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Alterations in the Adenine-plus-Thymine-Rich Region of *CEN3* Affect Centromere Function in *Saccharomyces cerevisiae*

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Centromere DNA from 11 of the 16 chromosomes of the yeast *Saccharomyces cerevisiae* have been analyzed and reveal three sequence elements common to each centromere, referred to as conserved centromere DNA elements (CDE). The adenine-plus-thymine (A+T)-rich central core element, CDE II, is flanked by two short conserved sequences, CDE I (8 base pairs [bp]) and CDE III (25 bp). Although no consensus sequence exists among the different CDE II regions, they do have three common features of sequence organization. First, the CDE II regions are similar in length, ranging from 78 to 86 bp measured from CDE I to the left boundary of CDE III. Second, the base composition is always greater than 90% A+T. Finally, the A and T residues in these segments are often arranged in runs of A and runs of T residues, sometimes with six or seven bases in a stretch. We constructed insertion, deletion, and replacement mutations in the CDE II region of the centromere from chromosome III, *CEN3*, designed to investigate the length and sequence requirements for function of the CDE II region of the centromere. We analyzed the effect of these altered centromeres on plasmid and chromosome segregation in *S. cerevisiae*. Our results show that increasing the length of CDE II from 84 to 154 bp causes a 100-fold increase in chromosome nondisjunction. Deletion mutations removing segments of the A+T-rich CDE II DNA also cause aberrant segregation. In some cases partial function could be restored by replacing the deleted DNA with fragments whose primary sequence or base composition is very different from that of the wild-type CDE II DNA. In addition, we found that identical mutations introduced into different positions in CDE II have very similar effects.

Successful cell division requires a highly reliable mechanism for the transmission of genetic information. We are only beginning to understand the molecular mechanisms of spindle formation and chromosome segregation. The interaction between the chromosome and the spindle fiber takes place at a specialized region of the chromosome called the centromere. Presumably specific DNA-binding proteins interact with the centromere DNA (*CEN*) to form a complex, the kinetochore, that is the site of attachment to the spindle fiber microtubule.

Functional centromeric DNA has been isolated from several chromosomes of the yeast *Saccharomyces cerevisiae*. These cloned *CEN* DNAs allow autonomously replicating plasmids to be maintained stably at low copy number during mitotic cell growth and to segregate properly during meiotic cell division (7, 10).

Centromere DNAs from different yeast chromosomes are similar in nucleic acid sequence and are functionally interchangeable (8). Sequence information from 11 of the 16 yeast centromeres is now available (12, 15, 20, 26, 31) and allows the conserved centromere sequence elements (Fig. 1) to be defined. Centromere DNA element I (CDE I) is an 8-base-pair (bp) sequence (5′-PuTCACPuTG-3′) present in all 11 *CEN* DNAs. The adenine-plus-thymine (A+T)-rich central core segment (CDE II) is 78 to 86 bp in length. The CDE III homology encompasses 25 bp and has the consensus sequence 5′-TGT-T-TG--TTCCGAA-----AAA-3′ (15). A deletion removing CDE I, II, and III abolishes *CEN* function, demonstrating that the conserved sequences are important for plasmid (11) and chromosome (8) stability. We showed (23) that single point mutations in conserved element III DNA can cause dramatic chromosome loss during mitosis. This suggests that at least one of the conserved sequence elements is indeed required for centromere function and probably defines sites for binding of the protein(s) involved in the actual spindle fiber attachment. Several investigators have proposed that a chromatin-protein complex containing these sequences functions as the yeast equivalent of a mammalian kinetochore (2–4, 11).

Although the central CDE II core segment is extremely A+T rich in the 11 known yeast centromeres, no statistically significant consensus sequence exists. However, the CDE II regions do have three common features of sequence organization. First, the A+T-rich regions are similar in length, extending from 78 to 86 bp from CDE I to the left boundary of CDE III (84 bp in *CEN3*; Fig. 1). Second, the base composition is always greater than 90% A+T. Finally, the A and T residues in these sequences are often arranged in runs of A and runs of T residues, sometimes with six or seven bases in a stretch.

We constructed mutations in the CDE II region of *CEN3*, the centromere from chromosome III, which were designed to investigate the length and sequence requirements for function of this segment of centromere DNA. We made a collection of CDE II insertion, deletion, and replacement mutations in *CEN3* DNA and analyzed the effect of these altered centromeres on plasmid and chromosome segregation in *S. cerevisiae*. We chose to work with *CEN3* because the CDE II region contains only three *Aha*III sites (Fig. 2), thereby simplifying the mutagenesis procedure.

When we began this work we fully expected that the CDE II region of yeast *CEN3* could not be altered without a significant loss of centromere function. The extreme conservation of length, high A+T base composition, and stretches of A and T sequence organization suggested that these characteristics might be critical for mitotic chromosome segregation. Instead we found that the CDE II region could be doubled in length and the altered centromere DNA still

