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The Centromere Enhancer Mediates Centromere Activation in *Schizosaccharomyces pombe*

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The centromere enhancer is a functionally important DNA region within the *Schizosaccharomyces pombe* centromeric K-type repeat. We have previously shown that addition of the enhancer and *cen2* centromeric central core to a circular minichromosome is sufficient to impart appreciable centromere function. A more detailed analysis of the enhancer shows that it is dispensable for centromere function in a *cen1*-derived minichromosome containing the central core and the remainder of the K-type repeat, indicating that the critical centromeric K-type repeat, like the central core, is characterized by functional redundancy. The centromeric enhancer is required, however, for a central core-carrying minichromosome to exhibit immediate centromere activity when the circular DNA is introduced via transformation into *S. pombe*. This immediate activation is probably a consequence of a centromere-targeted epigenetic system that governs the chromatin architecture of the region. Moreover, our studies show that two entirely different DNA sequences, consisting of elements derived from two native centromeres, can display centromere function. An *S. pombe* CENP-B-like protein, Abp1p/Cbp1p, which is required for proper chromosome segregation in vivo, binds in vitro to sites within and adjacent to the modular centromere enhancer, as well as within the centromeric central cores. These results provide direct evidence in fission yeast of a model, similar to one proposed for mammalian systems, whereby no specific sequence is necessary for centromere function but certain classes of sequences are competent to build the appropriate chromatin foundation upon which the centromere/kinetochore can be formed and activated.

The centromere, a chromosomal landmark governing the proper segregation of chromosomes during mitosis and meiosis, is the site of sister chromatid attachment and kinetochore assembly. Recent studies have indicated that the centromere is also involved in checkpoint control during the cell cycle, delaying the onset of the metaphase-to-anaphase transition if all mitotic chromosomes have not attained bipolar orientation on the spindle (4, 36). Thus, the centromere acts as a multifunctional region upon which successful cell division depends.

The structure and function of centromeres in a variety of eukaryotes have been extensively studied (reviewed in reference 35), including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and humans. The centromeres that have been best characterized are those in the yeasts. *S. cerevisiae* centromeres are examples of point centromeres in which all the DNA sequences necessary in *cis* for function are contained within a 125-bp stretch that is composed of three conserved elements, CDEI, CDEII, and CDEIII. CDEIII, a binding site for the centromere-protein complex CBF3 (21), is most critical to centromere function, as certain single-nucleotide changes within this element result in total inactivation of the centromere. Other studies have shown that the CDEII element plays a critical role in sister chromatid cohesion in meiosis (9, 38). Thus, it appears that the spindle attachment and sister chromatid cohesion aspects of centromere function can be uncoupled. The search for *S. cerevisiae* centromere-binding proteins has led to the identification of CBF1/CP1 (3) and the four-component complex, CBF3 (21, 44), which bind to CDEI and CDEIII, respectively. In addition, the kinesin-related motor protein Kar3p copurifies with the CBF3 complex (24). However, it is still unclear how these DNA and protein components interact with each other to establish an active *S. cerevisiae* centromere.

In contrast to the point centromeres in *S. cerevisiae*, those in the fission yeast *S. pombe* are much more complex and are considered the paradigm of the regional centromere found in higher eukaryotes (35). Native *S. pombe* centromeres encompass 40 to 100 kb of DNA containing extensive amounts of centromere-specific repeated sequences (5–7, 14, 17, 28, 32). Although the arrangement of the different repeated DNA elements differs among the three *S. pombe* centromeres, as well as among different fission yeast strains, the common structural motif consists of a 4- to 7-kb central core flanked by a large (~35-kb) inverted repeat, which itself is composed of centromeric repeat elements organized in a chromosome-specific manner (43, 45). The complexity of the fission yeast centromeres, however, is considerably lower than that of mammalian centromeres, which encompass hundreds to thousands of kilobases of DNA, the majority of which is highly repetitive satellite DNA (25, 39).

In spite of the complexity of mammalian centromeres, considerable progress toward their characterization has been made. Several human centromere proteins (CENPs) have been identified and characterized through the use of antisera from humans afflicted with the CREST syndrome of scleroderma (27) and monoclonal antibodies directed against fractionated chromosomal proteins (50). CENP-A is a centromere-specific histone (34), and CENP-B binds to a 17-bp motif, the CENP-B box, found in α-satellite DNA (23). CENP-E localizes to the outer kinetochore plate prior to the commencement of mitosis and, along with the inner kinetochore-associated kinesin-like protein MCAK (49), is thought to be a motor protein responsible for chromosome movement (50). CENP-C and CENP-D are both found tightly associated with the kinetochore plates,

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but their specific functions are not known. Two proteins which localize to the inner region between chromatids (INCENPs) have been identified and are thought to be involved in the regulation of sister chromatid separation (12). Although a number of protein components of the mammalian centromere-kinetochore complex have been identified, the mechanics of kinetochore formation and activity are still not understood, and characterization has been hindered by the presence, in the region, of highly repetitive satellite DNAs that vary dramatically in sequence among different species and by the lack of an artificial chromosome assay for centromere function.

Two distinct structural models have been suggested for mammalian centromeric DNA organization. One proposes that centromeric satellite DNA or some combination of repeated DNAs is an integral component of the functional centromere and is perhaps the only DNA required in *cis*, forming the foundation upon which the proteinaceous kinetochore is formed (16, 48). The other is a repeat-subunit model that postulates that the kinetochore is formed by folding together of specific microtubule-interacting DNA segments, which in turn are separated on the linear DNA by satellite linker segments (51). Unlike the former model, where highly repeated DNA is the foundation for kinetochore formation, in the subunit repeat model the satellite DNA plays a somewhat ancillary role, connecting nucleotide sequences that bind specific kinetochore proteins; thus, the regional centromere is equivalent to an organized collection of point centromeres. The major difference in the two models is that the latter predicts specific microtubule-interacting DNA regions, each somewhat analogous to the *S. cerevisiae* centromere locus and interspersed within satellite sequences, thereby incorporating properties of the simple centromere into the complex one, while the former model postulates a heterochromatin foundation formed perhaps from certain classes of DNA elements but without a strict requirement for a particular DNA sequence.

Because *S. pombe* centromeres share a number of characteristics with higher eukaryotic centromeres, including the presence of various classes of untranscribed repeated DNA sequences (reviewed in references 8 and 35), multiple spindle fiber attachments per kinetochore (11), and the capacity to be functionally regulated in vivo via a centromere-targeted epigenetic system (42), we have undertaken a detailed evaluation of the centromeric DNA requirements in this versatile yeast system to distinguish between the two models for higher eukaryotic centromeric structural organization described above. We have shown previously that two *S. pombe* centromeric DNA elements, the unique central core and portions of the centromere-specific K-type repeat, are important to centromere function and together on a minichromosome are sufficient to establish a functional centromere (1). The 2.1-kb *Kpn*I fragment within the K-type repeat, termed the centromere enhancer, can act in an orientation- and distance-independent manner to affect both centromere function and the chromatin structure of the central core (22). In addition, a structurally compromised centromere on a circular minichromosome can become functionally activated in *S. pombe* by a novel epigenetic mechanism (42). Specific protein-binding sites within the K-type repeat (1) and the centromeric central core DNA (18) (also see below) have also been identified. These findings have led us to propose a model for the establishment of a functional centromere (discussed in reference 8) that involves the DNA looping-facilitated, protein-mediated interaction of the *Kpn*I region (centromere enhancer) with the central core, such that the central core and its surrounding sequences become packaged into a higher-order chromatin structure that supports the various aspects of centromere function.

