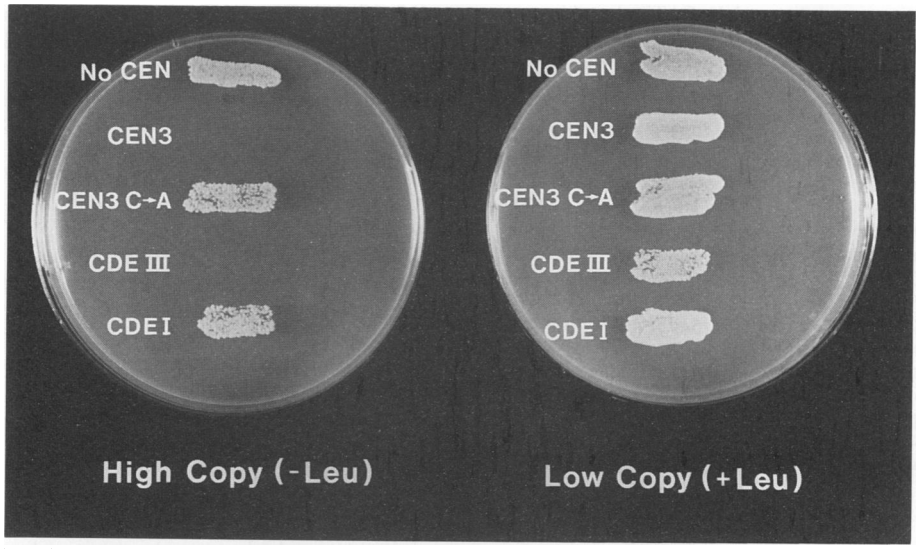

FIG. 2. Evidence that plasmids with wild-type CDE III sequences are subject to copy number control. Cells transformed with YEpFAT7 (no *CEN*), YEpFAT7-Loc1 (289-bp *CEN3*), YEpFAT7-Loc2 (*CEN3* C to A), YEpFAT7-1 (CDE III), or YEpFAT7-16 (CDE I) were grown overnight at 32°C in low-copy (+leucine) selective medium and then streaked on low-copy (+leucine) and high-copy (−leucine) selective plates. The plates were incubated for 3 days at 32°C and photographed.

percentage of the population carrying that plasmid. The lowest value was arbitrarily set at 1, and all other samples are reported relative to this value.

Colony sectoring assay. Single colonies of 5MO-1 transformed with the appropriate plasmid were inoculated into 5 ml of YALHKT low-copy medium (0.66% yeast nitrogen base, adenine [20 μg/ml], leucine [20 μg/ml], histidine [30 μg/ml], lysine [20 μg/ml], tryptophan [50 μg/ml], 2% glucose) and grown as described above. Appropriate dilutions were then plated on YALHKT low-copy plates (0.66% yeast nitrogen base, adenine [5 μg/ml], leucine [20 μg/ml], histidine [30 μg/ml], lysine [20 μg/ml], tryptophan [50 μg/ml], 2% glucose) and YAHKT high-copy plates (0.66% yeast nitrogen base, adenine [5 μg/ml], histidine [30 μg/ml], lysine [20 μg/ml], tryptophan [50 μg/ml], 2% glucose). These plates contain a reduced amount of adenine to allow detection of white, pink, and red colonies (24).

Quantitation of chromosome loss. Single colonies of 5MO-1 transformed with the appropriate plasmid were grown and plated as described above. After 2 days of growth at 32°C on low-copy plates or 3 days of growth at 32°C on high-copy plates, 10 single colonies were picked and the chromosome loss rate was determined as described by Hegemann et al. (19) and Shero et al. (35). Colonies were picked before sectoring was apparent (usually 4 to 5 days at 32°C) to ensure that a random sample was analyzed.

RESULTS

In eukaryotes, chromosomal copy number is controlled by several independent mechanisms. One mechanism ensures that each chromosome is replicated only once in a single cell cycle (for a review, see reference 18). A second mechanism, mediated by the centromere, guarantees the proper segregation of sister chromosomes to each daughter cell. The small and well-defined centromere of the budding yeast *S. cerevisiae* provides an ideal system for study of the contribution of centromere function to copy number control.

