Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 577–581, January 1989
Genetics

Construction of functional artificial minichromosomes in the fission yeast *Schizosaccharomyces pombe*

(centromeres/fission yeast/artificial minichromosomes)

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ABSTRACT **The centromere DNAs from chromosomes I and III of *Schizosaccharomyces pombe* have been cloned in an artificial chromosome vector in both budding and fission yeasts. In *S. pombe*, synthetic linear and circular minichromosomes containing an intact centromere are stable mitotically and behave as independent genetic linkage groups that segregate properly through meiosis. These experiments present a general strategy for the isolation of centromeres from other organisms.**

Analysis of centromeric DNA from the fission yeast *Schizosaccharomyces pombe* has revealed the presence of several classes of moderately repetitive DNA sequences (1–4). These DNA sequence repeats are present only in the centromere regions of the three *S. pombe* chromosomes and have been shown to be transcriptionally silent (1). Thus, with respect to their heterochromatic properties, the centromeres of *S. pombe* more closely resemble those of higher eukaryotes than the relatively simple centromeres of the budding yeast *Saccharomyces cerevisiae* (5).

In *S. cerevisiae*, small [150 base pairs (bp)] cloned centromere DNA sequences (designated here *CEN*) confer mitotic stability upon autonomously replicating (*ARS*) plasmids and enable them to segregate faithfully through meiosis (6). Previously, no discrete segment of *S. pombe* DNA had been found to confer mitotic or meiotic stability on *ARS* plasmids in *S. pombe* (1–3). In this study, we have developed a minichromosome assay system for centromere function in *S. pombe*. The *S. cerevisiae* yeast artificial chromosome (YAC) vector system (7) has been used to clone large restriction fragments from the *S. pombe* genome in *S. cerevisiae*. Linear artificial chromosomes containing the *S. pombe* centromeric regions from chromosomes I and III (designated here *cen1* and *cen3*) were obtained in *S. cerevisiae* and subsequently introduced by transformation into *S. pombe* and assayed for proper centromere function. Both linear and circular artificial chromosomes were recovered in *S. pombe*. These minichromosomes are mitotically stable and segregate properly through meiosis, indicating that the cloned fragments contain functional *S. pombe* centromeres. They are the first synthetic chromosomes found to be fully functional in an organism other than *Saccharomyces*. The methods described in this study should provide a general approach for the cloning of centromeres from other organisms as well.

MATERIALS AND METHODS

Strains, Transformations, and Genetic Manipulations. The genotypes of the *S. pombe* strains are given in the legends to Tables 1 and 2. *S. cerevisiae* strain AB1380 (*α ura3 trp1 ade2-1 can1-100 lys2-1 his5*) was kindly provided by David Burke (7). DNA transformations of *S. cerevisiae* (7) and *S. pombe* (10) and genetic manipulations of *S. pombe* (11, 12) were performed as described.

Enzymes, DNA Isolation, and Field-Inversion Gel Electrophoresis (FIGE). Restriction enzymes were from New England Biolabs, and T4 DNA ligase and calf intestinal alkaline phosphatase were from Boehringer Mannheim Biochemicals. Genomic DNA from *S. cerevisiae* (8) and *S. pombe* (13) was isolated as described. FIGE was carried out as described (14) in 1% agarose gels at 180 V for 18 hr with a linear ramp consisting of a beginning pulse time of 0.5 sec forward and 0.2 sec reverse and an ending pulse time of 5 sec forward and 2 sec reverse. The gels were stained with ethidium bromide.

RESULTS

Cloning of *S. pombe* Centromere Regions in *S. cerevisiae*. Previous studies in *S. pombe* have demonstrated that the centromere regions reside on three large genomic *Sal* I restriction fragments of 50, 90, and 120 kilobases (kb), corresponding to chromosomes I, II, and III, respectively (1). Further FIGE analysis in this study indicates that the sizes of these fragments are closer to 65 kb (chromosome I), 100 kb (chromosome II), and 150 kb (chromosome III). These three genomic *Sal* I fragments contain various numbers of copies of the centromere-specific sequence repeats, designated K, L, and B (2, 3). The most well-characterized *S. pombe* centromere region is that of chromosome II in which the repeated sequences are organized in a large inverted repeat ≈35 kb long (1). Our previous attempts to clone intact *S. pombe* centromeric DNA from chromosome II in *E. coli* have been unsuccessful, presumably because of the inability of *E. coli* to replicate inverted repeat structures (15, 16).

