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Structure and Sequence of the Centromeric DNA of Chromosome 4 in *Saccharomyces cerevisiae*

CARL MANN AND RONALD W. DAVIS*

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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The *CEN4* sequences from chromosome 4 that impart mitotic stability to autonomously replicating (*ARS*) plasmids in yeast cells have been localized to a 1,755-base-pair (bp) fragment. This fragment could be cut in half to give two adjacent, nonoverlapping fragments, that each contained some mitotic stabilization sequences. One of the half-fragments worked as efficiently as the larger fragment from which it was derived, while the other half provided a much poorer degree of mitotic stabilization. Sequencing of 2,095 bp of DNA including this region revealed the presence of a centromere consensus sequence, elements I, II, and III (M. Fitzgerald-Hayes, L. Clarke, and J. Carbon, Cell 29:235–244, 1982), in the half-fragment providing high levels of mitotic stability. The poorly stabilizing half-fragment did not contain any obvious sequence homologies to other centromere sequences. Deletion analysis of the 1,755-bp fragment indicated that removal of the 14-bp element I plus 16 of the 82 bp of element II impaired mitotic stability. Removal of elements I and II eliminated the mitotic stability provided by the consensus sequence.

The ease with which DNA may be introduced into the yeast *Saccharomyces cerevisiae* has led to the detection of sequences that allow plasmids to undergo autonomous replication. These *ARS* plasmids are very unstable because they undergo nonrandom partitioning in mitosis (14). *S. cerevisiae* divides by budding, and *ARS* plasmids preferentially partition with the mother cell (11).

The instability of *ARS* plasmids is the basis for a functional assay for the isolation of yeast sequences which can stabilize these plasmids by correcting their partitioning defect (6, 13). These stabilizing sequences have been shown to be yeast centromeric DNA on the basis of the mitotic and meiotic properties they impart to *ARS* plasmids that contain them and because their deletion from a normal yeast chromosome renders that chromosome severely unstable (4).

The functional centromeric DNA has been localized to small segments of DNA whose sequences have been determined for the centromeres of chromosomes 3, 8, and 13 (5, 11). A set of sequence elements was found to be similar among these centromeres. A subcloning and deletion analysis for the centromere of chromosome 4 is presented here and shows the presence of the consensus sequence of elements I, II, and III. In addition, sequences adjacent to this centromere consensus sequence in chromosome 4 can provide a low level of mitotic stabilization to autonomously replicating plasmids.

MATERIALS AND METHODS

DNA sequencing. Sequencing was done by the chemical degradation method of Maxam and Gilbert (10). The strategy for sequencing involved the isolation of BAL 31 exonuclease-generated deletion derivatives of the fragment Sc4146 (Fig. 1). Deletions whose endpoints differed by 1 to 300 base pairs (bp) were isolated by cutting plasmid pNN280 (see Fig. 4) with *Pvu*II and digesting it with BAL 31 as described by Johnston and Davis (8). 10-mer *Bam*HI linkers (Collaborative Research, Inc.) were ligated onto blunt duplex ends left after BAL 31 digestion and treatment with DNA polymerase I. The plasmids were then digested with *Eco*RI, to release *CEN4* fragments that contained various-sized deletions starting from the *Pvu*II site of Sc4146, and marked with *Bam*HI linkers. These fragments were recloned into the *Eco*RI-*Bam*HI portion of the vector YRp17 (see Fig. 4). Some BAL 31 deletions were also generated by cleaving Sc4146 with *Eco*RI and digesting it with BAL 31 toward the *Pvu*II site. In this case, *Eco*RI linkers were ligated to the blunt-end deletion fragments. These cloned fragments served as a nested set of sequences that each contained a convenient *Bam*HI or *Eco*RI linker site, marking the deletion endpoint, that could be 5′ or 3′ end labeled with ^{32}P for sequencing (13). All of the sequence in Fig. 2 was obtained either from both DNA strands or from overlapping BAL 31 deletions on a single DNA strand. The sequence was analyzed for homologous internal sequencing and sequencing between other centromeres by using the SEQ homology search program (2).