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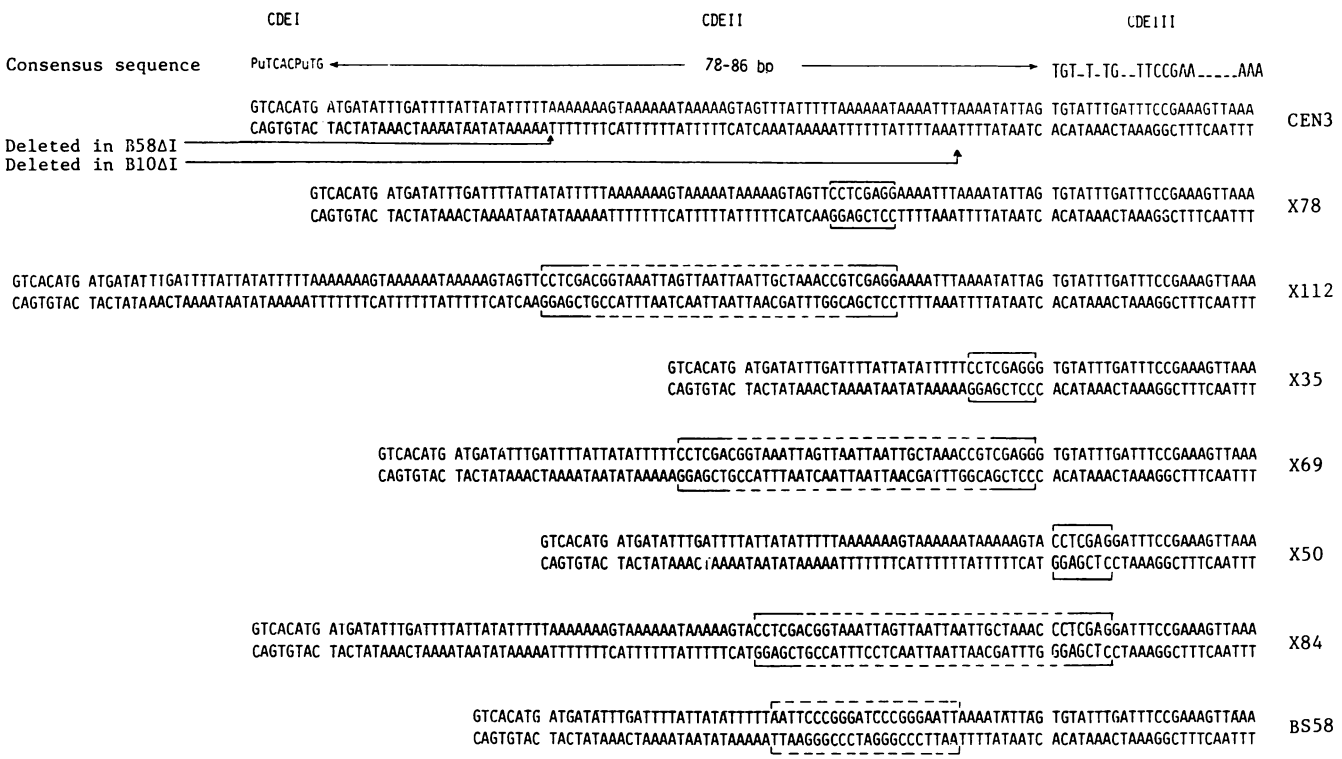

FIG. 1. Summary of deletion and replacement mutants. The organization of conserved centromere elements CDE I, II, and III is shown at the top of the figure. The CDE III consensus sequence represents nucleotide positions that are conserved in 8 of the 11 centromere DNAs analyzed (15). The sequence of the wild-type centromere DNA from chromosome III is shown, and the *CEN3* DNA deleted in mutants B58ΔI and B10ΔI is indicated by arrows on the sequence. Note that these mutants are also deleted for CDE I. The solid brackets indicate the *Xho*I linkers at the endpoints of the deletions in X78, X50, and X35. The 34-bp oligonucleotide insertions in replacement mutants X112, X69, and X84 are enclosed by dashed lines, as is the 22-bp palindrome insertion in BS58. The deletion mutation in X50 removes 34 bp of CDE II and the first seven bp of CDE III.

retained the ability to assemble into a partially functional kinetochore. Deletion mutations removing CDE II sequences proved to have a more deleterious effect. We found that the deleted sequences could be replaced with segments of DNA which have little similarity to the wild-type arrangement of A and T residues and still form a partially functional mitotic centromere. Also, insertion of identical 46-bp sequences into different positions in CDE II had very similar effects.

MATERIALS AND METHODS

Bacteria and yeast strains. *Escherichia coli* JM16 (S1179: F⁻ Δ*lacU169 dam-3 rpsL*) was used for transformation in the construction of the *Bcl*I linker insertion mutants. *E. coli* JM1 [F⁻ Δ*lacx74 araD139* Δ(*araBOIC-leu*)*7679 galU galK rpsL*] was used for the selection of kanamycin-resistant transformants carrying K1000-1 or K1000-3. *E. coli* JM14 (SK1592: F⁻ *gal* T1ʳ *endA sbcB hsdR4 hsdM*⁺) was used for transformation and amplification of all other plasmids. JM103 [Δ(*lac-pro*) *supE thi rpsL endA sbcB15 hsdR4*/F′ *traD36 proA*⁺*B*⁺ *lacI*ᵠ Δ*lacZM15*] was used as the host for M13 cloning and DNA sequencing. *S. cerevisiae* SB9882-4CR (*MATα*/*MAT***a** *trp1-289*/*trp1-289 ura3-52*/*ura3-52 LEU2*/*leu2-3,112 HIS4*/*his4-519*) (8) and SB7883-1C (*MATα*/*MAT***a** *trp1-289*/*trp1-289 ura3-52*/*ura3-52 LEU2*/*leu2-3 CRY1*ˢ/*cry1*ʳ *met14*/*MET14 ade1*/*ADE1*) were used for the genomic substitution experiments and YP47 (*MATα ura3-52 ade2-101 lys2-801* Δ*trp1* [*psi*⁻]) was used as the host for the plasmid transformations and plasmid mitotic stability assays.

