The Centromeric K-Type Repeat and the Central Core Are Together Sufficient to Establish a Functional *Schizosaccharomyces pombe* Centromere

Mary Baum, Vivian K. Ngan, and Louise Clarke

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

Submitted December 14, 1993; Accepted May 31, 1994

Monitoring Editor: Tim Hunt

The DNA requirements for centromere function in fission yeast have been investigated using a minichromosome assay system. Critical elements of *Schizosaccharomyces pombe* centromeric DNA are portions of the centromeric central core and sequences within a 2.1-kilobase segment found on all three chromosomes as part of the K-type (K/K"/dg) centromeric repeat. The *S. pombe* centromeric central core contains DNA sequences that appear functionally redundant, and the inverted repeat motif that flanks the central core in all native fission yeast centromeres is not essential for centromere function in circular minichromosomes. Tandem copies of centromeric repeat K", in conjunction with the central core, exert an additive effect on centromere function, increasing minichromosome mitotic stability with each additional copy. Centromeric repeats B and L, however, and parts of the central core and its core-associated repeat are dispensable and cannot substitute for K-type sequences. Several specific protein binding sites have been identified within the centromeric K-type repeat, consistent with a recently proposed model for centromere/kinetochore function in *S. pombe*.

INTRODUCTION

Faithful and equal distribution of eukaryotic chromosomes during cell division is dependent on centromere function. The centromere serves not only as the attachment site for spindle microtubules but also as a point of attachment for sister chromatids, holding them together through the first meiotic division. As the organizing center for kinetochore proteins, the centromere interfaces with one or as many as several dozen spindle microtubules, depending on the complexity of the organism, effecting chromosome movement and proper segregation in mitosis and meiosis.

In the budding yeast *Saccharomyces cerevisiae*, a small 125-basepair (bp) region on each chromosome contains all of the DNA sequences required in *cis* for full centromere function (Clarke and Carbon, 1985; Cottarel *et al.*, 1989). This region has been divided into three functionally distinct centromeric DNA elements (CDEI–CDEIII) based on nucleotide sequence and genetic assays of altered centromere regions in the native chromosomes and on minichromosomes (reviewed in Clarke and Carbon, 1985). Several proteins have been identified to date that interact with the *S. cerevisiae* centromere (Baker *et al.*, 1989; Cai and Davis, 1989; Jiang and Phi-

lippsen, 1989; Lechner and Carbon, 1991; Jiang *et al.*, 1993), including a motor activity capable of moving CEN3-complexed beads along microtubules (Hyman *et al.*, 1992). A single microtubule is thought to attach to the budding yeast kinetochore structure (Peterson and Ris, 1976).

In marked contrast to *S. cerevisiae*, centromeric DNA in primates encompasses 300–5000 kilobase pairs (kb), representing several percent of the genome (Waye and Willard, 1987; Willard, 1990), and is highly repetitive, consisting of tandem 171-bp monomers arranged into large arrays of α -satellite DNA that are chromosome-specific (Waye and Willard, 1987). A spatial analysis of the human centromere/kinetochore has been made with autoimmune antibodies from patients with CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) (Earnshaw and Rothfield, 1985; reviewed by Willard, 1990), leading to the identification of five centromere-binding proteins (CENP-A to -E). The CENP-B protein can bind two DNA molecules in vitro, each containing a 17-bp motif (the CENP-B box) found in a subset of the 171-bp α -satellite monomers, and possibly modulates a higher order structure at the centromere (Muro

et al., 1992). In the trilaminar kinetochore, the centromeric DNA appears to be associated with the inner kinetochore plate only, implying that this inner plate acts as the framework for assembly of the outer kinetochore plate (Cooke *et al.*, 1993). A repeat subunit model for centromere/kinetochore organization has been proposed by Zinkowski *et al.* (1991), who have demonstrated that mammalian kinetochores can be fragmented into many small units or "minikinetochores," which are still capable of spindle attachment and progress through mitosis in the usual manner. The functional analysis of mammalian centromeric DNA, however, is limited by the lack of an artificial chromosome assay.

The centromere of the fission yeast *Schizosaccharomyces pombe* is not as complex as that of higher eukaryotes and is more amenable to genetic and biochemical characterization. The three centromeric DNAs in *S. pombe* span ~40–100 kb (~1–3% of the chromosome length) and contain distinct classes of centromere-specific repeated DNA arranged entirely or partially into a large inverted repeat (Figure 1A). The organization of repeats within larger arrays (e.g., K/L/B in *cen2*) is chromosome-specific, and the number of arrays is variable and strain-dependent, somewhat analogous to centromere repeat organization in higher eukaryotes (Clarke and Baum, 1990; Hahnenberger *et al.*, 1991; Steiner *et al.*, 1993). The arms of the inverted repeat at each *S. pombe* centromere are separated by a central core (4–7 kb) of asymmetric DNA. The central cores are further differentiated at the chromatin level. Unlike the inverted repeat sequences, which are packaged into regularly spaced nucleosomes, the central core chromatin lacks regular nucleosomal packaging and is nuclease resistant (Polizzi and Clarke, 1991; Takahashi *et al.*, 1992). The central core of *cen2* (cc2) is unique in the *S. pombe* genome, but cc1 and cc3 contain a large homologous region, in addition to a small amount of unique sequences (Takahashi *et al.*, 1992).

Centromeric DNAs of each of the three *S. pombe* chromosomes (*cen1*, *cen2*, or *cen3*), cloned as large restriction fragments into yeast artificial chromosomes, exhibit the hallmarks of normal chromosomal centromeres when these constructs are introduced into *S. pombe*. A minichromosome that carries a complete *S. pombe* centromeric DNA region is mitotically very stable (loss rate of 10^{-3} – 10^{-4}) and segregates faithfully through meiosis I and II (Hahnenberger *et al.*, 1989; Clarke and Baum, 1990). It behaves essentially as a fourth genetic linkage group in *S. pombe*. Functional assays have also been performed on smaller contiguous portions of *S. pombe* centromeric DNA (7–38 kb) that have been recovered as circular plasmids by integration/excision strategies (Clarke and Baum, 1990; Hahnenberger *et al.*, 1991) or gap repair (Matsumoto *et al.*, 1990; Takahashi *et al.*, 1992). All functional minichromosomes derived in this way from *cen1*, *cen2*, or *cen3* share structural similarities. They contain the centromeric central core with all, or nearly all, of one arm of the centromere-

specific repeat array on one side and at least several kb of the complementary arm of the repeat array on the other side, forming a small inverted repeat. Such constructs are lost in *S. pombe* in approximately one in 100 mitotic cell divisions and predominantly segregate to two of the four meiotic products (2+:2–) in crosses in which the *cen*-plasmid is segregating as an aneuploid minichromosome ($n \times n + \text{minichromosome}$). However, they tend to undergo sister chromatid separation in the first rather than the second meiotic division (Clarke and Baum, 1990; Hahnenberger *et al.*, 1991; Takahashi *et al.*, 1992). Therefore, centromeric repeat sequences appear to play a role in maintaining sister chromatid attachment in meiosis I. If the centromeric repeat array and the central core region are assayed separately (Clarke and Baum, 1990), the individual constructs behave as *Ars* plasmids lacking centromeres and are lost in one of every two or three cell divisions. Thus, these observations suggest that several centromeric DNA elements, encompassing many kilobase pairs, together make up functional *S. pombe* centromeric DNA.

Although the DNA sequence of the centromeric repeats and central core regions is known, this information has not been helpful in determining which centromeric DNA elements, unique or repeated, are necessary and sufficient for centromere function, and no centromere-binding proteins or sites for such proteins have been described for *S. pombe* (Clarke *et al.*, 1986; Nakaseko *et al.*, 1986, 1987; Chikashige *et al.*, 1989; Clarke and Baum, 1990; Takahashi *et al.*, 1992). Deletion or replacement of centromeric sequences in minichromosomes has, however, demonstrated that establishment of an active centromere requires K repeat and central core sequences (Hahnenberger *et al.*, 1991; Takahashi *et al.*, 1992).

We present here a comprehensive analysis of combinations of centromeric DNA sequences derived from *cen1* and *cen2* that are assayed as circular minichromosomes for the centromere-defining parameters of mitotic stability and 2+:2– meiotic segregation. The results of these analyses show that many functional combinations are possible, but key components for centromere function are portions of the central core, which are functionally redundant, and a 2.1-kb *Kpn* I-*Kpn* I fragment found at all three native centromeres within each K-type repeat. We also show that the centromeric inverted repeat motif is not essential for centromere function in circular minichromosomes. In addition, we report the identification of several protein binding sites within the critical centromeric K-type repeat.

MATERIALS AND METHODS

Strains, Transformations, and Genetic Manipulations

S. pombe strains used in this study are Sp223 (*h⁻ ade6.216 leu1.32 ura4.294*, gift from D. Beach, Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), SBP120390 (*h⁻*

ade6.704 leu1.32 ura4.294, from this laboratory), SBPD400 (*h⁻/h⁻ ade6.210/ade6.216 leu1.32/leu1.32 ura4.294/ura4.294 can^s/can^s*) (Fishel *et al.*, 1988), SBP32588 (*h⁺ leu1.32 ura4.294 tps13.1 lys1.1 LEU2⁺* [III]) (Hahnenberger *et al.*, 1989), SBP41786-2 (*h⁺ leu1.32 ura4.294 tps13.1 lys1.1 fur1.1*) (Hahnenberger *et al.*, 1991), and SBP42192 (*mat1-P mat2.3Δ::LEU2⁺::his2 lys1.1 tps13.1 ura4.294*). SBP42192 was constructed in this laboratory with strains kindly provided by Amar Klar (ABL-Basic Research Program, Laboratory of Eukaryotic Gene Expression, Frederick, MD). Because *mat2* and *mat3* are deleted (Klar and Miglio, 1986), the mating type of SBP42192 (*mat1-P*) is stable. Growth media and conditions were as described (Gutz *et al.*, 1974; Moreno *et al.*, 1991). DNA transformations were performed as previously described (Ito *et al.*, 1983) or with an alkali-cation yeast transformation kit (Bio 101, La Jolla, CA).