Mitotic stability assay. Lithium acetate-mediated DNA transformation of *S. cerevisiae* YNN281 (a *trp1Δ his3Δ200 ura3-52 lys2-801 ade2-101*) was performed as described by Ito et al. (7), in a manner selecting for Trp^+ cells. Transformants were grown to saturation in 1 ml of minimal medium containing all required nutrients except for tryptophan. The percentage of plasmid-bearing cells was determined by diluting and plating from 1 to 200 cells on YPD medium, followed by replication on plates lacking tryptophan. This was repeated after diluting the transformants grown under selection into rich YPD medium and growing 17 generations nonselectively. The percentage of plasmid-containing cells was obtained for two growouts of two independent transformants for each plasmid. The average of the four values is presented below (see Fig. 4). Individual determinations could differ from the average by as much as a factor of two.

RESULTS

The isolation and initial localization of the functional *CEN4* sequences to a 3.6-kilobase (kb) *Bam*HI-*Eco*RI fragment (Sc4137) have been described previously (13), and the initial localization is shown in Fig. 1. A further localization

* Corresponding author.

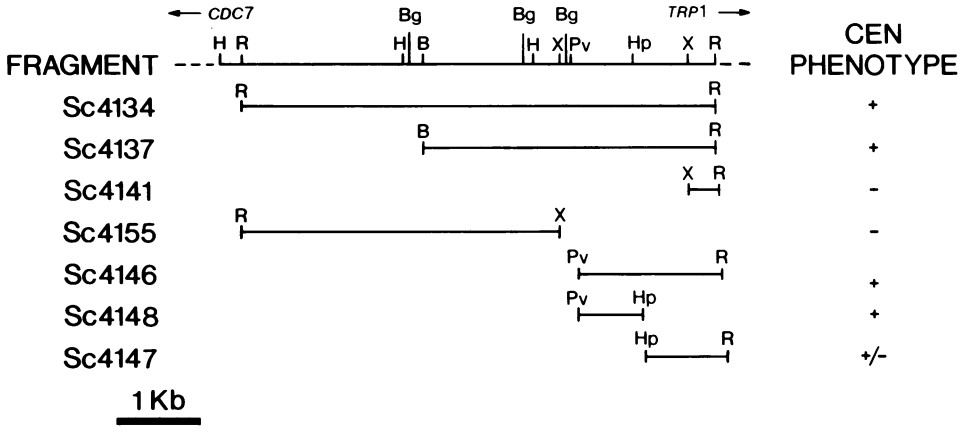


FIG. 1. Restriction map of *CEN4* region subclones and qualitative mitotic stabilities. The indicated fragments of DNA from the centromere region of chromosome 4 were cloned into the vector YRp17 and qualitatively examined for their ability to provide mitotic stability by methods described in the text and in a previous publication. Abbreviations: H, *Hin*dIII; R, *Eco*RI; Bg, *Bgl*II; B, *Bam*HI; X, *Xho*I; Pv, *Pvu*II.

of sequences that provide mitotic stability to autonomously replicating plasmids is described here. A 1,755-bp *Pvu*II-*Eco*RI fragment (Sc4146) was qualitatively observed to stabilize the mitotic segregation of the *ARS1* plasmid YRp17 (13). This was accomplished by streaking colonies from the transformation plate onto nonselective YPD plates and then determining the percentage of cells that contained the plasmid after replica plating to selective plates. DNA flanking the Sc4146 fragment provided no mitotic stability. Four kilobases of DNA toward the CDC7 gene and 10 kb toward the TRP1 gene were tested (13). The Sc4146 fragment was further subcloned into two adjacent, nonoverlapping half-fragments with the enzyme *Hpa*I. The 905-bp *Hpa*I-*Eco*RI fragment (Sc4147) and the 850-bp *Pvu*II-*Hpa*I fragment (Sc4148) both provided some mitotic stability to the plasmid YRp17. The sequence of the entire region (Sc4146) containing mitotic stabilizing sequences was thus determined.

