

Single Base-Pair Mutations in Centromere Element III Cause Aberrant Chromosome Segregation in *Saccharomyces cerevisiae*

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In this paper we show that a 211-base pair segment of *CEN3* DNA is sufficient to confer wild-type centromere function in the yeast *Saccharomyces cerevisiae*. We used site-directed mutagenesis of the 211-base pair fragment to examine the sequence-specific functional requirements of a conserved 11-base pair segment of centromere DNA, element III (5'-TG[†]TT[†]CCGAA-3'). Element III is the most highly conserved of the centromeric DNA sequences, differing by only a single adenine · thymine base pair among the four centromere DNAs sequenced thus far. All of the element III sequences contain specific cytosine-guanine base pairs, including a 5'-CCG-3' arrangement, which we targeted for single cytosine-to-thymine mutations by using sodium bisulfite. The effects of element III mutations on plasmid and chromosome segregation were determined by mitotic stability assays. Conversion of CCG to CTG completely abolished centromere function both in plasmids and in chromosome III, whereas conversion of CCG to TCG decreased plasmid and chromosome stability moderately. The other two guanine · cytosine base pairs in element III could be independently converted to adenine · thymine base pairs without affecting plasmid or chromosome stability. We concluded that while some specific nucleotides within the conserved element III sequence are essential for proper centromere function, other conserved nucleotides can be changed.

Saccharomyces cerevisiae is the only organism from which centromere DNA has been identified and sequenced. The centromere is a specialized segment of each chromosome which functions at each cell division to ensure proper chromosome segregation. Centromere DNA has been isolated and mapped to six different yeast chromosomes, *CEN3* (8), *CEN11* (12), *CEN6* (27), *CEN4* (33), *CEN5* (21), and *CEN14* (M. Neitz and J. Carbon, unpublished data). Four of these *CEN* DNAs have been sequenced and have been shown to contain three common features of sequence organization (5, 12, 27). All contain an extremely adenine · thymine-rich region which is 82 to 89 base pairs (bp) long (element II) and is flanked on either side by short regions of partial homology, elements I and III. As Fig. 1 shows, the element III sequence is identical in *CEN3* and *CEN11* and differs by only one adenine · thymine base pair in *CEN4* and *CEN6*.

At least three lines of evidence suggest that these sequence elements are involved in centromere function. Centromere plasmids containing the element I through III region are stably maintained in yeasts, and complete deletion of these sequences results in dramatic plasmid instability (3, 13). Plasmids containing two *CEN* DNAs (dicentric) are unstable in mitosis and suffer deletions involving the conserved *CEN* sequence elements, suggesting that there is a physical interaction between the conserved *CEN* DNA and the spindle apparatus (23). Recently, Clarke and Carbon (9) demonstrated that an acentric chromosome which was generated by complete deletion of sequence elements I, II, and III from chromosome III was extremely unstable. Therefore, it is now certain that elements I, II, and III code for a centromeric DNA.

In this study, we investigated the role of a specific centromere sequence, element III (5' TG[†]TT[†]CCGAA-3'),

in segregation of plasmids and chromosomes. In addition to being highly conserved, the element III sequence also contains the only cluster of cytosine and guanine residues within the surrounding adenine · thymine-rich centromere DNA. In a single-stranded form, the cytosine residues are amenable to alteration by treatment with sodium bisulfite. Therefore, we used this reagent to produce cytosine-to-thymine mutations in the element III sequence in *CEN3* DNA.

The segregation of plasmids and chromosomes bearing these centromere mutations (*CEN**) was assayed in the yeast *S. cerevisiae*. We found that a single-base pair mutation which changed CCG to CTG was sufficient to completely abolish the mitotic centromere function of both plasmids and chromosomes. Conversion of CCG to TCG affected plasmid stability slightly but increased chromosome nondisjunction 1,000-fold. Two other cytosine-to-thymine mutations which we studied did not significantly alter plasmid stability in yeast cells. We conclude that element III is essential for mitotic centromere function and that certain nucleotide positions within element III are critical.

MATERIALS AND METHODS

Strains, plasmids, media, and enzymes. *Escherichia coli* strains JM14 (SK1592; F⁻ gal T1^r endA *sbcb* *hsdR4* *hsdM*⁺) and JA221 (C600; *lacY* *leuB6* *trpE5* *hsdR* *hsdM*⁺ *recA1*) were used for transformation and amplification of plasmid DNAs; strain JM103 [Δ (*lac-pro*) *supE* *thi* *rpsL* *endA* *sbcb15* *hsdR4* *F'**traD36* *proA*⁺ *B*⁺ *lacI*^Q Δ *lacZM15*] was used as a host for M13 cloning and propagation of bisulfite-mutagenized DNA. *S. cerevisiae* strain SB9882-4CR (*MATa*/*MATa* *trp1-289/trp1-289* *ura3-52/ura3-52* *LEU2/leu2-3,112* *HIS4/his4-519*) (9) was used for genomic substitution experiments, and strain YP47 (*MATa* *ura3-52* *ade2-101* *lys2-801* *trp1* Δ) which was constructed by P. Hieter and R. W. Davis, was used for plasmid mitotic stability assays. The media used for bacterial and yeast growth have been described previously by Hsiao

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III

<u>CEN</u> 3	T G T A T T	T G A T T T C C G A A	A G T T A A A A
<u>CEN</u> 4	T G T T T A	T G A T T A C C G A A	A C A T A A A A
<u>CEN</u> 11	T G T T C A	T G A T T T C C G A A	C G T A T A A A
<u>CEN</u> 6	A G T T T T	T G T T T T C C G A A	G A T G T A A A

FIG. 1. DNA sequence comparison of the conserved element III region. The DNA sequences surrounding element III are shown for *CEN4*, *CEN3*, *CEN11*, and *CEN6*. The 11-bp element III core sequences are indicated by a box. Additional short segments of perfect homology among the four centromere DNAs are located on either side of element III.

and Carbon (18). Restriction endonucleases, T4 DNA ligase, and Klenow DNA polymerase I were obtained from New England BioLabs, Inc., or International Biotechnologies, Inc. The M13 universal primer and the *Bam*HI site primer were purchased from New England BioLabs, Inc.; dideoxynucleotide stocks were obtained from P-L Biochemicals, Inc. The buffers and reaction conditions used were those specified by the supplier. [α - 32 P]ATP (specific activity, 300 to 400 Ci/mmol) was purchased from Amersham Corp. The M13 cloning protocols, replicative-form (RF) DNA preparations, and dideoxynucleotide sequencing reaction procedures used have been described by Amersham and Sanger et al. (30). Most plasmid cloning procedures and DNA manipulations were performed by the methods of Maniatis et al. (22).

