A Centromere DNA-binding Protein from Fission Yeast Affects Chromosome Segregation and Has Homology to Human CENP-B

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Abstract. Genetic and biochemical strategies have been used to identify *Schizosaccharomyces pombe* proteins with roles in centromere function. One protein, identified by both approaches, shows significant homology to the human centromere DNA-binding protein, CENP-B, and is identical to Abp1p (autonomously replicating sequence-binding protein 1) (Murakami, Y., J.A. Huberman, and J. Hurwitz. 1996. *Proc. Natl. Acad. Sci. USA.* 93:502–507). Abp1p binds in vitro specifically to at least three sites in centromeric central core DNA of *S. pombe* chromosome II (cc2). Overexpression of *abp1* affects mitotic chromosome stability in *S. pombe.* Although inactivation of the *abp1* gene is not lethal, the *abp1* null strain displays marked mitotic chromosome instability and a pronounced meiotic defect. The identification of a CENP-B–related centromere DNA-binding protein in *S. pombe* strongly supports the hypothesis that fission yeast centromeres are structurally and functionally related to the centromeres of higher eukaryotes.

The centromere is a highly specialized region found on eukaryotic chromosomes. Centromeric DNA, and the kinetochore proteins that associate with it, orchestrate the segregation of condensed chromosomal DNA during cell division. Functional defects in centromeric DNA or centromere DNA-binding proteins may produce aneuploidy, a chromosomal abnormality often associated with genetic disease. Whereas centromere function is universally required by all eukaryotic species, the complexity of centromeric DNA elements required to carry out this function is not always as well conserved.

The budding yeast *Saccharomyces cerevisiae* accomplishes proper chromosome segregation using a point centromere consisting of only 125 bp of nonrepetitive DNA (Clarke and Carbon, 1985; Cottarel et al., 1989; for review see Pluta et al., 1995). Despite their functional similarity, the point centromeres in *S. cerevisiae* bear little structural resemblance to the larger, regional centromeres found in higher eukaryotes. Regional centromeres are structurally complex, span many kilobases of DNA, and contain both unique and highly repetitive DNA elements (Pluta et al., 1995).

Centromeres in fission yeast encompass 40–100 kb and have a conserved structural arrangement consisting of a 4–7-kb central core of DNA surrounded by a variable organization of inverted and direct repeats and thus can be classified as relatively simple regional centromeres (Chikashige et al., 1989; Hahnenberger et al., 1989, 1991; Clarke and Baum, 1990; Murakami et al., 1991; Takahashi et al., 1992; Steiner et al., 1993; see Fig. 3 *A*). Fission yeast minichromosome constructs require both nonrepetitive central core DNA sequences and portions of centromeric K-type (K/K″/dg) repeat DNA for centromere function (Clarke and Baum, 1990; Matsumoto et al., 1990; Hahnenberger et al., 1991; Murakami et al., 1991; Takahashi et al., 1992; Baum et al., 1994). Both the central core and the K-type repeat DNAs appear to contain functionally redundant sequence elements as well as a number of sequence-specific protein binding sites (Baum et al., 1994; Ngan, V.K., and L. Clarke, manuscript submitted for publication). A centromere-nonfunctional minichromosome construct, containing only central core DNA, becomes functional with the addition of a K-type element (Baum et al., 1994). Furthermore, addition of the K-type repeat induces the central core DNA to adopt an unusual chromatin structure analogous to the core chromatin structure observed in native centromeres (Clarke et al., 1993; Marschall and Clarke, 1995). It has been proposed that central core and K sequences interact via DNA looping, stabilized by centromeric DNA-binding proteins (Clarke et al., 1993; Baum et al., 1994; Marschall and Clarke, 1995). Some minichromosome constructs containing portions of centromeric DNA are able to undergo epigenetic activation from a nonfunctional to a functional state (Steiner and Clarke, 1994; Ngan, V.K., and L. Clarke, manuscript submitted for publication), further evidence that higher order DNA structure is integral to centromere function. Although the K-type element appears to be the only repeat element containing sequences essential for mitotic centromere

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function, additional repeat elements are likely to play a role in maintaining meiotic sister chromatid cohesion (Clarke and Baum, 1990; Hahnenberger et al., 1991). Similar to the heterochromatic DNA found at the centromeres of most organisms, *Schizosaccharomyces pombe* centromeric DNA is not transcribed. *S. pombe* centromeric DNA is the site of a position effect variegation in the expression of reporter genes inserted into the centromere region (Allshire et al., 1994). Mutations in several genes derepress centromeric reporter gene expression and concomitantly elevate chromosome loss (Allshire et al., 1995), suggesting that higher order DNA structure may play a role in centromere function. One of these gene products, Swi6p, has been localized to regions of heterochromatic DNA, including the centromere (Ekwall et al., 1995).

Heterochromatic human centromeres contain a large region of repetitive DNA elements, including higher order α-satellite arrays that are based on a 171-bp consensus monomer (Choo et al., 1991). Analogous to the structure of the *S. pombe* centromere, the repeat organization within the α-satellite arrays is chromosome specific and variable among individuals (Willard and Waye, 1987; Wevrick and Willard, 1989; Steiner et al., 1993). In certain contexts, α-satellite DNA alone apparently dictates some level of centromere function (Haaf et al., 1992; Larin et al., 1994); however, unique DNA elements may enhance centromere activity (Brown et al., 1994). A 17-bp protein-binding motif designated the "CENP-B box" is found within some classes of human α-satellite DNA and in *Mus musculus* minor satellite DNA (Wong and Rattner, 1988; Masumoto et al., 1989) and is the binding site for the 80-kD protein CENP-B (Earnshaw et al., 1987).

Human CENP-B is antigenic for centromere-specific antibodies present in the serum of patients with the CREST syndrome (Calcinosis, Raynaud's phenomenon, esophageal dysmotility, and telangiectasia) of progressive systemic sclerosis (Moroi et al., 1980) and localizes throughout the centromeric heterochromatin beneath the kinetochore (Cooke et al., 1990). CENP-B binds to DNA as a monomer, is able to form homodimers, and in vitro can form a stable complex containing two DNA molecules and two CENP-B monomers (Kitagawa et al., 1995). It has been hypothesized that CENP-B plays a role in establishing a higher order chromatin structure that may be necessary for mammalian centromere function (Ikeno et al., 1994). CENP-B binding is not detected at all functional centromeres, suggesting that CENP-B is not essential, or that some centromeres have small, undetectable levels of CENP-B, or that some centromeres use functionally redundant proteins (Pluta et al., 1992, 1995). In support of the latter statements, the Asian mouse, *Mus caroli*, contains a CENP-B gene that can be detected in genomic DNA by PCR but lacks a protein detected by antibodies directed against CENP-B. In addition, *M. caroli* has no CENP-B box but contains a related DNA element that is competent to bind CENP-B (Kipling et al., 1995). When present, CENP-B binding is not diagnostic for centromere activity. Apparently, complete centromere function requires additional genetic and/or epigenetic factors (Earnshaw et al., 1989; Tyler-Smith and Willard, 1993).

In an effort to identify *S. pombe* proteins with roles in centromere function, we have taken both genetic and biochemical approaches. Meeks-Wagner and Hartwell (1986) demonstrated that overexpression of certain genes in *S. cerevisiae*, presumably creating an abnormal stoichiometry of protein components in a cellular complex, can result in increased frequency of chromosome loss. This approach was used to identify the putative *S. cerevisiae* kinetochore protein Mif2p (Meluh and Koshland, 1995). We used a similar method and screened an *S. pombe* genomic library, constructed in a multiple copy number vector, for plasmids that destabilized a *cen1* linear minichromosome. Using this assay, we identified a gene with significant homology to the gene specifying human centromere DNA-binding protein, CENP-B. The product of the *S. pombe* gene is identical to Abp1p (autonomously replicating sequence-binding protein 1),[1] a protein isolated independently by Murakami et al. (1996) through its ability to bind to the AT-rich *S. pombe ars* consensus sequence defined by Maundrell et al. (1988; see Discussion). Simultaneously with our genetic experiments, we have pursued isolation of *S. pombe* centromere proteins using centromere DNA-specific affinity chromatography. A 62-kD protein was purified and shown to bind specifically to at least three sites in centromeric central core DNA of *S. pombe* chromosome II (cc2). This protein is also identical to Abp1p; an *abp1* null strain completely lacks the specific DNA-binding activity that is enriched in Abp1p-overexpressing strains, and antibody to Abp1p causes a supershift of the cc2 fragments bound by p62. The identification of a CENP-B–related *S. pombe* protein that binds specifically to centromeric DNA in vitro and affects chromosome segregation in vivo sustains our hypothesis that the fission yeast centromere constitutes an excellent model for the mammalian kinetochore and provides a valuable tool for elucidating more about conserved aspects of centromeric structure and function among eukaryotes.

Materials and Methods

Strains, Media and abp1 Gene Disruption

The *Escherichia coli* strain used in this study is DH5α (*recA1*; GIBCO BRL, Gaithersburg, MD). Procedures for *E. coli* transformations, cloning techniques, and plasmid isolations were as previously described (Maniatis et al., 1982). The *S. pombe* strains used in this study were Sp223 (h^- *ade6.216 leu1.32 ura4.294*, a gift from D. Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); AL91 (h^{90} *ura4.D18 swi6::ura4*$^+$ *ade6.M216*; Lorentz et al., 1994), EG388 (h^{90} *rik1-304 leu1 ura4.D18*; Egel et al., 1989), KE108 (h^{90} *clr4.55 ura4 ade6.M216*; Ekwall and Ruusala, 1994), SBP120390 (h^- *ade6.704 leu1.32 ura4.294*), SBP32590/pSp(cen1)-7L-sup3E (h^- *ade6.704 leu1.32 ura4.294*/*ura4*$^+$ *sup3E URA3 TRP1*), SBP070196-F5B (h^+ *ade6.210 leu1.32 ura4.D18*), SBP070196-A7C (h^- *ade6.216 leu1.32 ura4.D18*), SBP070196-F8B (h^+ *ade6.210 leu1.32 ura4.D18 abp1::ura4*$^+$), SBP070196-F8C, -F5C (h^- *ade6.216 leu1.32 ura4.D18 abp1::ura4*$^+$), SBP082996/pSp(cen1)-7L-sup3E (*ade6.704 leu1.32 ura4.294 abp1::ura4*$^+$/*ura4*$^+$ *sup3E URA3 TRP1*), and SBP051596 (h^+/h^- *ade6.210/ade6.216 leu1.32*/*leu1.32 ura4.D18*/*ura4.D18*). The diploid strain SBP051596 was generated in this laboratory by crossing strains ED666 (h^- *ade6.210 leu1.32 ura4.D18*) and ED667 (h^+ *ade6.216 leu1.32 ura4.D18*, both gifts from R. Allshire, Medical Research Council Human Genetics Unit, Edinburgh, Scotland, UK). Growth media and conditions were as described (Gutz et al., 1974; Moreno et al., 1991).

