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

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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 ~1–2 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 (Wevrick 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 both mitotically and meiotically 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).

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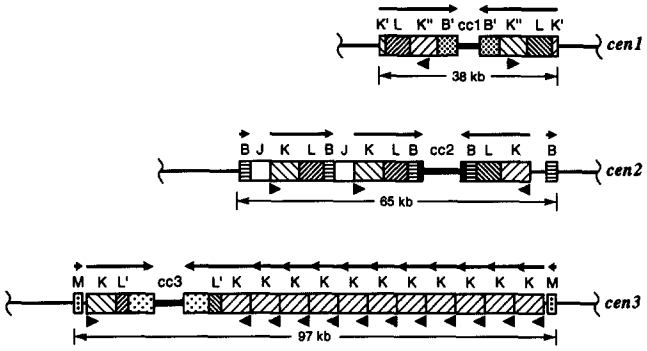

Figure 1. Schematic representation of the centromeric DNAs from chromosomes I (*cen1*), II (*cen2*), and III (*cen3*) of *S. pombe* strain Sp223. All three centromeres contain a large inverted repeat flanking a chromosome-specific central core (*cc*). The inverted repeat consists of centromere-specific repeated elements (*K* and *L*) as well as chromosome-specific repeated elements (*B*, *B'*, and *M*). The 6.4-kb K repeats in *cen2* are similar to the *cen3* K repeats with respect to orientation and structure (Steiner et al., 1993). The K-type repeat in *cen1* is divided into two parts (*K'* and *K"*); the orientation of K" is reversed relative to that of K' (Hahnenberger et al., 1991). Sequences within the K repeat have been designated as the dg repeat by Nakaseko et al. (1986, 1987). The *cen3* L' repeat is a truncated (~3 kb) version of the 4.5-kb L repeat (Steiner et al., 1993). The *cen1* central core (*cc1*) is 4.1 kb, the *cen2* central core (*cc2*) is 6.8 kb, and the *cen3* central core (*cc3*) is ~5 kb. cc1 and cc3 share ~3.3 kb of homology; cc2 is comprised of DNA sequences that are unique in the *S. pombe* genome. Chromosome-specific repeats flank the central core of each centromere (*cen1* structure from Hahnenberger et al. [1991]; *cen2* and *cen3* structures from Steiner et al. [1993]). The location and orientation of the 2.1-kb KpnI fragment that contains the centromere enhancer is indicated by the solid arrowhead under each K-type repeat.

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 Carbon, 1982; Schulman and Bloom, 1991). Single base pair mutations that inactivate the centromere completely disrupt the nuclease-resistant core structure (Schulman and Bloom, 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 central 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 leu1.32 ura4.294*; gift from D. Beach) and SBP120390 (*h- ade6.704 leu1.32 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 HincII 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 (Ito et al., 1983). The construction of *cen1*-derived minichromosomes has been described (pSp[*cen1*])-BHp, Hahnenberger et al., 1991; pSp-BHpΔKpn, 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 H_2O. 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 PMSF), and then lysed in lysis buffer (20 mM Pipes, pH 6.8, 18% Ficoll 400, 0.5 mM $MgCl_2$, 1 mM PMSF). 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 $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM PMSF). The resuspended nuclei were prewarmed at 32°C for 3 min and micrococcal nuclease (Boehringer Mannheim Biochemicals, Inc.) was added to 250 U/ml. Aliquots were removed after various times of incubation at 32°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 (Bio101), and ^{32}P-labeled by random priming to a specific activity of $>10^8$ 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′(*cen1*), 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 et al., 1991).

