Construction of artificial chromosomes in yeast

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Fifty-five-kilobase long artificial chromosomes containing cloned genes, replicators, centromeres and telomeres have been constructed in yeast. These molecules have many of the properties of natural yeast chromosomes. Centromere function is impaired on short (less than 20 kilobases) artificial chromosomes.

CONTINUING attempts have been made to relate the behaviour of chromosomes in mitotic and meiotic cell cycles to their structure. These studies have largely been performed by manipulation of naturally occurring chromosomes by classical genetic techniques[1–3]. The availability of recombinant DNA technology allows an alternative approach: the construction of artificial chromosomes from cloned fragments of DNA. We have used this strategy to investigate the structural requirements for normal chromosome function in the yeast *Saccharomyces cerevisiae*.

Four different classes of functional elements have been identified on natural chromosomes: genes[4], replication origins[5,6], centromeres[7,8] and telomeres[9].

Stinchcomb, Struhl and Davis[5] and Carbon and Hsaio[6] identified sequences in the yeast genome which conferred on plasmids the ability to replicate extrachromosomally. These *ARS* (autonomously replicating sequence) elements are presumed to be chromosomal replication origins although there is no proof for this assertion. Plasmids which contain *ARS* elements are mitotically unstable and are lost rapidly when selective pressure is relaxed[5,6]. This observation allowed the identification of functional yeast centromeres (abbreviated *CEN*) as sequences which increased the mitotic stability of *ARS*-bearing plasmids[7,8]. Mitotic stability has been measured in three ways: (1) by determining the fraction of plasmid-bearing cells in selectively grown cultures[5,6], (2) by measuring the rate at which the plasmid is lost following the relaxation of selective pressure[10], or (3) by pedigree analysis in which individual cell lineages are followed and plasmid loss is detected as the inability of single cells to give rise to colonies on selective medium[11]. Pedigree analysis allows the direct determination of the segregation frequency, which is defined as the fraction of cell divisions (of plasmid-bearing cells) in which only one of the progeny cells receives the plasmid. For circular *ARS* plasmids 5–20% of selectively grown cells contain the plasmid[5,6], and the segregation frequency ranges from 0.3 to 0.6 (ref. 11). The addition of a centromere increases the mitotic stability such that the fraction of plasmid-bearing cells is 90% and the segregation frequency 0.01 (refs 7, 8, 10). In addition, the presence of a centromere causes the copy number to decrease from 20–50 to 1–2 plasmids per cell[7,8].

The ends of chromosomes are specialized structures known as telomeres. Analysis of linear DNA plasmids from the macronuclei of protozoans has shown that their termini consist of a tandem array of a simple repetitive sequence[12]. These sequences function as telomeres on linear plasmids in yeast[9]. Using such plasmids Szostak and Blackburn[9] have cloned a yeast telomere whose structure seems to resemble that of the *Tetrahymena* ribosomal DNA end. For technical reasons we have used the *Tetrahymena* rDNA termini (which we abbreviate Tr ends) rather than yeast telomeres in our experiments. The Tr ends we have used also function as *ARS* elements in yeast[13].

Our approach has been to construct linear centromeric plasmids containing various combinations of cloned chromosomal elements, and to compare their behaviour with that of natural yeast chromosomes. Their mitotic properties have been analysed by measuring plasmid copy number and mitotic stability, while we have used tetrad analysis to follow their behaviour in meiosis. Natural chromosomes are lost mitotically at a frequency of 10^{-4} to 10^{-5} (refs 14–16) and are maintained at one copy per cell in haploid strains. We find that centromeric linear plasmids between 7 and 15 kilobases (kb) long are present in many copies per cell and are much less mitotically stable than are circular *ARS* centromeric plasmids. In contrast, 55-kb centromeric linear plasmids show a high level of mitotic stability, are maintained at low copy number and behave meiotically like natural chromosomes.

