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Genetics

Mitotic and meiotic stability of linear plasmids in yeast

(chromosome structure/telomeres/centromeres)

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ABSTRACT **Circular recombinant DNA plasmids that contain autonomously replicating sequences (*ARS*s) are maintained in extrachromosomal form in transformed yeast cells. However, these plasmids are unstable, being rapidly lost from cells growing without selection. Although the stability of such a plasmid can be increased by the presence of yeast centromere DNA (*CEN*), even *CEN* plasmids are lost at a high rate compared to a *bona fide* yeast chromosome. Natural yeast chromosomes are linear molecules; therefore, we have asked if linearization can improve the stability of recombinant DNA plasmids. Linear plasmids with and without yeast *CEN*s were constructed *in vitro* by using termini from the extrachromosomal ribosomal DNA (rDNA) of the ciliated protozoan *Tetrahymena thermophila* as "telomeres." These linear plasmids transformed yeast at high frequency and were maintained as linear extrachromosomal molecules during mitotic growth. Moreover, linear plasmids containing *CEN*s were also transmitted through meiosis: these plasmids segregate predominantly 2+:2− at the first meiotic division, indicating that *Tetrahymena* rDNA termini can provide telomere function during yeast meiosis. Linear plasmids without *CEN*s were about as stable in mitosis as the comparable circular plasmid. Thus, the *Tetrahymena* rDNA termini have no marked positive or negative effect on the mitotic stability of *ARS* plasmids. However, linear plasmids containing *CEN*s are three to four times less stable in mitotic cells than circular *CEN* plasmids. This decrease in stability is not due to a functional change in the centromere itself; rather, linearization of a *CEN* plasmid has a direct detrimental effect on its mitotic stability. These results may reflect the existence of spatial constraints on the positions of centromeres and telomeres, contraints which must be satisfied to achieve stable segregation of chromosomes during mitosis.**

Eukaryotic chromosomes, unlike those of bacteria, are linear structures. Cytological and genetic evidence indicates that telomeres, the physical ends of eukaryotic chromosomes, are specialized structures that are essential for maintenance of chromosomes. Biochemical considerations also dictate that ends of chromosomes be specialized in some way: because DNA polymerases cannot initiate DNA chains *de novo* and can only synthesize DNA in a 5′ to 3′ direction, replication of ends requires special mechanisms or structures to avoid the production of 5′-terminal gaps at each end of the chromosome. Most eukaryotic chromosomes also contain a centromere, a discrete locus that is distinguished cytologically as the site at which spindle microtubules attach. Like telomeres, centromeres are essential structural components of chromosomes which are required for proper disjunction during both mitosis and meiosis.

Yeast can be transformed by recombinant DNA plasmids containing a selectable gene (1). Plasmids containing autonomously replicating sequence (*ARS*) elements transform yeast at high frequencies (10^3–10^5 transformants per μg) and are maintained in extrachromosomal form presumably because the *ARS* serves as an origin of DNA replication (2). Most *ARS* plasmids are found in only a subset of the cells in a population growing under selection for the plasmid and are quickly lost from the population when selection is removed. This instability appears to be due to nonrandom segregation rather than defective replication of *ARS* plasmids (3). Sequences from the centromere regions of yeast chromosomes improve the segregation of *ARS* plasmids during both mitosis and meiosis (4). However, even plasmids containing yeast centromere DNAs (*CEN*s) are lost at a high rate (≈3% loss per generation; this work) compared to a *bona fide* yeast chromosome (0.001% loss per generation; L. Hartwell, personal communication).

We have constructed linear plasmids with and without yeast centromeres. We show that small linear centromere plasmids can be maintained during both mitosis and meiosis. We also demonstrate that linearization of a centromere plasmid decreases its mitotic stability.