To determine the centromere DNA sequence requirements for centromere-mediated plasmid copy number control, the plasmids described in Fig. 1 were transformed into the yeast strain KR36-60 (31). The parental plasmid YEpFAT7 (31) contains a promoter-deficient allele of *LEU2*, *leu2d*, which allows complementation of leucine auxotrophy only when present in 50 to 100 copies per cell (15, 31). The 2 μm circle origin of replication and the *URA3* gene (complementing uracil auxotrophy in single copy) are also present on the plasmid for propagation in yeast cells. YEpFAT7-Loc1 and YEpFAT7-Loc2 differ from YEpFAT7 by the introduction of a copy of *CEN3* adjacent to the *GAL1-10* promoter (see Materials and Methods). Induction of transcription from this promoter by growth in galactose inactivates the ability of the centromere to mediate proper segregation (20, 21). YEpFAT7-Loc2 differs from YEpFAT7-Loc1 by the introduction of a single-base-pair mutation in CDE III (change of the conserved central C residue to an A). Centromeres with this mutation exhibit a greater than 10^4-fold increase in chromosome loss (17) and do not assemble a nuclease-resistant complex as determined by indirect end labeling (32). The other plasmids used in this study (YEpFAT7-1, -6, -7, -16, -21, -31, -42, -150, -C+Fl, and -Fl) were constructed by inserting the centromere sequences shown in Fig. 1 into the unique *Sph*I site of YEpFAT7 (see Materials and Methods). These centromere sequences exhibit a wide range of centromere function (12, 17, 20) (Fig. 1). Importantly, neither YEpFAT7-1 (CDE III and 16 bp of CDE II) nor YEpFAT7-16 (CDE I and 24 bp of CDE II) are competent for chromosome segregation (greater than 10^4-fold increase in chromosome loss [12, 17, 19]).

After transformation with *URA3* as the selectable marker (low-copy selection; −uracil, +leucine), positive transformants were grown in low-copy selective liquid medium (−uracil, +leucine) and then streaked on both low- and high-copy (−uracil, −leucine) selective plates. Figure 2 shows the analysis of five different centromere sequences with this assay. While all five plasmids confer growth on low-copy plates, plasmids with a functional centromere (YEpFAT7-Loc1) or CDE III alone (YEpFAT7-1) do not confer growth on high-copy selective plates (Fig. 2). The

TABLE 1. Mitotic stability of YEpFAT7 plasmids

Plasmid	*CEN*	Plasmid loss/generation[a]	
		Glucose	Galactose
YEpFAT7	None	2.0×10^{-2}	3.0×10^{-2}
YEpFAT7-Loc2	Conditional 289-bp C to A	2.0×10^{-2}	3.0×10^{-2}
YEpFAT-16	CDE I	1.5×10^{-2}	ND
YEpFAT7-1	CDE III	2.5×10^{-2}	ND
YEpFAT7-Loc1	Conditional 289-bp *CEN3*	1.0×10^{-3}	2.5×10^{-2}
YEpFAT7-6	627-bp *CEN3*	2.5×10^{-3}	ND
YEpFAT7-21	108-bp CDE II	5.5×10^{-3}	ND
YEpFAT7-42	CDE I deletion	1.5×10^{-2}	ND
YEpFAT7-7	44-bp CDE II deletion	2.0×10^{-2}	ND
YEpFAT7-31	CDE I + 34-bp CDE II deletion	3.0×10^{-2}	ND

[a] Two independent transformants were assayed for each plasmid. ND, not determined.

inability of YEpFAT7-Loc1 and YEpFAT7-1 to confer high-copy growth suggests that the copy number of plasmids with functional centromeres or with CDE III alone is maintained below the level required for high-copy growth. When a similar medium shift experiment using YEpFAT7-Loc1 is carried out in liquid medium, the population undergoes approximately two to three doublings before arresting as mostly unbudded cells (data not shown). The inability to form colonies on high-copy selective plates most likely results from starvation for leucine. This inability to confer high-copy growth is exhibited by every centromere sequence with a wild-type copy of CDE III (see Table 2).

The finding that the plasmid with only CDE III (YEpFAT7-1) fails to confer high-copy growth suggests that centromere copy control does not require proper segregation. CDE III, like the C-to-A point mutant (YEpFAT7-Loc2) and CDE I (YEpFAT7-16), will not function when used to replace an endogenous centromere in a chromosome and segregates as an *ARS* plasmid in plasmid stability assays (12, 17, 19) (Fig. 1). To determine whether CDE III mediates segregation when inserted into plasmid YEpFAT7, the mitotic stabilities of the YEpFAT7 derivatives were determined (see Materials and Methods). The results of this analysis, summarized in Table 1, indicate that the mitotic stability of YEpFAT7-1 (CDE III) does not significantly differ from that of YEpFAT7 (no *CEN*), YEpFAT7-Loc2 (C-to-A point mutant), and YEpFAT7-16 (CDE I). Plasmids with functional centromeres (YEpFAT7-Loc1 and YEpFAT7-6), however, are approximately 10-fold more stable than plasmids with nonfunctional centromeres. This result suggests that CDE III by itself has no segregation function when inserted into YEpFAT7. Although CDE III by itself does not appear to mediate proper segregation, it is assembled into a typical nuclease-resistant centromere complex (data not shown). A 34-bp sequence consisting of 3 bp to the left of CDE III, CDE III, and 6 bp to the right of CDE III will also compete for the binding of CBF3 in vitro (29). The results of the plating assay, mitotic stability assays, and nuclease mapping experiments together indicate that the ability to control plasmid copy number correlates with the ability to assemble a DNA-protein complex on CDE III but not with the ability of a centromere sequence to mediate proper segregation.

