Mutational Analysis of Meiotic and Mitotic Centromere Function in *Saccharomyces cerevisiae*

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ABSTRACT

A centromere (*CEN*) in *Saccharomyces cerevisiae* consists of approximately 150 bp of DNA and contains 3 conserved sequence elements: a high A + T region 78–86 bp in length (element II), flanked on the left by a conserved 8-bp element I sequence (PuTCACPuTG), and on the right by a conserved 25-bp element III sequence. We have carried out a structure-function analysis of the element I and II regions of *CEN3* by constructing mutations in these sequences and subsequently determining their effect on mitotic and meiotic chromosome segregation. We have also examined the mitotic and meiotic segregation behavior of *ARS* plasmids containing the structurally altered *CEN3* sequences. Replacing the periodic tracts of A residues within element II with random A + T sequences of equal length increases the frequency of mitotic chromosome nondisjunction only 4-fold; whereas, reducing the A + T content of element II while preserving the length results in a 40-fold increase in the frequence of chromosome nondisjunction. Structural alterations in the element II region that do not decrease the overall length have little effect on the meiotic segregation behavior of the altered chromosomes. Centromeres containing a deletion of element I or a portion of element II retain considerable mitotic activity, yet plasmids carrying these same mutations segregate randomly during meiosis I, indicating these sequences to be essential for maintaining attachment of the replicated sister chromatids during the first meiotic division. The presence of an intact element I sequence properly spaced from the element III region is absolutely essential for proper meiotic function of the centromere.

RECENT studies on centromeres in the budding yeast, *Saccharomyces cerevisiae*, have provided a detailed picture of the structure of the centromeric (*CEN*) DNA regions. To date, the functional centromere DNAs from 10 of the 17 yeast chromosomes have been isolated and sequenced (for reviews see BLACKBURN and SZOSTAK 1984; CLARKE 1985; CLARKE and CARBON 1985). Although these centromere sequences do not cross-hybridize, they display a striking organizational homology. Each centromere is divided into three sequence domains. Element II, the largest domain, is a 78–86-bp region of high A + T (>90%). The precise sequence of the element II region is not conserved; however, all element II regions contain short runs of A followed by blocks of T (in contrast to random or alternating A and T bases). Element II is flanked on the left by a conserved 8-bp (PuTCACPuTG) element I sequence. To the immediate right of element II lies the 25-bp conserved, partially palindromic sequence of element III (TGTTTT(T/A)TG·TTTCCGAAA····AAA).

Mutational analyses of the yeast centromere regions have been undertaken in several laboratories (CARBON and CLARKE 1984; PANZERI *et al.* 1985; MCGREW, DIEHL and FITZGERALD-HAYES 1986; NG, CUMBERLEDGE and CARBON 1986; HEGEMANN *et al.* 1986; GAUDET and FITZGERALD-HAYES 1987). Most of these studies have emphasized centromere function during mitosis. Structurally altered *CEN* sequences have been assayed either on *ARS* plasmids or by replacement of a genomic *CEN* sequence with a structurally altered copy. Analysis of the mitotic segregation behavior of altered chromosomes offers a 1000-fold increase in sensitivity when compared to measurements of *CEN-ARS* plasmid segregation rates (CLARKE and CARBON 1983), since wild-type chromosomes are lost approximately once in every 10^5 mitotic cell divisions, whereas *CEN-ARS* plasmids are 2–3 orders of magnitude less stable (CLARKE and CARBON 1980; NG, CUMBERLEDGE and CARBON 1986). Single base-pair changes in the palindromic element III sequence can increase the rate of chromosome loss 10,000-fold (MCGREW, DIEHL and FITZGERALD-HAYES 1986; NG, CUMBERLEDGE and CARBON 1986). Previous studies (CARBON and CLARKE 1984; GAUDET and FITZGERALD-HAYES 1987) have shown that duplications, rearrangements and partial deletions within sequence elements I and II result in a 10–1000-fold decrease in mitotic stability of the altered chromosome *III*. Because only certain structural features of element II are conserved, it is

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not known if this partial loss of function is a consequence of altering specific nucleotide sequences, changing the AT:GC ratio, and/or changing the length of the region. PANZERI *et al.* (1985) have reported that *CEN-ARS* plasmids carrying small duplications within element II or small increases in the GC content of element II show no measurable effect on plasmid mitotic stability. However, small decreases in mitotic stability may not be detected when assayed on the relatively unstable, small *CEN* plasmids.

Less is known about the relationship between centromere structure and function during meiotic cell divisions. However, deletions and rearrangements spanning elements I and II can have a considerably greater effect on meiotic than on mitotic functions of the centromere (CARBON and CLARKE 1984). When only one copy of chromosome *III* carries the *CEN* mutation in a diploid, the meiotic segregation behavior of the altered chromosome appears to be normal. However, if both copies of chromosome *III* contain a structurally altered centromere, a high percentage of the meiotic progeny are inviable, and the segregation pattern of the altered chromosome *III* indicates precocious separation of sister chromatids during the first meiotic division. While deletions and rearrangements within elements I and II preferentially disrupt meiotic segregation, PANZERI *et al.* (1985) have reported that *CEN-ARS* plasmids containing a duplication of the element II region exhibit normal meiotic segregation behavior, yet show a pronounced decrease in mitotic stability.

In this paper we have attempted more clearly to define the role of the *CEN* element I sequence in mitosis and meiosis, and to ascertain the relative importance of the length, (A + T)-content, and the sequence specificity of the high A + T element II region.

MATERIALS AND METHODS

Media and enzymes: Media for bacterial and yeast growth have been described previously (TSCHUMPER and CARBON 1983). 5-Fluoro-orotic acid was obtained from Specialty Chemicals, Gainesville, Florida. Most enzymes were obtained from New England Biolabs (Beverly, Massachusetts). Calf intestine alkaline phosphatase was from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. Zymolyase was a gift from Kirin Brewery Co. (Applied Bioscience Laboratory, Takasaki, Gumma Pref., Japan). Buffers and reaction conditions were those specified by the manufacturer.

