A Novel Cis-Acting Centromeric DNA Element Affects S. pombe Centromeric Chromatin Structure at a Distance

Laura G. Marschall and Louise Clarke
Department of Biological Sciences, University of California, Santa Barbara, California 93106

Abstract. The chromatin structure of the central core region of Schizosaccharomyces pombe centromeric DNA is unusual. This distinctive chromatin structure is associated only with central core sequences in a functional context and is modulated by a novel cis-acting DNA element (centromere enhancer) within the functionally critical K centromeric repeat, which is found in multiple copies in all three S. pombe centromeres. The centromere enhancer alters central core chromatin structure from a distance and in an orientation-independent manner without altering the nucleosomal packaging of sequences between the enhancer and the central core. These findings suggest a functionally relevant structural interaction between the enhancer and the centromeric central core brought about by DNA looping.

The centromere is a specialized region of the eukaryotic chromosome that ensures the proper segregation of the chromosome during mitotic and meiotic cell divisions. The centromere functions in the context of a nucleoprotein complex to mediate attachment to the spindle and effect the movement of chromosomes along the spindle microtubules. This region also serves as a point of attachment for sister chromatids, ensuring their attachment in mitotic metaphase and throughout the first meiotic division.

The centromeric DNAs of the fission yeast Schizosaccharomyces pombe are complex, span many kilobases (40-100 kb) of DNA, and are characterized by several classes of centromere-specific repeated DNA elements (Clarke et al., 1986; Nakaseko et al., 1986, 1987). These regions do not appear to be transcribed into RNA (Fishel et al., 1988; Polizzi and Clarke, 1991), and are reminiscent of higher eukaryotic centromeric DNAs, which contain very large regions of heterochromatic, repetitive DNA sequences (Singer, 1982; Miklos, 1985). The structures of the centromeric DNAs of all three S. pombe chromosomes in several common laboratory strains have been well characterized; each contains a large inverted repeat motif composed of centromere-specific repeat elements designated K (or dg), L (or dh), B, and M, ranging in size from \( \sim l \) to 2 kb (B and M) to 5-6 kb (K and L), flanking a 4-7 kb central core (cc) that is chromosome specific (Fishel et al., 1988; Chikashige et al., 1989; Clarke and Baum, 1990; Hahnenberger et al., 1991; Murakami et al., 1991; Steiner et al., 1993; Fig. 1).

The organization of repeats at each centromere differs among chromosomes and strains, but the basic inverted repeat configuration appears to be conserved (Steiner et al., 1993). Thus, in S. pombe, as in higher eukaryotes (Wervick and Willard, 1989), the centromere of a particular chromosome can vary with regard to the gross organization of repetitive sequences.

The centromeric DNAs of S. pombe, when carried on artificial minichromosomes, are fully functional with respect to centromere activity (Hahnenberger et al., 1989). Minichromosome assays have been used to assess centromere function in S. pombe and have revealed that sequences within the centromeric central core and the K-type repeat are absolutely essential for centromere function, but that neither the central core nor K-type repeat alone is sufficient to confer function (Hahnenberger et al., 1991; Baum et al., 1994). The centromeric repeats in S. pombe have also been shown to play a role in maintenance of sister chromatid attachment in meiosis I (Clarke and Baum, 1990; Hahnenberger et al., 1991). Recently, it has been shown that between two and four microtubules are attached to each S. pombe centromere in mitosis (Ding et al., 1993).

Examination of the chromatin structure of S. pombe centromeres has revealed that most of the centromere-specific repeats are packaged into a regular periodic nucleosomal array typical of bulk chromatin (Chikashige et al., 1989; Polizzi and Clarke, 1991). In contrast, the central core sequences and a small portion of their associated repeats show an unusual chromatin structure that is essentially devoid of typical nucleosomal packaging and is distinct from that of the flanking centromere-specific repeats (Polizzi and Clarke, 1991; Takahashi et al., 1992). It appears that this unusual central core chromatin structure at S. pombe centromeres persists throughout the cell cycle (Polizzi and Clarke, 1991).
Chromatin organization is an important aspect of centromere structure and function. The budding yeast (Saccharomyces cerevisiae) centromere, which encompasses only 125 bp (Clarke and Carbon, 1980; Cottarel et al., 1989), has a distinct chromatin structure in which 150-200 bp of DNA is packaged into a highly nuclease-resistant core structure flanked by DNase I hypersensitive sites and an array of positioned nucleosomes (Bloom and Clarke, 1990). Single base pair mutations that inactive the centromere completely disrupt the nuclease-resistant core structure (Bloom and Carbon, 1991). Mammalian centromeric DNA is rich in highly repetitive sequences that are predominantly heterochromatic; the positioning of nucleosomes on the repetitive α satellite DNA of primate centromeres is highly ordered (Zhang et al., 1983; van Holde, 1989). In addition, the proper folding of chromosomal DNA through the centromere into a higher order chromatin structure is a prerequisite for a subunit repeat model proposed for the mammalian centromere-kinetochore complex (Zinkowski et al., 1991). It has also been reported that modification of the chromatin of one centromere on a dicentric Y chromosome leads to inactivation of this centromere (Maraschio et al., 1990), further supporting the idea that correct chromatin organization is essential for centromere function.

