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# Identification of DNA Regions Required for Mitotic and Meiotic Functions within the Centromere of *Schizosaccharomyces pombe* Chromosome I

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**We have determined the structural organization and functional roles of centromere-specific DNA sequence repeats in *cen1*, the centromere region from chromosome I of the fission yeast *Schizosaccharomyces pombe*. *cen1* is composed of various classes of repeated sequences designated K′, K″ (dgl), L, and B′, arranged in a 34-kb inverted repeat surrounding a 4- to 5-kb nonhomologous central core. Artificial chromosomes containing various portions of the *cen1* region were constructed and assayed for mitotic and meiotic centromere function in *S. pombe*. Deleting K′ and L from the distal portion of one arm of the inverted repeat had no effect on mitotic centromere function but resulted in greatly increased precocious sister chromatid separation in the first meiotic division. A centromere completely lacking K′ and L, but containing the central core, one copy of B′ and K″ in one arm, and approximately 2.5 kb of the core-proximal portion of B′ in the other arm, was also fully functional mitotically but again did not maintain sister chromatid attachment in meiosis I. However, deletion of K″ from this minichromosome resulted in complete loss of centromere function. Thus, one copy of at least a portion of the K″ (dgl) repeat is absolutely required but is not sufficient for *S. pombe* centromere function. The long centromeric inverted-repeat region must be relatively intact to maintain sister chromatid attachment in meiosis I.**

The centromere ensures the proper segregation of eukaryotic chromosomes by supplying at least two distinct functions. This highly specialized DNA region provides the spindle attachment point in both mitosis and meiosis; in addition, it maintains attachment of sister chromatids in the first meiotic division, resulting in their migration together to the same pole.

Functional centromeres from the budding yeast *Saccharomyces cerevisiae* have been isolated and extensively characterized (reviewed in references 4, 8, 12, and 16). The functional centromeric DNA (*CEN*) from this organism is approximately 125 bp in length (13) and lacks the large blocks of transcriptionally silent pericentric heterochromatin that are characteristic of the centromere regions of higher eukaryotes (30, 42). Comparison of the nucleotide sequences of 11 *S. cerevisiae* centromeres has revealed the presence of three conserved sequence elements: CDEI, CDEII, and CDEIII (17, 23). Mutations in the CDEI element of a centromere residing on an artificial chromosome in *S. cerevisiae* can result in increased precocious sister chromatid separation in the first meiotic division but have relatively minor effects on segregation in meiosis II or mitosis (14, 18, 36). In contrast, certain single-base changes in the CDEIII sequence lead to total inactivation of the centromere (21, 29, 33). These results indicate that two functions of the centromere, spindle attachment and maintenance of sister chromatid attachment in meiosis I, can be differentially affected by mutations in different DNA sequence elements within the centromere.

The centromere regions of the fission yeast *Schizosaccharomyces pombe* are more characteristic of those of higher eukaryotes in containing several classes of repetitive DNA sequences that span many kilobases (9, 15, 32). These repeated sequences are present only at the three centromere regions, and no polyadenylated transcripts from these regions have been detected (15, 37). The three *S. pombe* centromeres (*cen1*, *cen2*, and *cen3*) have been cloned on large genomic *Sal*I restriction fragments in yeast artificial chromosome (YAC) vectors, and a minichromosome assay system has been developed to determine the minimum arrangement of DNA sequences necessary for centromere function in this organism (10, 20).

Recently, a plasmid integration-excision strategy has been used to facilitate chromosome walking through regions containing repetitive DNA (10). This method was used to clone and map over 80 kb of DNA from the centromere region of *S. pombe* chromosome II (*cen2*). The *cen2* region contains four tandem units of the centromere-specific K, L, and B repeats, two of which are arranged in a 31-kb inverted repeat flanking a 7-kb central core sequence that is unique to *cen2* (10). It was found that the 1.5-kb core-proximal portion of the inverted repeat is also unique to *cen2* and has been designated the *cen2* core-associated repeat. A long-range genomic mapping technique has recently demonstrated the presence of an inverted repeat in the *cen1* and *cen3* regions as well (6).

In this study, the plasmid integration-excision strategy has been used to analyze the entire 65-kb *Sal*I fragment previously shown to contain the functional centromere from chromosome I of *S. pombe* (20). The *cen1* region contains a 34-kb inverted repeat flanking a 4- to 5-kb central core. As previously observed for *cen2* (10) and *cen3* (34), deletion of a portion of the inverted repeat in *cen1* results in increased precocious sister chromatid separation in the first meiotic division but has no effect on mitotic stability or the second meiotic division. All mitotic and meiotic centromere function is abolished, however, when most of the K″ (dgl) repeat sequences are deleted from *cen1*. These studies suggest that at least a portion of the K″ repeat is essential for centromere

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function and that these sequences are likely to play a role in spindle attachment, either directly in kinetochore protein binding or by providing an essential chromatin conformation.

## MATERIALS AND METHODS

**Strains, transformations, and genetic manipulations.** The *S. pombe* strains used in this study and their genotypes are Sp223 (*h⁻ leu1.32 ura4.294 ade6.216*; gift from D. Beach), SBP32588 (*h⁺ leu1.32 ura4.294 tps13.1 lys1.1/LEU2⁺* [III]; 20), and SBP41786-2 (*h⁺ leu1.32 ura4.294 tps13.1 lys1.1 fur1.1*; this laboratory). *S. cerevisiae* AB1380 (**a** *ura3 trp1 ade2-1 can1-100 lys2-1 his5*) was a gift from D. Burke (3), and strain GTSC14 (**a** *ura3 his4 trp1 leu2*) was provided by G. Tschumper. Growth media and conditions for *S. pombe* (19) and *S. cerevisiae* (41) were as previously described. DNA transformations were performed as previously described (24).

Plasmids were integrated by site-directed homologous recombination (35) into either *S. pombe* Sp223 or *S. cerevisiae* GTSC14 containing the 78-kb linear *cen1* minichromosome pSp(*cen1*)-7L (20). Minichromosome pSp(*cen1*)-3C was made larger by site-directed integration of a pBR322-derived vector containing the *S. cerevisiae URA3* and *LEU2* genes into the *URA3* sequences present on the minichromosome. The size of minichromosome pSp(*cen1*)-BHp was increased by site-directed integration into pBR322 sequences of plasmid pMB-neo (10), which contains the *S. cerevisiae LEU2* gene. The *S. cerevisiae LEU2* gene complements the *S. pombe leu1.32* mutation, but as a result of the poor expression of the *LEU2* gene in *S. pombe*, most transformants selected contain multiple copies of the plasmids integrated in tandem (10). Minichromosome pSp(*cen1*)-3XB was constructed by replacing the deleted portion of the left arm of the inverted repeat in pSp(*cen1*)-3C by fragment-mediated transplacement (38). A pBR322-derived vector was constructed that contains the K′ and L repeats on a 7-kb *Xho*I-*Bam*HI fragment (see Fig. 2A) and the *S. cerevisiae URA3* gene interrupted by the *S. pombe leu1* gene. This plasmid was digested with *Pst*I and *Bam*HI, liberating a 10-kb fragment containing the K′ and L repeats and the disrupted *URA3* gene. This DNA fragment was gel purified and used to transform strain Sp223/pSp(*cen1*)-3C, selecting for leucine prototrophy and subsequently screening colonies for uracil auxotrophy.