Hill and Bloom (20) have shown that transcription from the *GAL1-10* promoter inhibits the ability of an adjacent centromere to mediate proper chromosome segregation. Nevertheless, the inactivated centromere is still assembled into a typical nuclease-resistant complex. Transformants with YEpFAT7 (no *CEN*), YEpFAT7-Loc1 (wild-type *CEN*), and YEpFAT7-Loc2 (CDE III point mutant) were grown in low-copy selective medium (−uracil, +leucine) with galactose as the sole carbon source to induce transcription from the adjacent *GAL1-10* promoter. Under these conditions, the conditional centromere no longer confers increased mitotic stability to YEpFAT7-Loc1 (Table 1). Cells were then streaked on both low- and high-copy (−uracil, −leucine) selective galactose plates. The failure of a conditionally inactivated centromere (YEpFAT7-Loc1) to confer high-copy growth in galactose (Fig. 3) supports the

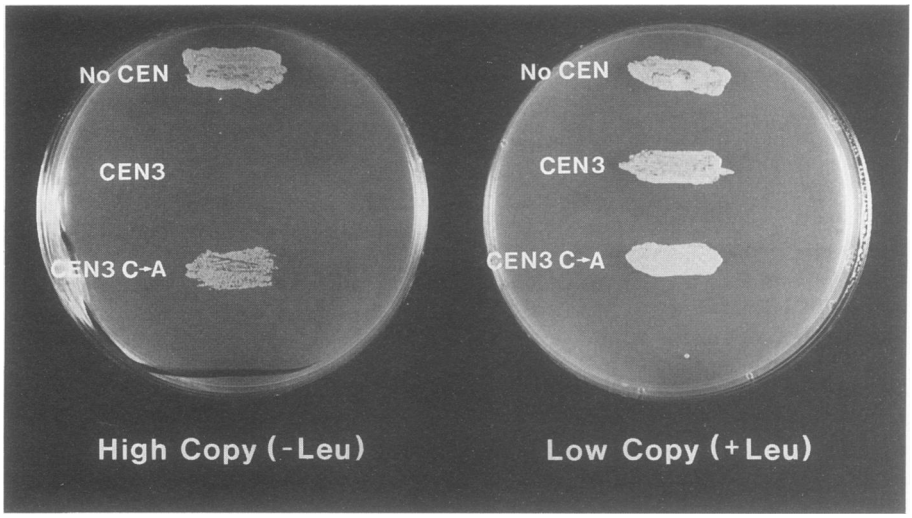

FIG. 3. Evidence that inactive conditional centromeres are subject to copy number control. Cells transformed with YEpFAT7 (no *CEN*), YEpFAT7-Loc1 (289-bp *CEN3*), or YEpFAT7-Loc2 (*CEN3* C to A) were grown overnight at 32°C in low-copy (+leucine) selective galactose medium and then streaked on low-copy (+leucine) and high-copy (−leucine) selective galactose plates. The plates were incubated for 3 days at 32°C and photographed.

TABLE 2. Copy number control values

Plasmid	*CEN*	Copy no. control value[a]			
		Glucose	n^b	Galactose	n
YEpFAT7-Loc1	Conditional 289-bp *CEN3*	1.8×10^{-6} (± 1.4)[c]	5	1.1×10^{-2} (± 0.2)	4
YEpFAT7-Loc2	Conditional 289-bp C to A	3.5×10^{-1} (± 5.0)	7	3.1×10^{-1} (± 0.3)	4
YEPFAT7	None	7.7×10^{-1} (± 1.6)	7	9.2×10^{-1} (± 0.6)	3
YEpFAT7-150	150-bp *CEN3*	1.7×10^{-7} (± 1.7)	3	ND[d]	
YEpFAT7-6	627-bp *CEN3*	2.4×10^{-7} (± 0.7)	3	ND	
YEpFAT7-C+Fl	*CEN3* + 1-kb flanking sequence	6.5×10^{-8} (± 3.3)	3	ND	
YEpFAT7-21	108-bp CDE II	2.1×10^{-6} (± 1.3)	2	ND	
YEpFAT7-42	CDE I deletion	3.7×10^{-4} (± 3.0)	3	ND	
YEpFAT7-7	48-bp CDE II deletion	2.9×10^{-3} (± 0.7)	2	ND	
YEpFAT7-31	CDE I + 38-bp CDE II deletion	1.8×10^{-2} (± 0.7)	3	ND	
YEpFAT7-1	CDE III	6.0×10^{-3} (± 5.0)	6	ND	
YEpFAT7-16	CDE I	7.5×10^{-1} (± 0.9)	3	ND	
YEpFAT7-Fl	1-kb flanking sequence	6.4×10^{-1} (± 5.0)	3	ND	

[a] Expressed as colonies on −leucine/colonies on +leucine.
[b] *n*, number of independent transformants assayed.
[c] Standard deviations (given in parentheses) are the same order of magnitude as the reported values.
[d] ND, not determined.

conclusion that the ability of a sequence to bind centromere proteins, but not proper interaction with the segregation machinery, is required for copy number control of centromeric plasmids.