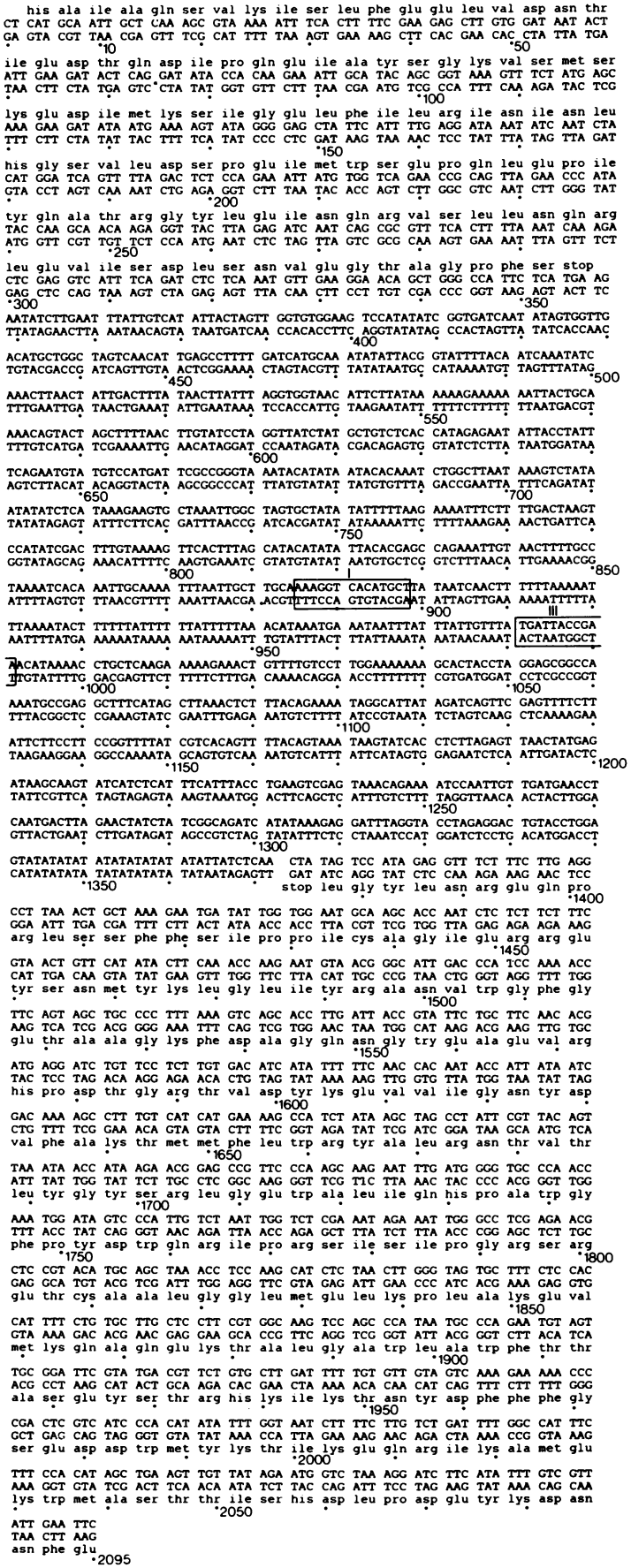
CEN4 DNA sequence. The sequence of 2,095 bp of DNA including the Sc4146 fragment is shown in Fig. 2. The consensus sequence elements I, II, and III found in *CEN3*, *CEN6*, and *CEN11* were also observed for *CEN4*. There is no sequence in *CEN4* that resembles the 10-bp element IV sequence of *CEN3* and *CEN11*, nor is there a particularly good match to this sequence in *CEN6* (5, 12). A consensus sequence for all four centromeres is presented in Fig. 3.

There were two large open reading frames (ORFs) which flanked the *CEN4* consensus sequence. The coding strand in each case was in the 5′-to-3′ direction toward the consensus sequence. In both instances, the ORF continued past the endpoints of DNA sequence.