Plasmids were integrated by site-directed homologous recombination into the *cen2* region of SBPD400 or Sp223 as previously described (Clarke and Baum, 1990). Plasmid pSp-CSal was generated by site-directed integration at the *EcoRI* sites in *cc2* (Figure 2B, site *c*) of a pBR322-derived *ura4* vector containing a 1.3-kb *Cla* I-*Sph* I *cc2* restriction fragment. Genomic DNA was digested with *Sal* I to liberate a fragment containing the pBR322-derived integrated plasmid along with flanking genomic sequences. For plasmid recovery, the digested DNA was ligated and used to select *S. pombe* Ura⁺ transformants.

A series of plasmids, modeled after pSp3-Sal (Clarke and Baum, 1990), that either lack *cc2* or contain small substitutions in place of specific *cc2* restriction fragments were generated by an integration/excision scheme to delete *cc2*, followed by fragment-mediated replacement of portions of *cc2*. First, a small central core deletion plasmid carrying a single *Spe* I site (pSp3-SNcΔSp) was constructed from a partial *Spe* I digestion of a pSp3-SNc-derived plasmid (Figures 2A and 4) (Clarke and Baum, 1990) in which *ura4* was replaced by *leu1*. Plasmid pSp3-SNcΔSp is missing the 6.8-kb region between the *Spe* I site in the left core-associated repeat and the *Spe* I site that is nearest the right end of *cc2* and carries only 0.5 kb of *cc2* sequences. The remaining *Spe* I site (Figure 2B, site *r*) allowed site-directed integration of pSp3-SNcΔSp into the *cen2* core-associated repeat of *S. pombe* strain Sp223. Digestion of the genomic DNA with *Sal* I, circularization of the restriction fragments, and subsequent transformation into *S. pombe* allowed the rescue of a 39-kb plasmid, pSp3-SalΔSp (Figure 2B), which lacks most of *cc2*. The latter plasmid was the recipient for fragment-mediated replacements to generate central core alterations.

DNA segments containing altered *cc2* regions were constructed in vitro and then introduced into minichromosomes by fragment-mediated transplacement. First, a 8.6-kb *Nco* I-*Nco* I fragment encompassing *cc2* was cloned in a pBluescript-derived vector (pKNS[-]), gift from R. Kuhn and J. Carbon, University of California, Santa Barbara, CA) in which the *Sma* I site is replaced by a *Nco* I site to form pKNS-*cc2*. A 2.8-kb *Cla* I-BamHI *ura4::sup3-e* cassette (gift from J. Carbon) was then substituted for *cc2* restriction fragments in pKNS-*cc2*. The *sup3-e* gene (Hofer *et al.*, 1979; Kohli *et al.*, 1989) was kindly provided by Ian Willis (Albert Einstein College of Medicine, Bronx, NY). To replace a larger portion of *cc2*, a phage λ *EcoRV*-*Sna*BI restriction fragment (nucleotides 8822–12002), ligated to the *ura4::sup3-e* cassette, was cloned into pKNS-*cc2* between the *Nde* I and rightmost *Sna*BI sites of the central core (Figure 2B). The *Nco* I fragment from these plasmids with an altered central core region was used for fragment-mediated replacement into the plasmid pSp3-SalΔSp in SBP120390 (Rothstein, 1991), creating a series of pSp3-Sal-like plasmids with various *cc2* alterations.

Centromere-inactive small minichromosomes and those with marginal centromere function were made larger by the site-directed integration of linearized pMB-neo (8.5 kb) at the *Sca* I site in the Ap^r gene of the vector (Clarke and Baum, 1990). The *S. cerevisiae* *LEU2* gene in pMB-neo complements the *S. pombe* *leu1.32* mutation, although presumably poorly, because tandem integrations of the plasmid are usually observed (Fishel *et al.*, 1988). The number of copies of pMB-neo inserted into the minichromosome was determined by *Not* I digestion of transformant DNA and Southern analysis.

The mitotic stability of minichromosomes was assayed by two methods. The replica-plating method (Clarke and Baum, 1990) was

used to assay for unstable plasmids (loss rate $\geq 1 \times 10^{-1}$) or stable minichromosomes that carried only the *ura4* marker. The mitotic stability is expressed as the apparent mitotic loss frequency, which is calculated as the ratio of the number of cells that have lost the plasmid and hence are unable to grow on selective media. Alternatively, a color assay method using an opal nonsense suppressor tRNA gene, *sup3-e*, as a marker for the minichromosome allowed for a more accurate quantitation of loss rates for stable minichromosomes (Hieter *et al.*, 1985). In the latter method, serial dilutions of a single colony that had been grown under selection for the minichromosome were plated on rich media. Because adenine is limiting in the medium, colonies that lose the minichromosome accumulate a dark red pigment because of the *ade6.704* mutation in the host strain. Cells that retain the minichromosome are white because of suppression of the mutation. Half-sector colonies indicate loss of the minichromosome in the first cell division after plating. The loss rate equals the number of half-sector colonies divided by the sum of half-sector plus white colonies, as described by Hieter *et al.* (1985).

We have observed that mitotic loss rates of plasmids assayed by the above methods appear 10-fold higher than those for the same plasmids assayed by the 5-fluoroorotic acid (FOA) method used in previous studies (Hahnenberger *et al.*, 1989, 1991; Clarke and Baum, 1990), probably because of an artifact of the FOA assay. FOA must have a toxic effect, even on some cells that have lost the minichromosome, resulting in fewer colonies and a skewed mitotic loss rate. The colony color assay and the replica-plating method for determining mitotic stability are more reliable, as results from these assays are comparable and never differ by more than twofold.

The meiotic behavior of constructs was assayed through tetrad analysis as described by Hahnenberger *et al.* (1989). The genes *lys1.1* and *tps13.1* are tightly linked to *cen1* and *cen2*, respectively, whereas *fur1.1* (SBP42192) and *LEU2⁺* (SBP32588) are linked to *cen3*, and thus serve as markers for the three *S. pombe* chromosomes. The genes *ura4* or *leu1* serve as a marker for the minichromosome, which essentially behaves as a fourth genetic linkage group. We assume that tetrads with a 4+0- and 3+1- distribution of the minichromosome marker arose from diploids that carried more than one copy of the minichromosome before meiosis. Tetrads that do not contain the minichromosome (0+4-) result from cells in the population that lost the minichromosome before mating or progeny that received the minichromosome but lost it during the ~30 mitotic divisions between meiosis and the assay for the minichromosome.

Minichromosomes Constructed In Vitro

Plasmids were constructed using standard techniques (Maniatis *et al.*, 1982) and were propagated in *Escherichia coli* strain DH5α (*recA1*, Bethesda Research Laboratories, Bethesda, MD). Circular minichromosomes were constructed by in vitro ligation of various centromeric DNA sequences. Plasmids derived from pSp3-SNc (Clarke and Baum, 1990) include pSp3-SNc+K and pSp3-SNc+Kopp, which contain the K repeat as a 6.4-kb *Cla* I fragment from *cen3* (Figure 1B) cloned into the BamHI site of pSp3-SNc (Clarke *et al.*, 1993). Plasmids pSp3-SNc+K⁺, pSp3-SNc+K⁺opp, and pSp3-SNc+K⁺K⁺K⁺ (Figure 4) were constructed in an identical manner and contain the *cen1* K⁺ repeat as a 5.6-kb BamHI-*Nco* I fragment cloned into the BamHI site of pSp3-SNc.

A similar set of plasmids was derived from pSp-*cc2* (Figure 4). pSp-*cc2* was constructed from pKNS-*cc2* by replacing the *Nco* I site near the right side of *cc2* with a *Sal* I site to allow cloning of the *ura4::sup3-e* cassette as a 2.3-kb *Cla* I-*Kpn* I fragment into the *Sal* I-*Kpn* I sites in the pBluescript polylinker. Plasmid pSp-*cc2*+Kpn was constructed by cloning the 2.1-kb *Kpn* I-*Kpn* I K⁺ fragment into the *Kpn* I site of pSp-*cc2* such that the *ura4::sup3-e* cassette lies between the right core-associated repeat sequences and the 2.1-kb fragment. pSp-*cc2*+K⁺ was constructed by cloning the K⁺ repeat into the BamHI-*Nco* I sites in the Bluescript polylinker, placing the K⁺ repeat at the left boundary of core-associated repeat sequences. pSp-*cc2*+K⁺K⁺ was constructed by adding a second copy of the K⁺ repeat, through blunt-end ligation, at the *Nco* I site of pSp-*cc2*+K⁺. pSp-*cc2*+K⁺K⁺K⁺ was constructed by excision of the tandem K⁺ repeat units from pSp3-SNc+K⁺K⁺K⁺ as a

Figure 1. Centromeric DNA regions in *S. pombe* strain Sp223. (A) Diagrammatic representation of the DNA organization in the three *S. pombe* centromeres. A large inverted repeat (bold arrows) flanking an asymmetrical central core (cc) is a conserved motif at each centromere. The repeated DNA elements B, J, K, L, and M are organized into centromere-specific arrays. The number of arrays is variable and strain-dependent (Steiner *et al.*, 1993). The repeated DNA found immediately adjacent to the central core (core-associated repeat; $\square$, in *cen1* and *cen3*; $\blacksquare$, in *cen2*) is specific to that particular centromere, except for the inclusion of several tRNA genes (Kuhn *et al.*, 1991; Takahashi *et al.*, 1991). It is not known if the tRNA genes are transcribed, but there is no evidence for transcription of any other centromeric sequences in *S. pombe* (Fishel *et al.*, 1988; Polizzi *et al.*, 1991). K' sequences are found as contiguous sequences within *cen2* and *cen3*, as indicated by the brackets, and are in an orientation opposite to those in *cen1* (see below). (B) The structural relationship between K-type repeats from the three *S. pombe* centromeres. The sequences representing K-type repeats and adjacent regions in *cen1*, *cen2*, and *cen3* are depicted diagrammatically. Location of some restriction sites are shown for reference. Although the K repeats in *cen2* and *cen3* appear to be identical, the *cen1* K' repeat is defined as a 5.6-kb region (*Bam*HI-*Nco* I) that is essentially equivalent to the 4.4-kb tail of the K repeat plus an additional 1.2-kb region (*Kpn* I-*Nco* I), most of which is identical to the head of the J repeat. A 300-bp stretch adjacent to the *Kpn* I site in this 1.2-kb region

is duplicated at the very tip of the head of the K repeat (Chikashige *et al.*, 1989; Ngan and Clarke, unpublished data). The 150 bp at the *Nco* I end of the K' repeat appears to be *cen1*-specific and is more accurately designated as part of the *cen1* core-associated repeat sequences (Hahnenberger *et al.*, 1991). The sequences present in the alternatively designated dg repeats (Nakaseko *et al.*, 1986; Chikashige *et al.*, 1989; Murakami *et al.*, 1991) are shown as arrows over the diagrammatic representations. Other arrows define centromeric repeat elements J, L, M, and B'. The direction in which the central core is located, with respect to the K-type repeats is indicated for each centromere. Restriction sites are as follows: B, *Bam*HI; C, *Cla* I; E, *Eco*RI; H, *Hind*III; K, *Kpn* I; Nc, *Nco* I; and S, *Sph* I. Not all *Sph* I sites are shown.