To evaluate the contribution made by the K-type repeat to overall centromere function and the validity of our model in the context of the two models for the higher eukaryotic centromeric DNA organization described above, we performed a characterization of the K-repeat region directed toward identifying the functionally critical sequence(s), addressing the functional importance of various protein-binding sites, localizing these sites, and identifying the proteins binding to them. We report here that the centromeric K-type repeat, like the centromeric central core, is characterized by functional redundancies and that portions of the repeat are dispensable for centromere function of minichromosome constructs. We also show that a CENP-B-like *S. pombe* protein (Abp1p/Cbp1p) binds in vitro to several sites within K-repeat DNA and adjacent sequences, as well as within the central core, suggesting a mechanistic link between the *S. pombe* and human centromeres. Importantly, our results provide, for the first time, direct evidence for an *S. pombe* model similar to one described above for the mammalian centromere, where no single sequence is absolutely necessary for function but, rather, certain classes of DNA elements are competent for providing the appropriate chromatin foundation for centromere/kinetochore formation and activation.

MATERIALS AND METHODS

Strains, culturing, transformations, and genetic manipulations. The *S. pombe* strains used in this study are Sp223 (*h*$^-$ *ade6-216 leu1-32 ura4-294*; a gift from D. Beach), SBP120390 (*h*$^-$ *ade6-704 leu1-32 ura4-294*; from our laboratory), SBP42192 (*mat1-P mat2,3Δ::LEU2*$^+$*::his2 lys1-1 tps13-1 ura4-294*; from our laboratory [1]), and SBP070196-F5C (*h*$^-$ *ade6-216 leu1-32 ura4-D18 abp1Δ::ura4*$^+$; from our laboratory [18]). The growth media and conditions used were as described by Gutz et al. (15) and Moreno et al. (26). DNA transformations were performed with an alkali-cation yeast transformation kit (Bio 101, La Jolla, Calif.), with the modification that the incubation time in the lithium-cesium acetate solution at 30°C was extended from 30 min to at least 1 h. The mitotic stabilities of the constructs were assayed by the replica-plating method (7) with the *S. pombe ura4* gene as the genetic marker. The mitotic stability is expressed as the apparent mitotic loss frequency, which is calculated as the proportion of all cells that have lost the plasmid and hence are unable to grow on selective media. The meiotic behavior of the constructs was assayed by tetrad analysis with SBP42192 as the mater strain (1).

The centromere activation test was first described by Steiner and Clarke (42) and consists of prolonged propagation (at least 30 doublings) of the *S. pombe* strain carrying the minichromosome of interest in sequentially larger volumes of selective media, followed by an assay of the mitotic stability of the minichromosome in the cells that still carry it. A representative sample of the final culture is serially diluted for plating onto the same selective media. This plating step recovers the cells which still carry the minichromosome. Single colonies from these selective plates are subjected to mitotic stability assays to determine whether the mitotic loss frequency of the minichromosome has changed. This test is effective in recovering cell lines in which the centromere on the minichromosome of interest becomes activated, because those cells attain a growth advantage in selective media once the minichromosome with its markers has been mitotically stabilized.

In vitro construction of minichromosomes. The circular minichromosomes described below were constructed by standard DNA manipulation techniques as described by Sambrook et al. (37). All plasmid intermediates were propagated in *Escherichia coli* DH5α (*rec*A1) (Bethesda Research Laboratories, Gaithersburg, Md.).

The *cen1*-derived minichromosome constructs were made in vitro by inserting a *Kpn*I cassette with the appropriate deletion into the pSp-BHpΔKpn construct to yield the series of pSp(*cen1*)-BHp-based deletion constructs (ΔN-S, ΔS-P, ΔP-C, ΔC-K, ΔB-R, and ΔB-R+ΔP-C [Fig. 1]). The derivation of pSp-BHpΔKpn was described by Baum et al. (1). By using pKNS-Kpn, a pBluescript-based plasmid with the 2.1-kb *Kpn*I cassette cloned into the *Kpn*I site of the polylinker (1), deletions within this cassette were generated by either dropping out the desired segment from the plasmid and religating or isolating the portions of the 2.1-kb *Kpn*I cassette on each side of the deletion and sequentially subcloning the portions into pBluescript KS (Stratagene, La Jolla, Calif.) to regenerate a *Kpn*I cassette. The exceptions to these methods were the ΔB-R-containing deletion constructs. Because the endpoints of this deletion are at restriction sites *Bsm*I and *Rsa*I, both of which are not unique within the 2.1-kb *Kpn*I region, PCR was used to amplify a 425-bp *Nar*I-*Bsm*I fragment and a 70-bp *Rsa*I-*Sna*BI fragment, which were then ligated in the appropriate orientation to generate a *Nar*I-*Sna*BI fragment containing the 65-bp *Bsm*I-*Rsa*I deletion. This *Nar*I-*Sna*BIΔ*Bsm*I-*Rsa*I

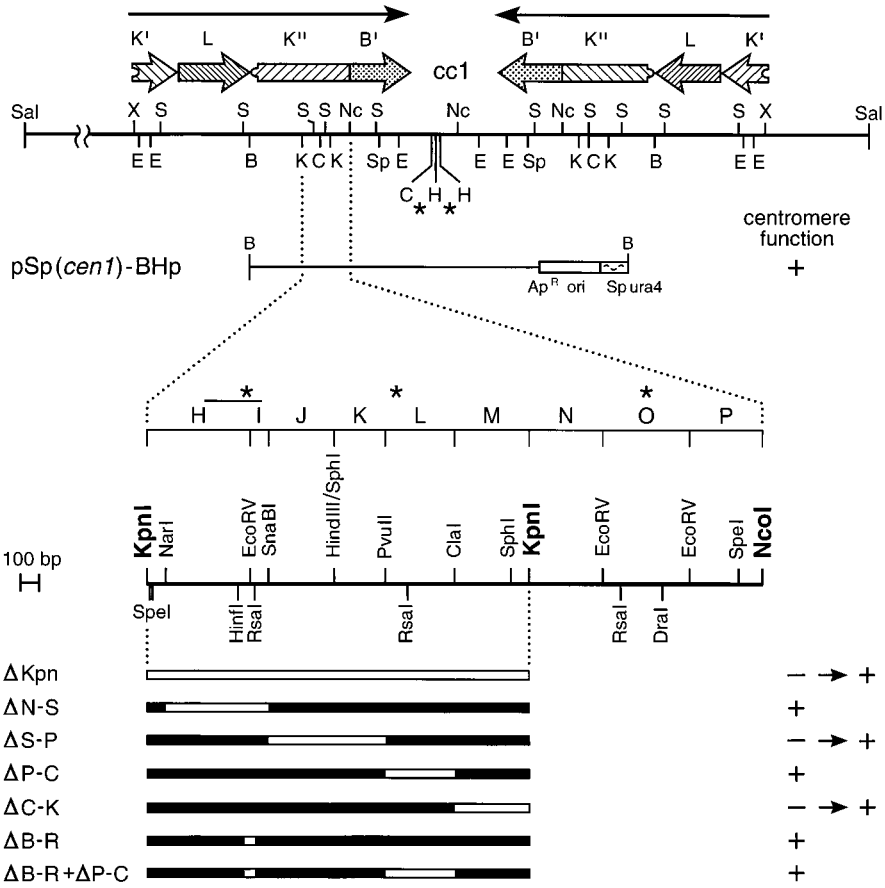

FIG. 1. pSp(*cen1*)-BHp-derived deletion constructs. Structures of the deletion constructs derived from the *cen1* minichromosome pSp(*cen1*)-BHp are shown. The upper portion of the figure represents a partial restriction map of the 65-kb *Sal*I fragment containing the entire *cen1* region, with the centromeric DNA elements marked above the map and the centromere DNA present in the pSp(*cen1*)-BHp minichromosome shown below. The restriction sites are defined as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Nc, *Nco*I; S, *Sph*I; Sal, *Sal*I; Sp, *Spe*I; X, *Xba*I. Not all *Hin*dIII sites are shown. The lower portion of the figure shows an enlarged view of the restriction map of the 3.3-kb *Kpn*I-*Kpn*I-*Nco*I region of the K″ repeat, with the fragments used in previously described gel mobility shift assays with *S. pombe* extracts (1) marked above the map. The bars aligned below the map represent centromere enhancer sequences that are present (solid) or deleted (open) in the various deletion constructs. The presence (+) or absence (−) of centromere function exhibited by each of the deletion constructs is indicated at the right. The locations of specific protein-binding sites within the K″ repeat and cc1 are marked by asterisks.