Determination of sequences important for mitotic stability. The *CEN4* consensus sequence was entirely contained within the 850-bp *Pvu*II-*Hpa*I fragment Sc4148 (contained in plasmid pNN281) (Fig. 4). The adjacent 905-bp *Hpa*I-*Eco*RI fragment Sc4147 (contained in plasmid pNN285) exhibited no obvious sequence homology to the Sc4148 fragment or to any other available centromeric DNA sequence but nevertheless contained some mitotic stabilization sequences. To obtain a more quantitative estimate of the degree of mitotic stability provided by these fragments, *ARS1* plasmids containing different DNA sequences from the *CEN4* region were transformed into the haploid strain YNN281 in a manner selecting for the gene *TRP1* on the plasmid. Transformants were grown to saturation in medium selecting for the plasmid, and the percentage of plasmid-containing cells was determined. Cells from the selection medium were also diluted and grown for 17 generations in rich YPD medium, and the percentage of plasmid-containing cells was again determined.

The mitotic stability was determined for plasmids containing the fragments Sc4147 and Sc4148 and for several BAL 31 deletion fragments that eliminated portions of the *CEN4* consensus sequence (Fig. 4). Plasmids containing an intact *CEN4* consensus sequence (such as pNN280, 281, and 282 in Fig. 4) all showed similar stabilities. The plasmid pNN282 contained a BAL 31 deletion which removed DNA up to ~200 bp in front of element I. This deletion might have created a plasmid with slightly impaired mitotic stability, as there were fewer cells which contained this plasmid at zero generations of nonselective growth compared with plasmids pNN280 and 281 (55% versus ~80%), but at 17 generations of nonselective growth, the percentages of cells containing each plasmid were essentially equivalent. In contrast, the plasmid pNN283 contained a BAL 31 deletion that removed all of element I plus 16 of 82 bp of the AT-rich element II. This plasmid showed a clearly impaired mitotic stability relative to plasmids containing an intact consensus sequence. Mitotic stabilization was decreased ca. twofold. Elimination of an additional 55 bp of AT-rich DNA in the plasmid pNN284 further reduced its mitotic stability. In fact, the presence of element III seemed to confer no significantly greater mitotic stability to this plasmid than was provided by the Sc4147 fragment alone in pNN285, which contained no sequence homology to fragment Sc4148 or to other centromere sequences. Plasmids containing the *CEN4* consensus sequence had approximately fivefold-higher mitotic stabilities compared with plasmids containing the Sc4147 fragment. Nevertheless, the Sc4147 fragment imparted eas-

FIG. 2. Nucleotide sequence of *CEN4* DNA. Shown in boxes are the conserved centromere elements I (14 bp) and III (11 bp) surrounding an 82-bp AT-rich element II. Also shown are the translated amino acid sequences for two ORFs that are tightly linked to this consensus sequence. The coding strands for the ORFs are both 5′ to 3′ in the direction of the consensus sequence. The ORFs continue past the endpoints of the DNA sequence we have determined. The strategy used for determining the DNA sequence is described in the Materials and Methods.