MATERIALS AND METHODS

The yeast strains used were 3482-16-1 (a *met*2, *his*3∇-1, *leu*2-3, *leu*2-112, *trp*1-289, *ura*3-52; from L. Hartwell and ED109-16D (α *ade*2-1, *ade*8-10, *ura*3; from S. Henikoff). *Tetrahymena* ribosomal DNA (rDNA) was prepared as described (5) from *Tetrahymena thermophila* strain C3V (from E. Blackburn). The recombinant DNA plasmids used are diagramed in Fig. 1. DNA restriction fragments were eluted from agarose gels onto DEAE paper (6). DNA was extracted from yeast cells, subjected to electrophoresis, and transferred to nitrocellulose as described (7). Transformed yeast cells were grown in Y complete medium lacking uracil and adenine (hereafter called YC-ura-ade), which was prepared as described for Y complete without tryptophan (7), except that tryptophan was added to 0.1 g per liter and uracil and adenine were omitted. For mitotic stability studies, cells were transferred to YC-ura-ade to which was added uracil at 20 μg/ml. To determine the stability of plasmids in meiosis, haploid yeast cells containing circular or linear *CEN*4 plasmids were mated to haploid cells lacking plasmid DNA. The diploids were grown to stationary phase in YC-ura-ade and then were transferred to sporulation medium (0.3% potassium acetate). At the time of transfer, the culture was diluted and plated on YC and YC-ura-ade plates to determine the fraction of cells containing plasmid.

RESULTS

Construction of Linear Plasmids. Yeast can be transformed with linear DNA molecules but the transforming DNA is usually integrated into chromosomal DNA (8). To maintain a plasmid in linear form, specialized structures at the ends of the DNA molecule are required (9). Terminal restriction fragments from the macronuclear rDNA of the ciliated protozoan *T. thermo-*

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Abbreviations: *ARS*, autonomously replicating sequence; *CEN*, centromere DNA; rDNA, DNA encoding rRNA; kb, kilobase(s).

phila (Fig. 1) that have been shown to provide end function for *ARS* plasmids in mitotic yeast cells (9) were used as the source of ends for linear plasmids. Because there are about 600 copies of the rRNA genes per haploid genome in the *Tetrahymena* macronucleus and the rDNA can be isolated readily by differential centrifugation in CsCl (5), *Tetrahymena* rDNA termini provide a convenient source of DNA ends for *in vitro* construction of linear plasmids.

Linear plasmids with and without centromeres were constructed by ligating *Tetrahymena* rDNA termini to derivatives of the circular plasmid YCp19 (ref. 12; Fig. 1*b*). Three linear plasmids were constructed (Fig. 1*c*). For construction of plasmids containing *CEN4* (LYT-C-1 and LYT-C-2), YCp19 was linearized by digestion with either *Bam*HI or *Eco*RI and ligated to gel-purified *Bam*HI or *Eco*RI end fragments from *Tetrahymena* rDNA, and linear plasmids containing two *Tetrahymena* rDNA ends were purified by agarose gel electrophoresis. A linear plasmid without a centromere (LYT-1) was constructed by digesting YCp19 with *Eco*RI and *Bam*HI and then purifying the 6.5-kb fragment that lacks *CEN4* by agarose gel electrophoresis. This fragment was ligated to a mixture of *Eco*RI- and *Bam*HI-digested gel-purified *Tetrahymena* rDNA end fragments and a plasmid containing one *Eco*RI and one *Bam*HI rDNA end isolated by agarose gel electrophoresis.

The three linear plasmids (LYT-C-1, LYT-C-2, and LYT-1) and two control circular plasmids (YCp19 and YRp12) were used to transform yeast strain 3482-16-1. All five plasmids transformed at high frequencies (Table 1). Two criteria were used to verify the linearity of LYT-C-1, LYT-C-2, and LYT-1 in transformed cells (Fig. 2): (*i*) A single band was detected by hybridization to undigested DNA from cells transformed with linear molecules. In contrast, multiple bands representing covalently closed circles, nicked circles, linear, and (for YRp12) multimer forms were detected by hybridization to undigested DNA from YRp12 and YCp19 transformed cells. (*ii*) Digestion with *Kpn* I, which has one recognition site in YCp19, produced one band for circular plasmids and two for linear molecules. Both fragments from the linear plasmids hybridized to an end-specific *Tetrahymena* rDNA probe isolated from pRP7 (data not shown).