fragment was used to replace the native *Nar*I-*Sna*BI fragment in the 2.1-kb *Kpn*I cassette to yield the ΔB-R deletion. The *Kpn*I cassette carrying the ΔB-R+ΔP-C double deletion was generated by isolating the *Kpn*I-*Pvu*II*ΔBsm*I-*Rsa*I fragment from the *Kpn*I*ΔBsm*I-*Rsa*I cassette and ligating it in the correct orientation to the *Cla*I-*Kpn*I fragment to yield the *Kpn*IΔB-R+ΔP-C cassette, which was then cloned into pBluescript KS. The various *Kpn*I cassettes, each containing a different deletion(s), were then isolated as *Kpn*I fragments and cloned into the *Kpn*I site of pSp-BHpΔKpn.

The *cen2*-derived constructs were all made in vitro. The vector pSp-ars-cc2 was constructed by cloning cc2 with 0.9 kb of core-associated repeat flanking each end as a *Nco*I-*Sal*I fragment (from pSp-cc2 [1]) into the *Nco*I and *Sal*I sites of the pBluescript-based plasmid pKNS-SUA. This plasmid carries the *S. pombe sup3-e* and *ura4* markers as well as the 1.2-kb *Eco*RI fragment from *S. pombe ars1* (42). Although there are sequences that can act as origins of replication within the central core region, we added a separate origin to the vector to ensure that one was present and readily usable without compromising the ability of the vector to support both centromere activity and replication when the appropriate centromeric sequences were added. To the pSp-ars-cc2 vector (Fig. 2), one of the following fragments from the 2.1-kb *Kpn*I region of the K-type repeat was cloned into the unique *Kpn*I site: a 1.1-kb fragment containing the localized H-I binding site and the *Pvu*II-*Cla*I fragment (fragment L), the 0.66-kb *Kpn*I-*Sna*BI fragment, the 1-kb *Kpn*I-*Hin*dIII fragment, or the 1-kb *Hin*dIII-*Kpn*I fragment. The last two fragments represent the left and right halves of the 2.1-kb *Kpn*I region, respectively. The 1.1-kb fragment was constructed in vitro by sequential cloning of the individual components, i.e., the 90-bp *Hin*fI-*Rsa*I fragment containing the H-I binding site(s), the 375-bp *Pvu*II-*Cla*I fragment containing the L binding site(s), and the 620-bp *Hin*dIII-*Sal*I pBR322 spacer fragment, into pBluescript KS so that the resulting 1.1-kb fragment could be released as an *Eco*RI-*Xho*I fragment for cloning into the pSp-ars-cc2 plasmid vector. To ensure that the potential of these recombinant pSp-ars-cc2 plasmids to exhibit centromere activity was not masked by their small size (discussed in reference 1), each recombinant plasmid was enlarged by site-directed integration of one copy of the *Sca*I-linearized pMB-neo plasmid (8.5 kb) into the Ap^r gene of the vector as described by Clarke and Baum (7).

Structural analysis of the minichromosome constructs. The structure of each minichromosome construct in *S. pombe* after transformation, centromere activation, and, in certain cases, tetrad analysis was verified through Southern analysis. Total *S. pombe* DNA was isolated as described by Beach and Klar (2) and manipulated with only cut-off micropipette tips to avoid shearing the genomic DNA. Analysis of total genomic DNA included an examination of (i) uncut DNA to determine whether the plasmid was present as a free circle or had integrated into the *S. pombe* genome, (ii) DNA restricted with the appropriate enzyme to linearize the plasmid in question [*Bam*HI or *Sal*I for the pSp(*cen1*)-BHp-based deletion constructs, and *Sal*I for the unenlarged or *Not*I for the enlarged recombinant pSp-ars-cc2 plasmids] to determine the overall length of the plasmid, (iii) DNA restricted with *Bam*HI and *Nco*I to determine the size of the K″ element in the pSp(*cen1*)-BHp-based deletion constructs, and (iv) DNA restricted with the appropriate enzyme to linearize the plasmid in question and then partially digested with a restriction enzyme that cuts several times along the length of the plasmid to examine the structural integrity of the plasmid. Restriction digests, electrophoresis of the digestion products, transfer of the DNA to ZetaProbe GT nylon membrane, and hybridization were performed as described by Steiner et al. (43). The following ^{32}P-labeled probes were used in hybridization: pBR322 or pBluescript KS to reveal the circular or linear minichromosome or its fragments; or a 1.7-kb *Bam*HI-*Spe*I fragment to identify the *Bam*HI-*Nco*I K″ repeat in the minichromosome. Because of sequence similarity, the *Bam*HI-*Spe*I fragment also hybridizes to and thus identifies all the genomic fragments containing the K-type repeat.

Primers and PCR. The synthetic oligonucleotides used in this study were made by Operon Technologies (Alameda, Calif.) and are as follows: Nar-5′, 5′-cagga tcCACCAGTATCGGCGCCACAT-3′ (27-mer); Bsm-3′, 5′-gaattCAAATACT AATACTATATATC-3′ (26-mer); Rsa-5′, 5′-gaatTCTTCTACATCATTCAAA CAT-3′ (25-mer); and SnaB-3′, 5′-ccgtcgacCAGACAATACGTATGCGATT-3′ (28-mer). The *Bam*HI, *Eco*RI, and *Sal*I ends (underlined) on the primers were added to facilitate the cloning of the amplified product. The lowercase nucleotides are not found in the genome and were added to form the 5′ restriction sites. The primers were used to amplify, by PCR, a 425-bp *Nar*I-*Bsm*I fragment and a 70-bp *Rsa*I-*Sna*BI fragment from the 2.1-kb *Kpn*I region of the K-type repeat. PCR amplification was performed as follows. The reaction mixture consisted of 2 ng of template DNA (linearized pBluescript with the K″ repeat cloned into it), 1 μg of each primer (approximately 1 μM), 0.2 mM each deoxynucleoside triphosphate, and 10 U of *Taq* polymerase (AmpliTaq; Cetus) in a 100-μl volume. The amplification parameters used were 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 68°C for 1 min (3 min for the last cycle).

PCR was also used to amplify the 320-bp H-I fragment from the 2.1-kb *Kpn*I region for gel mobility shift assays and localization of the protein-binding site(s) within the H-I fragment. The primers, reaction conditions, and parameters used for this PCR amplification were previously described by Baum et al. (1).