1. *Abbreviations used in this paper*: Abp1p, autonomously replicating sequence-binding protein; ARS, autonomously replicating sequence; cc2, central core DNA of *Schizosaccharomyces pombe* chromosome II; DAPI, 4′, 6-Diamidino-2-phenylindole; IS, insertion sequence.

DNA transformations and fragment-mediated transplacements were performed with an alkali–cation yeast transformation kit (BIO 101, Inc., Vista, CA). The *sup3E*-marked *cen1* linear minichromosome pSp(cen1)-7L-sup3E was created by fragment-mediated transplacement of *sup3E* into pSp(cen1)-7L (Hahnenberger et al., 1989) adjacent to *ura4.*

For disruption of the *abp1* gene, a 1.8-kb HindIII–BamHI fragment containing the entire *abp1* coding region (Fig. 1 *A*) was subcloned into pBluescript KS-. The 1.3-kb BglII–EcoRI region was dropped out of this fragment, leaving 95 and 165 bp of the NH_2- and COOH-terminal coding regions of *abp1*, respectively. In its place, a 1.8-kb fragment containing the *S. pombe ura4* gene was end-filled with the Klenow fragment of DNA polymerase I and inserted by blunt end ligation. Double digestion of this plasmid with HindIII and BamHI produced a 2.3-kb fragment containing the *S. pombe ura4* gene and flanking sequences homologous to the *abp1* region of the genome. This linear fragment was used for fragment-mediated transplacement (Rothstein, 1983) in the *S. pombe* diploid strain SBP051596. Stable Ura^+ transformants were analyzed by Southern blot hybridization for heterozygous disruption of *abp1*. Diploids heterozygous for *abp1* were sporulated and dissected to separate meiotic spores using a dissecting microscope (MSM System, series 200; Singer Instruments, Somerset, UK).

Determination of Vector Copy Number

The pSp200 vector, a pBR322-based plasmid, contains *S. pombe ars1* on a 1.2-kb EcoRI–EcoRI fragment and *S. cerevisiae LEU2* on a 2.2-kb XhoI–SalI fragment cloned into the EcoRI and SalI sites, respectively, of pBR322. *S. pombe* strain Sp223 was transformed with pSp200 and grown under leucine selection. Genomic DNA was prepared from six Leu^+ transformants, digested with SalI to linearize pSp200, fractionated by FIGE (field inversion gel electrophoresis), and subjected to Southern blot analysis as previously described (Steiner and Clarke, 1994), using a ^{32}P-labeled *ars1* probe. Exposed film was scanned with a laser densitometer (model LKB 2222-010 UltroScan XL; Bromma, Sweden). On average, the plasmid band was observed to be ~20 times more intense than the genomic *ars1* band.

S. pombe Genomic Library Construction

Total *S. pombe* DNA was isolated by the method of Beach and Klar (1984) and manipulated with cutoff micropipette tips. Approximately 30 μg of isolated DNA was partially digested with 1 U of Sau3AI for 10 min at 37°C to obtain an average size of 10 kb. Sucrose gradient size–selected DNA was cloned into the BamHI site of pSp200 at a 2:1 weight ratio of insert to vector, respectively, and transformed into *E. coli.* Of 18 random transformants tested, all had inserts with an average size of 6.8 kb and a range of 1 to 18 kb. Overall, 25,240 transformants representing ~12 *S. pombe* genome equivalents were obtained.

Minichromosome Destabilization Assay

The *S. pombe* library was transformed into SBP120390/pSp(cen1)-7L-sup3E, and Leu^+ transformants were selected on SD (0.67% yeast nitrogen base without amino acids, 2% glucose) + adenine (10 mg/liter) + uracil (50 mg/liter). Under these conditions, loss of the *cen1* minichromosome results in red colony color or red sectoring in a white background (Baum et al., 1994). Of the ~32,500 colonies obtained, 562 colonies exhibited a sectored phenotype. These isolates were subjected to successive rounds of restreaking on colony color indicator media. Plasmid DNAs from 26 isolates that sectored reproducibly were rescued into *E. coli* and retransformed into *S. pombe.* Of these, 25 continued to produce sectoring above background levels and were grouped into classes based on the level of sectoring observed.

The mitotic stability of the pSp(cen1)-7L-sup3E minichromosome was determined in wild-type cells transformed with vector pSp200 or pSp200-*abp1* by colony color assay or replica plating, respectively, and measured in *abp1* null cells by colony color assay (Clarke and Baum, 1990; Baum et al., 1994).

Gel Mobility Shift and Supershift Assays

DNA fragments examined for bandshifts were small (<400 bp) restriction fragments from cc2, as indicated in Fig. 3 *A*. The gel mobility shift assay was performed as previously described (Baum et al., 1994). End-labeled fragments (5 fmol) were mixed with protein extract and incubated for 20 min at room temperature in binding buffer containing 10 mM Hepes (pH 8.0), 6 mM $MgCl_2$, 2 mM NaF, 100 mM KCl, 0.5 mM DTT, 10% glycerol, and 1–2 μg poly(dI-dC) (~2,000-fold excess [wt/wt]; Amersham/USB, Cleveland, OH). Binding specificity was determined by adding 125 fmol (25-fold excess) of the unlabeled DNA fragment as competitor in the binding reaction.

Supershift assays were performed with polyclonal antibody to Abp1p (kindly provided by J.-K. Lee, J.P. Sanchez, and J. Hurwitz, Memorial Sloan-Kettering Cancer Center, New York, NY; Murakami et al., 1996). A 0.4-M KCl chromatin extract or an affinity-purified protein fraction was preincubated in binding buffer with antibody for 20 min at room temperature. Labeled DNA fragment was added, and the mix was incubated at room temperature for an additional 20 min, followed by electrophoresis.

Preparation of the c2-10_{61} DNA Affinity Column

A DNA fragment containing a 57-bp region of fragment c2-10 was amplified by PCR using the following primers: 5′(BglII)-gaagatcTAACTCGACTTTATAAAATTT-3′ and 5′(BamHI)-gaggatcCAAATTAAACATTGCTAATAA-3′. Digestion with BamHI and BglII yielded a 61-bp fragment that was concatamerized at 15°C with T4 DNA ligase, redigested with BamHI and BglII, and cloned into the BamHI site of pBluescript KNS- (Baum et al., 1994). $[c2\text{-}10_{61}]_4$, a clone containing four tandem copies of the insert, was identified and sequenced (Sequenase kit; Amersham/USB). The $[c2\text{-}10_{61}]_4$ construct was excised as a 266-bp fragment with BamHI and EcoRI. The fragment was biotinylated at the EcoRI end with biotin-16-dUTP (Sigma Chemical Co., St. Louis, MO) and coupled to streptavidin–agarose (GIBCO BRL) as described previously (Lechner and Carbon, 1991). A small aliquot of biotinylated DNA that was ^{32}P end-labeled at the BamHI site was included in the reaction to determine the coupling efficiency to the column. Approximately 75% of the DNA fragments remained bound to the column. The columns were prepared, run, and stored as described by Lechner and Carbon (1991).

Purification of Abp1p

The following buffers were used: B (50 mM KPO_4 [pH 7.0], 100 mM β-glycerophosphate, 10 mM NaF, 10 mM EDTA, 10 mM EGTA, 0.5 M DTT, 1 mM PMSF, 2.5 μg/ml leupeptin, and 2.5 μg/ml pepstatin A); D (10 mM Hepes [pH 8.0], 20% glycerol, 6 mM $MgCl_2$, 2 mM NaF, 1 mM DTT, 0.2 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin A); E (10 mM Hepes [pH 8.0], 20% glycerol, 2 mM NaF, 1 mM DTT, and 0.5 M KCl). 4 liters of the overexpressing strain carrying pSp200-*abp1* were grown to mid-log phase (A_{600} ~0.6) in SD + A + U. Freshly harvested cell paste (13 g) was washed with water, and the washed pellet was packed into a 60-ml syringe and extruded into a beaker of liquid nitrogen. Cells were mechanically disrupted in liquid nitrogen (Sorger et al., 1989) using a blender at high speed for 20 min. All subsequent steps were performed at 4°C and as previously described (Lechner and Carbon, 1991) with the following modifications. The freeze-dried powder was resuspended in 24 ml of buffer B and stirred gently for 30 min. After centrifugation (14,000 *g* for 30 min), the supernatant (low salt extract) was flash frozen in small aliquots and stored at −70°C, and the pellet (chromatin) was further extracted. The pellet was resuspended in 20 ml of buffer B, KCl was added to 400 mM, and the mixture was incubated for 30 min. After centrifugation (34,000 *g* for 20 min), the soluble material (0.4 M KCl chromatin extract) was further clarified (100,000 *g* for 1 h). 17 ml of the supernatant was mixed with 28 ml 2× buffer D and 0.15 mg/ml poly(dI-dC) in a final volume of 56 ml to yield 120 mM KCl. Insoluble material was removed by centrifugation (20,000 *g* for 30 min followed by 100,000 *g* for 30 min), and the supernatant was passed through a $[c2\text{-}10_{61}]_4$ DNA affinity column (1 ml, described above, equilibrated in buffer D, 120 mM KCl). The column was washed with 15 ml buffer D, 150 mM KCl, and eluted with six 0.5-ml aliquots of buffer E. Elution fractions were flash frozen and stored at −70°C.

