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In Vivo Analysis of the *Saccharomyces cerevisiae* Centromere CDEIII Sequence: Requirements for Mitotic Chromosome Segregation

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In the yeast *Saccharomyces cerevisiae*, the complete information needed in *cis* to specify a fully functional mitotic and meiotic centromere is contained within 120 bp arranged in the three conserved centromeric (*CEN*) DNA elements CDEI, -II, and -III. The 25-bp CDEIII is most important for faithful chromosome segregation. We have constructed single- and double-base substitutions in all highly conserved residues and one nonconserved residue of this element and analyzed the mitotic in vivo function of the mutated *CEN* DNAs, using an artificial chromosome. The effects of the mutations on chromosome segregation vary between wild-type-like activity (chromosome loss rate of 4.8×10^{-4}) and a complete loss of *CEN* function. Data obtained by saturation mutagenesis of the palindromic core sequence suggest asymmetric involvement of the palindromic half-sites in mitotic *CEN* function. The poor *CEN* activity of certain single mutations could be improved by introducing an additional single mutation. These second-site suppressors can be found at conserved and nonconserved positions in CDEIII. Our suppression data are discussed in the context of natural CDEIII sequence variations found in the *CEN* sequences of different yeast chromosomes.

One of the essential events in the eucaryotic cell cycle is the faithful segregation of sister chromatids to the two daughter cells. Essential for this process is the attachment of the mitotic spindle to the kinetochore located on the chromosome. Kinetochore functions include maintenance of a dynamic association with the mitotic spindle and its microtubules (microtubule capture [40]). In addition, kinetochores have been implied to carry mechanochemical motors required for chromosome movements (20, 49, 50, 56). Chromosomal localization and assembly of the kinetochore are mediated by the centromere. Centromeric (*CEN*) DNA sequences, which define genetically the site of kinetochore assembly, have been identified in the budding yeast *Saccharomyces cerevisiae* (12, 43) and the fission yeast *Schizosaccharomyces pombe* (11, 42). The *S. pombe* sequences are large complex structures with different long and short repeating elements (10). In the budding yeast *S. cerevisiae*, the isolation and sequence analysis of 13 different *CEN* DNAs established the existence of a short consensus sequence of about 120 bp in length, which can be subdivided into the three *CEN* DNA elements CDEI, -II, and -III (Fig. 1). Chromatin studies have uncovered a specific centromere structure (4). A detailed analysis revealed a region of approximately 160 bp of protected DNA including CDEI, -II, and -III (19). The *CEN* consensus sequence consists of a 78- to 86-bp-long central sequence composed of about 90% A+T, termed CDEII, flanked on one side by an 8-bp conserved CDEI sequence and on the other side by a 25-bp conserved sequence termed CDEIII. Deletion of all three elements from a yeast chromosome results in an acentric chromosome that is lost at frequencies of greater than 0.1 per cell division (13). Removing sequences outside these elements does not abolish *CEN* function (7, 21, 45, 48). A 125-bp DNA fragment composed of these three elements is sufficient to fulfill complete meiotic and mitotic *CEN* function (14). Removal of CDEI or CDEI plus parts of CDEII diminishes the quality of chromosome transmission significantly but does not abolish it (15, 48). Similarly, deletion of *CPF1*, a gene whose product binds specifically to CDEI, has a minor effect on *CEN* function (2, 6, 39).

In contrast, deletion of CDEIII results in a total loss of *CEN* activity, revealing the essential importance of CDEIII for centromere function (48). CDEIII is the only *CEN* element for which single-base-pair changes have been shown to abolish *CEN* function (22, 38). Several lines of evidence indicate specific binding of proteins to CDEIII. Retention assays have identified protein binding to oligonucleotide duplexes carrying the CDEIII sequence (21, 44). Exonuclease III experiments using crude protein extracts revealed that the CDEIII region was protected against digestion (44). In vivo footprint experiments identified specific highly conserved G nucleotides within CDEIII protected against methylation (16, 58). The isolation of a multisubunit protein complex that binds to a region of the *CEN* DNA including CDEIII has recently been reported (35).

Despite the evidence illustrating the essential importance of CDEIII for *CEN* function, the relevance and possible role of the conserved base pairs within CDEIII for complete mitotic *CEN* function are still unclear. Mutagenesis of four highly conserved nucleotides within CDEIII had shown that the absolute conservation of base pairs does not necessarily reflect their importance in mitotic *CEN* function (22, 38, 44). The work presented in this report determines the contribution of all conserved nucleotides within CDEIII for faithful mitotic chromosome segregation. A detailed knowledge of the CDEIII nucleotide requirements will help our understanding of CDEIII DNA-protein interactions.

The assay for measuring chromosome segregation efficiency is highly sensitive and is based on the chromosome fragment assay (22, 55), which was modified to facilitate determination of chromosome loss rates. The results de-

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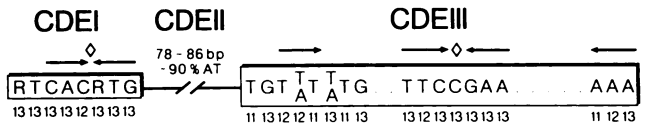

FIG. 1. Centromere consensus sequence. The *S. cerevisiae CEN* consensus sequence was derived from comparison of 13 different *CEN* DNAs (24, 28; sequence of *CEN2* from Jäger [29a]). Numbers below the sequences indicate the degree of conservation (e.g., 13 indicates that all 13 *CEN* DNAs carry the indicated nucleotide at the indicated position). Dots mark nonconserved positions, the diamond indicates the twofold axis going through this C · G base pair, and arrows mark regions of dyadic symmetry. R, purine.

scribed here point to possible CDEIII contact sites with centromere-binding proteins. Data obtained by saturation mutagenesis of the highly conserved 7-bp palindromic core sequence of CDEIII show an asymmetric involvement of the half-sites in mitotic *CEN* function. Most of the conserved and several of the nonconserved base pairs surrounding the core sequence also have an important influence on mitotic chromosome segregation. Finally, we found evidence for second-site suppression within CDEIII. This finding too is indicative of the unusual character of this DNA-protein complex compared with other palindromic protein-binding sites (46).