Localization of the protein-binding site(s) within fragments. All four fragments, H-I, L, O, and c1-8, had previously been subcloned into pBluescript KS. To localize the protein-binding site(s), each fragment was released from the plasmid (or, in the case of H-I, PCR amplified) and subjected to digestion by other restriction enzymes to yield subfragments. Each of these subfragments was used as a specific competitor of the complexes formed by the full-length fragment in gel mobility shift assays (see below). A subfragment that was able to specifically compete out the formation of all full-length complexes was considered to contain the protein-binding site(s) present on the full-length fragment. The subfragment was then used as a probe in a gel mobility shift assay to confirm that it formed the same number of complexes as the full-length fragment and that these complexes could be specifically competed by the subfragment as well as by the full-length fragment. The two H-I *Eco*RV subfragments were not effective competitors of the full-length H-I complexes, and so it was presumed that the H-I-binding site(s) either included or was immediately adjacent to the *Eco*RV site. A 90-bp *Hin*fI-*Rsa*I H-I subfragment, which encompasses the *Eco*RV site (Fig. 1), was found to be a very effective competitor of the full-length H-I complexes, thereby localizing the H-I-binding site(s) to a 90-bp *Hin*fI-*Rsa*I subfragment. The protein-binding site(s) within the full-length c1-8 fragment could not be delimited by these methods.

Gel mobility shift assays. The DNA probes used were the H-I, L, and O fragments or the subfragments containing the localized protein-binding sites from the K″ repeat and the c1-8 fragment (a 265-bp *Cla*I-*Hin*dIII fragment) from the *cen1* central core. These probes were radiolabeled by filling the 5′-overhanging restriction ends with ^{32}P-labeled dATP and dCTP with the Klenow fragment of DNA polymerase I (New England BioLabs, Beverly, Mass.). Gel mobility shift assays were performed as described by Baum et al. (1) with two modifications: the concentration of KCl was reduced to 50 mM, which optimizes complex formation by these fragments, and the amount of the nonspecific competitor poly(dI-dC) used was a standard 2 μg per 20-μl reaction mixture. The source of protein was a 400 mM KCl whole-cell extract from either a wild-type strain (Sp223) or the Δ*abp1* strain (SBP070196-F5C).

Preparation of *S. pombe* protein extracts. The protein extracts used in the studies described here were either a crude nuclear extract or a 400 mM KCl whole-cell extract from the *S. pombe* Sp223 wild-type strain or the Δ*abp1* strain (SBP070196-F5C). Preparation of a crude nuclear extract of *S. pombe* proteins was described previously by Baum et al. (1). The 400 mM KCl extract of whole cells was prepared by a modified version of the mechanical-disruption-in-liquid-nitrogen method (41) and is described in detail elsewhere (18).

Affinity purification of a centromere-binding protein. The details of the affinity purification of the 62-kDa protein, which was independently identified as Abp1p (29), are described elsewhere (18).

Gel filtration chromatography. The sizes of the activities binding to the H-I, L, O, and c1-8 fragments were determined by gel filtration chromatography. A crude nuclear extract was prepared from 1 liter of *S. pombe* Sp223 cells and loaded onto an equilibrated Sephacryl S300 (Pharmacia Biotech, Piscataway, N.J.) column, with 400 mM NaCl–600 mM KCl–10 mM HEPES-KOH (pH 8.0)–1.5 mM $MgCl_2$–0.1 mM EGTA–0.5 mM dithiothreitol–5% glycerol–0.5 mM phenylmethylsulfonyl fluoride–2 mM NaF as the running buffer. The column was calibrated with gel filtration molecular size standards (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, albumin, carbonic anhydrase, and cytochrome *c*; kit MW-GF-1000 [Sigma, St. Louis, Mo.]).

RESULTS

The *S. pombe* 2.1-kb *Kpn*I centromere enhancer region is dispensable for centromere function in the presence of other centromere repeats. We have reported previously that the *S. pombe* centromeric K-type repeat (~6 kb) and the *cen2* central core (cc2; ~7 kb) are two centromere-specific DNA elements that together are sufficient for partial centromere function on a minichromosome in fission yeast (1). Minichromosomes exhibiting partial centromere function retain appreciable mitotic stability and segregate through meiosis to two of the four haploid products but exhibit precocious sister chromatid separation in meiosis I. Functionally important sequences of the K-type repeat element are contained within the 2.1-kb *Kpn*I region (Fig. 1), since a single copy of this region, in conjunction with cc2 sequences, can constitute a partially functional centromere. This region of the K-type repeat has also been designated the centromere enhancer because it affects the central core chromatin structure at a distance and in an orientation-independent manner (22).

Our laboratory has also described an epigenetic system that can target and alter the centromere function of minichromosomes introduced by DNA transformation into *S. pombe* (42). A centromere DNA-containing minichromosome, when introduced by transformation, does not always immediately assume a centromere-functional conformation in vivo, presumably because the DNA construct has not yet adopted the necessary higher-order chromatin structure that must be established within the cell. As a result of this finding, we asked whether the centromeric sequences on the deletion construct pSp-BHpΔKpn, a *cen1*-derived minichromosome that lacks the 2.1-kb *Kpn*I centromere enhancer region (Fig. 1), could become functionally activated. The pSp-BHpΔKpn construct was previously judged inactive with respect to centromere function (1) and includes the following centromeric DNA elements: from left to right, the remainder of the K″ repeat, the B′ repeat, cc1, and a small part of B′ on the right side of cc1, thereby generating approximately 3 kb of inverted repeat around cc1.

Minichromosome pSp-BHpΔKpn was subjected to an in vivo centromere activation test, which consists of propagating a transformed fission yeast isolate on selective media over approximately 30 generations, followed by assaying the mitotic loss frequency of the minichromosome in several representative isolates of the population that have retained the construct. We found that some, but not all, of the cells in the population after prolonged propagation appeared to carry a centromere-activated minichromosome. The activated, and hence stable, form of the ΔKpn construct in one isolate chosen for further examination exhibits a mitotic loss frequency of 7.9% and partial centromere function through meiosis (33% of the tetrads segregated in a 2+:2− pattern), indicating that the activated centromere is stable through both mitotic and meiotic cell divisions (Table 1). The minichromosome in the isolate tested, however, was present in more than one copy, as indicated by the high proportion (64%) of the 4+:0− and 3+:1− classes of segregants (Table 1). Nevertheless, the overall centromere activity of the stable form of the ΔKpn minichromosome greatly contrasts the activity of the unstable form, which exhibited a mitotic loss frequency of 74% and was completely lost in 93% of meiotic tetrads examined (Table 1). Extensive analysis of the DNA structure of this stable form of the ΔKpn construct showed that it had not undergone DNA rearrangement through the course of propagation and that the observed activation of the centromere was not the result of plasmid integration into the genome or of restoration of the 2.1-kb *Kpn*I region by gene conversion (data not shown).

To address whether the activation of the centromere on the ΔKpn construct is the result of an epigenetic mechanism or of a mutational or modification event altering the plasmid DNA, we isolated the stable, centromere-activated form of the ΔKpn construct from the corresponding *S. pombe* strain and trans-

TABLE 1. Centromere activity exhibited by *cen1*-derived constructs in mitosis and meiosis

Construct	Mitotic loss frequency	Distribution of construct:					
		In meiotic products					To sister spores[b]
		4+:0−	3+:1−	2+:2−[a]	1+:3−	0+:4−	
Immediate activation							
pSp(*cen1*)-BHp[c]	0.037	1	5	13 (50%)	2	5	2/13 (15%)
pSp(*cen1*)-BHpΔN-S	0.082	5	16	20 (38%)	4	7	1/20 (5%)
pSp(*cen1*)-BHpΔP-C	0.12	4	6	8 (40%)	1	1	4/8 (50%)
pSp(*cen1*)-BHpΔB-R	0.083	3	7	8 (36%)	2	2	2/8 (25%)
pSp(*cen1*)-BHpΔB-R+ΔP-C	0.079	2	13	13 (35%)	4	5	1/13 (8%)
Slow activation							
pSp-BHpΔKpn[d]							
Unstable	0.74	0	0	1 (2%)	3	51 (93%)	1/1
Stable	0.079	7	16	12 (33%)	1	0	9/12 (75%)
Stable-dimer		0	1	28 (80%)	4	2	13/28 (46%)
pSp(*cen1*)-BHpΔS-P							
Unstable	0.88						
Stable	0.14	0	0	49 (80%)	4	8	36/49 (74%)
pSp(*cen1*)-BHpΔC-K							
Unstable	0.59						
Stable	0.077	0	7	18 (53%)	1	8	4/18 (22%)