Western Blot Analysis

S. pombe crude extract or affinity-purified fractions were electrophoresed on a 10% SDS–polyacrylamide gel in 25 mM Tris, 190 mM glycine, and 0.1% SDS (pH 8.3) at 12.5 V/cm for 3 h. Proteins were electroblotted onto nitrocellulose in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol. Membranes were blocked in 2% milk/TBS-T (20 mM Tris, 137 mM NaCl [pH 7.6], and 0.1% Tween 20) overnight at 4°C followed by two rinses with TBS-T. Polyclonal antibody to Abp1p (1:1,000) was incubated with the blocked membrane in 5% milk/TBS-T at room temperature for 1 h

followed by three 10 min washes with 2% milk/TBS-T. Goat anti–rabbit IgG–alkaline phosphatase secondary antibody (1:1,000; Bio-Rad Laboratories, Richmond, CA) was incubated with the membrane in 5% milk/TBS-T at room temperature for 1 h followed by three 15-min washes with TBS-T. Bands were detected with NBT/BCIP (Promega Corp., Madison, WI) (Harlow and Lane, 1988).

Results

A Screen for S. pombe Genes That in Elevated Dosage Increase the Loss Rate of a Stable cen1 Minichromosome

A number of multicomponent structures, including the centromere/kinetochore, the DNA replication complex, the mitotic chromosomes, and the mitotic spindle, are likely to be made up of interacting proteins that must be present in precise stoichiometry to yield a functional complex. Overexpression of one component may disturb the stoichiometric relationship and cause chromosome instability because of improper replication or segregation of chromosomal DNA (Meeks-Wagner and Hartwell, 1986). To identify gene products with potential roles in chromosome segregation, we screened an *S. pombe* genomic library, constructed in the multicopy vector pSp200 (Fig. 1 *A*), for genes that in elevated dosage cause high loss rates of a stable *cen1* linear minichromosome. The library was used to transform strain SBP32590/pSp(cen1)-7L-sup3E. Because of an *opal* mutation in the *ade6* gene, *S. pombe* strain SBP32590 ac-

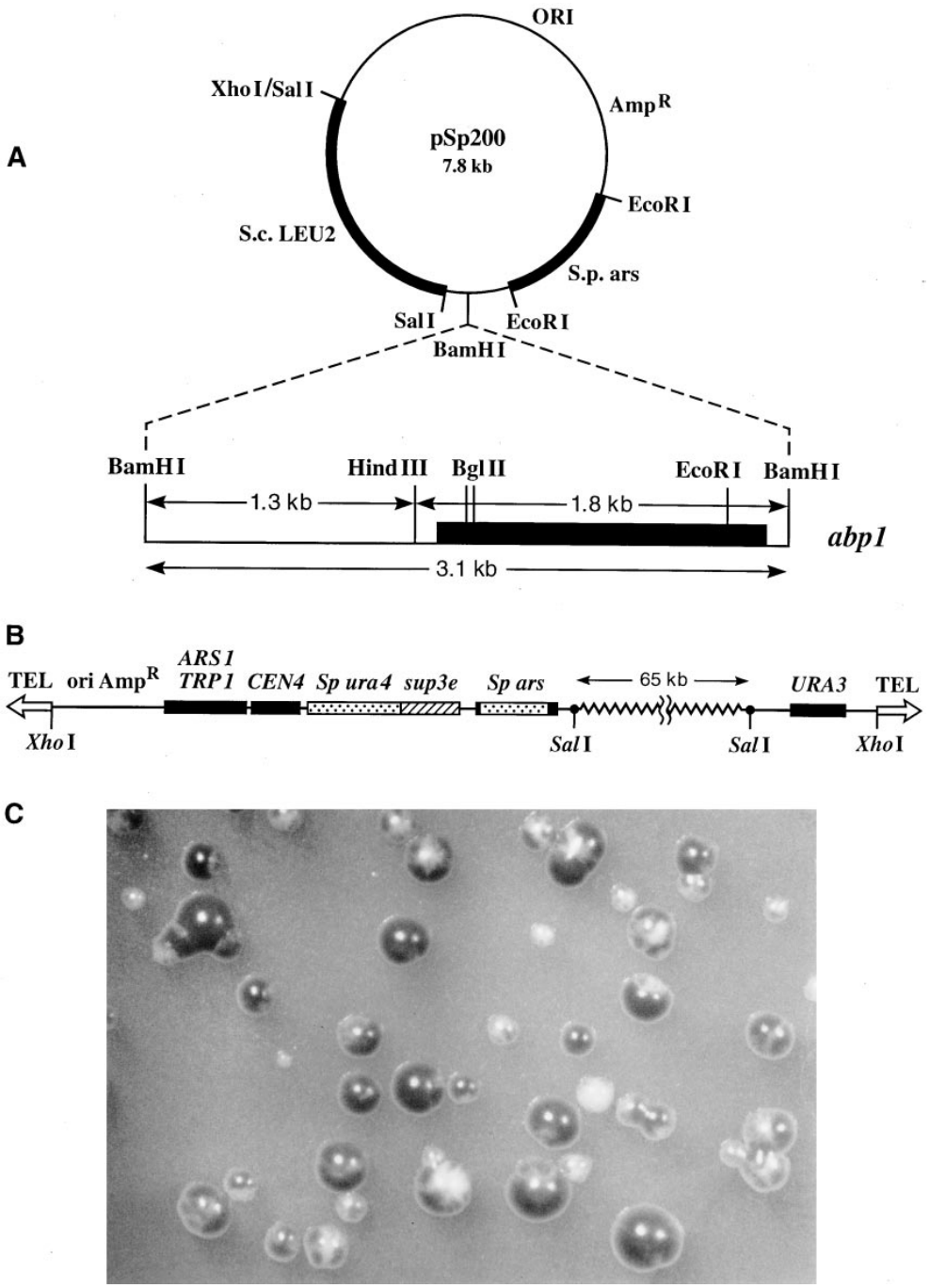

Figure 1. A screen for *S. pombe* genes that decrease minichromosome stability when present in multiple copies identifies *abp1*. (*A*) Structure of pSp200-*abp1* and (*B*) the 78-kb linear minichromosome pSp(cen1)-7L-sup3E, which contains the 65-kb *cen1* SalI fragment cloned in the YAC vector pMB-1 (Hahnenberger et al., 1989). (*C*) SBP32590/pSp(cen1)-7L-sup3E cells transformed with pSp200-*abp1*. The pSp200 vector has no effect on minichromosome stability, and transformed colonies are predominantly white (data not shown), whereas *abp1* in multiple copies causes high level (class III) minichromosome loss, observed as dark sectors.

cumulates a red pigment when grown on media containing low concentrations of adenine and the colony color is red. When the strain also carries the in vitro–constructed minichromosome pSp(cen1)-7L-sup3E, containing a tRNA nonsense suppressor of *ade6* (Fig. 1 *B*), colony color is white. Chromosome loss events, due to either missegregation or replication defects, result in red-sectored colonies (Hofer et al., 1979; Kohli et al., 1989). Approximately 32,500 transformant colonies representing 16 genome equivalents were screened for colonies containing readily discernible red sectors. Roughly 0.07% of the transformed colonies reproducibly displayed increased red sectoring and were grouped into three classes: 9 isolates exhibited very high levels of sectoring in every progeny colony (class III; Fig. 1 *C*); 5 others showed high levels of sectoring in almost every progeny colony (class II); and 11 isolates exhibited sectoring above background levels, although >75% of progeny colonies were white (class I).

In this screen, elevated numbers of centromeric DNA binding sites for critical proteins might also cause minichromosome instability by sequestering a centromere protein from the kinetochore complex. Thus, plasmid DNAs from each class were first screened by Southern blot hybridization for the presence of DNA sequences derived from centromeric central core and K-repeat DNA elements; however, no significant hybridization was detected (data not shown).

One class III sequence, isolated three times, was localized to a 3.1-kb BamHI fragment (Fig. 1 *A*). A BamHI and HindIII double digest was used to separate this region into two fragments encompassing 1.3 and 1.8 kb. The presence of the 1.8-kb fragment in the pSp200 vector caused a high rate of minichromosome loss in SBP32590/pSp(cen1)-7L-sup3E, although not as severe as that caused by the original 3.1-kb fragment. On the other hand, the 1.3-kb fragment had a negligible effect on minichromosome stability. The nucleotide sequence of the entire 3.1-kb fragment was determined. In the 1.8-kb region, analysis of the sequence revealed a 1.6-kb open reading frame (Fig. 1 *A*, *shaded box*) specifying a protein with significant homology to the mammalian centromere DNA-binding protein CENP-B (see below) and identical to *S. pombe* Abp1p (Murakami et al., 1996). Further characterization of *abp1* was carried out on cells transformed with pSp200-*abp1*, the pSp200 vector containing the entire 3.1-kb BamHI fragment. In cells newly transformed with pSp200-*abp1*, the loss frequency of the pSp(cen1)-7L-sup3E minichromosome was 200-fold higher than in transformed cells carrying the vector alone (Table I).

A different class III destabilizing DNA segment was localized to a 2.4-kb BamHI fragment, sequenced, and found to encode *S. pombe* Nim1p, a negative regulator of the *S. pombe* mitotic inhibitor Wee1p (Russell and Nurse, 1987*a*,*b*). Increased expression of *nim1* induces premature mitotic entry, disrupting the temporal ordering of mitotic events and resulting in elevated chromosome loss.

Table I. Alterations in the Cellular Level of Abp1p Affect Minichromosome Stability

Strain	*abp1* Genotype	Mitotic Loss Frequency*
SBP32590/pSp(cen1)-7L-sup3E	$abp1^{+}$	4.4×10^{-4}‡
SBP32590/pSp(cen1)-7L-sup3E/pSp200	$abp1^{+}$	2.6×10^{-4}
SBP32590/pSp(cen1)-7L-sup3E/pSp200 *abp1*	$abp1^{OE}$	5.2×10^{-2}
SBP082996/pSp(cen1)-7L-sup3E	*Δabp1*	3.8×10^{-2}

*See Materials and Methods.
‡Data from Hahnenberger et al. (1991).

