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A 125-Base-Pair *CEN6* DNA Fragment Is Sufficient for Complete Meiotic and Mitotic Centromere Functions in *Saccharomyces cerevisiae*

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***Saccharomyces cerevisiae* centromeres contain a conserved region ranging from 111 to 119 base pairs (bp) in length, which is characterized by the three conserved DNA elements CDEI, CDEII, and CDEIII. We isolated a 125-bp *CEN6* DNA fragment (named ML *CEN6*) containing only these conserved elements and assayed it completely separated from its chromosomal context on circular minichromosomes and on a large linear chromosome fragment. The results show that this 125-bp *CEN6* DNA fragment is by itself sufficient for complete mitotic and meiotic centromere functions.**

The stable maintenance of genetic information during meiosis and mitosis depends on highly accurate mechanisms of chromosome segregation. In the yeast *Saccharomyces cerevisiae*, missegregation of a chromosome is an extremely rare event, occurring only once in every 100,000 mitotic cell divisions (11) and once in every 10,000 meiotic cell divisions (29). Of essential importance to this high fidelity of chromosome transmission is the centromere region of the chromosome. The centromere (kinetochore), consisting of the chromosomal DNA segment (*CEN* DNA) and specific proteins (*CEN* proteins), provides the site of attachment to the mitotic and meiotic spindles.

In *S. cerevisiae* the *CEN* DNA of 12 of the 16 chromosomes has been cloned (4, 9, 15, 17, 20, 25). Sequence comparison revealed three conserved centromere DNA elements (CDEs) present in a segment that varied from 111 to 119 base pairs (bp) in length (9, 15, 17). The central portion consists of highly A+T-rich CDEII (78 to 86 bp), flanked 5′ by 8-bp CDEI (uTCACuTG) and 3′ by 25-bp CDEIII (TGT$^{T}_{A}$ T$^{T}_{A}$TG..TTCCGAA.....AAA). A variety of approaches have been used to analyze the DNA sequence requirements for centromere function in mitosis. Deletion of a 627-bp genomic *CEN* sequence that includes the three conserved DNA elements from chromosome III led to extreme instability of the resulting acentric chromosome (5). Mutational analysis, including point mutations, deletions, and insertions, confirmed and further characterized the importance of these three elements for centromere function (6–8, 10, 12, 13, 21, 22, 24). Less is known about the DNA sequence requirements for *CEN* function in meiosis. Meiotic cell divisions involve two types of centromere-mediated chromosome segregation. In meiosis I, reductional division occurs (sister chromatid pairs separate); in meiosis II, equational division occurs (sister chromatids separate), a process similar to mitotic chromosome segregation. It is as yet unclear to what extent *CEN* DNA sequences required in meiosis and mitosis are identical or different. It has recently been shown by deletion analysis that CDEI is important for maintaining the association of sister chromatids in meiosis I when analyzed on circular minichromosomes (7).

The length of the conserved segment containing CDEI, CDEII, and CDEIII (111 to 119 bp) is considerably shorter than the region of 220 to 250 bp which was shown to be protected in nuclease digestion experiments (1). Therefore, it was possible that additional sequences flanking the conserved elements were needed in *cis* to specify complete mitotic and meiotic centromere functions. Previous work to delimit the functional borders of *CEN* DNA have included two basic approaches: deletional analysis on circular minichromosomes and genomic deletion/substitution experiments on chromosome III. With the first approach it was shown that undirectional deletions up to, but not including, the boundaries of CDEI or CDEIII had no effect on plasmid stability (12, 24). Because circular minichromosomes are lost at a rate of once in 100 cell divisions (3 orders of magnitude more frequently than authentic chromosomes), it could not be concluded that all sequences flanking the conserved elements are dispensable on authentic chromosomes. In the second approach a 627-bp *CEN3* fragment on chromosome III is replaced by a *CEN* deletion derivative and the effect on segregation of the resultant chromosome III derivative is tested. It was shown that replacement by a 289-bp fragment (that includes 8 bp to the left of CDEI and 170 bp to the right of CDEIII) or a 211-bp fragment (that includes 64 bp to the left of CDEI and 38 bp to the right of CDEIII) yielded a mitotically stable chromosome III derivative (3, 21). These experiments show that sequences between the endpoints of the 627-bp *CEN3* fragment being replaced and the endpoints of the deletion fragments being inserted are not required for mitotic *CEN* function. The potential role of sequences in the chromosomal centromere region that are present outside the 627-bp *CEN3* fragment cannot be assessed since these sequences are retained in the deletion/substitution chromosome III derivatives.

To determine the minimal-length centromere sequence (ML *CEN*) required for full centromere activity in both mitosis and meiosis, we isolated a 125-bp *CEN6* DNA fragment (ML *CEN6*), using conveniently located restriction sites. Testing ML *CEN6* with the recently developed chromosome fragment assay (P. Hieter, manuscript in preparation; 13) and on plasmids allows us to conclude that this *CEN* DNA fragment is, by itself, sufficient to fulfill complete

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mitotic centromere function. Plasmid data presented here suggest that the high A+T content normally found immediately surrounding the conserved CDEs is not important in determining *CEN* function. Furthermore, the meiotic activity of ML *CEN6* is indistinguishable from that of a 1,160-bp *CEN6* DNA fragment when placed on linear artificial chromosomes.

