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Characterization of *Schizosaccharomyces pombe* minichromosome deletion derivatives and a functional allocation of their centromere

Osami Niwa, Tomohiro Matsumoto[1], Yuji Chikashige and Mitsuhiro Yanagida

Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606, Japan

[1]Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA

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A 530 kb long *Schizosaccharomyces pombe* linear minichromosome, Ch16, containing a centric region of chromosome III, has previously been made. In the present study, we constructed a number of deletions in the right and/or left arms of Ch16, and compared their structure and behaviour with Ch16. The functional centromere, *cen3*, is allocated within a 120 kb long region which is covered by the shortest derivative, Ch10, and is comprised mostly of centromeric repeating sequences. The shortest minichromosome is stable in mitosis and the copy number control is apparently precise. In monosomic meiosis it segregates normally. In disomic meioses, however, the frequency of non-disjunction is very high, suggesting that it may not form a pair. The mitotic loss rate of one of the left-arm deletions, ChR32, which lacks a part of the centromeric repeating sequence, is the highest of all the deletions. This deletion also exhibits the highest precocious sister chromatid separation in meiosis I, suggesting that sister chromatid association might become weakened in ChR32. Our results indicate that the proper meiotic segregation of *S.pombe* minichromosomes is dependent upon the formation of a bivalent. *S.pombe* may not have the 'distributive segregation' found with *Saccharomyces cerevisiae* minichromosomes.

Key words: centromere/*S.pombe*/minichromosome

Introduction

As a chromosomal domain responsible for faithful transmission of eukaryotic chromosomes, the centromere has long been a subject of great interest. Analysis of the cloned DNA fragments of *Saccharomyces cerevisiae* has localized the functional centromere within a short segment <200 bp long (Clarke and Carbon, 1985; Panzeri *et al.*, 1985). This centromeric DNA, however, does not function in the distantly related fission yeast *Schizosaccharomyces pombe* (Clarke and Carbon, 1985), suggesting structural diversification of the centromeres. *S.pombe* centromeric DNAs have structural features not found in *S.cerevisiae*. They are large (40-120 kb, Nakaseko *et al.*, 1986; Fishel *et al.*, 1988; Chikashige *et al.*, 1989; Hahnenberger *et al.*, 1989) and complex, being rich in repeating sequences (called dg and dh or K and L; Nakaseko *et al.*, 1986, 1987; Clarke *et al.*, 1986). These highly conserved sequences appear to be required for chromosome stability and segregation (Chikashige *et al.*, 1989).

We previously isolated a minichromosome of *S.pombe* (designated Ch16), which is stably maintained, in addition to the three regular chromosomes (Niwa *et al.*, 1986). Ch16 was made by irradiating an unstable aneuploid disomic for chromosome III with gamma-rays, followed by screening stable partial aneuploids. Pulsed field gel (PFG) electrophoresis (Schwartz and Cantor, 1984; Chu *et al.*, 1986) shows that it is 530 kb long (Niwa *et al.*, 1986), which is roughly one-sixth of the size of the smallest regular chromosome III (Fan *et al.*, 1989). Because the ends of this linear minichromosomes contains ~300 bp terminal sequences hybridizing to the *S.pombe* telomeres (Matsumoto *et al.*, 1987), we conclude that the broken ends are healed by the *de novo* addition of the telomere sequence. Genetical and physical characterizations indicate that Ch16 contains an intact centromere III and the two centromere-linked loci, *ade6* and *fur1*, on the right and left arms of Ch16 respectively (Niwa *et al.*, 1986). To localize and characterize the regions involved in the maintenance and segregation of this minichromosome, we made a series of further deletions either in the right, or left, arm of Ch16 using the strategy described in this paper. A number of the deletion derivatives obtained were analysed in regard with their structure and behaviour in mitosis and meiosis.

Results

Deletions in Ch16-23R

Deletions in the left or the right arm of the minichromosome Ch16 were isolated by the method schematized in Figure 1a. A host strain HM348-23R (genotype shown in Table III), which contains a minichromosome Ch16-23R (with *S.cerevisiae LEU2* gene integrated onto the left arm of Ch16), and is Fu^s (fluorouracil sensitive) Leu$^+$ Ade$^+$ (due to intragenic complementation between *ade6-210* and *ade6-216*), was gamma-ray irradiated to induce chromosome breakage. Certain left-arm deletions should have the phenotype Fu^r Leu$^-$ Ade$^+$ (case A in Figure 1a). Forty-eight strains with such phenotype were selected and examined by PFG electrophoresis. Among those, only five were found to be significantly shorter than the original Ch16-23R (530 kb), as is shown in Figure 1b where they (lanes f-j) are run along with the standard size markers of lambda phage DNA oligomers (lane a) and Ch16 (lane k). Their lengths are 340 (lane f, ChR32), 390 (g, ChR33), 400 (h, ChR14), 470 (i, ChR27) and 490 kb (j, ChR36). The minichromosomes in 26 other strains, however, are approximately identical in size to Ch16-23R, and those in four are longer. Minichromsomal bands were not seen in the remaining 13 strains.