Micrococcal nuclease, which under partial digestion conditions cuts specifically in the linker DNA regions between nucleosome core particles, is a powerful reagent for probing the nucleosomal structure of eukaryotic chromatin (for review see Kornberg, 1977; van Holde, 1989). The micrococcal nuclease digestion pattern of central core chromatin from the centromere of chromosome II (*cen2*) is shown in Fig. 2 *B*. When a DNA blot of this chromatin is probed with a unique 850-bp *cen2* central core EcoRI fragment (Fig. 2 *A*), a broad smear lacking any evidence of a periodic nucleosomal array (DNA ladder) is revealed (Fig. 2 *B, right*). This pattern is in sharp contrast to the periodic nucleosomal array evident in the ethidium bromide–stained gel derived from bulk chromatin of *S. pombe* (Fig. 2 *B, left*). The absence of a periodic nucleosomal array is revealed with probes from all regions of the *cen1* and *cen2* central cores (Polizzi and 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^{-1}; 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 *cen1* 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″ Alters Central 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^{-2} and 4.3×10^{-2}, 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

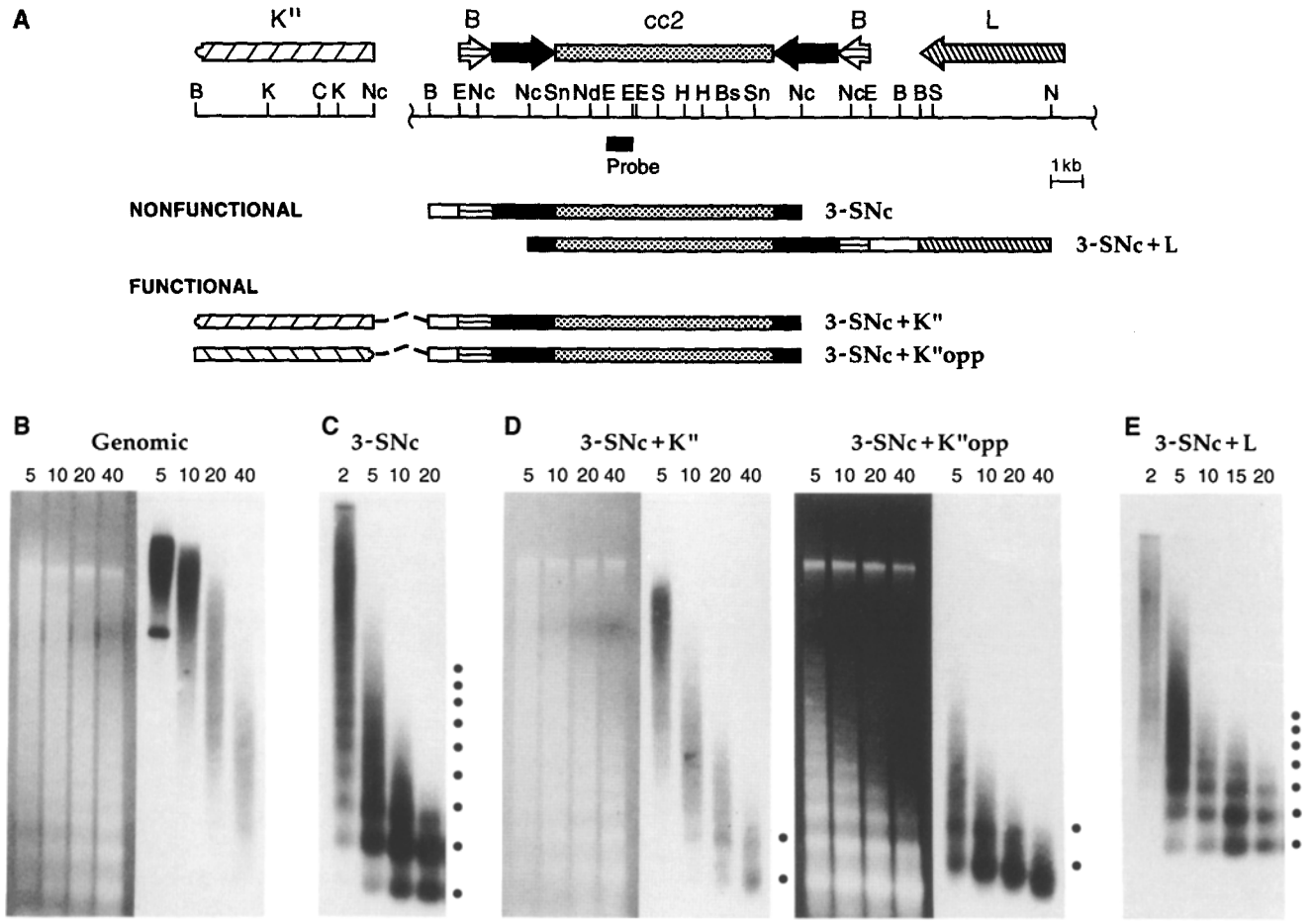

Figure 2. Centromeric central core chromatin structure of centromere-inactive and centromere-active minichromosomes in *S. pombe*. (*A*) Structures of circular *cen2*-derived minichromosomes aligned with the restriction maps of the K″ repeat and a portion of *cen2*. In the functional minichromosomes, 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