To determine the relationship between central core chromatin structure and centromere function in S. pombe, we have examined the chromatin structure in centromere-active and centromere-inactive circular minichromosomes that contain various portions of S. pombe centromeric DNA, using partial nuclease digestion to reveal nucleosomal organization. We report here that centromeric core sequences in a nonfunctional context on minichromosomes in S. pombe are packaged into a periodic nucleosomal array typical of bulk chromatin. Central core sequences of minichromosomes carrying functional centromeres, on the other hand, display an unusual chromatin structure also seen at native centromeres revealing no evidence of a regular periodic nucleosomal array; this unique chromatin structure absolutely correlates with centromere function. Furthermore, DNA sequences within a 2.1-kb region from the centromere-specific K-type repeat, which is essential for centromere function in S. pombe, are necessary and sufficient to alter the chromatin structure of the centromeric central core. Thus, the S. pombe K-type repeat contains a novel cis-acting DNA element (centromere enhancer) that is capable of altering centromeric central core chromatin structure from a distance and in an orientation-independent manner. The cis interaction between two centromeric DNA elements, which are critical for centromere function and separated by a distance of 6–12 kb in native chromosomes, implies that a looping of the DNA sequences between them is an important aspect of centromere structure/function and chromatin organization in fission yeast. Such a system provides an excellent model for the study of centromere formation in higher eukaryotes.

**Materials and Methods**

**Strains, Minichromosomes, and DNA Manipulations**

S. pombe strains Sp223 (h- ade6.216 leu3.2 ura4.204; gift from D. Beach) and SBP120390 (h- ade6.704 leu3.32 ura4.294; this laboratory) were used in this study. S. pombe growth media and culturing conditions were as described (Gutz et al., 1974).

The cen2-derived minichromosome pSp3-SNc has been described (Clarke and Baum, 1990). The construction of minichromosomes derived from pSp3-SNc, with the exception of pSp3-SNc+K*+leu, is described in Baum et al. (1994). Construction of pSp3-SNc+K*+leu was as follows. BamHI linkers (New England Biolabs, Beverly, MA) were added to the blunt ends of the 660-bp HindII fragment from the S. cerevisiae LEU2 gene and the fragment was cloned into the unique BamHI site of pSp3-SNc+K* via standard techniques (Maniatis et al., 1982). The resulting minichromosome has heterologous S. cerevisiae LEU2 sequences inserted between the K* and B centromeric repeat elements. Plasmid pSp3-SNc+K*+leu was transformed into Sp223 using the lithium acetate procedure (Sko et al., 1983). The construction of cen1-derived minichromosomes has been described (pSp[cen1])-BHp, Hahnengerber et al., 1991; pSp-BHpAKpn, Baum et al., 1994).

Restriction enzymes were from New England Biolabs or Boehringer Mannheim Biochemicals, Inc. (Indianapolis, IN). Calf intestinal phosphatase and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals, Inc.

**Nuclei Isolation, Digestion of Chromatin, and DNA Purification**

Nuclei were prepared from 500-ml cultures as described by Polizzi and Clarke (1991). Briefly, cells grown to logarithmic phase were harvested and washed in H2O. Cells were converted to spheroplasts by resuspending in SH buffer (20 mM Hepes, pH 7.4, 1.2 M sorbitol, 0.5 mM PMSF) and digesting with lysing enzymes (Sigma Chem. Co., St. Louis, MO) and zymolyase 100T (ICN Biomedicals, Costa Mesa, CA). Spheroplasts were washed in SP buffer (20...
mM Pipes, pH 6.8, 1.2 M sorbitol, 1 mM PMFSF), and then lysed in lysis buffer (20 mM Pipes, pH 6.4, 18% Ficoll 400, 0.5 mM MgCl₂, 1 mM PMFSF). Nuclei were harvested by centrifugation through a glycerol/Ficoll cushion.