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CT CAT GCA ATT GCT CAA AGC GTA AAA ATT TCA CTT TTC GAA GAG CTT GTG GAT AAT ACT
GA GTA CGT TAA CGA GTT TCG CAT TTT TAA AGT GAA AAG CTT CAC GAA CAC CTA TTA TGA
10 50
ile glu asp thr gln asp ile pro gln glu ile ala tyr ser gly lys val ser met ser
ATT GAA GAT ACT CAG GAT ATA CCA CAA GAA ATT GCA TAC AGC GGT AAA GTT TCT ATG AGC
TAA CTT CTA TGA GTC CTA TAT GGT GTT CTT TAA CGA ATG TCG CCA TTT CAA AGA TAC TCG
100
lys glu asp ile met lys ser ile gly glu leu phe ile leu arg ile asn ile asn leu
AAA GAA GAT ATA ATG AAA AGT ATA GGG GAG CTA TTC ATT TTG AGG ATA AAT ATC AAT CTA
TTT CTT CTA TAT TAC TTT TCA TAT CCC CTC GAT AAG TAA AAC TCC TAT TTA TAG TTA GAT
150
his gly ser val leu asp ser pro glu ile met trp ser glu pro gln leu glu pro ile
CAT GGA TCA GTT TTA GAC TCT CCA GAA ATT ATG TGG TCA GAA CCG CAG TTA GAA CCC ATA
GTA CCT AGT CAA AAT CTG AGA GGT CTT TAA TAC ACC AGT CTT GGC GTC AAT CTT GGG TAT
200
tyr gln ala thr arg gly tyr leu glu ile asn gln arg val ser leu leu asn gln arg
TAC CAA GCA ACA AGA GGT TAC TTA GAG ATC AAT CAG CGC GTT TCA CTT TTA AAT CAA AGA
ATG GTT CGT TGT TCT CCA ATG AAT CTC TAG TTA GTC GCG CAA AGT GAA AAT TTA GTT TCT
250
leu glu val ile ser asp leu ser asn val glu gly thr ala gly pro phe ser stop
CTC GAG GTC ATT TCA GAT CTC TCA AAT GTT GAA GGA ACA GCT GGG CCA TTC TCA TGA AG
GAG CTC CAG TAA AGT CTA GAG AGT TTA CAA CTT CCT TGT CGA CCC GGT AAG AGT ACT TC
300 350
AATATCTTGAAT TTATTGTCAT ATTACTAGTT GGTGTGGAAG TCCATATATC GGTGATCAAT ATAGTGGTTG
TTATAGAACTTA AATAACAGTA TAATGATCAA CCACACCTTC AGGTATATAG CCACTAGTTA TATCACCAAC
400
ACATGCTGGC TAGTCAACAT TGAGCCTTTT GATCATGCAA ATATATTACG GTATTTTACA ATCAAATATC
TGTACGACCG ATCAGTTGTA ACTCGGAAAA CTAGTACGTT TATATAATGC CATAAAATGT TAGTTTATAG
450 500
AAACTTAACT ATTGACTTTA TAACTTATTT AGGTGGTAAC ATTCTTATAA AAAAGAAAAA AATTACTGCA
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TCAGAATGTA TGTCCATGAT TCGCCGGGTA AATACATATA ATACACAAAT CTGGCTTAAT AAAGTCTATA
AGTCTTACAT ACAGGTACTA AGCGGCCCAT TTATGTATAT TATGTGTTTA GACCGAATTA TTTCAGATAT
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750
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GGTATAGCAG AAACATTTTC AAGTGAAATC GTATGTATAT AATGTGCTCG GTCTTTAACA TTGAAAACGG
800 850
TAAAATCACA AATTGCAAAA TTTAATTGCT TGCAAAAGGT CACATGCTTA TAATCAACTT TTTTAAAAAT
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900
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950
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1000 1050
AAATGCCGAG GCTTTCATAG CTTAAACTCT TTACAGAAAA TAGGCATTAT AGATCAGTTC GAGTTTTCTT
TTTACGGCTC CGAAAGTATC GAATTTGAGA AATGTCTTTT ATCCGTAATA TCTAGTCAAG CTCAAAAGAA
1100
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1150 1200
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1250
CAATGACTTA GAACTATCTA TCGGCAGATC ATATAAAGAG GATTTAGGTA CCTAGAGGAC TGTACCTGGA
GTTACTGAAT CTTGATAGAT AGCCGTCTAG TATATTTCTC CTAAATCCAT GGATCTCCTG ACATGGACCT
1300