Strains and plasmids: The *Escherichia coli* strains JA226 (C600 *hsdR*$^-$ *hsdM*$^+$ *recBC lop-11 thi leuB6 strR*) and JM101 (*Δlacpro supE thi*/F′ *traD36 proAB lacZΔM15*) were used for *E. coli* transformations, plasmid preparations and single stranded phage preparations. Yeast strains 419-25d (**a** *leu2 trp1 his4 ura3-52*), 514C (*α adel met14 trp1 ura3-52*), and 843-9C (*α adel met14 trp1 ura3-52*) were constructed in this laboratory by A. J. CARPOUSIS. The strain 419-25d contains a copy of the plasmid pAC22 integrated near the *leu2* locus, distal to the centromere. Plasmid pAC22 contains a 3.9-kb *Hin*dIII fragment containing the wild-type *ADE1* gene (A. J. CARPOUSIS, personal communication). The diploid strains SB51486 and SB121385 were constructed by crossing 419-25d with 514C and 842-9C, respectively. Both diploids are homozygous *ade1/ade1* on chromosome *I* and contain the wild-type *ADE1* gene integrated on one copy of chromosome *III*; thus loss of the marked chromosome *III* gives a red colony phenotype.

Plasmid pLCJ (which contains a deletion of the *Aha*III-*Hin*dIII fragment of pKC7 (RAO and ROGERS 1979) was obtained from L. CLARKE; plasmid pRNmp7-30-1 (which contains a 290-bp *Rsa*1-*Alu*1 *CEN3* fragment cloned into the *Hin*cII site of mp7) was provided by R. NG; plasmids pJC303-7, pJC303-31, and pJC303-6 were provided by L. CLARKE (CARBON and CLARKE 1984); plasmid pS5-M4 was provided by W. FANGMAN (FANGMAN and DUJON 1984).

Construction of *CEN3* mutations—element II substitutions: The plasmid pLCJS-A7 was constructed by subcloning a 586 bp *Sau*3A *CEN3* fragment from pJC303-7 (CARBON and CLARKE 1984) into pLCJ. This *CEN3* fragment contains a 48-bp deletion within the element II region. pLCJ-A7 contains a unique *Aha*III site located in the middle of element II, providing a convenient cloning site for insertion of various DNA fragments within element II. To obtain random AT-rich sequences to insert within element II, a 1065-bp *Msp*I fragment from pS5-M4 (containing AT-rich mitochondrial DNA (FANGMAN and DUJON 1984)) was first partially digested with DNase I. Similarly, to obtain random pBR322 sequences for insertion within element II, a 622-bp *Msp*I fragment from pBR322 was partially digested with DNase I. The DNase treated DNA fragments were fractionated on 2% agarose gels, and the 50–100-bp size fragments were isolated, treated with Klenow polymerase I, and ligated into the unique *Aha*III site within pLCJS-A7. The DNA was used to transform *E. coli* strain JA226 and recombinant plasmids carrying either mitochondrial or pBR322 DNA were identified by restriction endonuclease analysis.

Construction of *CEN3* mutations—element I deletion: A 14-bp deletion spanning the conserved element I region of *CEN3* was constructed by oligonucleotide site-directed mutagenesis. A 27-mer deletion oligodeoxynucleotide, GAAAAATAGTACAAGATATTTGATTTT, was synthesized using an Applied Biosystems 380A DNA Synthesizer. The oligonucleotide was gel purified and then hybridized to pRNmp7-30-1 DNA at 25°. Hybridization and subsequent treatment with DNA polymerase I, T4 DNA ligase and transformation into strain JM101 were performed following the procedure of NEWMAN, OGDEN and ABELSON (1983). Plaques containing phage bearing a deletion of element I were identified by differential hybridization to the deletion oligonucleotide and an element I oligonucleotide (CAAATAAGTC(T,G,C)CATGATG) at 25° and 32°.

DNA sequencing: All element I and II mutations were subcloned into M13 phages mp7 or mp18. The sequence of each *CEN* mutation was determined by the method of SANGER, NICKLEN and COULSON (1977), using a *CEN*-specific oligonucleotide primer.

Genomic substitutions and Southern blots: Each *CEN3* construct was inserted into the centromere replacement vector pJC3-13 (CLARKE and CARBON 1983). Four of the mutant *CEN3* regions (303-2, 303-7, 303-17, and 303-16) were subcloned on *Rsa*I fragments into the *Bam*HI site of pJC3-13. The *Rsa*I sites lie 8 bp to the left of element I and 198 bp to the right of element III. The plasmids pJC303-31 and pSC303-100 were constructed directly in pJC3-13; in both cases, the *Bam*HI site of pJC3-13 lies to the immediate left of the element II sequences shown in Figure 1.

The wild-type copy of *CEN3* in chromosome *III* was replaced with the altered *CEN3* sequence using the fragment-mediated transformation method of ROTHSTEIN (1983). The *ura3/ura3* diploid strain SB51486 or SB121385 was transformed with a linear *Eco*RI fragment from pJC3-13. This *Eco*RI fragment contains the wild-type *URA3* gene, the altered *CEN3* sequence, and both the left and right flanking DNA surrounding *CEN3* (CLARKE and CARBON 1983). Transformation of the diploid with this fragment can both rescue the Ura$^-$ phenotype and replace the wild-type 624 bp *CEN3* region of chromosome *III* with the altered copy. SOUTHERN (1975) blot analysis of yeast genomic DNA was carried out as described in CLARKE and CARBON (1983).

Each *CEN3* mutation was also subcloned on a *Rsa*I fragment into the *Bam*HI site of pJC3-13-*ARS*. The plasmid pJC3-13-*ARS* was constructed by inserting a 624-bp *Xho*I fragment containing *ARS2* (TSCHUMPER and CARBON 1982) into the *Sal*I site of pJC3-13. The *CEN-ARS* plasmid DNAs were used to transform the *ura3* haploid strain 419-25d. All yeast transformations were done using the lithium acetate method (ITO *et al.* 1983). The resulting *URA3*$^+$ transformants were mated with either strain 514C or 843-9C to give diploid cells bearing the *CEN3-ARS* plasmids.

