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Functional Analysis of a Centromere from Fission Yeast: A Role for Centromere-Specific Repeated DNA Sequences

LOUISE CLARKE* AND MARY P. BAUM

Department of Biological Sciences, University of California, Santa Barbara, California 93106

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A circular minichromosome carrying functional centromere sequences (*cen2*) from *Schizosaccharomyces pombe* chromosome II behaves as a stable, independent genetic linkage group in *S. pombe*. The *cen2* region was found to be organized into four large tandemly repeated sequence units which span over 80 kilobase pairs (kb) of untranscribed DNA. Two of these units occurred in a 31-kb inverted repeat that flanked a 7-kb central core of nonhomology. The inverted repeat region had centromere function, but neither the central core alone nor one arm of the inverted repeat was functional. Deletion of a portion of the repeated sequences that flank the central core had no effect on mitotic segregation functions or on meiotic segregation of a minichromosome to two of the four haploid progeny, but drastically impaired centromere-mediated maintenance of sister chromatid attachment in meiosis I. This requirement for centromere-specific repeated sequences could not be satisfied by introduction of random DNA sequences. These observations suggest a function for the heterochromatic repeated DNA sequences found in the centromere regions of higher eucaryotes.

The centromeres of higher eucaryotic chromosomes are large and complex and include not only the kinetochore or spindle attachment site(s), but often extensive regions of pericentric heterochromatin that can span megabases of DNA. In spite of numerous attempts, a functional role has yet to be applied to centromeric heterochromatin (3, 17, 27). There does seem to be a strong functional association, however, between a class of satellite repeat sequence arrays and the *responder* of *segregation distorter* in *Drosophila melanogaster* (33).

The centromeres of budding yeast are relatively small (150 base pairs [bp] of DNA) and are free of heterochromatic DNA sequences (8, 9). In contrast, however, the centromere regions of the fission yeast *Schizosaccharomyces pombe* resemble those of higher eucaryotes in containing several classes of repeated DNA sequences which encompass many kilobases (kb) of DNA on each of the three *S. pombe* chromosomes (6, 7, 10, 24). Centromeric repeats designated K, L, and B are heterochromatic-like and untranscribed (10). The centromere regions of the three *S. pombe* chromosomes, including all the centromere-specific DNA sequence repeats, reside on three large genomic *Sal*I restriction fragments of 65, 100, and 150 kb, derived from chromosomes I, II, and III, respectively (10).

Recently, we have developed a minichromosome assay system for centromere function in *S. pombe* that uses a yeast artificial chromosome (YAC) vector modified for selection and replication both in *Saccharomyces cerevisiae* and in *S. pombe* (14). This system has enabled us to demonstrate that functional centromere sequences are contained within the 65-kb and 150-kb *Sal*I restriction fragments from the centromeric regions of chromosomes I and III. The availability of an assay system for centromere function makes *S. pombe* an excellent model system in which to assess the role of repetitive DNA sequences in centromere function. We show here that functional centromere sequences are contained within a 100-kb *Sal*I genomic restriction fragment from the centromere region of chromosome II; in addition, we present for the first time a structure-function analysis of an *S. pombe* centromere.

A plasmid integration-excision strategy has been used to clone over 80 kb of the *cen2* region in *Escherichia coli*. The centromere-specific repeats in *cen2* are organized into four 14-kb units, two of which occur as part of an inverted repeat configuration flanking a 7-kb region of nonhomology. At least a portion of the inverted repeat, along with the 7-kb central core, specifies stable mitotic and meiotic segregation (spindle fiber attachment) of genetically marked minichromosomes. However, minichromosomes containing deletions of portions of the centromere-specific repeat sequences display a very high degree of sister chromatid separation in the first meiotic division. Thus, two major roles of the centromere in meiosis (ensuring stable distribution through the two meiotic divisions of the minichromosome of two of the four haploid progeny and maintaining sister chromatid attachment in meiosis I) have been functionally separated. These results have significant implications with regard to the role of heterochromatic DNA sequence repeats at the centromeres of higher eucaryotic chromosomes.

MATERIALS AND METHODS

Strains, transformations, and genetic manipulations. *S. pombe* diploid strain SBPD400 (h^-/h^- *ade6.210/ade6.216 leu1.32/leu1.32 ura4.294/ura4.294* can^s/can^r [10]) was the recipient for all site-directed integrations. The *S. pombe* haploid strain Sp223 (h^- *leu1.32 ura4.294 ade6.216*; gift from D. Beach) was the host for all DNA transformations with excised plasmids. Growth media and conditions were those described by Gutz et al. (12). DNA transformations of *S. pombe* were performed as described before (16). Stable Ura^+ transformants obtained by site-directed integration of pSp500 (Fig. 1) into SBPD400 were identified as follows. A total of 240 Ura^+ transformant colonies were picked onto nonselective medium and subsequently replica-plated for seven rounds of nonselective growth before they were replica-plated to medium selecting for the Ura^+ phenotype. Those clones (14 total) that were still Ura^+ after several rounds of nonselective growth and that contained sequences hybridizing to pBR322 were characterized further. Two

* Corresponding author.

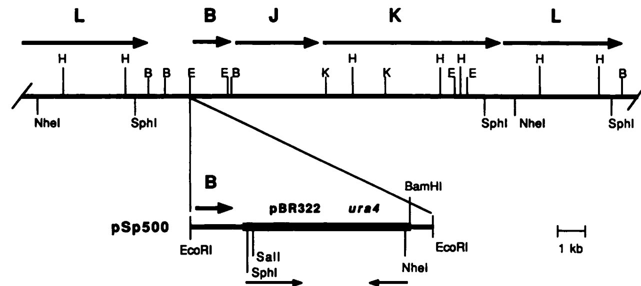


FIG. 1. Mapping strategy with the integration-excision plasmid pSp500. Plasmid pSp500 was restricted at a unique *Eco*RI site within the *S. pombe* sequences and introduced by site-directed homologous integration into the *cen2* region near the B repeats of one copy of chromosome II in the *S. pombe* diploid SBPD400. The plasmid was constructed so that excision from total integrant DNA with *Sal*I, *Sph*I, or *Nhe*I would release both the vector and neighboring centromeric sequences. The arrows at the top of the figure delineate centromere-specific repeats L, B, J, and K. Restriction sites are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I.

carried multiple copies of pSp500. The remaining 12 contained only a single integrated copy of pSp500 and fell into the five integrant classes described below.

Mitotic stabilities of various plasmids in *S. pombe* were measured either by plating selectively grown cells for single colonies onto nonselective medium and replica-plating to medium selecting for the Ura$^+$ phenotype to determine the fraction of cells that retained the plasmid or by plating dilutions of selectively grown cells onto SD plates (31) supplemented with adenine, leucine, and uracil in the presence or absence of 5-fluoroorotic acid (1 mg/ml) as described by Hahnenberger et al. (14). The mitotic stability of minichromosomes is represented as the frequency of Ura$^-$ segregants within a population of selectively grown cells. The number of cells in a population derived from a single cell is approximately equal to the total number of cell divisions that have occurred. Thus, assuming that growth of cells which lose a plasmid or minichromosome is minimal in the absence of uracil, the frequency of Ura$^-$ segregants within a population of selectively grown cells is approximately equal to the frequency of minichromosome loss per cell division.