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MATERIALS AND METHODS

Strains, media, and enzymes. The following *Escherichia coli* strains were used for in vitro mutagenesis and plasmid amplification: BMH71-18mutS [*thi supE* Δ(*lac-pro*) *recA galE rpsI* F′ *lacI*^{q} *lacZΔM15 proA*$^+$*B*$^+$ *mutS215*::Tn*10*) (source, H. J. Fritz), JM101 [*supE thi* Δ(*lac-pro*) F′ *traD36 proAB lacI*^{q} *lacZΔM15*] (source, J. Messing), MK30-3 [Δ(*lac-pro*) *recA galE rpsL* F′ *lacI*^{q} *lacZΔM15 proA*$^+$*B*$^+$] (source, H. J. Fritz), and XL1-Blue (*recA*1 *lac endA*1 *gyrA96 thi hsdR17 supE44 relA*1 F′ *lacI*^{q} *lacZΔM15* Tn*10*) (source, Stratagene). To prepare specific mutations, bacteriophages M13mp9 (source, J. Messing) and M13mp9rev (source, H. J. Fritz) were used. Chromosomal fragmentation transformation was done in *S. cerevisiae* YJH6 (a/α *ura3-52/ura3-52 lys2-801*^{amber}/*lys2-801*^{amber} *ade2-101*^{ochre}/*ade2-101*^{ochre} *trp1-Δ1/trp1-Δ1cyh*^{r}*2/cyh*^{r}*2*) (59). Media for bacterial and yeast growth were prepared as described elsewhere (37, 41, 54). The nonselective synthetic minimal yeast medium SD+6 is SD medium supplemented with six bases and amino acids: adenine, leucine, uracil, tryptophan, histidine, and lysine. The selective medium SD+6-Ura lacks uracil. Fluctuation analysis was done on YPD plates supplemented with cycloheximide (10 μg/ml): Sigma. The enzymes used were from various companies. Buffers and reaction conditions were as specified by the manufacturer. [γ-^{32}P]ATP was purchased from Amersham Corp. DNA manipulations, plasmid cloning and sequencing, and bacterial transformation were done as described by Maniatis et al. (37). Yeast transformation was done according to Ito et al. (29, 51).

Oligonucleotides. The oligonucleotides designed for directed mutagenesis are between 17 and 21 bp in length and were synthesized on a model 380B Applied Biosystems DNA synthesizer. All mutants were verified by sequencing, using as the primer oligonucleotide XIX, which is homologous to a DNA sequence 78 bp upstream of CDEI (22). After precipitation, all oligonucleotides were 5′-phosphorylated by using polynucleotide kinase and purified by column chromatography using Sephadex G-50 (Pharmacia).

Oligonucleotide-directed mutagenesis. All single-point mutations were introduced by the gapped-duplex approach (31). To construct the gapped-duplex DNA, single-stranded DNA of bacteriophage M13mp9 carrying a 1.16-kb *Sal*I-*Bam*HI *CEN6* fragment was mixed with double-stranded M13mp9 rev DNA, which was digested with restriction enzymes *Eco*RI and *Hin*dIII. By denaturing and renaturating, a double-stranded gapped-duplex molecule carrying the *CEN6* insert in the gap was created. This gap was the target site for an annealing reaction with the 5′-phosphorylated mutagenic oligonucleotide. The resulting heteroduplex DNA is characterized by a mismatch inside the region of the *CEN6* fragment. The gaps were filled in by a polymerase/ligase reaction. Subsequently, the constructs were transformed in *E. coli* BMH71-18mutS and then reinfected in strain MK30-3. Dot blot analysis identified potential mutants, which were then verified for the specific mutation by sequencing according to Sanger et al. (52). The two mutants CDEIII(2-C) and CDEIII(2-T) have been described previously (22). Double mutants were constructed essentially in the same way except that preexisting point mutations were used to generate the gapped-duplex DNA.

Plasmid construction. The in vivo *CEN* activity of the in vitro-generated *CEN* mutants was analyzed on chromosome fragments (CF). To construct such artificial chromosomes, the centromere point mutants were cloned into the shuttle vector pKE5. Replicative-form DNA of M13 clones harboring the various mutations was cleaved with *Bam*HI and *Sal*I, and the resulting 1.16-kb *CEN6* fragment was inserted into *Bam*HI-*Sal*I-cut pKE5. Plasmid pKE5 was derived from pYCF5 (22) and carries in addition the cycloheximide sensitivity gene *CYH*^{S}*2* cloned as a 1.4-kb *Bam*HI-*Hin*dIII fragment (source, J. R. Warner). pKE5 contains the ampicillin resistance gene for selection in *E. coli* and *URA3* and *SUP11* as marker genes in *S. cerevisiae*. This vector also carries the Y′a region and the D8B element, which are needed for sequence-specific recombination to generate artificial CF (22). The cloning of all 32 1.16-kb fragments carrying the different *CEN6*-CDEIII single-point mutations leads to the following constructs: pBJ1-CDEIII(16-G), pBJ2-CDEIII(16-T), pBJ3-CDEIII(16-C), pBJ4-CDEIII(17-G), pBJ5-CDEIII (17-T), pBJ6-CDEIII(17-C), pBJ7-CDEIII(13-T), pBJ8-CDEIII(13-G), pBJ9-CDEIII(13-A), pBJ10-CDEIII(12-C), pBJ11-CDEIII(12-G), pBJ12-CDEIII(12-A), pBJ13-CDEIII (11-G), pBJ14-CDEIII(11-A), pBJ15-CDEIII(11-C), pBJ16-CDEIII(7-G), pBJ17-CDEIII(6-G), pBJ18-CDEIII(5-A), pBJ19-CDEIII(5-G), pBJ20-CDEIII(4-A), pBJ21-CDEIII (4-G), pBJ22-CDEIII(3-A), pBJ23-CDEIII(1-G), pBJ24-CDEIII(1-T), pBJ25-CDEIII(1-C), pBJ26-CDEIII(23-G), pBJ27-CDEIII(24-T), pBJ28-CDEIII(25-G), pBJ29-CDEIII (25-C), pBJ30-CDEIII(2-T), pBJ31-CDEIII(2-C), and pRN32-CDEIII(15-T).

The five double mutants are named pRN15-CDEIII(1-T,15-T), pRN20-CDEIII(1-T,15-A), pRN16-CDEIII(15-T, 18-A), pRN17-CDEIII(15-C,18-A), and pRN21-CDEIII(15-A, 18-A).

In vitro generation of artificial CF. To generate artificial CF in yeast cells, the various pKE5 clones containing specific *CEN6*-CDEIII mutations were linearized at the unique *Not*I site and transformed into YJH6 by the lithium acetate method (29). In every case, eight independent Ura$^+$ transformants were checked for a stable pink phenotype by restreaking the colonies on selective SD+6-Ura plates. Two

of these colonies were then chosen for orthogonal-field-alternation gel electrophoresis (OFAGE) analysis (8, 9) to determine the specific karyotype. OFAGE conditions were as follows: time, 12 h; temperature, 4°C; voltage, 300 V; pulse time, 40 s.