[a] The number in parentheses represents the percentage of tetrads exhibiting the '2+:2−' pattern of minichromosome distribution.
[b] This column represents the fraction of 2+:2− tetrads in which the minichromosome segregated to sister spores, and the number in parentheses represents the percentage.
[c] The meiotic data on pSp(*cen1*)-BHp were previously published by Hahnenberger et al. (17).
[d] The data on the unstable form of pSp-BHpΔKpn were previously published by Baum et al. (1).

formed the plasmid DNA directly back into the original *S. pombe* host strain, Sp223. If the stability of ΔKpn arose from a mutational event or from modification of the plasmid DNA, such a change would be heritable and all back-transformants should carry a stable construct. However, if an epigenetic mechanism is responsible for activation of the minichromosome centromere after propagation in vivo, resulting in the stable form of the construct, the centromere-inactive form should be recoverable in the back-transformants, as has been demonstrated for other centromere constructs by Steiner and Clarke (42). A total of eight back-transformants were examined, and they all exhibited minichromosome mitotic loss frequencies that ranged between 75 and 92% (averaging 81%), as well as the expected, unaltered plasmid DNA structure as determined by rigorous restriction analyses (data not shown). Therefore, because we can readily identify a centromere-inactive form of ΔKpn by transforming *S. pombe* with minichromosomal DNA recovered from cells carrying the centromere-active form, we conclude that activation of the centromere on the ΔKpn construct is indeed a consequence of a centromere-targeted epigenetic effect.

To evaluate the centromere function of a single copy of the stable ΔKpn construct in meiosis, one of the haploid meiotic products with the appropriate genotype from a tetrad in the 4+:0− class of segregants in the first mating was chosen for a second cross with our tester strain (see Materials and Methods). It was anticipated that in this meiotic product the stable ΔKpn construct would be present in single copy. Results from this second mating showed that the construct was present in single copy and exhibited appreciable centromere function, with 80% of the tetrads segregating in the 2+:2− pattern, and in 46% of these tetrads, the minichromosome segregated to sister spores (Table 1). These data suggest that the overall centromere activity of the stable ΔKpn construct is even better than that of the undeleted pSp(*cen1*)-BHp parent (Fig. 1 [derivation previously described in reference 17]; Table 1). However, rigorous analysis of meiotic products from several tetrads of this second cross revealed that in these haploid progeny the ΔKpn construct has become a head-to-tail dimer, most probably resulting from an in vivo homologous recombination event during meiosis. It is noteworthy that a minichromosome with this structure exhibits such a high level of centromere activity, because dicentric configurations in most species lead to chromosome instability. Dicentric configurations in *S. pombe* have not yet been analyzed, however.

Results from the studies described above indicate that the *Kpn*I region of the K-type repeat is dispensable for centromere function in the context of ΔKpn but is required for a functional centromere to be assembled immediately following transformation with naked minichromosomal DNA. This character and the effects of this region on central core chromatin structure (22) confirm that the 2.1-kb *Kpn*I region of the K repeat is indeed an enhancer of centromere function. However, the observation that the enhancer region is functionally dispensable indicates that other sequences on the ΔKpn minichromosome, most probably those within the remainder of the K-type repeat, can substitute, albeit poorly, for the centromere enhancer. Because neither a deletion of the entire enhancer when other K-type sequences are present nor deletions across the centromeric central core (1, 42) significantly affect centromere function once a centromere is activated in vivo, we conclude that there is not a single unique or essential sequence solely responsible for the contribution to centromere function made by either the K-type repeat or the central core but, rather, a number of separate sequences, such that a certain number and/or combination of them must be removed to drastically reduce function. Importantly, these observations argue against a repeat subunit model for the *S. pombe* centromere and support a model for centromere organization in fission yeast that does not demand a strict sequence requirement.

The organization of the centromere enhancer is modular, with separate regions contributing to function. Six other pSp(*cen1*)-BHp-derived constructs, which carry small (≤620-bp) deletions spanning the 2.1-kb *Kpn*I centromere enhancer

region, were generated to localize the functionally important sequences associated with centromere activation (Fig. 1). In four of these constructs (ΔN-S, ΔB-R, ΔP-C, and ΔB-R+ΔP-C), the regions deleted eliminate sequences that have been demonstrated in vitro to include either one or both of two previously identified protein-binding sites (in fragments H-I and L [Fig. 1] (1; also see below). Evaluation of the effects of the six deletions on centromere activity reveals that in both mitosis and meiosis, the ΔN-S, ΔB-R, ΔP-C, and ΔB-R+ΔP-C deletion constructs exhibit a level of centromere activity not significantly changed from that of the undeleted pSp(*cen1*)-BHp parental minichromosome (Table 1; Fig. 1). Therefore, it appears that these deleted sequences are dispensable for centromere activation in this minichromosome context.

In contrast, the centromere activity of the ΔS-P and ΔC-K constructs in mitosis was initially drastically reduced in comparison to the parental minichromosome (mitotic loss frequencies of 88 and 59% for ΔS-P and ΔC-K, respectively, versus 3.7% for pSp(*cen1*)-BHp [Table 1]). These mitotic loss frequencies are similar to that originally reported for pSp-BHpΔKpn. We subjected the ΔS-P and ΔC-K constructs to the centromere activation test and, as anticipated, found that the centromeres on both ΔS-P and ΔC-K constructs became activated upon propagation, as exhibited by their new mitotic loss frequencies (14% for ΔS-P and 7.7% for ΔC-K [Table 1]). Again, extensive analysis of the DNA structure of the stable forms of the two constructs showed that the structures had not undergone DNA rearrangement through the course of propagation and that the observed activation of the centromere was not the result of plasmid integration into the genome or of restoration of the deleted sequences by gene conversion. In addition, the meiotic behavior of the stable forms of the constructs was equivalent to or better than that of the undeleted pSp(*cen1*)-BHp parent (Table 1). While the sequences deleted in ΔS-P and ΔC-K are not absolutely required for centromere function, their absence, like deletion of the entire 2.1-kb *Kpn*I region, leads to difficulty in establishing an active centromere when the minichromosomes are introduced via DNA transformation, a consequence that again is most probably attributable to the previously described centromere-targeted epigenetic mechanism. Thus, at least two separate regions within the centromere enhancer contribute to enhancer function (i.e., immediate activation of the centromere), and a deletion of either results in slow activation of the centromere on the minichromosome (Table 1), analogous to that seen with a deletion of the entire region. This finding indicates that the functionality of the centromere enhancer is modular in nature.