Mitotic analysis: The frequency of mitotic chromosome loss was measured by following the loss of the integrated *URA3* marker on chromosome *III* using a positive selection for Ura$^-$ auxotrophy (BOEKE, LA CROUTE and FINK 1984). Diploid cells (containing the *URA3*$^+$ altered *CEN3* substitution in one copy of chromosome *III*) were grown on YEPD to stationary phase. The cells were then plated at the appropriate dilutions on SD plates supplemented with adenine, uracil, and the required amino acids, in the presence and absence of 5-fluoro-orotic acid (FOA). Loss of orotidine-5′-phosphate decarboxylase activity (*URA3*) permits yeast to grow on media containing FOA (BOEKE, LA CROUTE and FINK 1984). The frequency of chromosome loss was calculated by determining the fraction of Ura$^-$ cells within the population.

Meiotic analysis: Yeast matings, sporulations and dissections were carried out as described previously (TSCHUMPER and CARBON 1983), with one modification; tetrads were incubated in 0.5% Zymolyase 100T at 25° for 30 minutes prior to dissection.

RESULTS

Construction of structurally altered *CEN* DNAs: We have chosen to examine six different *CEN3* constructs containing mutations in the conserved element I and II sequences. Each of the wild-type and structurally altered *CEN3* sequences was inserted into the *Bam*HI site of the centromere replacement vector pJC3-13; thus the DNA sequences surrounding the wild-type and mutant *CEN3* constructs are identical. The DNA sequences of the structurally altered centromeres are shown in Figure 1 and in diagrammatic form in Figure 3. Three of the *CEN3* constructs contain deletions within element I and II: pSC303-17 contains a 14 bp deletion spanning the element I region, pJC303-7 contains a 48-bp deletion within element II, and pJC303-31 has an 89-bp deletion which includes all of element I and most of the element II region. To examine the function(s) of the conserved features of the element II region (*i.e.*, the conserved length, the high A + T content, and the alternating blocks of A followed by blocks of T), we also constructed four *CEN3* plasmids that contain alterations in each of these characteristics. We have replaced 48 bp of element II in *CEN3* with 49 bp of mitochondrial DNA (pSC303-2). This 49-bp fragment is greater than 90% A + T; however, it consists primarily of alternating A and T residues and lacks the runs of A_{4-6} T_{4-6} found in the wild-type sequence. In pSC303-16, 48 bp of *CEN3* element II have been replaced by 57 bp of pBR322 sequences containing 44% A + T, thus lowering the A + T content of element II from 93 to 62%. One of the plasmids we constructed contains a fortuitous *Bam*HI site within element II (not shown). Plasmid pSC303-100 was constructed by deleting the *Bam*HI fragment extending from the *Bam*HI site in pJC3-13 to the new *Bam*HI site within element II in this plasmid. Thus, pBR322 sequences flanking the *Bam*HI site of pJC3-13 (CLARKE and CARBON 1983) occur immediately adjacent to the element II region in pSC303-100. This *CEN3* construct contains element III plus 10 bp of wild-type element II sequence and 37 bp of random A + T, but lacks element I. No fortuitous element I consensus sequences occur within the 340 bp of pBR322 sequences that flank element II in pSC303-100.

Genomic substitutions of *CEN3*: To assay the phenotype of each *CEN3* mutation *in vivo*, we have used fragment-mediated transformation (ROTHSTEIN 1983) of yeast diploids to replace the wild-type 624 bp *CEN3* sequence on one copy of chromosome *III* with the altered *CEN3* sequence. Each of the altered *CEN3* plasmids (pJC303-31, pJC303-7, pSC303-2, pSC303-17, pSC303-16, and pSC303-100) was digested with *Eco*RI to give a 5-kb fragment containing DNA sequences occurring to the left of *CEN3* in the genome, the *URA3* gene, the altered *CEN* sequence and DNA sequences to the right of *CEN3*. A *ura3/ura3* diploid yeast strain was then transformed with the various *Eco*RI fragments, giving Ura$^+$ transformants in which the 624 bp *CEN3* region of one copy of chromosome *III* has been replaced by the *URA3* gene and the structurally altered *CEN3* sequence (Figure 1) (CLARKE and CARBON 1983).

Confirmation of the genomic structure of the heterocentric substitution strains was obtained by hybridization experiments. Genomic DNA preparations isolated from the Ura$^+$ transformants and each of the 4 haploid meiotic progeny of the Ura$^+$ diploids were restricted with *Hin*dIII, electrophoresed on 1% agarose gels, blotted onto nitrocellulose filters and probed with a mixture of ^{32}P-labeled 624-bp *CEN3* DNA and ^{32}P-labeled pBR322 DNA. The *Hin*dIII digestion of genomic DNA isolated from diploids carrying the *CEN* substitution should yield a 6.9-kb