17.7-kb *Nhe* I-*Eco*RI fragment and subsequent cloning into the *Xba* I site of pSp-cc2. pSp-cc2 Δ NcSn+K''K''K'' was constructed by isolation of the 7.6-kb *Sna*BI-*Nco* I portion of pSp-cc2, through a complete *Nco* I/partial *Sna*BI digest and ligation to the 17.7-kb fragment containing the tandem K'' repeat units. From pSp-cc2 Δ NcSn+K''K''K'', two deletion plasmids, pSp-cc2 Δ NcNd+K''K''K'' and pSp-cc2 Δ NcSn Δ BsSal+K''K''K'', were derived by digestion with the appropriate restriction enzymes and recircularization. pSp-cc2 Δ SSal+K''K''K''opp was constructed by replacement of the 8.6-kb *Nco* I-*Nco* I fragment in pSp-cc2 with the left half of cc2 (*Nco* I-*Sph* I) as a 4.3-kb *Nco* I-*Sal* I fragment from pSp3-S (Clarke and Baum, 1990) and addition of three tandem K'' repeat units. Southern analysis of transformant DNA with indirect end-labeling indicated that this plasmid suffered a deletion of one copy of K'' when transformed into *S. pombe*.

Plasmids containing specific deletions within the K'' repeat (Figure 5) were derived from the pSp(*cen1*)-BHp minichromosome (Hahnenberger *et al.*, 1991). The pSp(*cen1*)-BHp Δ B-K construct, which carries a deletion of the 2.3-kb *Bam*HI-*Kpn* I portion of the K'' repeat, was made by digesting pSp(*cen1*)-BHp with *Bam*HI and *Kpn* I and, subsequently, adding back the 2.1-kb K'' *Kpn* I-Kpn I fragment. This K'' *Kpn* I-Kpn I fragment, previously subcloned into pKNS(-),

was liberated as a *Bam*HI-*Kpn* I fragment for directional ligation to the *Kpn* I-*Bam*HI portion of pSp(*cen1*)-BHp so that the original orientation could be preserved. pSp-BHp Δ Kpn was made by digesting pSp(*cen1*)-BHp with *Kpn* I to drop out the 2.1-kb K'' *Kpn* I-Kpn I fragment and religating the ends to form a circular plasmid. pSp(*cen1*)-BHp Δ K-Nc was constructed by removing the entire K'' repeat (the 5.6-kb *Bam*HI-*Nco* I fragment) from pSp(*cen1*)-BHp through *Bam*HI complete and *Nco* I partial digests and then adding back the 4.4-kb *Bam*HI-*Kpn* I-Kpn I portion of K''. This 4.4-kb fragment was first subcloned into pKNS(-) so that it could be isolated as a *Bam*HI-*Nco* I fragment for directional ligation to the 17.5-kb *Nco* I-*Bam*HI portion of pSp(*cen1*)-BHp.

DNA Isolation, Field Inversion Gel Electrophoresis (FIGE), and Southern Analysis

The structure of each minichromosome in *S. pombe* was verified by Southern analysis with indirect end-labeling. Total DNA was isolated by the method of Beach and Klar (1984) and manipulated with cut-off micropipette tips. Following complete digestion at a unique re-

striction site adjacent to vector sequences (20–40 U/ μ g, 37°C, 3 h), serial threefold dilutions of a second enzyme were used to produce partial digestion products (37°C, 20 min). Digestion products were electrophoresed on a 1% agarose field inversion gel (Carle *et al.*, 1986) at 180 V for 17 h as described by Hahnenberger *et al.* (1991). After FIGE, the DNA was hybridized to ³²P-pBR322 as described by Steiner *et al.* (1993).

Primers and Polymerase Chain Reaction (PCR) Amplification

The synthetic oligonucleotide primers used in this study are the following:

5'-GAATTCGCTCTTATACTTGCCAA-3' (G-H 5'),
5'-GAATTCAGCCAATAATGACAAG-3' (G-H 3'),
5'-GAATTCGACTTTGGAGTAAATA-3' (H-I 5'), and
5'-GAATTCATTTATGATTCTTCC-3' (H-I 3').

The primers were synthesized by Operon Technologies (Alameda, CA). The *Eco*RI ends on the primers were added to facilitate cloning of the amplified product. PCR amplification of the G-H and H-I fragments was performed as follows. The reaction consisted of 1 ng of template DNA (the 5.6-kb *Bam*HI-*Nco*I K' repeat cloned into a pBluescript-derived vector), 200 ng (0.67 μ M) of each primer, 0.2 mM of each dNTP, and 0.6 U of *Taq* polymerase (AmpliTaq, Cetus) in a 20- μ l volume. The amplification parameters used were 25 cycles of denaturation at 94°C for 1 min and annealing/extension at 45°C for 2 min. The amplified products are a 250-bp fragment spanning approximately the left half of fragment H, and a 320-bp fragment encompassing the *Eco*RV site between K' restriction fragments H and I (Figure 6A).

Crude Nuclear Extract Isolation

Crude nuclear extract from a 2-L culture of *S. pombe* strain Sp223 was prepared as follows. While on ice, harvested nuclei (prepared as described by Polizzi and Clarke [1991]) were resuspended in a hypotonic buffer (400 mM NaCl, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid [HEPES]-KOH (pH 7.9), 1.5 mM MgCl₂, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.5 mM dithiothreitol [DTT], 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM NaF; Newman *et al.*, 1985) and disrupted with a Dounce homogenizer (10 strokes using pestle B). The homogenate was adjusted to 1 M salt by the addition of KCl, stirred slowly on ice for 30 min, and centrifuged for 1 h at 100 000 $\times$ g and 4°C to pellet nuclear debris. The supernatant fraction (crude nuclear extract) was divided into small aliquots, flash frozen in liquid nitrogen, and stored at -70°C. The protein concentration of the extract was determined to be 9.4 mg/ml by a Bradford assay (Bradford, 1976).

Gel Mobility Shift Assays

The DNA probes used in the gel mobility shift assays were small (<500 bp) fragments of the K' repeat isolated as restriction fragments (Figure 6A) with the exception of fragments G-H and H-I, which were generated as described above by PCR amplification. These fragments were radiolabeled by filling in 5'-overhanging restriction ends with ³²P-labeled dNTPs using the Klenow fragment of DNA polymerase I. Gel mobility shift assays were performed as described by Garner and Revzin (1981) with the modifications of Lechner and Carbon (1991). The conditions for DNA/protein binding were 10 mM HEPES (pH 8.0), 6 mM MgCl₂, 2 mM NaF, 0.5 mM DTT, 10% glycerol, and 100 mM KCl. In a 20- μ l volume, ~5 fmol of labeled fragment was allowed to bind to proteins in the crude nuclear extract (~5 μ g total protein). Different amounts (0.5, 0.75, 1, and 2 μ g) of poly(dI-dC) were used as the nonspecific competitor. To show that a mobility shift was because of specific protein binding, 125 fmol (25-fold excess) of the unlabeled DNA fragment was used to compete with the labeled fragment in the binding reaction. The binding reactions were electrophoresed through a 4% nondenaturing polyacrylamide gel in 1 $\times$

tris(hydroxymethyl)aminomethane (50 mM)/glycine (380 mM) buffer (pH 8.5) at 12 V/cm for 2.5 h.

RESULTS

Centromeric Central Core Sequences Are Necessary for Centromere Function in *S. pombe* and Show some Functional Redundancy

The unusual structure of *S. pombe* centromeric central core chromatin and the lack of regularly spaced nucleosomes in this region (Polizzi *et al.*, 1991; Takahashi *et al.*, 1992) led us to speculate that the centromeric central core might be a critical region involved in kinetochore formation. Additionally, all previously described *S. pombe* minichromosomes that are active with respect to centromere function contain the central core. Numerous attempts to study the effect of eliminating *cen2* central core (cc2) sequences via fragment-mediated DNA transplacement were unsuccessful, although this technique had been used successfully for *cen3*, resulting in centromere inactivation of *cen3*-derived minichromosomes (Takahashi *et al.*, 1992). We found that DNA fragment-mediated conversion was possible near the B repeat flanking cc2 (Figure 2A) but did not propagate across the central core, indicating this region may be protected, and thus inaccessible, by virtue of its unique architecture. A plasmid lacking cc2, but otherwise identical to the centromere-active minichromosome pSp3-Sal (Figure 2A) (Clarke and Baum, 1990), was obtained using an integration/excision scheme (see MATERIALS AND METHODS). The resultant plasmid, pSp3-Sal Δ Sp (Figure 2B), lacks 0.5 kb of the left core-associated repeat and 6.3 kb of cc2. When introduced into *S. pombe*, pSp3-Sal Δ Sp was mitotically unstable and only rarely survived meiosis (Table 1). Because the arms of the inverted repeat in this plasmid are separated by only 1.1 kb, this close proximity may sterically preclude kinetochore formation. Alternatively, the deletion of specific cc2 sequences may remove a critical aspect of the structural framework necessary for kinetochore assembly.