Plasmid *dl314* is a deletion derivative of pYe(*CEN3*)30 (12). pYe(*CEN3*)30 contains the 624-bp *Sau*3A *CEN3* fragment cloned into the *Bam*HI site of YRp7', regenerating a single *Bam*HI site at one yeast-pBR322 junction. Linear molecules generated by *Bam*HI digestion of pYe(*CEN3*)30 DNA were treated with BAL 31 exonuclease, religated in vitro, and amplified by transformation into *E. coli* (as described for *CEN11* deletion mutants by Fitzgerald-Hayes and Carbon [13]). Plasmid DNA isolates were characterized by restriction analysis and sequenced (24).

Yeast transformation. Plasmid DNAs that were uncut or were digested with *Eco*RI were introduced into yeast cells by using the lithium acetate transformation procedure (19) or the spheroplast method (17). For fragment-mediated genomic transplacements, 10 μ g of plasmid DNA was cut to completion with *Eco*RI and added to 2×10^8 competent yeast cells. Ura⁺ transformants were visible on the appropriate selective agar plates after a 48 to 72 h of incubation at 32°C.

Bisulfite mutagenesis. The mutagenesis procedure which we used (see Fig. 3) was a modification of the procedure described by Everett and Chambon (11), except that we used different annealing conditions (20) with 5 μ g of RF DNA and 2 μ g of single-stranded M13 DNA (mp8-314 or mp9-314). The precipitated heteroduplex DNA was treated with 3 M sodium bisulfite at 37°C for 15 min, and the bisulfite was removed by two successive Sephadex G-25 spun column steps (22) in place of the dialysis used by Kalderon et al. (20). An equal volume of 0.4 M Tris hydrochloride (pH 9.2)–0.1 M NaCl–4 mM disodium EDTA was added to the effluent, and the sample was incubated at 37°C overnight. Following ethanol precipitation in the presence of 0.4 M sodium acetate and 0.5 μ g of added carrier pBR322 DNA, the mutagenized DNA was suspended in 30 μ l of 10 mM Tris hydrochloride (pH 8.0)–1 mM disodium EDTA. Each repair reaction mixture contained 1 mM dATP, 1 mM dGTP, 1 mM dCTP, and

1 mM dTTP in 20 mM Tris hydrochloride (pH 7.5)–10 mM MgCl₂–10 mM dithiothreitol containing 5 U of Klenow DNA polymerase I and was incubated at room temperature for 30 min. The mutagenized, repaired DNA was used to transform *E. coli* JM103 cells, and single-stranded DNAs prepared from plaque-purified isolates were screened for mutations by using the dideoxynucleotide sequencing method (30). The universal M13 primer was used for sequencing mp8-314 templates, and the *Bam*HI site primer was used to sequence mutations on the opposite DNA strand in mp9-314 templates. This primer hybridized to the pBR322 DNA near the *Bam*HI site and permitted easy sequencing of the adjacent centromere insertion without reading through the pBR322 sequences. About 10% of the templates had mutations in the *CEN* DNA, but the frequency of multiple mutations depended on the number of cytosine residues in the DNA strand.

Plasmid and chromosome stability assays. For plating assays, Trp⁺ or Ura⁺ yeast transformants were grown from single colonies in 5 ml of YPD medium (2% peptone, 1% yeast extract, 2% glucose) supplemented with either 50 μ g of tryptophan per ml or 20 μ g of uracil per ml overnight at 32°C. Cell density was measured by determining *A*₆₆₀ and appropriate amounts were diluted in sterile water to yield 100 to 500 colonies when suitable portions were spread onto YPD agar plates. After overnight incubation at 32°C, the colonies were tested for either the Trp⁺ or Ura⁺ phenotype by replica plating onto yeast minimal plates (Difco yeast nitrogen base containing 2% glucose, 20 μ g of adenine per ml, and 0.5% Casamino Acids) with and without added tryptophan (or uracil). The numbers of Trp⁺ and Trp⁻ (or Ura⁺ and Ura⁻) colonies were scored after overnight incubation at 32°C. Under the conditions described above, 50 to 80% of the cells retained centromere-containing plasmids after several generations of nonselective growth. Typically, YRp7' and other noncentromeric *ARS* plasmids were retained in less than 10% of the cells. This assay provided a rapid means for analyzing mutagenized centromere DNAs for the ability to stabilize plasmids during mitosis.

For quantitative mating tests, cells of the diploid strain to be tested were grown for about 10 generations in nonselective medium. Approximately 3×10^6 of these cells were mixed with 4.5×10^6 cells of the appropriate haploid tester strain, either strain 262a(*MATa thr*) or strain 262 α (*MAT α thr*). The cell mixture was filtered onto sterile 0.45- μ m type HA membrane filters (Millipore Corp.). The filters were incubated cell side up on YPD agar plates for 3 to 4 h at 32°C. The cells were removed from the filters by vortexing in 10 ml of 1 M sorbitol, pelleted, and suspended in 0.4 ml of 1 M sorbitol. Samples of the appropriate dilutions were plated onto minimal agar plates (Difco yeast nitrogen base containing 2% glucose). Colonies were counted after incubation for 48 h at 32°C. All sampling and plating dilution tests were done in duplicate.

Southern transfer and hybridization. Total yeast DNA was prepared and deproteinized by the method of Bloom and Carbon (2). DNA samples were analyzed on 1% agarose gels and transferred to nitrocellulose paper (type BA85; Schleicher & Schuell, Inc.) as described by Southern (32). The filters were baked in a vacuum oven at 80°C for 2 h. DNA fragments for hybridization probes were isolated from restriction enzyme-cut JC313 plasmid DNA, separated on and eluted from polyacrylamide gels, and 32 P-labeled by nick translation (7, 28). Autoradiography was performed by exposing Kodak XAR-5 film with a Du Pont Cronex Lightning-Plus intensifying screen for 24 to 72 h at -80°C.