303-6							
GTACAAATAA	GTCACATGAT	GATATTTGAT	TTTATTATAT	TTTTAAAAAA	AGTAAAAAAT	AAAAAGTAGT	TTATTTTTAA
AAAATAAAAT	TTAAAATATT	AGTGTATTTG	ATTTCCGAAA	GTTAAAA			
303-31							
GT**********	**********	**********	**********	****AAAAAA	AGTAAAAAAT	AAAAAGTAGT	TTATTTTT**
**********	**AAAATATT	AGTGTATTTG	ATTTCCGAAA	GTTAAAA			
303-17							
GTACAA****	**********	GATATTTGAT	TTTATTATAT	TTTTAAAAAAA	AGTAAAAAAT	AAAAAGTAGT	TTATTTTTAA
AAAATAAAAT	TTAAAATATT	AGTGTATTTG	ATTTCCGAAA	GTTAAAA			
303-7							
GTACAAATAA	GTCACATGAT	GATATTTGAT	TTTATTATAT	TTTT******	**********	**********	**********
**********	**AAAATATT	AGTGTATTTG	ATTTCCGAAA	GTTAAAA			
303-2							
GTACAAATAA	GTCACATGAT	GATATTTGAT	TTTATTATAT	TTTTGATCGC	TTATATAATT	TATTATTATA	TATATATATA
TTATATTAAA	AATAAAATAT	TAGTGTATTT	GATTTCCGAA	AGTTAAAA			
303-16							
GTACAAATAA	GTCACATGAT	GATATTTGAT	TTTATTATAT	TTTTCTCATC	AGCGTGGTCG	TGAAGCGATT	CACAGATGTC
TGCCTGTTCA	TCCGCGTCCA	GAAAATATTA	GTGTATTTGA	TTTCCGAAAG	TTAAAA		
303-100							
**********	**********	**********	**********	****CCGCGT	TATATAATTT	ATTATTATAT	ATATATATAT
TATATTAAAA	TATTAGTGTA	TTTGATTTCC	GAAAGTTAAA	A			

FIGURE 1.—Nucleotide sequences of the structurally altered centromeres (*CEN3*) constructed in this study. The sequence of the top strand (elements I-II-III oriented from left to right) of wild-type *CEN3* (303-6) and each of the constructs are shown. Each sequence begins at the *Rsa*I cleavage site 10 bp to the left of element I; nucleotide #57 of the *CEN3* sequence shown in FITZGERALD-HAYES, CLARKE and CARBON (1982). These sequences were inserted into the centromere replacement vector pJC3-13. The rightward boundary of the 303-7, 303-2, 303-17, and 303-16 inserts lies at a *Rsa*I site located 198 bp beyond element III. The 303-6, 303-31, and 303-100 inserts extend 438 bp past element III to a *Bam*HI site [see FITZGERALD-HAYES, CLARKE and CARBON (1982) for a complete sequence of the *CEN3* region]. *Bold face:* 8 bp element I and 25 bp element III conserved sequences. *Stars:* nucleotides that have been deleted from the wild-type sequence. *Underline:* nucleotides that have been inserted or substituted into the wild-type sequence.

*Hin*dIII fragment containing the wild-type *CEN3* sequence and a 1.6-kb fragment containing the altered *CEN3* sequence (CLARKE and CARBON 1983). The results from Southern analysis of the 303-2 construction are shown in Figure 2. As expected, genomic DNA isolated from the untransformed parental diploid produces two *Hin*dIII fragments that hybridize to the *CEN3* +pBR322 probe (7.5 kb and 6.9 kb; lane a). The 7.5-kb band contains the pBR322 and *ADE1* sequences that are integrated near *leu2*, and the 6.9-kb band contains the wild-type *CEN3* sequence. As predicted from the structure of the transforming DNA fragments, hybridization of the *CEN3* +pBR322 probe to DNA isolated from the 303-2 diploid transformant (lane b) reveals three bands: the 7.2-kb and 6.9-kb bands plus the smaller 1.6-kb band. Genomic DNAs isolated from each of the four haploid meiotic progeny of the 303-2 strain were also subjected to Southern analysis (lanes c–f). As expected, two of the haploid progeny contain the 6.9-kb wt *Hin*dIII *CEN3* fragment and the other two contain the smaller 1.6-kb 303-3 *CEN* substitution fragment. Analogous results were obtained from Southern analysis of each of the mutant strains constructed in this work (data not shown).

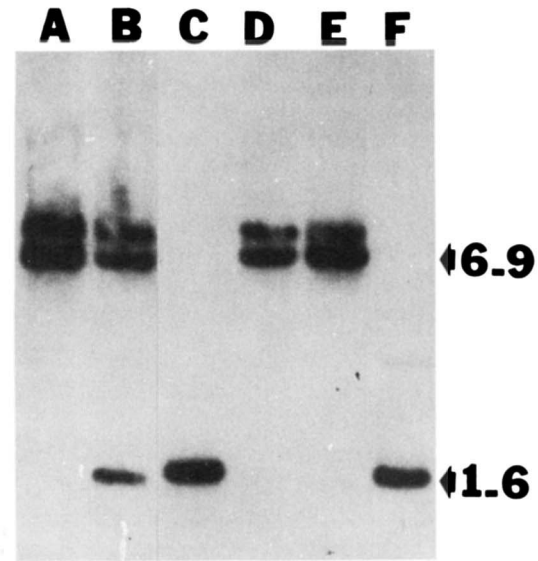

FIGURE 2.—Southern analysis of genomic DNAs from *CEN3* substitution strains. A blot hybridization autoradiogram of total yeast DNAs from the diploid transformant 303-2 and the haploid meiotic progeny is shown. All DNAs were restricted with *Hin*dIII. The blot was hybridized with a mixed probe containing ^{32}P-labeled pBR322 DNA and a 624 bp *CEN3* restriction fragment. Total genomic DNAs were from (lane A): the parent, untransformed diploid; (lane B): the 303-2 *URA3*$^+$ transformant; (lanes C–F): the four haploid progeny from a single tetrad produced by sporulation of the 303-2 diploid strain.