To test whether specific cc2 sequences are essential for centromere function, cc2 was replaced by an equivalent amount of heterologous DNA. Unlike plasmid pSp3-Sal, which is refractory to transplacements in the central core, the remaining central core sequences of the centromere-inactive plasmid pSp3-Sal Δ Sp could be manipulated in *S. pombe* by fragment-mediated conversion to either restore cc2 or to replace it with heterologous DNA. A 6-kb segment of DNA, consisting of the 2.8-kb *ura4:sup3-e* cassette (MATERIALS AND METHODS) and 3.2 kb of phage λ sequences, was substituted for the native 5.1-kb *Nde*I-*Sna*BI region of cc2 to maintain the proper spacing between the core-associated repeats. The resultant plasmid pSp3-Sal Δ NdSn+ λ (Figure 2B) contains only a small amount of cc2 sequences, 1.1 and 0.6 kb at the left and right sides of the central core, respectively. This plasmid is totally inactive with regard to mitotic centromere activity

Figure 2. In vivo-generated circular minichromosomes derived from *cen2*. (A) Previously described minichromosomes (Clarke and Baum, 1990) are shown aligned with a schematic map of *cen2*. (B) An enlarged view of *cc2* flanked by the core-associated repeat (black arrow) and the B repeat (striped arrow). The structure of minichromosomes with deletions in *cc2* or flanking regions as well as minichromosomes containing replacements for parts of *cc2* are aligned with the restriction map above to show the boundaries of centromeric DNA contained on the minichromosomes. The centromeric DNA is depicted as a solid line in functional minichromosomes and as a dashed line in nonfunctional minichromosomes. Those minichromosomes designated as functional are mitotically stable, segregate predominantly 2+2— through meiosis, but demonstrate a high degree of precocious sister chromatid separation at meiosis I. The dotted line in pSp3-SalΔSp shows the extent of the deletion in this plasmid. Central core replacements use the *ura4:sup3-e* cassette, with or without phage λ DNA to substitute for missing *cc2* sequences. Rotating arrows at the ends of the *cc2* region in the pSp3-SalΔNdE construct indicate that the central core is inverted in orientation relative to the native chromosome II. The construction of all the minichromosomes shown is described in MATERIALS AND METHODS. Vector sequences ori/Ap^R/leu1 and ori/Ap^R/ura4 are not drawn to scale and are 7.1 and 5.9 kb, respectively. Arrows at *c* and *r* indicate integration sites used in plasmid integration/excision strategies. Restriction sites are as follows: B, *Bam*HI; Bs, *Bst*EII; C, *Cla* I; E, *Eco*RI; H, *Hind*III; Nc, *Nco* I; Nd, *Nde* I; S, *Sph* I; Sn, *Sna*BI; and Sp, *Spe* I; not all *Sna*BI or *Spe* I sites are shown.

and is not detectable after meiosis (Table 1). These results suggest that a substantial portion of the central core may be necessary for function or that a particular region contained within this central 5.1 kb of DNA may be required. Alternatively, transcription of the *ura4:sup3-e* cassette within the central core may interfere with centromere function. However, the presence of these genes within *cc2* has no effect on minichromosome segregation, as shown by the similar mitotic loss rates and meiotic segregation patterns exhibited by pSp3-Sal and pSp3-Sal-us (Figure 2, A and B, and Table 1) (Clarke and Baum, 1990). Both constructs are mitotically stable, as is expected of minichromosomes with functional centromeres. In addition, both are predominantly seen in two of the four meiotic products in crosses where the minichromosome is segregating as an aneuploid chromosome ($n \times n +$ minichromosome, see MATERIALS AND METHODS). In a majority of the meiotic events,

however, these minichromosomes and all minichromosomes that lack portions of the centromere-specific repeats are found in nonsister spores, indicating premature separation of sister chromatids in meiosis I. Furthermore, an in vivo insertion of ~9 kb of pBR322-derived plasmid DNA into *cc2* within the native chromosome II also has no detectable effect on chromosome stability. Thus, the insertion of transcribed genes or heterologous sequences into the central core does not appear to significantly alter or inhibit centromere function.

To determine whether a specific sequence within the central core is uniquely required for centromere activity, we systematically restored regions of *cc2* to pSp3-SalΔSp, while maintaining the proper spacing between the arms of the inverted repeat. The 2.8-kb *ura4:sup3-e* cassette was used as a substitute for the missing *cc2* portion. A series of plasmids containing small deletions

Table 1. Mitotic stability and meiotic segregation of *cen1*- and *cen2*-derived minichromosomes

Host	Minichromosome	Size (kb)	Mitotic loss frequency	Meiotic distribution of the minichromosome					Sister:nonsister distribution
				4+:0-	3+:1-	2+:2-	1+3:-	0+:4-	
Functional									
I.	SBP120390 pSp3-Sal-us	48.4	9.1×10^{-3}	0	0	11 (69%)	4	1	2:9
	SBP120390 pSp3-SalΔNdE	47.0	1.3×10^{-2}	0	0	13 (72%)	2	3	6:7
	SPB120390 pSp3-SalΔEH	45.7	2.0×10^{-2}	0	0	14 (74%)	3	1	6:8
	SBP120390 pSp3-SalΔHBs	47.2	1.0×10^{-2}	0	0	16 (89%)	0	2	8:8
II.	Sp223 pSp3-SNc+K	23.9	6.4×10^{-2}	4	8	8 (33%)	3	1	3:5
	Sp223 pSp3-SNc+Kopp	23.9	8.1×10^{-2}	2	6	6 (38%)	1	1	1:5
	Sp223 pSp3-SNc+K"	23.1	6.9×10^{-2}	3	8	28 (48%)	6	14	10:18
	Sp223 pSp3-SNc+K"opp	23.1	4.3×10^{-2}	2	8	14 (50%)	3	1	4:10
	Sp223 pSp3-SNc+K"K"K"	34.3	1.3×10^{-3}	0	2	40 (85%)	5	0	16:24
III.	SBP120390 pSp-cc2+K"	19.4	1.2×10^{-1}	1	1	12 (43%)	4	10	3:9
	SBP120390 pSp-cc2+K"+neo ₃	44.9	1.8×10^{-2}	0	3	29 (60%)	4	12	4:25
	SBP120390 pSp-cc2+K"K"	25.0	8.6×10^{-3}	0	0	18 (60%)	8	4	9:9
	SBP120390 pSp-cc2+K"K"K"	31.5	6.6×10^{-3}	0	0	33 (73%)	5	7	15:18
IV.	Sp223 pSp(<i>cen1</i>)-BHp ^a	23.2	3.7×10^{-2}	1	5	13 (50%)	2	5	2:11
	Sp223 pSp(<i>cen1</i>)-BHpΔB-K	20.9	4.8×10^{-2}	10	10	18 (37%)	2	9	4:14
	Sp223 pSp(<i>cen1</i>)-BHpΔK-Nc	22.0	1.1×10^{-1}	7	16	22 (35%)	3	15	1:21
	SBP120390 pSp-cc2+Kpn+neo ₁	24.4	1.4×10^{-2}	0	0	33 (67%)	12	4	24:9
V.	SBP120390 pSp-cc2ΔNcSn+K"K"K"	30.6	6.1×10^{-3}	0	1	34 (79%)	4	4	19:15
	SBP120390 pSp-cc2ΔNcNd+K"K"K"	29.5	5.1×10^{-3}	0	0	46 (92%)	2	2	23:23
	SBP120390 pSp-cc2ΔNcSnΔBsSal+K"K"K"	28.6	4.3×10^{-3}	0	0	26 (90%)	2	1	16:10
	SBP120390 pSp-cc2ΔSSal+K"K"opp	22.0	2.3×10^{-2}	0	1	40 (62%)	8	16	19:21
	Sp223 pSp-CSal	38.9	1.1×10^{-2}	3	0	49 (82%)	4	4	33:16
Nonfunctional									
	Sp223 pSp3-SalΔSp	39.0	8.8×10^{-1}	0	0	0	1	13 (93%)	—
	SBP120390 pSp3-SalΔNdSn+λ	49.4	9.3×10^{-1}	0	0	0	0	17 (100%)	—
	Sp223 pSp3-SNc+L	20.7	4.8×10^{-1}	1	0	0	2	30 (91%)	—
	Sp223 pSp-BHpΔKpn	21.1	7.4×10^{-1}	0	0	1	3	51 (93%)	1:0
	SBP120390 pSp-cc2+Kpn	15.9	2.7×10^{-1}	0	0	1	1	6 (75%)	0:1
Vectors									
	SBP120390 pSp-cc2	13.8	7.1×10^{-1}			nd			
	Sp223 pMB-1 (<i>ars1</i>) ^b	12.8	2.3×10^{-1}			nd			
	Sp223 pSp300 (<i>ars1</i>)	7.4	3.1×10^{-1}			nd			

Diagrammatic representations of these plasmids are shown in Figures 2, 4, and 5, with the exception of pSp3-SNc+K and pSp3-SNc+Kopp described by Clarke *et al.* (1993). Centromere-active minichromosomes are grouped according to structure. The contribution of centromeric DNA elements to centromere function has been examined as follows: Group I, cc2; II, III, and IV, K-type sequences; V, the inverted repeat motif. Methods used to determine the mitotic loss frequency are described in MATERIALS AND METHODS. Mitotic loss rates of the functional minichromosomes in the host SBP120390 were measured by the colony color assay, with the exception of pSp-cc2+K", which was assayed by the replica plating method. Plasmids pSp3-SNc+K and pSp3-SNc+Kopp were shuttled into SBP120390 and sup3-e was transplanted into pBR322 sequences in these constructs to utilize the colony color assay to measure the mitotic loss rate of these plasmids. Mitotic loss rates of the remaining functional and nonfunctional minichromosomes, and the vectors, were assayed by the replica plating method. The *ura4* marker was used to follow the meiotic segregation of the minichromosomes, except for pSp3-SalΔSp, which carries only the *leu1* gene. The percentage of tetrads is only shown for the predominant class, 2+:2- or 0+:4- for functional or nonfunctional minichromosomes, respectively. Distribution of the *ura4* or *leu1* marker to sister spores represent first division segregants; distribution to nonsister spores are second division segregants. The meiotic segregation behavior of vector plasmids was not determined (nd).

^a Meiotic data from Hahnenberger *et al.* (1991).

^b Data from Clarke and Baum (1990).

within the central core was generated by fragment-mediated transplacement of pSp3-SalΔSp (Figure 2B). Because both ends of the transplacement fragment contain identical core-associated repeat sequences, the targeted region in pSp3-SalΔSp could undergo conversion in one of two orientations. Plasmids were screened by complete *Sal* I and partial *Spe* I digestion and mapped by Southern analysis to establish the orientation of the replacement. All of the transformants identified for pSp3-SalΔNdE contained the central core in an orientation opposite to

that in the native chromosome II. Both orientations were observed among stable transformants for the other replacements, but only those constructs exhibiting the orientation shown in Figure 2 were analyzed meiotically. Conversion in the central core of the native *cen2* was never observed.