Mitotic Stability of Linear and Circular Yeast Centromere Plasmids. The mitotic stability of circular and linear plasmids in transformed cells was determined in strain 3482-16-1. Logarithmic phase cultures were grown at 30°C in YC-ura-ade. Ali-

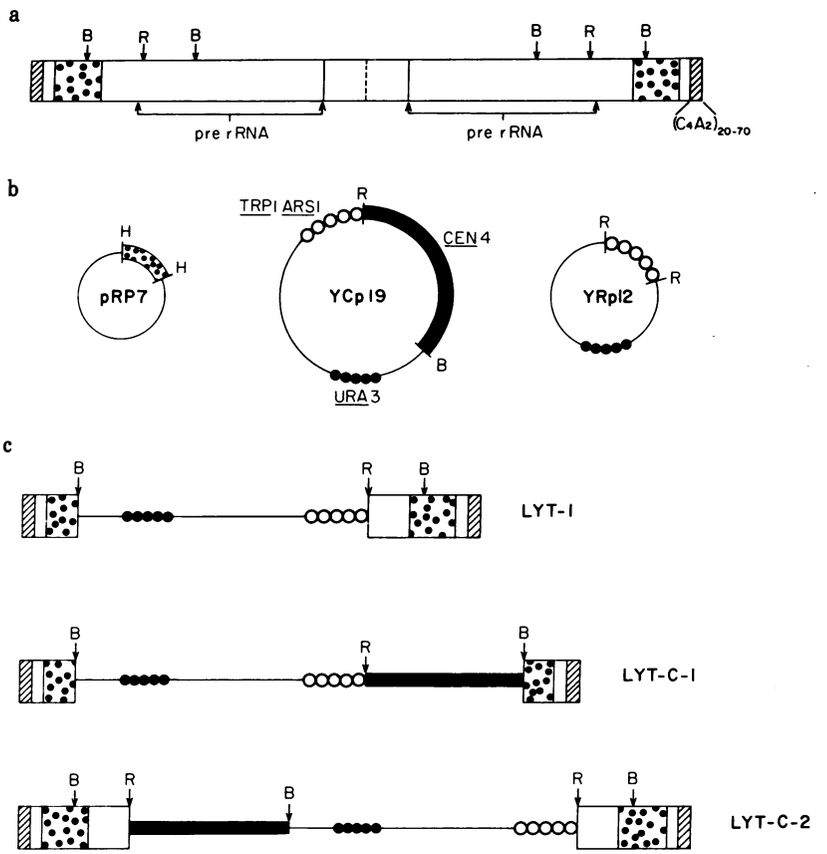

FIG. 1. Structure of DNAs. (*a*) Macronuclear rDNA from *T. thermophila* strain C3V (see ref. 10). The macronuclear rDNA of *Tetrahymena* exists in extrachromosomal, palindromic molecules of about 20 kilobases (kb). The center of symmetry in the molecule is marked by the dotted line. The portion of the molecule transcribed into the rRNA precursor is indicated. The terminal *Bam*HI (B, 1.3 kb) and *Eco*RI (R, 2.6 kb) restriction fragments were used to construct the linear plasmids diagramed in *c*. The terminal 100–400 base pairs at each end of the molecule is composed of 5′(C_4A_2)3′ repeats (hatched region) with single nucleotide gaps every few repeats. The dotted segment represents the portion of the molecule contained in the end-specific hybridization probe (isolated from pRP7; *b*). (*b*) Recombinant DNA plasmids. pRP7 contains a 1.05-kb *Hin*dIII (*H*) fragment from the noncoding region near the end of the *Tetrahymena* rDNA molecule (dotted region; *a*) inserted into pBR322 (supplied by R. Pearlman; ref. 11). YCp19 (10.1 kb) contains a 3.6-kb *Eco*RI (R)–*Bam*HI (B) fragment isolated from yeast centromere IV (*CEN4*; ■), a 1.45-kb region from yeast chromosome IV containing the *TRP1* and *ARS1* loci (○○○), and a 1.1-kb segment from yeast chromosome V, which contains the *URA3* gene (●●●) (supplied by R. Davis and D. Stinchcomb; ref. 12). The *Eco*RI site adjacent to the 5′ end of the *TRP1* gene was eliminated during the construction of YCp19. YRp12 (6.9 kb) contains the yeast *TRP1 ARS1* 1.45-kb *Eco*RI (R) fragment and the *URA3* gene in pBR322. (*c*) Linear plasmids. LYT-1 (10.4 kb), LYT-C-1 (12.7 kb), and LYT-C-2 (15.3 kb) were constructed by ligating the agarose gel-purified *Bam*HI (B) or *Eco*RI (R) terminal restriction fragments from *Tetrahymena* macronuclear rDNA to linearized YCp19 (or YCp19 from which *CEN4* was removed). Symbols are the same as those used in *a* and *b*.