To circumvent this problem, we have made use of the YAC vector system described by Burke *et al.* (7) to clone in *S. cerevisiae* the *Sal* I fragments containing the *S. pombe* centromere regions. Very large fragments of DNA can be ligated *in vitro* between the two arms of a YAC vector and subsequently used to transform *S. cerevisiae*. The resulting linear plasmids behave as functional minichromosomes in *S. cerevisiae* because they carry *S. cerevisiae CEN* and *ARS* elements and presumably telomeres, which are added *in vivo* (7). To permit the introduction of these minichromosomes into *S. pombe*, the *E. coli–S. cerevisiae–S. pombe* shuttle vector pMB-1 was constructed (Fig. 1). The vector pYAC4 (7), which contains the *ARS1* element, *CEN4*, and the *TRP1* and *URA3* genes of *S. cerevisiae*, was modified for use in *S. pombe* by insertion of a *S. pombe ars* element (Sp *ars*) and a *S. pombe* selectable marker (Sp *ura4*) to form pMB-1 as described in Fig. 1.

Size-fractionated *Sal* I genomic restriction fragments from *S. pombe* strain Sp223 were ligated with the arms of vector

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Abbreviations: *ARS/ars* elements, autonomously replicating sequences; FIGE, field-inversion gel electrophoresis; YAC, yeast artificial chromosome.

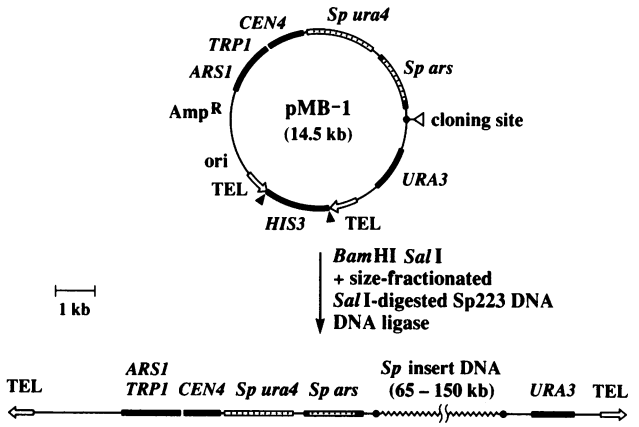

FIG. 1. For construction of vector pMB-1, the yeast artificial chromosome vector pYAC4 (7) was modified as follows. A 1.8-kb *Hin*dIII fragment containing the *S. pombe ura4* gene (L.C., unpublished data) was cloned into the unique *Cla* I site of pYAC4 by filling in both sites with the Klenow fragment of *E. coli* DNA polymerase I, followed by blunt-end ligation. A 1.2-kb *Eco*RI fragment containing a *S. pombe ars* element (17) was cloned into the unique *Eco*RI site in the *S. cerevisiae SUP4* gene. The resulting plasmid, pMB-1, was digested with *Bam*HI and *Sal* I to release the two arms (9.5 and 3.3 kb). The arms were treated with calf intestinal alkaline phosphatase to prevent their religation (18). Genomic DNA (60 μg) from Sp223 was digested with *Sal* I and fractionated on linear 10–40% sucrose gradients. Fractions containing the 65-kb, 100-kb, and 150-kb *Sal* I fragments, which include the *S. pombe* centromere regions, were identified by analyzing aliquots by FIGE, followed by hybridization of the dried gel to a ^{32}P-labeled probe containing the 6.4-kb K repeat sequence (1). The purified *Sal* I genomic DNA fragments were ligated into the pMB-1 vector arms by the method of Burke *et al.* (7) with minor modifications. The ligation mixtures were used to transform *S. cerevisiae* strain AB1380 (α *ura3 trp1 ade2-1 can1-100 lys2-1 his5*) as described (7). Ura$^+$ Trp$^+$ transformants were screened by colony hybridization (19) with a ^{32}P-labeled K repeat probe. Solid boxes, *S. cerevisiae* DNA; stippled boxes, *S. pombe* DNA; open arrows, *Tetrahymena* telomeres; ●, *Sal* I; ▲, *Bam*HI; Amp^R, ampicillin resistant.

pMB-1, and the DNA was used to transform *S. cerevisiae* strain AB1380 (Fig. 1). A total of four transformants containing the *S. pombe* centromeric *Sal* I fragments were identified by colony hybridization with a *S. pombe* K repeat probe. Total genomic DNA was isolated from these transformants and analyzed by FIGE. Three of the transformants contained a linear minichromosome designated pSp(cen3)-10L (Fig. 2*D*, lane b), which carries the 150-kb *Sal* I fragment from chromosome III of *S. pombe*, and the other transformant contained a linear minichromosome designated pSp(cen1)-7L (Fig. 2*A*, lane a), which carries the 65-kb *Sal* I fragment from chromosome I. In addition, transformants have recently been identified that contain the 100-kb *Sal* I fragment carrying the centromere region of chromosome II.