The mitotic stability of minichromosomes was assayed by plating dilutions of selectively grown cells of the *S. pombe* transformants on minimal plates supplemented with adenine, leucine, and uracil in the presence and absence of 5-fluoroorotic acid (1 mg/ml) as previously described (2, 20). The frequency of Ura⁻ segregants obtained in this assay is approximately equal to the frequency of minichromosome loss per cell division (10). Meiotic analyses were performed as described previously (20). Segregation of the minichromosome was assayed by scoring for the *S. pombe ura4* gene or the *S. cerevisiae URA3* gene (which complements the *S. pombe ura4.294* mutation) present on the plasmid, except for minichromosome pSp(*cen1*)-3XB, which contains the *S. pombe leu1* gene as its genetic marker. The sister:nonsister distribution of the minichromosomes was determined by scoring the tetrads for known centromere-linked markers on each of the three *S. pombe* chromosomes. The *lys1*, *tps13*, and *fur1* genes are tightly linked to the centromeres of chromosomes I, II, and III, respectively (26). Strain SBP32588 contains 8 to 10 copies of the *S. cerevisiae LEU2* gene tandemly integrated near the centromere of chromosome III (15, 20); hence, in crosses with this strain, the *LEU2* gene was scored as the centromere-linked marker for chromosome III. In each cross, approximately equal numbers of parental ditype and nonparental ditype asci were obtained, indicating that the minichromosomes behaved as independent linkage groups and were not integrated at any of the three native centromere regions. Tetrads in which the minichromosome marker distributed 2+:2− and showed a tetratype distribution relative to known centromere-linked markers indicated that the minichromosome underwent precocious sister chromatid separation in the first meiotic division. We assume that tetrads in which the minichromosome marker distributed 4+:0− or 3+:1− resulted from diploids that contained more than one copy of the minichromosome.

**Enzymes, DNA isolation, plasmid rescue, and field inversion gel electrophoresis (FIGE).** Restriction enzymes were from New England BioLabs or Boehringer Mannheim Biochemicals. T4 DNA ligase, calf intestinal alkaline phosphatase, T4 DNA polymerase, and Klenow fragment were from Boehringer Mannheim. Genomic DNA from *S. cerevisiae* (44) and *S. pombe* (1) was isolated as described, using cutoff pipette tips to prevent shearing. Plasmids were excised from genomic DNA prepared from *S. pombe* integrant strains by the method of Clarke and Baum (10). Genomic DNA was digested to completion with the desired restriction enzyme to liberate a fragment containing the pBR322-derived integrated plasmid along with surrounding genomic sequences. For plasmid recovery, the digested DNA was then diluted, ligated, and used to transform *Escherichia coli* DH5α (*recA1*; Bethesda Research Laboratories) to ampicillin resistance (10).

FIGE was carried out as described previously (5) in 1% agarose gels at 180 V for 17 h with a linear ramp consisting of a beginning pulse time of 0.5 s forward and 0.2 s reverse and an ending pulse time of 5 s forward and 2 s reverse. High-molecular-weight DNA markers from Bethesda Research Laboratories were used as size standards.

**Indirect end-label mapping of large minichromosomes.** To map the breakpoint in the 40-kb circular deletion minichromosome pSp(*cen1*)-3C (20), genomic DNA from the *S. pombe* transformant was digested to completion with *Sal*I, which linearizes the minichromosome, leaving the pBR322 and *URA3* sequences at one end. The *Sal*I-digested DNA was then partially digested with *Bam*HI, *Nhe*I, or *Xba*I, and the resulting fragments were separated by FIGE. The gels were dried, hybridized to $^{32}$P-labeled pBR322 containing the *URA3* gene, and subjected to autoradiography. For each partial digest, the number of fragments hybridizing to the *URA3* probe indicated the number of restriction sites for that enzyme present in the minichromosome, and the sizes of these fragments allowed the placement of these sites relative to the terminal *Sal*I site. Nick translations were performed by using a kit from Bethesda Research Laboratories.

## RESULTS

**The centromere-specific repeats in the *cen1* region are organized into a 34-kb inverted repeat flanking a 4- to 5-kb central core.** Recently, a plasmid integration-excision strategy has been developed to chromosome walk through regions that contain repetitive DNA sequences (10). In this method, a pBR322-derived plasmid containing an *S. pombe* selectable marker is integrated by site-directed homologous recombination into the genome in the region of interest.

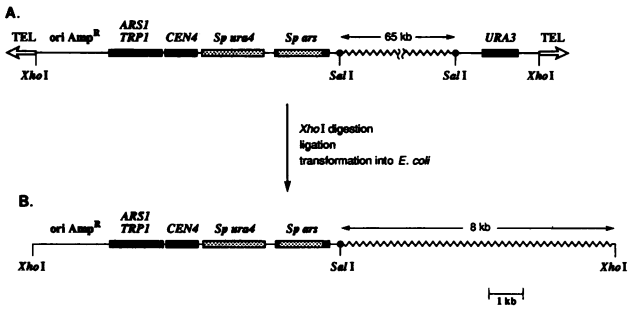


FIG. 1. Schematic showing the strategy used to clone a unique end fragment from the 65-kb *cen1*-containing *Sal*I fragment. (A) Structure of the 78-kb linear minichromosome pSp(*cen1*)-7L, which contains the *cen1 Sal*I fragment cloned in the YAC vector pMB-1 (20). Total genomic DNA from *S. cerevisiae* AB1380/pSp(*cen1*)-7L was digested with *Xho*I, diluted, ligated, and used to transform *E. coli* DH5α. The resulting plasmid (B) contains all of the large arm of pMB-1 except the telomere sequence and also contains an 8-kb *Sal*I-*Xho*I fragment from the left end of the genomic *cen1 Sal*I fragment.

Genomic DNA from the integrant strain is then digested with various restriction enzymes to release the plasmid along with surrounding genomic sequences, and the resulting plasmid is circularized by ligation in vitro and finally rescued in *E. coli*. This strategy has been used to determine the structural organization of the 100-kb genomic *Sal*I restriction fragment containing the *S. pombe cen2* region (10). In the present study, this method has been used to determine the number of copies and arrangement of the centromere-specific DNA sequence repeats in the *cen1* region.