If a single terminal deletion has occurred generating the

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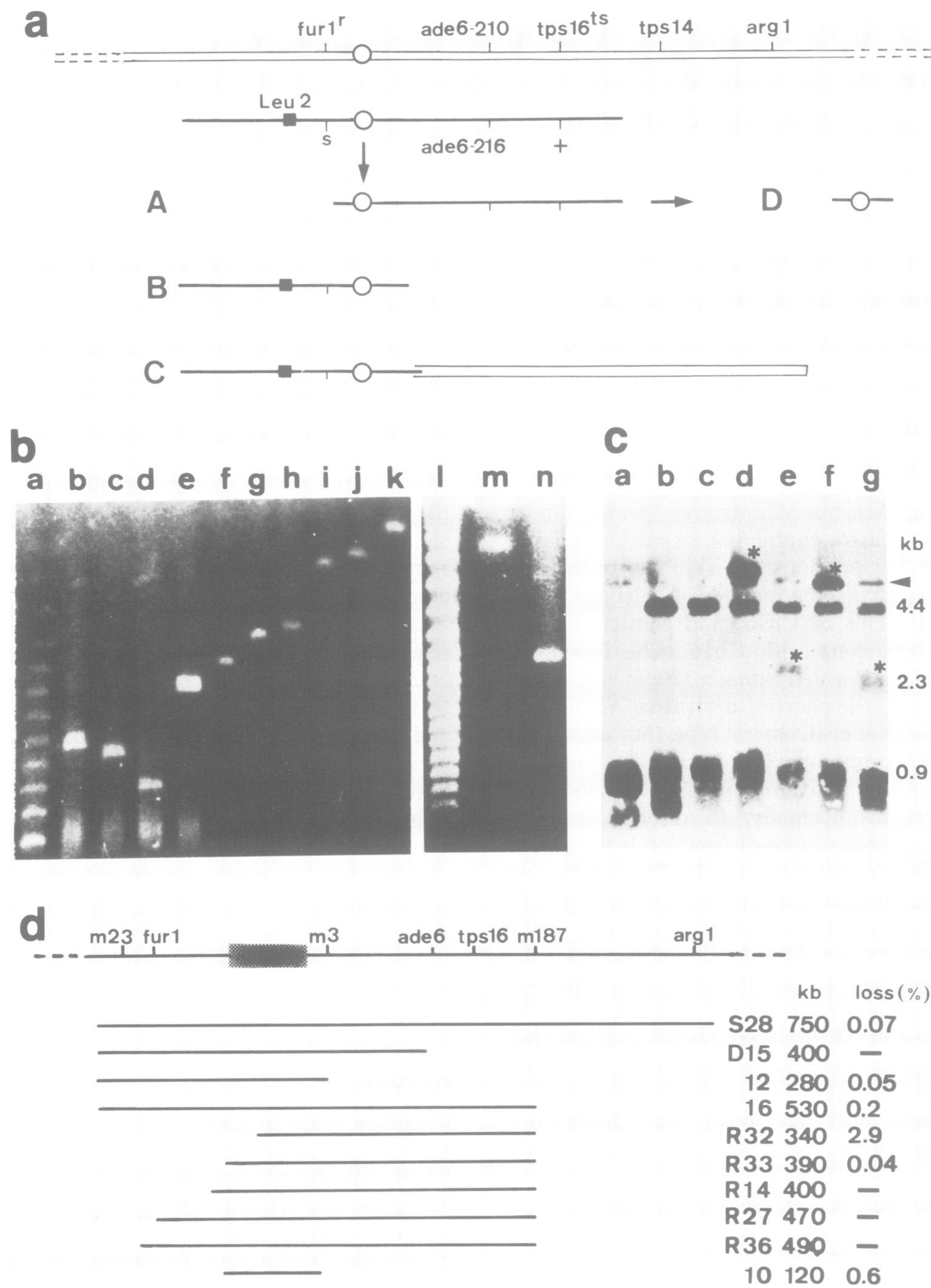

Fig. 1. Isolation and characterization of minichromosome deletion derivatives. (**a**) The strategy for isolation of minichromosomes deleted in the left or right arm. The parent strain (HM348-23R) contains a minichromosome Ch16-23R and has the genotype h^+ *leu1 fur1-1*^{r} *ade6-210 tps16-112*^{ts} Ch16-23R (*fur1*$^+$ *ade6-216 tps16*$^+$ m23 :: *LEU2*); its phenotype is Leu$^+$ Fu^s Ade$^+$ Ts$^+$. The figure shows the possible events giving rise to the phenotypes screened for as described in the text. (**A**) If breakage takes place at a site between *cen3* and the *fur1*$^+$ gene in the left arm of Ch16-23R, the resulting strain should be Leu$^-$ Fu^r Ade$^+$ Ts$^+$. (**B**) If a deletion occurs by a break in the right arm between *cen3* and *ade6*, the resulting strain should be Leu$^+$ Fu^s Ade$^-$ Ts$^-$. (**C**) By mitotic recombination, minichromosomes with increased length may be formed. ChS28 has the genetic structure as shown here. (**D**) A second step deletion produces the shortest minichromosome. (**b**) PFG gel electrophoresis of minichromosomes. **Lanes**: **b**, ChN9; **c**, ChN6; **d**, Ch10; **e**, Ch12; **f**, ChR32; **g**, ChR33; **h**, ChR14; **i**, ChR27; **j**, ChR36; **m**, ChS28; **k**, **n**, Ch16. The size standards are oligomeric bacteriophage lambda DNA (monomer: 42.5 kb) (**lanes a** and **l**). PFG electrophoresis was done in 1% agarose gel and the pulse time was 30 s for lanes a–k and 70 s for lanes l–n. (**c**) Telomeres of minichromosomes. Genomic DNAs were digested with *Eco*RI and probed with telomere sequence (pSTP16). **Lanes**: **a**, wild-type strain 972 containing no minichromosomes; **b**, ChR32; **c**, ChR33; **d**, ChR14; **e**, ChR27; **f**, ChR36; and **g**, Ch16. The 4.4 kb band represents the right end of the minichromosomes, whilst bands indicated by the asterisks represent the left ends. The weak bands indicated by the arrowhead are non-telomeric fragments. (**d**) The structure of minichromosomes derived by deletion of Ch16 along the *cen3*. At the top of the figure the structure of the original chromosome III is shown, with the relative location of the marker *fur1*, *ade6*, *tps16* and *arg1*. Below it the relative structure of deletion derivatives are shown along with their size (in kb) and the frequency of mitotic chromosome loss (in %) on the right-hand side. m23, m3 and m187 are the sequences used for integration. The shaded rectangle represents centromeric repetitive region.

shorter minichromosomes, one end may be altered whilst the other remains intact. To determine this, genomic DNAs were isolated from each of the five strains, digested with *Eco*RI and hybridized with the *S.pombe* cloned telomere sequence pSPT16 (Figure 1c). It was previously shown that in the wild-type, without a minichromosome, normal telomeres produced a single broad 0.9 kb *Eco*RI band (Figure 1c, lane a; Matsumoto *et al.*, 1987). However, in the strain containing Ch16, two additional telomeric bands were obtained, one at 4.4 and the other at 2.3 kb (lane g). A weakly hybridizing band at 7 kb (indicated by the arrowhead) is not related to the minichromosome telomeres as it exists in strains not containing minichromosomes. Evidence has previously been presented showing that the bands at 4.4 and 2.3 kb represent the telomeres of the right and left arms of Ch16 respectively (Matsumoto *et al.*, 1987). Consistently, the 4.4 kb telomeric band is preserved and the 2.3 kb band disappeared in the five strains containing the presumed left-arm deletions: novel hybridizing bands (indicated by the asterisks) are seen for the three shorter derivatives (ChR14, lane d; ChR27, lane e; ChR36, lane f). The other two, namely ChR32 (lane b) and ChR33 (lane c), however, show only the 4.4 kb band; the new band might either have co-migrated with the 4.4 kb band, or be missing altogether, or the left-arm telomere may only contain very short telomeric sequences. Thus left-arm deletions have taken place, followed by telomere healing in at least three of the derivatives.