Structural Analysis of the Artificial Chromosomes in *S. pombe*. To assay the cloned 65-kb and 150-kb fragments for centromere activity in *S. pombe*, we isolated and used total genomic DNAs from AB1380/pSp(cen1)-7L and AB1380/pSp(cen3)-10L to transform *S. pombe* strain Sp223 (10), selecting for the *ura4* marker contained on the minichromosomes. The structure of the minichromosomes in *S. pombe* was determined by analyzing genomic DNA from various transformants by FIGE. This analysis revealed that in most of the transformants, the linear minichromosomes had undergone structural rearrangements. The most common rearrangement observed was circularization of the linear chromosome, often accompanied by deletion of sequences as well. In a control experiment, the vector pMB-1 linearized with *Bam*HI was used to transform *S. cerevisiae* strain AB1380. Total genomic DNA from this strain was isolated,

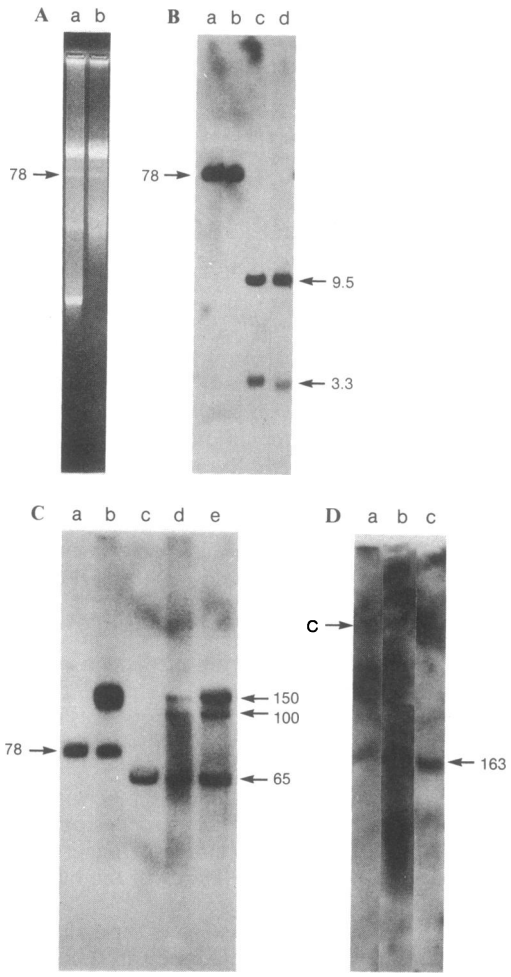

FIG. 2. (*A*) Visualization of the linear artificial chromosome pSp(cen1)-7L in genomic DNA from *S. cerevisiae* and *S. pombe* transformants. Undigested genomic DNA from *S. cerevisiae* strain AB1380 containing pSp(cen1)-7L (lane a) and from *S. pombe* strain Sp223 containing pSp(cen1)-7L (lane b) were subjected to FIGE (14). The arrow indicates the position of the 78-kb linear minichromosome, which migrates ahead of the main chromosomal band. (*B*) Autoradiogram of genomic DNAs from *S. cerevisiae* strain AB1380/pSp(cen1)-7L and *S. pombe* strain Sp223/pSp(cen1)-7L fractionated by FIGE and hybridized to ^{32}P-labeled pBR322 DNA. Lanes: a and c, genomic DNA from AB1380/pSp(cen1)-7L undigested and digested with *Sal* I, respectively; b and d, genomic DNA from Sp223/pSp(cen1)-7L undigested and digested with *Sal* I, respectively. *Sal* I digestion releases the 9.5-kb and 3.3-kb arms of the vector pMB-1 (indicated by the arrows on the right). (*C*) Identification of the 65-kb *Sal* I centromeric DNA insert in pSp(cen1)-7L. Undigested genomic DNA from AB1380/pSp(cen1)-7L (lane a) and Sp223/pSp(cen1)-7L (lane b) and *Sal* I-digested genomic DNA from AB1380/pSp(cen1)-7L (lane c), Sp223 (lane d), and Sp223/pSp(cen1)-7L (lane e) were fractionated by FIGE. The gel was dried, hybridized to a ^{32}P-labeled K repeat probe, and subjected to autoradiography. Arrows indicate the position of the 65-, 100-, and 150-kb *Sal* I centromeric DNA fragments. (*D*) FIGE analysis of genomic DNA isolated from *S. cerevisiae* and *S. pombe* transformants containing the *S. pombe cen3* minichromosome. Undigested genomic DNA from Sp223/pSp(cen3)-10C (lane a) and AB1380/pSp(cen3)-10L (lane b) and *Sal* I-digested genomic DNA from Sp223/pSp(cen3)-10C (lane c) were fractionated by FIGE as described except that the beginning pulse time was 0.3 sec forward and 0.1 sec reverse and the ending pulse time was 15 sec forward and 5 sec reverse. The gel was dried, hybridized to ^{32}P-labeled pBR322, and subjected to autoradiography. Arrows indicate the position of the circular (lane a) and linear (lanes b and c) forms of the *cen3* minichromosome.