Determination of the loss rate of artificial CF per mitotic cell division by using the cycloheximide resistance/sensitivity (R/S) system. The influence of a specific *CEN6*-CDEIII mutation on the mitotic stability of an artificial CF was quantified by a newly developed assay that is based on the colony color assay (23) and the CF assay (22). Pink Ura$^+$ transformants were checked by OFAGE analysis for the karyotype 2n+CF. For the assay, cells were taken from selective SD+6-Ura plates and diluted, and 150 to 300 cells were plated on nonselective SD+6 plates and incubated for 36 to 40 h at 30°C. Yeast colonies were allowed to grow to an average size of 50,000 cells per colony. The size of the colonies was checked under the microscope. Ten colonies of equal size were picked and resuspended in sterile water. Then 98% of the cells were plated out on two YPD plates containing cycloheximide, whereas the remaining 2% of the cells were plated on five YPD plates. After incubation for 3 to 4 days, the colonies were grown up and the number of cultivated yeast colonies could be determined. By using fluctuation analysis as described by Lea and Coulson (34), the total number of yeast colonies grown on YPD medium was correlated with the number of colonies that were resistant to cycloheximide. This allowed quantitative determination of the CF loss rate per mitotic cell division. In cases for which loss rates were determined several times, they diverged no more than 10% from each other. For some of the double mutants, we used a modified system that gave the percentage of cells carrying under selection the chromosome fragment. Ten different cultures were inoculated (cells were taken from many different colonies from a selective plate) for 3 days in selective liquid medium. Equal amounts of cells (100 to 200) from each culture were plated on two SD+6 and two SD+6-Ura plates. From the number of grown colonies we calculated the percentage and standard deviation of cells that carry a CF under selective conditions.

The mitotic CF loss rate for wild-type (wt) *CEN6* in yeast strain YJH6 was determined to be 4.8×10^{-4}, using the R/S system or the colony color assay (data not shown). Earlier, the loss rate of wt *CEN6* was quantified at 1.9×10^{-4}, using yeast strain YPH49 (22). For the compilation of all *CEN6* CDEIII mutations (see Fig. 4), we multiplied the earlier data by the factor 2.53 to adjust all loss rates to the same basal wt level.

RESULTS

Generation of mutations in CDEIII. The complete DNA sequence information needed in *cis* to specify *CEN* function in *S. cerevisiae* is contained within a 120-bp DNA segment (Fig. 1). CDEIII, which plays a key role in *CEN* function, is a 25-bp-long element consisting of an interrupted palindromic structure (Fig. 1). The degree of conservation of individual conserved nucleotides within this element differs. The core sequence 5′-TTCCGAA-3′ from position CDEIII(11) to CDEIII(17) is fully conserved in all centromeres analyzed with the exception of position 12, where in one *CEN* DNA (*CEN4*) a T nucleotide has been replaced by an A nucleotide. In addition, the core sequence carries the central part of an inverted repeat centered around the C nucleotide at position 14 (Fig. 1). Nucleotides outside the core sequence include the second part of the interrupted palindrome and show differences in conservation. Deletion analysis of sequences to the right of CDEIII defined the right boundary of this element (14, 21). The outmost left boundary of CDEIII has not been characterized well and is defined only by the position of the outermost left conserved nucleotide named position 1 (Fig. 1).

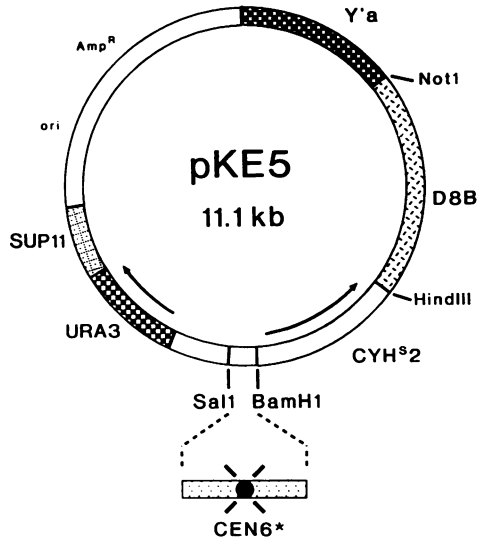

FIG. 2. Map of the fragmentation vector pKE5. This plasmid is derived from pYCF5 (22) and carries in addition the cycloheximide sensitivity allele *CYH^{S}2*. Directed fragmentation occurs via Y′a and D8B sequences. All *CEN6* mutants were inserted as 1.16-kb fragments into the unique *Bam*HI-*Sal*I sites. The orientation of transcription for *URA3* and *CYH^{S}2* is indicated by arrows.

Complete analysis of the CDEIII core sequence required saturation mutagenesis of the base pairs at positions 11 to 17. Changes at positions 14 and 15 of the *CEN6* core sequence have been analyzed previously (22). We report the introduction of the 15 remaining single-nucleotide substitutions into this sequence. In addition, we determined the specific contribution of all other conserved nucleotides within CDEIII for mitotic *CEN* function by generating another 15 single-point mutations. The nucleotide changes were generated by using the appropriate oligonucleotides and the gapped-duplex mutagenesis protocol developed by Kramer et al. (31).

Test system to measure mitotic *CEN* activity. All *CEN6* mutants were assayed for their ability to perform faithful mitotic chromosome segregation. The presence of a mutation was verified by sequence analysis. All *CEN6* mutants were cloned as 1.16-kb *Bam*HI-*Sal*I fragments into the chromosome fragmentation vector pKE5 (Fig. 2). This vector is a derivative of pYCF5 described earlier (22) and allows the generation of a 125-kb artificial, nonessential CF that carries the *CEN* DNA mutants as outlined in Fig. 3. Upon transformation of the *Not*I-linearized plasmid into yeast cells, the two vector ends carrying telomere-adjacent Y′a DNA and a 2.7-kb unique sequence from the left arm of chromosome III called D8B undergo homologous recombination events. This leads to the formation of a CF with a Y′-containing telomere at one side and the entire left part of chromosome III distal from the D8B sequence at the other side (Fig. 3). This new structure was inferred to be a telocentric chromosome. Originally, chromosome loss rates were measured by a visual assay that utilizes the red pigment phenotype of yeast colonies carrying the *ade2-101* ochre