Media and enzymes. Media for bacterial and yeast growth were as described previously (16). Restriction endonucleases, RNA ligase and T4 DNA ligase, polynucleotide kinase, and Klenow DNA polymerase I were from New England BioLabs, Inc. (Beverly, Mass.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and Promega Biotech (Madison, Wis.). Buffers and reaction conditions were as specified by the manufacturer. [α-^{32}P]ATP was purchased from Amersham Corp., (Arlington Heights, Ill.). The M13 cloning protocols and dideoxynucleotide sequencing reactions were as described by Amersham Corp. and Sanger et al. (29). Dideoxynucleotides were purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.). Most plasmid cloning procedures and DNA manipulations were performed as described by Maniatis et al. (21).

Construction of CDE II centromere mutations. Most of the centromere mutations described in this study were constructed from the parent plasmid, pYe(*CEN3*)30 (12). This plasmid contains the 624-bp *CEN3 Sau*3A fragment cloned into the *Bam*HI site of YRp7′ (32). Many of the mutations we constructed in *CEN* DNA made use of the three *Aha*III sites located in CDE II (Fig. 2). All the mutants were named according to the length of their CDE II regions (measured from CDE I to CDE III as shown in Fig. 1).

Insertion mutations. To construct insertion mutants B92-1, B92-2, and B108-2, pYe(*CEN3*)30 plasmid DNA was partially digested with *Aha*III to produce linear molecules and then ligated to 8-bp *Bcl*I linkers (5′-CTGATCAG-3′; Collaborative Research, Inc., Waltham, Mass.). After ligation, the

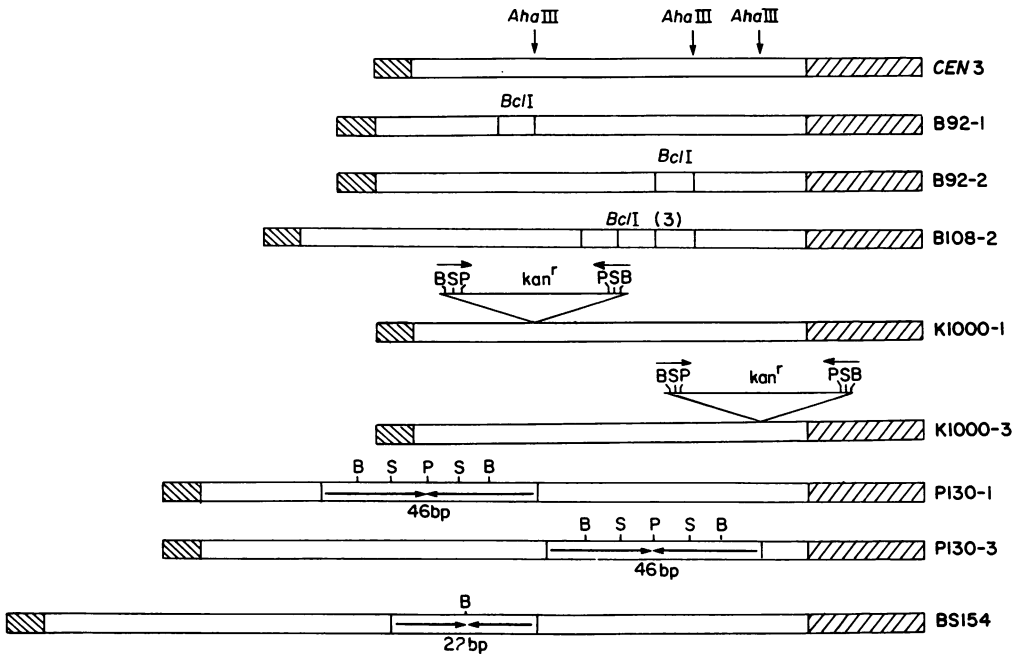

FIG. 2. Summary of insertion mutants. The relative positions and sizes of insertions into CDE II are shown. CDE I and III are indicated by hatched boxes. Mutants B92-1, B92-2, and B108-2 were constructed by inserting *Bcl*I linkers into the *Aha*III sites (5'-TTTAAA-3') indicated in CDE II. The positions of restriction sites for *Bam*HI (B), *Sal*I (S), and *Pst*I (P) are indicated. Palindromic sequences are indicated by arrows. Details of the mutant constructions are given in Materials and Methods.

reaction mixture was treated with *Bcl*I and then religated before transformation of *E. coli* JM16. DNA was prepared from ampicillin-resistant transformants and screened by restriction enzyme digestion to identify the correct plasmids. Centromere mutant B92-1 was shown by DNA sequence analysis (29) to contain one *Bcl*I linker in the first *Aha*III site (leftmost in CDE II; Fig. 2), B92-2 has one linker in the second *Aha*III site, and B108-2 has three linkers in the second *Aha*III site.

Larger insertions were created by blunt-end ligation of a 1.1-kilobase DNA fragment conferring kanamycin resistance (24) onto pYe(*CEN3*)30 *Aha*III linear molecules to produce mutants with the Kan^r fragment inserted into either the first (K1000-1) or the third (K1000-3) *Aha*III site. Mutants P130-1 and P130-3 were constructed by cleaving K1000-1 or K1000-3 plasmid DNAs at the *Pst*I sites flanking the Kan^r inserts. Religation resulted in retention of a 46-bp segment of the polylinker sequences in either the first (P130-1) or the third (P130-3) *Aha*III site in CDE II. The 46-bp insertion has the palindromic sequence 5'-AATTCCCGGGATCCGT CGACCTGCAGGTCGACGGATCCCGGGAATT-3', and is 61% guanine plus cytosine (G+C) and 39% A+T in base composition.