and the linearity of the plasmid was confirmed. This genomic DNA was then used to transform Sp223, and in all 20 of the transformants analyzed, the plasmid had circularized. Simi-

larly, Sugawara and Szostak (20) have reported that when *S. pombe* is transformed with a linear plasmid containing *Tetrahymena* telomeres, only 1 of 11 transformants contains a linear plasmid. The most likely explanation for the observed high frequency of circularization of the minichromosomes in *S. pombe* is that during propagation in AB1380, *S. cerevisiae* telomeres are added onto the ends, and presumably the *S. pombe* telomerase cannot efficiently use these sequences as primers for telomere addition.

Of the 20 *S. pombe* Sp223/pSp(cen1)-7L transformants analyzed, 1 was found to contain an intact linear copy of the minichromosome. The linear minichromosome was visible when undigested genomic DNAs from the *S. cerevisiae* AB1380 and *S. pombe* Sp223 transformants were fractionated by FIGE and stained with ethidium bromide (Fig. 2*A*). The 78-kb minichromosome appeared as a single band migrating ahead of the main band of chromosomal DNA (Fig. 2*A*). To confirm that the minichromosome in *S. pombe* was the correct linear structure, undigested DNA and *Sal* I-digested genomic DNA from Sp223/pSp(cen1)-7L were fractionated by FIGE and hybridized to labeled pBR322 DNA (Fig. 2*B*). In undigested genomic DNA from both the *S. cerevisiae* transformant (Fig. 2*B*, lane a) and *S. pombe* transformant (Fig. 2*B*, lane b), the probe hybridized to the 78-kb linear minichromosome because of the presence of pBR322 sequences in the vector pMB-1. Furthermore, *Sal* I digestion released the two arms of the vector from the minichromosome in DNA from both the *S. cerevisiae* (Fig. 2*B*, lane c) and *S. pombe* (Fig. 2*B*, lane d) transformants.

The integrity of the 65-kb *Sal* I insert in pSp(cen1)-7L was determined by hybridizing undigested DNA and *Sal* I-digested DNA from Sp223/pSp(cen1)-7L to a K repeat probe (Fig. 2*C*). In undigested DNA from the *S. cerevisiae* transformant (Fig. 2*C*, lane a) as well as the *S. pombe* transformant (Fig. 2*C*, lane b), the K repeat probe hybridized to the 78-kb minichromosome. However, for Sp223/pSp(cen1)-7L, the K repeat also hybridized to the main band of undigested chromosomal DNA because of the presence of the K repeat at the centromeres of all three native *S. pombe* chromosomes (Fig. 2*C*, lane b). In *Sal* I-digested DNA from untransformed *S. pombe*, the K repeat hybridized to the three centromeric fragments of 65, 100, and 150 kb (Fig. 2*C*, lane d). In genomic DNA from the *S. cerevisiae* transformant, *Sal* I digestion released the 65-kb insert from the minichromosome (Fig. 2*C*, lane c). In genomic DNA from Sp223/pSp(cen1)-7L digested with *Sal* I, the K repeat hybridized only to the 65-, 100-, and 150-kb *Sal* I fragments (Fig. 2*C*, lane e); however, the 65-kb band appeared to be more intense than in the untransformed strain (Fig. 2*C*, lane d). This analysis indicates that the Sp223 transformant contains an intact copy of the linear minichromosome pSp(cen1)-7L.