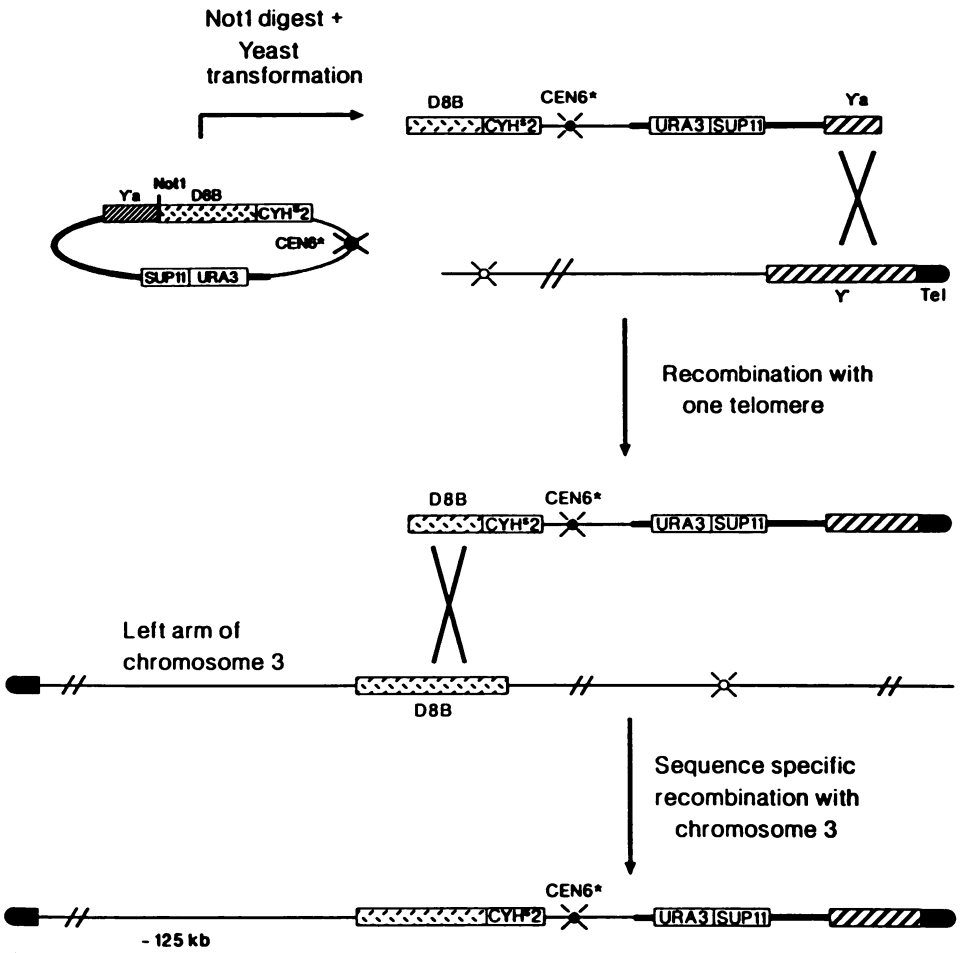

FIG. 3. Scheme of the in vivo generation of artificial CF. The scheme illustrates the proposed events leading to the generation of artificial CF by sequence-specific recombination events. After digestion with *Not*I, the linearized plasmid is transformed into yeast strain YJH6, where the free vector ends recombine with their homologous genomic DNA sequences of chromosome III. The resulting artificial CF has a length of about 125 kb.

mutation (23). Thus, a diploid yeast with a chromosome fragment carrying the ochre suppressor tRNA gene *SUP11* in the *ade2-101* background partly prevents the accumulation of pigment resulting in a pink colony color; loss of the CF during colony growth allowed the accumulation of pigment in these cells, resulting in red sectors. To improve the assay, we introduced the cycloheximide sensitivity allele CYH^S2 into pYCF5 as an additional marker (pKE5; Fig. 2). Since transcription toward a *CEN* DNA can interfere with *CEN* function (25, 47), we verified that *URA3* and CYH^S2 were transcribed away from the *CEN* DNA. The linearized plasmids carrying the different *CEN* mutants were transformed in the diploid yeast strain YJH6 homozygous for the recessive cycloheximide resistance gene cyh^r2. Transformants carrying the CF are sensitive to cycloheximide and do not grow on plates containing 10 μg of cycloheximide per ml. Loss of the chromosome fragment leads to growth on cycloheximide-containing plates, and chromosome loss events can be assessed by selection for resistance to cycloheximide. The new system is referred to as the R/S system. Thus, the CF system allows quantification of mitotic loss rates by using three different parameters: the colony color assay and the two positive selection systems based on resistance to 5-fluoroorotic acid (5) and cycloheximide, respectively. To compare the *SUP11*-based color assay and the cycloheximide assay, we used both assay systems to measure the mitotic loss rate of the CF carrying a wt *CEN* sequence (*CEN6*) (data not shown). Chromosome loss rates showed nearly identical frequencies, and thus a direct comparison of results from both systems can be made. The high accuracy of the CF system in combination with the fluctuation analysis is illustrated by the fact that in cases for which loss rates were determined several times, they diverged by no more than a factor of 1.1 (data not shown).

A new artificial CF was generated upon yeast transformation with the linearized plasmid DNA. This was verified by the pink color of the Ura^+ transformants and by the appearance of a chromosomal band of approximately 125 kb in the OFAGE analysis (Fig. 3) (data not shown). To determine chromosome loss rates, transformants with the karyotype 2n+CF were used. The mitotic loss rate per cell division was determined as described in Materials and Methods. The data obtained for wt *CEN6* and all single-point mutants are summarized in Table 1. Several independent experiments yielded a mitotic loss per cell division of 4.8×10^{-4} for wt *CEN6*, a rate that is 2.53-fold higher than that obtained in earlier experiments (22) and probably reflects strain differences.