Short centromeric linear plasmids

The construction of yeast linear plasmids is complicated by their inability to replicate in *Escherichia coli*. Our approach has been to construct circular plasmids in *E. coli* which contain yeast genes, replication origins and centromeres. We then add sequences that will function as telomeres by ligation *in vitro*[17], transform the resulting ligation mix into yeast and screen the transformants to find those which harbour plasmids of the predicted structure. Figure 1 illustrates this method as applied to the construction of the linear plasmid YLp4. The circular plasmid A75p9 was constructed by standard recombinant DNA techniques and contains the *LEU2* gene, *CEN3* and *ARS1* in the cloning vector pBR322. The Tr ends were added by ligating an inverted repeat of the terminal 0.7 kb of the *Tetrahymena* rDNA plasmid into the single *Sal*I site of A75p9. Previous experiments have shown that this inverted repeat resolves *in vivo*, converting circular molecules which bear it into linear plasmids having two stable ends[17]. After transformation of the ligation mix into yeast, Leu^+ transformants were isolated, DNA was prepared from them and the plasmids they contained were mapped with restriction enzymes by Southern blotting[18]. The transformants TA708 and TA712 contain a linear plasmid of 10.7 kb whose restriction map is as predicted from those of its constituent elements (data not shown).

The mitotic stability of the linear plasmid YLp4 was measured by determining the fraction of plasmid-bearing cells. As shown in Table 1, the mitotic stability of YLp4 is much lower than that of the circular *ARS1*, *CEN3* plasmid A75p9, from which it was derived. YLp4 is no more stable than the acentric linear plasmid YLp30, which is identical to YLp4 except that it lacks the *CEN3* fragment.

To measure the copy number of circular and linear centromeric plasmids, we ran *Hpa*I-digested DNA on an agarose gel and Southern-blotted it using radiolabelled DNA from the plasmid pSZ57 (ref. 19), which contains the *LEU2* gene and pBR322, as probe. The copy number was assessed from the intensity of the hybridization to the fragment containing the chromosomal *LEU2* gene relative to the *LEU2*-containing fragment from the plasmid. In the strain containing A75p9, the intensities of the chromosomal and plasmid-derived *LEU2* bands are equal, confirming the observation that circular *ARS*, *CEN* plasmids are maintained at one copy per cell. The band derived from YLp4 hybridizes much more intensely than the

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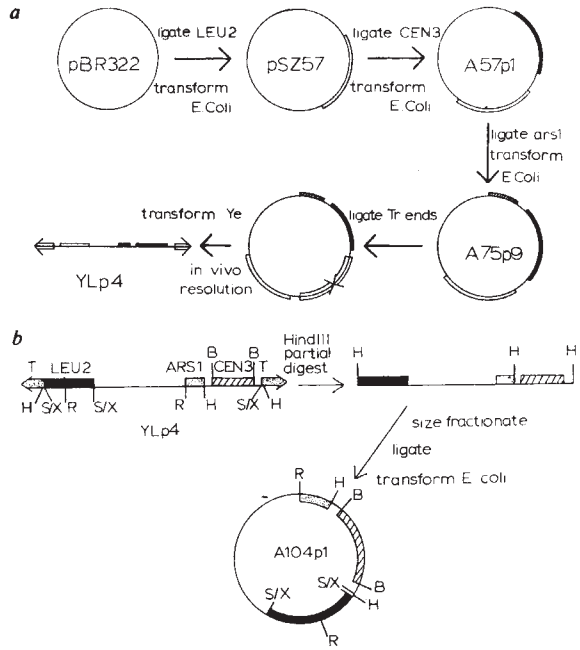

Fig. 1 *a*, Construction of the yeast linear plasmid YLp4. *b*, Recovery of a *CEN3*-containing circular plasmid from YLp4. Restriction enzyme sites: H, *Hin*dIII; S/X, *Sal*I–*Xho*I junction; R, *Eco*RI; B, *Bam*HI.
Methods: *a*, The construction of the *LEU2* plasmid pSZ57 has been described elsewhere[19]. pSZ57 was digested with *Bam*HI and ligated to a gel-purified 1.9-kb *Bam*HI *CEN3* fragment derived from the plasmid YCp82 (J. Hicks, personal communication). We identified A57p1 among the ampicillin-resistant transformants from this ligation mix by screening rapid DNA preparations by restriction enzyme digestion[36]. A75p9 was made by ligating A57p1 cleaved with *Pvu*I and *Hin*dIII to A71p4 (a pBR322 derivative containing *ARS1*, *LEU2* and the *TCM1* gene (A. W. M., unpublished)) cut with the same enzymes. The inverted Tr end repeat was prepared by gel-purifying a 0.7-kb *Xho*I–*Hha*I fragment containing the terminal sequences of the *Tetrahymena* rDNA and 14 bp of pBR322, derived from the plasmid pSZ222 (ref. 17), ligating this to itself and then recutting with *Xho*I after inactivation of the T4 DNA ligase. This head-to-head dimer was ligated to *Sal*I-cut A75p9 in the presence of both *Sal*I and *Xho*I in order to drive the reaction in favour of the desired product. The ligation mix was transformed into D234.3B with selection for Leu$^+$ transformants. Plasmid mapping was performed on Southern blots of restriction enzyme-cleaved DNA. *b*, DNA was size-fractionated on a 0.5% agarose gel and recovered by electrophoresis into and subsequent high salt elution from DEAE paper. The structure of A104p1 and three other independent isolates was verified by restriction enzyme mapping DNA prepared from *E. coli*. Note that the *Hin*dIII site, in A104p2, adjacent to the *LEU2* gene, is derived from a site within the Tr ends and therefore is absent from A75p9. This excludes the possibility that the recovered circular plasmids arose from contamination of DNA preparations with A75p9.