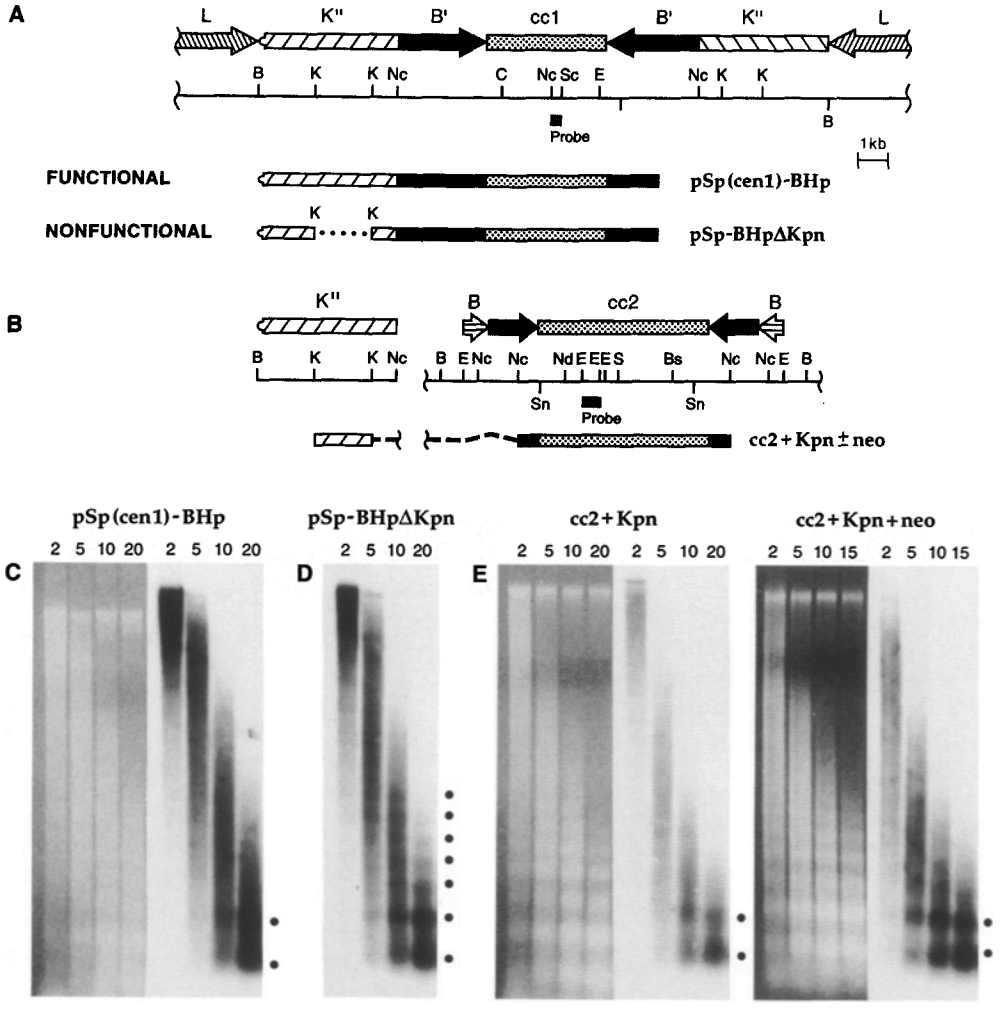

Figure 3. Central core chromatin structure is affected by the presence of a 2.1-kb region from the K-type centromeric repeat. (*A*) The structures of *cenl*-derived minichromosomes aligned with a restriction map of *cenl* from strain Sp223. pSp-BHpΔKpn was derived from pSp(*cenl*)-BHp and lacks the 2.1-kb KpnI–KpnI fragment of the K-type repeat, which is critical for centromere function (Baum et al., 1994). The bar under the restriction map indicates the 350-bp NcoI–ScaI fragment used to probe the blots in *C* and *D*. Restriction site designations are *B*, BamHI; *C*, ClaI; *E*, EcoRI; *K*, KpnI; *N*, NcoI; *Sc*, ScaI. Not all ClaI or ScaI sites are shown. (*B*) Structure of *cen2* central core–containing minichromosomes pSp-cc2+Kpn and pSp-cc2+Kpn+neo aligned with the restriction map of *cen2* and K″ repeat sequences. Dashed lines indicate adjoining of DNA segments that are normally noncontiguous in genomic centromeric DNA. Restriction site designations are *B*, BamHI; *Bs*, BstEII; *E*, EcoRI; *K*, KpnI; *Nc*, NcoI; *Nd*, NdeI; *S*, SphI; *Sn*, SnaBI. Not all ClaI, SphI, or SnaBI sites are shown. (*C*) (*Left*) Ethidium bromide–stained gel revealing the nucleosomal DNA ladder generated by micrococcal nuclease digestion of bulk Sp223/pSp(*cenl*)-BHp chromatin. (*Right*) Autoradiogram of blot (*left*) probed with the 350-bp NcoI–ScaI fragment from the *cenl* central core. (*D*) Autoradiogram of DNA blot from micrococcal nuclease–digested Sp223/pSp-BHpΔKpn chromatin probed with the 350-bp NcoI–ScaI fragment from the *cenl* central core. A distinct nucleosomal DNA ladder is superimposed on the broad smear attributable to hybridization to the genomic copies of *cenl* and *cen3*. (*E*) (*Left panel, cc2+Kpn* set) Ethidium bromide–stained gel showing the nucleosomal DNA ladder generated by micrococcal nuclease digestion of bulk SBP120390/pSp-cc2+Kpn chromatin, and (*right panel*) autoradiogram of the blot. (*Left panel, cc2+Kpn+neo* set) Ethidium bromide–stained gel showing the nucleosomal