Functional Redundancies, Distinct Localizations and Interactions Among Three Fission Yeast Homologs of Centromere Protein-B

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ABSTRACT

Several members of protein families that are conserved in higher eukaryotes are known to play a role in centromere function in the fission yeast *Schizosaccharomyces pombe*, including two homologs of the mammalian centromere protein CENP-B, Abp1p and Cbh1p. Here we characterize a third *S. pombe* CENP-B homolog, Cbh2p (*C*ENP-*B h*omolog 2). *cbh2*Δ strains exhibited a modest elevation in minichromosome loss, similar to *cbh1*Δ or *abp1*Δ strains. *cbh2*Δ *cbh1*Δ strains showed little difference in growth or minichromosome loss rate when compared to single deletion strains. In contrast, *cbh2*Δ *abp1*Δ strains displayed dramatic morphological and chromosome segregation defects, as well as enhancement of the slow-growth phenotype of *abp1*Δ strains, indicating partial functional redundancy between these proteins. Both *cbh2*Δ *abp1*Δ and *cbh1*Δ *abp1*Δ strains also showed strongly enhanced sensitivity to a microtubule-destabilizing drug, consistent with a mitotic function for these proteins. Cbh2p was localized to the central core and core-associated repeat regions of centromeric heterochromatin, but not at several other centromeric and arm locations tested. Thus, like its mammalian counterpart, Cbh2p appeared to be localized exclusively to a portion of centromeric heterochromatin. In contrast, Abp1p was detected in both centromeric heterochromatin and in chromatin at two of three replication origins tested. Cbh2p and Abp1p homodimerized in the budding yeast two-hybrid assay, but did not interact with each other. These results suggest that indirect cooperation between different CENP-B-like DNA binding proteins with partially overlapping chromatin distributions helps to establish a functional centromere.

EUKARYOTIC centromeres can be divided into two categories, "point" centromeres, which involve short, discrete DNA sequences of easily identifiable function, and "regional" centromeres, which include large blocks of repetitive sequences and have more complex strucutral and functional characteristics (Pluta *et al.* 1995). Only budding yeasts are known to have point centromeres, while organisms ranging from fission yeast to humans use regional centromeres of varying sizes and complexities. The three regional centromeres of the fission yeast *Schizosaccharomyces pombe* consist of 40–100 kb of DNA, including a variable number of repetitive sequence elements (reviewed in Clarke 1998). These elements are in turn arranged in an inverted repeat orientation around a region of 4–7 kb of unique sequences, where it is likely that the kinetochore is assembled. Like the centromeres of more complex eukaryotes, fission yeast centromeres exhibit epigenetic regulation (Steiner and Clarke 1994; Ekwall *et al.* 1997) and partial functional redundancy among the DNA elements: progressive removal of repetitive sequence elements from minichromosome constructs resulted in progressive disruption of sister chromatid cohesion in meiosis and reductions in mitotic segregation fidelity (Hahnenberger *et al.* 1991; Baum *et al.* 1994). Many of the proteins involved in centromere function in humans are conserved in *S. pombe*, including histone modifying proteins (Grewal *et al.* 1998; Freeman-Cook *et al.* 1999), chromodomain proteins (Ekwall *et al.* 1995; Doe *et al.* 1998; Halverson *et al.* 2000; Thon and Verhein-Hansen 2000), a centromere protein (CENP)-A homolog (Takahashi *et al.* 2000), and two CENP-B homologs (Murakami *et al.* 1996; Halverson *et al.* 1997; Lee *et al.* 1997; Baum and Clarke 2000).

The CENP-B family of proteins is highly conserved, especially among mammals, yet the function of CENP-B at the centromere is not completely understood (reviewed in Choo 1997). CENP-B acts as a dimer and binds to a site in the highly repetitive 171-bp α-satellite sequence found in most human centromeres. By virtue of these DNA binding and dimerization abilities, CENP-B is capable of bringing together distant copies of the α-satellite repeat *in vitro*, suggesting a possible involvement in packaging of centromeric heterochromatin (Yoda *et al.* 1998). However, the human Y chromosome and certain epigenetically activated neocentromeres lack detectable CENP-B (du Sart *et al.* 1997; Wandall *et al.* 1998). Furthermore, several strains of CENP-B null mice have no detectable mitotic defect, although some strains do exhibit reproductive and mild growth defects (Hudson *et al.* 1998; Kapoor *et al.* 1998;

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Perez-Castro *et al.* 1998; Fowler *et al.* 2000). These results suggest that either CENP-B has a centromere function that is redundant with other centromere binding proteins or CENP-B is dispensable for centromere function, in which case the high degree of sequence conservation might be explained by a role in the evolution or maintenance of satellite DNA (Kipling and Warburton 1997).