We previously described the cloning in *S. cerevisiae* of a 65-kb *Sal*I genomic restriction fragment containing the functional centromere from chromosome I of *S. pombe* with use of a YAC vector (20). An analysis of the structural organization of the *cen1* region was initiated by cloning in *E. coli* a unique fragment from one end of this 65-kb genomic *Sal*I fragment. A schematic of the 78-kb linear minichromosome pSp(*cen1*)-7L containing the *cen1 Sal*I fragment in the YAC vector pMB-1 (20) is shown in Fig. 1A. Digestion of the artificial chromosome with *Xho*I releases a fragment containing all of the large arm of the vector except for the telomere as well as insert DNA to the first *Xho*I site (Fig. 1; 3). Thus, from a *Xho*I digest of genomic DNA of *S. cerevisiae*

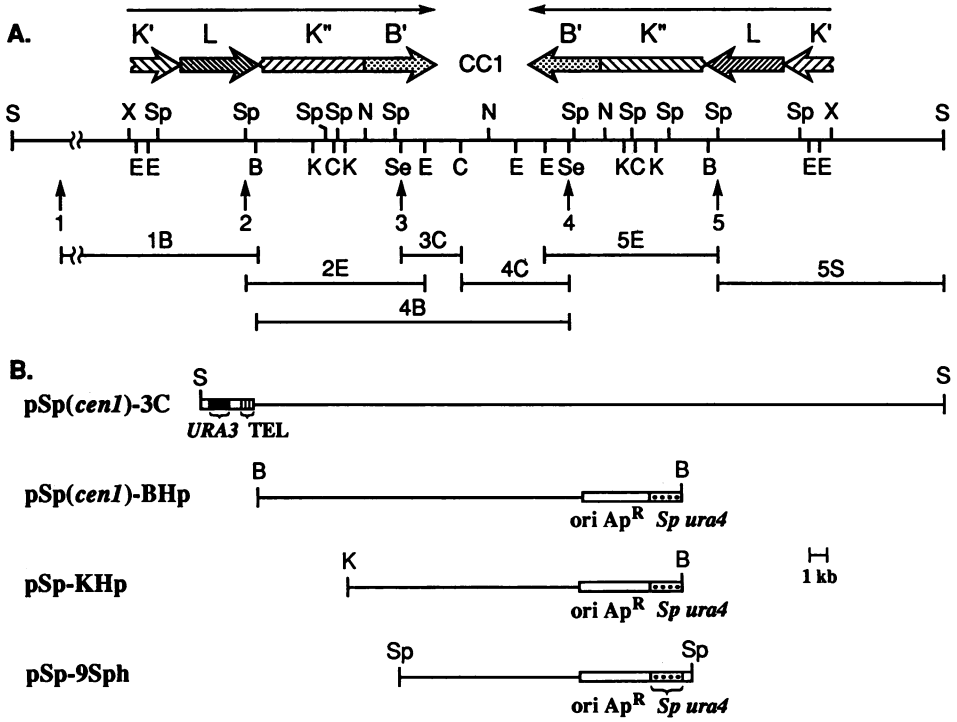


FIG. 2. Structural organization of the *S. pombe cen1* region. (A) Restriction map of the 65-kb *cen1 Sal*I fragment. Above the map is shown the location of the centromere-specific repeats B′, K′, K″, and L. In *cen1*, the 6.4-kb K repeat (9, 15) is divided into two fragments designated K′ and K″, with the orientation of K″ reversed relative to that of K′. The K′-L region and K″ have also been designated dhl and dgl, respectively (6). The B′ element from *cen1* and the B element from *cen2* contain the same $tRNA^{Ala}$ and $tRNA^{Ile}$ genes (27). The repeated DNA sequence elements are organized in a 34-kb inverted repeat that flanks a 4- to 5-kb central core (CC1). Below the restriction map are shown the sites of plasmid integration (1 to 5) and the DNA segments isolated by plasmid excision and rescue in *E. coli* (1B to 5S). (B) Structures of *cen1* minichromosomes. The 40-kb minichromosome pSp(*cen1*)-3C is a deletion derivative of the 78-kb linear minichromosome pSp(*cen1*)-7L (20) and contains the small arm of the YAC vector pMB-1 and *cen1* insert sequences from the rightward *Sal*I site to approximately 200 bp to the left of the leftmost *Bam*HI site. The deletion breakpoint in pSp(*cen1*)-3C was determined by indirect end-label mapping as described in Materials and Methods. The minichromosome pSp(*cen1*)-BHp consists of pBR322, the *S. pombe ura4* gene, and a 17-kb *Bam*HI-*Hpa*I fragment containing one copy of K″, 2.5 kb of inverted repeat, and the central core. Minichromosome pSp-KHp was derived from pSp(*cen1*)-BHp by deletion of the 5-kb *Bam*HI-*Kpn*I fragment. pSp-9Sph was constructed by subcloning the 9-kb *Sph*I-*Sph*I fragment containing the central core and the core-proximal portions of both copies of the B′ repeat into pBR322 containing the *S. pombe ura4* gene. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; S, *Sal*I; Se, *Spe*I; Sp, *Sph*I; X, *Xho*I. Not all *Spe*I and *Xho*I sites are shown.

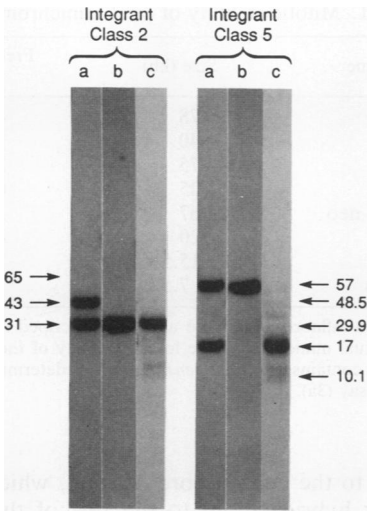


FIG. 3. FIGE analysis of integrant classes 2 and 5. For each integrant class, lanes a, b, and c contain *Sal*I-digested genomic DNA electrophoresed as described in Materials and Methods. The gel was dried, hybridized to $^{32}$P-labeled probes, and subjected to autoradiography. Lanes a were hybridized to a 7-kb *Xho*I-*Bam*HI fragment that includes the insert from the plasmid integrated into classes 2 and 5. Lanes b were hybridized to the unique *Xho*I-*Sal*I fragment from the left end of the 65-kb *cen1 Sal*I fragment. Lanes c were hybridized to the *S. cerevisiae LEU2* gene. Arrows point to the 43- and 31-kb (class 2) and 57- and 17-kb (class 5) *Sal*I fragments spanning the *cen1* region containing the integrated plasmid and to the positions of DNA size markers (in kilobases).