BS154 and BS58 were constructed by digesting P130-1 and P130-3 plasmid DNAs to completion with *Bam*HI and *Sal*I, mixing them together, religating, and transforming *E. coli* JM14. Plasmid DNAs isolated from ampicillin-resistant transformants were screened for the desired constructions by restriction digestion. BS154 has the *Bam*HI-*Sal*I fragment containing CDE I from P130-3 and the *Bam*HI-*Sal*I fragment containing CDE III from P130-1. This resulted in a duplication of 48 bp of CDE II DNA plus 22 bp of polylinker (5'-AATTCCCGGGATCCCGGGAATT-3'), thus enlarging CDE II to 154 bp. BS58 is the converse construction which removed 48 bp of CDE II and added 22 bp of polylinker, thereby shortening CDE II to 58 bp.

Deletion mutations. Two deletion mutants were derived from K1000-1 and K1000-3 (Fig. 2) by using the *Bam*HI site in the polylinker region flanking the Kan^r insert. B58ΔI was constructed by digesting K1000-1 with *Bam*HI and ligating the fragment containing 58 bp of CDE II, all of CDE III, and the remainder of the 624-bp yeast DNA (but deleted for CDE I) into the *Bam*HI site of YRp7'. B10ΔI was constructed by cloning the corresponding *Bam*HI fragment of K1000-3 (containing 10 bp of CDE II, all of CDE III, and the remainder of the 624 bp of yeast DNA) into the *Bam*HI site of YRp7'.

Additional deletion mutations were constructed by digesting plasmid B92-2 (Fig. 2) to completion with *Bcl*I and treating the linear molecules with *Bal* 31 exonuclease. The DNA was then incubated with Klenow DNA polymerase I to repair the ends resulting from *Bal* 31 digestion and ligated to 8-bp *Xho*I linker DNA (5'-CCTCGAGG-3'; Collaborative Research Inc.). After the excess linkers were trimmed off by digestion with *Xho*I, the linear molecules were religated and used to transform *E. coli* JM14. DNA was prepared from single-colony isolates, and the desired deletion mutations were verified by cloning into M13 vectors for dideoxynucleotide sequencing (29). Three deletion mutants were chosen for further study (Fig. 1): X78 (a 14-bp deletion and 8-bp *Xho*I linker insertion), X35 (a 57-bp deletion and *Xho*I linker addition), and X50 (a 41-bp deletion and *Xho*I linker insertion in which seven bp of CDE III were deleted).

Replacement mutations. The unique *Xho*I restriction sites in the deletion mutants were used to construct derivatives in which the deleted CDE II DNA was replaced with short synthetic oligonucleotides. For the replacement fragment we used a 34-bp oligonucleotide (generously provided by

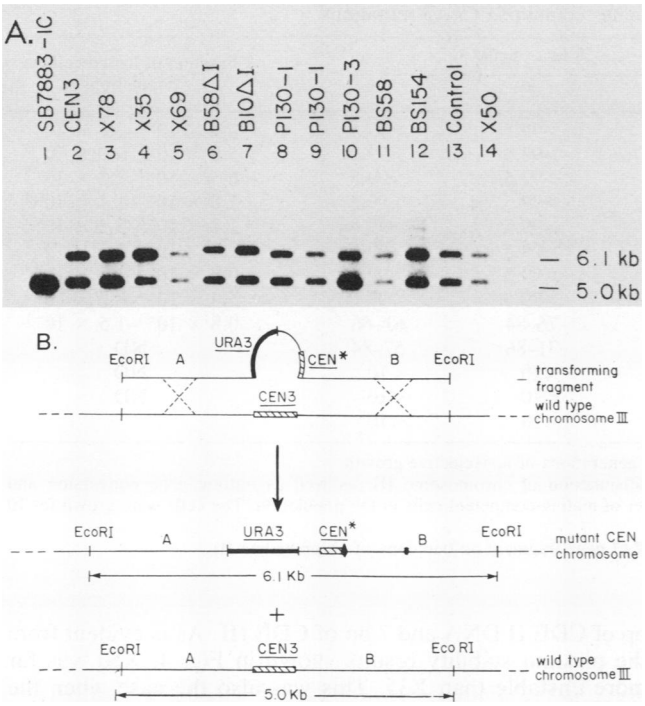

FIG. 3. Southern blot of genomic substitutions in chromosome III. (A) Total yeast DNA was prepared from each genomic transformant and subjected to Southern analysis. *Eco*RI-cut DNA from the untransformed diploid yeast strain SB7883-1C (lane 1) shows the 5.0-kilobase (kb) band expected for the wild-type copies of chromosome III. Lanes 2 to 12 and lane 14 show *Eco*RI-cut DNAs from transformed strains which have a mutant centromere and the *URA3* gene transplaced into one copy of chromosome III resulting in an *Eco*RI fragment of about 6.1 kilobases. The 900-bp *Bam*HI-*Hin*dIII fragment from flanking region B (shown in panel B) was radioactively labeled by nick translation with [α-^{32}P]ATP and used as a hybridization probe in these experiments. The control in lane 13 is *Eco*RI-cut DNA isolated from a previously characterized chromosome III genomic substitution strain. (B) For each construction the DNA fragment containing the mutant centromere was cloned into the *Bam*HI site of JC313. Yeast cells from diploid strain SB7883-1C were transformed with *Eco*RI-digested DNA from each of the *CEN**-JC313 derivatives. Recombination between the homologous flanking regions A and B of one resident copy of chromosome III and the incoming *Eco*RI restriction fragment results in replacement of a wild-type centromere with a mutant centromere plus *URA3* gene; the other copy of chromosome III remains unaltered. Not all *Bam*HI (◆) or *Hin*dIII (●) sites are shown.