In the case of the *cen3* minichromosome, pSp(cen3)-10L, none of the 20 transformants analyzed contained an intact linear copy of the minichromosome. However, 1 of the transformants was found to contain a circular form of the minichromosome in which the DNA insert appeared to be intact. In the original *S. cerevisiae* transformant, pSp(cen3)-10L appeared as a 163-kb band when undigested DNA was hybridized to pBR322 (Fig. 2*D*, lane b). In undigested DNA from the Sp223 transformant (Fig. 2*D*, lane a), the probe hybridized faintly to a doublet band that migrated very slowly in the field inversion gel. This slow migration in pulsed field gels is typical of circular DNA molecules (21). In *Sal* I-digested DNA from the Sp223 transformant (Fig. 2*D*, lane c), a single fragment of the same size as the linear form of the minichromosome in *S. cerevisiae* (lane b) hybridized to pBR322, and this same fragment also hybridized to the K repeat probe (data not shown). This indicates that the *S. pombe* transformant carries a circular form of pSp(cen3)-10L that contains a single *Sal* I site and is approximately the same size as the original linear plasmid identified in *S. cerevisiae*. This circular minichromosome in Sp223 is designated pSp(cen3)-10C.

Linear and Circular Centromeric Minichromosomes Are Mitotically Stable in *S. pombe*. In *S. cerevisiae*, cloned centromeres confer mitotic stability to autonomously replicating plasmids (6). Mitotic stability assays were performed on the *S. pombe* transformants by plating dilutions of cells on SD plates (8) supplemented with adenine, uracil, and leucine in the presence and absence of 5-fluoroorotic acid. The *ura4* gene present on the minichromosome encodes orotidine-5′-phosphate decarboxylase, which converts 5-fluoroorotic acid nucleotide to the toxic compound 5′-fluorouridylic acid. Thus, cells that have lost the minichromosome are able to grow in the presence of 5-fluoroorotic acid (9). The minichromosomes pSp(cen3)-10C and pSp(cen1)-7L were both found to be extremely stable, exhibiting loss rates per cell division of 4.8×10^{-4} and 5.0×10^{-3}, respectively (Table 1). A 40-kb circular *cen1* minichromosome, pSp(cen1)-3C, exhibited a loss rate of 1.6×10^{-2}. This minichromosome is missing the large arm of the vector, including *S. cerevisiae CEN4*, and about 28 kb of the *S. pombe* insert DNA. In contrast, the loss rate of the vector pMB-1 was 3.2×10^{-1} (Table 1), which indicates that the *S. cerevisiae CEN4* element is not functional in *S. pombe*. In addition, many minichromosomes that had undergone deletions and rearrangements were found to be mitotically unstable, even though *S. cerevisiae CEN4* had been retained. Minichromosome pSp(cen1)-7L was also introduced into the *S. pombe* diploid strain SBPD400 (1), and its loss rate in this strain was determined to be 6.4×10^{-3} (Table 1), indicating that the *cen1* minichromosome is equally stable in both haploid and diploid strains of *S. pombe*.

Meiotic Behavior of the Minichromosomes in *S. pombe*. The minichromosomes were also assayed for proper meiotic segregation in *S. pombe*. The Sp223 transformants were mated to a strain of the opposite mating type containing centromere-linked markers on all three chromosomes; the diploids sporulated, and the resulting asci were dissected and scored for genotype. pSp(cen3)-10C behaved as a functional chromosome, segregating 2+:2− in all tetrads dissected (Table 2). The *cen1* minichromosomes, pSp(cen1)-7L and pSp(cen1)-3C, also exhibited proper centromere function, segregating 2+:2− in 58% and 93% of the tetrads analyzed, respectively (Table 2). This is in marked contrast to the behavior in *S. pombe* of a plasmid containing a *S. cerevisiae CEN3* element, pYe(*CEN3*)41, which was lost completely in 68% of the tetrads (Table 2) and segregated 2+:2− in only 4 of 19 tetrads. The minichromosome pSp(cen1)-7L segregated 4+:0− in 11% of the tetrads, indicating that in some cases the copy number exceeds one per cell. This segregation pattern also has been observed with centromere plasmids in *S. cerevisiae* (6).