AB1380/pSp(*cen1*)-7L, a plasmid was recovered in *E. coli* that contained an 8-kb *Sal*I-*Xho*I fragment of *S. pombe* DNA from the left end of the 65-kb *Sal*I fragment (Fig. 1B). Use of this insert to probe *Sal*I-restricted *S. pombe* genomic DNA demonstrated that this fragment is unique to the *cen1* region (not shown). A 2.5-kb *Hpa*I-*Xho*I fragment contained within the 8-kb *Sal*I-*Xho*I fragment was subcloned into pBR322 carrying the *S. pombe ura4* gene. This plasmid was restricted at the unique *Sph*I site within the insert and was introduced by site-directed integration (35) into the centromere region of chromosome I of haploid strain Sp223 at integration site 1 shown in Fig. 2A. Genomic DNA from the integrant was restricted with *Bam*HI, diluted, ligated, and used to transform *E. coli*. A 25-kb plasmid was recovered that contained vector sequences as well as 17 kb of new genomic sequences extending rightward to the first *Bam*HI site in the 65-kb *Sal*I fragment (1B; Fig. 2A). Restriction mapping and Southern hybridization analysis of fragment 1B indicated that the terminal 7-kb *Xho*I-*Bam*HI fragment was not unique to *cen1* but contained sequences homologous to the *cen2* K and L repeats. Further restriction analysis of this fragment revealed that it contains a complete L repeat and a 2.2-kb portion (K′; Fig. 2A) of the 6.4-kb K repeat (10, 15). This combination of K′ and L repeats has been designated dhl by Chikashige et al. (6).

To continue walking along the 65-kb *Sal*I fragment, the terminal 1.1-kb *Hin*dIII-*Bam*HI fragment from the walk described above was used to introduce a plasmid by site-directed integration into the *cen1* region. However, because this fragment is part of the L repeat, integration could not be directed exclusively into chromosome I of *S. pombe* because of the copies of the L repeat present in the *cen2* and *cen3* regions. Instead, the plasmid was integrated into the 78-kb linear *cen1* minichromosome pSp(*cen1*)-7L residing in *S. cerevisiae* GTSC14 (see Materials and Methods). The 1.1-kb *Hin*dIII-*Bam*HI fragment was subcloned into pBR322 containing the *S. cerevisiae LEU2* gene. The plasmid was linearized at the unique *Sph*I site within the L-repeat sequences and used to transform GTSC14/pSp(*cen1*)-7L, selecting for the *URA3* and *TRP1* markers on the minichromosome and the *LEU2* gene on the integrating plasmid. In this experiment, it was expected that more than one integrant class would be obtained, depending on how many copies of the L repeat are present in the *cen1* region (10). FIGE analysis of genomic DNA from the transformant strains demonstrated that two distinct classes of integrants had been obtained, represented by integration sites 2 and 5 (Fig. 2A and 3). Because of the presence of a single *Sal*I site in the integrated plasmid, digestion of genomic DNA from the integrant strains with *Sal*I cleaves the *cen1* region into two fragments, which were identified by hybridization to the 7-kb *Xho*I-*Bam*HI fragment (Fig. 2A) that spans the integration sites (Fig. 3, lanes a). The sizes of these fragments were then used to determine the exact positions of the integration sites. Hybridization of *Sal*I-digested genomic DNA from integrant classes 2 and 5 with the unique 8-kb *Xho*I-*Sal*I fragment from the left end of the 65-kb *cen1 Sal*I fragment (Fig. 3, lanes b) identified fragments of 31 and 57 kb, respectively, and indicates the distance of the integration site for each class from the leftward *Sal*I site. *Sal*I-digested genomic DNA from both integrant classes was also hybridized to *LEU2* (Fig. 3, lanes c). For integrant class 2, the *LEU2* probe hybridized to the same *Sal*I fragment as the *cen1* unique probe (lanes b and c), indicating that these two sequences are on the same side of the *Sal*I site in the integrated plasmid. However, for integrant class 5, the *LEU2* probe and the *cen1* unique probe hybridized to different *Sal*I fragments (lanes b and c), indicating that they are on opposite sides of the *Sal*I site in the integrated plasmid. Thus, in the two integrant classes the plasmid had integrated in opposite orientations, and the two copies of the L repeat present in *cen1* are organized into an inverted repeat.

Excision from genomic DNA of integrant classes 2 and 5 yielded two plasmids, both containing within their respective inserts a 9-kb *Bam*HI-*Eco*RI fragment (2E and 5E; Fig. 2A). The restriction maps of both *Bam*HI-*Eco*RI fragments were identical except for the location of one of the three *Sph*I sites (Fig. 2A), indicating that the entire *Bam*HI-*Eco*RI fragment is part of the inverted repeat. Restriction mapping and Southern hybridization analysis further revealed that the cloned fragments contained both B and K hybridizing sequences. The 6-kb portion of the fragments extending from the *Bam*HI site to the *Nco*I site contained the remaining portion of the K repeat and has been designated K″ in Fig. 2A. K′ and K″ together comprise a complete K repeat as found in *cen2* (10, 15; Fig. 4), however, the orientation of K″ is reversed relative to that of K′. K″ has been designated dgl by Chikashige et al. (6), and its reversed orientation relative to that of dhl (K′+L) has been previously noted. The 1.5-kb central core-proximal *Nco*I-*Sph*I fragment from both 2E and 5E contains sequences homologous to the B repeat from *cen2* and has been designated a part of the *cen1* B′ repeat (Fig. 2A and 4). Sequencing analysis indicates that this homology is limited to a single copy of a tRNA$^{Ala}$ gene present in each repeat (27). Southern hybridization analysis demonstrated that the terminal 1.7-kb *Sph*I-*Eco*RI portion of 2E and 5E, also designated part of the B′ repeat in Fig. 2A, is unique to *cen1*, indicating that, as in *cen2*, the *cen1* inverted repeat contains sequences flanking the central core

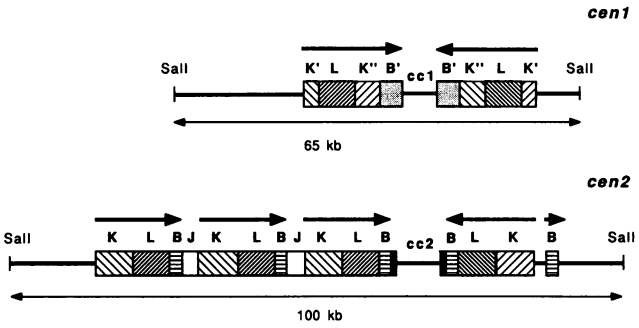


FIG. 4. Schematic comparison of the overall structural organizations of the centromeres from chromosomes I and II of *S. pombe*. Shown are the 65-kb *SalI* fragment containing *cen1* and the 100-kb *SalI* fragment containing *cen2*. Both centromeres contain two blocks of the centromere-specific DNA sequence repeats organized in an inverted repeat flanking a central core (cc). The *cen2* region also contains two additional tandem blocks of the B, K, and L repeats as well as an isolated copy of the B repeat and two copies of the J repeat (10). The innermost portion of the inverted repeat in both centromeres consists of mostly unique core-associated repeats of approximately 4 kb in *cen1* (stippled boxes) and 1.4 kb in *cen2* (black boxes). Although the entire K repeat is present in *cen1*, it is fragmented into two pieces designated K′ and K″. The K″ portion (dgl), which is core distal in *cen2*, is located between the B′ and L repeats in *cen1*, and its orientation is reversed relative to that in *cen2*.

that are not found at the other two centromere regions (Fig. 2A and 4).