Two right-arm markers, *ade6* and *tps16*, were used in a strategy designed to select deletions in the right arm of Ch16 (Figure 1a, case B). The parental strain HM348-23R is Ade$^+$ and Ts$^+$ (*ade6-210* and *tps16-112*^{ts} on the regular chromosome III are complemented by *ade6-216* and *tps16*$^+$ on Ch16-23R), whilst the predicted phenotype of strains harbouring deletions of the right arm to a site proximal to *ade6* would be Ade$^-$ Ts$^-$ Leu$^+$. After gamma-ray irradiation and selection, we obtained 28 strains with such phenotypes. By PFG electrophoresis, 19 of the 28 were found to contain shorter minichromosomes (280–400 kb). Three of them, ChD15 (400 kb), ChD1 (380 kb) and Ch12 (280 kb, shown in Figure 1b, lane e), were employed for genetical analysis.

Further deletions

To shorten further the minichromosomes, we employed ChR33 for generating secondary deletions (Figure 1a, case D). ChR33 is one of the shortest of the left-arm deletion derivatives. The parental strains HM348-R33 and HM396-Tr29 (the genotype shown in Table III) containing ChR33 (390 kb) and ChR33-Tr29 (450 kb; the length increase is due to multiple integration of the *LEU2* gene in the centromeric repetitive region, Materials and methods) respectively were constructed and gamma-ray irradiated. We selected Ade$^-$ Ts$^-$ survivors from HM348-R33 and Leu$^+$ Ade$^-$ from HM396-Tr29, and these were examined by PFG electrophoresis. One very short (120 kb) minichromosome designated Ch10 was obtained from HM348-R33 (Figure 1b, lane d), whilst two relatively short ones ChN6 (lane c, 180 kb) and ChN9 (lane b, 200 kb) were derived from ChR33-Tr29. ChN6 was 60 kb longer than Ch10, perhaps due to the multiple integration with the *LEU2* gene, so that the regions covered by Ch10 and ChN6 are probably similar.

To determine whether Ch10 contains the centromeric repeat sequences and the centromere-linked markers (Nakaseko *et al.*, 1986, 1987; Chikashige *et al.*, 1989), the PFG band of Ch10 was probed with the dg-dh repeat element, the cloned *fur1*$^+$ gene and the m3 sequence (Figure 1d). The latter two encompass the *cen3* and are the most proximal unique sequences mapped so far. The results of the hybridization indicate that the dg-dh repeat elements are at least partially present in Ch10, but the *fur1*$^+$ and the m3 sequences are not (data not shown), suggesting that Ch10 lacks most of the sequences present on the arms of Ch16.

A longer derivative

ChS28 is longer (750 kb, Figure 1b, lane m) than the original Ch16 (lane n; standard phage lambda oligomers shown in lane l). Tetrad analysis indicated that ChS28 contains distal markers on the right arm of chromosome III not present in Ch16; *tps14*$^+$ and *arg1*$^+$ loci exist but not *aro4*$^+$, *cdc11*$^+$ and *ade5*$^+$ (data not shown). *tps14* and *arg1* are mapped 20 and 50 cM respectively distal to m187 sequence, which marks the right end of Ch16 (Matsumoto *et al.*, 1987). The following results strongly suggest that mitotic recombination between the right arms of Ch16 and regular chromosome III as well as chromosome breakage is involved in the formation of ChS28 (Figure 1a, case C). (i) ChS28 contains the right-arm alleles, *ade6-216* and *tps16-112*^{ts}, which are present in the regular chromosome III but not in Ch16-23R. Thus the strain carrying ChS28 has the selection phenotype Ade$^-$ Ts$^-$. (ii) The left arm of Ch16 seems to be intact in ChS28, because *fur1*$^+$, the *LEU2* gene integrated at m23, is present but *ade10*$^+$, a more distal marker, is not present in ChS28.

Mitotic stability

Previous work showed that the original minichromosome Ch16 is relatively stable and its copy number appears to be precisely maintained when a single minichromosome is introduced into cells (Niwa *et al.*, 1986). In the present study the percentage frequency of cells that had lost the minichromosome in mitotic cell cultures (Materials and methods) was defined as the mitotic stability of the minichromosome deletions (the value was ~10 times higher than that of chromosome loss per cell division. The deletions were found to be as stable as Ch16 (0.2%) with the exception of ChR32 (Figure 1d). The frequencies of loss are 0.04–0.07% for ChS28, Ch12LE and ChR33 but 2.9% for ChR32. The shortest minichromosome Ch10 also appears to be stable. Because it has no selectable marker, PFG electrophoresis was used to monitor the presence of Ch10; all of the eight sibling colonies from the original isolate and the further eight siblings contained Ch10. The mitotic stability of a derivative of Ch10, namely Ch10-CN2 (integrated with the *sup3-5* gene by homologous recombination using plasmid pYC12-dgIII; Materials and methods) could be quantitatively measured and was 0.6%—somewhat higher than that of Ch16. It should be mentioned that longer minichromosomes are not more stable than shorter ones. There does not appear to be a simple relation between the length of minichromosomes and mitotic stability.