The largest substitution removed 2.9 kb from the middle of the central core (pSp3-SalΔEH). Two flanking substitutions removed 1.6 kb (pSp3-SalΔNdE) and 1.4 kb (pSp3-SalΔHBs) of cc2. All three of the constructs,

however, showed equivalent centromere activity when compared to pSp3-Sal-us (Table 1). Overall, 4.5 kb of cc2 from the *Nde* I site to the *Bst*EII site can be eliminated in 1- to 3-kb portions without further compromising mitotic or meiotic centromere function in these minichromosomes.

To address the possibility that central core sequences were restored in these constructs in vivo during meiosis, DNAs from progeny of representative tetrads were examined. Total DNA was digested with *Sal* I, which linearizes the minichromosome, subjected to FIGE, and probed with a fragment representing the deleted region. Two probes were used (Figure 2B, open bars), a 0.8-kb *Eco*RI-*Eco*RI restriction fragment that is absent in pSp3-Sal Δ NdE and pSp3-Sal Δ EH, but is present in pSp3-Sal Δ HBs, and a 0.9-kb *Hind*III-*Bst*EII fragment that is absent in pSp3-Sal Δ HBs, but present in the other two plasmids. The minichromosomes migrate as the smallest bands (46–47 kb) on the blot shown in Figure 3 and yield the expected hybridization pattern in each case, confirming that small deletions within cc2 do not inactivate the centromere. The probes also hybridize to the genomic *cen2* *Sal* I restriction fragment, 100 or 70 kb, respectively, in *S. pombe* strain SBP32588 or SBP120390 (Steiner *et al.*, 1993). In each tetrad examined, the hybridization pattern of the genomic *cen2* *Sal* I bands illustrates that the minichromosome segregated to nonsister spores.

A functional requirement for sequences within the remaining untested central core regions, the 1.1-kb *Sna*BI-*Nde* I fragment at the left end of cc2 and the 1.1-kb region to the right of the *Bst*EII site (Figure 2B), was examined using minichromosomes constructed in vitro, pSp-cc2 Δ NcNd+K"K"K" and pSp-cc2 Δ NcSn Δ BsSal+K"K"K" (Figure 4). These minichromosomes also exhibit centromere activity equivalent to that of pSp3-Sal-us (Table 1). In addition, two minichromosomes that contain different "halves" of cc2, pSp-CSal (Figure 2B) and pSp-cc2 Δ SSal+K"K"opp (Figure 4), are not further compromised for mitotic or meiotic function (Table 1). Plasmid pSp-CSal contains the rightmost 4.7 kb out of a total of 6.8 kb of cc2 sequences, whereas pSp-cc2 Δ SSal+K"K"opp contains the leftmost 3.3 kb of cc2 sequences. Although they share 1.3 kb of cc2 sequences (*Cla*I-*Sph*I), this region of overlap has also been shown to be nonessential (pSp3-Sal Δ EH), demonstrating that the central core of *cen2* contains several functionally interchangeable regions and that the minimal sequence limit has not yet been defined.

The results of these analyses lead us to conclude that only a portion of the centromeric central core is necessary but not sufficient for centromere function in *S. pombe*. Because 1- to 3-kb regions across cc2 are dispensable for centromere function, there is not one specific site in the central core that is essential for establishing an active centromere. Instead, a series of redundant sites distributed across the length of the central core is consistent with the above results.

Figure 3. Meiotic progeny from minichromosomes with a substituted cc2 region yield DNA hybridization patterns consistent with deletions of predicted cc2 sequences. For each minichromosome, total DNA from the parental strain (P), which was mated with SBP32588 (see MATERIALS AND METHODS), is compared with two *Ura*⁺ spores (1 and 2) from a representative tetrad in which the minichromosome segregated 2+:2-. Total DNA was digested with *Sal* I and subjected to FIGE and Southern analysis. Two ³²P-labeled cc2 fragments were used as probes; (A) a 0.8-kb *Eco*RI-*Eco*RI fragment from the left side of cc2, and (B) a 0.9-kb *Hind*III-*Bst*EII fragment from the right side of cc2 (□, Figure 2B). DNAs of the three cc2-substituted minichromosomes (pSp3-Sal Δ NdE, pSp3-Sal Δ EH, and pSp3-Sal Δ HBs) migrate at 46–47 kb. The native *cen2* regions in SBP32588 (derived from Sp223) and SBP120390 migrate as 100- and 70-kb *Sal* I fragments, respectively.

Repeats K and K" Show Comparable Contributions to Centromere Activity and Repeat L Cannot Substitute for K Sequences

In previous studies, the *cen1* K" repeat (Figure 1) has been shown to contain sequences essential for centromere function of minichromosomes derived from native *cen1* (Hahnenberger *et al.*, 1991). In *S. pombe* strain Sp223 used in this study, the K" repeat sequences are found not only in *cen1* but also as contiguous stretches of DNA, twice in *cen2* and twice in *cen3* (indicated by brackets, Figure 1A). Figure 1B consolidates new information with that previously published and depicts the relationship between the *S. pombe* K" repeat, which is specific to *cen1* (Hahnenberger *et al.*, 1991), and the K repeat along with flanking sequences, which is found in both *cen2* and *cen3* (Clarke and Baum, 1990; Steiner *et al.*, 1993). Also shown is the relationship among the sequences defined by our laboratory as K and K" and those defined by another laboratory as the dg repeats (Nakaseko *et al.*, 1986; Chikashige *et al.*, 1989; Murakami *et al.*, 1991). Detailed examination of the K" repeat through restriction mapping, Southern analysis, and DNA sequencing reveals that this repeat is comprised of sequences found in the centromeric K and J repeats of *cen2* and *cen3* as well as a small amount of sequences making up a portion of the *cen1*-specific central core-associated repeat B' (Clarke *et al.*, 1986; Nakaseko *et al.*,

Figure 4. *cen2*-derived circular minichromosomes. Diagrammatic representations of all constructs are aligned under the restriction map of *cen2* and *K''* sequences. pSp3-SNc has been described (Clarke and Baum, 1990). The nonfunctional plasmid, pSp3-SNc+L, contains a contiguous 16.3 kb of *cen2* sequences including 4.1 kb of the 4.5-kb centromeric L repeat (Clarke *et al.*, 1993). The centromere-active minichromosomes were constructed by in vitro ligation of centromeric elements that are not normally found adjacent to one another in native *cen2* (see MATERIALS AND METHODS). Dashed lines indicate ligation junctions. Either pSp3-SNc or pSp-cc2 was ligated to single or multiple copies of the 5.6-kb *Bam*HI-*Nco*I restriction fragment representing the *K''* repeat. Subscripts indicate the number of tandem copies of *K''*. Multiple copies of *K''* are arranged head-to-tail forming direct repeats. Patterned boxes

represent those centromere elements that are shown above the restriction map. The *cen2* core-associated repeat is indicated by a black arrow or box. □, an unnamed *cen2*-specific repeat that is present in pSp3-SNc and in some constructs with multiple copies of *K''*. Vector sequences (ori/Ap^R) and *S. pombe* genes (*ura4* or the *ura4:sup3-e* cassette) are indicated by a thick line. Plasmid pSp-cc2+Kpn+neo₁ contains one copy of pMB-neo that was integrated by homologous recombination. Restriction site abbreviations are as follows: B, *Bam*HI; Bs, *Bst*EII; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; N, *Nhe*I; Nc, *Nco*I; Nd, *Nde*I; S, *Sph*I; and Sn, *Sna*BI. Not all *Eco*RI, *Sph*I, or *Sna*BI sites are shown.

1986; Fishel *et al.*, 1988; Chikashige *et al.*, 1989; Clarke and Baum, 1990; Steiner *et al.*, 1993; Hahnenberger, Ngan, and Clarke, unpublished data). The *dgl* region corresponds to the 4.4-kb *Bam*HI-*Kpn*I-*Kpn*I region in *cen1* (Figure 1B) and is common to both the K and *K''* repeats. The remaining 1.2-kb *Kpn*I-*Nco*I region of the *cen1* *K''* repeat, with the exception of the 150-bp stretch at the *Nco*I end, corresponds to the "head" region of the J repeat that is found adjacent to the K repeat tail in *cen2* and, in part, in *cen3* (Figure 1B). Therefore, a contiguous stretch of ~5.4 kb of *K''* sequences, including the *dg* region, is common to all three *S. pombe* centromeres in strain Sp223.

Because we have shown that the central core is an essential component of the *S. pombe* centromere, we examined the ability of a single centromeric repeat, either L, K, or *K''*, to confer centromere activity to a nonfunctional central core plasmid, pSp3-SNc (Clarke and Baum, 1990) or pSp-cc2 (Figure 4). Plasmid pSp3-SNc+L (Clarke *et al.*, 1993) contains 4.1 kb of the 4.5 kb *cen2* L repeat and is inactive with regard to centromere activity (Table 1). Its mitotic loss rate (4.8×10^{-1}) is nearly indistinguishable from that of pSp3-SNc (4.9×10^{-1}) (Clarke and Baum, 1990) and segregates similar to an *ars1* plasmid (Table 1). In contrast, the K or *K''* repeat, added to pSp3-SNc in either orientation, resulted

in plasmids (pSp3-SNc+K, pSp3-SNc+Kopp, pSp3-SNc+K'', and pSp3-SNc+K''opp) that exhibit considerable centromere activity in *S. pombe* (Table 1). The addition of K-type sequences, regardless of their orientation, to pSp3-SNc increases plasmid mitotic stability 6- to 10-fold. However, the minichromosomes are probably not maintained in single copy in populations of cells undergoing meiosis, as judged by the number of tetrads analyzed in which *ura4*⁺ segregated 4+:0- and 3+:1-. Because these plasmids are circular, very little difference would be anticipated between the two orientations of K or *K''*, unless the spacing between K-type repeats in relation to the B repeat, the core-associated repeats, or the central core is crucial. Thus, K and *K''* exhibit a comparable ability to establish a functional *S. pombe* centromere, when combined with central core sequences, and a major portion of the centromeric L repeat cannot substitute for K-type sequences in this respect.