MATERIALS AND METHODS

Enzymes and media. Restriction enzymes, T4 DNA polymerase, Klenow polymerase, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals and used according to the specifications of the manufacturer. Zymolyase (20T) was purchased from ICN Biomedicals. Plasmid DNA preparations and other routine bacteriological and cloning techniques were done according to Maniatis et al. (19) with minor modifications. Yeast media and growth conditions were as described previously (28).

Mitotic plasmid stability assays. Plasmids were transformed into yeast strain SX1-2. Transformants were colony purified on minimal agar medium and six independent transformants were then chosen for testing. The *CEN* plasmid mitotic stability assay was performed as previously described (12, 13). The percentages of Trp$^+$ cells present in cultures were determined after 10 generations of growth in nonselective liquid medium.

Bacterial and yeast strains. *Escherichia coli* MM294 [*endA1 thi hsdR* Δ(*srlR-recA*)*306*], JM83 [*ara* Δ(*lac-proAB*) *rpsL h80 lacZ*Δ*M15*], and JM101 [*supE thi* Δ(*lac-proAB*) *F' traD36 proAB lacI*^{q} *lacZ*Δ*M15*] were used for *E. coli* transformations, plasmid preparations, and single-stranded bacteriophage preparation. The *S. cerevisiae* strains used were SX1-2 (α *trp1 his3 ura3-52 gal2 gal10*) (source, R. Davis), YPH49 [**a**/α *ura3-52/ura3-52 ade2-101/ade2-101 lys2-801/lys2-801 trp1-901/trp-901* (*psi*$^-$)], and YPH4 (**a** *ura3-52 lys2-801 ade2-101 his3-D200*).

Plasmid constructions. A 392-bp *Taq*I-*Taq*I DNA fragment containing *CEN6* was isolated from *CEN* plasmid pAS2 (24) and cloned into the *Acc*I site of pUC18 (29), yielding plasmid pUC18-*CEN6*:31. This plasmid was digested with *Pst*I and *Hin*dIII, and the 410-bp fragment (bearing the 392 bp of *CEN6*) was isolated and further digested with *Mbo*II. The 125-bp *Mbo*II-*Mbo*II *CEN6* fragment (called ML *CEN6* for minimal-length centromere 6) was submitted to the exonuclease activity of T4 DNA polymerase to remove the one-base 3′ overhanging ends and then cloned into the *Hin*cII site of the cloning area of pUC19 (30), yielding plasmid pUC19-*CEN6*:32. The orientation of this 125-bp *CEN6* DNA fragment was determined by cloning an *Eco*RI-*Hin*dIII fragment of pUC19-*CEN6*:32 into the corresponding sites of M13mp8 (30) and sequencing it (26). Blunt-ended 125-bp ML *CEN6* was cloned into the unique *Pvu*II site of yeast shuttle plasmid pLA427 (25) and into the unique *Cla*I site (blunt ended by fill-in with the Klenow polymerase) of pLA427, yielding pGC1 and pGC2, respectively. In addition, the *Eco*RI-*Hin*dIII fragment of pUC19-*CEN6*:32 bearing ML *CEN6* was cloned into the corresponding sites of pUC18, yielding pUC18-*CEN6*:40. From this plasmid a 447-bp *Pvu*II fragment (containing the 125 bp of *CEN6* in the cloning area of pUC19) was isolated and inserted into the unique *Pvu*II site of pLA427, yielding pGC3. Plasmid pJS27 was constructed by isolating the 155-bp *Bam*HI-*Hin*dIII fragment (containing ML *CEN6*) from pUC19-*CEN6*:32 and cloning it into the *Bam*HI-*Hin*dIII sites of pYCF5 (13).

Generation of CFs and determination of mitotic stability by fluctuation analysis. The parental plasmid used to generate a 150-kilobase (kb) chromosomal fragment (CF) was pYCF5 (13). The plasmids carrying the various *CEN* constructs were linearized at the *Not*I or *Eco*RI site and introduced into yeast strain YPH49 by the lithium acetate procedure, and URA$^+$ transformants were selected. Transformants were streaked onto nonselective plates to verify a stable pink phenotype resulting from one copy of the *SUP11* gene per diploid genome (14). The presence of the expected CF was confirmed by OFAGE (27) analysis of chromosome-sized DNA. Fluctuation analysis was performed as previously described (13). The rate of loss per cell division was obtained by determining the median number of cells without the CF in 10 test colonies. This median value was used to calculate the mean of CF loss events during the growth of the test colonies by using the mathematical expression derived from Lea and Coulson (18). The rate of loss per cell division is the ratio of the number of loss events (entirely red colonies) to the total number of plated cells (red, pink, and white colonies).

Meiotic analysis. Diploid strains YPH281 and YPH301, carrying one copy of the CF containing a 1.16-kb *CEN6* DNA fragment (13) and the 125-bp ML *CEN6* (this study), respectively, were sporulated and dissected. URA$^+$ spores were isolated and mated to strain YPH4. The resultant diploids, YJH2 and YJH3, were heterozygous for the chromosomal centromere-linked *TRP1* gene. All sporulations were done as previously described (28). Cells from a nonselective plate were spread onto sporulation plates and incubated for 4 to 5 days. Tetrads were suspended in 100 μl of 1 M sorbitol and treated with 1 μl of zymolyase (20 mg/ml, 20,000 U/g) for 10 to 12 min at room temperature. After the addition of 1 ml of 1 M sorbitol, the tetrad suspension was stored on ice and samples were placed on YPD (28) plates for dissection. The genotypes of the resulting spores were determined by replica plating.