Micrococcal nuclease digestion was performed by resuspending the pelleted nuclei in PC buffer (20 mM Pipes, pH 6.4, 0.1 mM CaCl₂, 0.5 mM Pipes, pH 6.8, 1.2 M sorbitol, 1 mM PMSF), and then lysed in lysis buffer. Nuclei were harvested by centrifugation through a glycerol/Ficoll cushion. Micrococcal nuclease (Boehringer Mannheim Biochemicals, Inc.) was added to 250 U/ml. Aliquots were removed after various times of incubation at 30°C (times of incubation are indicated in the figures). Nuclease digestion was stopped by adjusting aliquots to 2% SDS and 20 mM EDTA. DNA from chromatin preparations was purified using the method of Polizzi and Clarke (1991).

**Gel Electrophoresis and Southern Transfer**

DNA was electrophoresed with buffer recirculation on 1.4% agarose gels in 1× TAE (Maniatis et al., 1982) at 200 V for 2 h. Resolved DNA was transferred to a nylon membrane (Zeta Probe GT; BioRad Labs., Hercules, CA) following manufacturer's instructions using a positive pressure blotting system (Posiblot Pressure Blotter; Stratagene, La Jolla, CA). Transferred DNA was UV-cross-linked to the membrane using a Genelinker (BioRad Labs.).

**DNA Probes and Hybridization**

*S. pombe* centromeric DNA fragments were obtained from plasmids whose isolation has been described (Clarke and Baum, 1990; Hahnenberger et al., 1991). Fragments were gel purified, isolated using GeneClean (BioOil), and 32P-labeled by random priming to a specific activity of >10⁶ cpm/μg (Prime-it kit, Stratagene). Radiolabeled DNA was hybridized to blots for 40 h at 65°C in 1× Zeta hybridization buffer (100 mM sodium phosphate, 500 mM NaCl, 5% SDS). Blots were washed twice in 2× SSC/1% SDS and once in 0.5× SSC/1% SDS at 45-50°C.

**Results**

**Centromeric Central Core DNA Sequences Do Not Preclude Regular Nucleosomal Packaging in *S. pombe***

The structural organization of the centromeric DNA regions in one *S. pombe* strain, Sp223, is shown in Fig. 1 (Clarke and Baum, 1990; Hahnenberger et al., 1991; Steiner et al., 1993). Each centromeric DNA is composed entirely or in part of a large inverted repeat flanking a 4–7-kb chromosome-specific central core. The inverted repeat contains centromere-specific repeated elements (K and L) found at all three *S. pombe* centromeres and chromosome-specific repeated elements found only at the centromere of a particular chromosome (B, B', M). Repeats B (cen2), B' (cenl), and the core proximal portion of the cen3 repeat share homology that is restricted to a few transfer RNA genes present in these elements (Kuhn et al., 1991; Takahashi and coworkers, 1992) for *cenl* central core sequences isolated on a plasmid in *S. pombe*. Clearly central core sequences themselves, carried on circular plasmids, do not preclude formation of a regular periodic nucleosomal array in *S. pombe*.

**Centromeric Repeat K* Precludes Core Chromatin Structure from a Distance and in an Orientation-independent Manner**

The nucleosomal array in central core chromatin of the centromere-inactive minichromosome pSp3-SNc suggests that the lack of typical nucleosomal packaging seen in genomic central core sequences (Fig. 2 B) may be correlated with centromere activity. To address this issue we have examined the central core chromatin structure of a number of centromere-active minichromosomes. The addition of the K-type centromeric repeat K* to pSp3-SNc in either orientation (Fig. 2 A, 3-SNc+K* and 3-SNc+K*opp) restores centromere function to the centromere-inactive minichromosome, supporting the conclusion that both central core and K repeat elements are necessary for centromere function in *S. pombe* (Clarke et al., 1993; Baum et al., 1994). Minichromosomes pSp3-SNc+K* and pSp3-SNc+K*opp show appreciable mitotic stability in *S. pombe* (mitotic loss frequencies of 6.9 × 10⁻⁴ and 4.3 × 10⁻⁴, respectively, which are 7–10 × lower than that of the centromere-inactive pSP3-SNc) and segregate properly to the majority of the haploid products of meiosis (Baum et al., 1994). The micrococcal nuclease digestion patterns of pSp3-SNc+K* and pSp3-SNc+K*opp chromatin, determined by probing DNA blots