DNA ladder generated by micrococcal nuclease digestion of bulk SBP120390/pSp-cc2+Kpn+ neo chromatin, and (*right*) autoradiogram of the blot. Both blots are probed with the 850-bp EcoRI fragment from the *cen2* central core. In panels *C–E*, the numbers above each lane indicate the length of time (min) of micrococcal nuclease digestion and dots next to the autoradiograms indicate the positions of detectable nucleosomal DNA bands.

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 *cenl*-derived minichromosome pSp(*cenl*)-BHp contains the entire *cenl* central core, *cenl*-specific core-associated repeat sequences, and one copy of repeat K″ (Fig. 3 *A*, mitotic loss rate of 3.7×10^{-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 7.1×10^{-1}; Baum et al., 1994). Furthermore, the addition of this 2.1-kb KpnI fragment confers 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 1.4×10^{-2}; 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* 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(*cenI*)-BHp and the centromere-inactive minichromosome pSp-BHpΔKpn. The DNA blot of partially digested chromatin from a strain carrying pSp(*cenI*)-BHp, probed with a 350-bp NcoI–ScaI fragment near the center of the *cenI* 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 *cenI* central core fragment reveals a distinct DNA ladder, with evidence of stretches of at least seven regularly spaced nucleosomes (Fig. 3 *D*). The *cenI* central core probe hybridizes to central core sequences in *cenI* and *cen3* (Hahnenberger et al., 1991; Takahashi et al., 1992). Therefore, the ladder ascribed to the central core of pSp-BHpΔKpn is seen superimposed on a broad smear attributable to hybridization to the genomic copies of *cenI* and *cen3*. Thus, deletion of the 2.1-kb KpnI fragment from the sole copy of the K-type repeat in a centromere-active minichromosome 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 B, 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 4.8×10^{-1}), 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