Alterations in the Cellular Level of Abp1p Affect Mitotic Division and Meiosis

To determine if Abp1p is essential for cell viability, one entire chromosomal copy of the *abp1* gene was disrupted by *ura4* gene replacement in *S. pombe* diploid strain SBP051596 (Materials and Methods; Rothstein, 1983). Subsequent sporulation of the heterozygous diploid yielded mostly asci with four viable spores, indicating that Abp1p is not essential for mitotic growth. However, Δ*abp1* cells grow more slowly than wild-type cells, and this growth difference is accentuated at low temperatures (Fig. 2 *A*). Similarly, cells containing pSp200-*abp1* grow more slowly than cells with vector pSp200, suggesting that alterations in the normal level of *abp1* expression affect mitotic growth.

Flow cytometry profiles of Δ*abp1* cells grown at 30 and 17°C were indistinguishable from wild-type profiles and never revealed an increased population of cells with a DNA content less than 2N, indicating that the slow growth phenotype was not caused by a replication defect (data not shown). In contrast, wild-type cells transformed with pSp200-*abp1* and Δ*abp1* cells lost the *cen1* minichromosome at a much higher rate than did wild-type cells, suggesting that slowed cellular growth might result from a defect in chromosome segregation (Table I). Because diploid *S. pombe* strains are unstable, it is difficult to measure directly the loss frequencies of native chromosomes. However, if native chromosomes are lost at a frequency similar to that of the *cen1* minichromosome, this loss would easily account for slower growth rates observed in *abp1* null and overexpressing cultures at 30°C. Because aneuploid cells fail to divide, the effect observed on a population of cells stained with DAPI would be minimal, and the population as a whole might look relatively normal. Consistent with this, DAPI staining patterns of Δ*abp1* cells and pSp200-*abp1*–transformed cells grown at 30°C are not markedly different from those of wild-type cells grown under similar conditions.

DAPI stains of wild-type cells and pSp200 vector-transformed cells grown at 17°C revealed a majority of normal interphase cells with a single nucleus and a lesser number of dividing cells with two nuclei and a septum (Fig. 2 *B* and data not shown). In contrast, Δ*abp1* cells and pSp200-*abp1*–transformed wild-type cells grown at 17°C displayed a number of morphological abnormalities. Both Δ*abp1* cells and pSp200-*abp1* transformants are generally more rounded and irregularly shaped than wild-type and pSp200-transformed cells. A majority of Δ*abp1* cells and pSp200-*abp1*–transformed cells had a single, centrally located DNA mass.

Some additional unusual phenotypes are observed in Δ*abp1* cells. Approximately 1% of Δ*abp1* cells grown at 17°C were highly elongated (Fig. 2, *C* and *D*). Most lengthened Δ*abp1* cells had a single mass of DNA stretched to varying degrees across the center of the cell (Fig. 2 *C*, *ar-*

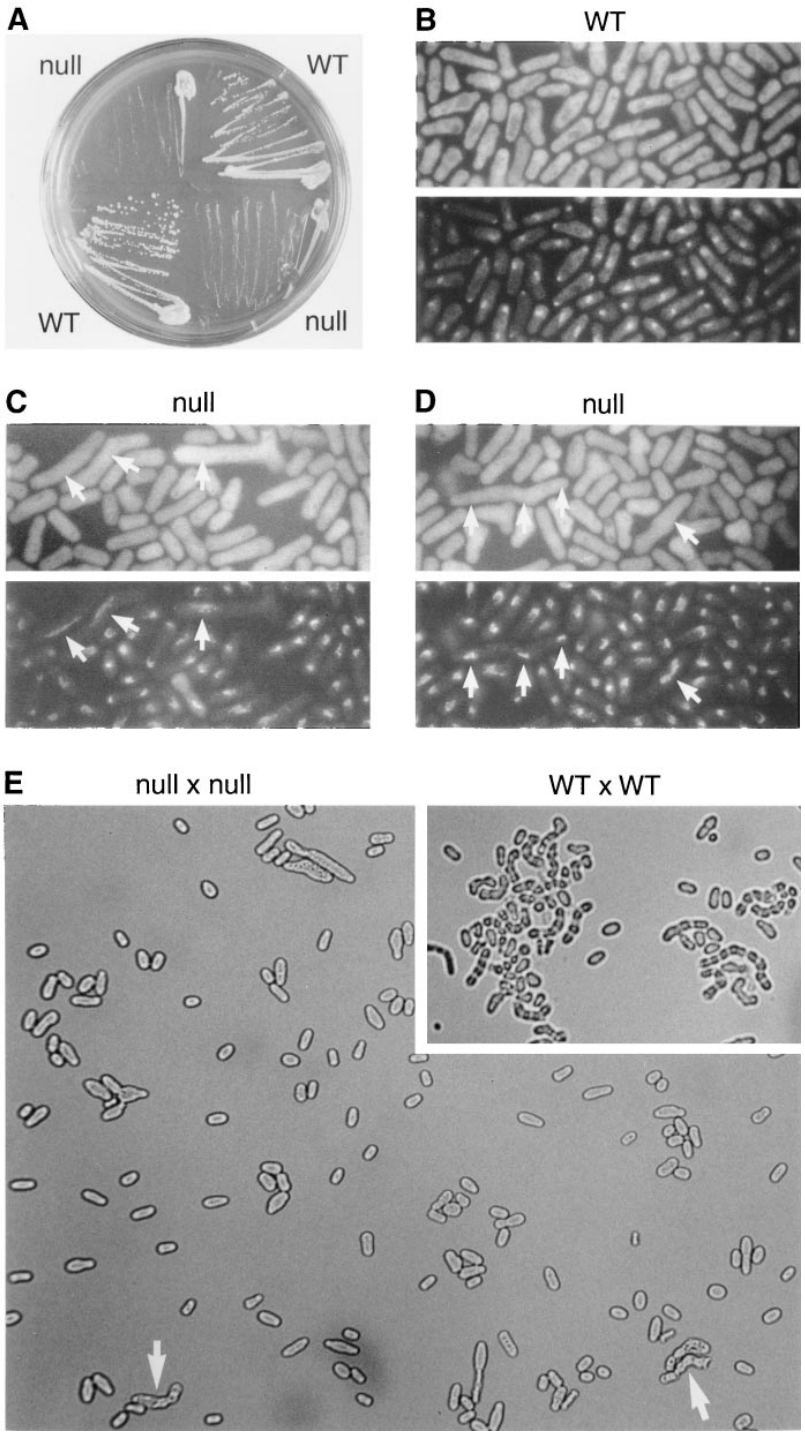

Figure 2. Alterations in the cellular level of Abp1p affect mitotic cell division and meiosis. (*A*) Δ*abp1* (null; SBP070196-F8B,-F8C) and wild-type (SBP070196-F8A,-F8D) haploid strains derived from the four meiotic progeny of diploid strain SBP051596 after heterozygous disruption of *abp1*. Null cells grow more slowly than wild-type cells on rich media at 17°C. Wild-type (SBP070196-F5A) (*B*) and Δ*abp1* (null; SBP070196-F5C) (*C* and *D*) cells grown exponentially in rich media at 17°C. Upper panels show cells exhibiting autofluorescence when viewed at a wavelength of 488 nm. Lower panels show the same cells stained with DAPI. Abnormal cellular and nuclear morphologies are indicated by arrows (see text). (*E*) Conjugation and sporulation products resulting from a Δ*abp1* homozygous cross (SBP070196-F8B × SBP070196-F8C) and (*inset*) a wild-type homozygous cross (SBP070196-F5B × SBP070196-A7C). Examples of rare asci seen in the Δ*abp1* cross are indicated by arrows.

rows); others displayed two chromatin masses positioned at opposite ends of the cell plus additional, apparently unsegregated, DNA at more central positions (Fig. 2 *D*, *arrows*). Generally, even in smaller cells, the DAPI-staining DNA mass is less compact in Δ*abp1* cells than in wild-type cells (Fig. 2, *C* and *D*). These phenotypes suggest that mitosis occurs abnormally in some Δ*abp1* and pSp200-*abp1*–transformed cells grown at low temperature.

To ascertain whether Abp1p is essential for meiosis, several +/Δ*abp1* heterozygous diploids were sporulated and the asci dissected. Haploid progeny were tested for mating type and *abp1* disruption by Southern blotting. Wild-type ($Abp1^+$) cells of opposite mating types were crossed and appeared to undergo meiosis normally, forming large numbers of asci (Fig. 2 *E*, *inset*). In contrast, when Δ*abp1* sister spores of opposite mating types are induced to undergo conjugation and sporulation, relatively normal asci are extremely rare; 0–0.7% of cells were observed to form asci out of 2,000–6,000 cells examined for each of 10 separate matings. In addition, aberrant asci-like forms were observed that contained unusually shaped spores (Fig. 2 *E*, *arrows*). The meiotic defect is recessive, because these same Δ*abp1* cells were able to conjugate and sporulate normally in crosses with wild-type cells, although fewer