Meiotic analyses (12) were carried out as described in Hahnenberger et al. (14). *S. pombe* strain Sp223 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* LEU-L (10), which contains 8 to 10 copies of the *S. cerevisiae LEU2* gene tandemly integrated near the centromere of chromosome III. The *LEU2* gene serves as a tightly centromere-linked marker for chromosome III. The *lys1.1* and *tps13.1* loci are tightly linked to centromeres I and II, respectively (18). Segregation of the minichromosomes was assayed by scoring for the *ura4* marker on the vector pSp500 or, in the case of pSp(cen2)52-C, for the *S. cerevisiae URA3* marker on the vector pMB-1.

Plasmids described in the final section of Results were made larger by site-directed integration (26) at a unique *Sca*I site of various numbers of copies of plasmid pMB-neo into the pBR322 sequences within the minichromosomes in *S. pombe*. Plasmid pMB-neo is a pBR322-derived vector which carries the *S. cerevisiae LEU2* gene (complements the *leu1.32* mutation in *S. pombe*) and a fragment from plasmid pKO-neo (32) containing the *E. coli lac*UV5-95 promoter, the simian virus 40 (SV40) early promoter, the transposon Tn*5* neomycin resistance gene, the SV40 small T antigen intron, and the SV40 poly(A) addition site. Plasmid pMB-neo (8.5 kb) does not replicate in *S. pombe* (M. Baum and L. Clarke, unpublished), and because *LEU2* is relatively poorly expressed in the heterologous system, integrants with multiple copies of pMB-neo integrated in tandem into the resident plasmid within the Sp223 (*leu1.32*) host are easily selected.

Enzymes, recombinant DNA technology, and field inversion gel electrophoresis. Restriction enzymes, T4 DNA ligase, and DNA polymerase were purchased from New England BioLabs and were used according to the vendor's instructions. Procedures for *E. coli* transformations, cloning techniques, and plasmid isolations were as described by Maniatis et al. (21). *S. pombe* DNA was prepared by the method of Beach and Klar (1), using wide-bore pipettes and snipped-off micropipette tips for all operations. For plasmid excisions, DNA was restricted to completion with *Sal*I, *Sph*I, or *Nhe*I in order to generate linear DNA fragments carrying both pSp500 (vector) and neighboring centromere sequences; the restricted DNA was diluted to approximately 1 μg/ml, ligated, and used to transform *E. coli* DH5α (*recA1*; Bethesda Research Laboratories) to ampicillin resistance.

Field inversion gel electrophoresis was carried out as described before (5) in 1% agarose gels at 180 V for 18 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.

Electron microscopy, polymerase chain reaction, and DNA sequencing. Electron microscopy was carried out exactly as described in Fishel et al. (10). All DNA sequencing reactions were performed on double-stranded DNA with the Sequenase kit (United States Biochemical Corp.) according to the manufacturer's instructions. Polymerase chain reaction (28) was carried out on 10 mg of *Sal*I-digested Sp223 DNA. Two 25-base oligomers (20 pmol each), corresponding to positions 40 to 64 and 259 to 283 in the sequence shown in Fig. 6b, directed the synthesis of a 244-bp fragment with the GeneAmp DNA Amplification kit (Perkin Elmer Cetus) and a Perkin Elmer Cetus thermal cycler. The amplification reaction was allowed to proceed for 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and extension at 68°C for 3 min. The amplified band was gel purified and isolated with Geneclean (Bio101). Then, 100 pg of this DNA was reamplified and spun through a Centricon 30 microconcentrator (Amicon) to remove excess dNTPs and primers, and a total of 350 ng of genomic DNA or 10 μg of pSp3-SNc DNA was used in DNA sequencing reactions primed by the oligomers described above.

RESULTS

A 100-kb genomic *Sal*I restriction fragment contains the functional centromere sequences from *S. pombe* chromosome II. A minichromosome assay system for centromere function in *S. pombe* has recently been developed (14). The centromere regions of the three *S. pombe* chromosomes, including all the centromere-specific repeated sequences, are contained on large genomic *Sal*I restriction fragments of 65 kb (chromosome I), 100 kb (chromosome II), and 150 kb (chromosome III) (10). Therefore, a YAC vector system (4), modified for replication and selection in both *S. cerevisiae* and *S. pombe*, was chosen in order to accommodate the cloning of large DNA fragments. This system uses *S. cerevisiae*, not *E. coli*, as the cloning host and thus avoids problems with replication of repeated sequences encountered with *S. pombe* centromeric DNA in the bacterial host

TABLE 1. Mitotic stability of *cen2*-related plasmids[a]

Minichromosome or vector	Size (kb)	Frequency of Ura⁻ segregants
Active		
pSp(cen2)52-C	105	7.4×10^{-4}
pSp3-Sal	44	1.4×10^{-3}
pSp3-Sal-neo$_{(5)}$	86	4.4×10^{-5}
pSp3-Sal-neo$_{(9)}$	120	1.1×10^{-4}
Inactive		
pSp3-Sal-dBst	28	4.3×10^{-1}
pSp3-S	13	3.6×10^{-1}
pSp3-S-neo$_{(3)}$	38	1.6×10^{-1}
pSp4-S	13	4.0×10^{-1}
pSp4-S-neo$_{(5)}$	55	2.6×10^{-1}
pSp3-SNc	17	4.9×10^{-1}
pSp3-SNc-neo$_{(2)}$	34	1.6×10^{-1}
pSp3-SNc-neo$_{(4)}$	50	2.2×10^{-1}
pSp1-N-b (pSp2-N-b)	30	8.5×10^{-1}
pSp5-N-b	30	4.9×10^{-1}
Vectors		
pMB-1 (YAC vector)	13	2.3×10^{-1}
pSp500 (integration vector)	9	3.2×10^{-1}

[a] Frequency of Ura⁻ segregants was measured as described in Materials and Methods.

(7). When shuttled from *S. cerevisiae* to *S. pombe*, minichromosomes carrying the *cen1* or *cen3* regions were shown to behave as independent genetic linkage groups (14).