TABLE 1. Mitotic CF loss rates

Yeast strain	Mutation	Chromsome loss/ cell division[a]
YRN35	*CEN6* (wt)	4.8×10^{-4}
YBJ23	CDEIII(1-G)	6.3×10^{-4}
YBJ24	CDEIII(1-T)	9.1×10^{-4}
YBJ25	CDEIII(1-C)	1.2×10^{-3}
YBJ30	CDEIII(2-T)	6.7×10^{-4}
YBJ31	CDEIII(2-C)	8.1×10^{-4}
YBJ22	CDEIII(3-A)	4.0×10^{-3}
YBJ21	CDEIII(4-G)	2.6×10^{-3}
YBJ20	CDEIII(4-A)	5.5×10^{-4}
YBJ18	CDEIII(5-A)	8.1×10^{-4}
YBJ19	CDEIII(5-G)	5.3×10^{-4}
YBJ17	CDEIII(6-G)	5.1×10^{-4}
YBJ16	CDEIII(7-G)	8.0×10^{-4}
YBJ13	CDEIII(11-G)	5.8×10^{-4}
YBJ14	CDEIII(11-A)	1.7×10^{-3}
YBJ15	CDEIII(11-C)	5.7×10^{-4}
YBJ10	CDEIII(12-C)	9.7×10^{-4}
YBJ11	CDEIII(12-G)	1.7×10^{-2}
YBJ12	CDEIII(12-A)	1.3×10^{-3}
YBJ7	CDEIII(13-T)	1.9×10^{-2}
YBJ8	CDEIII(13-G)	3.6×10^{-3}
YBJ9	CDEIII(13-A)	2.2×10^{-2}
YBJ1	CDEIII(16-G)	1.0×10^{-3}
YBJ2	CDEIII(16-T)	1.3×10^{-2}
YBJ3	CDEIII(16-C)	3.0×10^{-2}
YBJ4	CDEIII(17-G)	1.2×10^{-3}
YBJ5	CDEIII(17-T)	3.4×10^{-3}
YBJ6	CDEIII(17-C)	1.2×10^{-3}
YRN10	CDEIII(18-A)	4.8×10^{-4}
YBJ26	CDEIII(23-G)	6.6×10^{-4}
YBJ27	CDEIII(24-T)	5.0×10^{-4}
YBJ28	CDEIII(25-G)	8.4×10^{-4}
YBJ29	CDEIII(25-C)	8.8×10^{-4}

[a] Loss rates of artificial CF per mitotic cell division. The data were obtained by the R/S system and calculated according to the fluctuation analysis of Lea and Coulson (34).

All single-point mutations in the CDEIII core sequence lead to a reduction in in vivo *CEN* function. All point mutations in the core CDEIII DNA segment adversely affect *CEN* function, but the different rates of loss vary by up to 2 orders of magnitude. While the change of the T · A base pair at position 11 to a C · G or G · C base pair [CDEIII(11-C) and CDEIII(11-G), respectively] results in a mitotic loss rate close to the wt rate, other mutants such as CDEIII(16-C) show a chromosome loss rate of 3.0×10^{-2}, which is 63-fold higher than the wt loss rate. The negative effects of base pair substitutions on centromere efficiency increase gradually from the T · A base pair at position 11 [loss rate of 5.7×10^{-4} for mutant CDEIII(11-C)] to the C · G base pair at position 13 [loss rate of 3.6×10^{-3} for CDEIII(13-G)]. The same pattern can be seen for the right half-site of the core sequence. The chromosome fragment loss rates increase gradually from the A · T base pair at position 17 [loss rate of 3.4×10^{-3} for CDEIII(17-T)] to the A · T base pair at position 16 [loss rate of 1.3×10^{-2} for CDEIII(16-T)] and even more to the C · G base pair at position 15 [loss rate of 1.1×10^{-1} for CDEIII(15-T)] (Fig. 4) (22). The extent to which all possible three base pair changes at a particular position within the core sequence influence *CEN* function is rather diverse. The greatest differences were found for mutations at position 16, where the mutation CDEIII(16-C) shows a 30-fold-higher loss rate than does CDEIII(16-G). We had shown previously that the central base pair of the palindromic sequence at position 14 (C · G) is essential for *CEN* function: changing the base pair at this position abolishes function (22). Analysis of all core sequence mutants reveals that transversions within the core sequence give rise to the most drastic decreases in *CEN* function. At positions 11, 12, 16, and 17, both transversions have a greater influence on *CEN* activity than does the corresponding transition.

The palindromic CDEIII core half-sites have different impacts on mitotic chromosome segregation. The palindromic CDEIII core sequence TTCCGAA is highly conserved among *S. cerevisiae CEN* DNAs (Fig. 1). Base pair changes in the right half-site of this inverted repeat have a stronger effect on *CEN* function than do changes in the left half-site of the CDEIII core sequence. Moreover, a comparison of symmetrical changes reveals that all base pair substitutions in the right palindromic half-site have a more severe effect on the mitotic stability of the artificial chromosomes than do corresponding changes in the left half-site. For example, the mitotic loss rate for mutant CDEIII(17-G) is 1.2×10^{-3}, and the corresponding symmetrical change CDEIII(11-C) shows a loss rate of 5.7×10^{-4}. Other symmetrical changes show an even greater difference in their mitotic loss rates. For instance, we observe a 10-fold-higher CF loss rate for mutant CDEIII(16-T) (1.3×10^{-2}) than for the symmetrical mutant CDEIII(12-A) (1.3×10^{-3}) (Table 1). These results indicate an asymmetrical involvement of the palindromic half-sites in mitotic *CEN* function.

Conserved base pairs outside the CDEIII core sequence contribute significantly to mitotic *CEN* function. In contrast to the very high conservation of base pairs of the CDEIII core sequences, the remaining nucleotides left and right of positions 11 to 17 show a diverse degree of preservation, with nonconserved base pairs at positions 9 to 10 and 18 to 22. We introduced single-base-pair changes at all conserved positions outside the core sequence. Since the analysis of the CDEIII core sequence had revealed transversions to be more detrimental to *CEN* function than transitions, preferential transversion mutagenesis of nucleotides outside the CDEIII core sequence was carried out. Mutating the fully conserved nucleotides at positions 2, 6, and 25 has a minor effect on *CEN* activity; the chromosome loss rates increase by a factor of only 2 (Table 1). A characteristic of CDEIII is its interrupted palindrome. The outward regions comprise the three T · A base pairs at positions 3 to 5 and the three A · T base pairs at position 23 to 25 (Fig. 1). Mutations at these positions lead to moderately reduced *CEN* activity. Changes affecting the left outward region (positions 3 to 5) have a stronger effect on *CEN* activity than do the corresponding mutations in the right half-site. Mutant CDEIII(3-A) shows an eightfold increase in chromosome loss (4.0×10^{-3}), the highest loss rate so far determined for a point mutant outside the core sequence. In general, despite the relatively high conservation of certain base pairs outside the CDEIII core sequence, several mutations at these positions exhibit almost wt *CEN* function.