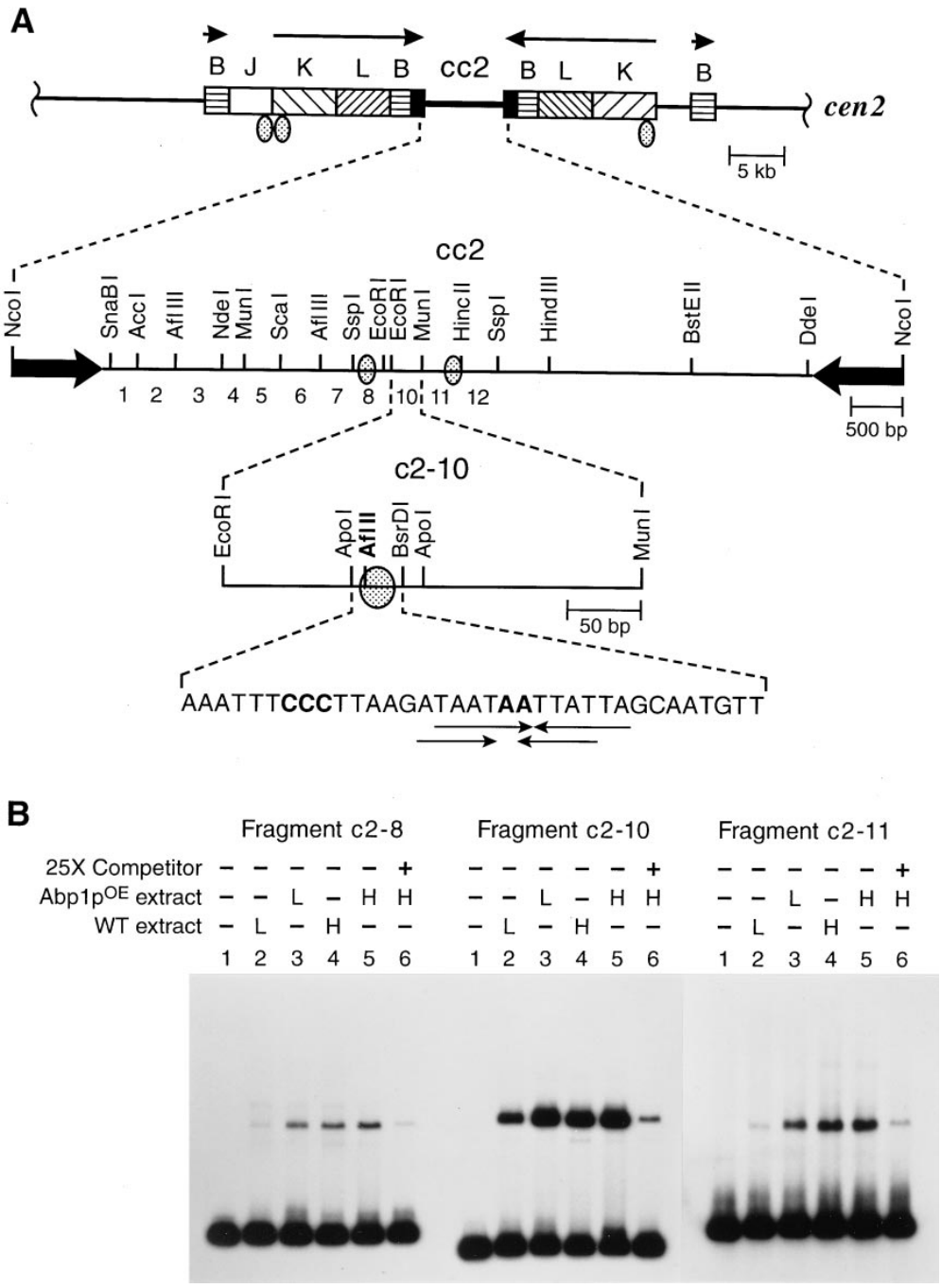

Figure 3. The *cen2* centromeric central core, essential for *cen* function, contains at least five specific protein-binding sites. (*A*) A schematic representation of the *cen2* region in *S. pombe* strain SBP120390 shows the location of *cen*-specific repeats B, J, K, and L, which are arranged in a strain-dependent, chromosome-specific array to form an inverted repeat (*bold arrows*) centered around an asymmetric central core (*cc2*; Steiner et al., 1993). An enlarged view of cc2 shows numbered restriction fragments (<400 bp) used as probes in gel mobility shift assays to detect centromere-binding proteins. Fragments c2-1, -2, -8, -10, and -11 exhibit specific mobility shifts (see Materials and Methods). Fragment c2-10 is enlarged to show relevant restriction sites. Below, one strand of the 35-bp ApoI–BsrDI DNA sequence from c2-10 is shown. Thin arrows mark regions of dyad symmetry. Bold letters in the sequence indicate homology with conserved bases that are essential for CENP-B binding to the mammalian CENP-B box (T**CCCG**PyT-**T**CCAA**CGAA**Pu; Masumoto et al., 1989, 1993). Shaded ovals depict the approximate position of Abp1p binding sites determined in this study and by V.K. Ngan and L. Clarke (manuscript submitted for publication). (*B*) Gel mobility shift assays with fragments c2-8 (332 bp), c2-10 (283 bp), and c2-11 (368 bp), with low salt extract (*L*; 6 μg protein) or 0.4 M KCl chromatin extract (*H*; 1.2 μg protein) prepared from wild-type (*WT*) or Abp1p-overexpressing strains (see Materials and Methods), indicate that increased binding to c2-8, -10, and -11 correlates with overproduction of Abp1p. Competition in lane *6* indicates sequence-specific DNA binding.

asci were observed than in crosses between wild-type cells. Out of 17 asci dissected from a Δ*abp1* homozygous mating, one ascus gave rise to four viable spores, and several others gave rise to one to three viable spores. Of the spores dissected, most that appeared normal were viable, whereas those with abnormal morphology failed to produce colonies. Therefore, Abp1p appears to play an essential role in the meiotic process, although rare competent spores are occasionally observed.

The Functionally Essential cc2 Region Contains Specific Protein-binding Sites

In a separate approach to identifying proteins with roles in centromere function, we pursued purification of centromere DNA-binding proteins using sequence-specific affinity chromatography. We demonstrated previously that half of the *cen2* central core (cc2), along with K-type repeat sequences, are sufficient to establish a functional, although somewhat compromised, *S. pombe* centromere on a circu-

lar minichromosome (Baum et al., 1994). Such a construct is ~30-fold less stable in mitosis than a circular minichromosome carrying the entire *cen2* region and segregates to two of the four meiotic products as expected, but it exhibits precocious sister chromatid separation in meiosis I. Using convenient restriction sites, this half of cc2 was cleaved into fragments <400 bp in length (Fig. 3 *A*), and each fragment was tested individually for sequence-specific protein binding activity in gel mobility shift assays (Garner and Revzin, 1981). A 0.4-M KCl extract of chromatin from the wild-type *S. pombe* strain SBP120390 was found to contain specific binding activity for five fragments from the left half of cc2: c2-1, c2-2, c2-8, c2-10, and c2-11 (Fig. 3, *A* and *B*, and data not shown). Based on total binding units, fragment c2-10 binding activity was found in equal proportions in the low salt and 0.4-M KCl extracts, which represent non–chromatin- and chromatin-associated proteins, respectively (Fig. 3 *B*, lanes *2* and *4*, and Table II). Because fewer proteins are in the chromatin fraction, the specific activity was enriched >10-fold in the chromatin extract compared with the low-salt extract.

Extracts from an Abp1p-overexpressing Strain Are Enriched for Specific Centromere DNA-binding Activity

In an effort to identify centromere-binding proteins associated with those cc2 fragments that exhibit specific gel mobility shifts, we screened extracts prepared from strains containing minichromosome-destabilizing plasmids identified in the genetic screen reported above and from strains containing mutations that derepress expression of a reporter gene embedded in the heterochromatin-like centromeric DNA or that cause an elevated rate of chromosome loss (Allshire et al., 1995). To date, three proteins, Swi6p, Rik1p, and Clr4p, appear to play a role at the centromere (Allshire et al., 1995; Ekwall et al., 1995) as well as at the silent mating type and other loci in fission yeast (Egel et al., 1989; Ekwall and Ruusula, 1994; Lorentz et al., 1994). Mutations in *rik1* and *clr4* have been shown to alleviate repression of a reporter gene inserted within the centromeric repeated DNA but do not affect repression of genes inserted within the central core DNA (Allshire et al., 1995). Gel shift assays performed with cc2 fragments and extracts prepared from a *swi6* null strain and from strains with mutations in *rik1* and *clr4* did not exhibit any alteration of the gel mobility shift profile compared with extract prepared from a wild-type strain (data not shown). On the other hand, for three of the cc2 fragments, c2-8, c2-10, and c2-11, specific DNA-binding activity was elevated in an Abp1p-overexpressing strain (Fig. 3 *B*, lanes *3* and *5*). An increase in binding activity was apparent in both the low-salt and 0.4-M KCl chromatin extract from whole cells. The majority of the overproduced protein was not chromatin associated in vivo, although excess Abp1p appears to favor increased formation of the DNA–protein complex (Fig. 3 *B*, lanes *3*, and Table II). Compared with a wild-type strain, however, c2-10 binding activity that is chromatin associated in vivo was elevated less than twofold in an Abp1p-overexpressing strain, as judged by specific activity (Table II). This increase may be accounted for by saturation of Abp1p binding sites and/or inappropriate binding to lower affinity sites.

Table II. Specific c2-10 DNA-binding Activity Is Elevated in an Abp1p-overexpressing Strain

Strain/Extract	Volume	Total Protein	Binding Units	Specific Activity	Enrichment‡
	ml	*mg*	*U**	*U/μg*	
WT/Low Salt	13.1	86.2	9,630	0.11	1.0
$Abp1p^{OE}$/Low Salt	13.4	102.9	30,010	0.29	2.7
WT/0.4 M KCl	14.4	6.1	8,130	1.33	12.1
$Abp1p^{OE}$/0.4 M KCl	14.6	8.8	15,960	1.81	16.5

Extract was prepared from a 2-liter culture of wild-type (*WT*) or Abp1p-overexpressing cells.
*1 U binds 1 fmol c2-10 DNA in a gel mobility shift assay.
‡Normalized to the specific activity of low-salt extract from WT cells.

Purification of a 62-kD Protein That Binds Specifically to cc2 DNA In Vitro and Is Identical to Abp1p

Using bandshift assays, the location of a DNA binding site within a given labeled DNA fragment can be approximated by competition with excess unlabeled smaller fragments that step across the region. In the case of fragment c2-10, digestion of the full-length EcoRI–MunI fragment with AflII yields two fragments that when mixed together did not compete with the full-length fragment for binding (Fig. 3 *A*). In contrast, digestion of the full-length fragment with ApoI yields three fragments, two of which did not compete for binding, but one, a 49-bp ApoI–ApoI fragment containing the AflII site, did compete. Further competition studies suggest that the binding site is located in a 35-bp region between the ApoI and BsrDI sites flanking AflII (Fig. 3 *A*, *bottom*). This region is 80% A+T, contains two overlapping short regions of dyad symmetry (Fig. 3 *A*, *arrows*), and shares very limited homology (five of nine essential bases; Fig. 3 *A*, **CCC** and **AA**) with the canonical mammalian CENP-B box (see Fig. 3 *A* legend; Masumoto et al., 1989, 1993). Moreover, AflII cleavage, which inactivates the c2-10 binding site, occurs between these conserved bases, leaving **CCC** and **AA** on separate DNA fragments.