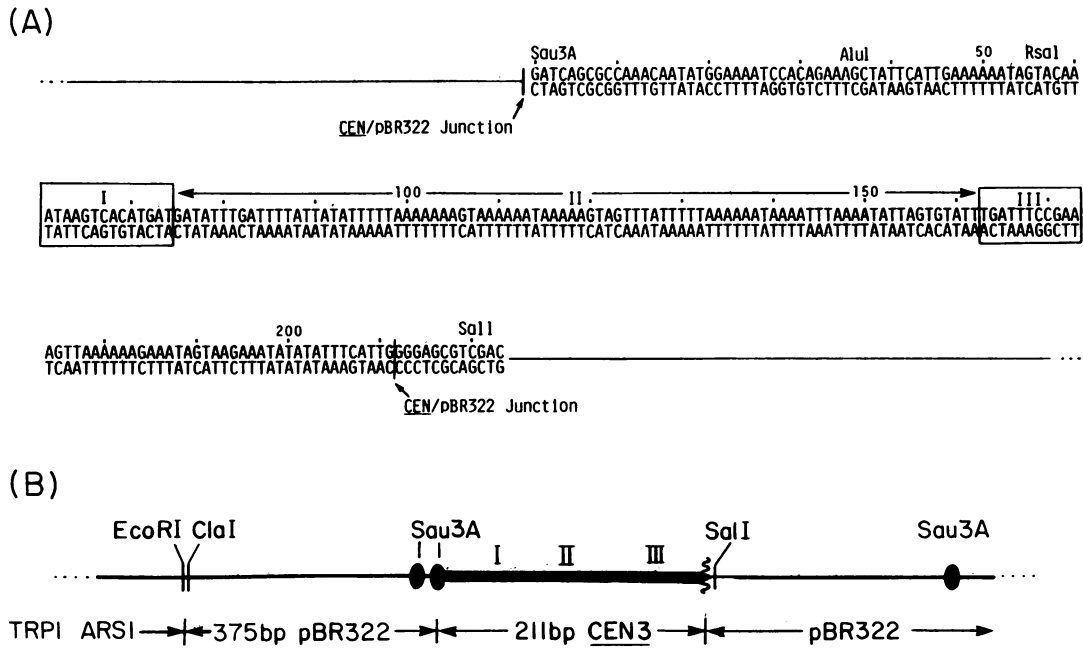


FIG. 2. Nucleotide sequence of the 211-bp *CEN3* DNA in plasmid *dl314*. (A) Sequence of the 211-bp *CEN3* DNA numbered 1 through 211 beginning at the leftmost *Sau3A* site shown. This pBR322-*CEN3* junction corresponds to position 375, and the *SalI* site corresponds to position 650 in pBR322 DNA (numbered as described by Sutcliffe [35]). The top DNA strand was mutagenized after cloning into M13 vector mp8 (mp8-314). The opposite strand was exposed to bisulfite as an M13 mp9 derivative (mp9-314). (B) Orientation of the 211-bp *CEN3* DNA segment relative to pBR322 DNA in *dl314*. The *EcoRI-SalI* fragment containing *CEN3* DNA was cloned into M13 vectors for mutagenesis. The *ClaI-SalI* *CEN** fragments were isolated from RF DNA and cloned into YRp7' for analysis in yeast cells. The *Sau3A* fragments containing the element III mutations were subsequently cloned into JC313 for fragment-mediated transformation of yeast cells.

RESULTS

To identify easily the single-base pair changes by using dideoxynucleotide sequence analysis, we mutagenized a short segment containing functional *CEN* DNA. Previous work had shown that wild-type centromere function can be conferred by a 624-bp *Sau3A* fragment [pYe(*CEN3*)30] (12), and subsequently a 289-bp *RsaI-AluI* fragment having *CEN3* function was identified (6). In this study, we worked with the 211-bp segment of *CEN3* DNA shown in Fig. 2A. The *CEN3* DNA extends from the *Sau3A* site which is located 60 bp before element I to the yeast-pBR322 junction sequences which are located 37 bp past element III. The plasmid containing this *CEN3* region, *dl314*, was constructed from plasmid pYe(*CEN3*)30 as described in Materials and Methods. The evidence presented in this study established that this 211-bp DNA segment containing *CEN3* is sufficient to confer wild-type centromere function, as measured by both plasmid and chromosome-mediated stability assays.

Construction of point mutations in element III (*CEN).** The 211-bp *CEN3* DNA fragment was subjected to bisulfite mutagenesis as shown in Fig. 3. Briefly, the 594-bp *EcoRI-SalI* fragment (Fig. 2B) was isolated from plasmid *dl314* DNA and cloned into M13 vectors mp8 and mp9. Single-stranded DNA was prepared and annealed to denatured mp8 or mp9 RF DNA that had been cut with *EcoRI* and *SalI*. The resulting heteroduplex DNA contained the 211 nucleotides of *CEN3* DNA in a single-stranded region where cytosine residues were susceptible to deamination by sodium bisulfite (31). Klenow DNA polymerase I was used to repair the single-stranded gap and to incorporate adenine residues opposite the uracil products of the bisulfite mutagenesis. The repaired DNA was used to transfect *E. coli* strain JM103.

Template DNAs were prepared from plaque-purified isolates, and mutants were identified by a dideoxynucleotide sequence analysis (30).

Four altered centromere DNAs (*CEN**) containing cytosine-to-thymine mutations in element III were identified and used for further study (Table 1). Two of the *CEN** mutants, mutants BCT2 and BCT4, contained only the single-base pair change in element III; the remainder of the 211-bp yeast DNA was wild type. However, mutant BCT1 had one additional change (cytosine to thymine at position 4), which rendered pBCT1 DNA resistant to cleavage at the *Sau3A* site at the lefthand pBR322-yeast junction (Fig. 2A). This additional point mutation was outside the region required for *CEN* function (6). The 211-bp segment of yeast DNA in pBCT3 contained two other changes (guanine to adenine at positions 120 and 190) in addition to the single point mutation in element III. The guanine-to-adenine mutation at position 120 fell in the center of element II, and the guanine-to-adenine change at position 190 was 17 bp past element III. How these additional alterations affect segregation will require construction of the appropriate single mutants. The *CEN** mutant DNA in pBCT1 contained a new *AsuII* recognition site (5'-TTCGAA-3') in the altered element III sequence. We used this unique *AsuII* site to produce a 2-bp insertion mutation in element III (mutant SD14, Table 1). Plasmid pBCT1 DNA was cut with *AsuII* to generate full-length linear molecules with two-base cohesive ends (5'-CG-3'). Repair of the cohesive ends with Klenow DNA polymerase I followed by blunt-end ligation resulted in a 2-bp insertion (5'-TTCGCGAA-3') and created a new *NruI* recognition site (5'-TCGCGA-3') within the altered element III sequence. The plasmid derivatives and yeast strains used in this study are listed in Table 2.