The results of Fig. 2 and 3 allow a qualitative assessment of the ability of specific centromere sequences to control plasmid copy number. To determine whether different centromere sequences could be distinguished in a quantitative assay, transformants with each plasmid were grown in liquid medium and then appropriate dilutions were plated for single colonies on low- and high-copy selective plates (see Materials and Methods). After growth, a copy number control value (high copy [−leucine]/low copy [+leucine]) was determined. This number represents the fraction of the plasmid-bearing population that can maintain the plasmid at a level sufficient to confer growth. As shown in Table 2, there is a 200,000-fold difference between the values observed for a wild-type functional centromere (YEpFAT7-Loc1 on glucose) compared with a mutant nonfunctional centromere (YEpFAT7-Loc2). This difference in copy number control value is similar to the difference observed between these sequences in chromosome segregation assays (17, 19, 22). There is also a 30- to 100-fold difference between the values observed for sequences that can assemble centromere complexes (YEpFAT7-Loc1 on galactose and YEpFAT7-1) and those that cannot (YEpFAT7-Loc2, YEpFAT7-16, YEpFAT7-Fl, and YEpFAT7). In a variety of segregation assays (endogenous chromosomes, nonessential chromosome fragments, and plasmids), these sequences cannot be distinguished, and all have been considered to be segregationally defective (12, 17, 19, 22) (Fig. 1 and Table 1). The copy number control assay, however, provides a measure of centromere function for those sequences unable to direct chromosome movement. The difference in copy number control values between functional centromeres (YEpFAT7-Loc1 on glucose) and assembled but segregationally impaired centromeres (YEpFAT7-Loc1 on galactose; and YEpFAT7-1) likely reflects the additional contribution of proper segregation to copy number control. This conclusion is supported by the finding that centromere sequences with chromosome loss rates between those of functional centromeres (10^{-6}) and CDE III (10^{-1}) have copy number control values that fall between the values reported for these two sequences (Table 2).

The conditional wild-type centromere (YEpFAT7-Loc1) exhibits a copy number control value that is 10- to 30-fold higher than other wild-type centromeres (YEpFAT7-C+F1, YEpFAT7-150, and YEpFAT7-6). The increased value observed for YEpFAT7-Loc1 more closely resembles the value for a centromere with a 108-bp CDE II (YEpFAT7-21). These two centromere sequences also have similar chromosome loss rates (Fig. 1). The increase in copy number control value observed for YEpFAT7-Loc1 may result from a low level of transcription from the *GAL1-10* promoter in glucose or other differences in the sequence context surrounding the centromeres. The finding that a 1.6-kb centromere (YEpFAT7-C+Fl) exhibits a copy number control value less than that of the 150- or 627-bp centromere (YEpFAT7-150 or -6) indicates that the length of chromosomal sequence flanking the centromere may contribute in part the difference in values.

To determine the relationship between the copy number control values of Table 2 and plasmid copy number, transformants with YEpFAT7, YEpFAT7-Loc1, YEpFAT7-Loc2, YEpFAT7-1, and YEpFAT7-16 were grown in low-copy selective medium (−uracil, +leucine). Total genomic DNA was isolated, and the relative plasmid copy number was determined by slot blot analysis as described in Materials and Methods. Figures 4 and 5 show that the relative copy numbers of the three copy-controlled sequences (YEpFAT7-Loc1 on glucose and galactose; YEpFAT7-1) are approximately equal and three- to sixfold less than that of plasmids not subject to copy number control (YEpFAT7, YEpFAT7-Loc2, and YEpFAT7-16). This analysis supports the results shown in Table 2, indicating that only a small percentage of the population bearing YEpFAT7-Loc1 or YEpFAT7-1 can maintain a plasmid copy number sufficient to confer high-copy growth. All other plasmids with a wild-type copy of CDE III are maintained at a copy number similar to that observed for YEpFAT7-Loc1 and YEpFAT7-1 (data not shown).

Interestingly, while the relative copy numbers of YEpFAT7 (no *CEN*) and YEpFAT7-16 (CDE I) are approximately equal when cells are grown in low-copy selective media, the copy number of YEpFAT7-Loc2 (CDE III point mutant) is slightly lower (approximately 2.0-fold; Fig. 5A). This difference in relative copy number increases to three- to fourfold when cells are grown in high-copy selective medium

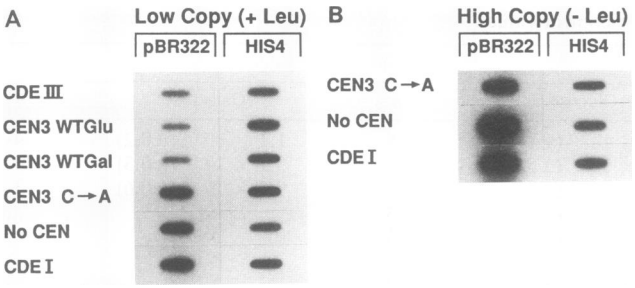

FIG. 4. Copy number determination of centromere plasmids. Cells transformed with YEpFAT7 (no *CEN*), YEpFAT7-Loc1 (289-bp *CEN3*), YEpFAT7-Loc2 (*CEN3* C to A), YEpFAT7-1 (CDE III), or YEpFAT7-16 (CDE I) were grown in low- or high-copy selective medium as described in Materials and Methods. DNA was isolated and used for slot blot analysis (see Materials and Methods). Duplicate blots were probed with nick-translated pBR322 to visualize plasmid sequences and a probe to the *HIS4* locus to correct for the amount of DNA loaded in each slot. The pBR322 blot was exposed to X-ray film for 2 h. The *HIS4* blot was exposed to X-ray film for 5 h.