Because repeats K and *K''* appear equivalent, we continued these studies with repeat *K''*, which can be isolated as a convenient restriction fragment (Hahnenberger *et al.*, 1991). Analysis of pSp-cc2-derived plasmids containing one, two, or three copies of *K''* indicates that multiple copies of these sequences exert an additive effect, enhancing centromere function with each

additional unit. Plasmid pSp-cc2+K" (Figure 4) exhibits poor mitotic stability (1.2×10^{-1}) (Table 1), however, this plasmid is relatively small (19.4 kb) and is at the lower end of the size range for retention of circular centromere plasmids in yeasts. We have observed that as with small circular and linear *CEN* plasmids in *S. cerevisiae* (Murray and Szostak, 1983; Hieter *et al.*, 1985), centromere plasmids in *S. pombe* are not stably maintained mitotically if they are smaller than a critical size. Increasing the size of plasmid pSp-cc2+K" to ~45 kb by integration of three tandem copies of pMB-neo sequences results in a 10-fold increase of its mitotic stability (pSp-cc2+K"+neo₃) (Table 1). The mitotic stability of pSp-cc2+K", however, increases even more dramatically (18-fold) with two additional copies of K" (pSp-cc2+K"K"K") (Table 1). Even one additional copy of K" increases mitotic stability 14-fold (pSp-cc2+K"K") (Table 1). To facilitate the cloning of the three tandem copies of K" into pSp-cc2, it was necessary to carry along a 0.9-kb *Bam*HI-*Eco*RI fragment adjacent to the B repeat (Figure 4). This small fragment apparently does not contribute significantly to centromere function, as it is not present in the construct with only two copies of K". The centromeric B repeat (Kuhn *et al.*, 1991; Takahashi *et al.*, 1991) does not contribute significantly to centromere function either, as demonstrated by the equivalent centromere activity exhibited by pSp-cc2+K"K"K" and pSp3-SNc+K"K"K" (Figure 4 and Table 1). Thus, each additional copy of K" results in an increase in mitotic stability of these constructs and a corresponding decrease in 0+:4- meiotic products, but these minichromosomes also exhibit a high degree of precocious sister chromatid separation in meiosis I.

A 2.1-kb Region of the *S. pombe* K-Type Repeat Is Necessary But Not Sufficient for Centromere Function

Repeats K and K" show a comparable ability to establish a functional centromere, when combined with centromeric central core sequences. This suggests that centromeric K-type repeats contain common critical sequences. Examination of the *cen1*-derived minichromosome pSp(*cen1*)-BHp (Figure 5) shows that this centromere construct, which lacks a portion of one arm of the inverted repeat, possesses an appreciable amount of mitotic activity and segregates in meiosis, although aberrantly (Table 1). Three deletion minichromosomes were derived from pSp(*cen1*)-BHp (Figure 5). These are pSp(*cen1*)-BHpΔB-K, pSp-BHpΔKpn, and pSp(*cen1*)-BHpΔK-Nc, which lack the 2.3-kb *Bam*HI-*Kpn* I fragment, the 2.1-kb *Kpn* I-*Kpn* I fragment, and the 1.2-kb *Kpn* I-*Nco* I fragment of the K" repeat, respectively. The pSp(*cen1*)-BHpΔB-K construct exhibits centromere activity very similar to that of the parent construct, pSp(*cen1*)-BHp (Table 1). Thus, the *Bam*HI-*Kpn* I deletion in repeat K" does not appear to reduce centromere

activity. The centromere activity of the pSp(*cen1*)-BHpΔK-Nc construct, however, is slightly reduced in comparison to that of the undeleted construct. Although the meiotic distributions of the two minichromosomes appear comparable, the mitotic loss frequency of pSp(*cen1*)-BHpΔK-Nc is consistently about threefold higher than that of pSp(*cen1*)-BHp. It appears then that the *Kpn* I-*Nco* I deletion in repeat K" leads to a mild reduction of centromere activity with respect to the frequency of chromosome loss. In marked contrast to the other two deletion plasmids, however, the pSp-BHpΔKpn construct exhibits a dramatic loss of centromere activity. The mitotic loss frequency of pSp-BHpΔKpn is 20-fold higher than that of pSp(*cen1*)-BHp (7.4×10^{-1} versus 3.7×10^{-2} , respectively), and pSp-BHpΔKpn only rarely survives meiosis. Hence deletion of the *Kpn* I-*Kpn* I region from the K" repeat of pSp(*cen1*)-BHp leads to a nonfunctional centromere. The results of these deletion analyses indicate that sequences within the 2.1 kb *Kpn* I-*Kpn* I region of the K-type repeat are essential for centromere activity, whereas sequences in the *Bam*HI-*Kpn* I and *Kpn* I-*Nco* I regions are not.

To confirm that the 2.1-kb *Kpn* I-*Kpn* I fragment encompasses the functionally active portion of K-type sequences, this fragment was assayed in the vector pSp-cc2. Plasmid pSp-cc2+Kpn (~16 kb) does not appear to contain a functional centromere, but if it is enlarged with a single copy of pMB-neo to ~24 kb (pSp-cc2+Kpn+neo₁) (Figure 4), centromere activity becomes apparent (Table 1). Mitotic stability of the enlarged plasmid increases ~20-fold, and the minichromosome is predominantly distributed 2+:2- in meiosis. Moreover, pSp-cc2+Kpn+neo₁ primarily segregates to sister spores in a manner similar to that of the fully functional artificial chromosome containing full length *cen2* (pSp(*cen2*)-52C, 92% [36/39] 2+:2-, 28:8 sister:nonsister distribution) (Clarke and Baum, 1990). In pSp-cc2+Kpn+neo₁, the *Kpn* I-*Kpn* I fragment is separated from the left core proximal repeat by 11.4 kb of pBluescript and pMB-neo sequences, approximating the spacing between these two regions in the native *cen2* (12.3 kb). Thus, pSp-cc2+Kpn+neo₁ may fortuitously contain optimal spacing between the functionally relevant cc2 sites and the critical *Kpn* I fragment from K-type repeats, or the increase in the overall length of the plasmid may be sufficient to account for its increased mitotic stability. The structure of this minichromosome was not altered during meiosis, as Southern analysis of total DNA from tetrads in which pSp-cc2+Kpn+neo₁ segregated 2+:2- was performed and yielded the expected hybridization pattern. Thus, the mitotic stability and the proper meiotic segregation exhibited by this minichromosome demonstrates that critical sequences required for centromere function are contained both in the central core and within the 2.1-kb *Kpn* I-*Kpn* I restriction fragment found at all K-type (K/K"/dg) repeats.

Figure 5. Minichromosome constructs derived from *cen1*. K' deletion constructs (see MATERIALS AND METHODS) derived from the pSp(*cen1*)-BHp minichromosome (Hahnenberger *et al.*, 1991) are aligned under a restriction map of the *cen1* region. Restriction sites are defined as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; Nc, *Nco*I; S, *Sph*I; Sal, *Sal*I; Sp, *Spe*I; and X, *Xba*I. Not all *Spe*I and *Xba*I sites are shown.

The Inverted Repeat Motif Is Unnecessary for Centromere Function in a Circular Minichromosome

The role of the inverted repeat motif, a conserved feature of all three native *S. pombe* centromeres (Figure 1A), was examined by removing either one or both copies of the core-associated repeat from circular *cen2*-derived minichromosomes. Constructs lacking the inverted repeat motif (pSp-CSal, Figure 2; pSp-cc2ΔNcSn+K''K''K'', pSp-cc2ΔNcNd+K''K''K'', pSp-cc2ΔNcSnΔBsSal+K''K''K'', pSp-cc2ΔSSal+K''K''opp, Figure 4) exhibit centromere activity similar to that of the minichromosomes pSp3-Sal-us and pSp-cc2+K''K''K'', which contain 3.9 kb and 0.9 kb of inverted repeat, respectively (Table 1). Thus, the inverted repeat motif is not essential for centromere function in a circular minichromosome. Moreover, plasmid pSp-cc2ΔNcSnΔBsSal+K''K''K'' does not contain any *cen2* core-associated repeat sequences and results of analyses with this construct demonstrate that centromeric K-type sequences in conjunction only with part of the central core are sufficient for appreciable centromere function.

Centromeric K Repeat Sequences Contain Specific Protein Binding Sites

The K-type repeat is not only essential for centromere function in *S. pombe* but also has a profound effect in *cis* and over a distance on the structure of centromeric central core chromatin in a manner that correlates directly with centromere function (Clarke *et al.*, 1993; Marshall and Clarke, unpublished data). In the native chromosomes and in centromere-active minichromosomes, central core sequences are in an unusual chromatin structure that lacks a regularly spaced nucleosomal array (Polizzi and Clarke, 1991; Takahashi *et al.*, 1992; Clarke *et al.*, 1993). The ability of the K-type repeat to influence central core chromatin structure suggests that this effect might be mediated by the interaction of proteins that bind to K-type and/or central core sequences. It has been shown that the binding of

one or more proteins to a DNA fragment can result in the retardation of the electrophoretic mobility of that fragment (Garner and Revzin, 1981). Hence, by assaying the electrophoretic mobility of small K'' DNA fragments in the presence and absence of *S. pombe* nuclear proteins, the approximate locations of protein binding sites can be determined.

We have examined the K'' repeat for centromere-specific protein binding sites by employing gel mobility shift assays, using specific K'' fragments and a *S. pombe* crude nuclear extract. The K'' repeat was divided into small (<500 bp) fragments for gel mobility shift assays (Figure 6A). All fragments examined are convenient restriction fragments with the exception of the PCR amplification products G-H and H-I, representing the left and right halves, respectively, of the 550 bp *Kpn*I-*Eco*RV K'' restriction fragment designated H. The mobility of each radiolabeled fragment was examined in the absence of protein and after incubation with *S. pombe* crude nuclear extract and varying amounts of poly(dI-dC), with or without unlabeled fragment as specific competitor.