Fumikyo Nagawa and Gerald Fink) with the sequence 5′-TCGACGGTAAATTAGTTAATTAATTGCTAAACCG-3′ (68% A+T) which was inserted by its *Sal*I ends into the *Xho*I site in the mutants. In this case the ligation reaction was digested with *Xho*I before transformation to linearize molecules which did not receive insertions. This was possible since the junction of the *Sal*I and *Xho*I ends produces a hybrid sequence which is not recognized by either enzyme. The three replacement mutants obtained by this approach are shown in Fig. 1 (X112, X69, and X84).

Plasmid and chromosome mitotic stability analyses. Centromere function was assessed for plasmid borne centromeres as well as in the genomic replacement strains by mitotic stability tests essentially as described in reference 23. Samples were removed for plating at 1 and 5 generations in the plasmid assays and at 7 and 13 generations in the chromosome assays. In these experiments approximately 70 to 80% of the cells retained a wild-type centromere plasmid while less than 10% of the cells retained the acentric YRp7′ plasmid after five to six generations of nonselective growth.

Genomic substitution vector and yeast transformation. To compare the effect of the mutations on plasmid and chromosome segregation, we analyzed the segregation of intact chromosome III copies bearing mutant centromeres (*CEN**). These strains were constructed by transforming *S. cerevisiae* with derivatives of the genomic substitution vector JC313 (provided by John Carbon [8]), which contains regions which flank the centromere on chromosome III (designated A and B in Fig. 3B), and a selectable gene, *URA3*. For fragment-mediated, site-directed targeting of these mutations into chromosome III, the JC313-*CEN** derivatives were cleaved with *Eco*RI, and the entire reaction was used to transform (17) yeast strain SB7883-1C to Ura$^+$ prototrophy (Fig. 3B) (28).

JC313-*CEN** derivatives were constructed as follows. *Bam*HI fragments containing centromere mutations B58ΔI and B10ΔI were cloned into the *Bam*HI site of JC313. B92-2 was recloned into JC313 on a *Hin*dIII-*Bam*HI fragment. The remaining genomic replacements, including wild-type *CEN3* as a control, were constructed by cloning the centromere-containing *Alu*I fragment bearing either *Bam*HI or *Bcl*I linkers into the *Bam*HI site of JC313. The 308-bp *Alu*I fragment of wild-type *CEN3* DNA was previously shown (5) to be functionally equivalent to the 624-bp *Sau*3A-*Bam*HI fragment contained in pYe(*CEN3*)30 (12). Potential integrants were streaked for single colonies on selective medium (without uracil), and the nature of the integration event was verified by Southern analysis (Fig. 3).

Quantitative mating experiments. Genomic transplacement strains which were indistinguishable from wild type by mitotic stability analysis (>99.5% Ura$^+$) were then tested by quantitative mating (9, 13). This assay is a more sensitive measure of chromosome stability and is used to detect aneuploid cells which have lost one copy of chromosome III and therefore have simultaneously become mating competent ($2n - 1$: *MAT***a** or *MAT*α). For this test a sample of diploid cells to be tested was grown nonselectively for 10 generations. Cells (3×10^6, determined by hemacytometer) were mixed with an excess (4×10^6 to 5×10^6) of an appropriate haploid tester strain of each mating type, either A544 (*MAT*α *ura2 gal mal*) or A545 (*MAT***a** *ura2 gal mal*). The cell mixture was filtered onto sterile filters (0.45-μm pore size; Millipore Corp., Bedford, Mass.) which were incubated cell-side up on YPD (2% peptone, 1% yeast extract, 2% glucose) plates for 4 h at 32°C. The cells were removed from the filters by vortexing in 5 ml of 1 M sorbitol, pelleted, and suspended in sterile water. Aliquots of serial dilutions were plated onto minimal agar plates (Difco yeast nitrogen base containing 2% glucose) so that only mated cells grew. Colonies were counted after 2 days of growth at 32°C. Usually two individual transformants were analyzed for each type of mutant. Quantitative mating assays were performed on two samples for each transformant, and the ranges of values obtained are reported in Table 1.

Southern transfer and hybridization. Total DNA was prepared from individual, single-colony-purified yeast transformants, digested overnight with *Eco*RI, and then electrophoresed through 0.7% agarose gels. The DNA was transferred to nitrocellulose paper (BA85; Schleicher & Schuell, Inc., Keene, N.H.) as described by Southern (30). The 900-bp *Bam*HI-*Hin*dIII DNA fragment from flanking region B was isolated from JC313 DNA or an equivalent plasmid by electroelution (International Biotechnologies,