(−uracil, −leucine; Fig. 4B and 5B). This result most likely accounts for the approximately twofold-lower copy number control value determined for YEpFAT7-Loc2 by plating (Table 2). The ability to detect copy number regulation of the C-to-A point mutant when its copy number is elevated suggests that this sequence has a measurable albeit low ability to mediate copy number control.

As described above, approximately 1% of a population of cells carrying YEpFAT7-1 (CDE III) are competent to form colonies when challenged with high-copy selection (Table 2). The colonies that arise on high-copy selective plates, however, grow significantly more slowly than colonies with YEpFAT7 or YEpFAT7-Loc2 and exhibit a marked heterogeneity in size (Fig. 6). This observation suggests that although a significant percentage of the population can escape selection against excess copies of CDE III, negative effects on cell growth can still be observed. Futcher and Carbon (16) and Runge et al. (30) have shown that cells forced to maintain multiple centromere plasmids exhibit increased rates of chromosome loss. Therefore, chromosome loss may be one component that contributes to the growth defects described above.

To quantitate the effect of increased copies of CDE III on chromosome loss, the diploid strain 5MO-1 (Materials and Methods) was transformed with YEpFAT7 (no *CEN*), YEpFAT7-1 (CDE III), and YEpFAT7-Loc1 (conditional centromere). 5MO-1 contains a nonessential YAC carrying the *S. cerevisiae SUP11* gene. In an *ade2-101* homozygous host cell, the *SUP11* gene allows the copy number of the YAC to be monitored by colony color. Colonies with one copy are pink, colonies with two copies are white, and those without the YAC are red. Thus, a chromosome loss event can be detected by the presence of a sectored colony; for instance, a red sector in a pink colony indicates a loss event (24, 35).

Pink transformants of 5MO-1 with either YEpFAT7, YEpFAT7-1, or YEpFAT7-Loc1 were grown in low-copy selective medium, and appropriate dilutions were plated for single colonies on low- and high-copy selective plates. After growth, the percentage of red, white, and sectored colonies was determined. As shown in Table 3 and Fig. 6, there is a dramatic increase in the percentage of sectored colonies when the high-copy competent colonies with YEpFAT7-1 are compared with the high-copy competent colonies with