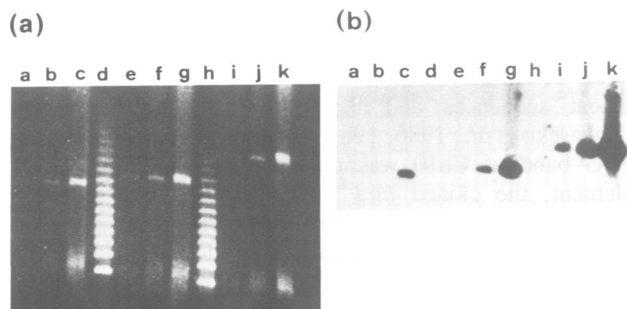

Fig. 2. The relative amounts of the repetitive sequence dg in ChR32, ChR33 and Ch16. Chromosomal DNAs were made for PFG electrophoresis from the cells of *S.pombe* strains containing ChR32 (**lanes a–c**), ChR33 (**lanes e–g**) or Ch16 (**lanes i–k**). The cells were diluted either 1/5 (**lanes a, e** and **i**) or 2/5 (**lanes b, f** and **j**), or undiluted (**lanes c, g, k**). **Lanes d** and **h** are the lambda phage DNA ladders. **Panel a** is an ethidium bromide-stained gel and **panel b** is a Southern blot hybridization of this gel probed with the dg sequence.

ChR32 partly lacks centromeric repeat sequences

ChR32 is the most mitotically unstable minichromosome (Figure 1d). It is the shortest among the left-arm deletions, suggesting that its centromere might be impaired. To determine whether ChR32 contains a full set of the centromeric repeat sequences in the *cen3* region, we estimated the relative numbers of dg and dh sequences in ChR32. Equal numbers of cells of strains carrying ChR32 (Figure 2, lanes a–c), ChR33 (lanes e–g) or Ch16 (lanes i–k) were diluted in the ratios 5:2:1 and subjected to PFG electrophoresis followed by ethidium bromide staining (Figure 2a) and Southern hybridization with the dg sequence (Figure 2b). The intensity ratios for Ch16, ChR33 and ChR32 were roughly 5:3:1. A similar result was obtained using dh as the probe (data not shown). Assuming that the total number of the dg-dh repeat motifs is 15 in the *cen3* region (Chikashige *et al.*, 1989) and that Ch16 contains an entire *cen3* region, we estimate that Ch16, ChR33 and ChR32 would contain 15, 9 and 3 repeat elements respectively. A similar experiment indicated that the shortest right-arm deletion Ch12, on the other hand, appears to contain the entire repeat elements of *cen3* (data not shown).

Mitotic non-disjunction of ChR32

We investigated the mode of minichromosome loss in mitotic division, which may be classified into three classes in terms of the number of a particular chromosome each daughter cell receives (Hieter *et al.*, 1985; Koshland *et al.*, 1985; Smith *et al.*, 1985): 2:0 (non-disjunction), 1:0 (loss) and 0:0 (complete loss). 2:0 and 1:0 segregation may be distinguished by crossing the Ade$^+$ daughter (containing one or two minichromosomes) with an Ade$^-$ tester strain, which does not have a minichromosome, followed by tetrad analysis. If the tested cells have two copies of the minichromosome, some of the resulting tetrads will exhibit $3^+:1^-$ or $4^+:0^-$ segregation, whilst, if the tested cells have only one copy, the diploid would produce only $2^+:2^-$ tetrads. The 0:0 class, however, cannot be distinguished from a division of the cell that did not receive minichromosome in some previous mitosis.

We examined 2028 individual cell divisions in exponentially growing culture of HM400 (*h*$^-$ *ade6-216* ChR32) using a micromanipulator and found that 1977 of them were normal, haivng two Ade$^+$ daughter colonies. In 11 divisions one daughter was Ade$^+$ and the other was Ade$^-$. Therefore the frequency of non-disjunction and/or loss is 0.55% per cell division. Tetrad analysis of the above Ade$^+$ daughter colonies using HM374 (*h*$^+$ *leu1 tps13*^{ts} *ade6-216*) as the tester showed that 2:0 segregation took place in eight cases. Thus the mitotic loss of ChR32 appears to be principally due to non-disjunction. In 40 divisions, both daughter cells were Ade$^-$. This was in good agreement with the separately determined value of 2.5% Ade$^-$ in the original cell culture.

Table I. Segregation patterns of monosomic minichromosomes in meiosis

Minichromosome	Reductional	Equational
(a) Ch16	83 (88%)	11 (12%)
ChS28	100 (92%)	9 (8%)
ChD15	65 (97%)	2 (3%)
Ch12	106 (96%)	4 (4%)
ChR33	75 (90%)	8 (10%)
ChN6	120 (100%)	0 (0%)
ChN9	118 (98%)	3 (2%)
ChR32	62 (48%)	67 (52%)
(b) Ch16	86 (85%)	15 (15%)
ChR32	54 (41%)	79 (59%)
(c) Ch16	74 (91%)	7 (9%)
ChR32	32 (40%)	49 (60%)

(a) *tps13* is tightly linked to *cen2* (Nakaseko *et al.*, 1986) and used as the centromere marker. Markers for minichromosome are *ade6* and/or *LEU2*. The frequency of reductional segregation is the sum of the parental and nonparental ditypes for *tsp13* and minichromosome markers. The frequency of equational segregation is the number of tetratypes. Crosses done are HM330 × HM337 for Ch16, HM374 × HM381 for ChS28, HM337 × HM355 for ChD15, HM337 × HM383 for Ch12LE, HM337 × HM396 for ChR33, ON394-1B × ON394-1A for ChN6, ON395-2A × ON395-1C for ChN9 and HM374 × HM400 for ChR32. Numbers in parentheses are the percentage frequencies for each type of segregation. (b) Segregation of Ch16 and ChR32 in the presence of *tws1* (see text). Crosses done for Ch16 and ChR32 are HM254 × HM369 and HM412 × HM413 respectively. (c) Segregation of ChR32 and Ch16LE in one cross, HM428 × HM429 (see text). Ade$^+$ and Leu$^+$ markers are used for ChR32 and Ch16LE respectively.