A second-site mutation can partly compensate loss of function of a primary mutation. The 13 analyzed *CEN* DNAs display some sequence variation in CDEIII. Each of the highly conserved CDEIII core sequences is embedded in a specific sequence background characteristic for each *CEN* DNA. The results of the single-point mutant analysis presented show that all base pairs of the core sequence are important for *CEN* activity. In contrast, mutations outside the core often result in a marginal decrease of *CEN* function. As little is known about the interactions and dependencies of

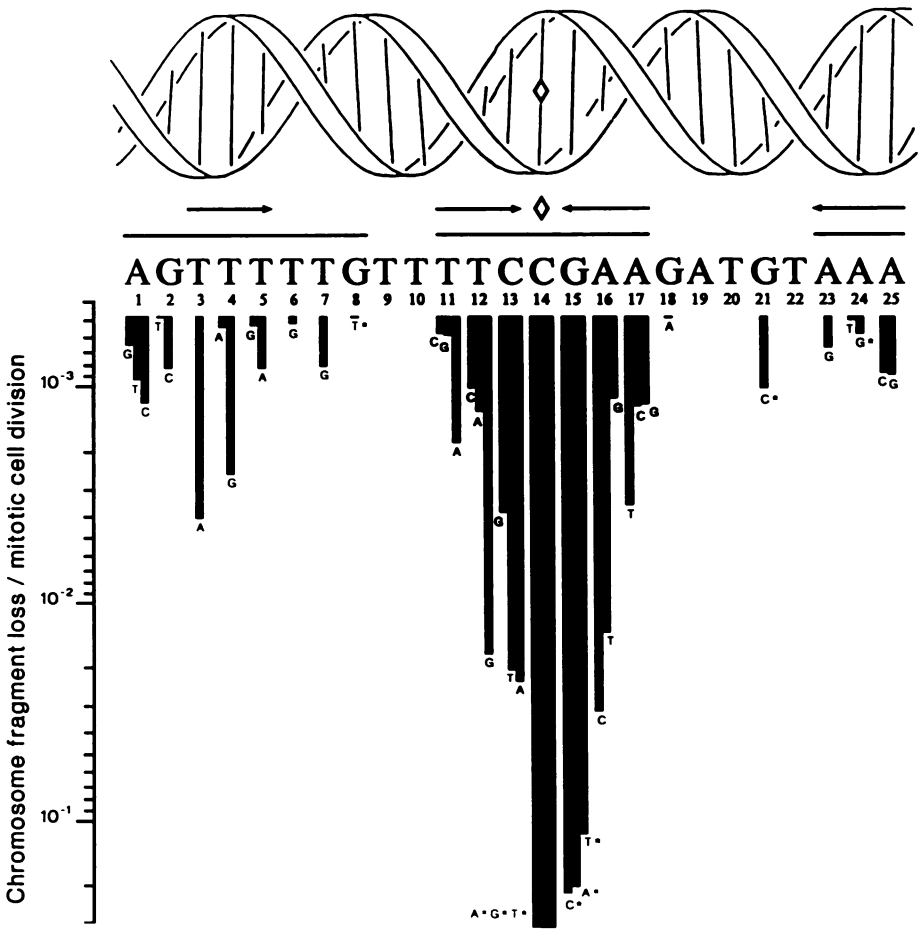

FIG. 4. Compilation of all single-point mutations in the *CEN* DNA element CDEIII from chromosome VI and their mitotic chromosome fragment loss rates. Data are taken from this study and from reference 22. Numbers below the sequence identify individual nucleotides, overlining marks indicate conserved regions, arrows indicate the palindromic structure, and the diamond indicates the twofold axis of symmetry. The B-DNA secondary structure as described elsewhere (46) is centered with its major groove on the CDEIII core sequence (diamond). Each bar represents the mitotic CF loss rate of a point mutation (Table 1), with the newly introduced nucleotide given below. Data for mutations marked by asterisks are from reference 22 and were recalculated as described in Materials and Methods.

the core sequence with the rest of the CDEIII sequence, double mutants within CDEIII were analyzed for their ability to function as a centromere. Core mutations at position 15 were chosen as the primary mutation. Changes at this position impair *CEN* function drastically (up to 300-fold-higher CF loss rate than for wt *CEN* [22]) but still allow quantification of mitotic chromosome segregation. For the second mutation, we chose positions 1 and 18, which in the case of *CEN6* do not carry the favorable base pairs found in other naturally occurring *CEN* sequences. At position 18 in eight other *CEN* DNAs an A · T base pair is found, and in *CEN6* the single-point mutant CDEIII(18-A) exhibits a loss rate identical to that of wt *CEN* (4.8×10^{-4}) (Table 1). The three double mutants CDEIII(15-T,18-A), CDEIII(15-A,18-A), and CDEIII(15-C,18-A) could not be analyzed with the CF stability assay, since their CF mitotic loss rates were too high. The percentage of cells carrying the CF was therefore measured after growth in selective medium as described in Materials and Methods. The results shown in Table 2 demonstrate that two double mutants exhibit a higher mitotic chromosome loss rate than the corresponding single mutant CDEIII(15-T). The third double mutant, CDEIII(15-A,18-A), shows approximately the same loss rate as CDEIII(15-T) alone. These results indicate that the effect of the primary mutations can be modulated by the introduction of a second mutation at position 18. We next asked what effect a second-site mutation in the other palindromic half-site (position 1) would have when combined with CDEIII(15) mutations. Introduction of CDEIII(1-T) into *CEN6* CDEIII increased the chromosome loss rate twofold (Table 1),

TABLE 2. Occurrence of CF in CDEIII double mutants

Yeast strain	Mutation	% ± SD of CF-carrying cells[a]
YRN32	CDEIII(15-T)	84 ± 6
YRN16	CDEIII(15-T,18-A)	59 ± 4
YRN21	CDEIII(15-A,18-A)	83 ± 6
YRN17	CDEIII(15-C,18-A)	45 ± 10

[a] Strains carrying chromosome fragments with the *CEN6* mutations indicated were grown in selective media as described in Materials and Methods. The cultures were diluted and plated on nonselective (SD+6) and selective (SD+6-Ura) plates to yield 200 colonies per plate. Ten different cultures were evaluated, and the percentage and standard deviation of cells bearing the CF was calculated.