Consistent with this modular organization of the centromere enhancer is the finding that there does not exist within the enhancer itself an essential sequence that is solely responsible for enhancer function, because smaller portions of this region are not sufficient to reconstitute an active centromere when added to a complete *cen2* central core (cc2) element (Fig. 2). Our laboratory has shown that the central core alone, or various combinations of centromere repeats in the absence of a central core, do not exhibit centromere activity, even after prolonged propagation of the host strain (1, 32a, 42). However, the addition of the entire 2.1-kb enhancer to a complete cc2 yields a construct that exhibits a high level of centromere activity—in mitosis, this construct is lost at a frequency of 1.4% (1). When small fragments representing various regions across the centromere enhancer were added singly or in combination to a centromere-nonfunctional pBluescript-based plasmid carrying cc2 (Fig. 2), the resulting constructs exhibited mitotic loss frequencies ranging from 13 to 46%, compared to 1.4% when the entire 2.1-kb *Kpn*I region was added (1). Therefore, to

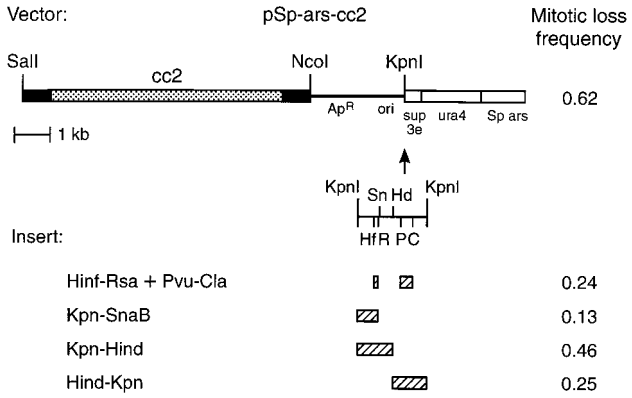

FIG. 2. *cen2*-derived in vitro-constructed minichromosomes. The *cen2*-derived minichromosomes were generated by the insertion of fragments from within the 2.1-kb *Kpn*I region of the K-type repeat into the pSp-ars-cc2 vector at the unique *Kpn*I site. The 14.8-kb pSp-ars-cc2 vector is a pBluescript-based plasmid carrying the entire cc2 (stippled box) along with 0.9 kb of core-associated repeat (black boxes) flanking each end of this region, as well as the *S. pombe* markers *sup3-e* and *ura4* and an *S. pombe* origin of replication, *ars1*. The location within the *Kpn*I region of each insert is indicated as hatched boxes aligned below a simplified restriction map of the region. Restriction sites are defined as follows: C, *Cla*I; Hd, *Hin*dIII; Hf, *Hin*fI; P, *Pvu*II; R, *Rsa*I; and Sn, *Sna*BI. The double insertion Hinf-Rsa + Pvu-Cla represents the addition of both previously identified H-I- and L-binding sites in which the normal spacing between these two regions has been preserved by including a 620-bp pBR322 fragment as a spacer between the *Hin*fI-*Rsa*I and *Pvu*II-*Cla*I fragments (see Materials and Methods). After fragment insertion, the resulting plasmids were enlarged by site-directed integration of the 8.5-kb pMB-neo plasmid into the Ap^r gene in the vector to ensure that the potential of these recombinant plasmids to exhibit centromere function was not masked by their small size (1). The mitotic loss frequency exhibited by each of these constructs is also shown. The mitotic loss frequency of the pSp-cc2+Kpn+neo construct, in which the entire *Kpn*I region was inserted into the pSp-cc2 vector, was previously determined to be 0.014 (1).

display centromere function, immediately following DNA transformation, a DNA segment containing more than half of the centromere enhancer, in conjunction with cc2, is required. This is not a surprising conclusion because neither half of the centromere enhancer contains both of the regions found associated with immediate centromere activation (i.e., the *Sna*BI-*Pvu*II and *Cla*I-*Kpn*I portions of the *Kpn*I region). Again, the finding that one specific small sequence is not solely responsible for centromere function within the regional centromeres of *S. pombe* contrasts markedly with the functionally critical 125-bp stretch of DNA that defines the point centromeres of *S. cerevisiae*.

A CENP-B-related *S. pombe* protein binds in vitro to specific sites in the centromere enhancer and its adjacent DNA and in cc1. The presence of protein-binding sites in fragments H-I, L, and O of the *cen1*-specific K″ repeat have been previously reported (1). These fragments are 320 to 450 bp in length, and H-I and L are within the 2.1-kb centromere enhancer region whereas O is adjacent to this region (Fig. 1). All three of these fragments form multiple complexes with crude protein extracts from *S. pombe* (Fig. 3). We have further localized the binding site(s) within these three fragments: the H-I protein-binding site(s) is localized to a 90-bp *Hin*fI-*Rsa*I subfragment which encompasses the *Eco*RV site separating restriction fragments H and I; the L-binding site(s) is localized to within a 122-bp *Pvu*II-*Rsa*I subfragment which represents the left one-third of fragment L; and the O-binding site(s) is localized to a central 227-bp *Rsa*I-*Dra*I subfragment (Fig. 1; see Materials and Methods).

One model for centromere organization and function in mammals proposed that the centromere protein CENP-B,

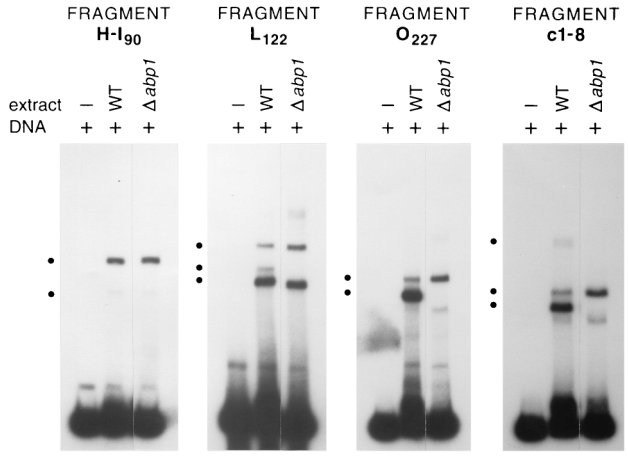

FIG. 3. Gel mobility shift assays of fragments H-I_{90}, L_{122}, O_{227}, and c1-8. Subfragments from the *cen1* K″ repeat (see Materials and Methods and Results), H-I_{90} (a 90-bp *Hin*fI-*Rsa*I fragment), L_{122} (a 122-bp *Pvu*II-*Rsa*I fragment), and O_{227} (a 227-bp *Rsa*I-*Dra*I fragment), and the full-length c1-8 fragment from cc1 were assayed for gel mobility shifts in the presence of whole-cell protein extracts (approximately 1 μg of total protein) from a wild-type (WT) Sp223 strain of *S. pombe* or a strain in which the *abp1* gene had been deleted (Δ*abp1*). The dots to the left of each panel indicate the specific complexes formed by each fragment.

which binds a consensus sequence that is found within α-satellite and is conserved among primate and rodent centromeric satellite DNAs (35), plays a role in organizing centromeric heterochromatin into the proper nucleoprotein foundation for kinetochore formation (30). CENP-B is associated with centromeric DNA in vivo in both a nonfunctional context and a functional, or activated, context (13). Using DNA affinity chromatography with a small fragment from the *cen2* central core, our laboratory has biochemically purified from a crude whole-cell extract of *S. pombe* a 62-kDa protein (Abp1p/Cbp1p) that has some striking similarities to human CENP-B (18). Coincidentally, the gene encoding this protein was identified in a genetic screen as a multicopy mitotic destabilizer of a nonessential, marked *S. pombe* minichromosome. Abp1p was originally isolated by Murakami et al. (29). Because Abp1p/Cbp1p binds in vitro to several sites in *cen2* central core DNA (18), we asked whether it also binds to sites within the essential K-type repeat, a predicted property if the protein plays a role in centromeric chromatin assembly similar to that proposed for CENP-B.