Monosome behaviour in meiosis

Previous tetrad and random spore analyses of Ch16 suggested that it is stably maintained and behaves independently of the regular chromosome III during meiosis (Niwa *et al.*, 1986). Meiotic and mitotic recombination rarely occurs between Ch16 and chromosome III. To investigate the behaviour of minichromosome deletion derivatives, we constructed diploids containing a single copy of each minichromosome (listed in Table I) and induced sporulation by nitrogen starvation (Materials and methods). Most tetrads (>96%) showed that $2^+:2^-$ segregation of the minichromosomes, indicating that, as in the case of Ch16, their meiotic loss hardly takes place.

The frequency of the precocious sister chromatid separation in meiosis I was measured for each deletion using *tps13* as the centromere marker (see the caption of Table I for the procedures and for the measurements; the frequency of equational segregation represents the precocious separation in meiosis I). This should be very low for normal meiotic chromosomes, but Ch16 showed a significantly high

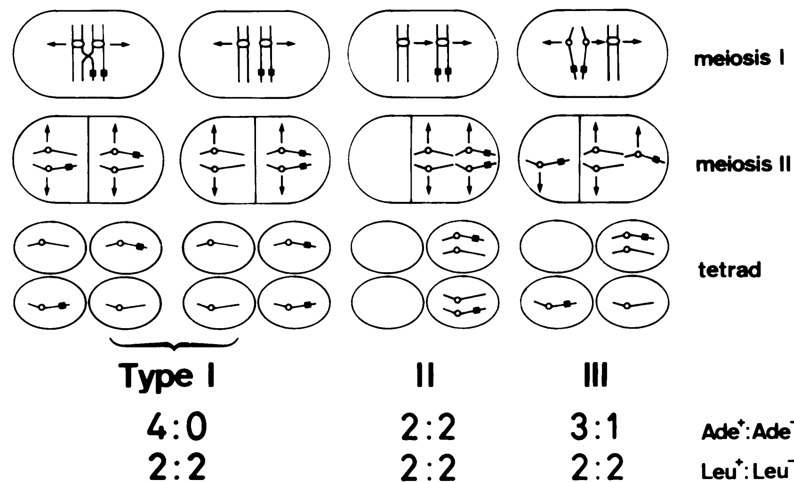

Fig. 3. Segregation patterns of two minichromosomes in meiosis (Niwa *et al.*, 1986). Type I tetrads ($4^+:0^-$): four spores each containing one minichromosome. Type II ($2^+:2^-$): two spores each with two minichromosomes and the other two spores without. Type III ($3^+:1^-$): one spore with two minichromosomes, two spores each with one minichromosome and the remaining spore without. Normal meiotic segregation through bivalent would produce type I tetrads. Independent non-paired segregation would result in the equal numbers of type I and type II segregation. Precocious sister chromatid disjunction would produce type III tetrads. In this figure both minichromosomes are marked by Ade^+ and one of them is marked by integrated Leu^+ (filled box).

Table II. Segregation patterns of disomic minichromosomes in meiosis

Cross[a]	Length	Segregation types		
		I ($4^+:0^-$)	II ($2^+:2^-$)	III ($3^+:1^-$)
1 ChS28 × ChS28	750 (kb)	199 (74%)	33 (12%)	37 (14%)
2 Ch16 × Ch16	530	252 (58%)	111 (25%)	73 (17%)
3 ChD15 × ChD15	400	134 (67%)	59 (30%)	6 (3%)
4 Ch12 × Ch12	280	94 (59%)	66 (41%)	0 (0%)
5 ChN9 × ChN9	200	80 (45%)	87 (49%)	12 (7%)
6 ChN6 × ChN6	180	83 (43%)	94 (49%)	15 (8%)
7 ChR33 × ChR33	390	87 (66%)	21 (16%)	23 (18%)
8 ChR32 × ChR32	340	20 (37%)	14 (26%)	20 (37%)
9 Ch16 × ChS28	530 × 750	47 (51%)	17 (18%)	29 (31%)
10 Ch16 × Ch12	530 × 280	106 (50%)	99 (47%)	7 (3%)
11 Ch16 × ChR32	530 × 340	22 (27%)	4 (5%)	55 (68%)
12 Ch12 × ChS28	280 × 750	85 (49%)	76 (44%)	11 (6%)

[a]Strains used for the crosses: (1) HM386 × ON321-1C; (2) HM339 × HM340; (3) HM355 × HM394; (4) HM382 × HM383; (5) ON395-2A × ON395-2D; (6) ON394-2A × ON394-1B; (7) HM396 × HM398; (8) HM400 × ON359-46B; (9) HM449 × HM462; (10) HM348 × HM354; (11) HM428 × HM429; (12) HM380 × HM381.

frequency (12%, Niwa *et al.*, 1986). The results for the deletion derivatives shown in Table Ia demonstrate two characteristic features. (i) ChR32 exhibits a strikingly high level of the precocious sister chromatid separation in meiosis I. (ii) The frequencies of this anomaly are low for shorter derivatives except ChR32. ChN6, the second shortest, showed very few precocious separations. Ch10, the shortest minichromosome, was not tested because a diploid containing Ch10-CN2 with the *sup3-5* marker did not produce asci suitable for tetrad analysis.

The high level of the precocious segregation in ChR32 was confirmed by using the *tws1* mutation (Nakaseko *et al.*, 1984; Niwa and Yanagida, 1988) in which only meiosis I takes place. As shown in Table Ib, in 59% of the cases ChR32 precociously segregates. This deficiency of ChR32 is *cis*-dominant, as shown in Table Ic. A diploid containing one Ch16 and one ChR32 was made and sporulated. Only ChR32 precociously segregates with a high frequency.

Malsegregation patterns in disomic meiosis

We previously showed two types of abnormalities in disomic meiosis of Ch16 (Niwa *et al.*, 1986). One type was apparent non-disjunction; two ascospores each contained two Ch16 and the other two lacked Ch16 (Figure 3). This type of malsegregation (designated type II segregation) represents 25% of the disomic tetrads examined (Table II). The other type was indicative of precocious sister chromatid separation in meiosis I producing the $3^+:1^-$ segregation (designated type III, Figure 3), which occurred with a frequency of 17% for Ch16 (Table II). The remaining tetrads (type I, Figure 3) were apparently normal, and represented 58% of those examined (Table II).