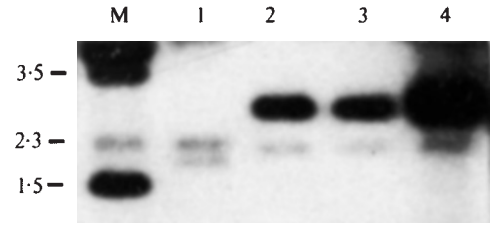

Fig. 2 Copy number of short linear plasmids. Lane M, molecular weight markers; 1, *Hpa*I–*Bam*HI-cleaved DNA from strain A281 carrying A75p9; 2, 3, *Hpa*I-cut DNA from strain A281 carrying YLp4; 4, *Hpa*I-cut DNA from strain A281 carrying YLp30. The chromosomal *LEU2* band is the upper band in lane 1 and the lower band in lanes 2–4. Molecular weights are shown in the left-hand margin.
Methods: DNA was run a 0.7% agarose gel, blotted according to Southern[18] and hybridized to nick-translated pSZ57[19] (pBR322 plus *LEU2*). The plasmid-derived bands which hybridize to pBR322 sequences are all greater than 5 kb and thus do not appear on the portion of the autoradiogram shown.

Table 1 Properties of small circular and linear plasmids

Plasmid	Form	Markers	% Plasmid-bearing cells	Segregation frequency	Copy no.
pSZ93	Circular	*ARS1, LEU2,*	5	0.34	50
A75p9	Circular	*ARS1, LEU2, CEN3*	90	0.02	1
YLp4	Linear	*ARS1, LEU2, CEN3*	61	0.11	15
YLp30	Linear	*ARS1, LEU2*	72	0.16	50

The copy number of the plasmids was measured from Southern blots (Fig. 2) and corrected for the fraction of plasmid-bearing cells. The percentage of plasmid-bearing cells was determined by plating a selectively grown culture on nonselective medium to give about 100 colonies per plate, replica plating to medium lacking leucine and measuring the fraction of colonies which grew. The segregation frequency (see text for definition) was measured by pedigree analysis. All plasmids were present in strain A281 (*α, leu2, his3–11,15, can1, cir°*). pSZ93 (ref. 34) is identical to A75p9 except that it lacks the *CEN3* fragment. Copy number is expressed as plasmid copy number per plasmid-bearing cell.

chromosomal *LEU2* gene. We estimate that the copy number of this linear centromeric plasmid is about 15 copies per plasmid-bearing cell. For the acentric linear plasmid YLp30, the plasmid copy number is even higher (Fig. 2, lane 4). Although the presence of a centromere does decrease the copy number of linear plasmids and may slightly increase their mitotic stability, both of these effects are much weaker than those seen on the addition of a centromere to circular *ARS* plasmids. Recently, similar findings have been reported for linear plasmids carrying *CEN4* (ref. 20).

Although the restriction map of YLp4 was as predicted, we were concerned that the *CEN3* sequence might have suffered some mutation which reduced its ability to function as a centromere. We reconstructed a circular *CEN* plasmid from YLp4 using a *Hin*dIII site within the Tr ends (Fig. 1*b*). DNA from TA708 was partially digested with *Hin*dIII and fragments between 9 and 11 kb were isolated, circularized and transformed into *E. coli*. DNA was prepared from four transformants and shown to have the expected restriction map. These circular plasmids from which the Tr ends had been removed were transformed into yeast. The Leu$^+$ transformants showed a level of mitotic stability indistinguishable from that of strains containing A75p9, proving that the *CEN3* sequences in YLp4 are still functional (data not shown). If the resolution of the Tr ends after replication in these plasmids was inhibited by the presence of a centromere, we should be able to detect unresolved replicated molecules as circular dimers on Southern blots. These were not seen even on overexposed autoradiograms. Thus, we conclude that the presence of the Tr ends inhibits the ability of *CEN3* to act as a functional centromere.