The first fission yeast homolog of CENP-B was isolated via its affinity *in vitro* for an autonomously replicating sequence (ARS) element and hence was named Abp1p (*A*RS *b*inding *p*rotein 1; Murakami *et al.* 1996). Abp1p is ~25% identical and 50% similar to CENP-B, binds with high affinity to centromeric DNA, and is an abundant nuclear protein (6000–13,000 chromatin-associated molecules per cell; Halverson *et al.* 1997; Baum and Clarke 2000). Overexpression of *abp1*$^+$ causes a dramatic increase in minichromosome loss, and deletion of *abp1*$^+$ results in a slow growth phenotype and moderately elevated minichromosome loss, as well as a sporulation defect (Halverson *et al.* 1997). Taken together, these results suggest that Abp1p is at least partially functionally analogous to human CENP-B.

A second CENP-B homolog, Cbh1p (originally designated Cbhp by Lee *et al.* 1997), was identified by affinity chromatography and localized by DNA footprinting to a specific sequence in centromeric repeat DNA and *ars3002*. Cbh1p is ~25% identical and 46% similar to CENP-B, and shows 40% identity and 67% similarity to Abp1p. Deletion of *cbh1*$^+$ results in no observable phenotype beyond a moderate increase in minichromosome loss, but deletion of both *cbh1*$^+$ and *abp1*$^+$ results in severe growth and chromosome segregation defects (Baum and Clarke 2000). This result suggests that these two fission yeast CENP-B homologs perform at least partially redundant functions in chromosome segregation and serves as a precedent for the possible existence of functionally redundant CENP-B family members in mammals.

Here we describe a third fission yeast gene with homology to human CENP-B, Cbh2p (*C*ENP-*B* *h*omolog 2). Like Cbh1p, Cbh2p is partially functionally redundant with Abp1p, as the double deletion (*cbh2*Δ *abp1*Δ) results in an enhancement of the slow-growth phenotype observed in *abp1*Δ strains as well as greatly increased missegregation of chromosomes and hypersensitivity to a microtubule-destabilizing drug. In contrast, a *cbh2*Δ strain shows no growth defects and a moderate increase in minichromosome loss, as does a *cbh2*Δ *cbh1*Δ strain. Although Abp1p, Cbh1p, and Cbh2p are structurally similar and display functionally redundant roles in chromosome segregation, these three CENP-B homologs show distinct *in vivo* localization profiles. Like Cbh1p, which is present at both centromeric and euchromatic regions *in vivo* (Baum and Clarke 2000), Abp1p localizes to centromeric heterochromatin and to replication origins, indicating that it, too, may have both centromeric and noncentromeric functions. In contrast, Cbh2p appears to localize specifically to centromeric heterochromatin in regions encompassing the unique central core and core-associated repeat DNAs. Our results suggest functionally redundant roles for CENP-B homologs in the organization of fission yeast centromeric heterochromatin, but distinct roles for these proteins outside the centromere region.

MATERIALS AND METHODS

Sequence analysis: Pairwise sequence comparisons were performed with the BESTFIT program (Wisconsin Package v. 10.0; Genetics Computer Group, Madison, WI), using default settings. The sequence alignment was performed with MACAW (Schuler *et al.* 1991).

Growth conditions, strains, and media: Fission yeast cultures were grown in minimal medium with appropriate supplements unless otherwise indicated (Gutz *et al.* 1974; Moreno *et al.* 1991). DAPI (4′,6-diamidino-2-phenylindole) staining was performed on cells grown to midlog phase in liquid culture and fixed by a 60-min incubation in 3% formaldehyde. Yeast transformations were performed by the alkali-cation method (Bio101, La Jolla, CA). Strains used in this study are listed in Table 1. Strains designated Spx34 were derived from a cross between strains SBP072098-21B7 and JIY49. Spx33-5B was derived from a cross between strains SBP082996 and JIY50. Strain Spx47-1 was derived from a cross between strains Spx36-6A and Spx40-E9, which was derived from a cross between strains SBP072098-21B7 and mis-6.