We reported previously that the centromere region from *S. pombe* chromosome II, which occurs on a 100-kb genomic *Sal*I restriction fragment (10), has been cloned in *S. cerevisiae* in the modified YAC vector pMB-1 (14). When a linear minichromosome containing this DNA fragment cloned into pMB-1 was transferred from *S. cerevisiae* into *S. pombe* via DNA transformation, all *S. pombe* transformants examined contained a circular derivative of the original linear plasmid. Circularization of minichromosomes, occasionally accompanied by deletions, has frequently been observed when linear minichromosomes are shuttled from *S. cerevisiae* to *S. pombe*, and presumably results from the inability of the *S. pombe* telomere terminal transferase to be primed efficiently by the *S. cerevisiae* telomeric repeats (14, 30). A circular *cen2* plasmid, pSp(cen2)-52C, identified in *S. pombe*, was shown by field inversion gel analysis to contain the entire 100-kb *Sal*I restriction fragment from the *cen2* region (not shown). Plasmid pSp(cen2)-52C is missing the large arm of the vector pMB-1 but retains the small arm, including the *S. cerevisiae URA3* gene, which complements *ura4* mutations in *S. pombe* (20). The loss of the large arm and retention of the small one in the circularization process is apparently a common resolution of the telomere incompatibility problem, as it has been observed with several of the pMB-1-derived centromere-containing minichromosomes we have shuttled from *S. cerevisiae* to *S. pombe* (K. Hahnenberger and L. Clarke, unpublished).

Plasmid pSp(cen2)-52C behaved as a stable and independent fourth genetic linkage group in *S. pombe*. The functional circular minichromosome was stable through mitotic cell divisions (7×10^{-4} frequency of Ura⁻ segregants; Table 1), and the genetic marker *URA3* on the minichromosome segregated properly and with high fidelity through both meiotic divisions (Tables 2 and 3). The plasmid segregated 2+:2− in 92% of the tetrads analyzed, and the genetic marker carried by pSp(cen2)-52C was centromere-linked and unlinked to the three parental chromosomes (Table 3). Sister chromatid separation of pSp(cen2)-52C in the first meiotic

TABLE 2. Meiotic sergregation of *cen2*-related plasmids[a]

Minichromosome	Size (kb)	No. (%) of tetrads with meiotic segregation pattern: 4+:0−	3+:1−	2+:2−	1+:3−	0+:4−
Active						
pSp(cen2)-52C	105	0	0	36 (92)	1	2
pSp3-Sal	44	0	1	16 (89)	0	1
pSp3-Sal-neo$_{(5)}$	86	2	2	14 (58)	3	3
pSp3-Sal-neo$_{(9)}$	120	0	0	13 (59)	4	5
Inactive						
pSp3-S	13	0	2	3	4	7 (44)
pSp4-S	13	0	0	2	7	17 (65)
pSp3-SNc	17	0	1	0	3	6 (60)
pSp1-N-b (pSp2-N-b)	30	0	1	0	0	14 (93)

[a] Meiotic analyses were carried out as described in Materials and Methods. Segregation of the minichromosomes was assayed by scoring for the *ura4* marker on the vector pSp500 or, in the case of pSp(cen2)52-C, for the *S. cerevisiae URA3* marker on the vector pMB-1.

division occurred in only about 20% of the tetrads analyzed. It is not known how frequently precocious sister chromatid separation takes place with the parental chromosomes in *S. pombe*. Thus, we conclude that functional *cen2* DNA sequences are included on the 100-kb genomic *Sal*I restriction fragment carried by pSp(cen2)-52C.

A Plasmid integration-excision strategy was used to map over 80 kb of the *cen2* region. The presence of repeated DNA sequences at all three centromeres in *S. pombe* makes conventional chromosome walking an impractical and inaccurate method of obtaining centromeric DNA for structural and functional analysis (7). Thus, a plasmid integration-excision strategy has been developed to obtain structural information across the *cen2* region. A 2-kb *Bam*HI-*Sau*3A restriction fragment was isolated from the *cen2* region plasmid pSp25-OB, which has been described previously (7). This DNA fragment, which includes a centromeric B repeat and an adjacent sequence that is unique to the *cen2* region and contains an *Eco*RI site, was cloned into a pBR322 vector carrying the *S. pombe ura4* gene. The *S. pombe* sequences carried by the integration plasmid pSp500 (Fig. 1) were

TABLE 3. Tetrad distributions of active minichromosomes[a]

Minichromosome	*cen*-linked marker (chromosome)	No. of tetrads: PD	NPD	T	Sister/nonsister distribution (PD + NPD:T)
pSp(cen2)52-C	*lys1* (I)	10	15	11	28:8
	tps13 (II)	12	16	8	
	LEU2 (III)	11	17	8	
pSp3-Sal	*lys1* (I)	1	3	12	4:12
	tps13 (II)	2	2	12	
	LEU2 (III)	2	2	12	
pSp3-Sal-neo$_{(5)}$	*lys1* (I)	5	2	7	7:7
	tps13 (II)	4	2	8	
	LEU2 (III)	7	1	6	
pSp3-Sal-neo$_{(9)}$	*lys1* (I)	4	0	9	4:9
	tps13 (II)	2	2	9	
	LEU2 (III)	1	2	10	

[a] 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: *lys1-cen1*, 3.6 centimorgans (cM); *tps13-cen2*, 0.5 cM; and *LEU2-cen3*, 0 cM (7, 10). PD, Parental ditype; NPD, nonparental ditype; T, tetratype.

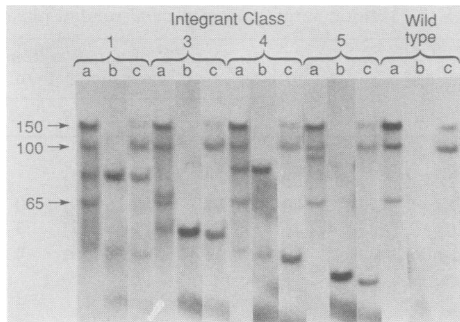


FIG. 2. Field inversion gel electrophoretic analysis of various pSp500 integrant classes. Integrant genomic DNAs were restricted with *Sal*I and subjected to electrophoresis as described in Materials and Methods. The gel was dried, hybridized to ^{32}P-labeled pBR322 probe, and autoradiographed (lanes b). The same gel was hybridized to ^{32}P-labeled K repeat probe and autoradiographed (lanes a). Finally, the gel was stripped by soaking overnight in 0.3 M NaOH at 37°C with gentle shaking, hybridized to ^{32}P-labeled *Hin*dIII-*Sal*I fragment from the *tps13*-proximal side of the 100-kb genomic *Sal*I fragment that includes the *cen2* region (7), and autoradiographed (lanes c). Numbers and arrows at the left of the figure indicate the positions of the 150-kb, 100-kb, and 65-kb genomic *Sal*I restriction fragments that contain the centromere regions of chromosomes III, II, and I, respectively (10).

known to occur at least twice within the *cen2* region, because these sequences make up a portion of the 31-kb inverted repeat (10). The cloned sequences also contained an *S. pombe ars* element, as the resulting plasmid pSp500 replicated well but was unstably maintained (Table 1) in *S. pombe*. Plasmid pSp500 was constructed to allow chromosome walking by plasmid excision from total integrant genomic DNA with *Sal*I, *Sph*I, or *Nhe*I from the sites of integration, followed by plasmid rescue via DNA transformation in *E. coli* (Fig. 1).

Plasmid pSp500 was restricted at the unique *Eco*RI site within the *Bam*HI-*Sau*3A fragment and introduced by site-directed integration (26) (Fig. 1) into the *cen2* region of one copy of chromosome II in the *S. pombe* diploid strain SBPD400. Five classes of stable Ura$^+$ transformants were obtained, which represent the products of homologous integration into each of five locations within the *cen2* region. These were identified by restricting total transformant DNAs with *Sal*I and analyzing digestion products by field inversion agarose gel electrophoresis and successive hybridizations to three labeled probes (Fig. 2).