TABLE 1. Mitotic stability of chromosomes containing *CEN3* mutations

Centromere	Length of CDE II (bp)	Ratio of A+T in CDE II (%)	% Ura$^+$ cells[a]			Fraction of mated cells in population[b]
			0	7	13	
CEN3[c]	84	93	>99.5	>99.5	>99.5	0.4×10^{-5}–2.7×10^{-5}
X78	78	84	>99.5	>99.5	>99.5	5.0×10^{-5}–8.0×10^{-5}
B92-2	92	89	>99.5	>99.5	>99.5	6.5×10^{-5}–7.5×10^{-5}
P130-3	130	74	>99.5	>99.5	>99.5	1.0×10^{-4}–1.1×10^{-4}
P130-1	130	74	>99.5	>99.5	>99.5	1.2×10^{-4}–3.3×10^{-4}
BS154	154	86	>99.5	>99.5	>99.5	1.3×10^{-3}–2.3×10^{-3}
X69	69	71	>99.5	>99.5	>99.5	0.8×10^{-3}–1.4×10^{-3}
BS58	58	75	>99.5	>99.5	97	5.4×10^{-3}–8.2×10^{-3}
X35	35	74	88–91	76–84	61–66	0.8×10^{-1}–1.5×10^{-1}
B58ΔI	58	93	86–90	71–86	57–84	ND
B10ΔI	10	90	34	<10	<10	ND
X50	50	86	0–12	<10	<10	ND
JC313[d]			10	<10	<10	

[a] Mitotic stability is expressed as the percentage of Ura$^+$ cells after 0, 7, and 13 generations of nonselective growth.
[b] The more sensitive quantitative mating assay reflects 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 in the population. The cells were grown for 10 generations before mating. ND, Not determined.
[c] This strain contains a 308-bp wild-type *CEN3 Alu*I fragment adjacent to the *URA3* gene fragment on one copy of chromosome III.
[d] Acentric.

Inc., electroelution apparatus) from polyacrylamide gels and labeled for hybridization by nick translation with [α-^{32}P]ATP (6, 27). Autoradiography was performed by exposing Kodak SR5 film with a Du Pont Cronex Lightning-Plus intensifying screen for 24 to 72 h at −80°C.

RESULTS

Deletions in CDE II reduce centromere function. The DNA sequences of several centromere deletion mutants are shown in Fig. 1. The deletion mutation in B58ΔI removed CDE I and the first 26 bp of CDE II. Analysis of the function of this and similar mutations made in centromeres from other yeast chromosomes (5, 18, 22, 25) demonstrates the importance of CDE I in centromere behavior. The B58ΔI mutation reduced plasmid stability by about 50% and decreased chromosome stability significantly (Fig. 4; Table 1). Despite this unstable behavior, B58ΔI still retained more centromere function than B10ΔI, which lacks an additional 48 bp of CDE II (Fig. 4; Table 1). The difference in function observed for these two mutants emphasizes the importance of CDE II function in centromere behavior.

Deletion mutations that remove 14 (X78), 41 (X50), or 57 (X35) bp of A+T DNA from CDE II without affecting CDE I were constructed and sequenced (Fig. 1). In all cases the endpoints of the deletion were marked with 8-bp *Xho*I linker DNA. Two of the CDE II deletions caused a marked loss in centromere function; only X78, with 14 bp removed, demonstrated almost wild-type behavior in both the plasmid (Fig. 4) and the chromosome (Table 1) stability assays. Therefore, the length of the CDE II region in *CEN3* can be reduced from 84 to 78 bp (the minimal length found so far in naturally occurring yeast centromeres) without an obvious effect on function.

When 57 bp of CDE II DNA immediately adjacent to the CDE III sequence were removed (X35; Fig. 1), the mitotic loss of chromosome III increased 10,000-fold (Table 1). The nondisjunction frequency for X35 was approximately 10-fold higher than that previously reported for a mutant with 36 bp of CDE II (303-7 [5]). This may reflect a decrease in function caused by deleting the nine bp of CDE II adjacent to CDE III (X35) which are still present in 303-7.

An even more dramatic effect on centromere function was observed for mutant X50 which has a deletion removing 34 bp of CDE II DNA and 7 bp of CDE III. As is evident from the plasmid stability results shown in Fig. 4, X50 was far more unstable than X35. This was also the case when the X50 mutation was contained on chromosome III (Table 1). X35 has less CDE II DNA but is more functional than X50, suggesting that the lack of centromere function in X50 results from the removal of sequences from the left-hand end of CDE III (Fig. 1) rather than from deletion of CDE II DNA.

Replacement mutations partially restore centromere function. We constructed three replacement mutants by inserting a 34-bp synthetic oligonucleotide (68% A+T) into the *Xho*I site of X35, X50, and X78 to produce X69, X84, and X112,

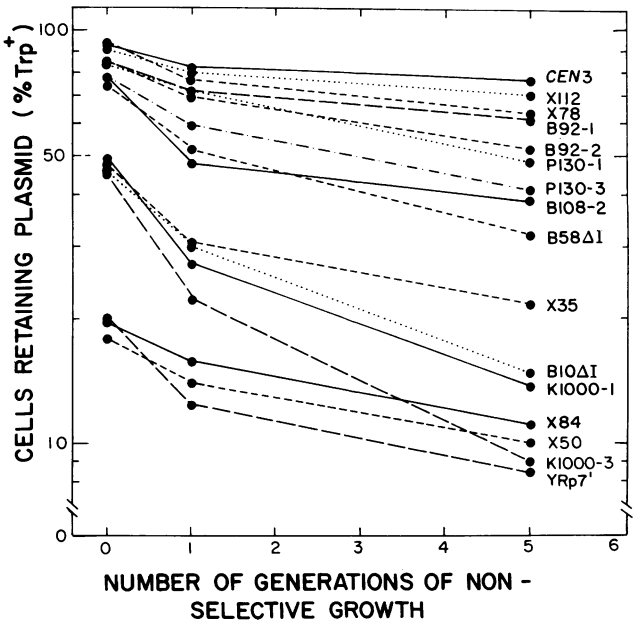

FIG. 4. Plasmid mitotic stabilities. Mitotic stabilities were determined by plating after several generations of growth in nonselective (YPD) medium. The percentage of Trp$^+$ cells represents the ratio of plasmid-containing cells in the population at the time the sample was removed from the nonselective culture.

respectively (Fig. 1). We found that an insertion increasing the length of CDE II from 78 to 112 bp (X112; Fig. 1) did not decrease mitotic plasmid stability (Fig. 4). Addition of 34 bp to X50 increased the distance between CDE I and the remainder of CDE III to 84 bp but failed to improve the mitotic stability of the plasmid (X84; Fig. 4). This observation supports the idea that the defect in X50 function is caused by the removal of the seven bp forming the left end of the CDE III dyad symmetry (15).