Marschall and Clarke. *A DNA Element Affecting Centromeric Chromatin*. 447

Clarke, 1991 and data not shown). It has been suggested that the central core may be the site of kinetochore assembly and that the apparently non-nucleosomal central core chromatin structure may reflect a higher order structural differentiation that is intimately related to centromere function (Polizzi and Clarke, 1991). To address the question of whether the unique central core chromatin structure is correlated with centromere function, we examined the chromatin structure of central core sequences in a non-functional context on a circular minichromosome in *S. pombe*. Plasmid pSp3-SNc, which contains the entire cen2 central core flanked by 850 bp of core-associated repeat on one side and a complete cen2 core-associated repeat unit and B repeat sequences on the other (Fig. 2 A), is nonfunctional with respect to centromere activity (mitotic loss rate of 4.9 × 10⁻⁴; Clarke et al., 1993). When chromatin from a *S. pombe* strain containing this minichromosome is subjected to partial micrococcal nuclease digestion and a blot of the isolated DNA is probed with the 850-bp EcoRI central core fragment, a DNA ladder characteristic of the periodic nucleosomal array typical of bulk chromatin is revealed (Fig. 2 C). Because this probe and all other central core probes used in this study also hybridize to functionally active, genomic central core sequences, the nuclease digestion pattern of the minichromosome chromatin is always superimposed on a broad smear of hybridization that is characteristic of the genomic central core sequences (Fig. 2 B). Probing this blot with a 500-bp HindIII fragment from the cen2 central core, which is located ∼2 kb from the EcoRI fragment (Fig. 2 A), also reveals a nucleosomal DNA ladder (data not shown). Similar results have been obtained by Takahashi and coworkers (1992) for *cenl* central core sequences isolated on a plasmid in *S. pombe*. Clearly central core sequences themselves, carried on circular plasmids, do not preclude formation of a regular periodic nucleosomal array in *S. pombe*.
Figure 2. Centromeric central core chromatin structure of centromere-inactive and centromere-active minichromosomes in S. pombe. (A) Structures of circular cen2-derived minichromosomes aligned with the restriction maps of the K" repeat and a portion of cen2. In the functional minichromosomes, dashed lines indicate the adjoining of centromeric elements that are not normally found adjacent to one another in cen2. The bar under the restriction map indicates the cen2 central core specific fragment used to probe the Southern blots below. Restriction site designations are: B, BamHI; Bs, BstEII; C, ClaI; E, EcoRI; K, KpnI; N, NheI; Nc, NcoI; Nd, NdeI; S, SphI; Sn, SnaBI. Not all ClaI, SphI, and SnaBI sites are shown. (B) (Left panel) Ethidium bromide-stained gel of total S. pombe DNA purified from chromatin that was partially digested with micrococcal nuclease. (Right panel) Autoradiogram of the blotted DNA (left) probed with a unique 850-bp EcoRI fragment from the cen2 central core. The hybridizing band in the left lane indicates a micrococcal nuclease hypersensitive site in the cen2 central core chromatin. (C) Autoradiogram of a DNA blot derived from micrococcal nuclease-digested chromatin of S. pombe strain Sp223/pSp3-SNc, probed with the 850-bp cen2 central core EcoRI fragment. A nucleosomal DNA ladder from the cen2 central core sequences on the minichromosome pSp3-SNc is superimposed on a broad smear attributable to the genomic copy of cen2. (D) (Left panel, 3-SNc+K" set) Ethidium bromide-stained gel of total DNA from micrococcal nuclease-digested chromatin of S. pombe strain Sp223/pSp3-SNc+K". (Right panel) Autoradiogram of the blotted DNA (left) probed with the 850-bp cen2 central core EcoRI fragment; a broad smear with bands characteristic of mononucleosomes and dinucleosomes derived from the centromeric central core sequences on the minichromosome is superimposed on a broad smear representing hybridization to the genomic copy of the cen2 central core. (Left panel, 3-SNc+K"opp set). Ethidium bromide-stained gel showing the nucleosomal DNA ladder generated by micrococcal nuclease digestion of chromatin from S. pombe strain Sp223/pSp3-SNc+K"opp. (Right panel) Autoradiogram of the blot (left) probed with the 850-bp cen2 central core EcoRI fragment; the micrococcal nuclease digestion pattern of central core chromatin in this strain is indistinguishable from the pattern seen with the strain carrying pSp3-SNc+K". (E) Autoradiogram of DNA blot from micrococcal nuclease-digested chromatin of strain Sp223/pSp3-SNc+L probed with the 850-bp cen2 central core EcoRI fragment. The pattern reveals a nucleosomal DNA ladder from the cen2 central core sequences on the minichromosome superimposed on a broad smear attributable to hybridization of central core sequences from the genomic copy of cen2. In panels B-E, the number above each lane indicates the length of time (min) of micrococcal nuclease digestion and the dots beside the autoradiograms indicate the position of detectable nucleosomal DNA fragments.

with the 850-bp cen2 central core EcoRI fragment, are shown in Fig. 2 D; their digestion patterns are essentially indistinguishable. The central core sequences of both minichromosomes exhibit a complete lack of a periodic nucleosomal array. Instead, a broad smear with detectable bands at the mononucleosome and dinucleosome positions is revealed (Fig. 2 D). The ethidium bromide-stained gels from which the blots were obtained confirm the nucleosomal integrity of the chromatin preparation (Fig. 2 D).