The integration plasmid pSp500 contains a unique *Sal*I site (Fig. 1) and thus introduces a new *Sal*I site into the *cen2* region at the point of integration, splitting the region into two *Sal*I fragments whose lengths may be used to determine the precise location of the integration site. The numbers at the top of Fig. 2 refer to the integrant classes. Lanes c were probed with a unique 4.2-kb *Hin*dIII-*Sal*I fragment from the right-most portion of the 100-kb genomic *Sal*I *cen2* fragment. The 4.2-kb probe sequence is located outside the region of centromeric repeats and within unique DNA on the right arm of chromosome II between the centromere and the *tps13* locus (7). This probe identified both the 100-kb *cen2* fragment from the unaltered homolog and the new *Sal*I fragment that contains these sequences for each integrant class. New *Sal*I fragments 78 kb, 61 kb (not shown), 43 kb, 27 kb, and 17 kb in length were identified from various transformant DNAs probed with the 4.2-kb *Hin*dIII-*Sal*I fragment (Fig. 2). Thus, five integration sites for pSp500 are localized within the 100-kb *cen2* fragment at these distances from the right-most *Sal*I site (Fig. 3).

Lanes a represent the same gel lanes as in c probed with the centromeric repeat K (7), which identifies for each integrant class the three *S. pombe* centromeric *Sal*I fragments of 150, 100, and 65 kb. The K repeat probe also identified in most cases the two new *Sal*I fragments generated by integration of pSp500 at a particular location within the *cen2* region of one copy of chromosome II, because repeat K occurred in multiple copies throughout the region. As anticipated from the results with the 4.2-kb probe above, integrant class 1 yielded two fragments of 78 kb and 30 kb; class 2, 61 kb and 48 kb (data not shown); class 3, 43 kb and 65 kb; class 4, 27 kb and 80 kb; and class 5, 91 kb. The 17-kb *Sal*I fragment from class 5 contained no K sequences and therefore was not identified by the K probe. For each integrant class, the two *Sal*I fragments added up to approximately 109 kb, the size expected from the 100-kb genomic fragment plus the 9-kb integrated plasmid.

Finally, lanes b are the same gel lanes described above probed with pBR322 DNA, which hybridizes to the new *Sal*I fragment in each transformant class that contains these vectors sequences and thereby identifies the orientation within the genome of the *S. pombe* sequences carried by the integrating plasmid. For example, integration sites 1 and 4 have the sequences that are adjacent to the B repeat and carried by the integrating plasmid organized in opposite orientations, because in the case of integrant class 1, pBR322 DNA hybridized to the 78-kb *Sal*I fragment that comprises the right portion of the centromere region in that class, and

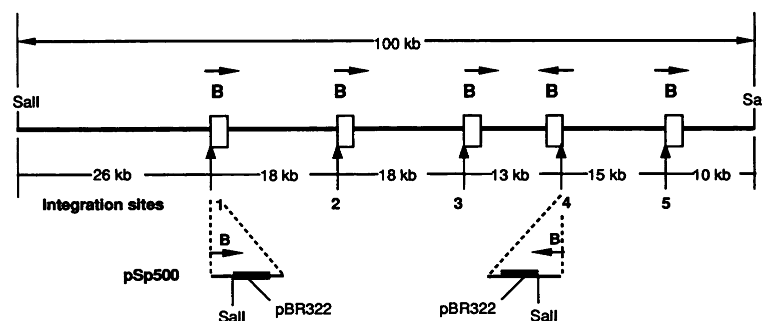


FIG. 3. Sites of integration of pSp500 into the *cen2* region. Five homologous integration sites for pSp500 are located as shown within the 100-kb genomic *Sal*I restriction fragment that includes the *cen2* region. Orientation of the integrated plasmid at each of the sites is given by the arrows above the B repeat locations.

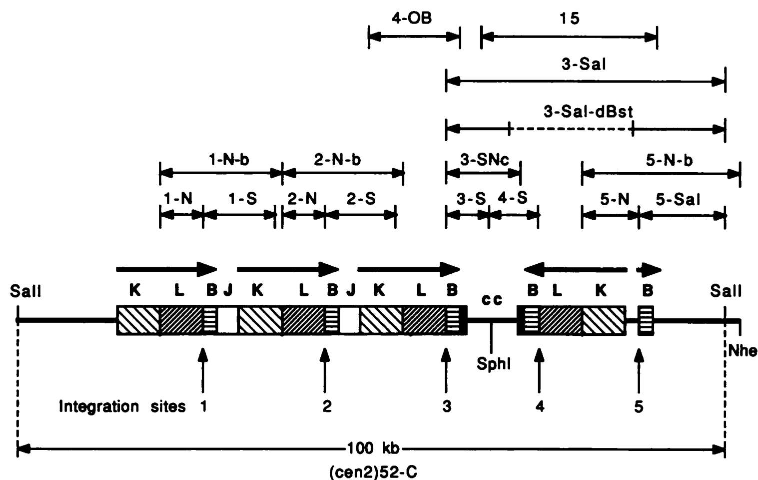


FIG. 4. Structure of the *cen2* region based on the structures of excised plasmids. The organization and orientation of centromeric repeats K, L, B, and J and the location of the nonhomologous 7-kb central core (cc) are shown. The solid black boxes directly flanking the central core represent 1.5 kb each of inverted repeat sequences that occur only at this location in the *S. pombe* genome. The right-most *SalI* site in the figure is *tps13*-proximal (7, 13). Plasmids pSp4-OB and pSp15 have been described, and neither possesses functional centromere activity in *S. pombe* (7, 10). Plasmids pSp3-Sal and pSp5-Sal were excised with *SalI* from integrant class 3 and 5 genomic DNAs, respectively. Plasmid pSp3-Sal-dBst is a derivative of pSp3-Sal that was obtained by deleting in vitro a large *BstEII* fragment from the latter, as indicated by the dotted line. Plasmids pSp1-N, pSp2-N, and pSp5-N were excised with *NheI* from integrant class 1, 2, and 5 genomic DNAs, respectively, and plasmids pSp1-N-b, pSp2-N-b, and pSp5-N-b were obtained in a similar manner from partial digestion products. Plasmids pSp1-S, pSp2-S, pSp3-S, and pSp4-S were obtained by excision with *SphI* from integrant class 1, 2, 3, and 4 genomic DNAs, respectively. The construction of pSp3-SNc and pSp(cen2)52-C is described in Results.

in the case of integrant class 4, it hybridized to the 80-kb fragment which is from the left side of the region. The locations of the plasmid integration sites and their relative orientations are summarized in Fig. 3.