Disruption of *cbh2*$^+$: Disruption of *cbh2*$^+$ was initiated by PCR amplification of 1.9 kb of sequences starting 41 bp upstream of the putative transcription start site (primers 5′-tag gatccGCGGCTGTCGTGG-3′ and 5′-aactgcagTGATTGGAG TAAGC-3′; lowercase indicates nonhomologous sequences containing restriction sites) and 2.1 kb of sequences starting 14 bp downstream of the putative stop codon (primers 5′-ttggatccGCAGATCGATTGTG-3′ and 5′-tatgagctcGCGACAT CCTTAGTGG-3′). The resulting products were cloned sequentially into the *Bam*HI/*Sac*I and *Bam*HI/*Pst*I sites of pBluescript$^-$ (Stratagene, La Jolla, CA), and then a 4.3-kb *Bgl*II-*Bam*HI fragment carrying the *hisG-ura4*$^+$*-hisG* cassette from pDM291 (McNabb *et al.* 1997) was ligated into *Bam*HI. The resulting plasmid was linearized with *Pst*I, transformed into the diploid strain SBPD021298-15, and resulting Ura$^+$ transformants were screened by Southern analysis and sporulated. Sibling h$^+$ (JIY49) and h$^-$ (JIY50) *cbh2*Δ haploid progeny from one of the resulting tetrads were verified by Southern analysis and used for subsequent analyses and strain constructions.

Minichromosome stability assay: Loss of a 78-kb linear *cen1* minichromosome, pSp(*cen1*)-7L-sup3E, was assayed as previously described (Baum and Clarke 2000). Briefly, cells grown under selection for the minichromosome were plated on low-adenine medium and the resulting colonies were scored for minichromosome loss frequency during the first division after plating, as indicated by the number of half-red colonies divided by the total number of colonies, excluding those that are entirely red.

Immunolocalization: Creation of a C-terminal Cbh2p-HA fusion was initiated by PCR amplification of the full-length *cbh2*$^+$ coding sequence with primers 5′-ttagatctgATGCCTC CATTGAG-3′ and 5′-gcggccgctCACAATCGATCTGC-3′. The *Bgl*II and *Not*I sites were used to insert this fragment into the *Bgl*II/*Not*I sites in plasmid pSGP72, a LEU2 derivative of the *nmt* promotor-based hemagglutinin (HA) tagging vector

TABLE 1

Yeast strains used in this study

Strain[a]	Genotype[b]	Source
JIY49	*h⁺ ade6-216 cbh2Δ::hisG-ura4⁺-hisG leu1-32 ura4-D18*	This study
JIY50	*h⁻ ade6-216 cbh2Δ::hisG-ura4⁺-hisG leu1-32 ura4-D18*	This study
Δmad-2	*h⁺ ade6-210 leu1-32 mad2Δ::ura4⁺ ura4D18*	S. Sazer
mis-6	*h⁻ mis6-302 leu1⁻*	M. Yanagida
PJ69-4A	*MAT***a** *GAL2-ADE2 gal4Δ gal80Δ his3-200 leu2-3,112 LYS2::GAL1-HIS3 met2::GAL7-lacZ trp1-901 ura3-52*	P. James
SBP072098-21B7[c]	*h⁻ ade6-704 cbh1Δ::hisG-ura4⁺-hisG leu1-32 ura4-294*	Lab collection
SBP072198-1C[c]	*h⁻ abp1Δ::ura4⁺ ade6-216 cbh1Δ::hisG-ura4⁺-hisG leu1-32 ura4-D18*	Lab collection
SBP082996[c]	*h⁻ abp1Δ::ura4⁺ ade6-704 leu1-32 ura4-294*	Lab collection
SBP32590[c]	*h⁻ ade6-704 leu1-32 ura4-294*	Lab collection
SBPD022198-15	*h⁺/h⁻ ade6-216/ade6-210 leu1-32/leu1-32 ura4-D18/ura4-D18*	Lab collection
Sp223	*h⁻ ade6-216 leu1-32 ura4-294*	Lab collection
Spx33-5B[c]	*h⁻ ade6-704 abp1Δ::ura4⁺ cbh2Δ::hisG-ura4⁺-hisG leu1-32 ura4⁻*	This study
Spx34-6A[c]	*h? ade6-704 cbh2Δ::hisG-ura4⁺-hisG leu1-32 ura4⁻*	This study
Spx34-7A[c]	*h? ade6-704 cbh1Δ::hisG-ura4⁺-hisG cbh2Δ::hisG-ura4⁺-hisG leu1-32 ura4⁻*	This study
Spx34-10[c]	*h? ade6-704 leu1-32 ura4⁻*	This study
Spx34-13[c]	*h? ade6-704 cbh1Δ::hisG-ura4⁺-hisG leu1-32 ura4⁻*	This study
Spx40-E9[c]	*h⁻ ade6-704 mis6-302*	This study
Spx47-1[c]	*h? ade6-704 cbh2Δ:hisG-ura4⁺-hisG mis6-302*	This study