An excision plasmid was also obtained from integration site 5 by using restriction endonuclease *SalI*. This plasmid contained a 12.8-kb insert extending to the rightward end of the 65-kb *SalI* fragment (5S; Fig. 2A). This insert was found to contain the same 7-kb *XhoI-BamHI* fragment described above containing the L and K′ repeats (Fig. 2A). The remainder of the insert DNA is unique in sequence and is presumably not part of the functional *cen1*.

A final site-directed integration was performed with a pBR322-derived plasmid carrying the *S. cerevisiae LEU2* gene and a 1.8-kb *HpaI-EcoRI* fragment derived from the core-associated B′ repeat. This plasmid was linearized at a unique *SpeI* site within the repeated sequence and was used to transform strain GTSC14/pSp(*cen1*)-7L. As expected, two classes of integrants were obtained (designated 3 and 4 in Fig. 2A) because of the presence of two copies of the B′ repeat in *cen1*. The remainder of the B′ repeats and the central core of *cen1* were cloned by excising plasmids from integrant classes 3 and 4 with *ClaI*. The inserts obtained from the two integrant classes (3C and 4C; Fig. 2A) were of different sizes, and further mapping indicated that the boundaries between the B′ repeats and the *cen1* central core are located within the 1.6-kb *EcoRI-ClaI* fragment (contained in 3C) at the left and the 1.5-kb *EcoRI* fragment (contained in 4C) at the right of the core (Fig. 2A). These fragments encode identical $tRNA^{Ile}$ genes located very close to the B′ repeat/central core boundary (27). Additional copies of the same tRNA gene are also found in the *cen2* B repeats (27). Another excision plasmid was obtained from integrant class 4 by using *BamHI*. This plasmid contains a 17-kb insert (4B; Fig. 2A) spanning the central core, 2.5 kb of the B′ repeat on the right of the core, and one complete copy of K″ and B′ on the left. Functional analysis of fragment 4B will be discussed below.

TABLE 1. Mitotic stability of *cen1* minichromosomes

| Minichromosome | Size (kb) | Frequency of Ura⁻ segregants[a] |
|---|---|---|
| pSp(*cen 1*)-7L | 78 | $4.4 \times 10^{-4}$ |
| pSp(*cen1*)-3C | 40 | $2.6 \times 10^{-3}$ |
| pSp(*cen1*)-75C | 75 | $2.8 \times 10^{-4}$ |
| pSp(*cen1*)-BHp | 25 | $6.3 \times 10^{-3}$ |
| pSp(*cen1*)-BHp-neo | 67 | $1.9 \times 10^{-4}$ |
| pSp-KHp | 20 | $4.0 \times 10^{-2}$ |
| pSp-9Sph | 15.5 | $2.6 \times 10^{-2}$ |
| pSp300 (vector) | 7.5 | $1.1 \times 10^{-2}$ |

[a] Measured by a 5-fluoro-orotic acid assay as described in Materials and Methods. The actual minichromosome loss frequency of the linear plasmid pSp(*cen1*)-7L that contains the entire *cen1* region was determined by a colony color sectoring assay (3a).

In contrast to the central core of *cen2*, which shows no homology (by hybridization) to the rest of the *S. pombe* genome, portions of the central core from *cen1* are not unique. The 3-kb *ClaI-EcoRI* fragment from the middle of the *cen1* central core (Fig. 2A) shows significant homology, by Southern hybridization, to the centromere region of chromosome III but not to *cen2* (not shown).

**Partial deletion of inverted repeat sequences in *cen1* impairs meiotic centromere function.** We previously described the isolation of a 40-kb *cen1* minichromosome, pSp(*cen1*)-3C, that exhibits partial centromere function (20). This circular minichromosome is a deletion derivative of the 78-kb linear minichromosome, pSp(*cen1*)-7L (Tables 1 and 2; 20), formed in vivo upon shuttling of the linear minichromosome from *S. cerevisiae* to *S. pombe*. pSp(*cen1*)-3C retains the small arm of the YAC vector pMB-1 containing the *S. cerevisiae URA3* gene, but it carries a deletion of the entire large arm of the vector as well as part of the 65-kb *SalI* insert. Circularization and deletion of linear minichromosomes upon transfer from *S. cerevisiae* to *S. pombe* is frequently observed and is presumably due to telomere incompatibility (10, 20, 43). Determination of the structural organization and restriction map of the entire *cen1* region as described above permitted precise mapping of the deletion breakpoint in the 40-kb minichromosome by an indirect end-label mapping technique (7). Genomic DNA from strain Sp223/pSp(*cen1*)-3C was digested to completion with *SalI*. This linearizes the

TABLE 2. Meiotic segregation of *cen1* minichromosomes[a]

| Minichromosome | Distribution of genetic marker on minichromosome | | | | | Sister:nonsister distribution (PD+NPD):T |
|---|---|---|---|---|---|---|
| | 4+:0− | 3+:1− | 2+:2− | 1+:3− | 0+:4− | |
| pSp(*cen1*)-7L | 6 (11) | 11 (20) | 31 (58) | 4 (8) | 1 (2) | 25:5 |
| pSp(*cen1*)-3C | 0 | 0 | 67 (83) | 8 (10) | 6 (7) | 28:39 |
| pSp(*cen1*)-75C | 0 | 1 (2) | 32 (78) | 4 (10) | 4 (10) | 17:14 |
| pSp(*cen1*)-3XB | 0 | 0 | 54 (95) | 2 (3) | 1 (2) | 41:13 |
| pSp(*cen1*)-BHp | 1 (4) | 5 (19) | 13 (50) | 2 (8) | 5 (19) | 2:11 |
| pSp(*cen1*)-BHp-neo | 5 (17) | 3 (10) | 13 (45) | 5 (17) | 3 (10) | 2:11 |
| pSp-KHp | 0 | 1 (3) | 0 | 2 (6) | 31 (91) | |
| pSp-9Sph | 0 | 0 | 4 (12) | 3 (9) | 27 (79) | 1:3 |

[a] Tetrads were dissected from crosses of *S. pombe* Sp223 containing the indicated minichromosomes to strain SBP32588 except for Sp223 containing the minichromosome pSp(*cen1*)-3XB, which was crossed to strain SBP41786-2. Genotypes of *S. pombe* strains are given in Materials and Methods. PD, Parental ditype; NPD, nonparental ditype; T, tetratype. Values in parentheses are percentages.