In the present study we examined the meiotic segregation patterns of minichromosome derivatives by crossing two strains, each containing one minichromosome. The results of 12 crosses are shown in Table II. In crosses 1–8 (between cells containing the same minichromosomes), the frequencies

of type II segregation appear to be inversely related to chromosome length; the maximal frequencies are ~50% for ChN6 and ChN9. The lowest frequency was 12% for the longest, ChS28. Two exceptions are ChR32 and ChR33 which are relatively short but give the low frequencies of type II. It should be mentioned that these two retain an intact right arm and lack a part of the centromeric repeat sequences (described above).

In the crosses 9 – 12 between different chromosomes, the shorter minichromosomes appear to determine the frequencies of type II segregation for each cross, although the reason for this is not understood. High-frequency type II segregation is obtained for the crosses 10 and 12, including Ch12 which, in a homologous cross, also gives a high level of type II segregation. The frequencies of type III, on the other hand, are high when ChR32 is involved. This is consistent with a high level of precocious sister chromatid separation of ChR32 in the monosomic meiosis. No definitive relation is found between frequency and minichromosome lengths in type III segregation, but it seems that the values of type III segregation are proportionally related to those for equational segregation in monosomic meiosis.

Meiotic recombination between minichromosomes

Previously we found that in disomic meiosis of Ch16 recombination could occur in type I segregants but not in type II (Niwa *et al.*, 1986). We investigated in the present study the meiotic recombination between Ch16 and ChS28, a long derivative, using the markers of the integrated *LEU2* and $arg1^+$ on ChS28 (Figure 1a). Type I tetrads indeed contained the recombinants (25 cases among 47 type I tetrads) but type II did not. The implications of this finding for the meiotic behaviour of minichromosomes are discussed below.

Discussion

Localization of the cen3 region in minichromosomes

The deletion series of the *S.pombe* minichromosome Ch16 made in the present study enabled us to delimit functionally the *cen3* region. Two derivatives, Ch12 and ChR33, with large deletions in the right and left arms respectively, contain a common 140 kb long region where a number of the repeating sequences dg-dh exist (Nakaseko *et al.*, 1986; Fishel *et al.*, 1988; Chikashige *et al.*, 1989; Hahnenberger *et al.*, 1989). Furthermore, the shortest minichromosome Ch10, largely lacking both arms, created by a two-step deletion, consists of a 120 kb long region sharing the same region that is in common between Ch12 and ChR33.

A greater part of Ch10 apparently consists of repeat sequences, and lacks the two unique marker sequences $fur1^+$ and m3 encompassing *cen3*. Recent direct mapping by a novel partial restriction method indicates that the entire *cen3* region is ~120 kb long, containing ~15 copies of the repeat motifs dg-dh arranged in an inverted fashion with a central flanking region (Chikashige *et al.*, 1989). The structures of the minichromosome are currently being investigated by this method. Preliminary analysis indicates that Ch10 contains most of the repeat motifs with a central flanking sequence (S.Murakami, unpublished result). Because Ch10 is relatively stable in mitosis and meiosis, it should contain the functional *cen3* region. ChR32 lacks a significant portion of the repeat motifs. It is more unstable than any other minichromosomes and separates precociously in meiosis I. Precise knowledge of the structure of ChR32 is required for the correct interpretation, because a structural rearrangment possibly exists in ChR32. Our results suggest that not all of the repeat motifs may be required for maintaining minichromosomes, rather their effects may be additive. Quantitative analysis on the contribution of different parts of the *cen3* region to the stability and segregation will require construction of minichromosomes with various deletions of defined areas.

Defective sister chromatid association

The 'weak' association of sister chromatids has been postulated to explain mitotic non-disjunction of chromosomes in *Drosophila* (Smith *et al.*, 1985). This may be consistent with our finding that the frequency of non-disjunction is high in ChR32, which deletes a large portion of the centromeric region. The holding of a pair of sister chromatids at the centromere might become weakened in the minichromosome.

The first meiotic division is characterized by the regulatory system that causes the absence of sister centromere separation. Malfunction of the system might result in the precocious sister chromatid separation in meiosis I. In the present study we show that some minichromosome deletions are impaired in such a system. The frequency of precocious chromatid separation in ChR32 is particularly high (~50%). The same defect in ChR32 may cause both mitotic loss and meiotic malsegregations.

Defect in disomic meiosis

Certain deletion derivatives, especially shorter ones, behave mostly normally in meiosis if a single minichromosome is introduced in zygotes. However, disomic minichromosomes show anomalous segregation. The high level of type II (2^+:2^-, Figure 3) segregation presented in this paper strikingly differs from the segregation pattern of the *S.cerevisiae* minichromosomes. In the budding yeast, both linear and circular minichromosomes faithfully disjoin in meiosis I in such a way (called 'distributive segregation'; Dawson *et al.*, 1986; Mann and Davis, 1986) that is independent of homology and recombination among chromosomes. It appears that *S.pombe* does not have the 'distributive segregation'.

Our interpretation for the cause of the type II segregation in Ch16 was that certain disomes fail to form a homologous pair (bivalent), followed by random movement to the poles in meiosis I (Niwa *et al.*, 1986). This implies that non-paired disomes move with an equal frequency to the same pole (apparent non-disjoining) or the opposite poles (apparent normal segregation). If this interpretation is correct, ~50% of the Ch16 disomes would move randomly to the poles, whereas ~30% (a part of type I) might segregate normally (Table II). Consistent with the presumed failure in type II for the bivalent formation, recombinants were found in type I segregants but not in type II.

