Construction and Behavior of Circularly Permutated and Telocentric Chromosomes in \textit{Saccharomyces cerevisiae}

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We developed techniques that allow us to construct novel variants of \textit{Saccharomyces cerevisiae} chromosomes. These modified chromosomes have precisely determined structures. A metacentric derivative of chromosome III which lacks the telomere-associated X and Y' elements, which are found at the telomeres of most yeast chromosomes, behaves normally in both mitosis and meiosis. We made a circularly permuted telocentric version of yeast chromosome III whose closest telocentre was 33 kilobases from the centromere. This telocentric chromosome was lost at a frequency of $1.6 \times 10^{-5}$ per cell compared with a frequency of $4.0 \times 10^{-6}$ for the natural metacentric version of chromosome III. An extremely telocentric chromosome whose closest telomere was only 3.5 kilobases from the centromere was lost at a frequency of $6.0 \times 10^{-5}$. The mitotic stability of telocentric chromosomes shows that the very high frequency of nondisjunction observed for short linear artificial chromosomes is not due to inadequate centromere-telomere separation.

We are attempting to define the structural requirements for accurate chromosome segregation in mitosis and meiosis (9a, 10). Examination of the \textit{Saccharomyces cerevisiae} genetic map (9) reveals that most yeast chromosomes are metacentric, that is, their centromeres lie roughly halfway between their telomeres. The known correlation between the physical and genetic maps (19) suggests that all the telomeres lie at least 100 kilobases (kb) from a centromere. These natural chromosomes are lost at a frequency of about $10^{-3}$ per cell division (5). In contrast, a 55-kb metacentric artificial chromosome, whose telomeres are about 25 kb from the centromere, is lost at a frequency of $10^{-2}$ per cell division, and 10- to 15-kb metacentric artificial chromosomes, whose telomeres are about 5 kb from the centromere, are lost at a frequency of $10^{-3}$ per cell division (2, 10). We wished to determine whether the difference in mitotic stability between natural and artificial chromosomes was related to the difference in centromere-telomere separation. To this end, we developed techniques that allow us to produce a wide variety of novel derivatives of yeast chromosome III. We produced both metacentric and telocentric chromosomes, all of which carry telomeres derived from those of the \textit{Tetrahymena} ribosomal DNA plasmid (23, 24) rather than those of natural yeast chromosomes.

We demonstrate that telocentric chromosomes are slightly more mitotically stable than metacentric natural chromosomes, but are much more stable than metacentric artificial chromosomes which have similar separations between their centromeres and the closest telomere. Telocentric chromosomes also segregate normally in meiosis. Finally, we show that the telomere-associated X and Y' sequences (1) are completely dispensable for normal chromosome segregation.

**MATERIALS AND METHODS**

Enzymes, chemicals, and media. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Inc. (Beverly, Mass.) and used as recommended by the manufacturer. Medium components were from Difco Laboratories (Detroit, Mich.) and Sigma Chemical Co. (St. Louis, Mo.), and media were prepared by the method of Sherman et al. (17).

**Strains.** The yeast strains used in this work are listed in Table 1. \textit{Escherichia coli} JA300 (25) and BA1 (\textit{leuB6 trpC1116 hisB thyA thi Str' Tc' hsdR hsdM}; A. W. Murray, unpublished data) were used for plasmid constructions. Bacterial plasmids used in this study were constructed by standard recombinant DNA techniques. Standard techniques were used for the construction of plasmids, sporulation, and tetrad analysis (17).

**Mitotic stability measurements.** The mitotic stability of derivatives of chromosome III was measured by a quantitative mating assay as described previously (Murray et al., in press). Cells were grown in leucine-free medium (to prevent early loss of the marked chromosome III derivative, which carries the only functional \textit{LEU2} gene) and then mated to a haploid tester strain before plating on medium on which only the products of the mating could grow. Because cells which have lost the marked chromosome III derivative can divide a small number of times on leucine-free medium, the results are expressed as the frequency of chromosome loss (chromosome loss events per cell division). In all cases true chromosome loss events were distinguished from a mitotic recombination or gene conversion event by monitoring the retention of a genetic marker on the opposite side of the centromere from \textit{MAT}. In each experiment at least 100 products of each mating were tested for chromosome loss in this way. The results shown in Table 2 are the average of at least three independent experiments.