We have examined nine other centromere-active minichromosomes, all containing K" and central core sequences, and all exhibit the unusual central core chromatin structure seen
A DNA Element Affecting Centromeric Chromatin

in Fig. 2 D for plasmids pSp3-SNc+K" and pSp3-SNc+K" opp (not all data shown). The presence of K" sequences on these circular minichromosomes alters the chromatin structure of central core sequences and this effect is independent of the orientation of K" with respect to the central core. In addition, K" sequences alter the central core chromatin from a distance, because as with native S. pombe centromeres, K" sequences are not directly adjacent to central core sequences in any of the minichromosome constructs examined (see below).

A 2.1-kb Region of the S. pombe K-Type Repeat Is Necessary and Sufficient to Alter Centromeric Central Core Chromatin Structure

Sequences within a 2.1-kb portion of the K-type centromeric repeat, along with centromeric central core sequences, are required for centromere activity in S. pombe (Baum et al., 1994). This 2.1-kb region is present within the K-type repeats, K and K", in multiple copies at all three native S. pombe centromeres (Fig. 1). The cen1-derived minichromosome pSp(cen1)-BHp contains the entire cen1 central core, cen1-specific core-associated repeat sequences, and one copy of repeat K" (Fig. 3 A, mitotic loss rate of 3.7 x 10^-2; Hahnenberger et al., 1991; Baum et al., 1994). The 2.3-kb BamHI-KpnI region from the left side of K" (see Fig. 3 A) or the 1.2-kb KpnI-NcoI region from the right can be deleted from the minichromosome without loss of centromere function. However, if the central 2.1-kb KpnI fragment of this K-type repeat is deleted, the resultant minichromosome, pSp-BHpΔKpn (Fig. 3 A), is completely nonfunctional with respect to centromere activity when introduced into S. pombe.
(mitotic loss frequency of $7.1 \times 10^{-4}$; Baum et al., 1994). Furthermore, the addition of this 2.1-kb KpnI fragment conveys centromere function to a centromere-inactive plasmid containing the entire cen2 central core and a small amount of core-associated repeat (Fig. 3 B, cc2+Kpn+neo, mitotic loss rate of $1.4 \times 10^{-4}$; Baum et al., 1994).

We have shown that a DNA element within repeat K* alters the chromatin structure of the centromeric central core and that the unique central core chromatin structure conferred by this element only occurs in functional centromeres. In light of these findings, we asked whether the critical 2.1-kb fragment occurring within K* and all $S. pombe$ cen2 K-type repeats is necessary and sufficient to alter the chromatin structure of the central core. Fig. 3 C and D show the micrococcal nuclease digestion pattern of central core sequences from the centromere-active minichromosome pSp(cc2)+Kpn and the centromere-inactive minichromosome pSp-BHpΔKpn. The DNA blot of partially digested chromatin from a strain carrying pSp(cc2)+BHp, probed with a 350-bp NcoI-ScaI fragment near the center of the cen2 central core (Fig. 3 A), reveals a broad smear with detectable bands representative of only mono- and dinucleosomes (Fig. 3 C, right); this is the same unusual chromatin structure present in central core sequences of all circular minichromosomes with functional centromeres. The ethidium bromide–stained gel from which the blot was obtained (Fig. 3 C, left) confirms the nucleosomal integrity of this chromatin preparation.

On the other hand, a Southern blot of DNA from partially digested chromatin from a strain carrying pSp-BHpΔKpn probed with the same cen2 central core fragment reveals a distinct DNA ladder, with evidence of stretches of at least seven regularly spaced nucleosomes (Fig. 3 D). The cen2 central core probe hybridizes to central core sequences in cen2 and cen3 (Hahnenberger et al., 1991; Takahashi et al., 1992). Therefore, the ascid to the central core of pSp-BHpΔKpn is seen superimposed on a broad smear attributable to hybridization to the genomic copies of cen2 and cen3. Thus, deletion of the 2.1-kb KpnI fragment from the sole copy of the K-type repeat in a centromere-active mini-chromosome not only results in loss of centromere function, but also abolishes the unique central core chromatin structure present in the functional centromere.