Deletion derivatives show various frequencies of the type II segregants and demonstrate the tendency for the shorter ones to have the higher frequencies (Table II). Infrequent formation of bivalents in ChN9 and ChN6 could account for the observed independent movement to the poles. Perhaps faithful meiotic segregation of minichromosome in *S.pombe*

Table III. *Schizosaccharomyces pombe* strains and minichromosomes made in the present study

Strains	Genotype
HM254	*h⁻ his2 tws1 ade6-210* Ch16
HM337	*h⁻ leu1 tps13ᵗˢ ade6-210*
HM348-23R	*h⁺ leu1 fur1-1 tps16-112ᵗˢ ade6-210* Ch16-23R
HM354	*h⁻ leu1 ade6-210* Ch12LE
HM355	*h⁺ leu1 fur1-1* ChD15
HM369	*h⁺ leu1 fur1-1 tws1 ade6-210*
HM374	*h⁺ leu1 tps13ᵗˢ ade6-216*
HM380	*h⁺ leu1 tps13ᵗˢ ade6-216* Ch12LE
HM381	*h⁻ leu1 ade6-216* ChS28
HM382	*h⁻ leu1* Ch12LE
HM383	*h⁺ leu1* Ch12LE
HM386	*h⁻ leu1* ChS28
HM394	*h⁻ leu1 tps13ᵗˢ ade6-210* ChD15
HM396	*h⁺ leu1 ade6-210* ChR33
HM397	*h⁺ ade6-210* ChR33
HM398	*h⁻ his2 ade6-210* ChR33
HM400	*h⁻ ade6-216* ChR32
HM412	*h⁺ tws1 ade6-216* ChR32
HM413	*h⁻ leu1 tws1 ade6-216*
HM428	*h⁻ leu1 tps13ᵗˢ ade6-216* ChR32
HM429	*h⁺ leu1 ade6-216* Ch16LE
HM449	*h⁻ leu1 ade6-216 arg1* ChS28
HM462	*h⁺ his2 leu1 arg1 fur1-1 ade6-210* Ch16FR
HM348-R33	*h⁺ leu1 fur1-1 tsp16-112ᵗˢ ade6-210* ChR33
HM396-Tr29	*h⁺ leu1 ade6-210* ChR33-Tr29
CN2	*h⁻ leu1 ade6-704* Ch10-CN2
ON321-1C	*h⁺ leu1* ChS28
ON359-46B	*h⁺* ade6-216 ChR32
ON394-1A	*h⁻ leu1 tps13ᵗˢ*
ON394-1B	*h⁺ his2 leu1* ChN6
ON394-2A	*h⁻ leu1 tps13ᵗˢ* ChN6
ON395-1C	*h⁺ leu1 tps13ᵗˢ*
ON395-2A	*h⁻ leu1* ChN9
ON395-2D	*h⁺ leu1 tps13ᵗˢ* ChN9

Minichromosomes	Genotype
Ch16	*fur1⁺ ade6-216 tps16⁺*
Ch16FR	*fur1-1 ade6-216 tps16⁺*
Ch16LE	*fur1⁺ ade6-216 LEU2* integrated near *ade6 tps16⁺*
Ch16-23R	m23 :: *LEU2 fur1⁺ ade6-216 tps16⁺*
Ch12LE	m23 :: *LEU2 fur1⁺*
ChD15	m23 :: *LEU2 fur1⁺*
ChS28	m23 :: *LEU2 fur1⁺ ade6-210 tps16-112ᵗˢ tps14⁺ arg1⁺*
ChR33	*ade6-216 tps16⁺*
ChR33-Tr29	*ade6-216 tps16⁺* dg :: *LEU2*
ChR32	*ade6-210* (*tps16*)[a]
ChN6	dg :: *LEU2*
ChN9	dg :: *LEU2*
Ch10-CN2	dg :: *sup3-5*
Ch10	no marker

[a]Allele not determined.

requires the homologous pairing (and recombination) as in many other organisms (Baker *et al.*, 1976; Surosky and Tye, 1988), a property greatly differing from that observed with budding yeast minichromosomes. The degree of inability of the *S.pombe* minichromosomes, made by the deletion methods to form the bivalent appears to be correlated with the decreasing length of either arm. It is easily conceivable that 'armless' minichromosomes such as Ch10 behave independently in meiosis I, producing high levels of the type II segregation.

Materials and methods

Yeast strains and plasmids

Yeast strains constructed and used in the present study are listed in Table III. Other strains previously described are HM235 (*h⁺ arg1 ade6-216*), HM248 (*h⁻ his2 ade6-210* Ch16), HM330 (*h⁺ leu1 fur1-1 ade6-210* Ch16FR), HM339 (*h⁻ his2 leu1 ade6-210* Ch16LE), HM340 (*h⁺ leu1 tps13ᵗˢ fur1-1 ade6-210* Ch16FR) (Niwa *et al.*, 1986) and HM348 (*h⁺ leu1 fur1-1 ade6-210 tps16-112ᵗˢ* Ch16) (Matsumoto *et al.*, 1987). They are derivatives of 972 *h⁻* and 975 *h⁺* (Kohli *et al.*, 1977). Integration plasmid YIp32 (Botstein *et al.*, 1979) carries the *S.cerevisiae LEU2* gene (which complements the *leu1* mutation in *S.pombe*). pSAm23 and pSAm3 were made by ligating YIp32 with *Hin*dIII fragments (m23 and m3 respectively) of Ch16 DNA which was isolated from agarose blocks containing the Ch16 band in PFG. The 1.2 kb m3 fragment is located on the right arm of chromosome III (5.5 cM distant from the centromere; Matsumoto *et al.*, 1987 and this work). The 2.7 kb m23 fragment was mapped in the present study on the left arm 2.0 cM distal to *fur1*. pSTP16 contains an *S.pombe* telomere sequence (N.Sugawara and J.W.Szostak quoted in Matsumoto *et al.*, 1987). pYC12-dgIII carries the *sup3-5* gene as the selection marker (capable of suppressing the *ade6-704* mutation; Hofer *et al.*, 1979) and a 6.0 kb long dg sequence derived from the *cen3* region (Nakaseko *et al.*, 1986).

Minichromosomes

The minichromosomes used in the present study are listed in Table III. They are derived from the original minichromosome Ch16 previously made by deleting a large part of chromosome III (Niwa *et al.*, 1986). Ch12LE carries pSAm23 integrated by homologous recombination but Ch12 does not (Matsumoto *et al.*, 1987). Ch16LE is a derivative of Ch16 with the *LEU2* gene integrated near to the *ade6* gene as described previously (Niwa *et al.*, 1986). Ch10-CN2 is a derivative of Ch10, which is integrated with pYC12-dgIII. ChR33-Tr29 is a derivative of ChR33. It is multiply integrated with pSS203, which consists of 6.0 kb *Eco*RI insert (containing dgIII) and an 8.4 long cosmid vector (carrying the *LEU2* marker), and is 60 kb longer than ChR33. Although the dgIII has ARS function, we could isolate a stable integrant that contained ChR33-Tr29.