Mitotic analysis: The frequency of mitotic loss of chromosome *III* was measured for each of the altered *CEN3* diploids by following loss of the *URA3*$^+$ marker. Loss of the marked copy of chromosome *III* gives a 2n-1 strain that is Ura$^-$. Yeast strains lacking orotidine-5′-phosphate decarboxylase activity (*Ura3*$^-$) are able to grow on media containing FOA, whereas *URA3*$^+$ strains cannot (BOEKE, LA CROUTE and FINK 1984). Using growth on FOA media as a positive

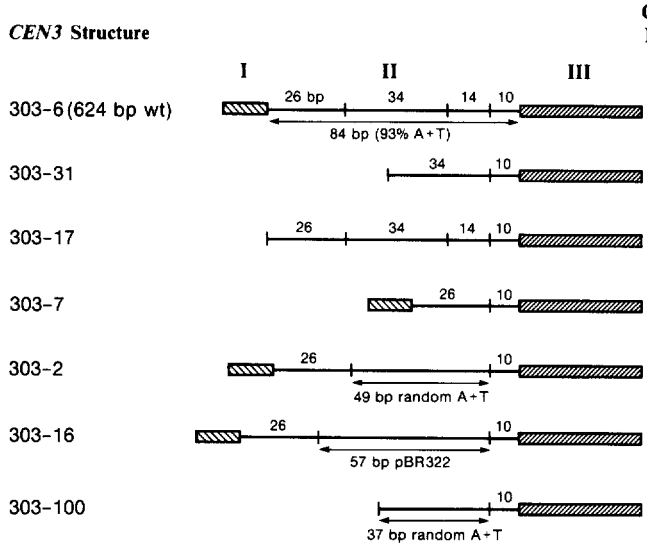

FIGURE 3.—Comparison of the mitotic and meiotic functions of various structurally altered centromeres. The shaded bars represent sequence elements I and III as indicated. The *Aha*III restriction sites within the element II region are shown (—|—). Distances are in base pairs. Assays for chromosome *III* loss and meiotic segregation in the *CEN3-ARS* plasmids are as described in the text and Table 4. The frequencies of chromosome *III* nondisjunction are the averages of three independent trials. The sister:nonsister values are the (PD+NPD):T ratios taken from Table 4.

selection for *Ura3* auxotrophy, we have calculated the maximum frequency of chromosome *III* loss by determining the fraction of Ura$^-$ cells within the population. Although the observed frequency of Ura$^-$ cells represents the sum of mitotic gene conversion and recombination at the *URA3* locus plus nondisjunction or loss of chromosome *III* to yield 2n-1 cells, less than 5 in 1×10^5 cells in the population are found to be Ura$^-$ without simultaneously losing markers on both arms of chromosome *III*. Therefore, we have estimated the maximum frequency of chromosome *III* loss for each of the altered *CEN3* diploids by determining the frequency of Ura$^-$ cells without correcting for the relatively minor contributions of mitotic recombination and gene conversion events (Figure 3). Chromosome nondisjunction during mitotic cell division occurs approximately once in 10^5 cell divisions (CLARKE and CARBON 1983). The rate of chromosome *III* loss in the 303-6 transformant, which contains the wild-type 624 bp *CEN3* sequence, is comparable to that of wild-type; the presence of the *URA3* gene immediately adjacent to the centromere sequences does not affect centromere function. Deleting element I plus half of the element II region (303-31) results in a 2000-fold increase in the frequency of chromosome *III* nondisjunction. However, deleting only the element I region (303-17) causes a relatively small drop in mitotic stability (20-fold). Evidently, the drastic loss of chromosome *III* stability observed for the 303-31 transformant is due primarily to the deletion within element II; the 303-7 construction (a 48-bp deletion within element II) shows a 400-fold increase in the frequency of mitotic nondisjunction of the altered chromosome *III*. Earlier studies, in which the mitotic stabilities of the chromosomes containing the 303-7 and 303-31 substitutions were determined by measuring the percentage of mating competent cells in the population, gave similar results (CARBON and CLARKE 1984).

To determine which structural features of *CEN3* element II are necessary for maintenance of proper chromosome segregation, we examined transformants containing several other *CEN3* mutations. The replacement of 48 bp of element II with a nearly equal length (49 bp) of another sequence rich in A + T (>90%) results in only a 4-fold drop in mitotic stability (303-2; Figure 3). However, when 48 bp of element II are replaced with a 57 bp DNA sequence containing only 44% A + T (303-16) the frequency of mitotic nondisjunction is increased 40-fold over wild-type levels. Neither of these two substitutions significantly alter the length of the element II region (wild-type element II sequences vary in length by 8 bp). The partial loss of function observed for the 303-2 and 303-16 centromere constructions can be attributed to changes in the sequential arrangement of A + T bases, and the total A + T content within element II, respectively. Centromere construction 303-100, containing the conserved element III sequence, 10 bp of the original element II, plus an adjacent stretch of 37 bp of random A + T nucleotides, shows significant mitotic stabilizing activity; chromosome *III* loss occurs only once in every 1000 cell divisions.

Meiotic analysis: We have also examined the meiotic behavior of each of the diploid transformants containing a structurally altered centromere. Diploid strains carrying one copy of chromosome *III* with the altered *CEN3* sequence in opposition to a chromosome *III* with wild-type *CEN3* (heterocentric substitutions) were sporulated and dissected. The haploid spores were analyzed using classical genetic techniques. The results are shown in Table 1. In all cases, the spore viability of the heterocentric constructions was very high (>85%). The frequency of recombination around the centromere region is not altered. The calculated map distances between the *LEU2* and *HIS4* loci and the *LEU2* and *MAT* loci on chromosome *III*

TABLE 1

Meiotic behavior of diploids containing heterozygous centromere mutations in chromosome *III*

a/α Diploid strain	Apparent map distance (cM)			Tetrads scored	Spore viability (%)
	URA3-MAT	LEU2-HIS4	URA3-LEU2		
303-6/*CEN3*	42	38	7	18	95
303-2/*CEN3*	70	45	7	24	87
303-16/*CEN3*	59	70	9	27	90
303-17/*CEN3*	92	55	8	19	96
303-100/*CEN3*	59	59	2	22	98

The heterocentric (*URA3*$^+$/*ura3*$^-$) diploids were constructed by transforming the strain SB121385 (**a** *leu2 trp1 his4 ura3-52/α ade1 met14 trp1 ura3-52*) with the centromere-substitution *Eco*RI fragments described in the text. The apparent map distances were calculated from recombinational frequencies as described by MORTIMER and HAWTHORNE (1966).