Figure 3*a* shows some of the other linear plasmids that we constructed. Their mitotic stability as measured by replica plating and by pedigree analysis is shown in Table 2. These plasmids, which range in length from 9.8 to 16.5 kb, all behave similarly to YLp4 in that they are mitotically unstable and are present in many copies.

One possibility that we considered was that the proximity of one of the Tr ends to *CEN3* in YLp4 could directly inhibit centromere function by some effect which is transmitted along the intervening DNA, such as nucleosome phasing. Centromeric DNA in yeast has been shown to have a specific chromatin structure[21], as have the Tr ends in *Tetrahymena*[22]. We examined this possibility by constructing plasmids in which the separation between the centromere and the Tr ends was varied.

The smallest linear centromeric plasmid we have constructed, YLp7, has only 0.3 kb of vector DNA separating *CEN3* from

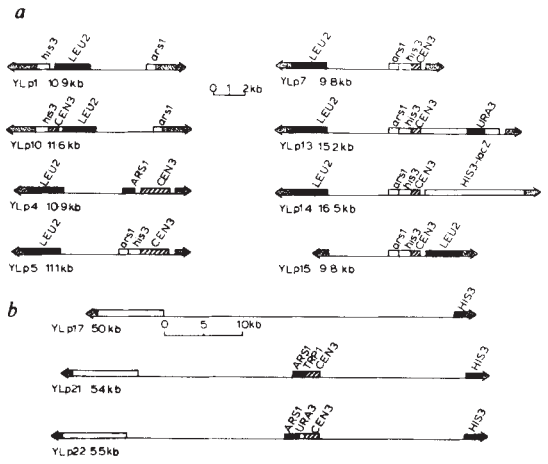

Fig. 3 Maps of linear plasmids. *a*, Short linear plasmids. The construction of YLp4 is described in Fig. 1 legend. The other plasmids were made using the same basic strategy. pBR322 sequences are shown as a single line and run through *ori* into the β-lactamase gene from left to right. Eukaryotic DNA is represented as thickened segments: ▬, selectable genes; ▭, *ARS* elements; ▭▶, Tr ends; ▭, *CEN3*. Non-functional genes or elements are represented in lower case letters. *b*, Long linear plasmids. Phage λ sequences are represented by a single line and run from *att* through *cos* to *J* running from left to right. Other symbols are as above.

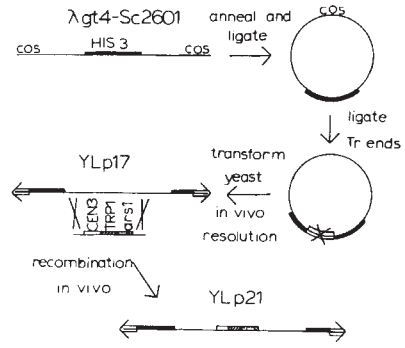

Fig. 4 Construction of a long linear plasmid. λgt4-Sc2601′ DNA[24] (1 μg ml^{-1}) was annealed at 37 °C for 24 h in 60 mM NaCl, 6 mM $MgCl_2$, 6 mM Tris-HCl *p*H 7.6, and then treated with T4 DNA ligase after the addition of ATP to 0.5 mM. After inactivation of the ligase, DNA was partially digested with *Xho*I, ethanol-precipitated and then ligated to the head-to-head *Xho*I dimer of the Tr ends. The ligation mix was transformed into D234.3B and His$^+$ transformants were selected. These were screened by restriction enzyme mapping on Southern blots to identify strains carrying YLp17. TA1016, a transformant of D234.3B carrying YLp17, was transformed with a *Cla*I–*Sph*I fragment which carries *CEN3*, *TRP1* and *ARS1* cloned into the *Bgl*II site at nucleotide 416 in the λ genome. This fragment was obtained by digesting the plasmid A106p1 (A.W.M., unpublished) with *Cla*I and *Sph*I. Trp$^+$, His$^+$ transformants were selected, screened for co-instability for these markers, and finally plasmids were restriction enzyme-mapped by Southern blotting.