The *cen2* region is composed of four tandem repeated units, two of which are organized into a 31-kb inverted repeat. Plasmid excisions from genomic DNA of each integrant class with *SphI*, *NheI*, or *SalI*, followed by ligation and recovery in *E. coli*, yielded a variety of plasmids, which were analyzed structurally by restriction mapping and reintroduced into *S. pombe*, where they were assayed for mitotic stability and proper meiotic function. The structure of the *cen2* region presented in Fig. 4 was deduced from the structures of the excised plasmids as diagrammed. Sequences were identified as being specific to a particular *S. pombe* centromere by hybridization analysis with dried-down field inversion agarose gels containing DNAs from *S. cerevisiae* clones transformed with minichromosomes carrying the functional *cen1*, *cen2*, and *cen3* regions (14).

The *cen2* region contained a variety of repeated sequences of various sizes. At the gross level, the region was organized into four 14-kb tandem repeats units (Fig. 4). Each 14-kb unit was in turn composed of repeated elements previously designated K, L, and B (7, 10). Repeat K was originally defined as a 6.4-kb sequence element occurring in tandemly arranged copies in *S. pombe* genomic cosmid clones derived from the *cen3* region (7). Repeat K does not exist as a 6.4-kb contiguous unit within *cen1*, however, but is interrupted by a portion of an L repeat (6; K. Hahnenberger and L. Clarke, unpublished). Repeat L is defined as the 5-kb repeated sequence within *cen2* that includes most of the region between K and B. The relationship of repeats K and L to the repeats designated dgII and dhII, whose nucleotide sequences have been reported by Nakeseko et al. (24, 25), is shown in Fig. 5. The exact size of the B repeat has not been determined, but is probably less than 1 kb (7). At least portions of K, L, and B are present at all three centromeres in *S. pombe* (7). The three left-most 14-kb units within *cen2* were separated internally by another repeat, designated J (Fig. 4 and 5), which was also found within *cen3*, but not within *cen1* (C. Polizzi and L. Clarke, unpublished). A detailed restriction map of a *cen2* K-L-B-J repeat unit is presented in the upper portion of Fig. 5.

A major structural feature of the region was the organization of two of the 14-kb K-L-B repeated units into part of a 31-kb inverted repeat which flanked a 7-kb region of nonhomology. The remainder of the inverted repeat included 1.5 kb of sequences (immediately adjacent to the B repeats) that directly flanked the central core on either side. A restriction map of the central core and surrounding inverted repeat sequences is presented in the lower portion of Fig. 5. The 1.5 kb of inverted repeat sequences that directly flanked the 7-kb central core appeared to be unique to this particular centromere (solid black boxes, Fig. 4 and 5), and, like the central core sequences, did not cross-hybridize to *cen1* and *cen3* DNAs under standard blot hybridization conditions.

Thus, the structural organization of the *S. pombe* centromere regions is complex. Repeats are present that are common among all three centromeres, but there are also repeated elements that appear to be specific to a particular centromere. The most striking feature of the overall organization of *cen2* is the presence of the large (31 kb) inverted repeat (10) (Fig. 4 and 5). Recently, it has been shown that an inverted repeat structure is also present in *cen1* and *cen3*, although the organization of the repeated elements appears to be different for each centromere (6).

A more exact measurement of the *cen2* nonhomologous central core was obtained by electron microscopy. A plasmid, pSp3-SNc, was constructed by inserting the 4.5-kb *SphI-NcoI* fragment from plasmid pSp4-S, which comprises approximately the right half of the central core plus a small amount of inverted repeat sequence, into the *SphI* site of plasmid pSp3-S, which includes the left half of the core and a small amount of inverted repeat (Fig. 4 and 5). Thus,

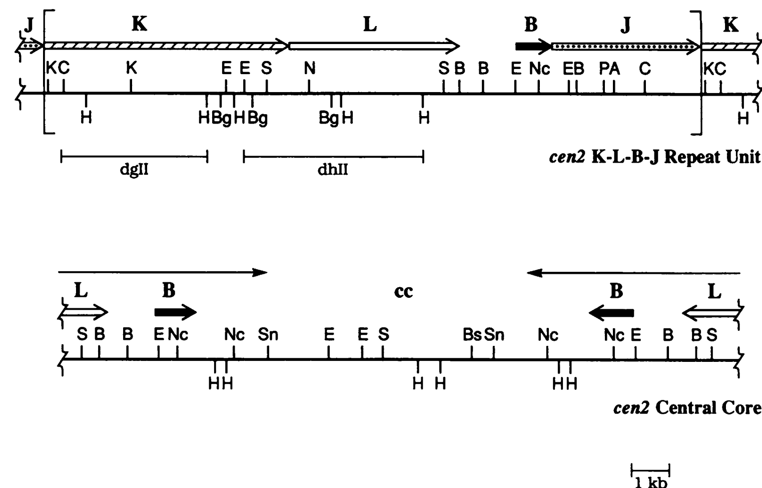


FIG. 5. Detailed restriction maps of the *cen2* K-L-B-J repeat unit and central core. Locations of the K, L, B, and J repeat sequences and central-core (cc) sequences are indicated. The relationship of repeats dgII and dhII, whose nucleotide sequences have been determined (24, 25), to repeats K and L is shown. The inverted repeat sequences flanking the central core extend to the upper arrowheads as shown in the lower portion of the figure. Restriction sites are as follows: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; C, *Cla*I (not all sites shown); E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; Nc, *Nco*I; P, *Pvu*II (not all sites shown); S, *Sph*I; Sn, *Sna*BI (not all sites shown).

pSp3-SNc is a reconstruction of the entire core region and also includes a small amount of the inverted repeat. Double-stranded plasmid pSp3-SNc DNA was restricted at a single *Nhe*I site within the pBR322 sequences, melted, allowed to reanneal rapidly, and spread for electron microscopy in the presence of formamide. Twenty single-stranded loop structures such as that shown in Fig. 6a were measured by using single-stranded ϕX174 DNA as a standard on the same grids. The size of the *cen2* central core was 7.0 ± 0.3 kb, and a detailed restriction map of the region is presented in Fig. 5. The central core itself did not contain any regions of obvious secondary structure (Fig. 6a).

Because the central core plasmid pSp3-SNc described above was constructed by joining inserts from the excised plasmids pSp3-S and pSp4-S, it was necessary to demonstrate that no genomic sequences were eliminated from the central core as a result of plasmid excisions and subsequent reconstruction. The DNA sequence of the region around the *Sph*I junction site within the central core was determined by using plasmids pSp3-S and pSp4-S as templates (see Materials and Methods). Two single-stranded DNA primers, 25 bases each, were constructed based on sequences from the regions about 100 to 120 bp on either side of the *Sph*I site, and a 244-bp sequence spanning the *Sph*I site was amplified via the polymerase chain reaction (28) process with total *S. pombe* genomic DNA as the template. The DNA sequence of the amplified fragment was identical to that obtained with pSp3-SNc as the template. This sequence is presented in Fig. 6b, along with the location of the specific primers used for polymerase chain reaction.