Five fragments (H-I, L, N, O, and P, see Figure 7) exhibited specific shifts in mobility when incubated with crude nuclear extract. Each shifted band most likely represents a complex between the radiolabeled fragment and one or more nuclear proteins. Treatment of the crude nuclear extract with heat (65°C) or with proteinase K abolishes the formation of these complexes, thereby indicating that it is probably the binding of proteins, and not other nuclear factors, that causes the mobility shift of the fragments assayed. The specificity of the interaction between the protein(s) and the fragments in the complexes formed was addressed by the comparison of the mobility shift profiles of each ³²P-labeled fragment in the presence and absence of excess unlabeled fragment, the specific competitor. The relative amount of a protein/³²P-labeled-DNA complex formed should be significantly reduced in the presence of the unlabeled specific competitor, if the formation of the complex re-

sults from the specific binding of protein(s) to that fragment. Examination of the mobility shift profiles of fragments **H-I**, **L**, **N**, **O**, and **P** shows that very large (≤ 2 μ g) amounts of poly(dI-dC) (Figure 7, lanes 2-5 of each panel) do not significantly reduce the relative amounts of the complexes formed, whereas the presence of a modest amount (25-fold excess, ~ 30 ng) of unlabeled fragment does (compare the rightmost two lanes in each panel). Thus, the results of specific competition indicate that all complexes formed by fragments **H-I** (two complexes), **L** (three complexes), **N** (two complexes), **O** (two complexes), and **P** (three complexes) result from the specific binding of protein to those fragments. The observed multiplicity of the specific complexes formed by each fragment suggests that all five fragments are bound by more than one protein or multiple units of the same protein. However, it cannot be ruled out that the lower molecular weight complexes represent the binding of the fragments by the degradation products of one protein. Therefore, it is clear from these results that fragments **H-I**, **L**, **N**, **O**, and **P** contain binding sites for a protein component of *S. pombe* nuclear extracts. These fragments are located within the 3.3-kb *Kpn* I-*Kpn* I-*Nco* I region of the *K''* repeat, and portions of this region have been identified in this study to play both major and minor roles in centromere function.

DISCUSSION

Previous structure/function studies of the *S. pombe* centromere have demonstrated that only minichromosomes containing nearly all of the full length inverted repeat motif flanking the central core (Figure 1) exhibit full centromere function, as judged by the centromere-defining parameters of mitotic stability, 2+:2- meiotic segregation, and sister chromatid separation during meiosis II (Hahnenberger *et al.*, 1989; Matsumoto *et al.*, 1990; Takahashi *et al.*, 1992). In previous studies, appreciable centromere function has been correlated also with minichromosomes that contain one copy of a *cen*-specific repeat array, the corresponding central core, and 3-4 kb of core-associated repeat sequences, forming a small inverted repeat (Clarke and Baum, 1990; Hahnenberger *et al.*, 1991; Takahashi *et al.*, 1992). Such

constructs are mitotically stable and segregate predominantly 2+:2- in meiosis, but in at least half of the meiotic events, the minichromosome is found in non-sister spores. This aberrant segregation is generally interpreted as precocious sister chromatid separation during meiosis I (Cumberledge and Carbon, 1987; Gaudet and Fitzgerald-Hayes, 1989). Thus, past studies have demonstrated that two parameters by which *S. pombe* centromere function is measured, mitotic stability and 2+:2- meiotic segregation, can be uncoupled from a third parameter, maintenance of sister chromatid attachment in meiosis I.

We conclude from results presented here that the *cen2* central core (cc2) and K-type (*K/K''/dg*) repeat DNA sequences are necessary and sufficient to establish a functional *S. pombe* centromere. Centromeric L, B, or the core-associated repeats, as well as other heterologous sequences, cannot substitute for the central core or K-type sequences in this capacity. The cc2 region exhibits functional redundancy because deletion of 1- to 3-kb portions from this 6.8-kb region does not significantly reduce centromere function, whereas removal of the entire cc2 region abolishes centromere activity. Thus, there does not appear to be a single critical site within cc2 that is functionally analogous to the CDEIII region of *S. cerevisiae* centromeres (Clarke and Carbon, 1985). The lack of a conserved sequence of any appreciable length among the cc1, cc2, and cc3 regions (Takahashi *et al.*, 1992; Marschall and Clarke, unpublished data) suggests that the functionally critical sequences may be quite small and/or degenerate.

In contrast to the functional redundancy exhibited by cc2, a major contribution to centromere function can be attributed to one portion of the K-type repeat. Although deletion of the 2.1-kb *Kpn* I-*Kpn* I region from the 5.6-kb *cen1* centromeric *K''* repeat abolishes centromere activity in a minichromosome, the addition of this 2.1-kb *Kpn* I region to the central core generates a functional *S. pombe* centromere as demonstrated by the results with plasmid pSp-cc2+*Kpn*+neo₁ (Table 1). The 2.1-kb *Kpn* I region has been shown by restriction mapping, Southern blotting, and DNA sequence analyses to be contained in all K-type repeats, which are present in two or more copies at all three fission yeast centromere loci

Figure 6. Location of DNA fragments that exhibit specific gel mobility shifts in the *K''* repeat unit. (A) Restriction fragments used in the gel shift assays are designated alphabetically. The bar indicates the **H-I** fragment, which was generated by PCR amplification (see MATERIALS AND METHODS). The asterisks mark those fragments that were found to exhibit specific gel mobility shifts upon incubation with *S. pombe* crude nuclear extract (see Figure 7). (B) A restriction map of the *cen1* 5.6-kb *K''* repeat.

Figure 7. Gel mobility shift assays of K' fragments. The gel mobility shift assays of fragments H-I (A), L (B), N (C), O (D), P (E), and K (F) are shown. For all panels, from left to right, lane 1 shows the gel mobility of the probe only, lanes 2–5 show the gel mobility of the protein/ 32 P-labeled-DNA complexes formed in the presence of increasing amounts (0.5–2 μ g) of the nonspecific DNA competitor poly (dI-dC), and lane 6 is equivalent to lane 5 but with the inclusion of a 25-fold excess (125 fmol) of unlabeled fragment as the specific DNA competitor. In all cases, 5 fmol of probe (equivalent to $\sim$ 1–1.5 ng of DNA) was used in the binding reaction. Fragment K does not form specific protein-DNA complexes and is shown as a negative control. Arrowheads mark the specific complexes formed.

(Clarke *et al.*, 1986; Nakaseko *et al.*, 1986, 1987; Chikashige *et al.*, 1989).

In addition, the inverted repeat motif does not appear to be necessary for centromere function in a circular minichromosome, because constructs lacking this motif are mitotically stable and segregate in meiosis, albeit aberrantly. However, no apparent correlation is observed between particular centromeric sequences studied here and a propensity to undergo precocious sister chromatid separation. In summary, essential portions sufficient for appreciable centromere activity have been identified and they total less than one-fifth of the entire *cen2* region. Furthermore, the functional component of the *S. pombe* centromeric DNA cannot be reduced to a small, contiguous region such as is the case in *S. cerevisiae*.

The existence of specific protein binding sites within the K' repeat is confirmed by gel mobility shift assays that identified five fragments within the K' repeat, H-I, L, N, O, and P, that are bound specifically by one or more nuclear proteins. Whereas fragments H-I and L are found within the functionally critical 2.1-kb *Kpn* I-*Kpn* I region, fragments N, O, and P are not, but these latter three fragments do lie in the adjacent *Kpn* I-*Nco* I region, which, when deleted, leads to some reduction

in centromere activity. Although the deletion of N, O, and P sequences does not totally eliminate centromere activity, it is likely that these sequences play some role in centromere function. Analysis of K' DNA sequence data shows that many short nucleotide stretches, ranging from 4 to 20 bp in length, are found repeated within the 5.6-kb K' region (Clarke *et al.*, 1986; Nakaseko *et al.*, 1986; Chikashige *et al.*, 1989), and some of these may represent the binding sites for nuclear protein(s) identified in this study. Whereas each of the five K' fragments exhibiting mobility shifts contains several different repeated motifs, there does not appear to be a repeated motif or a sequence common to all five fragments (Nakaseko *et al.*, 1986, 1987). However, when the sequences of these five fragments are compared in pairwise fashion, short stretches, ranging from 16 to 40 bp, can be found in common between any two fragments. Interestingly, fragments H-I, L, and O contain the 5'-TGGAAA-3' motif that represents the core portion of the 17-bp CENP-B box, the binding site for CENP-B, a human centromere-binding protein (Masumoto *et al.*, 1989; Muro *et al.*, 1992; Takahashi *et al.*, 1992).

A recent model has been proposed to describe the establishment of an active centromere, based on observations made from the native chromosomes and arti-

ficial minichromosomes (Clarke *et al.*, 1993). Two DNA sequence elements, the central core, which contains functionally redundant regions, and the 2.1-kb *Kpn* I-*Kpn* I portion of the K-type repeat, are required *in cis* for centromere function and are separated by at least several kilobases in the minichromosomes described here and in the native chromosomes. Therefore, any interaction between the two regions is likely to involve DNA looping, mediated by centromere binding proteins. The results of the studies presented here identify critical K-type repeat sequences that are likely to be involved in this protein-mediated interaction. The importance of repetitive DNA in centromere function is supported by the recent finding that the human α -satellite repeat appears to be directly involved in providing some aspect of centromere function (Haaf *et al.*, 1992). The atypical nucleosomal packaging of the native centromeric central core regions in *S. pombe* (Polizzi and Clarke, 1991; Takahashi *et al.*, 1992) suggests that the central core, along with the K-type repeat, may be further complexed with centromere specific proteins, resulting in the formation of a higher order chromatin structure that is essential for the establishment of an active centromere/kinetochore. It is perhaps to this higher order structure that spindle microtubules attach to effect chromosome movement during cell division. The diffuse, fibrillar chromosomal structure seen with electron microscopy (Ding *et al.*, 1993) and thought to be the *S. pombe* kinetochore may represent these proteins and additional proteins assembled on a framework that interfaces with the spindle microtubules. The functional redundancy observed within cc2 sequences and the presence of multiple K-type repeat elements at each native centromere, perhaps forming the framework for kinetochore assembly, is consistent with the observation by Ding *et al.* (1993) that two to four microtubules are associated with each *S. pombe* centromere in mitosis.

The fission yeast centromere is qualitatively similar to that of higher eukaryotes, containing ≤ 100 kb of nontranscribed heterochromatin-like repeated DNA. The functionally critical *Kpn* I-*Kpn* I fragment is distributed in two to 12 copies at each *S. pombe* centromere. A second critical region for *S. pombe* centromere function is located within the central core sequences, flanked by the inverted repeat, a motif that has yet to be identified in higher eukaryotic centromeres. In addition, the *S. pombe* centromere is attached to the mitotic apparatus by multiple (2 to 4) spindle fibers, although fewer than are associated with mammalian centromeres. Thus, several features of the *S. pombe* centromere are analogous to the mammalian centromere, on a smaller scale, making fission yeast an excellent model for study of eukaryotic centromeres.