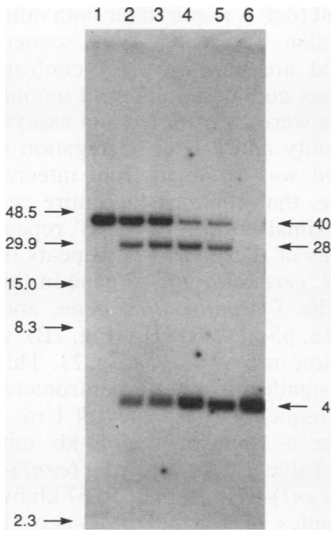


FIG. 5. Indirect end-label mapping of pSp(*cen1*)-3C. The deletion breakpoint in the 40-kb minichromosome was determined as described in Materials and Methods. Genomic DNA from strain Sp223/pSp(*cen1*)-3C was digested to completion with *Sal*I, 20 U of *Bam*HI was added, and aliquots of the digestion mixture were removed at 10 s (lane 2), 1 min (lane 3), 5 min (lane 4), and 10 min (lane 5). A separate sample of genomic DNA was digested to completion with both *Sal*I and *Bam*HI (lane 6). The digestion products were separated by FIGE, and the gel was dried and hybridized to $^{32}$P-labeled pBR322-*URA3*. Arrows indicate the complete *Sal*I digestion product (40 kb), the complete *Sal*I-partial *Bam*HI digestion product (28 kb), and the complete *Sal*I-*Bam*HI double-digestion product (4 kb), as well as the positions of DNA size markers (in kilobases).

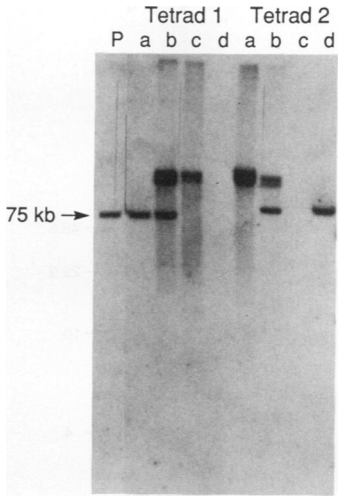


FIG. 6. FIGE analysis of tetrads from the cross of strain Sp223 containing the 75-kb minichromosome pSp(*cen1*)-75C to tester strain SBP32588. Genomic DNA from the parent strain Sp223/pSp(*cen1*)-75C (lane P) and from the haploid progeny of two tetrads in which the minichromosome had segregated to nonsister spores was digested with *Sac*I to linearize the minichromosome and subjected to electrophoresis as described in Materials and Methods. The gel was dried, hybridized to $^{32}$P-labeled pBR322, and subjected to autoradiography. The arrow indicates the position of the linearized 75-kb minichromosome. The higher-molecular-weight band that also hybridizes to the pBR322 probe in lanes b and c (tetrad 1) and lanes a and b (tetrad 2) is due to the presence of integrated copies of a pBR322-derived plasmid containing the *S. cerevisiae LEU2* gene at the centromere of chromosome III in strain SBP32588 (20).

minichromosome, leaving the small arm of pMB-1 at one end, proximal to the breakpoint (Fig. 2B). The *Sal*I-restricted DNA was then partially digested with several enzymes whose sites had been mapped and which cut infrequently in the 65-kb *Sal*I fragment (see Materials and Methods). The DNA fragment ladders thus generated were separated by FIGE and probed with pBR322-*URA3*. In this way, the presence or absence of restriction sites and their distances from the *Sal*I site in the small arm of the YAC vector were determined. An example of a DNA fragment ladder generated by total digestion of Sp223/pSp(*cen1*)-3C with *Sal*I followed by partial digestion with *Bam*HI is shown in Fig. 5. Hybridization with the pBR322-*URA3* probe revealed the expected 40-kb *Sal*I product, a 28-kb *Sal*I-partial *Bam*HI product, and a 4-kb *Sal*I-*Bam*HI complete digestion product. The indirect end-label mapping analysis indicated that the breakpoint in pSp(*cen1*)-3C occurred approximately 200 bp to the left of the *Bam*HI site in the left arm of the inverted repeat (Fig. 2B).

Functional studies of pSp(*cen1*)-3C had shown that the minichromosome exhibits mitotic stability and segregates predominantly 2+:2− through meiosis but undergoes precocious sister chromatid separation in the first meiotic division in more than 50% of the tetrads analyzed (Table 2; 20). This failure to maintain proper sister chromatid attachment could be the result of deletion of necessary centromeric sequences or could be due to the relatively small size of the minichromosome. It has been found that increasing the size of small centromeric plasmids in *S. cerevisiae* greatly improves their mitotic and meiotic function (22, 31). The effect of size on the behavior of pSp(*cen1*)-3C was addressed by integrating a pBR322-derived plasmid containing the *S. cerevisiae URA3* and *LEU2* genes into the vector sequences within the minichromosome by site-directed homologous recombination. An integrant was selected for analysis in which five copies of the plasmid had integrated in tandem, increasing the size of the 40-kb minichromosome to 75 kb. This larger minichromosome is designated pSp(*cen1*)-75C. Increasing the size of pSp(*cen1*)-3C had a marked effect on its mitotic stability, decreasing the frequency of minichromosome loss to approximately that of the plasmid pSp(*cen1*)-7L containing an intact *cen1* (Table 1). However, the increase in size had only a slight effect on the meiotic behavior of the minichromosome; pSp(*cen1*)-75C segregated predominantly 2+:2− through meiosis but still underwent precocious sister chromatid separation in the first meiotic division in a large number of the tetrads analyzed (45% tetratype asci; Table 2), although this frequency was somewhat lower than that observed for the 40-kb minichromosome (58% tetratype asci; Table 2).

To address the possibility that some or all of the tandem copies of the integrated plasmid had been lost during the meiotic process, genomic DNA from the haploid progeny of two of the tetrads in which the minichromosome segregated to nonsister spores was isolated and analyzed by FIGE. Digestion of genomic DNA from the parent strain Sp223/pSp(*cen1*)-75C with *Sac*I linearizes the 75-kb minichromosome (Fig. 6, lane P). In both tetrads analyzed, the two Ura$^+$ progeny contained the intact 75-kb minichromosome (tetrad 1, lanes a and b; tetrad 2, lanes b and d). The higher-molecular-weight bands that also hybridize to the pBR322 probe in Fig. 6 are due to the integrated copies of a pBR322-derived plasmid containing the *S. cerevisiae LEU2*

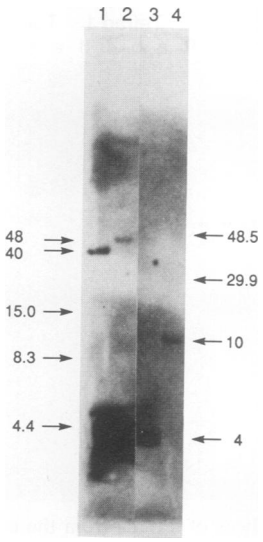


FIG. 7. Replacement of K′+L into pSp(*cen1*)-3C. The missing K′+L sequences in pSp(*cen1*)-3C were restored by fragment transplacement, as described in Materials and Methods, generating minichromosome pSp(*cen1*)-3XB. Genomic DNAs from strains Sp223/pSp(*cen1*)-3C and Sp223/pSp(*cen1*)-3XB were digested with *Sal*I (lanes 1 and 2, respectively) and with *Sal*I plus *Bam*HI (lanes 3 and 4, respectively). The digested DNA was separated by FIGE, and the gel was dried and hybridized to $^{32}$P-labeled pBR322-*URA3*. In both sets of digests, the hybridizing fragments (indicated by arrows) are increased in size by the expected amount, from 40 to 48 kb for the *Sal*I products and from 4 to 10 kb for the *Sal*I-*Bam*HI products (see Materials and Methods and Fig. 2A) as a result of introduction of the transplaced sequences. Arrows also locate positions of DNA size markers (in kilobases).

gene at the centromere of chromosome III in strain SBP32588 (20).