TABLE 3. Chromosome loss in CDEIII double mutants

Yeast strain	Mutation	Chromosome loss/ cell division $(10^{-2})^a$
YRN15	CDEIII(1-T,15-T)	3.0
YRN20	CDEIII(1-T,15-A)	1.4

[a] Determined as described in Materials and Methods by performing fluctuation analyses by the method of the median.

although a T · A base pair at position 1 is highly conserved (Fig. 1). The double mutants CDEIII(1-T,15-T) and CDEIII (1-T,15-A) exhibit a lower loss rate than do the corresponding single mutants CDEIII(15-T) and CDEIII(15-A) (Table 3). Mutant CDEIII(1-T,15-T) has a chromosome fragment loss rate of 3×10^{-2}, which is about 3.7-fold lower than the rate for CDEIII(15-T), and the loss rate for CDEIII(1-T,15-A) (1.4×10^{-2}) is about 12.9-fold lower than for mutant CDEIII(15-A) alone (see Fig. 5B). Therefore, it is possible to introduce second-site mutations into CDEIII which can partly compensate loss of function of a primary mutation within this element.

DISCUSSION

The conserved *CEN* DNA element CDEIII is an essential component of the *CEN* DNA required in *cis* to ensure faithful mitotic and meiotic chromosome segregation. The aim of our mutation analysis was to determine the importance of all conserved CDEIII nucleotides in mitotic *CEN* function. In vitro-generated *CEN6* CDEIII point mutations were placed on an artificial CF and assayed for in vivo *CEN* activity. Our system for measuring chromosome stability is based on the colony color assay (23) using chromosome fragments (22, 55) and was modified by using the R/S system. Colonies growing on cycloheximide plates were shown to be simultanously red (loss of the *SUP11* gene) and Ura$^-$ (loss of *URA3* gene), indicating that the CF was lost. One possible problem associated with selection systems is the appearance of a phenotypic lag for drug sensitivity (22). We therefore ascertained that after plating of cells on cycloheximide-containing plates, no microcolonies indicative of residual growth of cells with the CF (carrying the *CYH*^{S}*2* allele) could be observed. This phenomenon may in part be due to the rapid decay of excess amounts of ribosomal protein L29, which is encoded by the *CYH2* gene (36).

The CDEIII core sequence may be recognized asymmetrically. Our saturation mutagenesis of the core sequence revealed that each single-base-pair change at these positions affects *CEN* function adversely (Table 1 and Fig. 4). The negative effect on *CEN* function increases gradually from both ends of the palindrome (positions 11 and 17) toward the central base pair at position 14, whose substitution abolishes *CEN* activity completely (22). The strong negative effects observed for point mutations within the core sequence of CDEIII were also reported for four different mutations at positions 13 to 15 in the centromere of chromosome III of *S. cerevisiae* (38, 44).

The bilateral symmetry of the core sequence supports the idea that the proteins binding to this sequence use two possibly identical subunits to bind in similar ways to the right and left halves of the palindromic core sequence. However, our data do not support such a proposal. As an example, mutations CDEIII(15-C) and CDEIII(13-G) represent identical base changes at symmetric locations, yet the right mutation [CDEIII(15-C)] reduces *CEN* activity about 60-fold more than the left mutation [CDEIII(13-G)] (Fig. 4). At each of the symmetrical positions (11 + 17, 12 + 16, and 13 + 15), the identical base pair change in the right half-site is more deleterious to *CEN* function, suggesting a greater overall contribution to DNA-protein interactions by the right half of the CDEIII core sequence. Thus, the results of the mutational analysis suggest asymmetric protein binding.

Recently, a similar situation was found for the CDEI sequence, which is bound in vivo by the helix-loop-helix protein CPF1. In this case, a stronger involvement of the left palindromic half-site in mitotic *CEN* function was identified (45b). Thus, in both elements the half-sites facing CDEII are less important than the half-sites pointing away from the rest of the *CEN* DNA. The significance of this finding is unclear at present. Indications for asymmetry in the binding of the *lac* repressor to the symmetrical *lac* operator have been described (reviewed in reference 3).

As shown in Fig. 4, mutating the central C · G bp at position 14 to its symmetrical counterpart G · C abolishes *CEN* function. This finding suggests that the central base pair is part of the half-site and consequently that the half-sites overlap. A similar situation exists for the *S. cerevisiae* transcriptional activator GCN4, which binds its 9-bp palindromic recognition sequence as a dimer (27). The binding site was shown to be asymmetric since the central base pair is important, suggesting that the GCN4 monomers make different contacts to the nonequivalent half-sites (26, 53).

Mutations within a protein binding site but outside the direct protein-DNA contact regions may affect protein binding by influencing the DNA conformation, as shown for the phage 434 operator-repressor system (32, 33). The conformational flexibility of the DNA is utilized to interact with proteins, and subsequently the DNA structure may get deformed (57). In our case, the proteins binding to the core sequence prefer a specific base pair at each position, as a change to the other three possible base pairs always results in reduced *CEN* activity. We therefore propose that CDEIII core sequence mutations exert their effects by interfering directly with protein-DNA interactions. It is unclear at present whether this direct interaction occurs via indirect or direct readout recognition (1, 18, 30, 33).

CDEIII palindromic sequences are located in three adjacent major grooves at the same side of the helix. Projection of the 25-bp-long CDEIII sequence onto a B-DNA helix shows that the inverted repeats are located on the same side of the double-helical structure and occupy three adjacent major grooves (Fig. 4). It has been suggested that the major groove has about twice the information content of the minor groove applicable for sequence-specific DNA-binding proteins (17). Our mutational analysis strongly suggests direct DNA-protein interactions for the core sequence. Furthermore, in vivo footprint analysis of *CEN6* has revealed that within the core sequence the upper-strand G nucleotide and the two lower-strand nucleotides are completely protected against methylation (16, 58), indicating tight DNA-protein interactions at these positions. Changing base pairs in the outer halves of the interrupted palindrome leads to a small reduction in the rate of mitotic CF loss. The strongest effect found so far is an eightfold increase in loss rate for the mutant CDEIII(3-A) located in the center of the left major groove. In general, base pair changes in the left region have a greater impact on *CEN* function than do changes on the right. This is in accordance with the finding that the left side of CDEIII is more conserved than the right (Fig. 1).