TABLE 2

Meiotic behavior of diploids containing homozygous centromere mutations in chromosome *III*

a/α Diploid strain	Percent sporulation	Tetrads scored	Spore viability (%)
SB42886 (parent)	62	18	95
303-2/303-2	46	36	95
303-2/303-17	65	18	94
303-17/303-17, cross 1	11	28	48
303-17/303-17, cross 2	0		
303-17/303-17, cross 3	0		
303-100/303-100	0		
303-16/303-16	50	30	83

The homocentric diploids were obtained by back-crossing selected *URA3*$^+$ haploid progeny from the diploid transformants shown in Table 1. The genotypes are 303-2/303-2: **a** his4 leu2/α *met14*; 303-2/303-17: **a** *his4 leu2/α met14*; 303-17/300-17 cross 1: **a** *met14 leu2/α his4*; 303-17/303-17 cross 2: **a** *met14 leu2/α his4*; 303-17/303-17 cross 3: **a** *leu2/α his4 met14*; 303-16/303-16: **a** *met14 his4/α leu2*; 303-100/303-100: **a** *met14 his4/α leu2*.

fall within the range observed for the untransformed parental strain. The map distances between the *CEN3*-linked *URA3* gene and *leu2* fall within the normal values for *leu2* to *CEN3*. Since *URA3* is now tightly centromere-linked, the segregation pattern of *URA3* and the centromere XI-linked *met14* marker was also scored. No URA3:*met14* tetratypes were observed in any of the tetrads examined. By these criteria, the heterocentric constructions containing 1 copy of an altered *CEN3* sequence appear phenotypically normal during meiotic cell division.

The meiotic behavior of chromosome *III* in diploids carrying homozygous *CEN3* mutations (homocentric constructions) was also assayed. Haploid progeny containing the altered *CEN3* sequence (from the heterocentric diploids) were backcrossed to construct homocentric diploids. Four *CEN3* mutations, 303-2, 303-16, 303-17, and 303-100 were chosen for analysis. The **a**/α diploids were sporulated, and when possible the resulting asci were dissected and analyzed (results are shown in Table 2). The 303-2/303-2, 303-16/303-16 and 303-17/303-2 diploids sporulate as efficiently as the parental strain. However, diploids containing some of the homocentric constructions tested (303-17/303-17 and 303-100/303-100) either yield asci with extremely poor spore viability or frequently do not sporulate at all. A diploid obtained by back-crossing Ura$^-$ haploid progeny (normal *CEN3*) of the 303-17 heterocentric diploid sporulates readily, however (87% spore viability). Although it appears likely that the nonsporulation phenotype is a consequence of the homocentric centromere mutations found within these strains, we cannot exclude the possibility that it is due to some other factor. CARBON and CLARKE (1984) have obtained sporulation-competent diploids containing homocentric *CEN3* substitutions with element I or II deletions. However, the resulting asci contain a high percentage of inviable spores and many of the viable progeny are disomic for chromosome *III*, indicating pronounced defects in meiotic segregation of the altered chromosome *III*.

To measure directly the frequency of precocious separation of the replicated sister chromatids in meiosis I, without the complications of insufficient sporulation and spore lethality, we have examined the meiotic behavior of *ARS* plasmids carrying the altered *CEN3* sequences. Wild-type *CEN-ARS* plasmids segregate through meiosis as minichromosomes (CLARKE and CARBON 1980). After replication, the sister plasmids migrate to the same pole during meiosis I, then separate and segregate to opposite poles during meiosis II. A variable fraction (60–90%) of the tetrads show 2+:2− segregation of the *CEN* plasmid; however, all of the tetrads in which 2+:2− segregation of the *CEN* plasmid is observed also show tight centromere linkage for the plasmid marker. That is, the plasmids always segregate to sister spores; tetratypes with respect to known centromere markers are seldom if ever observed.

To obtain diploid cells carrying plasmids with the altered *CEN3* sequence, we first subcloned each *CEN3* construct into the plasmid pJC313AR. The recombinant plasmid, containing the *URA3* marker, an autonomously replicating sequence (*ARS2*; TSCHUMPER and CARBON 1982) and the altered *CEN3* sequence, was

TABLE 3

Relative mitotic stabilities of various mutant *CEN3-ARS* plasmids

Plasmid	Percent Ura$^+$ cells after 9–10 generations of nonselective growth
pJC303-6A	94
pJC303-31A	12
pSC303-17A	81
pJC303-7A	9
pSC303-16A	24
pSC303-100A	78
pJC3-13A	2

All mitotic stability experiments were performed on plasmids transformed into *S. cerevisiae* strain 419-25d. Colonies were picked from selective media, grown for 9–10 generations in nonselective media (YPD) and plated for single colonies on YPD plates. The fraction of cells lacking plasmid was determined by replica-plating the colonies onto minimal medium lacking uracil. Plasmid pJC303-6A contains a wild-type *CEN3*, whereas pJC3-13A completely lacks a centromere.

used to transform the *ura3* haploid strain 419-25d. Before examining the meiotic behavior of the altered *CEN-ARS* plasmids, the relative mitotic stability of each plasmid was measured (Table 3). The Ura$^+$ transformants were then mated with a suitably marked parent, sporulated and dissected. Each tetrad was scored for the presence of *URA3* and *met14* (which is tightly centromere-linked on chromosome *XI* and thus identifies sister spores). As expected, asci dissected from strains carrying plasmids with a *CEN3* mutation show a much higher frequency of 0+:4− plasmid segregation than is typically seen with strains carrying wild-type *CEN-ARS* plasmids. This is probably due to the higher rates of mitotic loss (Table 3) of the plasmids with altered *CEN* sequences, however it is possible that the *CEN* mutations in some way increase the frequency with which the plasmids are totally lost in the two meiotic divisions.