We found that Abp1p/Cbp1p indeed binds to sites identified in fragments L and O, which are within and adjacent to the centromere enhancer, respectively, as well as to sites in fragments c1-8 (the 265-bp *Cla*I-*Hin*dIII fragment [Fig. 1]) and c1-7 (the adjacent 315-bp *Hin*dIII-*Hin*dIII fragment [data not shown]) within the *cen1* central core. The interactions of specific binding proteins at sites in fragments L, O, and c1-8 appear to be complicated. Gel mobility shift assays show that the three fragments each form more than one protein-DNA complex in vitro: fragments L and c1-8 form three specific complexes, and fragment O forms two (Fig. 3) (1). However, not all of the complexes formed by the three fragments result from binding by Abp1p/Cbp1p. A comparison of the gel mobility shifts of these centromeric DNA fragments, resulting from specific binding to a protein(s) present in wild-type and Δ*abp1* whole-cell extracts (Fig. 3), reveals that Abp1p/Cbp1p is a component of the middle L complex, the predominant (smaller) O complex, and the predominant (smallest) and largest c1-8 complexes. Abp1p/Cbp1p is not a component of the H-I and other L complexes, indicating that binding sites for an as yet unidentified protein(s) are also in the centromere enhancer region. The nature of these Abp1p/Cbp1p-specific complexes are corroborated by results from gel mobility shift assays of the fragments in which DNA affinity-purified Abp1p/Cbp1p is used instead of whole-cell extract and in which a polyclonal antibody against Abp1p/Cbp1p is included in the reaction (data not shown). We conclude that while Abp1p/Cbp1p binds in vitro to fragments L, O, and c1-8, other proteins must bind as well to form the other specific complexes.

Additional evidence that other proteins are binding to these three fragments comes from the size fractionation, by gel filtration chromatography, of proteins binding to *S. pombe* centromeric DNA fragments (see Materials and Methods). The shift activity(ies) responsible for the formation of both O complexes and the middle and smallest c1-8 complexes elutes from a gel filtration column in a peak corresponding to approximately 125 to 130 kDa. The shift activities responsible for the formation of L complexes elute in two separate peaks, one at approximately 125 to 130 kDa, which gives rise to the top and middle L complexes, and the other at approximately 70 kDa, which gives rise to the smallest L complex. In complexes where Abp1p/Cbp1p is a component, it is most probably binding as a dimer because the protein species responsible for the formation of these complexes has been sized by gel filtration chromatography to be approximately 125 kDa. We conclude that a CENP-B-related protein, along with other as yet unidentified proteins in *S. pombe*, can bind in vitro to a number of sites within the central core and K repeat sequences (Fig. 4). These observations are consistent with a model where Abp1p/Cbp1p plays a role in organization of the K-type-repeat and central core, the two sequences important for *S. pombe* centromere function.

DISCUSSION

Our previous studies have indicated that two regions of *S. pombe* centromeric DNA, the central core (cc) and a 2.1-kb region of the K-type repeat, designated the centromere enhancer (22), are sufficient to establish partial centromere function on a minichromosome (pSp-cc2+Kpn+neo [1]). The same cc2 plasmid without the *Kpn*I region does not exhibit centromere function, even when tested for centromere activation after prolonged propagation of the host, indicating that the central core alone is not sufficient to support centromere function and that other DNA is also required, presumably to enable the region to assume a proper chromatin structure. Thus, we did not expect to find the centromere enhancer to be dispensable for function in the context of the pSp-BHpΔKpn construct.

The finding that deletion of the 2.1-kb *Kpn*I region from pSp(*cen1*)-BHp can yield a construct with a centromere capable of being activated in vivo indicates not only that the deleted region is not essential but also that centromeric sequences remaining on the pSp-BHpΔKpn minichromosome can substitute for the deleted centromere enhancer in establishing an active centromere. These sequences are most probably those within the remainder of K″, a 4.5-kb portion of the *cen1*-specific K-type repeat (17) (Fig. 1). The observation that other centromeric sequences can substitute for the enhancer demonstrates one level of functional redundancy between centromeric DNA elements. Another functionally redundant centromeric DNA region is the central core, because small deletions (1 to 3 kb) across the unique 7-kb cc2 do not adversely affect centromere function whereas a deletion of the entire region abolishes function (1).

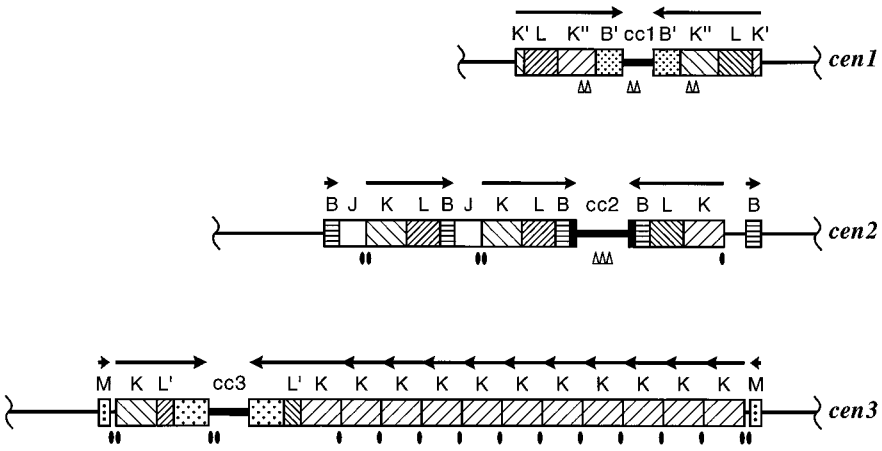

FIG. 4. Location of Abp1p/Cbp1p-binding sites within *S. pombe* centromeric DNA. The three *S. pombe* centromeric regions encompass approximately 40 to 150 kb of DNA. Abp1p/Cbp1p-binding sites determined in vitro by gel mobility shift assays of centromeric DNA fragments are indicated by open triangles and sites inferred by sequence homology to those centromeric DNA fragments exhibiting gel mobility shifts are indicated by solid ovals aligned below the diagrammatic representation of the three fission yeast centromeric DNAs of the strain Sp223.

We have now shown that both pSp-BHpΔKpn and pSp-cc2+Kpn+neo (carrying cc2 and the centromere enhancer [1]), consisting of different DNA sequences derived from *S. pombe cen1* and *cen2*, respectively, are capable of exhibiting centromere function. The lack of common centromere-derived sequences between these two minichromosomes again supports our conclusion that one essential DNA sequence solely responsible in *cis* for centromere function does not exist in fission yeast. That two different DNA sequences can support centromere function is indicative of yet another level of functional redundancy and argues against a subunit repeat model.

The existence of different levels of functional redundancy in centromeric DNA reflects the complexity of the regional centromeres of fission yeast. Another example of this complexity is apparent in the epigenetic mechanism that results in activation of the centromere, a phenomenon that is a manifestation at the chromatin level of the dynamic process whereby an active centromere becomes established. This process probably involves the assembly of centromeric DNA and protein components into the appropriate higher-order structure that is recognized in vivo as the site of sister chromatid attachment and kinetochore assembly. Although the entire 2.1-kb *Kpn*I region appears to be dispensable in the context of pSp(*cen1*)-BHp, our results from these studies demonstrate that its presence, in conjunction with the central core, is necessary for immediate centromere activation upon transformation with minichromosomal DNA, consistent with the results from all of our previous structure-function analyses. When centromeric DNA is introduced via transformation, it is presented to the host cell in a form not normally encountered in vivo, that is, stripped of all chromosomal proteins. The chromatin and specialized chromosomal regions such as the centromere kinetochore must presumably then be reassociated with proteins and restructured to assume the proper configuration. Once re-formed, however, this higher-order conformation appears stable through both mitotic and meiotic cell divisions.