These experiments indicate that the overall size of the 40-kb minichromosome is not the principal factor causing its aberrant meiotic behavior. It is likely, therefore, that the relatively small portion of the inverted repeat that was deleted in this minichromosome is required for full meiotic centromere function. This hypothesis was verified by restoring the missing portion (K′ and L) of the inverted repeat into pSp(*cen1*)-3C. The 7-kb *Xho*I-*Bam*HI fragment containing the K′ and L repeats (Fig. 2A) was introduced into pSp(*cen1*)-3C by fragment-mediated transplacement as described in Materials and Methods, thereby regenerating the entire inverted repeat. An analysis of this DNA sequence replacement is presented in Fig. 7. Minichromosome pSp(*cen1*)-3XB, in which the K′ and L sequences are restored, was assayed for proper meiotic centromere function by tetrad analysis. Restoring the missing portion of the inverted repeat to the 40-kb minichromosome reduces the frequency of segregation to nonsister spores from 58 to 24%, approximately the same frequency as that displayed by the functional minichromosomes containing the entire 65-kb centromeric *Sal*I fragment from chromosome I (16%; Table 2) and the 100-kb centromeric *Sal*I fragment containing *cen2* (22%; 10). These findings suggest that all or nearly all of the inverted repeat in *cen1* is required for wild-type meiotic centromere function, since removing less than half of one arm results in failure to maintain proper sister chromatid attachment in the first meiotic division, but restoring these sequences restores this defect to wild-type levels.

**The K″ element (dgl) is required for both mitotic and meiotic centromere function.** To identify DNA sequences within the *cen1* region that are necessary for centromere function, minichromosomes containing different amounts of the centromeric repeats were constructed and assayed in *S. pombe* for mitotic stability and proper segregation in meiosis. An excision plasmid was obtained from integrant class 4, as described above, that contains the entire central core, the 2.5-kb core-proximal portion of the B′ repeat on the right, and single copies of the B′ and K″ repeats on the left (4B; Fig. 2A). The *S. cerevisiae LEU2* gene in this plasmid was replaced with the *S. pombe ura4* gene, and the resulting minichromosome, pSp(*cen1*)-BHp (Fig. 2B), was introduced via transformation into *S. pombe* Sp223. This minichromosome displays significant mitotic centromere function, exhibiting a loss frequency (frequency of Ura$^-$ segregants) in the same range as that of the 40-kb minichromosome [pSp(*cen1*)-3C; Table 1]. As with pSp (*cen1*)-3C, increasing the size of pSp(*cen1*)-BHp from 25 to 67 kb by integration of four tandem copies of plasmid pMB-neo (10) resulted in restoration of mitotic stability to approximately that of plasmid pSp(*cen1*)-7L containing an intact *cen1* (Table 1). Meiotic analysis demonstrated that pSp(*cen1*)-BHp segregated 2+:2− in 50% of the tetrads analyzed but to nonsister spores in almost every case (Table 2). This finding suggests that deleting both copies of K′ and L and one copy of B′ and K″ has little or no effect on mitotic centromere function but somewhat impairs 2+:2− segregation in meiosis and, as would be predicted from the meiotic defect apparent in pSp(*cen1*)-3C, virtually abolishes maintenance of sister chromatid attachment in the first meiotic division.

To determine the minimum DNA sequences required for mitotic centromere function, we constructed minichromosome pSp-9Sph, which contains the 9-kb *Sph*I fragment, including the entire central core plus both copies of the 2.5-kb core-proximal portion of the B′ repeat (Fig. 2B). This 15.5-kb minichromosome was found to be very unstable mitotically, exhibiting a loss frequency in the same range as that of the *S. pombe ars* plasmid pSp300 (Table 1). pSp-9Sph was also found to lack any meiotic function, segregating 0+:4− in 79% of the tetrads analyzed (Table 2). These experiments indicate that the central core plus 2.5 kb of core-associated inverted repeat is insufficient to confer any centromere function on an artificial chromosome. Apparently, either the complete copy of the B′ or K″ repeat (or both) present in pSp(*cen1*)-BHp (Fig. 2B) is absolutely required for mitotic centromere function.

The importance of the K″ element was assessed by deleting a 5-kb *Bam*HI-*Kpn*I fragment containing most of the K″ sequences from pSp(*cen1*)-BHp to form pSp-KHp (Fig. 2B). This minichromosome was found to be as unstable mitotically as pSp-9Sph (Table 1) and, as expected, segregated 0+:4− in virtually every tetrad analyzed (Table 2). These results indicate that at least one copy of the K″ element is required for centromere function. The functional significance of the B′ repeat and the central core has yet to be determined, although previous experiments have shown that the K region alone has no centromere activity (15).

## DISCUSSION

The structural organization of the centromere-specific DNA sequence repeats in the 65-kb *Sal*I fragment containing the functional centromere from chromosome I of *S. pombe* has been determined. Fragments spanning the entire *cen1* region were recovered in *E. coli* by using a plasmid integra-

tion-excision strategy (10), and minichromosomes containing various portions of the *cen1* region were assayed for centromere function in *S. pombe*.

The centromere region from chromosome II of *S. pombe* has been shown to contain four tandem units of the K, L, and B repeats, and two of these 14-kb units are arranged in an inverted repeat flanking a 7-kb central core sequence that is unique to *cen2* (Fig. 4; 10). Long-range genomic mapping studies indicate that the *cen3* region contains even more copies of the repeats than does *cen2*, but their precise organization has not yet been determined (6, 9). Although the *cen1* region contains only two copies of the centromeric repeats, they are also organized in an inverted repeat flanking a 4- to 5-kb central core (Fig. 4; 6). As in *cen2* (10), the *cen1* region contains a mostly unique core-associated repeat (B′) directly flanking the central core sequence (Fig. 2A and 4). These findings plus the observation that the *cen3* region also contains an inverted-repeat configuration (6) strongly suggest that this structure is intimately involved in the functioning of the centromere in *S. pombe* and perhaps in the centromeres of higher eukaryotes as well, since the chromomeres in the pericentric heterochromatin of many plant chromosomes are arranged in a bilaterally symmetrical pattern about a central point (28).