GTATATATAT ATATATATAT ATATTATCTCAA CTA TAG TCC ATA GAG GTT TCT TTC TTG AGG
CATATATATA TATATATATA TATAATAGAGTT GAT ATC AGG TAT CTC CAA AGA AAG AAC TCC
1350 stop leu gly tyr leu asn arg glu gln pro
1400
CCT TAA ACT GCT AAA GAA TGA TAT TGG TGG AAT GCA AGC ACC AAT CTC TCT TCT TTC
GGA ATT TGA CGA TTT CTT ACT ATA ACC ACC TTA CGT TCG TGG TTA GAG AGA AAG AAG
arg leu ser ser phe phe ser ile pro pro ile cys ala gly ile glu arg arg glu
1450
GTA ACT GTT CAT ATA CTT CAA ACC AAG AAT GTA ACG GGC ATT GAC CCA TCC AAA ACC
CAT TGA CAA GTA TAT GAA GTT TGG TTC TTA CAT TGC CCG TAA CTG GGT AGG TTT TGG
tyr ser asn met tyr lys leu gly leu ile tyr arg ala asn val trp gly phe gly
1500
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glu thr ala ala gly lys phe asp ala gly gln asn gly try glu ala glu val arg
1550
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TAC TCC TAG ACA AGG AGA ACA CTG TAG TAT AAA AAG TTG GTG TTA TGG TAA TAT TAG
his pro asp thr gly arg thr val asp tyr lys glu val val ile gly asn tyr asp
1600
GAC AAA AGC CTT TGT CAT CAT GAA AAG CCA TCT ATA AGC TAG CCT ATT CGT TAC AGT
CTG TTT TCG GAA ACA GTA GTA CTT TTC GGT AGA TAT TCG ATC GGA TAA GCA ATG TCA
val phe ala lys thr met met phe leu trp arg tyr ala leu arg asn thr val thr
1650
TAA ATA ACC ATA AGA ACG GAG CCG TTC CCA AGC AAG AAT TTG ATG GGG TGC CCA ACC
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leu tyr gly tyr ser arg leu gly glu trp ala leu ile gln his pro ala trp gly
1700
AAA TGG ATA GTC CCA TTG TCT AAT TGG TCT CGA AAT AGA AAT TGG GCC TCG AGA ACG
TTT ACC TAT CAG GGT AAC AGA TTA ACC AGA GCT TTA TCT TTA ACC CGG AGC TCT TGC
phe pro tyr asp trp gln arg ile pro arg ser ile ser ile pro gly arg ser arg
1750 1800
CTC CGT ACA TGC AGC TAA ACC TCC AAG CAT CTC TAA CTT GGG TAG TGC TTT CTC CAC
GAG GCA TGT ACG TCG ATT TGG AGG TTC GTA GAG ATT GAA CCC ATC ACG AAA GAG GTG
glu thr cys ala ala leu gly gly leu met glu leu lys pro leu ala lys glu val
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CAT TTT CTG TGC TTG CTC CTT CGT GGC AAG TCC AGC CCA TAA TGC CCA GAA TGT AGT
GTA AAA GAC ACG AAC GAG GAA GCA CCG TTC AGG TCG GGT ATT ACG GGT CTT ACA TCA
met lys gln ala gln glu lys thr ala leu gly ala trp leu ala trp phe thr thr
1900
TGC GGA TTC GTA TGA CGT TCT GTG CTT GAT TTT TGT GTT GTA GTC AAA GAA AAA CCC
ACG CCT AAG CAT ACT GCA AGA CAC GAA CTA AAA ACA CAA CAT CAG TTT CTT TTT GGG
ala ser glu tyr ser thr arg his lys ile lys thr asn tyr asp phe phe phe gly
1950
CGA CTC GTC ATC CCA CAT ATA TTT GGT AAT CTT TTC TTG TCT GAT TTT GGC CAT TTC
GCT GAG CAG TAG GGT GTA TAT AAA CCA TTA GAA AAG AAC AGA CTA AAA CCG GTA AAG
ser glu asp asp trp met tyr lys thr ile lys glu gln arg ile lys ala met glu
2000
TTT CCA CAT AGC TGA AGT TGT TAT AGA ATG GTC TAA AGG ATC TTC ATA TTT GTC GTT
AAA GGT GTA TCG ACT TCA ACA ATA TCT TAC CAG ATT TCC TAG AAG TAT AAA CAG CAA
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2050
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asn phe glu
2095