Generally, minor-groove base pairs in CDEIII, defined

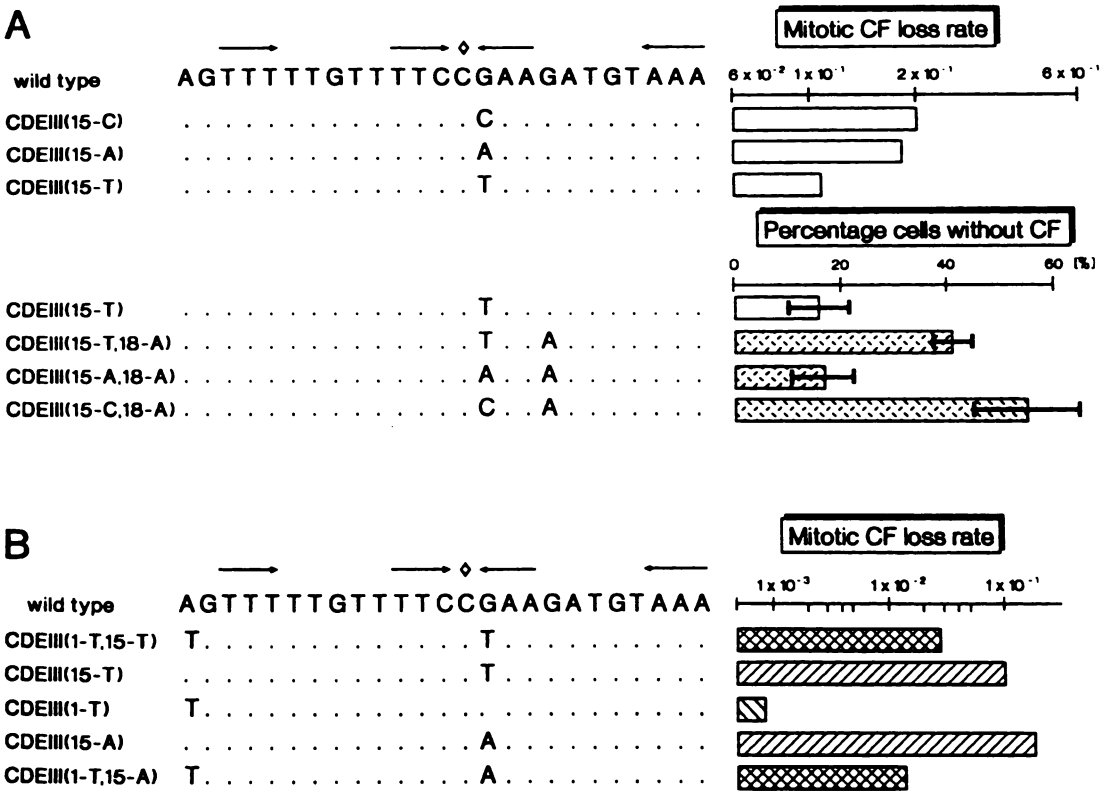

FIG. 5. Comparison of the effects of single and double mutations within CDEIII. The percentages of cells with no CF in panel A were recalculated from Table 2 to allow a better comparison to the corresponding CF loss rates. Error bars indicate standard deviations from the mean. In both panels, the mitotic CF loss rates for the single mutations CDEIII(15-T), CDEIII(15-A), and CDEIII(15-C) were taken from reference 22 and recalculated as described in Materials and Methods.

according to our scheme in Fig. 4, seem to play a subordinate role, and several of them are not conserved (Fig. 4). Nevertheless, these sequences may also contribute to *CEN* activity by playing a part in establishing the required local conformation of the CDEIII-carrying molecule (57). For example, a mutation of the nonconserved G · C bp at position 21 gives a twofold increase in mitotic chromosome loss rate (22), while a mutation at position 18 has no effect on *CEN* function, either because it does not contribute to centromere assembly or because the negative effect of this mutation is compensated by the extended palindromic core sequence. In addition, we find that point mutations of other highly conserved base pairs can have a very small effect on *CEN* function, e.g., CDEIII(2-T) in the major groove and CDEIII(8-T) in the minor groove. Furthermore, the change of CDEIII(8-G) to CDEIII(8-A) has no influence on mitotic *CEN* activity of *CEN3* (38, 44). Thus, the conserved G nucleotides at positions 2 and 8 behave identically in the in vivo mutational analysis. However, the in vivo footprint analysis reveals that the G nucleotide at position 2 is protected against methylation, while the G nucleotide at position 8 is accessible to dimethylsulfate (58). This discrepancy between mutation and protection analysis may be solved only after the protein components binding to CDEIII have been identified. Another possibility is the potential importance of these G · C base pairs for proper meiotic function of CDEIII.

Evidence for second-site suppression within CDEIII. The entire 25-bp-long CDEIII sequence appears to be involved in DNA-protein interactions, as second-site mutations are able to suppress primary mutations (Fig. 5). The single mutations at position 15 are localized in the major groove and show a strongly reduced *CEN* activity. In a double mutant with an additional mutation at position 1, the influence of the primary mutations at position 15 on *CEN* function changes significantly (Fig. 5B). The *CEN* activity of the double mutant CDEIII(1-T,15-A) is 12.9-fold higher than that of CDEIII(15-A) alone. Thus, the negative effects of base pair changes at positions most likely involved in specific DNA-protein interactions can be compensated by the introduction of a non-wt base pair at other positions (so-called intraelement suppression).

These results can be related to naturally occurring variations in *CEN* sequences. We had shown previously that the *CEN* activities of *CEN4* and *CEN6* are nearly identical (22). Recently, the centromere from chromosome II was isolated, and its mitotic chromosome loss rate was determined as 4.7×10^{-4}, using the R/S system (29a). Thus, *CEN2*, *CEN4*, and *CEN6* show almost identical *CEN* activity but exhibit differences in their CDEIII DNA sequences. We find four base pair changes between *CEN2* and *CEN6*, five between *CEN2* and *CEN4*, and nine between *CEN4* and *CEN6*. Most of these changes are in the nonconserved subregions of this element. If the different *CEN* DNAs are recognized by the same set of proteins and the levels of mitotic and meiotic centromere activity among all *CEN* DNAs are identical, then this finding indicates a degeneracy in recognition of CDEIII, which could help to explain how *CEN4*, which carries the only deviation from the conserved core sequence among all *CEN* DNAs [CDEIII(12-A) instead of CDEIII(12-T)], has

the same *CEN* activity as the other *CEN* DNAs. The rest of the *CEN4* CDEIII sequence may consist of a base pair composition which can compensate for the negative effect of this "wrong" base pair at position 12. It is also possible that the unusually short CDEII sequence in *CEN4* plays a role. In *CEN6*, mutant CDEIII(12-A) shows a 2.2-fold increase in mitotic chromosome loss. The sequence variations found in naturally occurring *CEN* sequences may in fact reflect a degeneracy in sequence recognition, and the double mutants described above are possibly the first step toward a novel synthetic *CEN* sequence with wt activity. The generation of mutants that display greater than wt mitotic *CEN* activity makes this a possibility (45a).

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