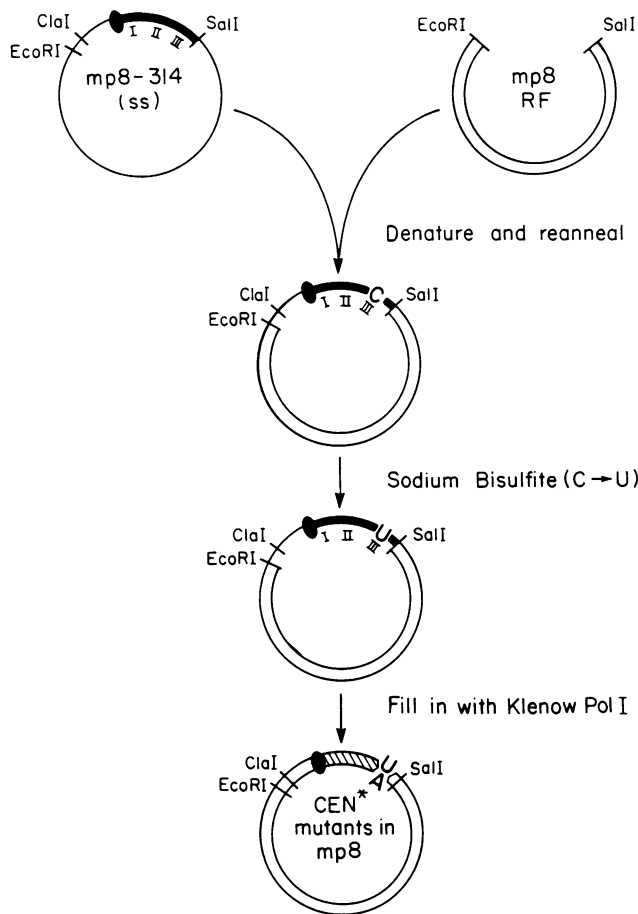


FIG. 3. Procedure for bisulfite mutagenesis. Single-stranded mp8-314 or mp9-314 DNA was annealed to denatured, *EcoRI*-*SalI*-cut mp8 or mp9 RF DNA and treated with sodium bisulfite. The heteroduplex molecules were repaired with Klenow DNA polymerase I. Following transfection of strain JM103, single-stranded templates were prepared and screened for mutations by dideoxynucleotide sequencing. The example shown is for mp8 clones. The same approach was used to mutagenize the opposite strand (see details in text). The cross-hatched segments indicate *CEN** DNA.

Mutations in element III decreased the mitotic stability of *CEN* plasmids. Once the *CEN** mutations were identified, it was necessary to clone the altered centromere DNAs into a replicating vector for segregation assays in yeast cells. The vector, YRp7', contained a fragment of yeast DNA that encoded *TRP1* and *ARS1* cloned into the unique *EcoRI* site of pBR322 (34, 36). *ARS* plasmids are very unstable during

TABLE 1. Mutations in *CEN3* element III

Centromere DNA	Element III sequence ^a
<i>CEN3</i> (624 bp)TGATTTCGGAA
<i>d314</i> (211 bp)TGATTTCGGAA
BCT1TGATTTCGGAA
BCT2TGATTTCGAA
BCT3TGATTTCGAA
BCT4TAATTTCGGAA
BCT4TGATTTCGAAA
SD14TGATTTCGCGAA

^a The wild-type and mutant element III sequences are shown. The nucleotides in element III that differ from the wild-type nucleotides in *CEN** mutants BCT1, BCT2, BCT3, BCT4, and SD14 are underlined. Mutant BCT1 DNA was used to generate the 2-bp insertion in mutant SD14 (see text).

TABLE 2. Plasmids and yeast strains

Centromere DNA fragment	Plasmids ^a		<i>CEN</i> chromosome III substitution yeast strain ^b
	YRp7' derivative (<i>TRP1 ARS1</i>)	JC313 derivative (<i>URA3</i>)	
<i>CEN3</i> , 624 bp	pYe(<i>CEN3</i>)30	p <i>CEN3</i> -13	Ye <i>CEN3</i> -13
<i>d314</i> , 211 bp	<i>d314</i>	p314-13	Ye314-13
BCT1, 211 bp	pBCT1	pBCT1-13	Ye1-13
BCT2, 211 bp	pBCT2	pBCT2-13	Ye2-13
BCT3, 211 bp	pBCT3	pBCT3-13	Ye3-13
BCT4, 211 bp	pBCT4	pBCT4-13	Ye4-13
SD14, 213 bp	pSD14	pSD14-13	Ye14-13

^a YRp7' (*TRP1 ARS1*) derivatives were used to assay plasmid stabilities in yeast cells.

^b Derivatives of chromosome III genomic substitution vector JC313 were used to construct the yeast strains by fragment-mediated transformation of strain SB9882-4CR (*MATa/MATa trp1-289/trp1-289 ura3-52/ura3-52 LEU2/leu2-3,112 HIS4/his4-519 cry/CRY*). All of the strains resulted from homologous integration of the appropriate *EcoRI* fragment into the *MATa LEU2 HIS4* copy of chromosome III.

mitotic cell division. This is a result of an extreme segregation bias which produces mother cells with multiple plasmid copies and daughter cells that completely lack the plasmid (25). Addition of cloned yeast centromere DNA to an *ARS* plasmid corrects this segregation bias and confers mitotic stability by ensuring the proper distribution of a plasmid copy to both mother and daughter cells at each division (8, 25).

*CEN** M13 RF DNAs were digested with *ClaI* and *SalI*, and isolated DNA fragments containing the *CEN** sequences were ligated to YRp7' DNA cut with *ClaI* and *SalI*. This manipulation generated *CEN** plasmids which were identical to the parent plasmid, *d314*, except for the *CEN** mutations (Table 2). Purified plasmid DNAs for each *CEN**-YRp7' derivative (pBCT1, pBCT2, pBCT3, pBCT4, and pSD14) were used to transform yeast strain YP47 (*MATa ura3-52 ade2-101 lys2-801 trp1Δ*). *Trp*⁺ yeast transformants were subjected to mitotic stability tests as described in Materials and Methods. Briefly, transformed yeast strains were grown nonselectively, and the percentage of *Trp*⁺ cells in the population was measured by replica plating onto agar with and without tryptophan (12). In many experiments in which we used at least two different yeast strains, we always obtained similar mitotic stability results for pYe(*CEN3*)30 (624-bp *CEN3*) and *d314* (211-bp *CEN3*). The centromere fragments on both of these plasmids appeared to be functioning properly in the plasmid assay. This result was confirmed by analyses of yeast strains containing the 211-bp *CEN3* fragment substituted for the wild-type 624-bp *CEN3* segment in chromosome III (see below). It is worth noting that neither pYe(*CEN3*)30 nor *d314* was present in 100% of the population, despite the functional centromere DNA on the plasmid. This was probably the result of the small plasmid size (5 to 6 kb) and not the result of a lack of *CEN* function. Hieter et al. (16) have shown that a small circular plasmid containing a functional *CEN* is less stable than a large circular plasmid containing the same *CEN* DNA.

The mitotic stabilities of the *CEN** plasmids, the parent plasmid *d314*, and YRp7' are compared in Table 3. The guanine-to-adenine element III mutation at position 171 in pBCT4 and the guanine-to-adenine transition at position 164 in pBCT3 had little or no effect on the mitotic stability of the plasmids in yeast cells. However, one of the *CEN** plasmids, plasmid pBCT1, was somewhat less stable than *d314* and was retained by fewer than 50% of the cells after overnight

growth in nonselective media. In contrast, two of the element III mutations caused extreme plasmid instability. The single cytosine-to-thymine mutation at position 170 in mutant BCT2 abolished mitotic centromere function. A similar result was obtained for cytosine-guanine insertion mutant pSD14, which was clearly less stable than the parent plasmid from which it was derived, plasmid pBCT1 (Table 3).