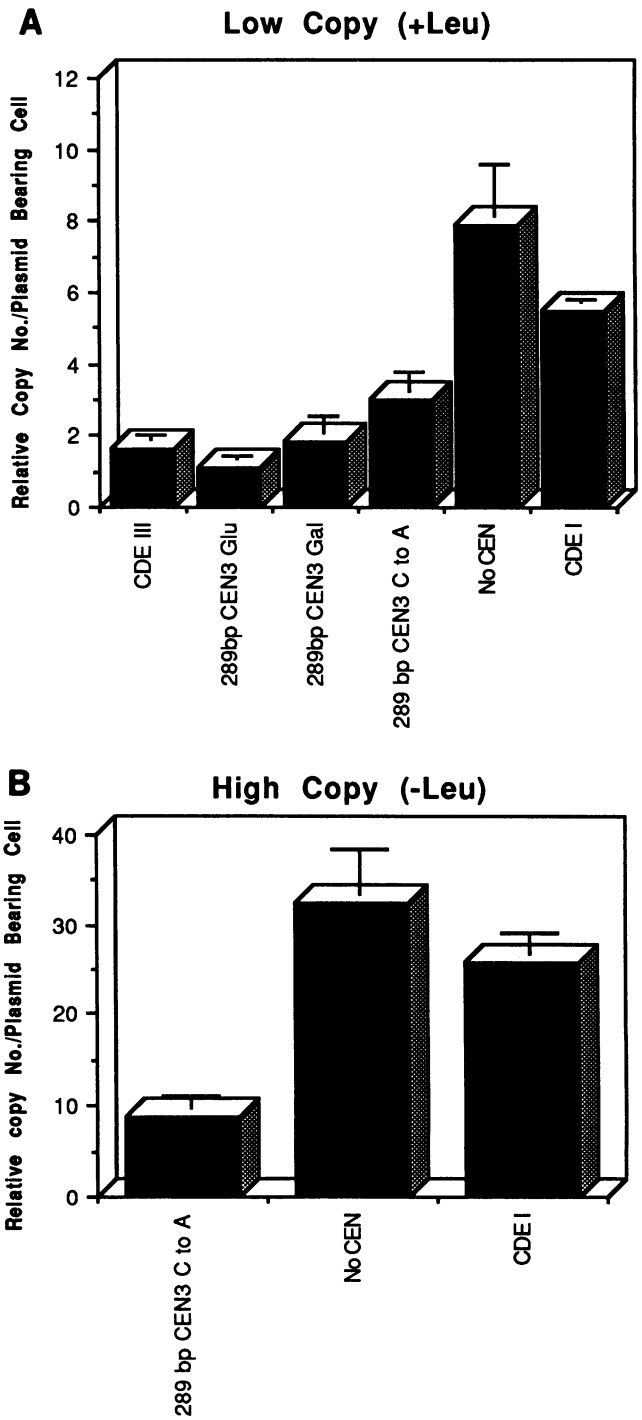

FIG. 5. Copy number quantitation. DNA was isolated from two independent transformants bearing YEpFAT7 (no *CEN*), YEpFAT7-Loc1 (289-bp *CEN3*), YEpFAT7-Loc2 (*CEN3* C to A), YEpFAT7-1 (CDE III), or YEpFAT7-16 (CDE I). The relative plasmid copy number per plasmid-bearing cell was determined for cells grown in low-copy (+leucine) or high-copy (−leucine) selective medium as described in Materials and Methods.

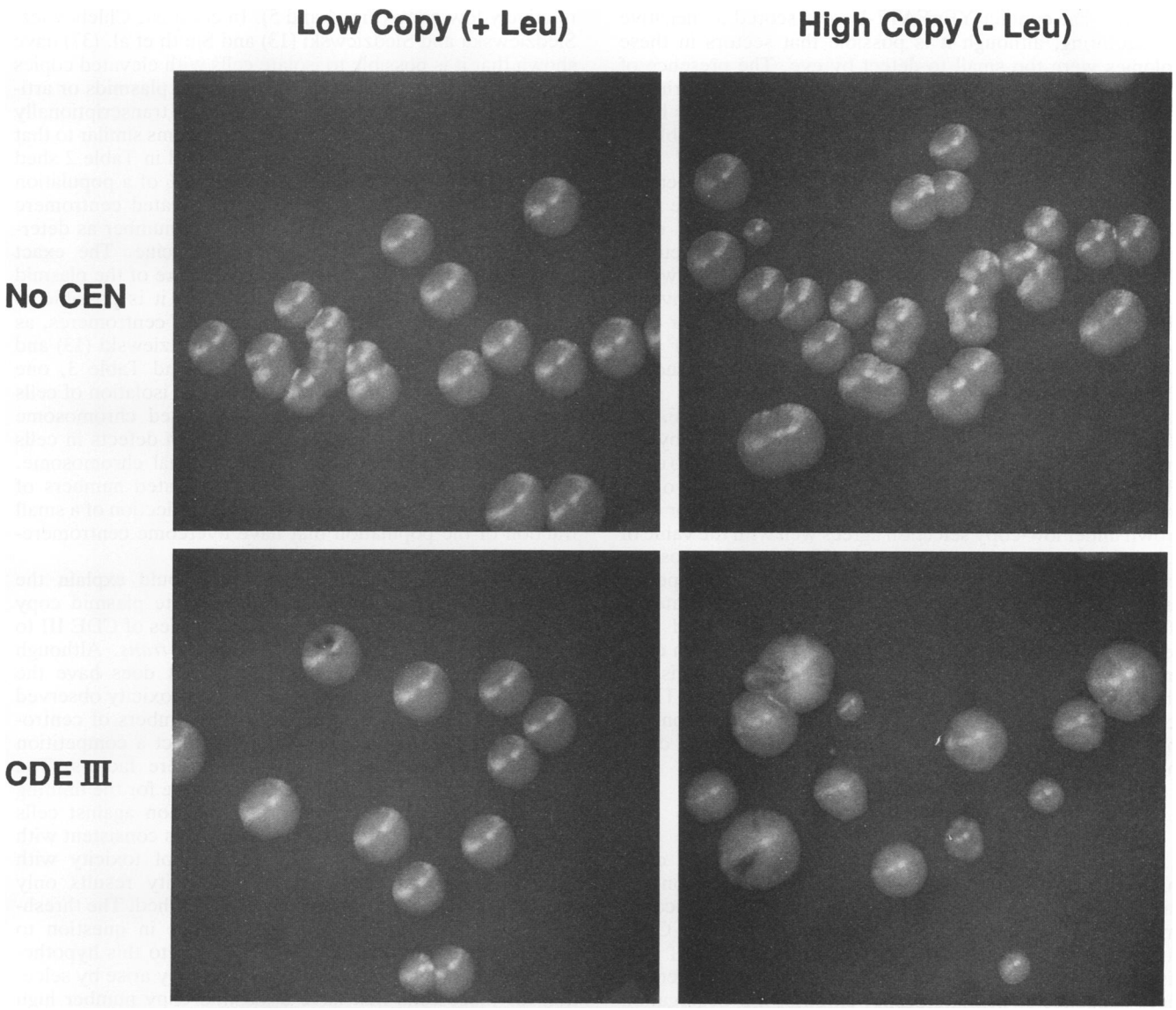

FIG. 6. Evidence that excess copies of CDE III result in increased chromosome loss. Transformants of the diploid strain 5MO-1 bearing either YEpFAT7 (no *CEN*), YEpFAT7-1 (CDE III), or YEpFAT7-Loc1 (289-bp *CEN3*) were grown in low-copy (+leucine) selective medium as described in Materials and Methods. Appropriate dilutions were then plated on low-copy (+leucine) and high-copy (−leucine) selective plates and incubated at 32°C for 6 days. Colonies were photographed with a Nikon SMZ-10 dissecting microscope. Magnification, ×2.5.