ACKNOWLEDGMENTS

We thank John Carbon, Kim Middleton, and Susan Myers for critical reading of this manuscript, and John Carbon, Kim Middleton, Karen

Hahnenberger, Laura Marschall, and Noemi Steiner for helpful discussions. Noemi Steiner, Kristina Jindra, Susan Myers, and Kristen Truell assisted with the construction of these plasmids. This work was supported by U.S. Public Health Services grant GM-33783 from the National Institutes of Health. V.K.N. was partially supported by a grant from the University of California Cancer Research Coordinating Committee.

REFERENCES

- Baker, R.E., Fitzgerald-Hayes, M., and O'Brien, T.C. (1989). Purification of the yeast centromere binding protein CP1 and a mutational analysis of its binding site. *J. Biol. Chem.* 264, 10843-10850.
- Beach, D.H., and Klar, A.J.S. (1984). Rearrangements of the transposable mating-type cassettes of fission yeast. *EMBO J.* 3, 603-610.
- Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
- Cai, M., and Davis, R.W. (1989). Purification of a yeast centromere binding protein that is able to distinguish single base-pair mutations in its recognition site. *Mol. Cell. Biol.* 9, 2544-2550.
- Carle, G.F., Frank, M., and Olson, M.V. (1986). Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science* 232, 65-68.
- Chikashige, Y., Kinoshita, N., Nakaseko, Y., Matsumoto, T., Murakami, S., Niwa, O., and Yanagida, M. (1989). Composite motifs and repeat symmetry in *S. pombe* centromeres: direct analysis by integration of *NotI* restriction sites. *Cell* 57, 739-751.
- Clarke, L., Amstutz, H., Fishel, B., and Carbon, J. (1986). Analysis of centromeric DNA in the fission yeast *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. USA* 83, 8253-8257.
- Clarke, L., and Baum, M.P. (1990). Functional analysis of a centromere from fission yeast: a role for centromere-specific repeated DNA sequences. *Mol. Cell. Biol.* 10, 1863-1872.
- Clarke, L., Baum, M., Marschall, L.G., Ngan, V.K., and Steiner, N.C. (1993). Structure and function of *Schizosaccharomyces pombe* centromeres. *Cold Spring Harbor Symp. Quant. Biol.* 58, 687-695.
- Clarke, L., and Carbon, J. (1985). The structure and function of yeast centromeres. *Annu. Rev. Genet.* 19, 29-56.
- Cooke, C.A., Bazett-Jones, D.P., Earnshaw, W.C., and Rattner, J.B. (1993). Mapping DNA within the mammalian kinetochore. *J. Cell Biol.* 120, 1083-1091.
- Cottarel, G., Shero, J.H., Hieter, P., and Hegemann, J.H. (1989). A 125-base-pair *CEN6* DNA fragment is sufficient for complete meiotic and mitotic centromere functions in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 9, 3342-3349.
- Cumberledge, S., and Carbon, J. (1987). Mutational analysis of meiotic and mitotic centromere function in *Saccharomyces cerevisiae*. *Genetics* 117, 203-212.
- Ding, R., McDonald, K.L., and McIntosh, J.R. (1993). Three-dimensional reconstruction and analysis of mitotic spindles from the yeast, *Schizosaccharomyces pombe*. *J. Cell Biol.* 120, 141-151.
- Earnshaw, W.C., and Rothfield, N. (1985). Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma* 91, 313-321.
- Fishel, B., Amstutz, H., Baum, M., Carbon, J., and Clarke, L. (1988). Structural organization and functional analysis of centromeric DNA in the fission yeast *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* 8, 754-763.
- Garner, M., and Revzin, A. (1981). A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *E. coli* lactose operon regulatory system. *Nucleic Acids Res.* 9, 3047-3060.

- Gaudet, A., and Fitzgerald-Hayes, M. (1989). Mutations in *CEN3* cause aberrant chromosome segregation during meiosis in *Saccharomyces cerevisiae*. *Genetics* 121, 477–489.
- Gutz, H., Heslot, H., Leupold, U., and Loprieno, N. (1974). *Schizosaccharomyces pombe*. In: Handbook of Genetics, ed. R.D. King, New York: Plenum Publishing, 395–446.
- Haaf, T., Warburton, P.E., and Willard, H.F. (1992). Integration of human α -satellite DNA into simian chromosomes: centromere protein binding and disruption of normal chromosome segregation. *Cell* 70, 681–696.
- Hahnenberger, K.M., Baum, M.P., Polizzi, C.M., Carbon, J., and Clarke, L. (1989). Construction of functional artificial minichromosomes in the fission yeast *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. USA* 86, 577–581.
- Hahnenberger, K.M., Carbon, J., and Clarke, L. (1991). Identification of DNA regions required for mitotic and meiotic functions within the centromere of *Schizosaccharomyces pombe* chromosome I. *Mol. Cell. Biol.* 11, 2206–2215.
- Hieter, P., Mann, C., Snyder, M., and Davis, R.W. (1985). Mitotic stability of yeast chromosomes: a colony color assay that measures nondisjunction and chromosome loss. *Cell* 40, 381–392.
- Hofer, F., Hollenstein, H., Janner, F., Minet, M., Thuriaux, P., and Leupold, U. (1979). The genetic fine structure of nonsense suppressors in *Schizosaccharomyces pombe*. *Curr. Genet.* 1, 45–61.
- Hyman, A.A., Middleton, K., Centola, M., Mitchison, T.J., and Carbon, J. (1992). Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature* 359, 533–536.
- Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact cells treated with alkali cations. *J. Bacteriol.* 153, 163–168.
- Jiang, W., Lechner, J., and Carbon, J. (1993). Isolation and characterization of a gene (*CBF2*) specifying a protein component of the budding yeast kinetochore. *J. Biol. Chem.* 268, 513–519.
- Jiang, W., and Philippsen, P. (1989). Purification of a protein binding to the CDEI subregion of *Saccharomyces cerevisiae* centromere DNA. *Mol. Cell. Biol.* 9, 5585–5593.
- Klar, A.J.S., and Miglio, L.M. (1986). Initiation of meiotic recombination by double-strand DNA breaks in *S. pombe*. *Cell* 46, 725–731.
- Kohli, J., Munz, P., and Söll, D. (1989). Informational suppression, transfer RNA, and intergenic conversion. In: Molecular Biology of the Fission Yeast, eds. A. Nasim, P. Young, and B.F. Johnson, San Diego: Academic Press, 75–96.
- Kuhn, R.M., Clarke, L., and Carbon, J. (1991). Clustered tRNA genes in *Schizosaccharomyces pombe* centromeric DNA sequence repeats. *Proc. Natl. Acad. Sci. USA* 88, 1306–1310.
- Lechner, J., and Carbon, J. (1991). A 240 kd multisubunit protein complex, CBF3, is a major component of the budding yeast centromere. *Cell* 64, 717–725.
- Maniatis, T., Fritsch, E.A., and Sambrook, J. (1982). Molecular Cloning. New York: Cold Spring Harbor Laboratory Press.
- Marschall, L.G., and Clarke, L. (1994). A novel cis-acting centromeric DNA element affects *S. pombe* centromeric chromatin structure at a distance. (submitted).
- Masumoto, H., Masukata, H., Muro, Y., Nozaki, N., and Okazaki, T. (1989). A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J. Cell Biol.* 109, 1963–1973.
- Matsumoto, T., Murakami, S., Niwa, O., and Yanagida, M. (1990). Construction and characterization of centric circular and acentric linear chromosomes in fission yeast. *Curr. Genet.* 18, 323–330.
- Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* 194, 795–823.
- Murakami, S., Matsumoto, T., Niwa, O., and Yanagida, M. (1991). Structure of the fission yeast centromere *cen3*: direct analysis of the reiterated inverted region. *Chromosoma* 101, 214–221.
- Muro, Y., Masumoto, H., Yoda, K., Nosaki, N., Ohashi, M., and Okazaki, T. (1992). Centromere protein B assembles human centromeric α -satellite DNA at the 17-bp sequence, CENP-B box. *J. Cell Biol.* 116, 585–596.
- Murray, A.W., and Szostak, J.W. (1983). Construction of artificial chromosomes in yeast. *Nature* 305, 189–193.
- Nakaseko, Y., Adachi, Y., Funahashi, S., Niwa, O., and Yanagida, M. (1986). Chromosome walking shows a highly homologous repetitive sequence present in all the centromere regions of fission yeast. *EMBO J.* 5, 1011–1021.
- Nakaseko, Y., Kinoshita, N., and Yanagida, M. (1987). A novel sequence common to the centromere regions of *Schizosaccharomyces pombe* chromosomes. *Nucleic Acids Res.* 15, 4705–4715.
- Newman, A.J., Lin, R.-J., Cheng, S.C., and Abelson, J. (1985). Molecular consequences of specific intron mutations on yeast mRNA splicing in vivo and in vitro. *Cell* 42, 335–344.
- Peterson, J.B., and Ris, H. (1976). Electron-microscopic study of the spindle and chromosome movement in the yeast *Saccharomyces cerevisiae*. *J. Cell Sci.* 22, 219–242.
- Polizzi, C., and Clarke, L. (1991). The chromatin structure of centromeres from fission yeast: differentiation of the central core that correlates with function. *J. Cell Biol.* 112, 191–201.
- Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* 194, 281–301.
- Steiner, N.C., Hahnenberger, K.M., and Clarke, L. (1993). Centromeres of the fission yeast *Schizosaccharomyces pombe* are highly variable genetic loci. *Mol. Cell. Biol.* 13, 4578–4587.
- Takahashi, K., Murakami, S., Chikashige, Y., Funabiki, H., Niwa, O., and Yanagida, M. (1992). A low copy number central sequence with strict symmetry and unusual chromatin structure in fission yeast centromere. *Mol. Biol. Cell* 3, 819–835.
- Takahashi, K., Murakami, S., Chikashige, Y., Niwa, O., and Yanagida, M. (1991). A large number of tRNA genes are symmetrically located in fission yeast centromeres. *J. Mol. Biol.* 218, 13–17.
- Waye, J.S., and Willard, H.F. (1987). Nucleotide sequence heterogeneity of alpha satellite repetitive DNA: a survey of alphoid sequences from different human chromosomes. *Nucleic Acids Res.* 15, 7549–7569.
- Willard, H.F. (1990). Centromeres of mammalian chromosomes. *Trends Genet.* 6, 410–416.
- Zinkowski, R.P., Meyne, J., and Brinkley, B.R. (1991). The centromere-kinetochore complex: a repeat subunit model. *J. Cell Biol.* 113, 1091–1110.