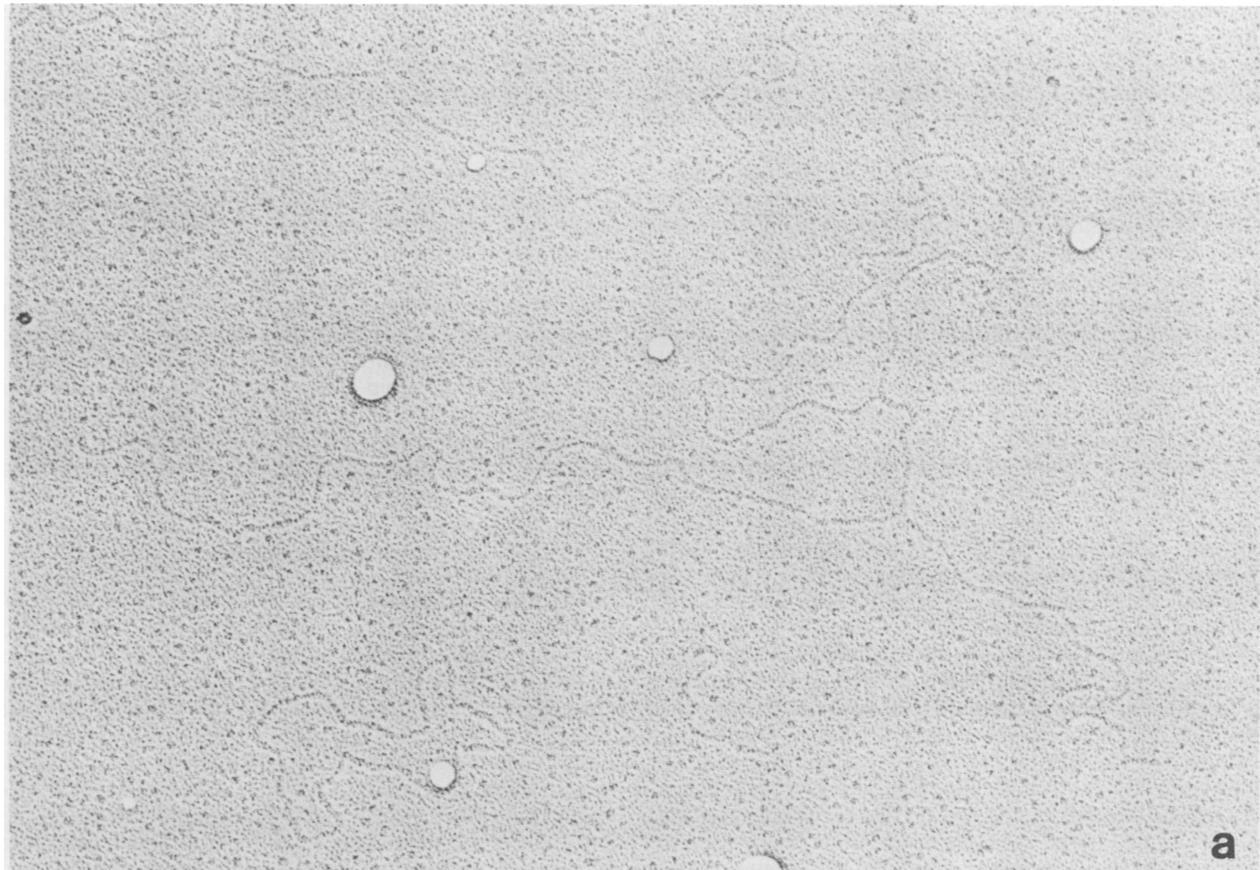
The region around the central-core *Sph*I site was approximately 75% A+T, in contrast to 65% for the genomic DNA as a whole (2). The sequence contained no extensive direct repeats, but did include several inverted repeats, the largest of which was 24 bases in length (positions 68 to 91, Fig. 6b). No homologies to the *S. cerevisiae* conserved centromere sequence elements CDEI and CDEIII (9) were apparent within this limited region.

Addition of heterologous sequences at various locations into the *cen2* region does not inactivate the centromere. The five integrant classes described above were derived in *S. pombe* diploid strain SBPD400 because it was anticipated that integration of additional sequences into the centromere region could inactivate the centromere and cause mitotic chromosome loss. The stable diploid SBPD400 was constructed by polyethylene glycol-induced fusion of two haploids of identical mating type (10), selecting for intragenic complementation of two mutant *ade6* alleles (12). Removal of adenine selection leads to gradual haploidization of the diploid population (10^{-3} frequency, Table 4), presumably because in *S. pombe* mitotic chromosome loss, which leads to formation of unstable aneuploids, results ultimately in the recovery of stable haploids (11, 18). Haploid colonies are easily distinguished from white diploid ones, as the presence of one or the other mutant *ade6* allele results in a pink or red colony. Thus, to assess the effect on mitotic chromosome stability of integration of heterologous sequences at various locations into the *cen2* region, each diploid Ade$^+$ integrant strain was grown on selective medium and then replated under nonselective conditions, and the rate of chromosome

TABLE 4. Chromosome stability in *cen2* integrant diploids[a]

Diploid strain	Integration site	Total no. of colonies scored	Frequency of haploidization and mitotic recombination (no. of half-sectored colonies)
SBPD400/9a	1	2,460	0.8×10^{-3}
SBPD400/3a	2	2,191	0.9×10^{-3}
SBPD400/10b	3	2,720	1.8×10^{-3}
SBPD400/12a	4	2,455	1.2×10^{-3}
SBPD400/11a	5	2,786	2.5×10^{-3}
SBPD400 (wild type)		2,373	1.3×10^{-3}

[a] Haploidization frequency of diploid strains was measured as described in Results.