Although the overall organization of the centromeric repeats in *cen1* and *cen2* is conserved, there are significant differences. In *cen1*, the K-repeat element (15) is split into two fragments by the L repeat (Fig. 2A). The portion of K that is core distal in *cen2* is core proximal in *cen1* and is reversed in orientation (termed K″ in Fig. 2A and 4; 6). Hybridization studies indicate the *cen2* central core to be unique (10); however, the central 3 kb of the *cen1* central core cross-hybridizes to the 150-kb genomic *Sal*I fragment containing *cen3*. Sequencing analysis of B and B′ repeats from *cen1* and *cen2* have revealed the presence of several tRNA genes (27). Whether these tRNA genes are actively transcribed has not yet been determined. Analysis of the chromatin structure of *S. pombe cen2* in vivo indicates that regions containing the B repeat exhibit an unusual nuclease cleavage pattern (37), possibly as a result of the tRNA gene cluster present in these regions.

The structural differences observed among the three *S. pombe* centromeres suggest that the precise arrangement of the repeated elements may not be critical to centromere function. The arrangement of the K repeat in *cen1* as compared with *cen2* indicates that this element, a portion of which is absolutely essential for centromere activity, can function in either orientation. Although the K repeat has not yet been implicated as a site of specific protein binding, many protein-binding sites, including the *S. cerevisiae* centromere, function in an orientation-independent manner (11). Studies of the organization of human centromeric alphoid repetitive sequences showed a very high degree of variability not only among different chromosomes but among different individuals as well, suggesting that the role of these sequences does not depend on their precise organization in the centromere region (25). It has also been shown that heterologous sequences can be integrated at various locations in the *S. pombe* centromere regions, including the inverted repeat and the central core, without affecting centromere function (10, 15). Apparently, the introduction of these foreign sequences does not significantly disrupt the local chromatin conformation or prevent the specific binding of proteins required for proper centromere function.

Previous studies have suggested a functional role for the *S. pombe* centromeric repeats. Deleting a portion of the repeated sequences in *cen2* (10) or in *cen3* (34) results in a slight decrease in mitotic stability and a dramatic increase in precocious sister chromatid separation in meiosis I. The results presented here for minichromosomes derived from the *cen1* region confirm these observations. The 40-kb circular minichromosome, pSp(*cen1*)-3C, was shown by restriction site mapping to have deleted one copy of the K′ and L repeats at the end of one arm of the inverted repeat (Fig. 2B). This minichromosome segregates predominantly 2+:2− through meiosis, but it is less stable mitotically and undergoes precocious sister chromatid separation in meiosis I more frequently than does the 78-kb linear minichromosome containing the entire *cen1* region (20). Increasing the size of pSp(*cen1*)-3C to 75 kb by integration of additional vector sequences restores its mitotic centromere function to approximately that of a plasmid containing an intact, wild-type *cen1* [pSp(*cen1*)-75C and pSp(*cen1*)-7L; Table 1] but has little effect on the aberrant meiotic behavior (Table 2). Similar results have been obtained for functional *cen2*-derived minichromosomes (10). However, replacing the deleted copy of K′ and L in pSp(*cen1*)-3C apparently repairs the meiotic defect; pSp(*cen1*)-3XB gives precocious sister chromatid separation at levels equivalent to those exhibited by the minichromosomes containing the entire *cen1* and *cen2* regions (Table 2; 10, 20). This finding suggests that pSp(*cen1*)-3C contains all of the sequences necessary for spindle attachment (mitotic stability and 2+:2− segregation in meiosis) and implicates the K′- and L-repeat elements in the maintenance of sister chromatid attachment in the first meiotic division. Alternatively, any large inverted repeat, rather than one composed of the K′ and L repeats specifically, may function to hold the sister chromatids together in meiosis I.

Minichromosome pSp(*cen1*)-BHp, which contains the central core, a 2.5-kb portion of B′ on the right of the core, and one copy each of B′ and K″ on the left, exhibits significant mitotic stability relative to *S. pombe ars* plasmids, and as found for other mitotically active constructs, increasing the size of this minichromosome results in an increase in mitotic stability to approximately wild-type levels (Table 1). Although pSp(*cen1*)-BHp segregates predominantly 2+:2− in meiosis (Table 2), it segregates to nonsister spores in 85% of the tetrads (Table 2). Separation of sister chromatids followed by random segregation in meiosis I would be expected to yield segregation of the minichromosome to nonsister spores in approximately 50% of the tetrads. The very high frequency of nonsister spore segregation observed for pSp(*cen1*)-BHp suggests that in meiosis I this minichromosome undergoes the equational division of meiosis II rather than the typical reductional division of meiosis I. It has recently been shown in *S. cerevisiae* that in certain genetic backgrounds different chromosomes show different segregation tendencies, with some chromosomes segregating reductionally and others segregating equationally during the same meiotic division (39). Further analysis indicates that the sequences responsible for the choice between reductional or equational division are located at the centromere (40).

Deleting most of the K″ sequences from pSp(*cen1*)-BHp to form pSp-KHp (Fig. 2B) results in the complete loss of centromere function. This construct exhibited a loss frequency in mitosis similar to that of an *ars* plasmid (Table 1) and rarely survived meiosis (Table 2). As expected, plasmid pSp-9Sph, which is missing all K″ and portions of the B′ sequences (Fig. 2B), is also totally inactive (Tables 1 and 2). These results strongly imply that the K″-sequence element is

absolutely required for centromere function, and thus it is possible that this DNA region is a component of the kinetochore, the DNA-protein complex containing the spindle attachment site. Comparison of the structural organizations of *cen1* and *cen2* (Fig. 4) suggests that the K″ sequences can function in both orientations and at different positions relative to the other repeats and the central core. Alternatively, the K″ repeat may be involved in conferring a specific chromatin conformation on the region that directly binds to the spindle fiber.

This study has permitted a preliminary assignment of repeats in the *cen1* region to the specific functions of the centromere in which they are involved. The K′ and L repeats appear to play a role in maintaining sister chromatid attachment in meiosis, while the K″-sequence element is implicated in spindle attachment. The smallest *cen1*-derived minichromosome identified so far that exhibits centromere function, pSp(*cen1*)-BHp, contains the central core, a portion of the core-associated B′ repeat at the right of the core, and single copies of B′ and K″ at the left. However, the functional significance of the central core and B′ repeats has not yet been directly addressed. The chromatin structure of the *cen1* and *cen2* central core regions is characterized by an absence of regular nucleosomal packaging, suggesting that these regions may be the sites of kinetochore assembly (37). Although the *cen2* central core does not cross-hybridize to the other two central core regions (10), it is possible that, as in *S. cerevisiae*, these regions contain short conserved sequence elements which serve as key protein-binding sites.

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