Table 1. Behavior of plasmids in mitotic cells

	Transformation frequency, transformants per μg	% cells containing plasmid	% loss per generation
YCp19	1.5×10^4	74 ± 13 ($n = 9$)	3 ± 1 (range, 2–6; $n = 8$)
LYT-C-1	3×10^3	55 ± 12 ($n = 3$)	10 ± 1 (range, 9–11; $n = 3$)
LYT-C-2	3×10^3	69 ± 14 ($n = 10$)	11 ± 3 (range, 8–15; $n = 7$)
YRp12	1.5×10^4	27 ± 3 ($n = 3$)	21 ($n = 2$)
LYT-1	3×10^3	38 ± 9 ($n = 5$)	18 ± 3 (range, 16–22; $n = 4$)

Cells (10^9) were incubated with each purified plasmid DNA. The transformation frequency (transformants/μg) per 5×10^7 viable cells in strain 3482-16-1 is presented. The lower transformation frequencies seen for linear plasmids are presumed to be due to the fact that these plasmids were isolated from agarose gels. Transformants were grown in YC-ura-ade and cells spread on both YC and YC-ura-ade plates to determine the percentage of cells containing plasmid for cells in logarithmic phase growth. The means and SDs of several determinations of the percentage of cells with plasmid and the number (n) of independent determinations are presented. Cells were transferred to medium containing uracil at 20 μg/ml and were maintained in logarithmic phase growth for 3 days. Aliquots were taken at various times and spread on YC and YC-ura-ade plates. The percentage of cells that lose plasmid per generation (X) was determined as follows: $X = 1 - e^r$, where $r = \ln(A/B)/N$. N, number of generations in uracil-containing medium; A, % of cells with plasmid at N generation; B, % of cells with plasmid at start. For rate of plasmid loss, the mean, range, SD, and number (n) of determinations are presented. Only two determinations were made for YRp12 because in other experiments the plasmid integrated into chromosomal DNA during the course of the experiment.

quots of each culture were diluted, sonicated briefly, and spread on both YC and YC-ura-ade plates to determine the percentage of cells containing plasmid DNA (Table 1). Both linear (LYT-C-1 and LYT-C-2) and circular (YCp19) centromere plasmids were found in a higher percentage of cells than plasmids without centromeres (LYT-1 and YRp12). Aliquots of logarithmic phase cultures were then diluted into YC-ura-ade to which uracil had been added at 20 μg/ml and maintained in logarithmic phase growth for 3 days. At 24-hr intervals, aliquots were taken and spread on both YC and YC-ura-ade plates. The percentage of cells containing plasmid at each time interval was determined (Fig. 3) and used to calculate the rate of loss of plasmid DNA (Table 1). Both linear (LYT-1) and circular (YRp12) plasmids without centromeres were lost at a rate of about 20% per generation (Table 1), although LYT-1 is marginally more stable than YRp12. Thus, linearization of an *ARS* plasmid has little effect on its mitotic stability. YCp19, the circular centromere plasmid, was lost at a rate of about 3% per generation. However, both linear *CEN* plasmids (LYT-C-1 and LYT-C-2) were lost at substantially higher rates (≈11% loss per generation; Fig. 3 *Right* and Table 1). Thus, linearization of the centromere plasmid has a detrimental effect on its mitotic stability.