Similarly, addition of the 2.1-kb KpnI fragment from the $S. pombe$ K-type repeat to a centromere-inactive central core–containing plasmid results in a minichromosome with a functional centromere (Baum et al., 1994). The 16-kb minichromosome pSp-cc2+Kpn (Fig. 3 B) appears inactive with regard to centromere function based on mitotic stability assays in $S. pombe$, but when it is enlarged to 24 kb with heterologous DNA sequences, the resulting minichromosome, pSp-cc2+Kpn+neo, is fully functional with respect to centromere activity (Baum et al., 1994). Centromere plasmids in $S. pombe$, like those in $S. cerevisiae$ (Murray and Szostak, 1983), are stably maintained mitotically only if they are of a sufficient overall size (Baum et al., 1994), although the basis for this phenomenon is not well understood. Minichromosomes pSp-cc2+Kpn and pSp-cc2+Kpn+neo both exhibit the unique central core chromatin structure present in other minichromosomes with functional centromeres (Fig. 3 E). The observation that both pSp-cc2+Kpn and pSp-cc2+Kpn+neo exhibit the same unique chromatin structure characteristic of functional centromeres supports the hypothesis that pSp-cc2+Kpn appears inactive as a result of its small size rather than the lack of an element essential for centromere activity. We propose the term "centromere enhancer" for the DNA sequences within the K repeat that affect chromatin structure of the central core region at a distance.

**Centromeric Repeat L Does Not Alter Central Core Chromatin Structure**

While the centromere enhancer can restore function to a centromere-inactive plasmid carrying only the central core region, other centromeric repeats cannot function in this capacity (Baum et al., 1994). In light of this observation, we investigated other centromeric repeats for the ability to alter the central core chromatin structure. Because a periodic nucleosomal array is observed in the central core sequences of pSp3-SNc and this minichromosome contains one copy of centromeric repeat L, a single copy of the B repeat is not sufficient to alter chromatin structure. We also examined the structure of pSp3-SNc+L, a centromere-inactive minichromosome (mitotic loss frequency of $4.8 \times 10^{-4}$), which contains 4.1 kb of the 4.5-kb cen2 L repeat in addition to pSp3-SNc sequences (Fig. 2 A; Baum et al., 1994). An autoradiogram of a DNA blot of micrococcal nuclease–digested chromatin from a strain carrying pSp3-SNc+L, probed with the cen2 central core 850-bp EcoRI fragment, is shown in Fig. 2 E. It is evident that the central core sequences of pSp3-SNc+L are packaged into a typical nucleosomal array, as is seen with the centromere-inactive minichromosome pSp3-SNc (Fig. 2 C). Thus, sequences from centromeric repeat L do not alter the chromatin structure of the central core, nor do they restore centromere function to this minichromosome. This result further corroborates the association between an unusual central core chromatin structure, a functionally active centromere, and DNA sequences from the essential K repeat.

**The Centromere Enhancer Alters Central Core Chromatin Structure Without Affecting Intervening DNA Sequences**

The above results, as well as the locations of the repeats in native $S. pombe$ centromeres, suggest that the centromere enhancer sequences alter centromeric central core chromatin structure from a distance. The possibility exists that the unusual central core chromatin structure of functional centromeres is due to a perturbation of a regular nucleosomal array that begins at the K-type repeat and is propagated along the DNA molecule into the central core. Previous studies have determined that K sequences are packaged into a regular periodic nucleosomal array (Chikashige et al., 1989; Polizzi and Clarke, 1991; Ngan, V., and L. Clarke, unpublished observation). To understand the mechanism by which the enhancer alters the chromatin structure of the central core, it is essential to determine the nature of the chromatin structure of sequences lying between the K-type repeat and the central core.

We examined the chromatin structure of pBR322-derived vector sequences in minichromosomes from $S. pombe$ strains carrying either pSp3-SNc+K or pSp3-SNc+K*opp, using a 750-bp EcoRI–PstI fragment from pBR322 as probe (Fig. 4). These sequences lie between K* and the central core.
on one side of the central core in the circular minichromosome. The autoradiogram of DNA from a strain carrying pSp3-SNc+K" is shown on the left and that from a strain carrying pSp3-SNc+K" opp is shown in the middle of Fig. 4 B. It is evident that the vector DNA sequences between K" and the central core are packaged into a nucleosomal array typical of bulk chromatin.

A derivative of pSp3-SNc+K" was constructed in order to examine only the sequences between K" and the central core on the non-vector side of this centromere-active circular minichromosome. A 660-bp HincII fragment from the *S. cerevisiae LEU2* gene was inserted into pSp3-SNc+K" at the unique BamHI site between K" and the central core (Fig. 4 A). This construction, designated pSp3-SNc+K" + leu, specifically allows the investigation of these heterologous sequences between K" and the central core without interference from sequences in the genome. The autoradiogram of a DNA blot of micrococcal nuclease-digested chromatin from a strain carrying pSp3-SNc+K"+leu, probed with a 660-bp HincII fragment, is shown in Fig. 4 B. A nucleosomal DNA ladder indicative of up to seven nucleosomes in a periodic array is revealed, indicating that these unique sequences between K" and the central core are packaged into nucleosomes typical of bulk chromatin. Central core sequences from pSp3-SNc+K"+leu, however, exhibit the same unusual chromatin structure seen with pSp3-
SNc+K" (data not shown), and both pSp3-SNc+K" and pSp3-SNc+K"+leu are equally stable mitotically in S. pombe (Baum, M., and L. Clarke, unpublished observations). Thus, sequences on both sides of a centromeric central core in a functional context are packaged into a regular periodic nucleosomal array, suggesting that K-type sequences interact with central core sequences specifically and from a distance to effect the distinct chromatin structure associated with centromere function.