Table 1. Mitotic behavior of the *S. pombe* minichromosomes

Host	Minichromosome	Length, kb	Loss frequency (per cell division)
Sp223	pSp(cen3)-10C	163	4.8×10^{-4}
Sp223	pSp(cen1)-7L	78	5.0×10^{-3}
SBPD400	pSp(cen1)-7L	78	6.4×10^{-3}
Sp223	pSp(cen1)-3C	40	1.6×10^{-2}
Sp223	pMB-1 (vector)	13	3.2×10^{-1}

The *S. pombe* strains and genotypes are: Sp223 (*h⁻ leu1.32 ura4-294 ade6.216*) and SBPD400 (*h⁻/h⁻ ade6.210/ade6.216 leu1.32/leu1.32 ura4-294/ura4-294 can^s/can^r*) (1). Loss of the minichromosomes was assayed by plating dilutions of selectively grown cells onto SD plates (8) supplemented with adenine, leucine, and uracil in the presence or absence of 5-fluoroorotic acid (1 mg/ml) as described in the text and in ref. 9.

Table 2. Meiotic segregation of the minichromosomes

Mini-chromosome	Distribution in tetrads of the genetic marker on minichromosome, %				
	4+:0−	3+:1−	2+:2−	1+:3−	0+:4−
pSp(cen3)-10C	0	0	21 (100%)	0	0
pSp(cen1)-7L	6 (11%)	11 (21%)	31 (58%)	4 (8%)	1 (2%)
pSp(cen1)-3C	0	0	13 (93%)	1 (7%)	0
pYe(CEN3)41	0	0	4 (21%)	2 (11%)	13 (68%)

In the first three crosses, *S. pombe* strain Sp223 (see the legend to Table 1) containing the minichromosome to be tested was crossed with strain SBP32588 [*h*$^+$ *leu1.32 ura4-294 tps13-1 lys1-1/ LEU2*(III)]. Strain SBP32588 is a derivative of *S. pombe* strain LEU-L, which contains 8–10 copies of the *S. cerevisiae LEU2* gene tandemly integrated near the centromere of chromosome III (1). This strain is Leu$^+$, and the *LEU2* gene serves as a tightly centromere-linked marker for chromosome III. Segregation of the minichromosomes was assayed by scoring for the *ura4* marker on the vector pMB-1. In the fourth cross, *S. pombe* strain Sp223 containing the *S. cerevisiae CEN3 ARS1 LEU2* plasmid pYe(CEN3)41 (6) was crossed with strain SPJ88 (*h*$^+$ *leu1.32 lys1-1 tps13-1 fur1-1*). In this case, segregation of the minichromosome was assayed by scoring the *LEU2* marker.

If the centromeres are indeed functional on these minichromosomes, the *ura4* marker should segregate as a centromere-linked gene, independent of centromere-linked markers on the three native *S. pombe* chromosomes. The centromere linkage of the *ura4* marker contained on the minichromosomes was analyzed by comparing its segregation to that of the *S. pombe* centromere-linked markers *lys1* (chromosome I), *tps13* (chromosome II), and *LEU2* (chromosome III). In the cross containing pSp(cen3)-10C, only parental ditype and nonparental ditype distributions of the *ura4* marker relative to the centromere-linked markers were observed, indicating that the replicated sister chromatids move to the same pole in the first meiotic division and properly segregate to sister spores in the second meiotic division (Table 3)—behavior typical of a chromosome containing a functional centromere. Approximately equal numbers of parental ditype and nonparental ditype asci were obtained, indicating that pSp(cen3)-10C behaves as an independent linkage group and, therefore, is not integrated at any of the three native centromere regions (Table 3).

Table 3. *S. pombe* minichromosome genes segregate as independent centromere-linked markers

Mini-chromosome	*cen*-linked marker (chromosome)	*ura4:cen*			Crossovers between *cen* marker:*cen*
		PD	NPD	T	
pSp(cen3)-10C	*lys1* (I)	9	8	4	4
	tps13 (II)	9	12	0	0
	LEU2 (III)	9	12	0	0
pSp(cen1)-7L	*lys1* (I)	13	10	7	2
	tps13 (II)	19	7	5	0
	LEU2 (III)	11	14	5	0
pSp(cen1)-3C	*lys1* (I)	2	4	7	0
	tps13 (II)	2	4	7	0
	LEU2 (III)	4	2	7	0
pYe(CEN3)41	*lys1* (I)	1	0	3	0
	tps13 (II)	1	0	3	0

Tetrads dissected from the crosses listed in Table 2 were scored for genetic markers on the minichromosome and known centromere-linked markers on chromosome I (*lys1*), chromosome II (*tps13*), and chromosome III (*LEU2*). Genetic distances between these markers and their corresponding centromeres are as follows in centimorgans (cM): *lys1–cen1*, 3.6 cM; *tps13–cen2*, 0.5 cM; and *LEU2–cen3*, 0 cM (refs. 1 and 2). PD, parental ditype; NPD, nonparental ditype; T, tetratype.