The instability of linear centromere plasmids was not due to alteration of the *CEN4* sequence. DNA was extracted from cells carrying LYT-C-2, digested with *Eco*RI to remove the *Tetrahymena* rDNA ends from the linear molecule, and ligated under conditions that favor intramolecular ligation (DNA diluted to 3 μg/ml). The ligation mix was used to transform yeast strain

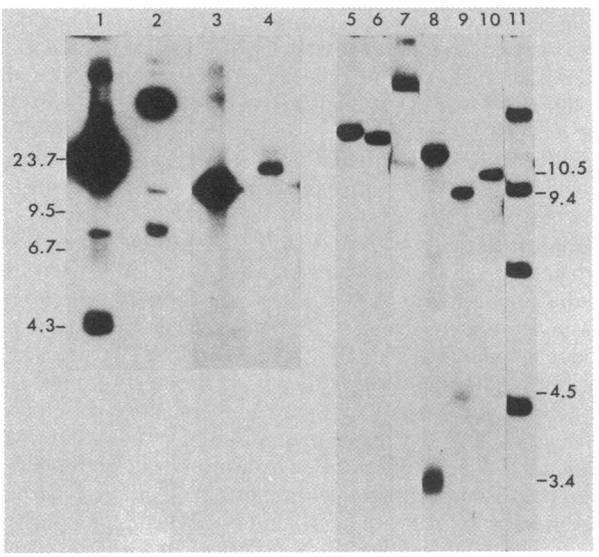

FIG. 2. Structure of circular and linear plasmids in yeast. DNA isolated from 3482-16-1 cells carrying circular or linear plasmids was analyzed by electrophoresis in 0.7% agarose gels. DNA was transferred to nitrocellulose and hybridized with nick-translated pRP7 (Fig. 1). Lanes 1–7 contain undigested DNA isolated from cells transformed by YRp12 (lane 1), YCp19 (lanes 2 and 7), LYT-1 (lane 3), LYT-C-2 (lanes 4 and 5), and LYT-C-1 (lane 6). The relative intensities of hybridization indicate that YRp12 and LYT-1 both exist in multiple copies per cell (lanes 1 and 3 each contain DNA from 3.5×10^7 cells). The DNAs in lanes 8–10 were digested with *Kpn* I, an enzyme with a single recognition site in YCp19 and no site in the *Eco*RI terminal fragment from *Tetrahymena* rDNA. DNAs are from cells containing LYT-C-2 (lane 8), LYT-C-1 (lane 9), and YCp19 (lane 10). Lane 11 contains nick-translated *Hin*dIII fragments of λ DNA. Sizes are in kb pairs.

3482-16-1. Transformants contained a plasmid whose size and pattern of digestion with restriction enzymes was indistinguishable from that of YCp19. Mitotic stability studies on four such transformants indicate that each of the plasmids was lost at a rate of 2–4% per generation. Thus, the *CEN* on the linear plasmids had not been functionally altered during the creation or propagation of linear plasmids.