Genetic procedures

The standard genetic procedures previously described for *S.pombe* were followed (Gutz *et al.*, 1974). YPD (complete medium; 2% polypeptone, 1% yeast extract and 2% glucose) and SD (minimal medium; 0.67% Difco yeast nitrogen bases without amino acids, 2% glucose) were used with 1.5% agar for plates. When needed, 40 μg/ml of the appropriate amino acids, 10 μg/ml adenine sulphate or 100 μg/ml 5-fluorouracil (5FU) were added. Crosses were done on SPA medium (Gutz *et al.*, 1974) at 26°C. *ade6⁻* mutants produce red-coloured colonies on YPD as well as on SD supplemented with a limited amount of adenine sulphate (10 μg/ml). This phenotype was used as an indicator of *ade6⁻* mutation. Intragenic complementation takes place between *ade6-210* and *ade6-216* (Gutz *et al.*, 1974). The centromere linkage of Leu⁺ pSAm3 integrated on the normal chromosome III was determined using the *tws1* mutant (Nakaseko *et al.*, 1984; Niwa and Yanagida, 1988). This mutant produces two-spored asci from a single-division meiosis (meiosis I is present, but meiosis II is absent). In *tws1*, a single exchange between the centromere and a given marker produces equational segregation for that marker; if there is no proximal exchange, then the marker segregates reductionally. This dyad analysis is convenient for mapping centromere-linked markers (Nakaseko *et al.*, 1986) and for genetic analysis of minichromosomes during meiosis.

Pulsed field gel electrophoresis (PFG)

The sizes of the minichromosomes were determined by PFG electrophoresis (Schwartz and Cantor, 1984). A LKB Pulsaphor system (Pharmacia-LKB) and a hand-made CHEF type apparatus (Chu *et al.*, 1986) were used. Specimen agarose blocks were prepared as described previously (Niwa *et al.*, 1986). *S.cerevisiae* chromosomal DNAs (Gardiner *et al.*, 1986) and oligomeric lambda DNA (Smith *et al.*, 1986) were used as size markers. Monomeric lambda *vir* DNA is 42.5 kb in length.

Isolation of deletion derivatives of the minichromosome Ch16

HM348 carrying Ch16 was transformed with pSAm23. The transformant and its minichromosome integrated with pSAm23 are designated HM348-23R and Ch16-23R respectively. The genotype of HM348-23R is *h⁺ leu1⁻*

fur1-1 tps16-112^{ts} ade6-210 Ch16-23R (m23 :: *LEU2 fur1$^+$ ade6-216 tps16$^+$*). The *LEU2* gene (integrated using the m23 target fragment) and the *fur1$^+$* gene are the markers on the left arm of Ch16-23R. The resistant *fur1-1* mutation on chromosome III is recessive to the wild-type, so that HM348-23R is sensitive to the drug due to the presence of Ch16-23R. The *ade6-216* and *tps16$^+$* alleles on Ch16 are capable of complementing *ade6-210* and *tps16-112^{ts}* mutations on chromosome III and are used as the markers for the right arm.

HM348-23R was irradiated with gamma-rays at a dose of 150 krad, followed by overnight incubation at 26°C in YPD medium; cells were then plated on SD supplemented with leucine and 5FU or with adenine sulphate. Two hundred and twenty-three Leu$^-$ and 5FU-resistant (Fu^r) colonies were obtained from the former plates. Among those, 144 appeared to contain a minichromosome, as judged by the fact that Ade$^-$ cells (an indicator of minichromosome loss) frequently segregated from them in the presence of 20 μg/ml thiabendazole, a tubulin inhibitor which causes chromosome loss. The sizes of the minichromosomes in 48 strains were determined by PFG electrophoresis. From the adenine-supplemented plates, on the other hand, Leu$^+$ Ade$^-$ red colonies were selected and then tested for their temperature-sensitive (Ts) phenotype. About a half of them were Ts$^-$. Twenty-eight Ade$^-$ Ts$^-$ strains were tested for heterozygosity of the *fur1* locus, and examined for the presence of minichromosomes by PFG electrophoresis. Twenty of these contained minichromosomes with the reduced sizes and in one the minichromosome was longer (ChS28).

Mitotic stability of deletion derivatives

Strains carrying minichromosomes were plated on YPD and incubated at 26°C for 2 days. Cells from a single colony were suspended in 1 ml YPD. The average number of cells per colony was ~15 000, or ~2^{14}. Cells were plated on YPD and incubated at 26°C for several days. The red Ade$^-$ phenotype was used as an indication of minichromosome loss for the strains HM248, HM397, HM381, HM400 and CN2 containing Ch16, ChR33, ChS28, ChR32 and Ch10-CN2 respectively. The loss of Ch12LE from HM383 was shown by the resulting leucine-requiring phenotype. The number of cells lacking Ch12LE was determined by replica plating.

Meiotic behaviour of the minichromosomes

To make diploids containing a monosomic minichromosome, strains carrying one copy of minichromosome were crossed with those not having a minichromosome. Diploids were sporulated and tetrads dissected using *tps13* as the centromere marker (Nakaseko *et al.*, 1984, 1986). In each cross, >85% of the dissected asci contained four viable spores, and >96% of the complete tetrads showed 2^+:2^- segregation for the presence of minichromosomes, indicating that meiotic loss rarely takes place. To analyse the behaviour of disomic minichromosomes, two strains each containing one minichromosome were crossed. In disomic meioses, >80% of the tetrads exhibited four viable spores. These tetrads were found to belong to one of the three types shown in Figure 3. They varied in the number of minichromosomes distributed among the four spores. Type I tetrads consists of the four spores each of which contains one minichromosome (4^+:0^-). Type II contains two spores with two minichromosomes and two spores without (2^+:2^-). In type III, one spore contains two minichromosomes, two spores contain one and the remaining spore contains none (3^+:1^-).

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