b

```
  1  CCATAAAGTT TTATGATAAT TGTTGTGATT ATCAACTTTA CTAATTTGAC  50
 51  TCCTAATCAG TTAGAATTAT TTTAATAAAT TTACTAAAAC ATTAAACAAA 100
                                          SphI
101  CAACGGCACA CTGTTTTTTT GGTCACAGCT TCTAAGCATG CAAATGAAAT 150
151  TACTCAAGGA ATTTGCTGGC TTAATAATTT TATTTAATAA ATAAAGATTA 200
201  TAGTAGAAAA GAATGAAAAA GTATTTAGTT GACGATAGTA TTTGTTACAA 250
251  ATTAAAAACT CAAAACCATT CTTGCAGTAA GTCAATCGTG ATTGACA    297
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FIG. 6. Molecular characterization of the *cen2* central core. (a) Electron micrograph of stem-and-loop structure formed by rapid reannealing of single-stranded plasmid pSp3-SNc DNA. Samples were spread in 40% formamide for electron microscopy as described in Materials and Methods. Double-stranded DNA appears relatively thick and straight, whereas single-stranded regions are thin and kinked. Single-stranded circular φX174 DNA was spread on the same grid as a size standard. The size of the *cen2* central core is approximately 7 kb. (b) Nucleotide sequence around the *Sph*I site within the central core. The identical nucleotide sequence was obtained for both plasmid pSp3-SNc DNA and for polymerase chain reaction-amplified fragment from total *S. pombe* DNA by using the 25-base primers derived from the sequences underlined (see Materials and Methods). The location of the central-core *Sph*I site is indicated.

loss was determined by the ratio of half-sectored pink-white or red-white colonies to the total number of colonies. (This assay does not distinguish between haploidization frequency and mitotic recombination events that lead to homozygosity of one or the other mutant *ade6* alleles.) No significant differences were observed in the frequency of haploidization and mitotic recombination events between any of the integrant strains and the parent diploid (Table 4). Even sequence disruption and insertion into the inverted repeat sequences close to the central core (integration sites 3 and 4, Fig. 3) did not appear, within the limits of this assay, to destabilize the chromosome. Similarly, the insertion of over 80 kb of foreign DNA into the K repeat regions at each of the three *S. pombe* centromeres did not interfere with centromere function (10).

Central core sequences are insufficient for *cen2* function; a role for centromere-specific repeats is implicated. With the exception of pSp(cen2)-52C (described above) and pSp3-Sal (described in detail below), none of the plasmids shown in Fig. 4 possessed functional centromere activity. When assayed in *S. pombe*, they were mitotically unstable, with loss frequencies indistinguishable from that of the vector pSp500 (Table 1), and they rarely survived the two meiotic divisions (Table 2). For all experiments described herein, the structure of each plasmid was confirmed by Southern blot analysis (29) when reintroduced into *S. pombe*, because plasmid sequence rearrangements are common, particularly within sequences from the centromere regions.

Plasmid pSp(cen2)-52C contains the entire region of centromere-specific repeats on chromosome II (10), and its mitotic and meiotic behavior generally reflects that of the parental chromosomes (Tables 1, 2, and 3). Plasmid pSp3-Sal comprises the central core, a small amount of inverted repeat to the left and right of the core, and an entire B-K-L unit plus an additional B repeat to the right of the core (Fig. 4). This truncated minichromosome was mitotically stable (1.4×10^{-3} frequency of Ura^- segregants; Table 1) and segregated predominately 2+:2− through meiosis (in 89% of tetrads; Table 2). It exhibited a high degree of sister chromatid separation in the first meiotic division, however, as predominantly tetratype configurations were obtained in tetrad analysis, indicating segregation of the minichromo-

some homologs to nonsister spores (Table 3). Thus, the centromere sequences on pSp3-Sal specify several of the major roles of the centromere in meiosis, including spindle attachment and copy number control (2+:2− segregation), but lack the property of holding the sister chromatids together in meiosis I.

Plasmid pSp3-Sal was restricted with *Bst*EII, and a deletion plasmid, pSp3-Sal-dBst, was recovered in *E. coli*. The resulting 28-kb plasmid lacked sequences extending from the *Bst*EII site in the right portion of the central core (Fig. 5) through the right-most B-K-L unit to a *Bst*EII site just to the left of integration site 5 (Fig. 3). Thus, plasmid pSp3-Sal-dBst retained most of the central core and a B repeat but was missing the entire right (and most of the left) portion of the inverted repeat. The plasmid was mitotically unstable in *S. pombe*; thus, deletion of these sequences functionally inactivates the centromere.

Plasmids that contained all or a portion of the central core sequences but lacked the B-K-L-J region (pSp3-S, pSp4-S, and pSp3-SNc) were mitotically unstable in *S. pombe* (Table 1) and only rarely survived the two meiotic divisions (Table 2). Similarly, plasmids containing the B-K-L-J region but lacking the central core (pSp1-N-b, pSp2-N-b) did not contain a functional centromere (Tables 1 and 2). Thus, functional centromere activity appears to require an extensive region on *S. pombe* chromosome II. The only functional centromere sequences identified to date include both the central core and the centromere-specific repeats. One B-K-L unit along with the central core (pSp3-Sal) did not seem to provide complete centromere function, however, because at least portions of another unit are needed for proper meiotic behavior. At this point, it is unclear whether this is due to specific sequence requirements within the B-K-L and central core regions or merely reflects a need for an inverted repeat structure surrounding the central core.

Partial deletion of centromere-specific DNA sequence repeats results in aberrant meiotic segregation patterns. Circular plasmid pSp3-Sal was defective in maintaining sister chromatid attachment in the first meiotic division. This precocious sister chromatid separation observed for pSp3-Sal could be a result of its relatively small size (44 kb) or a lack of specific repeat sequences necessary for full centromere function. Circular plasmid pSp(cen2)-52C (105 kb), described above, and plasmids pSp(cen1)-7L (78 kb; linear) and pSp(cen3)-10C (163 kb; circular), all of which carry the entire region of centromere-specific repeats from their respective chromosomes, only occasionally failed to maintain sister chromatid attachment in the first meiotic division (Table 3) (14). As in the case of pSp3-Sal, the frequency of sister chromatid separation was considerably higher (50%) for the smaller (40 kb) circular *cen1* minichromosome pSp(cen1)-3C (14), which otherwise segregated stably through mitosis and meiosis. In budding yeast, increasing the size of minichromosomes carrying a complete *S. cerevisiae* centromere dramatically increases their mitotic stability, and aberrant meiotic behavior of large centromere-containing minichromosomes is rare (15, 23). Thus, we examined the functional consequences of increasing the size of pSp3-Sal by addition of random vector sequences.

When 42 kb (five copies) or 76 kb (nine copies) of vector pMB-neo sequences were added to pSp3-Sal (44 kb) to generate plasmids pSp3-Sal-neo$_{(5)}$ (86 kb) and pSp3-Sal-neo$_{(9)}$ (120 kb), respectively (see Materials and Methods), the mitotic stability of the plasmids increased about 10- to 20-fold (Table 1). However, the increase in size did not alter the high frequency of precocious sister chromatid separation in meiosis I (Table 3). Interestingly, increased mitotic stability with increasing size appears to be a property of functional centromere plasmids and does not seem to be applicable to other plasmids in *S. pombe*. When the sizes of the central-core plasmids pSp3-S (13 kb), pSp4-S (13 kb), and pSp3-SNc (17 kb) were increased by integrating additional vector sequences to generate plasmids pSp3-S-neo$_{(3)}$ (38 kb), pSp4-S-neo$_{(5)}$ (55 kb), and pSp3-SNc-neo$_{(4)}$ (50 kb), only very modest increases in mitotic stability (two- to threefold) were seen (Table 1). Therefore, it can be concluded that sequences required for mitotic segregation functions (spindle attachment) and distribution of the minichromosome to two of the four haploid progeny are present on pSp3-Sal, but the centromeric repeated sequences that are deleted in this plasmid are necessary for maintaining sister chromatid attachment in meiosis I. This functional role cannot be fulfilled by the addition of random vector sequences that increase the overall size of the minichromosome.