[a] All strains are *S. pombe* except PJ69-4A, which is *S. cerevisiae*. Plasmid-bearing strains are described in MATERIALS AND METHODS.

[b] Strains designated *ura4⁻* carry either the *294* or *D18* allele. Strains designated *h?* were not tested for mating.

[c] These strains contain the 78-kb linear minichromosome pSp(cen1)-7L-sup3E (*cen1⁺ sup3E⁺ ura4⁺ TRP1 URA3*; HAHNENBERGER *et al.* 1991).

SLF172 (gift from S. Forsburg; FORSBURG and SHERMAN 1997). The resulting plasmid, pJI61, was transformed into strain SBP32590. Cells were grown to midlog phase in Edinburgh minimal medium (EMM; Stratagene) under repressing (25 μM thiamine) and nonrepressing (no thiamine) conditions. Expression of the fusion construct under both conditions was verified by Western analysis using an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL). Immunostaining was performed according to the method of EKWALL *et al.* (1996) with the following modifications: cells were fixed for 90 min in 3% formaldehyde plus 0.2% glutaraldehyde, and zymolyase digestion of the cell wall was carried out for 1 hr. The fusion protein was visualized with HA.11 (BabCo, Richmond, CA) followed by a fluorescein-conjugated secondary antibody (Molecular Probes, Eugene, OR). Cells were imaged using a Nikon Microphot-SA fluorescence microscope (A. G. Heinze Precision Optics, Irvine, CA) and Image-1 version 4.0 software (Universal Imaging Corporation, West Chester, PA). Images were processed in Adobe photoshop 5 and Illustrator 8 (Adobe Systems Incorporated, San Jose, CA).

Chromatin immunoprecipitation assay: Creation of the N-terminal GST-Cbh2p fusion was initiated by PCR amplification of the full-length *cbh2⁺* coding sequence with primers 5′-ttagatctgATGCCTCCATTGAG-3′ and 5′-gcggccgctCACA ATCGATCTGC-3′. The resulting *Bgl*II and *Not*I sites were used to insert this fragment into the *Bgl*II/*Not*I sites in plasmid pESP-1 (Stratagene), and the resulting plasmid, pJI76, was transformed into strain JIY49T. The N-terminal GST-Abp1p fusion was created similarly, using primers 5′-ttagatctgATGG GAAAAATCAAAAGAAG-3′ and 5′-gcggccgctTTAGGTGCTT CTCAAACGAG-3′ and inserting into the *Bgl*II/*Not*I sites in plasmid pESP-1 (Stratagene). The resulting plasmid, pESP-1-abp1, was transformed into strain SBP082996. In each case proper expression was verified by Western blotting on extracts from strains grown under repressing and nonrepressing conditions as described above. Cells from a midlog phase culture grown under repressing conditions were fixed by addition of one-tenth volume of fixation solution (11% formaldehyde, 0.1 M NaCl, 1 mM NaEDTA, 0.5 mM NaEGTA, 50 mM Tris-HCl, pH 8.0) followed by a 10-min incubation at 32°. The reaction was quenched with 120 mM glycine for 5 min at room temperature, and the cells were washed four times in extraction buffer (50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 5% Na-deoxycholate) and lysed by vortexing with glass beads (SAITOH *et al.* 1997). Chromatin DNA was sheared by sonication to an average length of 850 bp, and the glutathione S-transferase (GST)-tagged proteins were immunoprecipitated by incubation of 2.7×10^8 cell equivalents of chromatin with 11 mg of anti-GST polyclonal rabbit antibodies (product no. G-7781; Sigma, St. Louis) at 4° overnight, followed by capture of the immunocomplexes with Protein G-Sepharose beads for 2 hr at 4°. Immunoprecipitates were washed, bound chromatin was released, formaldehyde crosslinks were reversed by incubation at 65°, and the DNA was purified as previously described (BAUM and CLARKE 2000). Portions of total and immunoprecipitated DNA used for PCR amplification were adjusted for differences in copy number as follows: 1/15,000 and 1/100, respectively, for centromeric K repeat sequences AB (314 bp), HI (225 bp), and M (202 bp); 1/900 and 1/6, respectively, for central core c2-10/11 sequences (300 bp), and 1/1800 and 1/12, respectively, for the core-associated repeat (CAR; 286 bp), *ars1* (318 bp), *ars3002* (285 bp), *ars2004* (239 bp), *top2* (346 bp), *leu1* (315 bp), and *hyp* (272 bp) sequences. Products from limited PCR amplification (25–28 cycles) were electrophoresed on 2% NuSieve 3:1 agarose gels (FMC BioProducts, Rockland, ME) and visualized with ethidium bromide. Gels were digitally photographed and densitometry values were determined with an AlphaImager 2000 system (Alpha Innotech, San Leandro, CA).