To confirm that the single-base pair mutations in element III were not altered during propagation of the *CEN** plasmids in yeast cells, pBCT1 and pBCT2 DNAs were recovered by transforming *E. coli* strain JM14 to ampicillin resistance, using total DNAs from plasmid-bearing yeast strains. Centromere-containing DNA fragments were isolated from these plasmids and cloned into M13 vectors. A sequence analysis showed that the original element III mutations were present without additional alterations (data not shown).

Genomic substitution of *CEN mutations for the wild-type *CEN3* in yeast chromosome III.** The plasmid-mediated assay for centromere function provides useful information rapidly. However, it suffers from the limitation that centromere plasmids do not have the size, conformation, or telomere sequences of intact chromosomes and are typically less stable mitotically than yeast chromosomes are in vivo (16, 26). To circumvent these problems, Clarke and Carbon (9) use site-directed integration (29) to substitute an alternate *CEN* fragment for the wild-type centromere DNA in intact chromosome III. This approach provided us with a unique opportunity to assess the effects of the single-base pair element III mutations on the segregation of otherwise intact chromosome III in vivo.

As shown in Fig. 4A, *Sau3A* fragments containing the *CEN** DNAs were isolated from *dl314*, pBCT1, pBCT2, pBCT3, pBCT4, and pSD14 plasmid DNAs and cloned into the unique *Bam*HI site in substitution vector JC313 (9). As a control, a JC313 derivative containing the *CEN3* 624-bp *Sau3A* fragment was also constructed (p*CEN3*-13). Purified plasmid DNAs were prepared for each JC313 derivative (p*CEN3*-13, p314-13, pBCT1-13, pBCT2-13, and pSD14-13), and the constructions were confirmed by a restriction analysis. Diploid yeast strain SB9882-4CR (*MAT α ura3-52 trp1-289 leu2-3,112 his4-519 cry1/MAT α ura3-52 trp1-289 can1*) was transformed with *Eco*RI-cut DNA from each of these JC313 plasmid derivatives, and *Ura*⁺ transformants were selected. Figure 4B shows the recombination events that were responsible for integration of the transforming DNA fragment into chromosome III. About 200 *Ura*⁺ transformants were obtained for each 1 μ g of *Eco*RI-cut p*CEN3*-

TABLE 3. Mitotic stabilities of *CEN** plasmids in yeast cells

Plasmid	Element III sequence	% Of cells retaining plasmid ^a	
		Selective medium	Nonselective medium
<i>dl314</i>	TGATTTCCGAA	72	55
pBCT4	TGATTTCCAAA	74	55
pBCT3	TAATTTCCGAA	64	50
pBCT1	TGATTTTCGAA	62	43
pBCT2	TGATTTCTGAA	10	5
pSD14	TGATTTTCGCGAA	10	5
YRp7'		10	5

^a The mitotic stabilities of several *CEN** plasmids with element III mutations were compared with the mitotic stabilities of plasmids *dl314* and YRp7' in yeast strain YP47. Stability analyses were performed as described in Materials and Methods.

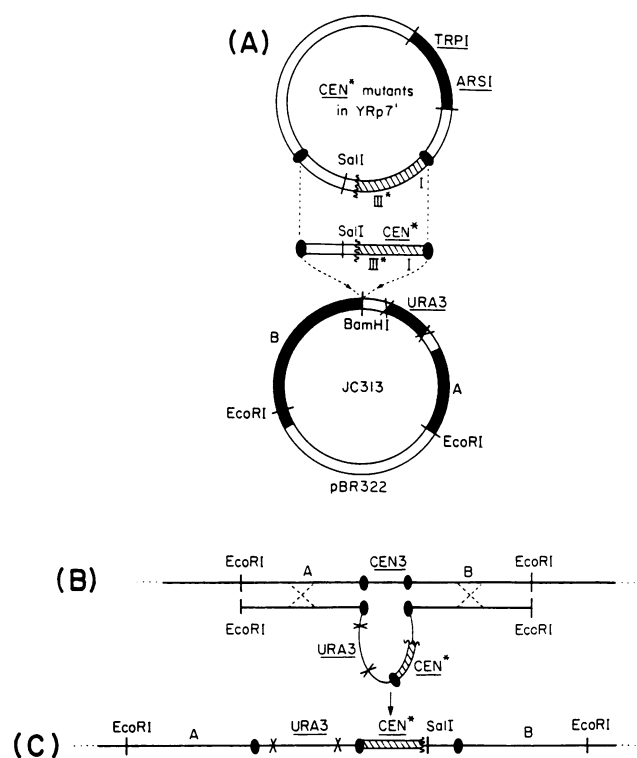


FIG. 4. Construction of plasmids for genomic substitution of *CEN** mutations. (A) *CEN** plasmid DNAs (pBCT1, pBCT2, pBCT3, pBCT4, and pSD14) were digested with *Sau3A*, and fragments containing the *CEN** regions were isolated from gels and ligated to *Bam*HI-cut JC313 DNA. The derivatives of JC313 all contained the 1.1-kb *Hind*III *URA3* fragment and regions A and B, segments of DNA that normally flank the 624-bp *CEN3* fragment in chromosome III (9). JC313 *CEN** plasmid DNAs were cleaved to completion at the two *Eco*RI sites and were used to transform diploid yeast strain SB9882-4CR (*MAT α /MAT α trp1-289/trp1-289 ura3-52/ura3-52 can1/CAN LEU2/leu2-3,112 HIS4/his4-519 cry/CRY*) to a *Ura*⁺ phenotype. The cross-hatched segments indicate *CEN** DNA. (B) Genomic substitution took place by recombination between regions A and B on the plasmid and the homologous chromosomal DNA (dashed lines). These events were stimulated by the double-stranded ends generated by *Eco*RI cleavage of the plasmid DNAs (29). (C) Generalized diagram of *CEN**-substituted chromosome III. Symbols: ●, *Sau3A* sites; ×, *Hind*III sites. Not all of the *Sau3A* and *Hind*III sites are shown.

13, p314-13, pBCT3-13, pBCT4-13, or pBCT1-13 plasmid DNA. Significantly lower frequencies (about 10 transformants per μ g) were obtained from transformation with *Eco*RI-digested pBCT2-13, pSD14-13, and JC313 plasmid DNAs. Several *Ura*⁺ colonies from each transformation experiment were analyzed further to confirm integration into chromosome III.