The meiotic analysis indicated that the *ura4* marker on pSp(*cen1*)-7L was centromere-linked and also was segregating independently of the three native *S. pombe* chromosomes. In five of the tetrads (17%), however, the minichromosome *ura4* marker gave a tetratype distribution in relation to the other centromere-linked markers, suggesting that the minichromosome sister chromatids are occasionally separating during the first division, resulting in their segregation to nonsister spores (Table 3). The frequency of precocious sister chromatid separation was even higher for the smaller, circular minichromosome pSp(cen1)-3C, which showed a tetratype distribution in half of the tetrads analyzed. However, this frequency was still much lower than that of the control plasmid pYe(*CEN3*)41, which gave a tetratype distribution relative to the centromere-linked markers in 75% of the tetrads in which the plasmid marker segregated 2+:2− (Table 3). The accurate and proper segregation of pSp(cen1)-7L and pSp(cen3)-10C through both meiotic divisions confirms that the minichromosomes contain the functional centromeres from *S. pombe* chromosomes I and III, respectively.

DISCUSSION

In this study, we report the isolation of functional centromeric DNA from two of the three chromosomes of *S. pombe* and the development of a minichromosome assay system for centromere activity in this organism. The 65-kb and 150-kb *Sal* I restriction fragments that contain the centromeric DNA sequence repeats from *S. pombe* chromosomes I and III were ligated into the yeast artificial chromosome vector pMB-1 and cloned in *S. cerevisiae*. The resulting minichromosomes were then introduced into *S. pombe* to assay them for centromere function.

When transferred via DNA transformation into *S. pombe*, it was observed that the linear minichromosomes often suffered structural rearrangements. In most cases the minichromosomes became circularized, and frequently sequences were deleted as well. Circularization presumably results from the inability of the *S. pombe* telomere terminal transferase to be primed efficiently by *S. cerevisiae* telomeric repeats (20).

When the minichromosomes containing either the intact 65-kb *Sal* I centromeric DNA fragment from chromosome I [pSp(cen1)-7L] or the 150-kb *Sal* I centromeric fragment from chromosome III [pSp(cen3)-10C] were assayed for proper centromere function in *S. pombe*, both were found to be mitotically 100- to 1000-fold more stable than the vector pMB-1 (Table 1). It is unlikely that this stability is due to the presence of the *S. cerevisiae CEN4* sequence on the vector because several large linear minichromosomes that had undergone rearrangement but retained *CEN4* on the large arm of the vector were found to be very unstable mitotically. Also, the smallest of the minichromosomes obtained in this study, a 40-kb circular *cen1* minichromosome, pSp(cen1)-3C, was found to lack the *CEN4* element but was 20-fold more stable mitotically than the vector pMB-1 (Table 1).

A more definitive and accurate assay for centromere function on minichromosomes in *S. pombe* is proper meiotic segregation. Meiotic analysis of pSp(cen3)-10C indicates that it behaves as a functional chromosome, existing in single copy in the parent diploid and segregating 2+:2− in sister spores in all tetrads analyzed (Tables 2 and 3). The minichromosome pSp(cen1)-7L also exhibits proper centromere function, segregating 2+:2− in sister spores in the majority of tetrads analyzed. Plasmids, such as pYe(CEN3)41, which contain a *S. cerevisiae* centromere, segregate predominantly 0+:4− in *S. pombe*, and in those tetrads where the plasmid segregates 2+:2−, most contain the plasmid in nonsister spores (Tables 2 and 3). Similarly, a plasmid containing the *S. cerevisiae CEN11* element and a *S. pombe ars* element was

found to segregate 0+:4− in 18 of 25 tetrads analyzed (data not shown). In a small number of tetrads, pSp(cen1)-7L segregated 4+:0− or 3+:1− (Table 2), suggesting that in some cases the copy number of the minichromosome in the parent diploid is greater than one.