the nearest Tr end. In YLp15 the *LEU2* fragment has been inverted so that it now lies between the centromere and the nearest Tr end. Thus, the centromere has been moved to a more central position without changing the length of the plasmid. The mitotic stabilities and copy numbers of YLp7 and YLp15 are similar (Table 2). In YLp13 a 5.2-kb fragment carrying the *URA3* gene has been inserted into YLp4 between *CEN3* and the nearest Tr end, while in YLp14 a fusion between the *E. coli lacZ* gene and the yeast *HIS3* gene (A.W.M., unpublished) has been inserted in the same position. Strains harbouring these plasmids show mitotic stabilities similar to that of YLp7 despite the greatly increased separation of the centromere from the Tr ends. In addition, Leu$^+$ transformants carrying YLp13 are also Ura$^+$, while those carrying YLp14 express the *HIS3–lacZ* fusion. Thus, genes on both sides of *CEN3* are expressed in these plasmids. If the Tr ends inhibit *CEN3* activity via effects which are transmitted along the DNA which lies between these elements, the inhibition is transmitted over at least 6 kb without affecting the expression of genes which lie between the Tr ends and the centromere.

Long linear centromeric plasmids

The estimated length of yeast chromosomes varies from 150 to 1,000 kb (using a value of 2.7 kb per centimorgan)[23]. The plasmids described above are at least an order of magnitude shorter than the smallest yeast chromosome. We wanted to determine whether the small size of these centromeric molecules was responsible for their mitotic instability. To construct larger linear plasmids we used the strategy illustrated in Fig. 4. The λ phage λgt4.Sc2601′ (ref. 24) carries a 10.1-kb fragment of yeast DNA which contains the *HIS3* gene but lacks an *ARS* element. Phage DNA was circularized by ligation of the cohesive ends and the inverted Tr repeat was introduced into the *Xho*I site within the yeast DNA insert. As the Tr ends carry an *ARS* element, their attachment to the *HIS3* phage DNA yields unstable His$^+$ transformants which contain linear plasmids. We named the 50-kb acentric linear plasmid YLp17 and confirmed its structure by restriction enzyme mapping on Southern blots.

The centromeric linear plasmid YLp21 was constructed by recombination *in vivo*. A strain carrying the long linear acentric plasmid YLp17 was transformed by a restriction fragment which bore *TRP1*, *ARS1* and *CEN3* inserted into a segment of λ DNA. A double cross-over or gene conversion event between the restriction fragment and YLp17 serves to integrate *TRP1*, *ARS1* and *CEN3* into the *HIS3* linear plasmid[25]. We selected transformants which were both Trp$^+$ and His$^+$ and screened these for ones in which the two markers co-segregated. Southern blot restriction enzyme mapping of DNA from these strains showed that they contained a 55-kb linear plasmid having the predicted restriction map. The plasmid YLp22 is similar to YLp21 but contains the *URA3* gene inserted into *TRP1*, thus inactivating it and yielding a *URA3*, *HIS3* linear plasmid of 56 kb.

The long linear centromeric plasmids YLp21 and YLp22 have mitotic segregation frequencies of 0.006 and 0.015, respectively. In selectively grown cultures, more than 95% of the cells express the plasmid-encoded markers. The long centromeric plasmids YLp21 and YLp22 are more mitotically stable than the circular *ARS*, *CEN* plasmids A106pl and A110pl, from which their centromeres were derived (Table 2). This contrasts strikingly with the behaviour of the short linear centromeric plasmids, which are much less stable than their circular parents. To measure the copy number of YLp21 and YLp22, DNA from diploid strains which carried both of the long linear centromeric plasmids was digested with *Pvu*II, run on an agarose gel and Southern-blotted using *CEN3* DNA as a probe (Fig. 5). In all eight DNA samples, representing four subclones each from the crosses DA173 and DA174, we consistently see stronger hybridization to the *CEN3* band derived from YLp21 than to that derived from chromosome III. We estimate that there are two to three copies of YLp21 per cell. In contrast, in some subclones, such as DA173S2 and DA173S4, the hybridization to the *CEN3*-containing band of the Ura$^+$ plasmid YLp22 is of the same intensity as that to the chromosomal band, while in other subclones (DA173S1, DA173S3) the plasmid band is clearly more intense (Fig. 5). This suggests that some subclones contain two copies of YLp22 while others have only one copy, and that such differences are stable enough to persist in cultures that have been grown for at least 20 generations. These results suggest that the long linear centromeric plasmids we have constructed are faithfully

segregated to both progeny cells in most mitotic divisions. They are lost more frequently and have a less rigorously controlled copy number than do natural chromosomes.