RESULTS

Cloning of ML *CEN6*. The length of the DNA segment containing the conserved DNA elements CDEI, CDEII, and CDEIII varies slightly among the 12 *CEN* sequences analyzed to date due to variations in the length of CDEII. The average size is 117 bp. In the case of *CEN6*, the three elements have a total length of 118 bp and are flanked by two *Mbo*II restriction sites (Fig. 1A). A 392-bp *Taq*I-*Taq*I *CEN6* fragment and a 125-bp *Mbo*II-*Mbo*II *CEN6* fragment (called ML *CEN6*) were subcloned into the multicloning areas of pUC18 and pUC19, respectively. Plasmids pUC18-*CEN6*:31 and pUC19-*CEN6*:32 allow 21 unique enzyme combinations to be used to reisolate either the 392-bp *CEN6* or the 125-bp ML *CEN6* DNA fragment (Fig. 1B).

Mitotic analysis of ML *CEN6* on plasmids. To perform the mitotic plasmid stability assay (12), the 125-bp ML *CEN6* fragment was cloned into vector pLA427. Although small circular *CEN* DNA-containing minichromosomes have a relatively high level of loss (loss rate, approximately 10^{-2} per cell division under nonselective conditions), this assay is fast and convenient and therefore useful in defining centromere function to a first approximation. We placed the ML *CEN6* DNA fragment into the unique *Cla*I restriction site of pLA427, resulting in plasmid pGC2, and into the unique *Pvu*II site of pLA427 in both orientations, resulting in plasmids pGC1 and PGC3 (Fig. 2A). (For details see Materials and Methods). The maps and orientations of CDEI, CDEII, and CDEIII for each plasmid are depicted in Fig. 2A. The main differences among the three constructs are the positions of ML *CEN6* within pLA427, which changes the

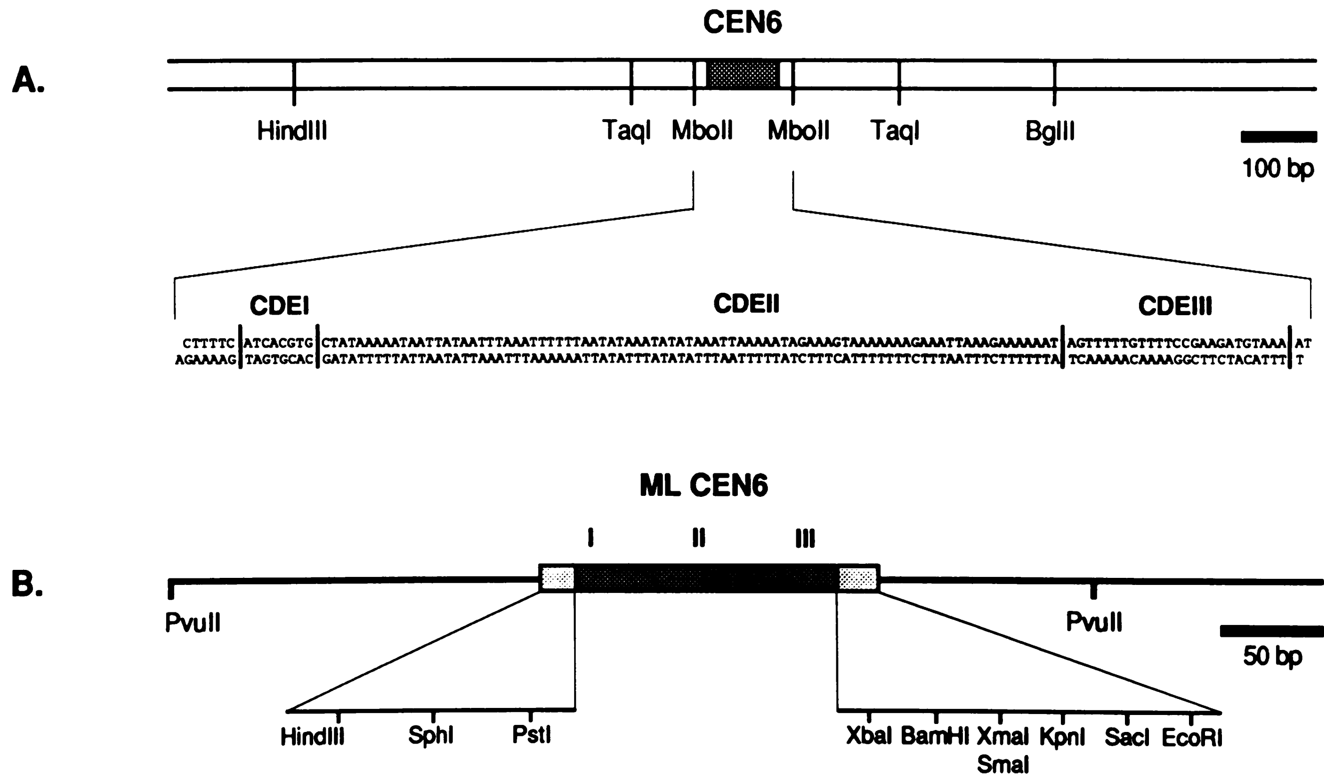

FIG. 1. Isolation of ML *CEN6*. (A) Map of the centromere region on chromosome VI, with the conserved *CEN* DNA elements indicated by the hatched box. Not all *Mbo*II and *Taq*I sites outside the central *Taq*I-*Taq*I fragment are shown. The sequence of the 125-bp *Mbo*II-*Mbo*II DNA fragment (ML *CEN6*) is shown below. (B) ML *CEN6* was cloned into the *Hin*cII site of pUC19, yielding plasmid pUC19-*CEN6*:32. Twenty-one unique restriction enzyme combinations can be used to isolate the *CEN6* DNA. The hatched region represents the conserved DNA elements, and the lightly shaded boxes shown at the bottom indicate the multicloning area from pUC19.