The 34-bp insertion into X35 expanded the CDE II region to 69 bp (X69; Fig. 1) and resulted in a 100-fold increase in chromosome stability when compared with analogous chromosomes bearing X35 centromeres (Table 1). This suggests that the 34-bp insertion into CDE II at least partially compensated for the deleterious effect of the mutation either by increasing the separation between CDE I and CDE III or by restoring A+T-rich DNA to this region of the centromere. However, the X69 centromere was still not wild type in function despite the fact that the length of its CDE II region is only nine bp shorter than that of the functional centromere, X78. For comparison, BS58, which has a 48-bp CDE II deletion replaced with a 22-bp palindrome to produce a 58-bp CDE II, exhibited a slightly lower mitotic stability than X69 (Table 1).

Insertion mutations to expand CDE II beyond 84 bp. The *CEN3* CDE II insertion mutants constructed in this study are diagrammed in Fig. 2. Plasmids with centromere mutations were constructed which contain an 8-bp *BclI* linker insertion in one of two different positions in CDE II (B92-1 and B92-2). Both of these altered centromere plasmids showed slightly reduced mitotic stabilities when compared with that of the parent plasmid, pYe(*CEN3*)30 (Fig. 4). However, intact chromosomes containing the B92-2 centromere were almost as stable as wild-type copies of chromosome III (Table 1). One centromere mutant, B108-2, contains an insertion of three adjacent *BclI* linkers in CDE II and consistently demonstrated a decrease in plasmid mitotic stability (Fig. 4). In this case the plasmid instability may be attributable to the triple tandem insertions rather than length since its CDE II region is 108 bp, close in length to the 112-bp CDE II DNA which functioned well in replacement mutant X112 (Fig. 4).

Our initial studies showed that small insertion mutations in CDE II failed to have a dramatic effect on mitotic centromere function. We therefore constructed two *CEN3* mutants, K1000-1 and K1000-3, each containing a 1.1-kilobase fragment encoding kanamycin resistance in either the first or third *AhaIII* site in CDE II (Fig. 2). Both plasmids were extremely unstable during mitotic cell division in nonselective medium (Fig. 4). However, these mutants may retain some residual centromere function since both plasmids were present in >40% of the cells after selective growth, whereas the acentric plasmid YRp7′ was retained in only about 20% of the cells under similar growth conditions.

P130-1 and P130-3 each contain inserts of 46 bp of polylinker DNA which expand the CDE II regions in these mutants to 130 bp (Fig. 2). The mitotic stabilities of both of these plasmids were consistently lower than that of the wild-type control, pYe(*CEN3*)30 (Fig. 4). The plasmid stability results suggested that there was a small difference in mitotic stability depending on whether the 46-bp insertion was in the *AhaIII* site nearest CDE I (P130-1) or nearest CDE III (P130-3) (Fig. 4). However, analysis of these centromere mutations as chromosome III derivatives revealed that both mutations increased chromosome loss by about a factor of 10 (Table 1). This could be due to the increased distance between CDE I and III, or to the G+C-rich composition (61% G+C, 39% A+T), or to the palindromic nature of the 46-bp insertion. To further investigate this, we used P130-1 and P130-3 to construct another centromere mutation, BS154, which has a duplication of DNA that increases the overall length of CDE II to 154 bp, but contains only 22 bp of palindromic insert (see Materials and Methods and Fig. 2). This mutation decreased chromosome stability 10-fold relative to P130-1 and P130-3 and 100-fold relative to chromosome III copies containing the wild-type *CEN3* (Table 1), suggesting that length is a primary factor contributing to loss of centromere function.

DISCUSSION

Importance of CDE II length. The importance of CDE II for centromere function is evident from the unstable segregation of plasmids and chromosomes containing centromeres which lack this region. Deletion of CDE I (B58ΔI) or deletion of most of CDE II (X35) caused about the same dramatic (10,000-fold) reduction in chromosome stability (Table 1). Replacement of the 57-bp CDE II deletion in X35 with 34 bp of A+T-rich DNA (X69) partially restored centromere function and increased chromosome stability by 100-fold (Table 1). Although this replacement mutant had a CDE II 69 bp long, it was still not a fully functional centromere. In fact, chromosomes bearing the X69 centromere were lost 20 times more frequently than chromosomes with a CDE II region only nine bp longer (X78; Table 1). This could be due to the difference in length of CDE II or to the primary sequences present in each CDE II.

Mitotic stabilities decreased with a reduction in the length of CDE II (compare the function of X78, X69, and BS58). Although X69 and BS58 have similar A+T base compositions (71 and 75%, respectively [Table 1]), the length and primary sequences of their CDE II regions are different. BS58, although shorter, contains more wild-type CDE II DNA yet is less stable than X69. These results suggest that the length of CDE II, or the physical separation of CDE I and III, is a more important factor contributing to centromere function than is extreme A+T richness or the alternating stretches of poly(dA)-poly(dT) DNA found in the CDE II of wild-type centromeres. Studies are in progress to evaluate the effect of altering the primary sequence of CDE II without changing the wild-type length of the element. Preliminary results suggest that replacing a large segment of CDE II with non-A+T DNA while retaining wild-type length results in centromere plasmids that exhibit some reduction in mitotic stability (A. Gaudet and M. Fitzgerald-Hayes, unpublished data).