Meiotic behaviour

When diploid yeast cells sporulate, each spore in a tetrad receives one copy of each chromosome. Sporulation of a diploid monosomic for a single chromosome produces tetrads in which two spores contain a copy of this chromosome, while the other two spores lack it and are non-viable. The behaviour of centromere-linked markers on other chromosomes demonstrates that the two viable spores are sister spores[26], that is, the sister chromatids of the monosomic chromosomes segregate from each other at the second meiotic division. Many monosomic strains are unstable and revert to diploidy at a high frequency[26]. These revertants produce tetrads in which all four spores are viable.

When strains carrying circular *ARS*, *CEN* plasmids pass through meiosis, the predominant segregation classes observed are $4^+:0^-$, $2^+:2^-$ and $0^+:4^-$ for the markers carried on the plasmid (Table 3)[7,8,10,27]. $4^+:0^-$ and $0^+:4^-$ tetrads are presumed to arise from the sporulation of cells which contained two or no copies of the plasmid respectively, due to errors in mitotic segregation. In tetrads where the plasmid segregates $2^+:2^-$, the two spores which receive it are sister spores, indicating that the two copies of the plasmid segregate from each other at the second meiotic division. The rare tetrads showing $3^+:1^-$ or $1^+:3^-$ segregation probably arise as a result of the failure of sister plasmid molecules to segregate from each other at the second meiotic division. Note that an odd number of sister or non-sister chromatid exchanges will create dicentric dimeric circles, leading to aberrant segregation.

We constructed several diploids which contained both of the long linear centromeric plasmids YLp21 and YLp22. We can regard these two molecules as being homologous artificial chromosomes because YLp22 differs from YLp21 only by the insertion of the *URA3* gene. Because this insertion lies within the *TRP1* gene, YLp21 is Trp^+ Ura^-, while YLp22 is Trp^- Ura^+, so that the segregation of both plasmids can be followed. If the artificial chromosomes behave like natural yeast chromosomes we would expect to find two Trp^+ Ura^- spores (containing YLp21) and two Trp^- Ura^+ spores (containing YLp22) in each tetrad. If gene conversion between the two homologous plasmids occurred, tetrads which showed either 3:1 or 1:3 Trp^+Ura^-:Trp^-Ura^+ segregation should be found.

Table 3 shows the results of tetrad analysis of dipoids containing both artificial chromosomes. For both plasmids, more than 90% of the tetrads show $4^+:0^-$, $2^+:2^-$ or $0^+:4^-$ segregation. YLp21 shows a much higher fraction of $4^+:0^-$ segregation than YLp22, consistent with its higher copy number in mitotic cells (Fig. 5*a*). In more than 90% of the tetrads where one or both of the artificial chromosomes segregates $2^+:2^-$, the two spores which contain the plasmid are sisters (Table 3). This shows that the two copies of the plasmid segregate from each other at the second meiotic division, as do natural sister chromatids.

In 21% of the tetrads, both artificial chromosomes show $2^+:2^-$ segregation. In 50 out of 55 such tetrads, the two plasmids segregate from each other, yielding two Trp^+ Ura^- spores and two Trp^- Ura^+ spores. Thus, the homologous artificial chromosomes segregate from each other at the first meiotic division as do natural homologues.

To investigate the correlation between the meiotic behaviour of artificial chromosomes and their copy number, we sporulated portions of the cultures used to make DNA from the subclones DA173S1 to DA173S4. The segregation of YLp21 (Trp^+Ura^-) and YLp22 (Trp^-Ura^+) in these subclones is shown in Fig. 5*b*. As expected for a copy number greater than one, YLp21 shows mainly $4^+:0^-$ and $2^+:2^-$ segregation. In DA173S2 and DA173S4, where YLp22 is present as a single copy, $2^+:2^-$ segregation of this artificial chromosome greatly exceeds $4^+:0^-$, whereas for DA173S1 and DA173S3, where YLp22 is present in more than one copy, there are as many $4^+:0^-$ tetrads as there are $2^+:2^-$. This suggests that the meiotic segregation of these plasmids is faithful and that the deviations from the segregation pattern expected for natural chromosomes are due to cells entering meiosis with too many or too few copies of the plasmids.