The structures of the chromosome III copies involved in the genomic substitution events are shown in Fig. 5. The region around *CEN3* in the wild-type chromosome III shown in Fig. 5 contained the 624-bp *CEN3* fragment and flanking regions A and B. The properties of substitution strains equivalent to Ye*CEN3*-13 and YeJC313 have been described previously by Clarke and Carbon (9). Yeast strain Ye314-13 contained the 211-bp *CEN3* fragment substituted for the wild-type centromere on the *MAT α* copy of chromosome III. The Ye*CEN** diagram in Fig. 5 shows the general organization of the *MAT α* chromosome III copies substituted with

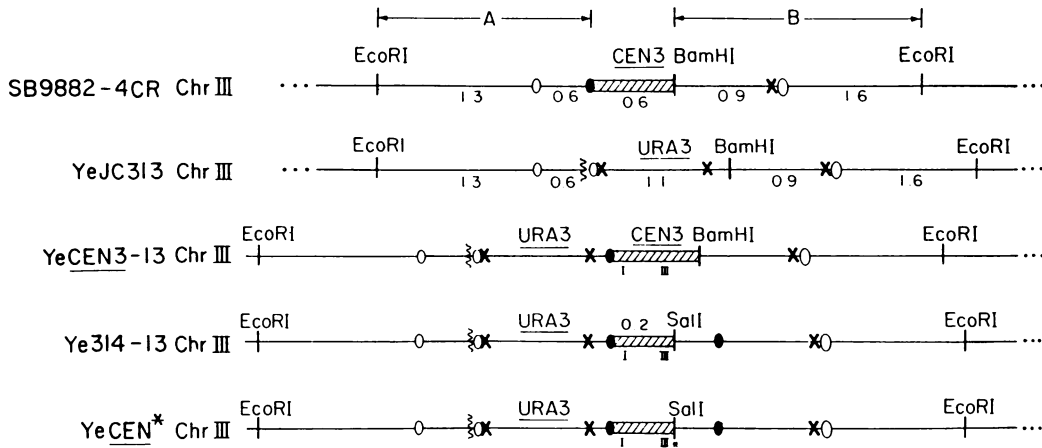


FIG. 5. Chromosome III variants constructed by fragment-mediated genomic substitution. The restriction maps of the chromosome III genomic substitution strains analyzed in this study are shown. A wild-type chromosome III analogous to either the *MATa* or *MATα* copy in yeast strain SB9882-4CR is diagrammed at the top; the position of the wild-type 624-bp *CEN3* region is indicated. Yeast strains YeJC313 and Ye*CEN3*-13 were generated as described by Clarke and Carbon (9). One copy of chromosome III in strain YeJC313 contains the *URA3* *HindIII* fragment in place of the *CEN3* 624-bp *Sau3A* fragment and is therefore acentric. One copy of chromosome III in Ye*CEN3*-13 contains the *URA3* gene insertion in addition to the *CEN3* 624-bp DNA and therefore serves as a wild-type chromosome III control marked with *URA3*. Strain Ye314-13 bears one chromosome III copy containing the *URA3* gene and the 211-bp *CEN3* DNA introduced on a 394-bp *Sau3A* fragment that includes the *SalI* site shown in Fig. 2. The chromosome III diagram at the bottom represents the *CEN**-substituted copies of chromosome III present in yeast strains Ye1-13(BCT1), Ye2-13(BCT2), Ye3-13(BCT3), Ye4-13(BCT4), and Ye14-13(SD14). In all cases the *Sau3A* fragment containing the *CEN* mutation was inserted in the same orientation as the fragment found in the wild-type chromosome. All of the *EcoRI*, *BamHI*, *SalI*, *HindIII* (×), and *ClaI* (○) sites are shown, but only the relevant *Sau3A* (●) sites are indicated. The *EcoRI*-*Sau3A* junction made in the original JC313 construction is indicated (‡). III* indicates the element III mutations in the *CEN** insertions and represents either an *AsuII* site in strain Ye1-13(BCT1) or an *NruI* site in strain Ye14-13(SD14). The fragment lengths are given in kilobase pairs, and the figure is not drawn to scale. The cross-hatched segments indicate *CEN* regions (624-bp *CEN3* in wild-type strain SB9882-4CR and strain Ye*CEN3*-13, 211-bp *CEN3* in strain Ye314-13 and *CEN** derivatives).

*CEN** mutant DNA. All of the chromosome III derivatives, as well as the wild-type chromosome III, contained the 900-bp *BamHI*-*HindIII* fragment (Fig. 5, region B) that was used as a hybridization probe in the Southern blot analyses

(Fig. 6). In the total DNA isolated from untransformed diploid strain SB9882-4CR (Fig. 6A, lane b), the probe hybridized to a 5.0-kilobase (kb) DNA fragment generated by *EcoRI* cleavage of both the *MATα* and *MATa* copies of

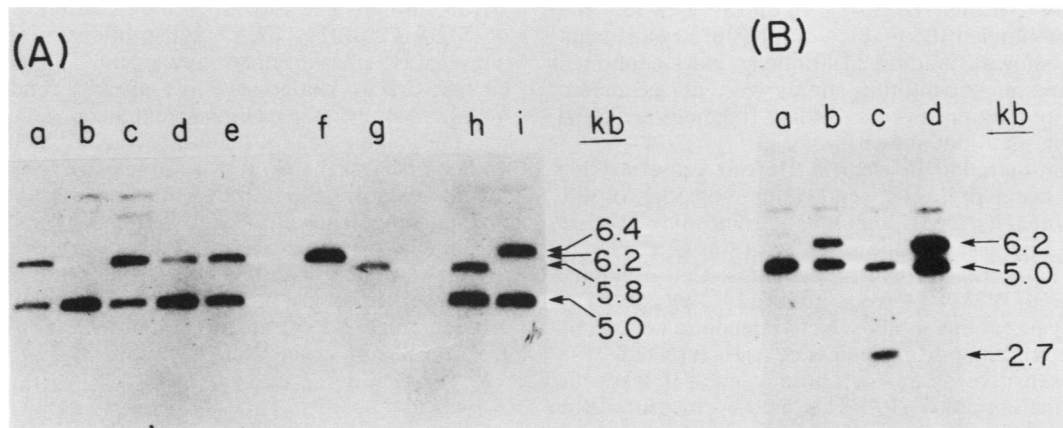


FIG. 6. Southern hybridization analyses of genomic substitution strains. (A) Total yeast DNAs were prepared from strains Ye314-13(*dl314*) (lane a), SB9882-4CR (lane b), Ye1-13(BCT1) (lane c), Ye2-13(BCT2) (lane d), Ye14-13(SD14) (lane e), YeJC313(JC313) (lane h), and Ye*CEN3*-13 (lane i), digested with *EcoRI*, electrophoresed, and transferred to nitrocellulose filter paper. *EcoRI* linear molecules of plasmid DNAs from p314-13 (6.2 kb) and JC313 (5.8 kb) were diluted 1:10⁵ and run in lanes f and g for molecular weight markers. The fastest-migrating band, 5 kb (lanes a through e, h, and i) represents the wild-type chromosome III copy. The sizes of the *EcoRI* fragments derived from each substituted chromosome III copy were as follows: 6.2 kb (lanes a, c, d, and e), 5.8 kb (lane h), and 6.4 kb (lane i). The 900-bp *BamHI*-*HindIII* fragment from flanking region B (see Fig. 5) was radioactively labeled by nick translation with [α -³²P]ATP and used as a hybridization probe in these experiments. (B) Total DNAs from yeast strains SB9882-4CR (lane a), Ye14-13 (lanes b and c), and Ye1-13 (lane d) were digested either with *EcoRI* alone (lanes a and b) or with *EcoRI* and *NruI* (lanes c and d), electrophoresed, transferred, and hybridized as described above. The *NruI* recognition site in mutant SD14 was generated by a 2-bp insertion into the element III sequence in mutant BCT1 (see text).