Tandem copies of a 61-bp PCR-amplified DNA fragment, $c2\text{-}10_{61}$, centered around the AflII site, were used for DNA affinity chromatography (see Materials and Methods and Table III). A 0.4-M KCl chromatin extract in binding buffer was applied to the DNA affinity column in the presence of excess poly(dI-dC) to reduce nonspecific binding. After a wash, bound protein was eluted with 0.5 M KCl. The peak of c2-10 binding activity contained a single predominant protein with an apparent molecular mass of 62 to 63 kD (Fig. 4 *A*, elution fraction 3).

Western blot analysis of affinity-purified elution fractions and crude extract indicates that Abp1p is a component of the affinity-purified p62 band. The polyclonal antibody to Abp1p recognized a single 62-kD protein in extracts from wild-type or Abp1p-overproducing strains (Fig. 4 *B*, lanes *1* and *2*) and in the eluted fractions from the affinity chromatography (Fig. 4 *B*, lanes *3* and *4*). In contrast, the cross-reacting protein was not detectable in extract prepared from a Δ*abp1* strain (null; Fig. 4 *B*, last lane), indicating that the antibody is specific for Abp1p. If polyclonal antibody to Abp1p was added to gel mobility

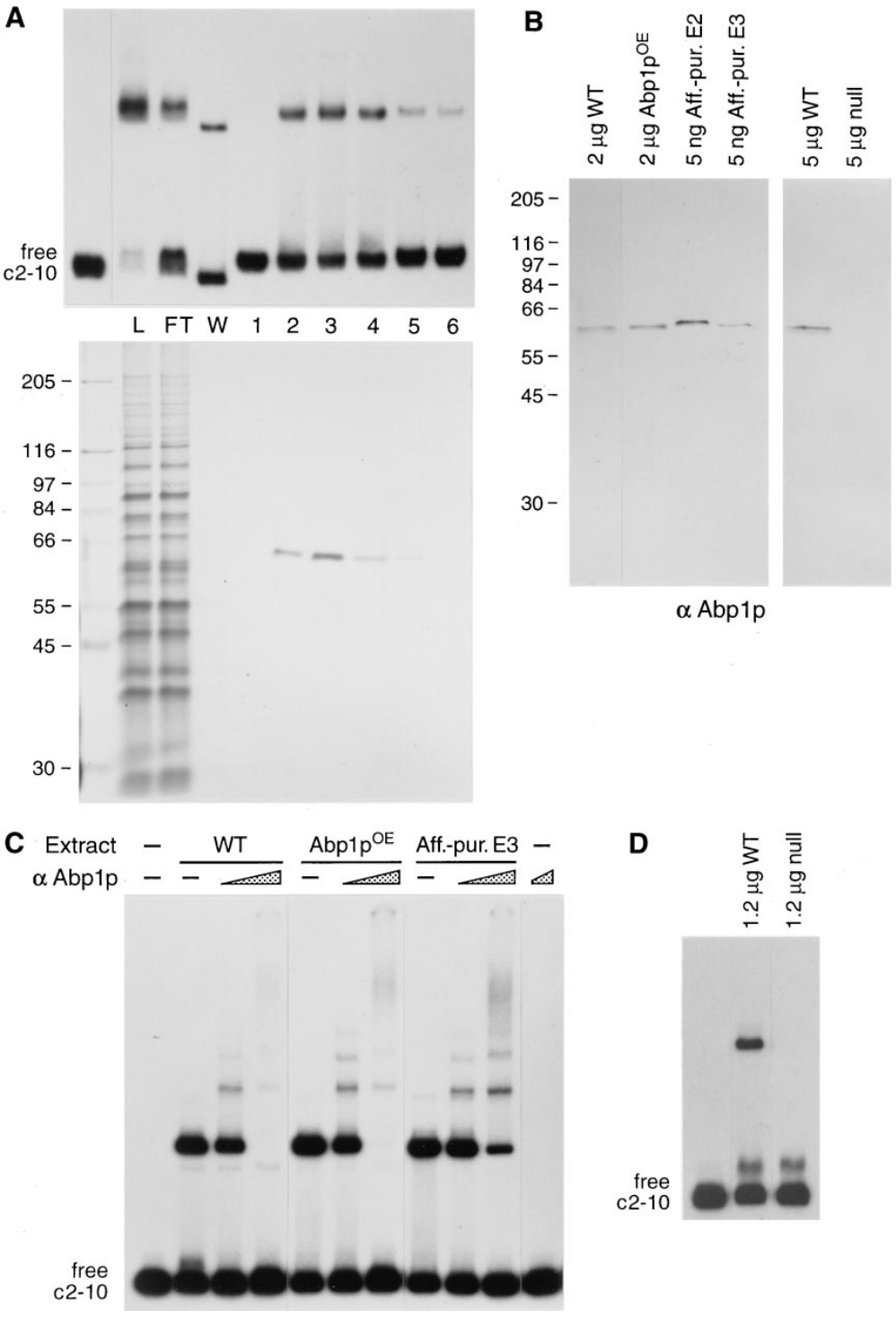

Figure 4. Purified Abp1p binds in vitro to c2-10 central core DNA. (*A*) Biochemical purification of the c2-10 binding activity was monitored by mobility shift assays (*top*) and silver-stained SDS-PAGE (*bottom*). See Materials and Methods for details of the purification procedure. Mobility shift assays used ^{32}P–c2-10 DNA as probe (*first lane*) and contained 20 μl of the diluted chromatin extract in binding cocktail applied to the affinity column (load; *L*), 20 μl of the column flowthrough (*FT*), 20 μl of the column wash fraction (*W*), or 2 μl of the 0.5-M KCl eluate fractions (lanes *1–6*). Amounts shown on the 7.5% SDS-PAGE gel are 2 μl of the column load, 2 μl of the column flowthrough, 5 μl of the column wash, or 12.5 μl of the 0.5-M KCl eluate fractions. Sizes indicated for the molecular mass standard (*first lane*) are in kilodaltons. Affinity chromatography resulted in an ~2,000-fold purification of Abp1p compared with the specific activity found in low-salt extract from whole cells (see Table III). (*B*) Western Blot analysis of affinity-purified elution fractions using a polyclonal antibody to Abp1p. αAbp1p recognizes a 62-kD protein band in 0.4 M KCl extract from wild-type SBP120390 or overexpressing SBP120390/pSp200-*abp1* cells as well as in eluate fractions 2 and 3 from the DNA affinity column (*left*), but it does not recognize a cross-reacting protein in 0.4-M KCl extract prepared from an *abp1* null (*right*). Size standards are not indicated for the right panel, but they migrated similarly to those shown for the left panel. (*C*) A supershift of fragment c2-10 is observed with αAbp1p and affinity-purified p62. Mobility shift assays were performed with 1.2 μg of protein in a 0.4-M KCl chromatin extract from wild-type SBP120390 or overexpressing SBP120390/pSp200-*abp1* cells, or with 1 ng elution fraction 3 protein mixed with increasing amounts of polyclonal antibody to Abp1p (0.01 or 0.1 μl). αAbp1, in the absence of *S. pombe* extract, has no effect. (*D*) A 0.4-M KCl chromatin extract prepared from an *abp1* null strain lacks c2-10 DNA-binding activity.

shift assays with fragment c2-10 and the affinity-purified protein or a 0.4-M KCl extract of chromatin prepared from wild-type or Abp1p-overexpressing strains, a supershift was observed (Fig. 4 *C*), indicating the presence of Abp1p in the shifted complex. Identical supershifted banding patterns were obtained with crude extracts from a wild-type strain and affinity-purified protein from an Abp1p-overproducing strain. In addition, a bandshift was not observed with extract from an *abp1* null strain (Fig. 4 *D*). Therefore, we conclude that Abp1p is a component of

Table III. Purification of Abp1p

Extract	Volume	Total Protein	c2-10 Binding Units	Specific Activity	Yield‡	Purification
	ml	*mg*	*U**	*U/μg*	*%*	
Low Salt	17.1	224.0	35,190	0.16	52	1
0.4 M KCl	17.1	30.8	32,400	1.05	48	7
Affinity Purified	1.5	0.003–0.006§	1,280	215–430	2	1,340–2,690

Extract was prepared from a 4-liter culture of an Abp1p-overexpressing strain (see Materials and Methods).
*1 U binds 1 fmol c2-10 DNA in a gel mobility shift assay.
‡The low-salt and 0.4-KCl chromatin extracts represent complementary fractions in the whole-cell extract. The assumption has been made that together they comprise 100% of total Abp1p activity (67,590 U).
§Affinity-purified protein was estimated by comparison to silver-stained standards.

the protein complex assembled on the c2-10 DNA fragment in vitro and that formation of the DNA–protein complex is Abp1p dependent. Furthermore, it is quite likely that the complex contains only Abp1p, because a single protein band is observed in the affinity-purified preparations. Supershift analysis of central core fragments c2-8 and c2-11 indicates that they are also bound by Abp1p (data not shown). Preliminary results from gel filtration chromatography of chromatin extracts indicate that a 175-kD protein complex binds to central core fragment c2-8 and a 125-kD complex binds to c2-10 and c2-11, suggesting that the c2-10 binding complex may represent a homodimer or heterodimer of Abp1p (Baum, M., and L. Clarke, unpublished results; Ngan, V.K., and L. Clarke, manuscript submitted for publication).

Sequence comparisons of Abp1p binding sites in cc2 imply that the binding motif is almost certainly AT rich but are otherwise uninformative. Bandshift competition assays in which a ^{32}P-labeled c2-10 fragment was competed with a 25-fold molar excess of an unlabeled native or mutationally altered sequence (Fig. 3 *A*, AAA substitutes for **CCC**) suggest that the binding sequence does not consist entirely of A and T. Competition with excess cold c2-10 DNA reduced binding to the radiolabeled native c2-10 fragment by 90%, whereas competition with the altered fragment resulted in only a 60% reduction in binding to the radiolabeled native c2-10 fragment. A competitor DNA sequence with a mutation in a critical residue would be expected to reduce binding of Abp1p to the ^{32}P-labeled c2-10 fragment by <10%. Thus, the intermediate value observed for the CCC to AAA alteration is a reflection of reduced binding affinity, but it also indicates that these residues are not essential for binding. Consistent with this finding, the motif shared between c2-10 and the CENP-B box does not appear to be conserved in other centromeric DNA fragments that bind Abp1p. Sequence comparisons between the 35-bp c2-10 region that contains the Abp1p binding site and other Abp1p-binding DNA fragments from the central cores and centromeric repeats (Fig. 3 *A*; Ngan, V.K., and L. Clarke, manuscript submitted for publication) suggest that the binding motif encompasses distinct AT-rich sequences interspersed with C and G residues. Identification of a consensus motif, however, has been complicated by the AT richness of the central cores (~70%) and the length of the DNA fragments implicated (100–400 bp).