Table 2 Mitotic stabilities of linear centromeric plasmids

Plasmid	Strain	% Plasmid-bearing cells	Segregation frequency
YLp4	A281	61	0.11
YLp5	A281	56	ND
YLp7	A281	67	0.10
YLp13	A281	66	0.13
YLp14	A281	65	0.17
YLp15	A281	73	0.07
YLp21	DA173, DA174	>99	0.006
A106p1	D234.3B	91	ND
YLp22	DA173, DA174	98	0.015
A110p1	D234.3B	92	ND

A106p1 is the circular plasmid from which the centromere-containing fragment of YLp21 was obtained, while A110p1 is the plasmid from which that of YLp22 was obtained. Strain genotypes: D234.3B, *α, leu2-3,112, his3-11,15, trp1, ura3, tcm1*; DA173, see Table 3. See Table 1 legend for other details. ND, not determined.

Table 3 Meiotic segregation of circular *CEN3* and long linear *CEN3* plasmids

Diploids	Plasmid	Segregation of plasmid-borne marker % Of tetrads showing: $4^+:0^-$	$3^+:1^-$	$2^+:2^-$	$1^+:3^-$	$0^+:4^-$	% FDS*
Published data for circular *CEN3* plasmids[7,10]		21	7	57	1	14	98
DA163	YLp21	50	1	42	1	6	100
DA170 to DA177	YLp21	56	5	33	2	3	95
DA170 to DA177	YLp22	18	5	58	2	17	93

Data for circular *CEN3* plasmids are from refs 7 and 10. Tetrad analysis was performed as described in ref. 35. The number of tetrads analysed for the crosses shown were: *CEN3* circles, 235; DA163, 74; DA170–DA177, 259. Strain genotypes: DA163 *a/α, ade1/+, leu2-3,112/+, his3-11,15/his3-11,15, trp1/trp1, ura3/+, tcm1/TCM1, YLp21*[*HIS3, TRP1*]; DA170 and DA172–DA177, *a/α, ade1/+, leu2-3,112/+, his3-11,15/his3-11,15, trp1/trp1, ura3/ura3, tcm1/TCM1, YLp21*[*HIS3, TRP1*], *YLp22*[*HIS3, URA3*]. DA171 has the same genotype as DA170 but is homozygous for trichodermin sensitivity: *TCM1/TCM1*. DA170 to DA177 were constructed by crossing Trp^+His^+ segregants from the cross DA163, to Ura^+His^+ segregants from the cross DA164, in which YLp22 was segregating. Diploids were constructed, sporulated and dissected by standard methods[35].

* Percentage of $2^+:2^-$ tetrads in which artificial chromosomes are found in sister spores (first division segregation, FDS) as determined by monitoring the segregation of the centromere-linked chromosomal markers *ade1* and *leu2*.

Discussion

We have constructed linear plasmids which contain all the elements known to be required for normal chromosome function. Plasmids which range from 9.8 to 16.5 kb in size are not mitotically stable and are present in many copies per cell. These plasmids behave more like acentric linear plasmids than centromeric circular ones. In contrast, linear centromeric plasmids of 55 kb constructed using phage λ DNA behave in mitosis and meiosis like circular centromeric plasmids, although they are considerably less stable than natural chromosomes. The simplest explanation of the difference in the behaviour of the two classes of linear centromeric plasmids is their difference in length. Size also affects the behaviour of natural chromosomes. The *chl* mutation leads to increased rates of mitotic loss for the smallest yeast chromosomes[15]. In zygotes in which nuclear fusion has been prevented by the *kar1* mutation, single chromosomes can be transferred from one nucleus to the other. The frequency of transfer decreases with increasing chromosome length[28].

We advance two possible explanations for the effect of length on the mitotic segregation of centromeric linear plasmids. The first is that a specific site for telomere resolution exists in the yeast nucleus, with which the ends of linear molecules remain associated even after telomere resolution. If this site is distant from the fibres of the mitotic spindle, the centromere of a plasmid or chromosome would be prevented from attaching to the spindle if the separation between the telomeres and the centromere was less than some critical distance. This would be consistent with the fact that all yeast chromosomes are metacentric as judged from their genetic maps[29]. Although telocentric chromosomes do occur in other organisms, these have much larger chromosomes, so that the centromere may be separated from the nearest telomere by tens or hundreds of kilobases while appearing cytologically adjacent to it. Because circular centromeric plasmids are not associated with the telomere binding site, they are not inhibited from attaching to the mitotic spindle. In the interphase nuclei of polytene *Drosophila* cells, the chromocentre (where the centromeres are believed to lie) lies on the opposite side of the nucleus from the telomeres[30].