Although all of the plasmids containing a *CEN3* mutation show occasional aberrant meiotic distribution patterns, four show very frequent tetratype segregation with respect to the *met14* centromere-linked marker (Table 4). Each of the *CEN3* plasmids that contain a deletion of element I (pSC303-17A, pSC303-100A, and pJC303-31A) or a deletion within element II (pJC303-7A) segregate aberrantly in more than 30% of the tetrads. A comparison between the mitotic and meiotic behavior of each of the altered centromeres is given in Tables 3 and 4 and Figure 3. Several of the *CEN* structural alterations that have only minor effects on the efficiency of mitotic chromosome or plasmid segregation strongly increase the frequency of tetratype plasmid segregation in meiosis. This is particularly true of mutations that delete the element I region. For example, chromosomes carrying a deletion of element I in *CEN3* (303-17) undergo mitotic nondisjunction only once in 10^4 cell divisions,

TABLE 4

Meiotic segregation of various mutant *CEN3-ARS* plasmids

Plasmid	Distribution in tetrads of the plasmid *URA3* gene					Centromere linkage of the plasmid *URA3* gene		
	4+:0−	3+:1−	2+:2−	1+:3−	0+:4−	PD	NPD	T
pJC 303-6A	6	1	25	1	15	12	13	0
pJC 303-31A	4	5	27	4	57	10	9	8
pSC 303-17A	2	3	16	1	6	2	6	8
pJC 303-7A	1	1	17	1	29	5	6	6
pSC 303-2A	0	0	11	1	18	6	4	1
pSC 303-16A	0	2	10	3	56	6	3	1
pSC 303-100A	2	0	4	3	41	1	0	3

In all crosses, the marker used to follow the plasmid (*URA3*) was wild type on the plasmid and mutant in both parents. The diploids used for sporulation were obtained by crossing the strain 419-25d/plasmid (**a** *leu2 trp1 his4 ura3-52/URA3*) with the strain 514C or strain 843-25d (both are *α adel met14 trp1 ura3-52*). The *CEN3* plasmid marker was scored *vs. met14* to determine centromere linkage. Abbreviations are: PD, parental ditype; NPD, nonparental ditype; and T, tetratype.

yet *CEN3-ARS* plasmids with the same element I deletion segregate randomly during meiosis. Deletion of 48 bp of element II leads to a tetratype distribution of the minichromosome in 35% of the 2+:2− tetrads. In contrast, however, *CEN3-ARS* plasmids with a substitution or insertion in element II segregate aberrantly in only 8–10% of the tetrads.

The unusual tetratype distribution of the mutant *CEN3-ARS* plasmids in the 2+:2− tetrads can be explained by either (1) precocious first division segregation of the replicated sister chromatids, or (2) simultaneous nondisjunction of two newly replicated *CEN-ARS* plasmids during meiosis II. Two lines of evidence indicate that precocious first division segregation is the correct model. First, the 303-2 and 303-17 constructs have roughly equivalent rates of mitotic nondisjunction, yet pSC303-17A segregates randomly during meiosis, while pSC303-2A segregates to nonsister spores in only 9% of the tetrads. It seems unlikely that the pronounced meiotic segregation defect could be explained by the low level of nondisjunction observed in mitosis, assuming that segregation in meiosis II is similar to a mitotic division. If pSC303-17A was present in multiple copies and undergoing frequent nondisjunction in meiosis II, then many of the tetrads should show 3+:1− plasmid segregation. In fact, only 10% of the tetrads scored show 3+:1− segregation (Table 4). Similarly, diploid cells harboring one copy of pSC303-17A that after replication fails to segregate properly in meiosis II would generate 1+:3− tetrads, yet only 4% of the tetrads scored show such a phenotype. Finally, CARBON and CLARKE (1983) have reported that 303-7 homocentric diploids show precocious first division segregation of the structurally altered chromosome *III* sister chromatids. Dip-

loid cells carrying the pJC303-7A plasmid also show frequent non-sister segregation of the plasmid in the 2+:2− tetrads.

DISCUSSION

We have examined the importance of the conserved element I and II sequences in centromere function during mitosis and meiosis. By altering the DNA sequence within the centromere on yeast chromosome *III*, we have been able to observe the effect of a variety of centromere mutations on the rate of chromosome *III* nondisjunction during mitotic cell division. We have measured the rate of chromosome *III* loss by following the loss of the integrated *CEN3*-linked *URA3* gene, using growth on media containing FOA as a positive selection for Ura$^-$ cells. This technique provides a simple and rapid method for measuring chromosome loss rates as low as 10^{-5} or as high as 10^{-1} per cell division.

Genomic substitution experiments have shown that a 289-bp *CEN3* fragment can act as a fully functional centromere in yeast (CLARKE and CARBON 1983). The highly conserved element III sequence of the centromere is essential for function; point mutations within the palindromic element III sequence can destroy all mitotic stabilizing activity (MCGREW, DIEHL and FITZGERALD-HAYES 1986; NG, CUMBERLEDGE and CARBON 1986). Element III is thought to be a protein binding site, necessary for the assembly of a functional kinetochore. Mutations in either of the conserved element I or II sequences can also affect mitotic function. In fact, the element II region of the centromere is essential for optimal mitotic function; deleting 48 bp of element II increases the rate of chromosome nondisjunction by 1000-fold (CARBON and CLARKE 1984). However, unlike the element I and III regions whose nucleotide sequences are strongly conserved, only certain structural features of the element II region are conserved, principally the overall length of the high A + T region, and the grouping of A residues in short consecutive runs, rather than in a random array. Structural alterations that affect these features reduce the efficiency of centromere function, yet the exact integrity of element II is not essential. The most important features of the element II region appear to be the overall length and the A + T content. We have found that changing the pattern of the A and T nucleotides, *e.g.*, eliminating the short runs of A_{4-7} T_{4-7} without reducing the overall A + T content or the length, increases the rate of chromosome *III* loss by only 4-fold, while reducing the overall A + T content of element II to 62% increases the rate of chromosome *III* loss 40-fold. Recently, GAUDET and FITZGERALD-HAYES (1987) have made similar observations. Our findings are in agreement with the interpretation that the minimum requirements for mitotic function include an element III sequence and an adjacent stretch of A + T residues (CARBON and CLARKE 1984). Recent studies in our laboratory (NG, NESS and CARBON 1986) suggest that the element II region in *CEN3* and *CEN14* is bent or "kinked," a property typical of double-stranded DNAs containing regularly spaced tracts of A and T residues (KOO, WU and CROTHERS 1986). It seems likely that the element II region may form a secondary structure whose conformation, rather than specific nucleotide sequence, is necessary for kinetochore assembly and/or function.