Abp1p Has Significant Homology to Human Centromere Protein CENP-B and to CENP-B–related Proteins

A database search using the BLAST program (Atschul et al., 1990) revealed that Abp1p has several domains with significant homology to human CENP-B and several other CENP-B–related proteins. Pairwise alignments were most significant between Abp1p and CENP-B from human and mouse (*P* values ~4.3×10^{-7}; Sullivan and Glass, 1991; Earnshaw et al., 1987) and between Abp1p and the CENP-B–related *jerky* gene product from *M. musculus* (*P* value 2.5×10^{-12}; Toth et al., 1995). CLUSTAL V alignments (Higgins et al., 1992) indicate that CENP-B (599 aa) and Abp1p (522 aa) are ~25% identical and 53% similar over 522 aa (Fig. 5), parameters similar to those reported by Murakami et al. (1996). A CLUSTAL V alignment of Abp1p and Jerky recognizes 20.8% identity and 48.5% similarity over 509 aa (data not shown). Not surprisingly, a similar level of homology has also been reported between CENP-B and Jerky (Toth et al., 1995). The large number of gaps and insertions required to generate the pairwise alignment between Abp1p and CENP-B suggests that the two proteins have similar origins but have diverged significantly from one another (Fig. 5). Interestingly, portions of the NH_2-terminal and central domains of Abp1p, CENP-B, and Jerky have significant homology to regions of the IS*630*-Tc1 family of transposases, *Drosophila pogo* transposase, and other *pogo*-related proteins (Fig. 6; Tudor et al., 1992; Doak et al., 1994; Toth et al., 1995; Smit and Riggs, 1996) including the "D,D35E" motif that is also found in

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Abp1p   MGKIKRRAITEHEKRALRHYFFQLQNRSG--QQDLIEWFREKFGKDISQPSVSQILSSKY
CENP-B  MGP-KRRQLTFREKSRIIQ---EVEENPDLRKGEIARRF------NIPPSTLSTILKNKR
        **  *** .* .** . .  .*** .. . .. .*      .*. ...* ** .*

Abp1p   SYLDNTVEKPWDV-----KRNRPPKYPLLEAALFEWQVQQGDDATLSGETIKRAAAILWH
CENP-B  AILAS--ERKYGVASTCRKTNKLSPYDKLEGLLIAW-FQQIRAAGLPVKGIILKEKAL--
        . * .  *.  .*     * *. . *  **. *. *  *  **   * *.   *      *

Abp1p   KIPEYQDQPVPNF--SNGWLEGFRKRHIL---HAINEQPTESVVLNNTEPPNDPLS----
CENP-B  RIAE--ELGMDDFTASNGWLDRFRRRHGVVSCSGVARARARNAAPRTPAAPASPAAVPSE
        .*.*  .  . .*  *****. **.** .    ..     . .       .*  * .

Abp1p   ----------RVYDVTRLTN---INDIFTMQETGLFWKLVPNGTPEVEDIKGITR-FKA
CENP-B  GSGGSTTGWRAREEQPPSVAEGYASQDVFSATETSLWYDFLPDQAAGLCGGDGRPRQATQ
                *  .   ...    .*.*.  **.*   ..*. .. . .  *  *

Abp1p   RITLTVCCNASGTERLPLWVIGYSQSPRVFRAANVKPEVMNFKWRSNGKASMTTAIMEEW
CENP-B  RLSVLLCANADGSEKLPPLVAGKSAKPRAGQAG------LPCDYTANSKGGVTTQALAKY
        *... .* ** *.*.**  * * *  **  .*.      .      .*.*...**  .

Abp1p   LRWFDACM--EGRKVILLIDSYTPHLRAVENIRNSGNDLRNTTVITLPSTSASISQPCSE
CENP-B  LKALDTRMAAESRRVLLLAGRLAAQSLDTSGLRH----------VQLAFFPPGTVHPLER
        *. .*. *  *.*.*.** . ...      .*.           . *.  ...  .*

Abp1p   GVIYALKASYRKHWVQYILEQNELGRNPYNTTNVLRAILWLVKA-W----TTDISPEIIE
CENP-B  GVVQQVKGHYRQAMLLKAMAALE-GQDPSGLQLGLTEALHFVAAAWQAVEPSDIAACFRE
        **.  .*. **.  .   .  . *.** *  * .* * *  .**.. . *

Abp1p   NAF----------NLSGVL
CENP-B  AGFGGGPNATITTSLKSEGEEEEEEEEEEEEEGEGEEEEEEGEEEEEEGGEGEELGEEE
         .*                                                .*.

Abp1p   GLFNESAVTSRALDEM----------IHPLRELVSEFSVQAAMRIED-----FISP
CENP-B  EVEEEGDVDSDEEEEEDEESSSEGLEAEDWAQGVVEAGGSFGAYGAQEEAQCPTLHFLEG
         . .*. * *  .*            . . *  . *.  .*    .      **

Abp1p   SEENIVDSSEDIINQIASQYMDDRAFETDEEESTEFQITT-KDAMKAIELLLNYEAQQPD
CENP-B  GEDSDSDSEEEDDEEEDDEDEDD---DDDEEDGDEVPVPSFGEAMAYFAMVKRYLTSFPI
        .*..  ** *.  ..   .  **   . ***.. *  . .  .**  . ..  * .  *

Abp1p   GNPAITISL-LNYQKLLEARGGNVNLSRLRS----T
CENP-B  DDRVQSHILHLEHDLVHVTRKNHARQAGVRGLGHQS
        ..   .  * *. . .  .*  .   . .*.    .
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Figure 5. Abp1p has homology to CENP-B. A CLUSTAL V alignment of human CENP-B (599 aa) and Abp1p (522 aa) is shown. Asterisks and dots indicate identical and similar residues, respectively.

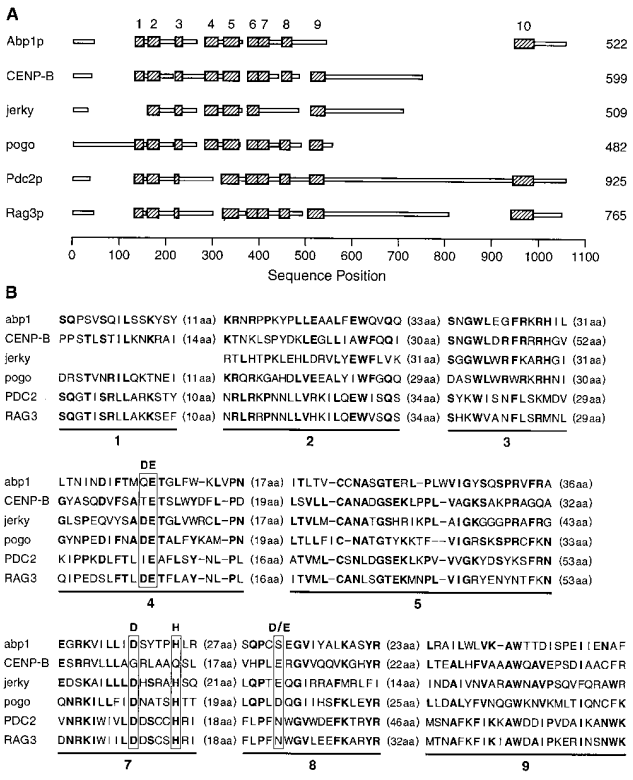

Figure 6. Abp1p and CENP-B have significant homology to transposase-related proteins. (*A*) Amino acid sequence alignment between *S. pombe* Abp1p, human CENP-B, *M. musculus jerky* gene product, *D. melanogaster pogo* transposase, and *S. cerevisiae* transcription factors Pdc2p and Rag3p. Hatched boxes represent blocks of significant homology as determined by MACAW 2.0.5. (Schuler et al., 1991). (*B*) Sequence comparison within blocks of significant homology indicated in *A*. Numbers in parentheses represent intervening amino acid sequences between the blocks shown. Dashes are gaps that were added to optimize sequence alignment. Residues in bold type represent identical matches in at least three of the proteins shown. Residues invariable in *pogo*-like and IS*630*-Tc1 transposases are boxed (Smit and Riggs, 1996).

bacterial insertion sequence (IS) transposases and retroviral integrases and is thought to form part of the transposase catalytic site (Kulkosky et al., 1992; Doak et al., 1994; Pollard and Chandler, 1995). When the MACAW 2.0.5. sequence alignment program is used to search for blocks of significant homology between CENP-B and Abp1p in a pairwise fashion, sequences corresponding to block 5 (Fig. 6 *B*, 32 aa) are recognized as significant, and sequences corresponding to block 3 (Fig. 6 *B*, 12 aa) are recognized as "maybe" significant (Schuler et al., 1991). The statistical significance of the homology in other regions of the two proteins greatly increases when additional transposase-like sequences are added to the alignment. When compared with typical transposase sequences, the amino acid sequences of CENP-B, Jerky, Abp1p, and the *pogo* transposase–related yeast transcription factors Rag3p (GenBank accession #X70186) and Pdc2p (Hohmann, 1993) are all somewhat degenerate, indicating that they may lack transposase activity (Fig. 6 *B*; Smit and Riggs, 1996). Although their exact functions remain to be determined, the homology shared between CENP-B and Abp1p suggests that they may play related roles at the centromere.

Discussion

We have used simultaneous genetic and biochemical approaches in an effort to identify *S. pombe* proteins with potential roles in centromere function. Both approaches led to the isolation of Abp1p, a protein with significant homology to the human centromere DNA-binding protein CENP-B. The gene for Abp1p was isolated by screening an *S. pombe* genomic library, constructed in a multicopy vector, for genes that caused high-frequency mitotic loss of a stable *cen1* linear minichromosome. Although Abp1p is not essential, cells overexpressing *abp1* as well as *abp1* null cells are slow growing and display mitotic defects when grown at low temperature, indicating that alterations in the cellular level of Abp1p affect normal mitotic growth. In addition, Abp1p is required for normal meiotic processes to occur in most cells. Using a separate, biochemical approach, we isolated Abp1p by sequence-specific affinity chromatography using a DNA sequence from the centromeric central core of *S. pombe* chromosome II. Furthermore, we have shown that Abp1p binds specifically in vitro to at least three sites in the *cen2* central core as well as within the K-type (K/K″/dg) repeat element (Fig. 3 *A*, *shaded ovals*; Ngan, V.K., and L. Clarke, manuscript submitted for publication), two elements that are required for centromere function (Baum et al., 1994).