The second explanation invokes a requirement for the physical proximity of the two centromeres of a pair of daughter molecules to ensure their attachment to opposite poles of the spindle. For circular plasmids this proximity would be achieved if replication resulted in the production of a catenated dimer, as has been observed in the replication of simian virus 40 (SV40)[31]. In contrast, the newly replicated copies of a short linear centromeric plasmid would not be topologically constrained to remain close to each other. However, as the length of such molecules increased, the rate at which the intertwined (but not interconnected) molecules separated from each other would decline, leading to increasingly faithful mitotic segregation. This model would predict that the fidelity of segregation of linear centromeric plasmids should increase gradually with length, while the telomere attachment site model might predict that there would be a critical size range in which the mitotic stability would increase rapidly with length.

The long linear centromeric plasmids YLp21 and YLp22 behave in many respects like genuine chromosomes. However, these artificial chromosomes show a frequency of mitotic loss two orders of magnitude higher than that of natural chromosomes and a less stringent control of copy number. These differences could reflect the absence from our molecules of some previously unknown element required for proper chromosome segregation. We favour the interpretation that they indicate the suboptimal functioning of one or more of the components they contain. For example, the Tr ends may not function exactly like yeast telomeres, a larger *CEN3* fragment might be required to ensure spindle attachment at each mitosis, a second *ARS* element may be required on the opposite side of the centromere from *ARS1*, or a further increase in the physical separation of the centromere and telomeres may be needed to ensure accurate segregation.

The availability of linear molecules of defined structure whose behaviour mimics that of natural chromosomes promises to assist investigations into the mechanisms of mitotic and meiotic chromosome segregation. By constructing artificial chromosomes which carry genetic markers near their termini, we can investigate the postulated requirement for recombination between homologous chromosomes for proper disjunction in the first meiotic division[32,33].

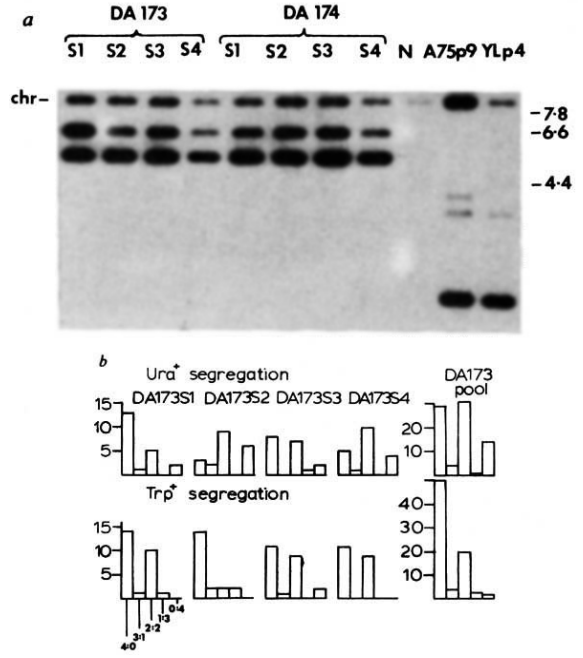

Fig. 5 *a*, Copy number of long linear plasmids. *b*, Meiotic segregation of long linear plasmids. DNA was digested with *Bam*HI plus *Pvu*II and run on a 0.5% agarose gel, Southern-blotted and then probed with a 1.2-kb *Bam*HI–*Cla*I *CEN3* nick-translated fragment. DA173 and DA174 subclones contain both YLp21 and YLp22. In these strains the lowest band is derived from the *CEN3*-containing fragment of YLp21, the middle band from that of YLp22 and the upper band from that of chromosome III. The chromosomal *CEN3* band (chr) is indicated in the left-hand margin, while the migration of molecular weight markers is shown in the right-hand margin. Other lanes: N, D234.3B without any plasmid; A75p9, strain A281 carrying the circular *ARS1*, *CEN3* plasmid A75p9; YLp4, strain A281 carrying the linear *ARS1*, *CEN3* plasmid YLp4. Faint bands in the last two lanes are due to contaminating pBR322 sequences in the radiolabelled probe. *b*, Meiotic segregation of long linear plasmids. Portions of the cultures used to make the DNAs analysed in *a* were sporulated and subjected to tetrad analysis[35].

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