surrounding DNA of the conserved elements. It has been shown before that a 392-bp *Taq*I *CEN6* DNA fragment cloned into the *Cla*I site of pLA427 (pAS2) exhibits mitotic function on circular minichromosomes (24). This vector was included as a positive control in our analysis. pAS2 has a size nearly identical to that of pGC3 and is only 300 bp bigger than pGC1 and pGC2. This is important since it is known that the mitotic stability of *CEN*-containing plasmids is reduced as the plasmids become smaller (12, 24). In pAS2 the DNA immediately surrounding CDEI, CDEII, and CDEIII is identical to the *CEN6* chromosomal situation. The A+T content is 75 and 80% for the first 40 bp to the left and to the right of the conserved elements, respectively. In Fig. 2B the first 40 nucleotides surrounding CDEI, CDEII, and CDEIII are shown for all four constructs. A comparison of the 12 sequenced *CEN* DNAs revealed that CDEI, CDEII, and CDEIII, on average, are surrounded by 75% A+T. In our plasmids this number goes down to as low as 40% A+T, depending on the particular construct. It has been shown previously that the orientation of a *CEN* DNA sequence has no influence on centromere activity when analyzed on an authentic chromosome (5). Plasmids pGC1 and pGC3 carry ML *CEN6* in opposite orientations (Fig. 2A). It should be noted that sequences from pBR322 linked to the residual *TRP1* promoter in pLA427 help to fulfill promoter function (2).

The results of the plasmid mitotic stability assay are presented in Table 1. Centromere function is tested by comparing the percentage of Trp^+ cells (which carry the plasmid) before and after growth for 10 generations in nonselective medium. The centromere activity of ML *CEN6* is quite similar in all constructs, although the results for pGC2 and pGC3 are slightly more than 1 standard deviation away from pAS2. This can be explained by the high fluctuation in plasmid stability especially obtained for small circular minichromosomes. The standard deviation is calculated from the measurements of five or six independent transformants for each construct. We conclude that ML *CEN6* functions nearly as well (or perhaps as well) as the 392-bp *CEN6* in pAS2 does. The data indicate that a high A+T content within the first 40 bp immediately left and right of the conserved elements is not important in determining *CEN* activity on plasmids. The results also show that ML *CEN6* can act independently of its orientation on plasmids. These assays are limited to detecting relatively large negative effects on centromere activity since circular *CEN* plasmids have a high background rate of loss (10^{-2} per cell division).

Mitotic behavior of ML *CEN6* when located on a linear artificial chromosome. The analysis of mitotic *CEN* activity on plasmids showed that ML *CEN6* exhibits approximately full *CEN* function when compared with *CEN6* DNA fragments of a larger size. To test ML *CEN6* on a chromosome, we used the far more sensitive CF assay (13). In this assay the *CEN* DNA of interest is cloned into plasmid pYCF5. Upon linearization, one end of the vector carries a 1.5-kb-fragment of the telomere-adjacent Y' region; the other end carries a 2.7-kb DNA fragment which is located on the left arm of chromosome III at a distance 10 kb from the centromere. After yeast transformation the ends undergo homologous recombination, generating a new artificial nonessential linear CF (~150 kb) (Fig. 3C). Since the artificial chromosome carries a tRNA suppressor (*SUP11*) and the