Discussion

Previous studies have indicated that central core sequences of functional centromeres in S. pombe have a unique chromatin structure that shows no evidence of a periodic nucleosomal array (Polizzi and Clarke, 1991; Takahashi et al., 1992). The results presented here establish that this unique chromatin structure absolutely correlates with centromere function and is modulated by a novel element within the functionally critical centromeric K repeat. We have examined the central core chromatin structure of thirteen functional minichromosomes containing portions of centromeric DNAs from all three S. pombe chromosomes and all show a central core structure lacking a regular periodic nucleosomal array (Figs. 2 D, 3, C and E and data not shown). In addition, we have examined three centromere-inactive plasmids containing sequences from cen1 and cen2 and found that in all cases the central core sequences present on these plasmids are packaged into a regular nucleosomal array indistinguishable from that of bulk chromatin (Figs. 2 C and E, 3 D).

The addition of specific sequences from the K centromeric repeat to central core containing plasmids not only confers centromere function to these otherwise centromere-inactive minichromosomes (Baum et al., 1994), but concomitantly alters central core chromatin structure (Fig. 2 D). A DNA element that resides within a 2.1-kb KpnI fragment from the centromeric K repeat is necessary and sufficient to confer this unique chromatin structure to central core sequences. Presumably, this is the same element that is responsible for the restoration of centromere activity to plasmids carrying these sequences. The deletion of this region of the K repeat specifically results in loss of the unique central core chromatin structure characteristic of functional centromeres (Fig. 3, C and D) and loss of centromere function. Sequences within other centromeric repeats cannot substitute for this element in altering central core chromatin structure (see Fig. 2, C and E) or conferring centromere activity (Baum et al., 1994).

The centromere enhancer within the K repeat appears to interact specifically with central core sequences; this is indicated by the presence of a periodic nucleosomal array for DNA sequences between the enhancer and central core and by the lack of any apparent effect of the enhancer on DNA sequences other than those of the central core (Fig. 4 B). Thus, this element is unique in altering central core chromatin structure from a distance, and in an orientation-independent manner, without altering the nucleosomal packaging of intervening sequences. The finding that the enhancer functions in either orientation is not surprising because K-type repeats are oriented differently with respect to the central core in the three S. pombe centromeres (Fig. 1; Chikashige et al., 1989; Clarke and Baum, 1990; Hahnenberger et al., 1991; Steiner et al., 1993; Baum et al., 1994).

What structural features are responsible for the non-nucleosomal appearance of the central core regions of functional S. pombe centromeres? Proteins required for the structure and function of active centromeres may bind extensively to central core DNA displacing histone subunits and preventing the formation of nucleosomal arrays. Apparently, nucleosomes occupy only a limited set of sites within the central core; in most cases the number of regularly spaced nucleosomes present in any one area is limited to two, because only mononucleosomes and dinucleosomes are seen for all centromere-functional minichromosomes tested. Alternatively, the central core may be packaged into a periodic nucleosomal array that is masked by the presence of a large number of centromere-specific proteins. S. pombe kinetochores are visible as small fibrillar structures by electron microscopy (Ding et al., 1993). The kinetochore proteins may limit access of the nuclease to the central core DNA resulting in an atypical nuclease digestion pattern.

A model of how the centromere enhancer may interact with central core sequences to yield a functional centromere is presented in Fig. 5. This model predicts that DNA sequences between the enhancer and the central core loop out, allowing interaction between these two elements. Proteins bound within the 2.1-kb KpnI fragment of the K-type repeat might contact the central core DNA directly or, more likely,
contact a factor bound to central core sequences. In this regard, several regions within the critical 2.1-kb region of the K repeat, as well as within the central core, have been shown to bind proteins present in S. pombe nuclear extracts (Baum et al., 1994; Ngan, V., D. Halverson, and L. Clarke, unpublished). Interaction between the enhancer and the central core alters the chromatin structure of the central core, either resulting in activation of centromere function or as a result of centromere function. Our data suggest that this interaction may be a prerequisite for the recruitment of kinetochore components that mediate or facilitate attachment to the mitotic and meiotic spindles. In human centromeres, for example, the localization of the centromere-binding protein CENP-C is restricted to active centromeres (Earnshaw et al., 1989). The model is somewhat analogous to the looping model proposed for the interaction of a transcriptional enhancer with its promoter (Pfahlne, 1988) or that of a locus control region/enhancer with its target sequences (Forrester et al., 1987; Groveld et al., 1987; Reittman et al., 1993). All three examples implicate DNA looping and affect the chromatin structure of the sequences involved.