A gradual decrease in centromere function was observed when the separation between CDE I and CDE III was expanded from 84 to 154 bp by insertion mutagenesis. Surprisingly, small insertions were accommodated with only moderate losses in chromosome stability. The 46-bp G+C-rich (61%) palindrome inserted into CDE II in P130-1 and P130-3 resulted in a small decrease in plasmid stability (Fig. 4) and only a 10-fold reduction in chromosome stability relative to wild type (Table 1). It has been reported previously that a duplication mutation in *CEN6* increased CDE II to 134 bp without an apparent effect on plasmid centromere function (pLA453 [25]). The same authors reported that a similar mutation expanding CDE II to 175 bp caused a significant reduction in plasmid stability. In this study we showed that increasing the length of CDE II to 154 bp decreased proper mitotic chromosome segregation by two

orders of magnitude (BS154; Table 1). Apparently the wild-type distance between CDE I and CDE III can be doubled before function of the altered centromere DNA is significantly impaired.

A 39-bp deletion (and 8-bp insertion) immediately adjacent to CDE I in *CEN6* decreased the length of CDE II from 85 to 54 bp and caused severe plasmid instability (25). Since two of the *CEN3* mutants of similar length (X69 and BS58) leave the leftmost part of CDE II unaltered and exhibit an intermediate level of centromere function (Table 1), it seemed possible that the end of CDE II nearest to CDE I might be especially sensitive to mutation. To test this possibility, we analyzed three insertion mutations introduced into different *Aha*III sites in the CDE II sequence (B92-1, B92-2, P130-1, P130-3, K1000-1, and K1000-3). We did not observe any differences in the effects of these mutations which correlated in any way with the position of the insertion in CDE II (Fig. 4; Table 1).

It is still possible that the CDE II sequences immediately adjacent to CDE I are particularly important for centromere function. If so, this could partially explain the dramatic instability associated with mutants lacking CDE I and the leftmost 26 bp of CDE II (B58ΔI [Fig. 4; Table 1]). However, at least some of the loss in function is due to deletion of CDE I DNA since a 2-bp insertion into CDE I causes a 10-fold reduction in chromosome stability (M. Fitzgerald-Hayes, unpublished data).

Centromere DNA element III is essential. We have previously shown that a single base-pair change altering a conserved C residue in CDE III can totally abolish centromere function on both plasmids and chromosomes (23). Also, deletions removing the right-hand end of the CDE III dyad symmetry have been shown to severely reduce centromere function on plasmids (25). Here we showed that a deletion removing 34 bp of CDE II and the leftmost 7 bp of CDE III also abolished centromere function (X50 [Fig. 1; Table 1]). An insertion of 34 bp (X84) failed to rescue the X50 mutation even though this same 34-bp fragment inserted into the CDE II mutant X35 resulted in a 100-fold increase in centromere function (X69; Table 1). Quite clearly, CDE III is the part of the centromere most sensitive to mutation and most essential for function of any kind. However, the functional relationship among the three conserved centromere DNA elements still remains to be resolved.

The plasmid and chromosome instabilities caused by the centromere mutations reported in this study could be the result of aberrant segregation, a failure to replicate, or physical loss of the chromosome during mitosis. Recently we have used the *SUP11*-based colony color assay (14) to demonstrate that both point mutations in CDE III (23) and deletion of either CDE II (X35) or CDE I (B58ΔI) DNA cause aberrant chromosome segregation (nondisjunction), thereby eliminating a replication defect as an explanation for our results (J. McGrew and M. Fitzgerald-Hayes, manuscript in preparation).

Possible roles for CDE II in CEN function. The conformation of CDE II DNA in vivo is not yet known. The A+T richness of this region suggests that it might adopt a single-stranded character in vivo, implying that the primary nucleotide sequence of CDE II is less important than its overall base composition. However, in wild-type centromere sequences the CDE II region is not random AT DNA but contains stretches of A and T residues. It may be that the A+T-rich DNA has a particular double-stranded helical conformation in vivo that is important for centromere function. Poly(dA)-poly(dT) DNA has unusual helical parameters (1). In kinetoplast DNA, stretches of five or six A residues separated by a half turn of the helix produce a bend in the duplex DNA (34). It has been predicted (33) that alternating stretches of poly(dA) and poly(dT) can cause a helical bend forming a superhelical structure. Although there is no evidence for a pronounced bend in the CDE II region, the *CEN3* fragment does migrate somewhat aberrantly in polyacrylamide gels (M. Fitzgerald-Hayes, unpublished data). A critical functional role for alternating runs of A and T residues remains in doubt since in this study we showed that chromosomes containing X69, which has most of the CDE II region replaced without any alternating A or T stretches, are at least partially stable. Segments of A+T DNA 80 bp long resist incorporation into nucleosome core particles, and short A+T-rich regions may play a role in the positioning of nucleosomes (19). Perhaps one function of the CDE II region is to exclude nucleosomes from the centromeric DNA to facilitate the binding and subsequent assembly of the kinetochore complex. It has been suggested that the CDE II region of the yeast centromere may have a more important function in meiotic chromosome disjunction (5) than in mitosis. Therefore we are currently extending these studies to investigate the meiotic behavior of chromosomes containing the CDE II centromere mutations.

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