**Construction of YLPb16.** The linear plasmid YLPb16 was constructed from the bacterial plasmid A90p2 and the terminal fragment of the \textit{Tetrahymena} ribosomal DNA plasmid (hereafter called the Tr end). A90p2 is a progenitor of the plasmid A164p2 (Fig. 1b), but lacks the inverted repeat of the Tr end flanking the yeast \textit{HIS3} gene present in A164p2. A90p2 was cut with \textit{SalI} and ligated to a gel-purified \textit{Xhol-HhaI} fragment containing the Tr end and derived from pSZ221 (23). This ligation mix was used to transform the \textit{leu2}
ura3 strain A288, and Leu+ Ura+ transformants were selected. These were restriction mapped on Southern blots to verify that they contained a plasmid of the correct structure. The transformant TA1145 was used to isolate TcIII-A as described below. The version of YLp16 in TA1145 contains a tandem head-to-tail duplication of the Tr end at its left end (Fig. 1c) which arose because the XhoI-HhaI Tr fragment was contaminated with an HhaI Tr fragment owing to incomplete XhoI digestion. This contaminating HhaI fragment then ligated onto the HhaI end of an XhoI-HhaI fragment which had already been ligated onto the SalI site of A90p2.

RESULTS

Directed chromosome breakage. One possible explanation for the failure of artificial chromosomes to segregate as faithfully as their natural counterparts is that they carry telomeres derived from those of the Tetrahymena ribosomal DNA plasmid (Tr ends [24]) rather than those found on natural yeast chromosomes. Although the Tr ends in yeasts are modified by the addition of several hundred base pairs of the yeast telomeric repeat sequence C12-A (16), they lack the telomere-associated sequences X and Y, which lie adjacent to the telomeric repeat sequences on many yeast chromosomes (1). To determine whether the X and Y elements play any role in normal chromosome behavior, we sought to replace the ends of chromosome III with Tr ends.

We took advantage of the existence of a circular derivative of chromosome III (Fig. 1a) created by recombination between the two silent mating-type cassettes, HML and HMR (4, 7). HML and HMR lie close to the left end right ends of chromosome III, respectively, and contain homologous sequences in direct orientation. Recombination between the silent cassettes creates a circular chromosome and a short linear molecule, which carries the chromosomal telomeres. This linear fragment lacks a centromere and is rapidly lost, but since none of the genetic information which it carries is essential, the strains carrying the circular chromosome III are viable as haploids. We relinearized chromosome III by directing a plasmid carrying an inverted repeat of the Tr ends to integrate at the HML-HMR fusion generated by the original recombination event (Fig. 1b). After the plasmid had integrated, the inverted repeat of the Tr ends resolved to give a pair of telomeres (23), generating a new version of chromosome III which we call chromosome III-Tr. This novel linear chromosome lacks the telomere-associated sequences which normally lie centromere distal to HML and HMR.

The plasmid A164p2 (Fig. 1b) carries the yeast HMRa locus, the selectable markers LEU2 and URA3, and an inverted repeat of the Tr ends separated by the yeast HIS3 gene. A164p2 DNA was cut with BamHI, which separates the HIS3 gene from the rest of the molecule, and then treated with DNA ligase to generate circular molecules which carried an uninterrupted inverted repeat of the Tr ends (we used this strategy because uninterrupted inverted repeats cannot be maintained in E. coli [8]). The circular DNA molecules were recut with XbaI, which cleaves the plasmid only in HMRa and therefore directs the plasmid to integrate at the HML-HMR fusion (12). The XbaI-cut DNA was transformed into the leu2 ura3 yeast strain TA1540 which carries the circular version of chromosome III, and Ura+ Leu+ transformants were selected.