Meiotic Stability of Linear and Circular Yeast Centromere Plasmids. During meiosis, plasmids such as YCp19 segregate predominantly 2+:2− and exhibit first division segregation (4, 12). Yeast cells transformed with YCp19 or LYT-C-2 were mated with strain ED109-16D. The diploid was maintained under selection, grown to stationary phase, and induced to sporulate. The percentage of the diploid cells with plasmid immediately prior to transfer to sporulation medium was 50% for diploids carrying YCp19 and 25% for diploids carrying LYT-C-2. Those diploids without plasmid that are capable of sporulation must necessarily yield tetrads in which the Ura^+ phenotype segregates 0+:4−. (Because for both YCp19 and LYT-C-2 there was a higher percentage of Ura^- presporulation diploids than 0+:4− tetrads, some of the Ura^- diploids must be incapable of sporulation.) Tetrads were dissected and the spores were tested for presence of plasmid DNA (Ura^+ phenotype; Table 2). Both the linear and circular centromere plasmids segregate predominantly 2+:2− in those tetrads with spores containing plasmid DNA (i.e., excluding 0+:4− tetrads). In those asci in which the plasmids segregated 2+:2−, segregation of plasmid DNA was also determined with respect to *LEU2*, a centromere-linked gene on chromosome III, which exhibits first division segregation in 87% of tetrads (13). In the majority of cases (88%, LYT-C-2; 78%, YCp19), the plasmids segregated at the same division as *LEU2*—that is, both LYT-C-2 and YCp19 segregate predomi-