The establishment of functional centromeres in S. pombe is clearly complex. Minimally, it requires the presence of two DNA elements, sequences present within a 2.1-kb region of the K-type repeat and portions of the central core (Baum et al., 1994), as well as an undetermined number of trans-acting centromere-specific binding factors and their regulators. The finding that S. pombe kinetochores and, indeed, those of sister chromatids, are not always associated with the same number of microtubules (Ding et al., 1993) in mitosis is consistent with the functional redundancy observed with centromeric central core sequences (Baum et al., 1994) and also with the hypothesis that kinetochore capture of spindle fibers emanating from the spindle pole bodies in prometaphase might occur in S. pombe, as in other organisms (Hayden et al., 1990). The unique chromatin structure of the central core may serve to differentiate that region from the bulk chromatin of the chromosomal arms, thus facilitating the successful capture of spindle microtubules.

The S. pombe system is an excellent model for centromere structure and function in higher eukaryotes. Centromere-specific repeats, which are present in many eukaryotes, are clearly implicated in centromere function in S. pombe. The finding that sequences within the central core of cen2 are functionally redundant (Baum et al., 1994) is particularly intriguing in light of the observation that mammalian centromeres can be fragmented into small units that are still capable of spindle attachment and progression through mitosis (Zinkowski et al., 1991). The unusual central core chromatin structure mediated by the cis-acting enhancer element within the K-type centromeric repeat is associated only with functional centromeres, suggesting an important role for this novel element in centromere structure/function.

We thank John Carbon, Mary Baum, Vivian Ngan, and Noemi Steiner for helpful discussions and critical reading of the manuscript. This work was supported by U.S. Public Health Services grant GM-33783 from the National Institutes of Health.

Received for publication 27 July 1994 and in revised form 25 October 1994.
Polizzi, C., and L. Clarke. 1991. The chromatin structure of centromeres from fission yeast: differentiation of the central core that correlates with function. J. Cell Biol. 112:191-201.

Ptashne, M. 1988. How transcriptional activators work. Nature (Lond.). 335:683-689.

Schulman, I., and K. S. Bloom. 1991. Centromeres: an integrated protein DNA complex required for chromosome movement. Annu. Rev. Cell Biol. 7:311-336.

Reitman, M., E. Lee, H. Westphal, and G. Felsenfeld. 1993. An enhancer/locus control region is not sufficient to open chromatin. Mol. Cell. Biol. 13:3990-3998.

Singer, M. 1982. Highly repeated sequences in mammalian genomes. Int. Rev. Cytol. 76:67-112.

Steiner, N. C., K. M. Hahnenberger, and L. Clarke. 1993. Centromeres of the fission yeast Schizosaccharomyces pombe are highly variable genetic loci.

Polizzi, C., and L. Clarke. 1991. The chromatin structure of centromeres from fission yeast: differentiation of the central core that correlates with function. J. Cell Biol. 112:191-201.

Ptashne, M. 1988. How transcriptional activators work. Nature (Lond.). 335:683-689.

Schulman, I., and K. S. Bloom. 1991. Centromeres: an integrated protein DNA complex required for chromosome movement. Annu. Rev. Cell Biol. 7:311-336.

Reitman, M., E. Lee, H. Westphal, and G. Felsenfeld. 1993. An enhancer/locus control region is not sufficient to open chromatin. Mol. Cell. Biol. 13:3990-3998.

Singer, M. 1982. Highly repeated sequences in mammalian genomes. Int. Rev. Cytol. 76:67-112.

Steiner, N. C., K. M. Hahnenberger, and L. Clarke. 1993. Centromeres of the fission yeast Schizosaccharomyces pombe are highly variable genetic loci.

van Holde, K. E. 1989. Chromatin. Springer-Verlag, New York. pp. 313-314.

Wevrick, R., and H. F. Willard. 1989. Long range organization of tandem arrays of α-satellite DNA at the centromeres of human chromosomes: high frequency array-length polymorphism and meiotic stability. Proc. Natl. Acad. Sci. USA. 86:9394-9398.

Zhang, X.-Y., F. Fittler, and W. Horz. 1983. Eight different highly specific nucleosome phases and α-satellite DNA in the African green monkey. Nucleic Acids Res. 11:4287-4306.

Zinkowoski, R. P., J. Meyne, and B. R. Brinkley. 1991. The centromerekinetocho complex: a repeat subunit model. J. Cell Biol. 113:1091-1110.