DISCUSSION

We have demonstrated that fully functional centromere activity is contained on a 100-kb genomic *Sal*I restriction fragment from chromosome II of *S. pombe*. A circular plasmid, pSp(cen2)52-C, bearing this fragment is stable through mitotic cell divisions (7.4×10^{-4} frequency of Ura$^-$ segregants; Table 1), segregates properly through both meiotic divisions, and thus behaves as a stable and independent genetic linkage group in *S. pombe*. Over 80 kb of the *cen2* region have been cloned as DNA fragments of various sizes on a set of plasmids derived by site-directed homologous integration of a vector into a repeated sequence located throughout the region, subsequent excision of the vector and surrounding centromere DNA, and recovery of the plasmids in *E. coli*. The structure of the *cen2* region was deduced from the structures of these plasmids, which have been reintroduced into *S. pombe* and assayed mitotically and meiotically to obtain a functional dissection of the centromere.

It has been shown previously that the centromere regions of *S. pombe* contain a number of centromere-specific repeats (6, 7, 10, 24, 25). Using the plasmid integration-excision strategy, we have identified an additional repeat unit, designated J, which is present twice within *cen2*, located between the leftmost K-L-B repeats, and in unknown locations within *cen3*. Several repeated regions within *cen2*, for example, the 0.9-kb *Bam*HI-*Eco*RI fragment directly adjacent to the B repeats (Fig. 1 and 5) and the 1.5-kb region directly flanking either side of the central core (Fig. 5), are repeated only within *cen2* and do not occur at the other *S. pombe* centromeres.

The repeated sequences in the *cen2* region are in part organized into a large inverted repeat (10). We show that the region contains four 14-kb tandem repeat units, two of which comprise most of the inverted repeat configuration that flanks a 7-kb central core of nonhomology. It is possible that a fifth 14-kb unit exists at the far left end of the region, but no integration sites in this region were identified. Chikashige et al. (6) have mapped only three large tandem repeat units in the *cen2* region; the discrepancies between their observations and ours may be due to strain differences. The frequency with which these tandemly repeated DNA sequences are lost by homologous recombination processes during mitotic and meiotic cell divisions has not been determined. It has recently been shown that an inverted repeat arrangement is also present at *cen1* and *cen3* (6). The presence of this

unusual palindromic configuration at all three centromeres in *S. pombe* argues that this structure serves a definite function. Early cytological studies with plant chromosomes have revealed that the arrangement of chromomeres and fibrillae in pericentric heterochromatin is bilaterally symmetrical about a central point, in the manner of a reversed repeat (19). Because this structural pattern is probably a reflection of underlying DNA sequence organization, pericentric DNA sequences could be arranged in a large palindromic repeat around a center of symmetry. Thus, the palindromic arrangements at *S. pombe* centromeres could be typical, on a reduced scale, of centromeric DNA structure in higher eucaryotes.

It has often been speculated what, if any, function centromeric heterochromatic sequences serve in higher eucaryotes (3, 17, 27). The *S. pombe* system provides an excellent model with which to investigate this question. We have shown that the 7-kb central core alone does not specify centromere function. Plasmids that contain portions or all of the core and only limited inverted repeat sequences (pSp3-SNc, for example) lack centromere function entirely, as do plasmids such as pSp1-N-b and pSp15 (Fig. 4) (10) that contain only a repeat unit or a repeat unit and a portion of the core. Plasmid pSp3-Sal, on the other hand, which contains the entire right arm of the inverted repeat (15.5 kb), a small portion of the left arm (3.5 kb), and the entire 7-kb central core, is stable in mitosis, segregates 2+:2− through meiosis, but still does not exhibit full function, as it shows a high degree of sister chromatid separation in meiosis I. Furthermore, precocious sister chromatid separation of pSp3-Sal occurs in most of the meioses in which the minichromosome segregates 2+:2−. One possible explanation for this behavior is that pSp3-Sal is undergoing a mitotic or meiosis II-like segregation in meiosis I. This aberrant meiotic behavior is not a property of the larger plasmid pSp(cen2)52-C, which carries all four large tandem repeat units, but was nevertheless observed if pSp3-Sal was simply made larger by integration of additional vector sequences. Thus, the evidence for a role for these heterochromatic-like, untranscribed repeats in centromere function in *S. pombe* is compelling. At least substantial portions of one large repeat unit plus the central core specify proper mitotic and meiotic segregation of minichromosomes, but portions or all of two units are required for full centromere function. It is unlikely that the tandem repeated units specify multiple spindle attachment sites, because pSp3-Sal retained mitotic and meiotic segregation functions and lacked all but one large repeat unit. Clearly, sequences that span a large region are involved in centromere function in *S. pombe*. It is possible that centromere function on all three *S. pombe* chromosomes is specified by the inverted repeat structure alone, as only this structure and no additional large tandem repeated units are present at *cen1* (6; K. Hahnenberger and L. Clarke, unpublished).

An obvious role to suggest for the *S. pombe* centromere central-core sequences would be that of specifying the spindle fiber attachment site(s). At the level of blot hybridizations, these sequences in *cen2* are unique in the *S. pombe* genome; they do not cross-hybridize to the other two centromeres and they are not transcribed (C. Polizzi and L. Clarke, unpublished). While centromeric repeats K and L are organized within *S. pombe* chromatin into nucleosomal arrays typical of bulk DNA, this nucleosomal pattern is completely disrupted for several kb within the central core and partially disrupted throughout the rest of the core (C. Polizzi and L. Clarke, unpublished). Thus, the central core is clearly a region of specialized structure within *S. pombe* chromatin.

It is unlikely that *S. pombe* centromere central-core or repeated sequences include spindle attachment sequences recognizable in the budding yeast *S. cerevisiae*. The three *S. pombe* centromere regions were originally cloned in *S. cerevisiae* in the linear YAC vector pMB-1, which carries *S. cerevisiae CEN4* (14). Plasmids containing two functional centromeres are very unstable in *S. cerevisiae* and rearrange to delete one or both of the centromere sequences (22). No rearrangements of the *S. pombe* centromere-containing minichromosomes were detected when propagated in *S. cerevisiae* for many generations. Either the *S. pombe* centromere is completely nonfunctional in *S. cerevisiae*, or the functional activity is too weak to be detected by the dicentric assay.

Previous work has shown that fully functional centromeres in *S. cerevisiae* are each specified by less than 150 bp of DNA that include two conserved sequence elements of 8 and 25 bp flanking an A+T-rich region of about 90 bp (8, 9). There are no heterochromatic repeated sequences in *S. cerevisiae* centromeres. This is in marked contrast to the *S. pombe* system, where functional *cen2* sequences span many kb of DNA. The possibility that a short sequence within the central core, for example, specifies centromere function is eliminated, because plasmids that contain all or portions of the core are inactive even if made considerably larger by integration of additional vector sequences. Furthermore, the active plasmid pSp3-Sal is rendered totally inactive by a large deletion of repeated sequences in pSp3-Sal-dBst, which retains nearly all of the central core. Thus, there appear to be two classes of centromeres in fungi; those of *S. cerevisiae*, which are relatively simple, and those of *S. pombe*, which are considerably more complex. It is possible that centromeres in budding yeast are indeed atypical, as those in *S. pombe* resemble, at least in their complexity and heterochromatic character, the centromeres of higher eucaryotes.

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