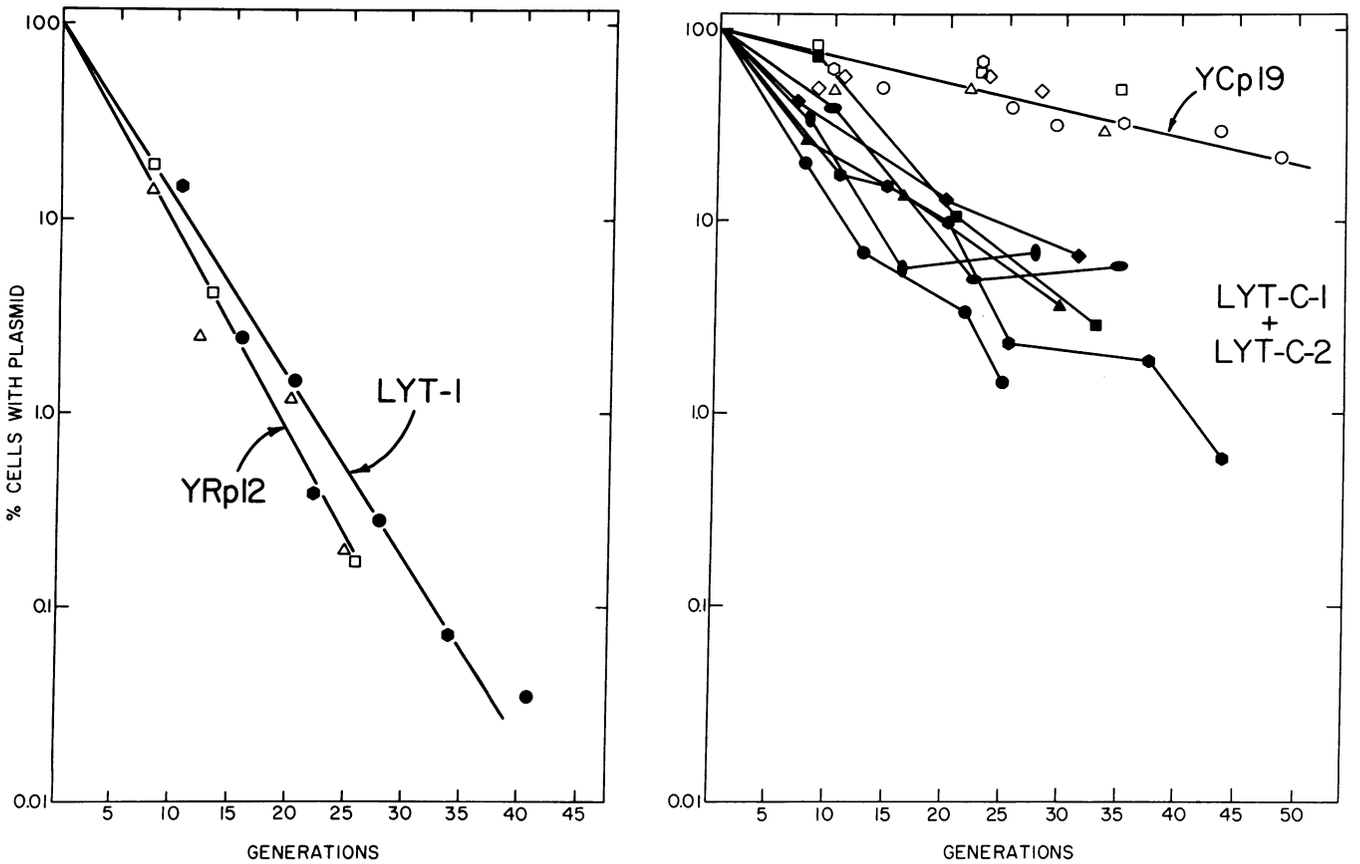

FIG. 3. Mitotic stability of circular and linear plasmids. Logarithmic phase cells containing circular or linear plasmids and growing under selective conditions for maintenance of plasmid (without uracil) were transferred to medium containing uracil. Cells were kept in logarithmic phase growth by dilution and aliquots were removed at various times, sonicated, and plated on uracil-containing and uracil-free plates to determine the percentage of cells containing plasmid. The percentage of cells containing plasmid at time 0 was normalized to 100%. The percentage of cells with plasmid versus the number of generations in uracil-containing medium is presented. Data representative of cultures carrying each of the plasmids are presented. Table 1 summarizes the data from all cultures examined. (*Left*) Plasmids without *CEN*4. YRp12 (□, △) and LYT-1 (●, ●). (*Right*) *CEN*4 plasmids. YCp19 (○, △, ◇, ○) and YCp19 constructed by removal of *Tetrahymena* termini from LYT-C-2, followed by self-ligation: (□); LYT-C-1 (●, ▲); LYT-C-2 (●, ●, ■, ●, ◆).

nantly at meiosis I. These data suggest that linear centromere plasmids function at least as well in meiosis as circular molecules.

DNA was extracted from colonies derived from four different spores from tetrads in which the linear plasmid had undergone 2+:2− segregation. The plasmid DNA from these cells retained the same size and pattern of digestion with restriction enzymes that was present prior to mating (data not shown). Thus, linear *CEN* plasmids are maintained as such through meiosis.

Table 2. Behavior of centromere plasmids in meiosis

Ura^+:Ura^-	LYT-C-2, %*	YCp19, %†
4+:0−	5	8
3+:1−	5	15
2+:2−	29	31
1+:3−	7	14
0+:4−	54	32

3482-16-1 cells carrying either LYT-C-2 or YCp19 were mated to ED109-16D cells. Diploids were sporulated and the resulting tetrads were dissected to determine which spores contained plasmid DNA (Ura^+ phenotype) and how the plasmid had segregated with respect to the centromere-linked gene *LEU2*. In 88% of the tetrads in which LYT-C-2 segregated 2+:2−, the plasmid segregated at the same division as *LEU2*. In 78% of the tetrads in which YCp19 segregated 2+:2−, the plasmid segregated at the same division as *LEU2*.

* 56 tetrads.

† 59 tetrads.

DISCUSSION

Centromeres and telomeres are essential components of the eukaryotic chromosome which function to insure its integrity and proper segregation during both mitosis and meiosis. In carrying out their functions, both of these chromosomal domains interact with other parts of the cell, such as microtubules, the nuclear membrane, and centromeres and telomeres on other chromosomes. Most circular *ARS* plasmids do not segregate to both progeny cells during mitosis. The presence of centromere DNA greatly improves segregation of circular plasmids, although even *CEN* plasmids are unstable compared to a *bona fide* yeast chromosome. Instability might be an inevitable consequence of circularity for small DNA molecules. For example, sister chromatid exchange between circular *CEN* plasmids could produce dicentric molecules that would presumably be lost during mitosis. Also rapid resolution of interlocked rings after replication is required for efficient segregation of low-copy-number circular DNAs.