We have now observed epigenetic activation of centromeres on minichromosome constructs in which the function of either the enhancer (this study) or the central core and/or its flanking core-associated repeat (42) has been compromised by in vitro manipulation. That perturbation of either of these elements leads to the same consequences is consistent with our hypothesis that the enhancer interacts with the central core and supports our model that formation of a higher-order chromatin structure provides the foundation for the establishment of a functional *S. pombe* centromere (8, 22). Our attempt to localize sequences within the enhancer that are associated with centromere activation led to the finding that the organization of this region is modular: the ΔS-P and ΔC-K deletions eliminate sequences that dictate the ability of the centromeric DNA on a minichromosome to display immediate centromere function, but the ΔN-S, ΔB-R, ΔP-C, and ΔB-R+ΔP-C deletions do not. These last deletions eliminate either one or both of the protein-binding sites located within the centromere enhancer (H-I and L). These sites may indeed be important, however, if the effects of the ΔS-P and ΔC-K deletions on immediate centromere activity are a consequence of alteration of the physical spacing between the two protein-binding sites or between these sites and sites in other centromeric DNA, such as the central core. We propose, therefore, that the centromere enhancer might facilitate the formation of an active centromere in somewhat the same way that enhancers of transcription elicit gene expression.

Transcriptional enhancers are thought to effect transcription by sequestering specific *trans*-acting factors and mediating their interaction, through DNA looping or bending, with a gene's promoter region and RNA polymerase. Detailed studies of enhancers (reviewed in reference 46) have revealed that they are modular, containing several binding sites for different transcription factors, which are themselves modular, and are composed of a specific DNA-binding domain, a multimerization domain, and a transcriptional activation domain. In some enhancers, the relative positions and orientations of these individual transcription factor-binding sites are critical to function. Furthermore, interactions between the different transcription factors binding to one enhancer are often crucial to enhancer activity. The enhancer of centromere function exhibits several characteristics that are similar to those of transcriptional enhancers. First, we have demonstrated that there are protein-binding sites, at least in vitro, for two or more different proteins, or protein complexes, within the region. Second, the effect of the ΔS-P and ΔC-K deletions within the enhancer region on overall centromere activity is consistent with the hypothesis that enhancer organization is modular. Third, the centromere enhancer is able to affect the chromatin organization of a distant target, namely, the central core, in an orien-

tation-independent manner (22), a process that most probably involves DNA looping and the physical interaction of proteins bound to the two centromeric elements, similar to the way transcriptional enhancers interact with their distant promoters.

A modular nature of the centromere enhancer would provide a simple explanation for the epigenetic activation of the centromere on the ΔS-P, ΔC-K, and ΔKpn deletion constructs. Structural alteration, brought about by either of the two smaller deletions, results in a partially disabled enhancer, perhaps through the disruption of the interactions between the proteins normally bound there. The consequence of a disabled enhancer is an inactive centromere. However, centromere activation can occur when the disrupted protein interactions are finally made or when the appropriate downstream events required for the establishment of an active centromere are triggered. For the ΔKpn deletion construct, in which the entire enhancer has been eliminated, the surrounding K″ repeat sequences may somehow compensate for the missing enhancer sequences and trigger the events necessary for active centromere formation.

The finding that the CENP-B-related *S. pombe* protein, Abp1p/Cbp1p, can bind in vitro to sites within and adjacent to the centromere enhancer, as well as to sites in both cc1 and cc2 (18), is also consistent with the enhancer-central core interaction hypothesis. Moreover, overexpression of Abp1p/Cbp1p in *S. pombe* cells dramatically affects the segregation of a marker minichromosome (18). Results from the size fractionation of the protein species binding to fragments L, O, and c1-8 indicate that Abp1p/Cbp1p may associate with these fragments as a dimer. It is conceivable that this dimer might act as a protein bridge between K and central core sequences, a role similar to that proposed for mammalian CENP-B in bringing together portions of α-satellite DNA (30). The observation that Abp1p/Cbp1p is not an essential protein suggests that other proteins are important for bringing together K and central core sequences and ultimately packaging centromeric DNA into a higher-order structure appropriate for centromere function. The involvement of other proteins reflects yet a fourth level of functional redundancy and is supported by the identification of binding sites for proteins other than Abp1p/Cbp1p within centromeric DNA (this work and unpublished results). Computer analysis of the centromeric DNA fragments that bind Abp1p/Cbp1p have not yet revealed a consensus binding site. Aside from AT richness, these fragments do not appear to have any obvious sequence motifs in common.

The recent finding that CENP-B exhibits significant sequence homology to the family of transposase and integrase proteins encoded by transposable elements (40, 47) leads us to speculate on the nature of the centromeric K-type repeats in *S. pombe*. We have previously examined in detail the K-type repeat sequences and reported the relationship among the different K-type repeats and their adjacent sequences found at the three *S. pombe* centromeres (1). The K-type repeat in *cen1* has an arrangement that separates (with an L repeat) the head of the repeat (K′) from its tail (K″) (Fig. 1), but the K-type repeats in *cen2* and *cen3* both have a structural motif where a 150-bp direct repeat is found at each end of the sequence (1). The presence of terminal direct repeats and the overall size of the K repeat element are reminiscent of retrotransposons, which are characterized by long terminal repeats at each end. While it is possible that the K repeat is derived from a retrotransposon, sequence analyses have not identified any open reading frames of reasonable length that represent protein products normally encoded by retrotransposons. Likewise, Northern blot analyses have not identified any transcripts from the K repeat region (14). The existence of retrotransposon elements in centromeres does, however, have precedents, as DNA segments representing portions of known retrotransposons have now been identified in the centromeres of *Drosophila* (20) as well as *Neurospora crassa* (3a). In addition, recent studies now implicate centromeric transposable elements, along with centric heterochromatin, in sister chromatid cohesion during meiosis I (10, 19). It is possible that transposable elements inserted into these regions of low recombination and, once there, eventually become recruited into some aspect of centromere function. Demonstration that the *S. pombe* protein Abp1p/Cbp1p binds specifically in vitro to several regions of centromeric K repeat and central core DNA, affects chromosome segregation when overexpressed, and shows significant homology to CENP-B, a component of mammalian centromeres that itself is homologous to the transposase/integrase family (29, 40), lends support to the retrotransposon hypothesis as a possible explanation for the origin of the K repeat in *S. pombe* centromeres.

The results of these studies further reveal that the regional centromeres of *S. pombe* are complex regions with similarities to centromeres of higher eukaryotes. The different levels of functional redundancy and the epigenetic effect on centromere function demonstrated here and previously (1, 42) are examples of this complexity. These observations suggest a hypothesis whereby many sequences within centromeric DNA are competent to participate in centromere formation. However, these sequences are not equally competent, and the overall activity of a centromere depends on the combination of sequences present, and probably on their spatial arrangement as well. An obvious question is to ask what makes a particular sequence centromere competent. Because centromeric DNA is not well-conserved among different organisms, it is likely that competence is governed by DNA character, such as AT richness or structural motifs, rather than by the presence of a particular sequence motif. If this is the case, centromere-competent sequences may exist elsewhere on the chromosome, and these sequences may be able to support centromere function in the absence of activity of the normal centromere. There is no evidence of potential secondary centromeres in *S. pombe*. However, the recent finding that deletion of the functionally critical sequences from a centromere-active minichromosome in *Drosophila* produces acentric derivatives which exhibit partial centromere function (31) supports the notion of additional centromere-competent sequences. Furthermore, stable dicentric human chromosomes have been identified with one active (centromere-functional) and one inactive primary constriction (13, 33). How centromere formation is regulated at these secondary sites as well as at the site of the native centromere remains to be determined. The presence of potential secondary centromeres in addition to the primary one adds yet another level of functional redundancy to this important chromosomal region.

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