TABLE 2. Loss frequencies of chromosome III derivatives

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Strain</th>
<th>Length (kb)</th>
<th>Telomere-centromere separation (kb)</th>
<th>Loss frequency events/cell (×10^-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal III</td>
<td>DA226</td>
<td>530±</td>
<td>250</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>III-Tr</td>
<td>DA247</td>
<td>500±</td>
<td>250</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>III-TcA</td>
<td>DA192</td>
<td>500±</td>
<td>33</td>
<td>1.6 ± 1.3</td>
</tr>
<tr>
<td>III-TcB</td>
<td>TA1545</td>
<td>480±</td>
<td>12</td>
<td>2.9 ± 1.6</td>
</tr>
<tr>
<td>III-TcC</td>
<td>TA1425</td>
<td>470±</td>
<td>3.5</td>
<td>6.0 ± 3.4</td>
</tr>
</tbody>
</table>

* The frequency of chromosome loss was measured by a quantitative mating assay as described in Materials and Methods.
* Separation between the centromere and the closest telomere. Figures for the metacentric chromosomes are estimates.
* From Schwartz and Cantor (15).
FIG. 1. Circularly permuted version of chromosome III. (a) Construction of the linear chromosome III-Tr. From top to bottom the figure shows: the normal version of chromosome III; the circular derivative of chromosome III (4); chromosome III-Tr which results from the resolution of an inverted repeat of the Tr ends into a pair of telomeres. Symbols: ->. yeast chromosomal telomeres and telomere-associated sequences; Tr ends (Tetrahymena telomeres as modified in yeast cells). (b) Restriction enzyme maps of A164p2, the HML-HMR fusion, and the chromosome ends produced by the integration and resolution of A164p2 (after removal of the HIS3 gene by BamHI digestion and religation and subsequent digestion with Xhol to target integration) at the HML-HMR fusion. The circular map of A164p2 is not to scale. Symbols: 222, LEU2; 222, URA3; 222, HMR sequences; 222, HML sequences; Tr ends. Restriction enzyme sites: B, BamHI, Bg, BglII; E, EcoRI; H, HindIII; P1, PvuI; P2, PvuII; Xb, Xhol. (c) Construction of the telocentric chromosome III-TcA. The integration event of the linear plasmid YLP16 at the leu2 gene of the circular chromosome III of strain A288 (kindly provided by J. Haber) is shown. See text for further details. The restriction maps of YLP16, the region around the chromosomal leu2 gene, and the products of integration are shown. YLP16 contains a BamHI and an Xhol site which are absent from A164p2. Symbols are as in panel b. (d) Restriction enzyme mapping of the chromosome ends in chromosomes III-Tr and III-TcA. Lanes: M, molecular weight markers (sizes [in kilobases] indicated in the left margin); 1. TA1340 (circle III); 2. TA1454-I (III-TcA); 3. TA1593 (III-Tr); 4. TA1594 (III-Tr). DNA was digested with the indicated restriction enzymes, run on a 0.5% agarose gel, Southern blotted, and probed with pBR322 plus the LEU2 gene. Fragments are marked as deriving from the telomere marked by the URA3 gene (Δ), the telomere marked by the LEU2 gene (□), or the endogenous leu2 gene (○). Note that in chromosome III-TcA the bands derived from the endogenous leu2 gene are absent, showing that integration of the Tr ends has occurred at this locus.

The restriction map of the telomeres of chromosome III-Tr can be predicted from the restriction map of A164p2 (Fig. 1b). Figure 1d shows the autoradiogram of a Southern blot which verifies that transformants TA1593 and TA1594 carry chromosome III-Tr. The LEU2 and URA3 genes are separated from each other by the resolution of the inverted repeat of the Tr ends which lies between them. Thus, the telomeres of chromosome III-Tr are genetically as well as physically marked: the left one by URA3 and the right by LEU2.

We used a quantitative mating assay (3) to investigate the mitotic stability of chromosome III-Tr. We made diploid strains by mating a MATa haploid carrying chromosome III-Tr to a MATa haploid which carried the normal version of chromosome III. Such diploid, MATa/MATα cells are incapable of mating with haploids. Loss of the MATα allele
carried on chromosome III-Tr generates an a-mating cell, which can be detected by its ability to mate to an a-mating tester strain. Because chromosome III-Tr carries the only functional alleles of the LEU2 and URA3 genes on its ends, genuine chromosome loss events can be distinguished from other events which produce cells which lack MATa information. Only in loss events will both the LEU2 and URA3 genes also be lost from the diploid. We compared the loss frequency of chromosome III-Tr with that of the natural version of chromosome III (Table 2). The loss frequency of chromosome III-Tr is nearly identical to that of the normal version of chromosome III, demonstrating that the absence of the telomere-associated X and Y' sequences does not destabilize a yeast chromosome in mitosis.