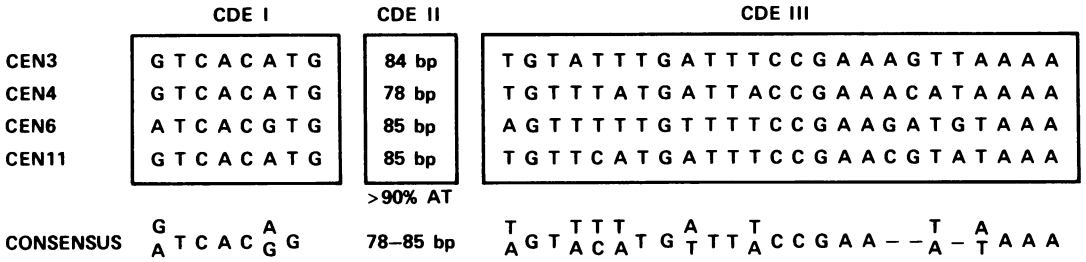

FIG. 3. Centromere consensus sequence. New consensus sequence elements CDEI, CDEII, and CDEIII are shown for each of the centromeres whose DNA sequence has been determined to date (5, 6). This represents a shortening of the old element I and lengthening of the old element III.

ily detectable mitotic stability to unstable *ARS* plasmids. Fragment Sc4147 was placed in numerous other *ARS* plasmids in different positions and, in all cases, conferred mitotic stability similar to that shown in Fig. 4. Furthermore, fragment Sc4147 has been incorporated into the plasmid for the color sector assay (5a) and for the CEN selection assay (5b) and shown significant stabilization but is too unstable for more accurate quantitation than that shown in Fig. 4.

Dicentric plasmid test of ability of Sc4147 to interact with the spindle apparatus. Dicentric plasmids, constructed in vitro, are unstable after introduction into yeast cells (9). These plasmids underwent deletion events that were consistent with their having been broken by the yeast spindle apparatus and then religated after variable amounts of exonuclease digestion of the broken ends. If the Sc4147 sequences responsible for mitotic stabilization act through the