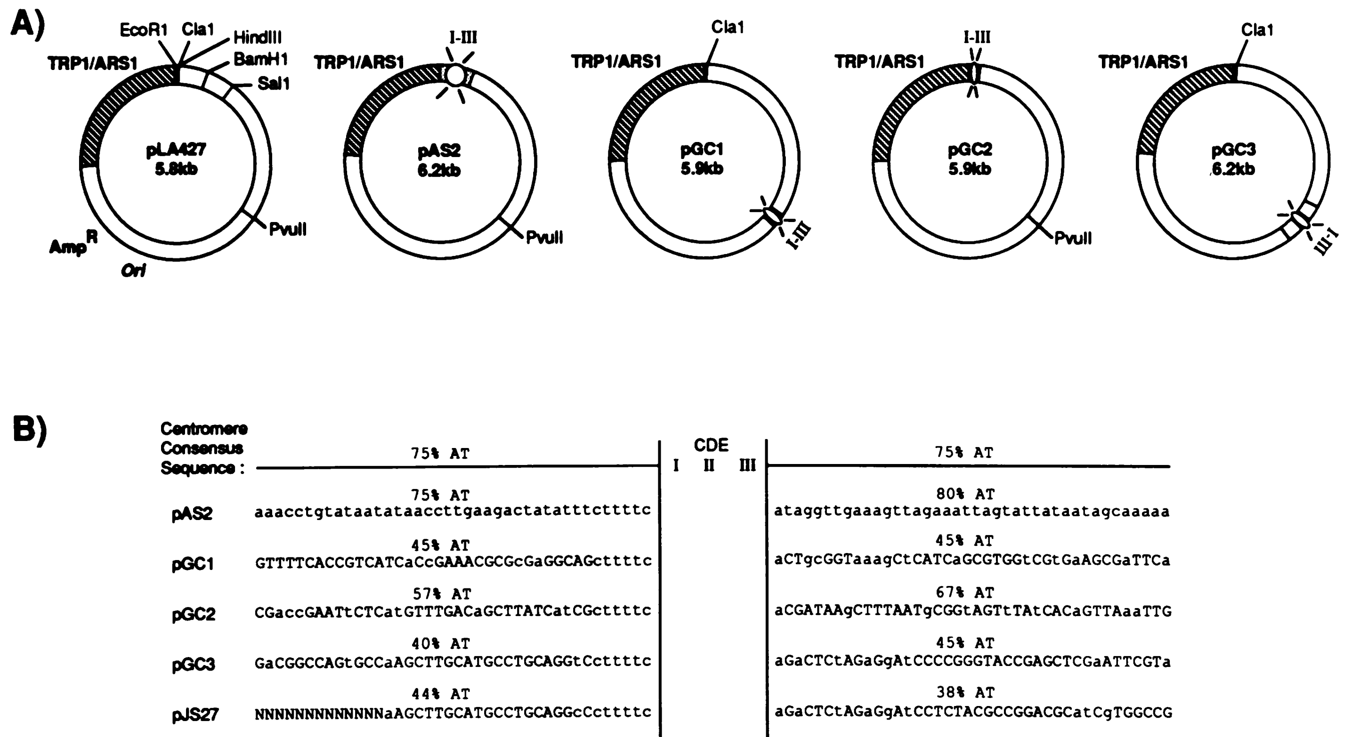

FIG. 2. Cloning of ML *CEN6* into a yeast vector. (A) The *E. coli*-yeast shuttle vector pLA427 is very similar to YRp7 (24). The ARS sequence allows autonomous replication of the plasmid in *S. cerevisiae*, while the *TRP1* gene is used to select for yeast transformants and as a marker in the mitotic plasmid stability assay. Plasmid pAS2 was generated by cloning the 392-bp *CEN6* DNA fragment into the unique *Cla*I site of pLA427. pGC1, pGC2, and pGC3 contain the 125-bp ML *CEN6* DNA fragment cloned into the unique *Pvu*II or *Cla*I site of pLA427 (for details see Materials and Methods). The orientation of ML *CEN6* in all constructs is indicated. (B) The sequence of the first 40 bp located immediately left and right of the conserved elements CDEI, CDEII, and CDEIII is shown for each construct. Lowercase letters indicate that a particular nucleotide is identical to the wild-type *CEN6* sequence at this position. For a comparison the DNA sequence surrounding ML *CEN6* in fragmentation vector pJS27 is presented. N, Nucleotides not determined.

yeast strain used is homozygous for *ade2-101* (and therefore gives rise to red colonies), the presence (pink color) or absence (red color) of the CF can be easily monitored by screening the color of the colonies. We had previously analyzed the mitotic stability of such a CF carrying the 1.16-kb *CEN6* DNA fragment. The loss rate of this artificial chromosome per mitotic cell division was calculated to be 1.9×10^{-4} (13). To apply this assay to ML *CEN6*, a 155-bp *Bam*HI-*Hin*dIII ML *CEN6* fragment from pUC19-*CEN6*:32 (Fig. 1B) was cloned into pYCF5, yielding pJS27 (Fig. 3A). This construct has an A+T content of 45 and 38% for the ML

TABLE 1. Mitotic plasmid stabilities[a]

Plasmid	% of Trp$^+$ cells after growth in nonselective medium	
	0 generation	10 generations
pAS2	79 ± 4	60 ± 5 (6)
pGC1	82 ± 5	54 ± 4 (6)
pGC2	71 ± 8	47 ± 7 (5)
pGC3	65 ± 6	45 ± 5 (5)
pLA427	2 ± 2	≤1 (6)

[a] The plasmids described in detail in the legend to Fig. 2 were transformed into haploid yeast strain SX1-2, and five or six independent transformants were chosen randomly to perform the mitotic plasmid stability assay as described earlier (13). The percentages of Trp$^+$ colonies, together with the standard deviations, are presented. The number of transformants tested is shown within parentheses. The negative control pLA427 lacks a centromere sequence.

CEN6-surrounding DNA in comparison with 75 and 80% for the genomic *CEN6* sequence (Fig. 2B). After cleavage with *Eco*RI, the linear DNA was transformed into diploid yeast strain YPH49, selecting for Ura$^+$ transformants. Electrophoretic karyotypes of transformants were determined by pulse-field gel electrophoresis of chromosome-sized DNA. Ethidium bromide-stained gels are shown for selected transformants in Fig. 3B. Parent strain YPH49 shows a length polymorphism for chromosome III indicated by the bracket. The newly generated CF in strains YPH281 (1.16-kb *CEN6*) and YPH301 (ML *CEN6*) can be seen at the bottom of the gel (arrow). As a result of the homologous recombination event, chromosome III is lost in about 10% of the transformants, leading to the karyotype $2n - 1 + CF$ (P. Hieter, unpublished results; see also reference 13). A transformant of the type $2n + CF$ (as shown in Fig. 2B) was chosen to measure the CF mitotic loss rates. The results of the fluctuation analysis for ML *CEN6* are listed in Table 2. A chromosome fragment containing ML *CEN6* is lost at a rate of 2.02×10^{-4}, which is indistinguishable from the rate associated with the 1.16-kb *CEN6* DNA fragment (1.9×10^{-4}). It has previously been shown that a 1.49-kb *CEN4* fragment gives a very similar CF loss rate of 1.7×10^{-4} (13). These results indicate that ML *CEN6* is sufficient for complete mitotic function on chromosomes. Therefore, wild-type *CEN* function in chromosomal centromere regions does not appear to rely on sequences flanking the 125-bp minimal DNA segment that contains CDEI, CDEII, and CDEIII.