Unlike elements II and III, element I is not absolutely required for centromere function during mitosis, but rather confers only a small enhancing effect on the efficiency of chromosome segregation. Deleting the entire element I region increases the rate of chromosome nondisjunction by only 20-fold. However, element I is absolutely required for proper meiotic function of the centromere (see below). Interestingly, the conserved eight base pair element I sequence occurs not only at the centromere but also in the 5′-untranslated region upstream of several transcription units (BRAM, LUE and KORNBERG 1986) and in the bovine 1.706 g/cm^3 satellite DNA (within the 23-bp repeat) (FITZGERALD-HAYES, CLARKE and CARBON 1982). BRAM and KORNBERG (1987) have recently identified a DNA binding protein from yeast and its counterpart from humans. This protein binds strongly to the element I consensus sequence. They postulate that it may enhance or facilitate the binding of other proteins at nearby sites on the DNA. The significance of this DNA binding protein in centromere function is unclear, however.

Certain structural alterations in the conserved element I or II sequences can produce a dramatic effect on centromere meiotic function without substantially affecting the fidelity of mitotic chromosome segregation. While heterocentric diploids, carrying mutations in the element I and II regions undergo relatively normal chromosome segregation during meiosis I (CARBON and CLARKE 1984; this work), the meiotic behavior of homocentric diploids, in which both copies of chromosome *III* contain the same mutation, is more complex. Homocentric diploids containing alterations in the nucleotide sequence of element II, but not in the length of element II, are phenotypically normal. That is, altering the periodic repetition of A residues or lowering the A + T content in the element II region does not significantly alter chromosome *III* segregation during meiosis I when assayed in either heterocentric or homocentric diploids. However, all of the homocentric diploids we have examined that contain a deletion of element I or a portion of element II either sporulate poorly or show extremely low spore viability. These homocentric diploids may also be defective in chromosome *III* segregation during

meiosis I. This hypothesis is supported by two observations: (1) structurally altered *CEN3-ARS* plasmids containing these same element I or element II deletions frequently segregate to nonsister spores (as discussed below); and (2) previous studies (CARBON and CLARKE 1984) on *CEN3*-altered chromosome *III* homocentric diploids have indicated that the sister chromatids, containing deletions in element II or both I and II, prematurely separate during the first meiotic division. It is unclear how the presence of a homolog containing the wild-type centromere is able to complement the defective *CEN* sequence in *trans*. Perhaps, when in a heterocentric configuration, the defective sister chromatids segregate normally during meiosis I by default; the wild-type chromosome *III* sister chromatids may bind to the spindle apparatus and undergo normal segregation, leaving the prematurely separated altered chromosomes behind. In the second meiotic division, segregation of the altered chromosomes would be relatively normal, since mitotic function is not drastically impaired by these *CEN* mutations. In this model, sister chromatids from both homologous chromosomes must be prematurely disjoined before random segregation may occur in meiosis I.

By following the segregation pattern of altered *CEN3-ARS* plasmids during meiosis we have been able to measure the frequency of precocious sister chromatid separation during meiosis I. This plasmid assay offers some advantages over the meiotic analysis of mutationally altered homocentric diploids, which can be complicated by low sporulation levels and high spore lethality. These studies indicate the element I region is more intimately involved in centromere function during meiosis I than during meiosis II or mitosis. All of the *CEN3* mutations we have examined that contain a deletion of the element I sequence show frequent premature sister chromatid separation in meiosis I. PANZERI *et al.* (1985) have examined the meiotic segregation of two plasmids that each contain a deletion of the element I region. One of the altered *CEN4-TRP1* plasmids shows a high frequency of tetratypes with respect to *ura3* and *MAT*, the other shows almost perfect segregation to sister spores in meiosis I. However, since the markers used to identify sister spores (*ura3* and *MAT*) are not as tightly *CEN*-linked as the *met14* marker used in our studies, the exact frequency of nonsister segregation of the altered *CEN4* plasmids would be difficult to determine. Deletions within the element II region also induce random segregation of the sister chromatids in the first meiotic division. Deleting 48 bp within the element II region of *CEN3* (303-7) increases the rate of mitotic chromosome loss to one in 100 cell divisions, yet *CEN3-ARS* plasmids containing this same element II deletion segregated to nonsister spores in 35% of the tetrads scored.

In meiosis I, the sister chromatids must remain together, bound at the centromere while they are paired and sorted. Subsequently during meiosis II the sister chromatids are segregated. The exact function of the sequence elements I and II in maintaining attachment of the replicated chromatids is still unclear. It is possible that the element I sequence is a binding site for a *trans*-acting factor that maintains the association of the centromeres of the sister chromatids by acting on both DNA molecules. Alternatively, the binding of a *trans*-acting factor at element I may delay DNA replication through the centromere, forcing the sister chromatids to remain attached during meiosis I (TSCHUMPER and CARBON 1983), or prevent topoisomerase action to untangle the replicated chromatids. The existence of *trans*-acting factors specific for meiosis I has been demonstrated in Drosophila. Two mutants, *mei-S322* and *ord* are known to cause the precocious separation of sister chromatids during meiosis I, leading to frequent equational (sister chromatid) nondisjunction (DAVIS 1971; MASON 1976; GOLDSTEIN 1980). Both of these mutations affect segregation of all of the chromosomes, and are thought to be involved in the maintenance of centromere structure or the process of sister-centromere separation during the first meiotic division.

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