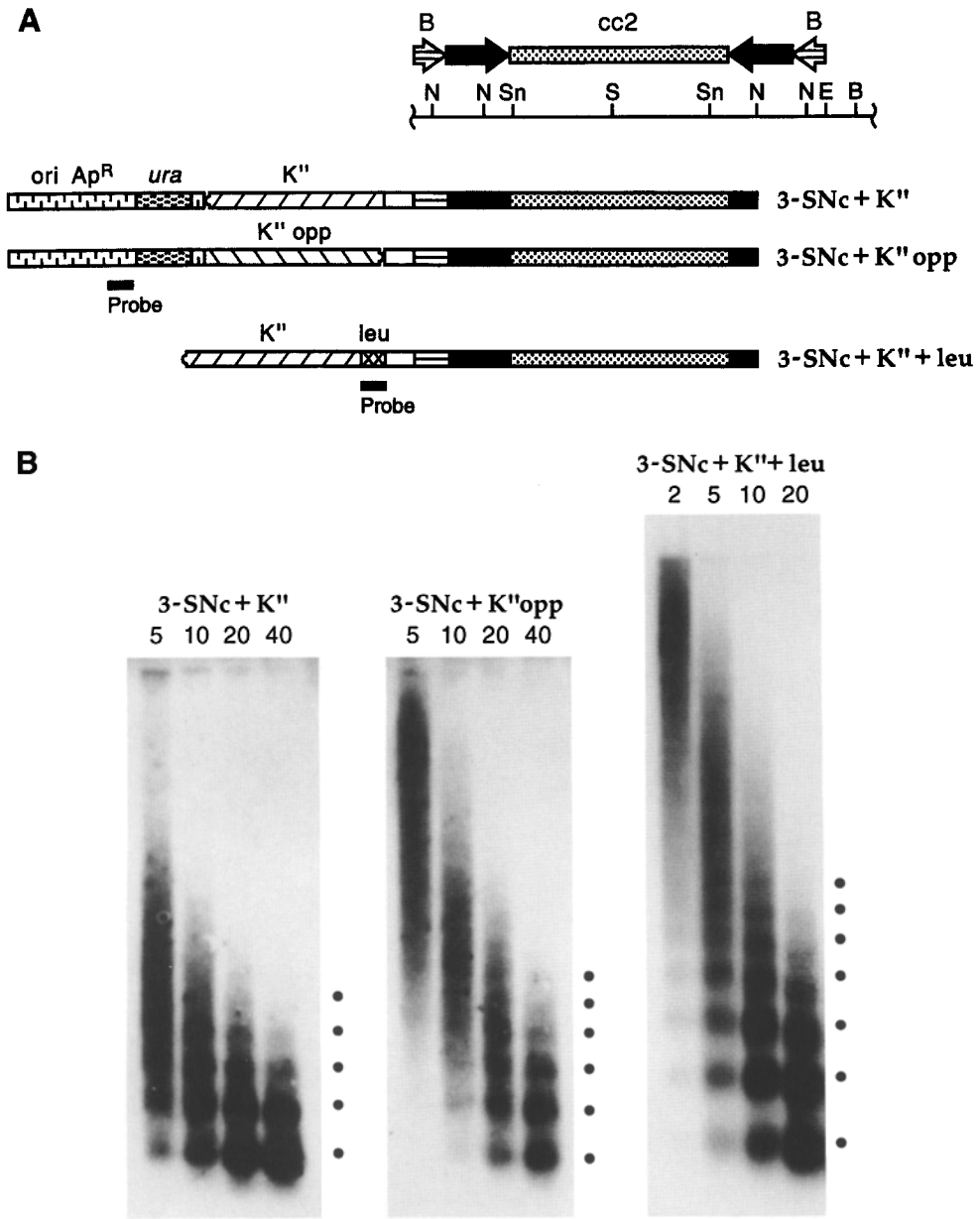

Figure 4. Chromatin structure of sequences located between the K-type repeat and the centromeric central core in centromere-active minichromosomes. (*A*) Minichromosomes used to examine chromatin structure of sequences between the K″ repeat and the central core. Shown are the structures of pSp3-SNc+K″, pSp3-SNc+K″opp, and pSp3-SNc+K″+leu aligned with a restriction map of the *cen2* central core and core-associated repeats. The bars under the structures indicate the fragments used to probe the DNA blots shown in *B*. Restriction site designations are *B*, BamHI; *E*, EcoRI; *N*, NcoI; *Sn*, SnaBI; *S*, SphI. Not all SnaBI or EcoRI sites are shown. (*B*) Blots of nucleosomal DNA probed with the fragments indicated in *A*. The minichromosome carried by the strain from which the chromatin was obtained is indicated above the autoradiograms. The blots of DNA from micrococcal nuclease digested chromatin of *S. pombe* strains Sp223/pSp3-SNc+K″ (*left*) and Sp223/pSp3-SNc+K″opp (*middle*) were probed with a 750-bp PstI–EcoRI fragment from pBR322. The blot of DNA from micrococcal nuclease–digested chromatin of strain Sp223/pSp3-SNc+K″+leu was probed with a 660-bp HincII fragment from the *S. cerevisiae LEU2* gene. The numbers above the lanes indicate the length of micrococcal nuclease digestion in minutes. Dots beside the autoradiograms mark the band positions of the nucleosomal DNA ladders.

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 *LEU2* 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 observation). 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 native 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,