We have investigated the effect of linearity on plasmid behavior by constructing linear plasmids with and without centromere DNA (*CEN*4). The termini from the extrachromosomal rDNA of *Tetrahymena* were used as "telomeres" because they can be obtained in sufficient quantity to allow construction *in vitro* of linear centromere plasmids. Each of the linear plasmids LYT-1, LYT-C-1, and LYT-C-2 transformed yeast at high frequency (Table 1) and were maintained in linear form during

mitotic growth (Fig. 2). Moreover, LYT-C-1 and LYT-C-2 were transmitted faithfully during meiosis. These results demonstrate that synthetic linear centromere plasmids can function during mitosis and meiosis. Like YCp19, LYT-C-1 and LYT-C-2 segregate predominantly 2+:2− in the first meiotic division. This pattern of segregation indicates that, like YCp19, the copy number of linear centromere plasmids is about 1 per cell. Likewise, because in the crosses described in Table 2, all spores were viable from tetrads carrying either YCp19 or LYT-C-2, neither of the mini-chromosomes induces nondisjunction of homologues—that is, there is no evidence for trivalent pairing for either circular or linear centromere plasmids. The linear centromere plasmids appear to function at least as well as circular centromere plasmids in meiosis (Table 2).

The mitotic properties of linear plasmids were also examined. Linear *ARS* plasmids containing *Tetrahymena* rDNA termini (LYT-1) were about as stable during mitosis as a circular *ARS* plasmid (Table 1). These results demonstrate that *Tetrahymena* rDNA termini have no inherent harmful effects on plasmid stability in yeast. However, linearization of a centromere plasmid markedly decreases its mitotic stability (Fig. 3 *Right*; Table 1). This decrease in stability is not due to a heritable change in the centromere itself because removal of the rDNA termini followed by circularization produces a plasmid with a stability equal to that of the circular control (YCp19). Moreover, it is unlikely that the *Tetrahymena* termini are themselves responsible for the instability because LYT-1 is as stable as YRp12 (Fig. 3 *Left*). Also, altering the relative positions of *ARS*1 and *CEN*4 on the linear chromosome does not improve its mitotic behavior (LYT-C-1 versus LYT-C-2; Fig. 1). However, the centromere on LYT-C-1 and LYT-C-2 must function to some extent because both linear *CEN* plasmids were more stable than either YRp12 or LYT-1.

We conclude that linearization itself has a detrimental effect on the mitotic stability of small centromere plasmids. We think it likely that there are structural constraints on telomere and centromere function in mitotic cells, constraints which are not satisfied by our small linear chromosomes. Perhaps a minimal overall size is required for maximal chromosome stability. It should be noted that even in yeast, in which the average chromosome (800 kb) is small, the smallest chromosome (150 kb) is still about 10 times larger than our synthetic linear chromosomes (14). Indeed, results from experiments monitoring chromosome loss in yeast strains defective either in nuclear fusion (15) or chromosome segregation (16) indicate that small chromosomes are more likely than large ones to be lost from mitotic cells. However, because YCp19 is more stable than the linear centromere plasmids, small size cannot be the sole explanation for the decreased stability of LYT-C-1 and LYT-C-2. Alternatively, a minimal spacing between telomeres and centromeres may be essential for chromosome stability. In support of this hypothesis, we note that no natural yeast chromosome is telocentric, and telocentric chromosomes are unstable in a variety of systems. Finally, chromatin structure and higher-order folding patterns might be disrupted in small linear chromosomes. Yeast centromere DNA is organized into very precisely aligned nucleosomes in chromosomes and on small circular plasmids (17). Although the chromatin organization of telomeres in yeast is not known, in *Tetrahymena* the rDNA termini are organized in a protected but non-nucleosomal conformation (18).

In summary, we have constructed small linear centromere plasmids and demonstrated that they can be maintained during both mitosis and meiosis in yeast. However, it is clear from the behavior of these plasmids that linearization of a small centromere plasmid is not itself sufficient to create a minichromosome with a stability equal to that of a genuine yeast chromosome.

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