Meiotic behavior of ML *CEN6*. The efficiency with which

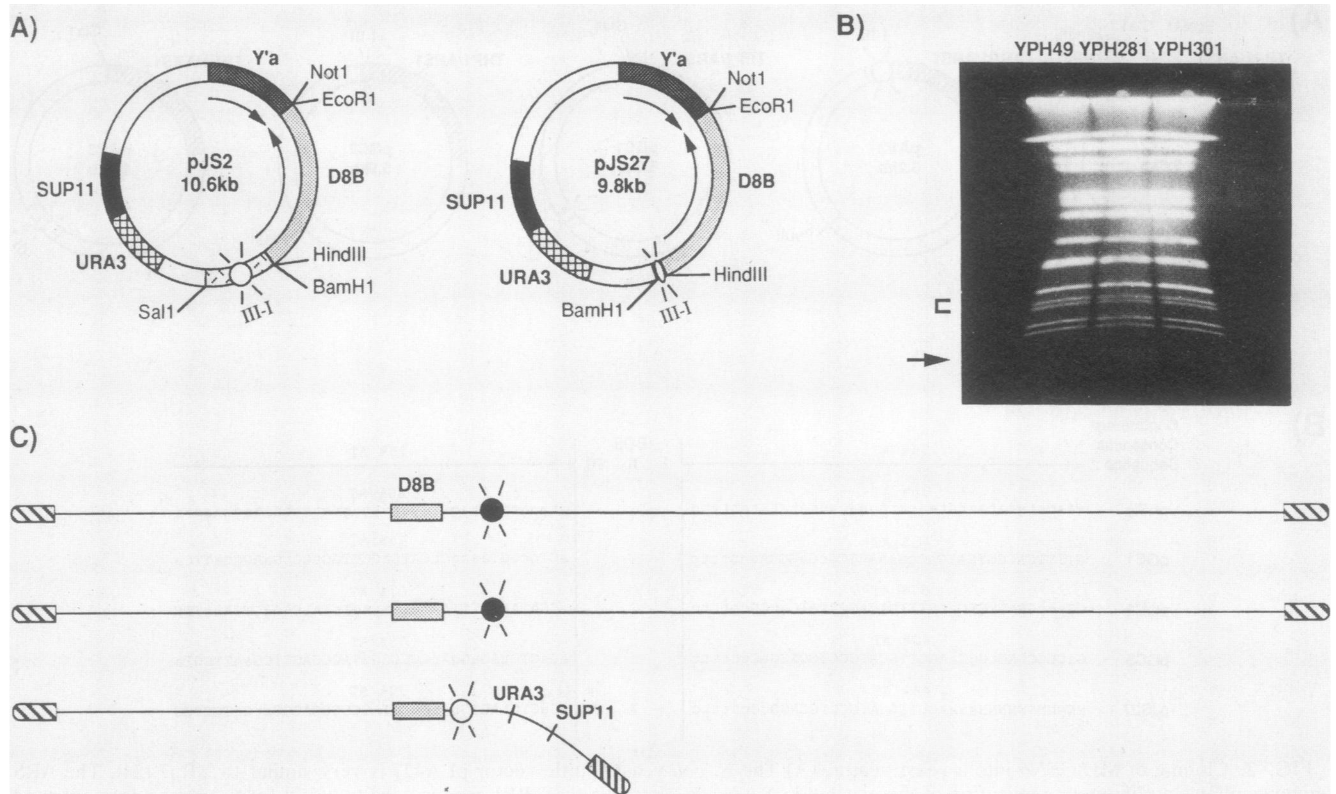

FIG. 3. Generation of CFs. (A) A 1.16-kb *Bam*HI-*Sal*I *CEN6* fragment and a 155-bp *Bam*HI-*Hin*dIII fragment (ML *CEN6*), including 30 bp of the pUC19 cloning area, were cloned into pYCF5 (13), yielding pJS2 and pJS27, respectively. Vector pYCF5 contains the genes *URA3* for genetic selection and *SUP11* for measuring the mitotic stability of the artificial chromosomes once they are generated. After linearization with *Eco*RI, the plasmids carry a fragment of the telomere-adjacent Y' region at one end and a unique fragment from chromosome III (called D8B) at the other end. Upon transformation each of the two ends undergoes a homologous recombination event with chromosomal sequences, which finally leads to a 150-kb telocentric CF. For details see Materials and Methods and reference 13. (B) OFAGE analysis of yeast transformants carrying CFs. YPH281 was generated by transformation of parental diploid strain YPH49 with pJS2 (1.16-kb *CEN6*) and YPH301 by transformation with pJS27 (ML *CEN6*). The bracket indicates the chromosome-length polymorphism of chromosome III; the arrow points to the generated artificial chromosome. (C) Shown schematically is the product of the fragmentation event leading to the in vivo generation of the CF. The chromosomal location of D8B on chromosome III is indicated, as well as the positions of *URA3* and *SUP11* on the artificial chromosome (not to scale), which are embedded in pBR322 sequences.