YEpFAT7. In over 2,500 colonies with YEpFAT7, no sectored colonies were observed. In contrast, 13% of the colonies with YEpFAT7-1 were sectored. Most of these colonies contained more than one sector, indicating that multiple loss events had occurred. Thus, selection for increased copies of CDE III results in increased rates of chromosome loss. Also, it should be pointed out that the value of 13% is most likely an underestimate. Many small

TABLE 3. Stimulation of loss of a nonessential chromosome by high-copy selection for CDE III (YEpFAT7-1)

Plasmid	*CEN*	Low copy (+Leu)[a]				High copy (−Leu)			
		% Red	% White	% Sectored	Chromosome loss rate	% Red	% White	% Sectored	Chromosome loss rate
YEpFAT7	None	0.2	0.05	0.06	4.9×10^{-4}	0.2	0.1	≤0.04	4.7×10^{-4}
YEpFAT7-1	CDE III	0.7	0.5	0.6	4.2×10^{-4}	1.0	0.2	13	1.9×10^{-2}
YEpFAT7-Loc1	289-bp *CEN3*	0.2	0.05	0.07	3.3×10^{-4}	ND	ND	ND	ND

[a] Percentages of red, white, and sectored colonies were determined by pooling the data from four independent transformants. At least 2,500 colonies were scored for each pool. Chromosome loss rate of the nonessential chromosome was determined as described in Materials and Methods. ND, not determined (too few colonies arise with YEpFAT7-Loc1 when challenged with high-copy selection [see Table 2] to allow reporting of accurate values).

colonies that contain YEpFAT7-1 were scored as negative for sectoring, although it is possible that sectors in these colonies were too small to detect by eye. The presence of YEpFAT7-1 also produces a slight increase in sectoring in the low-copy-selected population compared with cells bearing a plasmid without a centromere sequence (Table 3; compare YEpFAT7-1 [low copy] with YEpFAT7 [low copy]). The increase in sectoring observed in cells bearing YEpFAT7-1 under low-copy selection may reflect the fraction of the population with an increased plasmid copy number as determined by growth in the absence of leucine (6.0×10^{-3}; Table 2). This fraction of the population would be too small to produce a significant increase in the overall chromosome loss rate and would not be expected for cells bearing a functional centromere (fewer than 1 in 5×10^5 cells bearing YEpFAT7-Loc1 grow in the absence of leucine; Tables 2 and 3).

The chromosome loss rates of cells bearing YEpFAT7, YEpFAT7-1, and YEpFAT7-Loc1 were quantitated by the procedure described by Hegemann et al. (19) and Shero et al. (35). The value of approximately 4.1×10^{-4} (average of the three low-copy values reported in Table 3) obtained for cells grown under low-copy selection agrees well with the value of 4.8×10^{-4} reported by Jehn et al. (22) for a chromosome fragment constructed with *CEN6*. High-copy competent cells bearing YEpFAT7-1 (CDE III) exhibit approximately a 50-fold increase in chromosome loss rate compared with cells grown under low-copy selection (1.9×10^{-2} high copy and 4.2×10^{-2} low copy; Table 3). This increase is not observed for cells bearing YEpFAT7 (no *CEN*). These results thus provide critical evidence that selection for increased levels of CDE III results in an increase in chromosome loss.

DISCUSSION

This report has described a centromere plasmid copy number assay that measures centromere function on the basis of the ability of specific centromere DNA sequences to impart copy number control on an acentric plasmid. Copy number control has been determined to consist of two distinct but interrelated components. The ability of a centromere sequence to mediate proper chromosome segregation at each mitosis ensures that the plasmid copy number is maintained at a constant level. The finding that the number of segregationally impaired centromere sequences is maintained at a relatively low level, however, indicates that CDE III by itself fulfills the minimum requirement for centromere-mediated copy number control.

The results presented in this study extend the earlier work of Apostol and Greer (1). These authors demonstrated that transcriptionally inactivated centromeres can interfere with 2 μm circle-mediated plasmid amplification and concluded that transcriptionally inactivated centromeres retain partial function. The finding that CDE III by itself is competent to mediate copy number control suggests that the partial function retained by transcriptionally inactivated centromeres is due to CDE III.