Minichromosome pSp(cen1)-3C, which is only 20-fold more stable than pMB-1 mitotically (Table 1), was found to be very stable meiotically, segregating 2+:2− in 93% of the tetrads analyzed (Table 2). However, in half of these tetrads, pSp(cen1)-3C segregated to nonsister spores, indicating that the minichromosomes underwent precocious sister chromatid separation in the first meiotic division (Table 3). It is possible that this 40-kb circular minichromosome has suffered a deletion of centromeric DNA sequences that are necessary for optimal mitotic stability and for maintaining sister chromatid attachment during the first meiotic division but that are not required for overall meiotic stability and 2+:2− segregation. In *S. cerevisiae*, centromere mutations have been identified that cause random segregation in the first meiotic division but do not impair mitotic stability (22) or that decrease mitotic stability without affecting meiotic segregation (23). Alternatively, it is possible that pSp(cen1)-3C contains all of the sequences necessary for proper centromere function, but its relatively small size is responsible for its slightly aberrant mitotic and meiotic behavior (24, 25).

In this study, we have confirmed that *S. cerevisiae* centromere sequences do not function as centromeres in *S. pombe*. The data also indicate that *S. pombe* centromeres are not functional in *S. cerevisiae*. The minichromosomes pSp(cen1)-7L and pSp(cen3)-10L contain a *S. cerevisiae CEN4* element as well as a *S. pombe* centromere, yet when these minichromosomes are propagated in *S. cerevisiae*, no dicentric behavior is observed. Plasmids containing two centromeres are very unstable in *S. cerevisiae* and rearrange to delete one or both of the centromeres (26). However, no rearrangements of pSp(cen1)-7L or pSp(cen3)-10L were detected when propagated in *S. cerevisiae* strain AB1380 for many generations.

Niwa *et al.* (27) have used γ-irradiation of an unstable chromosome III disomic strain of *S. pombe* to produce by deletion a stable partial aneuploid that contains, in addition to the three normal chromosomes, a 500-kb linear minichromosome (Ch^{16}) that was derived from one copy of chromosome III and contained the pericentric region. The frequency of loss of the 500-kb linear Ch^{16} in mitosis is 10^{-4}, which is roughly equivalent to that of the artificial, circular 163-kb pSp(cen3)-10C described in these studies. The mitotic stability of both of these minichromosomes is 10- to 20-fold higher than that of the 78-kb linear pSp(cen1)-7L. All three minichromosomes segregate with high fidelity through meiosis. Thus, the unstable nature of aneuploidy in *S. pombe* is most likely due to gene dosage effects of an additional chromosome, rather than the presence of an additional centromere.

Now that DNA segments containing functional *S. pombe* centromeres have been identified and a minichromosome assay is available, it should be possible to determine the role of the B, K, and L DNA sequence repeats in centromere function. The availability of a cloned intact segment of each *S. pombe* centromere in a heterologous *S. cerevisiae* background should greatly expedite structural studies of these regions. The size of the functional centromeric DNA in *S. pombe* has not yet been determined, although the region of centromere-specific repeated sequences probably encompasses at least 30–50 kb on each chromosome (1, 3). There is no evidence as yet for a role of the repeated sequences in centromere function, and, indeed, they may serve only in an ancillary capacity to maintain, for example, a specific chromatin conformation, while the actual spindle attachment site may be quite small. The development of the minichromosome assay system described here should eventually lead to a determination of the minimum arrangement of sequences necessary for centromere function in *S. pombe*.

Finally, the techniques used in this study provide a general method for the isolation of centromeres from organisms for which a DNA transformation system and cloned centromere-linked genes are available. Existing yeast artificial chromosome vectors can be modified by insertion of an appropriate selectable marker from the organism of interest and then used to construct a library in *S. cerevisiae* containing very large fragments of the foreign genomic DNA. The minichromosomes containing centromere regions can be identified by yeast colony hybridization with a DNA fragment containing a known centromere-linked marker as the hybridization probe. If the cloned fragments are large enough, some should contain an intact functional centromere. The minichromosomes can then be introduced into the organism of interest and assayed for centromere function either as autonomously replicating plasmids or by observing dicentric behavior upon their integration into the host genome.

We thank Joy Wilkinson for constructing *S. pombe* strain SBP32588 and David Burke and Maynard Olson for helpful discussions and for providing plasmid pYAC4 and *S. cerevisiae* strain AB1380. K.M.H. was supported by a Damon Runyon–Walter Winchell Cancer Research Fund postdoctoral fellowship, DRG-949. This research was supported by National Institutes of Health Grant GM-33783.

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