ML *CEN6* can function during meiotic cell divisions was tested by following meiotic segregation of the CFs described above. Diploids YJH2 and YJH3 were constructed as described in Materials and Methods. The strains were induced to undergo meiosis and tetrads were dissected. Each strain is heterozygous for the *TRP1* locus, which is a marker tightly linked (1 centimorgan) to the centromere of chromosome IV. *TRP1* marks sister spores, the products of cell division in meiosis II. We examined the segregation of the chromosome fragment by following the *URA3* marker relative to the *TRP1* marker (Fig. 4). If meiotic segregation is faithful, the chromosome fragment should segregate to sister spores (parental or nonparental ditypes). The presence of *URA3* in nonsister spores (tetratype) would indicate a premature disjunction event in meiosis I. The presence of *URA3* in only one spore (1+:3−) would indicate chromosome loss or nondisjunction in meiosis II. Results of the meiotic analysis are presented in Table 3. For YJH2 (1.16-kb *CEN6* on the CF), three tetratypes were observed in the 117 four-spore tetrads that were analyzed (2.6%). For YJH3 (ML *CEN6* on the CF), only one tetratype was observed in the 167 tetrads analyzed (0.6%). The likelihood of finding tetratypes in our strains has a background of about 1% due to the 1-centimorgan distance of *TRP1* to its centromere. (It should be noted that the *URA3* gene is embedded in pBR322 sequences on the short arm of the CF. Thus, since the short arm has no homolog in these strains [i.e., is hemizygous], recombination between the *URA3* marker and the centromere on the CF cannot occur

TABLE 2. Mitotic CF loss rate of ML *CEN6*[a]

Test colony	Colony size	No. of red segregants
1	42,700	>600
2	61,100	90
3	55,800	72
4	52,400	62
5	52,300	36
6	44,300	32
7	36,400	24
8	26,400	20
9	49,100	18
10	58,000	12

[a] Yeast strain YPH301 carrying ML *CEN6* on the newly generated artificial chromosome was analyzed to determine the CF loss rate per cell division by fluctuation analysis, using the method of the median (18). Average colony size, 47,900; median number of red segregants, 34; mean number of red segregants, 9.7; CF loss rate = 9.7/47,900 = 2.02×10^{-4}.

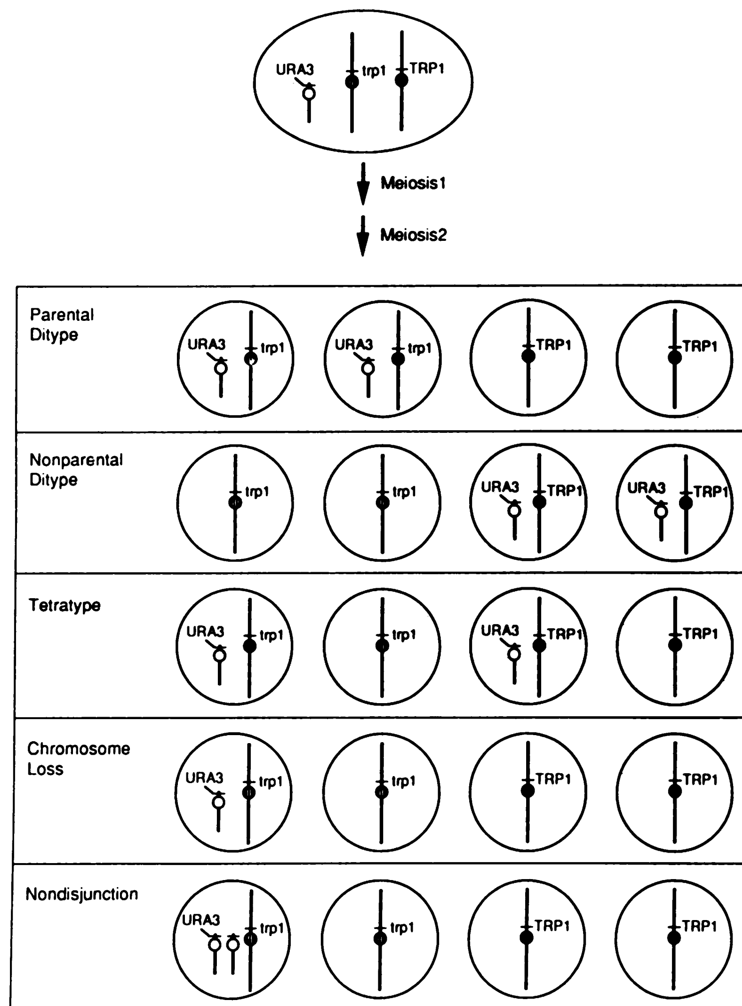

FIG. 4. Segregation behavior of CFs in meiosis. The distribution of the relevant centromere-linked markers are diagramed for normal (parental ditype, nonparental ditype) and abnormal (tetratypes, 1+:3−) segregation of the CF in meiosis.

and therefore does not contribute to the frequency of tetratype tetrads.) The frequency of 1+:3− tetrads for YJH2 and YJH3 was nearly identical (1.7 and 1.2%, respectively). The results show that ML *CEN6* is as efficient as a 1.16-kb *CEN6* DNA fragment in fulfilling centromere function in meiosis.