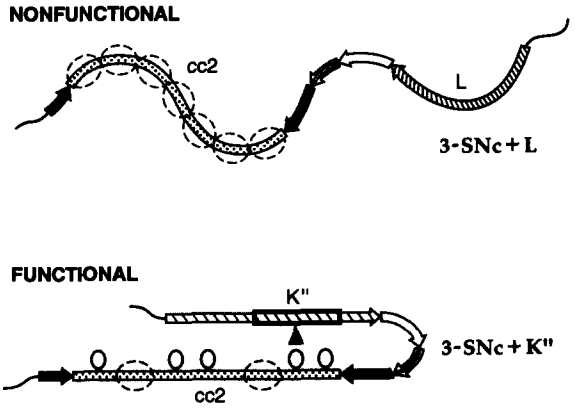

Figure 5. Model for the interaction between the centromere enhancer of the K-type repeat and the central core. Schematic representation of a proposed protein-mediated interaction between the centromere enhancer of the K-type repeat and the central core, resulting in the distinctive centromeric central core chromatin structure associated with centromere function. DNA sequences between the K-type repeat and the central core loop out, allowing interaction between a protein bound to the enhancer and a protein bound to central core sequences. The interaction between the centromere enhancer and the central core alters the central core chromatin structure (*3-SNc+K″*). Several regions within the 2.1-kb fragment of the K-type repeat that encompasses the enhancer and within the centromeric central core have been shown to bind proteins present in *S. pombe* nuclear extracts. When centromeric central core sequences are present in a nonfunctional context (*3-SNc+L*) in *S. pombe*, the central core is packaged into a periodic nucleosomal array typical of bulk chromatin. The dashed ovals represent nucleosomes (not drawn to scale).

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 (Ptashne, 1988) or that of a locus control region/enhancer with its target sequences (Forrester et al., 1987; Groveld et al., 1987; Reitman 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.

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