We also examined the meiotic behavior of chromosome III-Tr by crossing the haploid transformants TA1593 and TA1594 to strain DA164.9A which carries the natural version of chromosome III. The resulting diploids, DA235 and DA236, were sporulated and subjected to tetrad analysis to monitor the segregation of chromosome III. Because the two diploids behaved identically, the data from the two crosses were combined (Table 3). Several lines of evidence suggest that chromosome III segregation in these crosses is normal. First, 83% of the tetrads had four viable spores, a value similar to that of control crosses with two normal versions of chromosome III (data not shown). Second, among the tetradrs with only two or three viable spores, all the viable spores were capable of mating. If spore death was due to nondisjunction of chromosome III, some of the viable spores would contain two copies of chromosome III. A large fraction of such diploidal spores would carry both MATa and MATu information and be incapable of mating. Third, we could monitor the segregation of three genetic markers on chromosome III: MAT, and the terminal markers of chromosome III-Tr, LEU2 and URA3. Both MAT and URA3 showed classical Mendelian 2:2 segregation in 79 of 79 tetrads, while LEU2 showed 2:2 segregation in 77 tetrads and one case each of 3 LEU2:1 leu2 and 1 LEU2:3 leu2 segregation. Finally, we could monitor recombination between the telomeres of chromosome III-Tr. As expected from the genetic length of chromosome III (150 centimorgans [9]), terminal LEU2 and URA3 markers were not linked (the ratio of parental ditype:nonparental ditype:tetraplotype segregation was 9:11:55, which is not significantly different from the 1:1:4 ratio expected for unlinked genes).

**Mitotic stability of telocentric chromosomes.** A 55-kb linear artificial chromosome is lost a thousand times more frequently than a natural chromosome (10). This discrepancy could reflect the shorter overall size of the artificial chromosome, the smaller separation between its centromere and telomeres, or the presence on natural chromosomes of previously unidentified specialized sequences that are required for accurate chromosome segregation. We examined the role of telomere-centromere separation by analyzing the behavior of telocentric derivatives of chromosome III.

We made a full-length, telocentric chromosome by directing a linear plasmid to integrate close to the centromere of the circular version of chromosome III. The construction of the acentric linear yeast plasmid YLP16 is described in Materials and Methods. YLP16 was used to transform the leu2 ura3 strain A288, which carries the circular version of chromosome III. Because YLP16 carries ARS elements associated with the HMR locus and the Tr ends, it can replicate extrachromosomally, but is mitotically unstable. Integration of YLP16 into the circular version of chromosome III relinearizes the chromosome and makes the LEU2 and URA3 markers, originally carried by the plasmid, mitotically stable. Therefore, continuous growth in leucine-free medium enriches for integrants. We determined that the integrant TA1145-1 arose from recombination between the LEU2 sequences on YLP16 and the nonfunctional leu2 gene on chromosome III by showing that the restriction map of the chromosome ends agreed with the map predicted from the known restriction maps of YLP16 and the leu2 region (Fig. 1c and d). Because the leu2 gene is only 21 kb to the left of CEN3, integration of YLP16 at leu2 creates a full-length, circularly permuted, telocentric chromosome, chromosome III-Tca, whose left arm is only 34 kb long. The sequences from HML to LEU2 which are normally found on the left arm of chromosome III have been transferred by this series of manipulations to the right arm of chromosome III-Tca. Genetic tests indicated that the integration event had occurred such that the ends of chromosome III-Tca are uniquely marked: the left end carries URA3 and a nonfunctional leu2 gene, while the right end carries the wild-type LEU2 gene (data not shown).

The loss frequency of the telocentric chromosome was measured by performing quantitative mating assays on diploids that contained one normal and one telocentric version of chromosome III (Table 2). The loss frequency of chromosome III-Tca is 1.6 × 10−5, about four times that of the normal metacentric version of chromosome III. The separation between the centromere and the nearest telomere in chromosome III-Tca (34 kb) is similar to that in the 55-kb artificial chromosome YLP22 (23 kb; loss frequency, 1.5 × 10−5).
10^{-2}$ [10]. We conclude that the location of the centromere along the length of the chromosome is not a major determinant of mitotic chromosome behavior.