TABLE 3. Meiotic behavior of CFs containing either the 1,160-bp *CEN6* or the 125-bp ML *CEN6* fragment[a]

Determination	YJH2 (wild-type *CEN6*)		YJH3 (ML *CEN6*)	
	No. of tetrads	%	No. of tetrads	%
Parental ditype	54	46.1	81	48.5
Nonparental ditype	58	49.6	83	49.7
Tetratype	3	2.6	1	0.6
Chromosome loss or nondisjunction	2	1.7	2	1.2
Tetrads analyzed	117		167	
Four-spore-tetrad viability		65		62

[a] The rarely observed 1+:3− events were not analyzed further.

DISCUSSION

The aim of this work was to determine to what extent a minimal DNA fragment containing only the three conserved DNA elements CDEI, CDEII, and CDEIII is able to function as a centromere in both mitosis and meiosis. By taking advantage of two naturally occurring restriction sites located 6 bp to the left of CDEI and 1 bp to the right of CDEIII in *CEN6* DNA, we isolated a 125-bp fragment carrying CDEI, CDEII, and CDEIII and analyzed its centromere function on plasmids and on an artificial chromosome. In all assays the minimal *CEN6* sequence was embedded in sequences completely separated from its normal chromosomal context. Therefore, participation of any chromosomal sequences outside the conserved elements is excluded.

The mitotic stability of the *CEN* plasmids pAS2, pGC1, pGC2, and pGC3 was determined by measuring the percentage of plasmid-bearing cells after 10 generations of growth in nonselective medium. The analysis of ML *CEN6* on plasmids allows us to conclude that (i) the orientation of the *CEN* DNA on a plasmid does not seem to affect its activity and (ii) the A+T content within the first 40 nucleotides of the surrounding DNA can be lowered to 40% without drastically influencing the ability of ML *CEN6* to function as a cen-

tromere. ML *CEN6* was also analyzed for mitotic centromere function on a large linear artificial chromosome by testing the mitotic stability of *SUP11*-marked CFs. This method allows the quantitative measurement of mitotic centromere activity of a given *CEN* DNA over a three-log range (10^{-2} to 10^{-4}) (13). Of particular importance to this work, the *CEN* activity associated with a specific cloned *CEN* DNA segment is measured in a context completely separate from the chromosomal centromere region. Previously, we have analyzed the mitotic stability of CFs carrying wild-type *CEN6* (1.16-kb) or *CEN4* (1.49-kb) sequences. The CF loss rates per cell division associated with these *CEN* sequences are 1.87×10^{-4} (*CEN6*) and 1.68×10^{-4} (*CEN4*) (13). The results of the work described here show that a CF bearing ML *CEN6* exhibits wild-type segregation behavior (2.02×10^{-4}). The A+T content surrounding ML *CEN6* on the CF (44% left and 38% right) is lower than that of any of the plasmid constructs. Therefore, it seems that the natural average A+T content of 75% found for the surrounding DNA of the 12 sequenced *CEN* DNAs does not contribute significantly to mitotic centromere activity. Ng and Carbon fused foreign DNA at a point 4 bp from the right boundary of CDEIII of *CEN3* and observed a fivefold-reduced mitotic stability when analyzed on chromosomes by centromere substitution (22). This result is in contrast to our finding but might reflect the possible negative influence of a particular flanking foreign DNA on centromere activity.

The analysis of the meiotic segregation of *CEN* plasmids led to the conclusion that sequences outside a 392-bp *CEN6* or a 289-bp *CEN3* DNA fragment were not essential for proper meiotic centromere function on circular minichromosomes (3, 24). Genomic centromere deletion/substitution experiments showed that a 627-bp *CEN3* segment could be replaced by a 289-bp *CEN3* segment with no apparent effect on meiotic segregation of the resultant chromosome III derivative (3). These experiments are unable to delimit the minimal *CEN* DNA segment that is sufficient for segregation in meiosis. As with mitotic segregational analyses, the plasmid meiotic data are restricted to defining large negative effects because of the high background of meiotic segregational mistakes associated with circular minichromosomes. In the centromere substitution experiments, DNA sequences outside the 627-bp *CEN3* segment are retained on chromosome III, precluding an assessment of potential *cis*-acting sites in these flanking regions. From the work presented here, we can now limit the functional centromere sequence required in meiosis to 125-bp comprising just CDEI, CDEII, and CDEIII. A chromosome fragment carrying ML *CEN6* exhibited the same accuracy of segregation in meiosis I and II as did a chromosome fragment carrying a 1,160-bp *CEN6* DNA fragment.

The results of the present work argue against the active participation of specific DNA sequences outside the conserved elements in mitotic or meiotic centromere function. We cannot rule out the possibility that under certain circumstances sequences located outside the conserved elements may influence *CEN* activity. Such sequences could comprise unusual DNA conformations or promoter and terminator sequences. For example, it has been shown that placing a strong promoter adjacent to a *CEN* DNA can repress centromere activity (16, 23). The definition of the ML *CEN* sequence will help us to investigate the details of *CEN* protein-DNA interactions and the role of flanking DNA sequences in modulating the efficiency of centromere assembly or *CEN* action or both.

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