The results of this study resolve several paradoxical studies on centromere plasmid copy number control. Futcher and Carbon (16) and Runge et al. (30) have shown that cells forced to maintain increased numbers of centromere plasmids exhibit low viability, a mitotic pause, and an increase in chromosome loss. Previous work also has shown that 2 μm-based plasmids with functional (1, 39) or transcriptionally inactivated (1) centromeres are maintained at a relatively low copy (Fig. 4 and 5). In contrast, Chlebowicz-Śledziewska and Śledziewski (13) and Smith et al. (37) have shown that it is possible to isolate cells with elevated copies of either composite centromere-2μm circle plasmids or artificial chromosomes through the use of transcriptionally inactivated centromeres and selection systems similar to that described in this work. The data presented in Table 2 shed light on this disparity. Approximately 1.0% of a population of cells bearing a transcriptionally inactivated centromere can achieve an elevated centromere copy number as determined by growth in the absence of leucine. The exact percentage could be influenced by the nature of the plasmid construct and the strain used. Therefore, it is possible to select for cells with an elevated number of centromeres, as done by Chlebowicz-Śledziewska and Śledziewski (13) and Smith et al. (37). As shown in Fig. 6 and Table 3, one consequence of this type of selection is the isolation of cells exhibiting slow growth rates and increased chromosome loss. Smith et al. (37) also reported growth defects in cells selected for increased copies of an artificial chromosome. Thus, the identification of cells with elevated numbers of centromeres in earlier studies reflects the selection of a small fraction of the population that have overcome centromere-mediated copy number control.

There are several mechanisms that could explain the ability of centromere sequences to regulate plasmid copy number as well as the ability of excess copies of CDE III to interfere with chromosome segregation in *trans*. Although CDE III does not mediate segregation, it does have the capacity to bind centromere proteins. The toxicity observed in cells forced to maintained increased numbers of centromere plasmids has been suggested to reflect a competition among centromeres for limiting centromere factors. The ability of a centromere sequence to compete for the limiting factor(s) would provide a negative selection against cells with excess centromeres. This hypothesis is consistent with the conclusion that there is a gradient of toxicity with increasing plasmid copy number. Lethality results only when a certain threshold level has been reached. The threshold is set by the ability of the sequence in question to compete for the limiting factor. According to this hypothesis, the high-copy competent population may arise by selection for those cells that have a plasmid copy number high enough to satisfy the growth requirement but below the level resulting in lethality. The finding that sequences which can assemble a DNA-protein complex (kinetochore) but cannot mediate proper segregation (inactivated conditional centromere and CDE III) can compete at detectable levels would suggest that at least one of these limiting factors is required for kinetochore assembly. Furthermore, the results of this study suggest that it is a factor(s) involved directly or indirectly in the assembly of a DNA-protein complex on CDE III, the most essential DNA element.

A second mechanism for centromere-mediated copy number control is suggested by the finding that partially functional centromeres appear to activate a cell cycle checkpoint response (38). Excess nonfunctional centromeres or unassembled centromeres may activate a cell cycle check point response that blocks cell growth (18). The cells competent for high-copy growth would have to escape such a mitotic checkpoint. These cells would undergo mitotic divisions that bypassed the normal mechanisms ensuring the fidelity of chromosome segregation. Work on kinetochore assembly in higher eukaryotic cells (4, 5, 36) also supports a role for a checkpoint response in centromere-mediated copy number control. Microinjection of antikinetochore antisera into

higher eukaryotic cells in late G_2 results in a block in cell cycle progression at metaphase. This observation has led Bernat et al. (5) to speculate the metaphase block produced by late-G_2 microinjection of antikinetochore antisera is due to the failure of structurally perturbed kinetochores to signal the metaphase-to-anaphase transition.

The finding that functional and transcriptionally inactivated centromeres interfere with the 2 μm plasmid amplification system (1, 39) suggests the possibility that plasmid copy number control can be mediated by the ability of centromere sequences to interfere with the mechanism of copy number amplification. The results in Table 1, however, indicate that CDE III by itself does not strongly influence the mitotic stability of the plasmid used in this study. More importantly, Bitoun and Zamir (6) have shown that it is possible to select for cells with elevated copies of *CEN-ARS* plasmids by using a weak allele of *HIS3*, similar to the *leu2d* allele used in this study. Selection of cells with elevated copies of *CEN-ARS* plasmids by using *leu2d* has also been achieved (data not shown). Therefore similar copy number-based selection systems can be functional in the absence of the 2 μm circle amplification system.

The mechanisms for centromere-mediated plasmid copy number control discussed above are not mutually exclusive, and it may be combinations of these or others that together produce the reported effects. Regardless of the mechanism, the results of this study indicate that plasmid copy number control can be mediated by centromere sequences that do not mediate proper segregation. Thus, with use of this system, it is possible to identify a centromere function that is independent of the ability of the sequence under investigation to mediate chromosome segregation. The property shared among all centromere sequences competent to mediate copy number control is the ability to at least partially assemble a DNA-protein complex on CDE III. In this respect, segregationally impaired centromeres resemble inactive centromeres present on stable dicentric chromosomes in higher eukaryotic cells. Many of these inactive centromeres bind a subset of kinetochore proteins (for a brief review, see reference 33). The ability of assembled but nonfunctional kinetochores to affect cell growth and chromosome segregation in *trans* should allow a characterization of the process of kinetochore assembly by allowing the identification of factors that enhance or decrease the toxicity of exogenous centromere sequences.

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