To reduce the distance between the centromere and telomere still further, we constructed deletions which removed the DNA between LEU2 and CEN3 to yield the extremely telocentric chromosome, III-TcC. Figure 2a shows the strategy for making chromosome III-TcC by the one-step transplacement technique of Rothstein [14]. The diploid strain DA192 contains one copy of chromosome III and one copy of chromosome III-TcA and is homozygous for his3 and trpl. To create chromosome III-TcC, this strain was transformed with a 3.0-kb BamHI fragment derived from plasmid A233p1 (Fig. 2b), and His+ transformants were selected. One end of the fragment is homologous to sequences immediately to the right of CEN3, while the other is homologous to the Tetrahymena sequences at the telomeres of chromosome III-TcA. A double crossover (or gene conversion) between these regions of homology deletes all the sequences between CEN3 and the telomere of chromosome III-TcA including the URA3 gene. As a result, the distance from the left telomere to the functional centromere is only 3.2 kb plus the length of C13A repeats added to the Tr end (Fig. 2a). We screened 16 His+ transformants and found 6 of which had been Ura+. DNA from these was prepared, digested with BamHI, run on an agarose gel, Southern blotted, and probed with the plasmid pSZ64 (12) which contains pBR322 and the yeast HIS3 gene. Figure 2c shows that the 12-kb band derived from the left end of chromosome III-TcA disappeared and was replaced by a new band which ranged in size from 3.5 to 4.0 kb in different transformants. This length heterogeneity is typical of the terminal fragments of linear molecules [24]. The length of the C13A repeats at these chromosomal telomeres ranged from 0.3 to 0.7 kb, which is somewhat larger than the values reported for small linear plasmids [16, 24]. For two transformants, TA1424 and TA1425, the structure of the left end of the chromosome was confirmed by restriction mapping on Southern blots with HIS3 and CEN3 sequences as probes (data not shown).

The loss frequency of chromosome III-TcC was measured at 6.0 × 10^{-5} (Table 2), about 15 times higher than that of the normal version of chromosome III. A less telocentric chromosome, III-TcB (also derived from chromosome III-TcA by one-step transplacement), whose left end is 12 kb from the centromere, had a loss frequency of 2.9 × 10^{-3}. We conclude that as chromosomes are made progressively more telocentric, they segregate less faithfully in mitosis, although this effect is much less dramatic than that due to changes in overall chromosome length [6, 10, 21; Murray et al., in press]. Zakian et al. [26] and Surosky and Tye [22] have also shown that telocentric yeast chromosomes are only slightly less stable than normal metacentric chromosomes. The mitotic stability of telocentric chromosomes shows that a nearby telomere does not prevent the centromere from interacting normally with the mitotic spindle and implies that the instability of short artificial chromosomes is not due to their inability to attach to the spindle. We cannot rule out the possibility that one long chromosome arm is needed for attachment to the spindle at every division, although experiments with dicentric linear plasmids demonstrate that molecules with two very short arms attach in at least some fraction of mitoses (Murray et al., in press).

**Meiotic behavior of telocentric chromosomes.** Having established that telocentric chromosomes segregated normally in mitosis, we wished to examine their behavior in meiosis. As a first step, we crossed the haploid TA1425-1, which carries the telocentric chromosome III-TcA, to the haploid DA164.9A, which carries the natural version of chromosome III, to yield the diploid DA188. This diploid was sporulated and subjected to tetrad analysis (Table 3). Unlike the isogenic cross involving the metacentric chromosome III-Tr, the spore viability was poor: only 5 of 49 tetrads had four viable spores. We believe that this inviability is due to the generation of abnormal chromosomes, including duplications, deletions, dicentrics, and acentrics, by recombination between circularly permuted chromosomes. This interpretation is strongly supported by the observation that the terminal markers of chromosome III-TcA, LEU2 and URA3, are tightly linked in the tetrads with four viable spores, but not in tetrads with less than four viable spores (data not shown).