TABLE 4. Mitotic segregation of chromosomes containing *CEN** mutations

Yeast strain	Centromere	Element III sequence	Mitotic stability (%) ^a	Fraction of mated cells in population ^b
SB9882-4CR	Wild type	TGATTTCCGAA	NA	6.7×10^{-5}
Ye <i>CEN3</i> -13	<i>CEN3</i> (624 bp)	TGATTTCCGAA	>99.5	5.3×10^{-5}
Ye314-13	<i>d314</i> (211 bp)	TGATTTCCGAA	>99.5	9.2×10^{-5}
Ye3-13	BCT3	TAATTTCCGAA	>99.5	1.0×10^{-5}
Ye4-13	BCT4	TGATTTCCAAA	>99.5	1.0×10^{-5}
Ye1-13	BCT1	TGATTTTCGAA	80	3.6×10^{-2}
Ye2-13	BCT2	TGATTTCTGAA	1	ND
Ye14-13	SD14	TGATTTTCGCGAA	2	ND
YeJC313	Acentric		5	ND

^a Mitotic stability is expressed as the percentage of Ura⁺ cells after about 10 generations of nonselective growth. NA, Not applicable.

^b The more sensitive quantitative mating assay reflected the frequency of nondisjunction of chromosome III, as well as mitotic gene conversion and recombination at the *MAT* locus. The quantitative mating data represent the number of mating-competent cells present in a population. The cells were grown nonselectively for about 10 generations before mating. ND, Not determined.

chromosome III. As expected, this 5.0-kb fragment was also present in the preparations containing DNA from each genomic substitution strain (Fig. 6A, lanes a, c, d, e, h, and i); this represented cleavage of the copies of chromosome III which were not involved in the genomic substitution event. The DNA from each transformed strain also contained a larger *EcoRI* fragment that was generated from the copy of chromosome III containing the 1.1-kb *URA3 HindIII* fragment and the *CEN** DNA (Fig. 6A, lanes a, c, d, e, h, and i). This *EcoRI* fragment differed in length for each *CEN** substitution (Fig. 5). Similar results were obtained for strains Ye3-13 and Ye4-13 (data not shown).

The results of the Southern analysis shown in Fig. 6B confirmed the presence of a new *NruI* recognition sequence in chromosome substitution strain Ye14-13. The 6.2-kb *EcoRI* fragment containing the *CEN** region in strain Ye14-13 genomic DNA (Fig. 6B, lane b) was clearly cut by *NruI* to produce the expected 2.7-kb fragment (Fig. 6B, lane c). The analogous *EcoRI* fragment in strain Ye1-13 DNA was not cleaved by *NruI* (Fig. 6B, lane d). The genomic substitution strains (strains Ye314-13, Ye1-13, Ye2-13, and Ye14-13) all contained the predicted *SalI* site near element III (data not shown). Additional Southern blots confirmed that these genomic substitution strains were not generated by tandem integration events during fragment-mediated transformation (data not shown).

Single point mutation in element III can cause striking chromosome instability. The segregation behavior of the *CEN**-substituted (*URA3*) copies of chromosome III was first measured by assaying the stability of the Ura⁺ phenotype in individual transformant populations. Cell of strains Ye*CEN3*-13 and Ye314-13 were uniformly >99.5% Ura⁺ after 8 to 10 generations in nonselective medium containing uracil (Table 4). Supporting evidence was supplied by a much more sensitive assay for chromosome III loss, the quantitative mating assay (10). This method measured the number of mating-competent cells ($2n - 1$; *MATa* or *MATα*) that resulted from chromosome III loss, mitotic gene conversion, and recombination at *MAT* in a diploid ($2n$) population. These cells were mixed with an excess number of tester haploid cells of the appropriate mating type. The cells were incubated together and then plated onto media formulated to select against diploids, $2n - 1$ aneuploids, and the haploid tester cells and to permit only mated aneuploids to grow. In our quantitative mating experiments, the number of mating-competent cells per cell in control strain SB9882-4CR was 6.7×10^{-5} (Table 4). Similar results were obtained for chromosome III substitution strains Ye*CEN3*-13 (5.3×10^{-5}

mating-competent cell per cell) and Ye314-13 (9.2×10^{-5} mating-competent cell per cell).

Copies of chromosome III bearing the mutant BCT1 *CEN** mutation (strain Ye1-13) segregated aberrantly, as determined by both the plating test and the quantitative mating test (Table 4). After approximately 10 generations in medium supplemented with uracil, only about 80% of the cells in the population formed colonies on agar lacking uracil. As expected, the loss of the *MATα URA3 BCT1 LEU2 HIS4* copy of chromosome III simultaneously uncovered recessive loci on the *MATa* copy of chromosome III, and the mating-competent cells were phenotypically Ura⁻, Leu⁻, and His⁻. The quantitative mating data indicated that there was a high loss rate for the chromosome bearing the mutant BCT1 mutation. The fraction of strain Ye1-13 cells that were mating competent was 3.6×10^{-2} mating competent cell per cell, a significant increase compared with the fraction of mating-competent cells for parent strain Ye314-13.

Extreme chromosome III instability was observed for the *URA3* chromosome copies bearing either the BCT2 (Ye2-13) or SD14 (Ye14-13) *CEN** substitutions (Table 4). After nonselective growth, only a few of the cells grew on media lacking uracil. Under selective growth conditions, strain Ye2-13 and Ye14-13 cells had extended generation times, and the colonies had a heterogeneous nibbled morphology on minimal agar plates. It was not feasible to do quantitative mating tests on these strains, but the presence of $2n - 1$ mating-competent cells was confirmed by standard mating type patch tests, using tester stains. As reported previously (9), an extremely high level of chromosome instability was also observed for the acentric, Ura⁺ copies of chromosome III constructed by transformation with *EcoRI*-cut JC313 DNA (Table 4, strain YeJC313).

DISCUSSION

Functional boundaries of *CEN3* DNA. Previous work has shown that a 289-bp *CEN3* fragment stabilizes plasmids and chromosomes in yeast cells (6). In this study we found that a 211-bp segment of *CEN3* DNA encompassing elements I through III is sufficient to confer wild-type centromere function in two assays. Taken together, these results strongly suggest that the *CEN3* DNA which is necessary and sufficient for function is contained within a 155-bp region (Fig. 2, nucleotides 56 through 211), as delineated by the overlap of the 289- and 211-bp fragments.