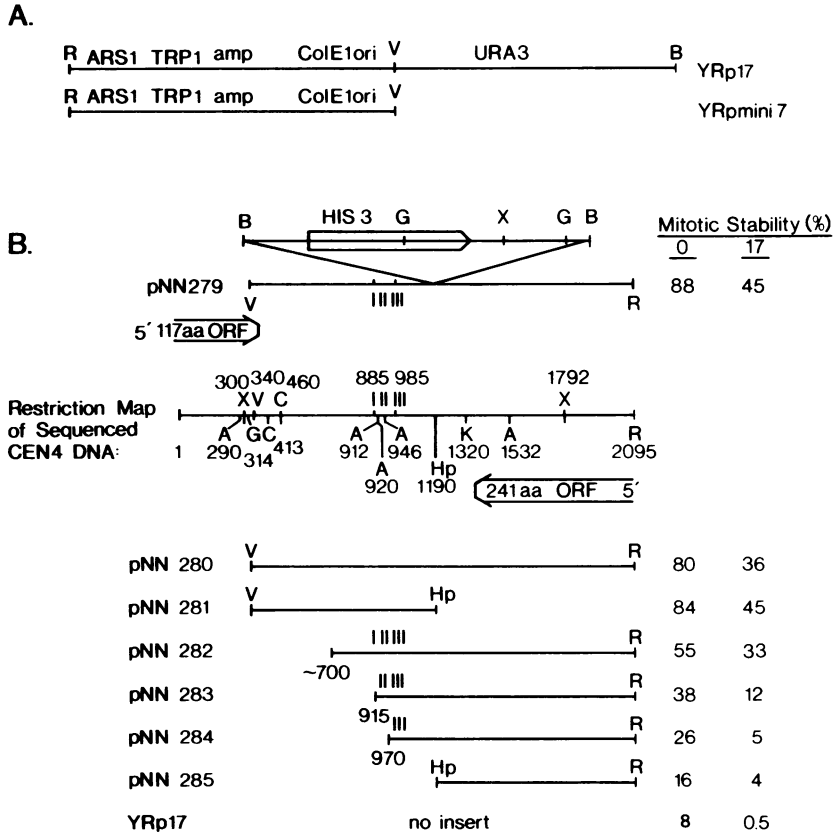

FIG. 4. Localization of sequences that provide mitotic stability. (A) Map of the two *ARS* plasmids into which *CEN4* restriction fragments were cloned to test mitotic stability. YRpmini7 was derived from YRp17 by cutting with *Eco*RI plus *Pvu*II. Only the large *Eco*RI-*Bam*HI fragment of YRp17 is shown, which was ligated with various *Eco*RI plus *Bam*HI *CEN4* fragments for cloning. (B) The restriction fragments shown for pNN279, pNN280, pNN281, and pNN285 were inserted as *Pvu*II-*Eco*RI or *Hpa*I-*Eco*RI fragments into the vector YRpmini7. For pNN281 and pNN285, a *Bam*HI linker was ligated to the blunt *Pvu*II or *Hpa*I ends so that a *Bam*HI site was present in the plasmids containing these subclones. The remaining BAL 31 deletion fragments were cloned as *Bam*HI-*Eco*RI fragments into YRp17. We found no significant differences in the mitotic stability provided by the Sc4146 fragment when it was present in the vectors YRpmini7 (pNN280) or YRp17 (not shown). The mitotic stabilities are expressed as percentages of plasmid-containing cells in the population immediately after growth with selection (0) and after 17 generations of nonselective growth and were determined as described in the Materials and Methods.

spindle apparatus, they presumably act in coordination with the *CEN4* consensus sequence of Sc4148. Increasing the separation of the two sequences might result in the two sequences acting as independent centromeres with the result that the plasmid would undergo occasional breakage and rearrangement events. This possibility was tested by inserting a 1.7-kb *Bam*HI *HIS3* gene fragment (15) into the *Hpa*I site of plasmid pNN280. The resulting plasmid, pNN279 (Fig. 4), was introduced into yeast cells by lithium acetate-mediated DNA transformation (7) selecting only for the gene *TRP1*. Transformants were then replicated to plates lacking histidine. The vast majority of the transformants grew well on the plates selecting for His^+ cells. Thus, there is no great inhibition of expression when the *HIS3* gene is placed within 400 bp of the *CEN4* consensus sequence. At frequencies of 0.1 to 1%, transformants were found that were Trp^+ His^-. Analysis of these transformants showed that they contained deletions of various extents that included the *HIS3* gene. However, these deletions are not specific to this plasmid. Deletions of unselected markers occur on both *ARS* and *CEN* plasmids at this frequency and appear to be due to the transformation process itself (3).

Dicentric plasmids undergo rapid deletion in all colonies after introduction into yeast cells, such that, by the time a colony is established on the transformation plate, it already contains a heterogeneous population of deleted plasmids (9). If plasmid pNN279 acts as an efficient dicentric plasmid, a large proportion of the cells in each colony on the transformation plate should contain plasmids in which the *HIS3* gene is inactivated by deletion. This was not observed. When colonies from the transformation plate were replated, it was found that the ~100 cells examined were all His^+. Similar results were obtained for plasmids containing the *HIS3* fragment in the opposite orientation relative to the *HIS3* fragment in pNN279 and for plasmids containing a tandem duplication of the *HIS3* fragment that separated Sc4147 and Sc4148 by 3.4 kb.

Since the Sc4147 fragment provided only poor mitotic stability relative to the Sc4148 fragment, the possibility remains that it interacts weakly with the spindle and cannot effectively compete for breakage with the higher-affinity Sc4148 fragment.

DISCUSSION

Sequences from the centromeric region of chromosome 4 that impart mitotic stability to autonomously replicating plasmids were localized to two fragments of 850 bp (Sc4148) and 905 bp (Sc4147). The Sc4148 fragment contained the consensus elements I, II, and III that have been found in all other centromeres examined to date and imparted the same mitotic stability to *ARS* plasmids as did a 627-bp *CEN3* fragment that contained a version of the consensus sequence found on chromosome 3. In contrast, the adjacent Sc4147 fragment provided a relatively poor mitotic stability and contained no sequences homologous to the centromere consensus sequence. How the Sc4147 fragment imparts its mitotic stability to plasmids is unclear. In particular, we do not know whether it acts through a weak interaction with the yeast spindle apparatus. We also do not know whether these sequences contained in chromosome 4 actually participate in the normal segregation of this chromosome. Deletions of the chromosomal Sc4147 and Sc4148 fragments are needed to assess the relative contributions of these fragments to the stability of chromosome 4. This may be done by the method of restriction fragment-mediated transplacement, as has been done for *CEN3* (4).

Deletions of element I plus 16 bp of element II DNA impaired *CEN4* function and removal of elements I and II abolished the mitotic stability provided by the *CEN4* consensus sequence. The high degree of sequence conservation of element III makes it seem likely that it too is required for centromere function (1).

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