The simplest interpretation of these data is that four viable spores can only be recovered from tetrads in which there is an even number of exchanges between any two chromatids (which leaves the terminal markers linked to each other). A similar situation is encountered in a diploid which contains one normal and one circular version of chromosome III (4). When this diploid was put through meiosis, only 46 of 216 tetrads had four viable spores. Fifteen of the four-spored tetrads had double crossovers on chromosome III, and all of these involved only two of the four chromatids.

We created a diploid with two copies of chromosome III-TcA by crossing the two Leu+ Ura+ spores of one of the tetrad with four viable spores from the multispored diploid, DA190, showed a high level of spore viability (85% of the tetrads had four viable spores), and MAT segregated...
DISCUSSION

This paper describes the development of new methods for making predetermined alterations in chromosome structure. We studied the mitotic properties of the manipulated chromosomes to explore the relationship between chromosome structure and behavior. Table 2 lists the mitotic loss frequencies of the molecules we constructed.

The telomeres of normal yeast chromosomes terminate in tandem copies of the telomere repeat sequence C13-A (1, 16, 24). Many chromosomal telomeres have one to four tandem repeats of the 7-kb Y' sequence adjacent to the telomere repeat sequence, and these in turn are adjacent to a single copy of the less well conserved X sequence (1). We found that these sequences are not required for normal chromosome function: a novel version of chromosome III whose telomeres are composed of the Y' ends plus the yeast telomeric repeat sequence and lack associated X or Y' elements shows normal behavior in mitosis and meiosis. The ability to introduce unique genetic and physical markers into the telomeres of particular chromosomes is likely to be generally useful in studies of chromosome structure and behavior.

In our previous analysis of the structural requirements for faithful chromosome segregation we suggested two explanations for the extreme mitotic instability of short (10 to 15 kb) linear artificial chromosomes (10): (i) that the telomeres of these molecules bind to some hypothetical telomere-binding site, which prevents centromeres which are physically close to a telomere from attaching to the mitotic spindle, and (ii) that catenation of sister chromatids provides a physical linkage which ensures their accurate segregation. The latter idea was first suggested by Sundin and Varshavsky (20) and is the basis of a detailed model of mitotic and meiotic chromosome segregation (11). The accurate segregation of telocentric chromosomes (22; this paper) argues strongly against the telomere-binding model. In addition, we have shown that a 16-kb dicentric artificial chromosome is rapidly rearranged to give monocentric derivatives (Murray et al., in press). This strongly suggests that both centromeres of the dicentric chromosome are capable of attaching to the mitotic spindle in a high fraction of cell divisions. If telomere binding prevented attachment of adjacent centromeres, the linear dicentric should rearrange slowly or not at all.

The effects of physical length on the mitotic segregation of circular and linear centromeric plasmids are distinct. Although the stability of circular centromeric plasmids increases with size, molecules as small as 3 kb still show proper disjunction in more than 95% of cell divisions (6; K. Bloom, personal communication). Short linear artificial chromosomes show a completely different behavior: they are present in many copies per cell and appear to segregate randomly at mitosis (2, 10). The accurate segregation of telocentric chromosomes and the rapid rearrangement of a short dicentric artificial chromosome imply that nearby telomeres do not affect the ability of the centromere to interact with the mitotic spindle. We suggest that the poor segregation of short linear artificial chromosomes is due to the fact that circular molecules are topologically different from linear ones, rather than the fact that linear chromosomes have telomeres while circular ones do not. Specifically, we propose that the catenation of sister chromatids directs their accurate segregation from each other and that this catenation can be maintained on topologically closed circular molecules but is resolved by rotation of linear molecules about each other (11). As the size of linear chromosomes (either artificial chromosomes [6, 10; Murray et al., in press] or fragments of natural chromosomes [21; Murray et al., in press]) increases, their copy number declines, and they begin to show the ordered segregation characteristic of natural chromosomes. We propose that this size dependence of chromosome segregation reflects the need to form topologically closed domains which can retain the catenation between sister chromatids that is generated during DNA replication (11, 20). Other lines of evidence that support the catenation-dependent model of chromosome segregation and its application to mitotic chromosome segregation are discussed elsewhere (11).

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LITERATURE CITED