Murakami et al. (1996) also independently isolated Abp1p by affinity chromatography using a target sequence containing four tandem repeats of an autonomously replicating sequence (ARS) consensus sequence that was identified in fragments capable of promoting efficient transformation in *S. pombe* (Maundrell et al., 1988). However, the functional significance of this 11-bp motif [(A/T)TAAATAAA(C/T)(A/T)] is unclear because deletion of this sequence has no apparent effect on the ability of such fragments to mediate autonomous replication (Maundrell et al., 1988), and some centromeric fragments, containing one or more consensus sequences, do not initiate replication at a detectable level in the chromosome (Smith et al., 1995). To detect Abp1p binding activity, Murakami et al. (1996) incubated *S. pombe* crude cell extracts with dimers or tetramers of a 31-bp oligonucleotide (5′-gaTCCAAATTAT-ATAAATAAATAATTTTTGa-3′) and monitored complex formation in gel mobility shift assays, suggesting that Abp1p binding sites are AT rich but might also contain G and C residues. In support of this, we have found that replacing a CCC motif in fragment c2-10 with AAA causes a minor reduction in Abp1p binding affinity. In addition, *S. pombe* centromeric central core DNA fragment c2-12 (Fig. 3 *A*) contains an ARS consensus sequence but lacks appreciable affinity for Abp1p (data not shown). Although the *S. pombe* centromere contains a 5–10-fold higher density of potential replication origins than does random fission yeast DNA, initiation signals occur at only a small minority of ARS elements present in the K and L repeats, and central core and core-associated repeat sequences lack ARS activity in a chromosomal context (Smith et al., 1995). The absence of ARS activity in central core sequences that have a demonstrated Abp1p binding activity

in vitro further suggests that Abp1p has a role in centromere function rather than in replication. Furthermore, FACScan® analysis of the Δ*abp1* strain gives no indication of a replication defect. To reflect its centromere-related roles, we suggest that a change in designation from *abp1* (Abp1p) to *cbp1* (Cbp1p; centromere binding protein) is appropriate.

Amino acid sequence comparisons suggest that Abp1p/Cbp1p shares appreciable structural homology with human CENP-B. CENP-B consists of at least three structural domains: an NH_2-terminal DNA-binding domain; a central domain of unknown function; and a COOH-terminal dimerization domain. The NH_2-terminal 125 amino acids of CENP-B delimit an experimentally determined DNA-binding domain that shows partial similarity to helix–loop–helix proteins, homeodomain proteins, and a number of transcription factors, although the exact motif responsible for DNA binding remains speculative (Sullivan and Glass, 1991; Yoda et al., 1992; Suzuki et al., 1995). Interestingly, significant homology is also observed between portions of the DNA-binding domain of CENP-B and the NH_2-terminal regions of Abp1p/Cbp1p and other proteins related to the IS*630*-Tc1 family of transposases, including *D. pogo* transposase and the two yeast transcription factors, Rag3p and Pdc2p (Fig. 6; Tudor et al., 1992; Hohmann, 1993; Smit and Riggs, 1996). Most likely, all of these proteins bind to DNA via their NH_2-terminal domains and may share a common DNA-binding motif.

In addition to a DNA-binding domain, CENP-B contains a homodimerization domain consisting of 59 COOH-terminal amino acids (Kitagawa et al., 1995). The COOH-terminal region of CENP-B lacks strong sequence conservation with Abp1p/Cbp1p as well as with the IS*630*-Tc1 family of transposases. However, Murakami et al. (1996) have suggested that the COOH-terminal domain of Abp1p/Cbp1p may function as a multimerization site. In addition, gel filtration data presented here and elsewhere suggest that Abp1p/Cbp1p binds to DNA as a dimer, either alone or as part of a multisubunit protein complex (Ngan, V.K., and L. Clarke, manuscript submitted for publication).

Separating the NH_2-terminal and COOH-terminal domains of CENP-B is a central region of unknown function with homology to the central domains of Abp1p/Cbp1p and the IS*630*-Tc1 transposase family (Fig. 6; Smit and Riggs, 1996). Although CENP-B and Abp1p/Cbp1p both contain mutations and/or altered spacing in the invariant residues of the D,D35E motif and may lack transposase catalytic activity, it is intriguing that both mammalian and fission yeast centromere DNA-binding proteins share homology to transposases. DNA sequencing has revealed the presence of a large number of transposons contained within the centric heterochromatin of functional *Drosophila* minichromosome derivatives (Le et al., 1995; Karpen et al., 1996) and within the centromeric region of *Neurospora crassa* chromosomes (Cambareri, E., R. Aisner, and J. Carbon, unpublished data). Notably, short and long interspersed nucleotide elements (SINEs and LINEs) are clustered at or near mammalian centromeres (Tyler-Smith and Willard, 1993). In addition, the size, structure, and head-to-tail arrangement of tandem repeat elements at the *S. pombe* centromere resemble known transposon arrangements, although these repeats contain no long open reading frames and no obvious sequence homologies to known transposons (Ngan, V.K., and L. Clarke, manuscript submitted for publication). It has been suggested that transposon repeats, either alone or in combination with satellite DNAs, may play a role in the formation and/or function of centromeric heterochromatin (Carmena and Gonzalez, 1995). Interestingly, fluorescent microscopy has shown that achiasmate chromosomes are held together by homologous regions of centric heterochromatin (Dernberg et al., 1996). These heterochromatic regions apparently contain multiple pairing elements that act in an additive fashion to promote efficient achiasmate meiotic chromosome disjunction in *Drosophila*, suggesting that cross-multimerization of intrinsic heterochromatic proteins or simultaneous binding to identical sequences on chromosome homologs by "special" proteins or complexes might mediate the pairing process (Karpen et al., 1996). Little is known about meiotic centromere function in *S. pombe.* One possibility is that multiple centric pairing proteins may maintain sister chromatid cohesion in meiosis I and throughout mitosis in a manner similar to that proposed for the achiasmate segregation elements in *Drosophila.* Abp1p/Cbp1p binds in vitro to multiple regions in *S. pombe* centromeric DNA and has apparent roles in both mitosis and meiosis. One potential role for Abp1p/Cbp1p would be to bind to transposon-like elements along the length of the centromere and facilitate the formation of a higher order *cis*- and/or *trans*-DNA structure via homodimerization and/or as part of a multicomponent protein complex.

Although Abp1p/Cbp1p is not absolutely essential for mitotic cell division, alterations in the cellular level of Abp1p/Cbp1p severely disrupt minichromosome segregation and have marked effects on cell growth and native chromosome segregation at low temperatures. The ability of Abp1p/Cbp1p to bind to centromeric DNA and to destabilize a minichromosome when expressed at elevated levels suggests that the stoichiometry of Abp1p/Cbp1p binding at the centromere is important for mitotic chromosome segregation. In addition, the severe meiotic defect observed in cells lacking Abp1p/Cbp1p suggests that Abp1p/Cbp1p performs an important role in the meiotic process. Because Abp1p/Cbp1p apparently binds specifically to both the centromeric central core and K-type repeat elements, and gel filtration experiments suggest that Abp1p/Cbp1p dimerizes in solution, it is intriguing to speculate that Abp1p/Cbp1p may play a role in bringing together K-repeat and central core DNA elements in the formation of a higher order structure at the *S. pombe* centromere (Clarke et al., 1993; Marschall and Clarke, 1995; Ngan, V.K., and L. Clarke, manuscript submitted for publication), a role similar to that proposed for CENP-B at the mammalian kinetochore. Computer comparisons of sequences that bind Abp1p/Cbp1p do not detect a consensus motif, although they are all AT-rich sequences with some G and C residues. Even though the binding site appears very degenerate, affinity-purified Abp1p/Cbp1p binds to the c2-10 sequence with higher affinity ($K_{app} > 10^9\ M^{-1}$; Baum, M., and L. Clarke, unpublished data) than CENP-B to the CENP-B box ($6 \times 10^8\ M^{-1}$; Muro et al., 1992). The ability of Abp1p/Cbp1p to bind to distinct AT-rich se-

quences in the central core regions, K repeats, and other centromeric repeated DNAs is consistent with the anticipated properties of an *S. pombe* centromere DNA-binding protein. The lack of appreciable sequence conservation among the *S. pombe* centromeric central core regions suggests that centromere DNA-binding proteins may recognize small and/or degenerate sequences or a conserved structural motif (Takahashi et al., 1992; Marschall, L., and L. Clarke, unpublished data). Because an *abp1/cbp1* deletion is not lethal, Abp1p/Cbp1p may be functionally redundant with one or more other centromere DNA-binding proteins. This is not surprising given the apparent overlapping functions of *S. pombe* centromeric DNA elements (Baum et al., 1994; Ngan, V.K., and L. Clarke, manuscript submitted for publication) and the potential functional redundancy of CENP-B at the human kinetochore.

Whether Abp1p/Cbp1p plays a role in the formation of higher order DNA structure at the *S. pombe* centromere and/or plays a role in chromosome pairing remains to be determined. Nevertheless, Abp1p/Cbp1p is the first fission yeast centromere DNA-binding protein to be isolated, and it provides a valuable tool for the identification of other *S. pombe* kinetochore proteins. Furthermore, the apparent structural and functional homologies shared between Abp1p/Cbp1p and CENP-B strongly suggest that knowledge gained from studying fission yeast centromeres will translate into an advanced understanding of the structure and functional organization of the higher eukaryotic kinetochore.

We thank Sean George for technical assistance, Vivian Ngan and Ed Cambareri for critical reading of this manuscript, and members of the L. Clarke and J. Carbon laboratories for helpful discussions.

This work was supported by U.S. Public Health Services grant GM-33783 from the National Institutes of Health.

Received for publication 4 October 1996 and in revised form 9 December 1996.

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