Studies involving centromere mutants have begun to de-

fine the essential region at higher resolution. Plasmids and chromosomes with element I DNA completely deleted but with some or all of element II intact exhibit elevated levels of aberrant segregation (6; A. Gaudet and M. Fitzgerald-Hayes, manuscript in preparation). However, plasmids with only the first 5 bp of element I deleted from *CEN11* DNA are still stable (13). The stable segregation behavior of plasmids (*dl314*) and chromosomes (strain Ye314-13) containing the 211-bp *CEN3* fragment indicates that the functional righthand boundary does not extend beyond the *CEN3*-pBR322 junction sequence that is located 37 bp past element III (Fig. 2). Some evidence that the functional centromere DNA extends beyond element III already exists. A single adenine-to-cytosine mutation introduced 7 bp downstream from element III (Fig. 2, adenine-to-cytosine change at position 180) results in a nondisjunction frequency of 10^{-3} per cell division for the altered chromosome (R. Ng and J. Carbon, personal communication). This places the righthand boundary somewhere between nucleotides 180 and 211 in Fig. 2. A comparison of the sequences in this region from four *CEN* DNAs reveals that while the most homologous segment is the 11-bp element III, the flanking sequences share partial homology as well (Fig. 1). It seems likely that the functional element III region includes conserved domains on either side of the central 11-bp sequence.

Integrity of element III is essential for centromere function. We investigated the effects of mutations that alter invariant nucleotide positions in *CEN3* element III DNA. The results of this study confirm that specific base pairs within element III are essential for proper mitotic centromere function. The aberrant segregation caused by the different *CEN** mutants allowed us to begin to elucidate which positions in element III are functionally most critical. The importance of the cytosine at position 170 is apparent from the dramatic effect of the mutant BCT2 alteration (cytosine-to-thymine mutation at position 170) on the segregation of *CEN* plasmids and chromosomes. None of the other *CEN** mutations changed the cytosine at position 170, and none of the other point mutations had such a dramatic effect on function. The nucleotide at position 169 (cytosine) is also important for stability. Strain Ye1-13 chromosomes bearing the mutant BCT1 *CEN** mutation (cytosine-to-thymine mutation at position 169) exhibited at least a 1,000-fold increase in the rate of chromosome loss. This effect was measurably diminished or obscured when the mutant BCT1 *CEN** mutation was carried on a plasmid (Table 3). Plasmids pBCT3 (guanine-to-adenine change at position 164) and pBCT4 (guanine-to-adenine change at position 171) are as mitotically stable as *dl314*, despite the additional point mutations in pBCT3 (guanine-to-adenine changes at positions 120 and 190). Chromosomes bearing the mutant BCT3 and BCT4 centromere mutations are as stable as the wild-type chromosome. These data indicate that some changes in the highly conserved element III sequence do not affect mitotic centromere function. The mutant SD14 *CEN** mutation contains a wild-type cytosine at position 170, but the destabilizing effect of the 2-bp insertion was apparent from the instability of mutant SD14 in both plasmid and chromosome stability assays (Tables 3 and 4). This mitotic instability could be the result of the combination of the adenine-to-cytosine change at position 172 and the adenine-to-guanine change at position 173, spatial disruption of element III, or perhaps interference from the extended palindrome created by the 2-bp insertion. We conclude that in an otherwise wild-type element III background, the cytosine at position 170 is essential.

Mitotic versus meiotic centromere function. In the presence

of a wild-type element III sequence, deletion mutations removing segments of the adenine · thymine-rich element II DNA result in mitotic instability of both plasmids and chromosomes in yeast cells (6; Gaudet and Fitzgerald-Hayes, unpublished data). A 48-bp deletion of element II DNA causes the altered chromosome III to undergo nondisjunction at a rate of 1.2×10^{-2} per cell division (6). This rate of chromosome loss is the same order of magnitude as the rate observed in this study for chromosomes bearing the mutant BCT1 point mutation in element III (strain Ye1-13) (Table 4). Plasmids with large deletions of element II DNA segregate poorly, but they are still significantly more stable than plasmids bearing element III point mutations. Homocentric yeast strains with the same element II deletion in both copies of chromosome III exhibit aberrant meiotic chromosome III segregation as well, suggesting that the adenine · thymine-rich DNA may function in both mitotic and meiotic chromosome movement (6).

A tetrad analysis of strain Ye314-13 showed completely normal meiotic segregation of the chromosome III copy containing the 211-bp *CEN3* fragment (K. Bourgaize and M. Fitzgerald-Hayes, unpublished data). However, meiotic analyses of some yeast strains bearing *CEN** mutations in one copy of chromosome III have been complicated by the extreme instability of the altered chromosomes. Heterocentric yeast strains Ye2-13(BCT2) and Ye14-13(SD14) sporulate very poorly, presumably because many cells in the population are aneuploid for chromosome III. Sporulation of Ye1-13(BCT1) diploid cells resulted in a large proportion of tetrads that scored $4^+ : 0^-$ for segregation of the Ura^+ phenotype and ascospore colonies that demonstrated unusual mating behavior. This aberrant behavior presumably resulted from aneuploids with extra copies of chromosome III generated by nondisjunction and inherited by the haploid progeny, further complicating the meiotic analysis. A tetrad analysis of strains Ye3-13 and Ye4-13 indicated that neither mutation greatly affects meiotic centromere function, although strain Ye4-13 may have a slightly elevated rate of recombination near the centromere (data not shown).

Assembly and maintenance of the attachment site. It is clear that the primary DNA sequence and the spatial arrangement of elements I through III are important for proper chromosome movement. How these regions interact to form a functional spindle fiber attachment site is not yet clear. It is reasonable to believe that the 11-bp element III sequence may form at least part of the primary binding site for a sequence-specific protein whose function is essential for efficient chromosome spindle fiber attachment. Chromatin mapping experiments (2) have shown that in vivo the centromeric element I through III region is located within a nuclease-resistant, protected section of chromosome III encompassing about 250 bp of *CEN3* DNA. This protected region is likely to be a structural feature common to all yeast centromeres (1) and is absent from chromatin associated with plasmids where elements I through III are completely deleted (3). We suspect that the element III region, probably interacting with a specific protein(s), is involved in the assembly or maintenance or both of this unusual chromatin configuration. The putative element III binding protein(s) may in fact be responsible for at least some of the centromere-specific DNA binding activity partially purified from yeast chromatin (1, 3). We are presently investigating the in vivo chromatin structure associated with the altered centromere DNA in the *CEN**-substituted chromosomes. If the chromatin configuration is also altered, as is likely, a correlation among specific *CEN* DNA sequences, centromere

meric chromosome structure, and the efficiency of spindle fiber attachment will be firmly established.

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ADDENDUM IN PROOF

The sequence of *CEN14* was recently published (M. Neitz and J. Carbon, *Mol. Cell. Biol.* 5:2887-2893, 1985).

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