Two-hybrid assay: Fusion proteins were created by insertion of the full-length coding sequence of the relevant fission yeast gene into either pGBDU-C1 (for GAL4 DNA binding domain fusions) or pGAD-C1 (for GAL4 transcription activation domain fusions; James *et al.* 1996). For *cbh2*$^+$, the coding sequence was amplified by PCR with primers 5′-ttggatccATGCC TCCATTGAG-3′ and 5′-gaagatctCACAATCGATCTGC-3′ and inserted into the *Bam*HI/*Bgl*II sites of the vectors. For *abp1*$^+$, the coding sequence was amplified by PCR with primers 5′-aaggatccGGAAAAATCAAAAGAAGAGCC-3′ and 5′-aaactgcag TTAGGTGCTTCTCAAACG-3′ and inserted into the *Bam*HI/*Pst*I sites in the vectors. For *cbh1*$^+$, the coding sequence was isolated as an *Xma*I fragment from plasmid pBKSII-cbh1 and inserted into the *Xma*I site of the vectors. pBKSII-cbh1 was created by PCR amplification using primers 5′-tccccccggg CCTCCTATACGCCAGGCCGTC-3′ and 5′-tccccccgggTCC GTAAGTGGATCACGCTAT-3′ and insertion into the *Xma*I site of pBKSII$^-$ (M. Baum and L. Clarke, unpublished data). Pairwise combinations of the resulting plasmids were cotransformed into strain PJ69-4A and the resulting transformants were tested on dropout media as described (James *et al.* 1996).

RESULTS

Sequence analysis of a new *S. pombe* CENP-B-related gene: The third fission yeast CENP-B homolog, *cbh2*$^+$, was identified by the *S. pombe* sequencing project as gene SPBC14F5.12c (EMBL accession no. AL023780). The predicted open reading frame is 514 amino acids, with no introns, producing an expected 59-kD protein. Cbh2p shows extensive homology along its entire length with the other two fission yeast homologs and to a lesser extent with human CENP-B (Figure 1). BESTFIT analysis revealed that Cbh2p is closest in homology to Abp1p, at 48% identity and 61% similarity (6 gaps), followed by Cbh1p at 42% identity and 53% similarity (6 gaps). Cbh2p is 31% identical and 40% similar to CENP-B (19 gaps). A BLAST search against the genome databases revealed that, like the other CENP-B family members, Cbh2p shares significant homology with several protein families of diverse function: the *jerky* family of mammalian proteins, which are involved in epileptic seizures (Toth *et al.* 1995); a protein of unknown function from *Arabidopsis thaliana* (EMBL accession no. AAD14510), Pdc2p and related transcription factors from budding yeasts (Hohmann 1993), and transposases from the *pogo* superfamily of transposable elements (Robertson 1996; Smit and Riggs 1996). Although the functional significance of the transposase homology is not known, it should be noted that Cbh2p contains four of five residues, including the presumptive catalytic site motif, known as "D,D35E", found to be invariant in the IS*630*-Tc1 transposases and highly conserved in other *pogo* superfamily members (Figure 1; Robertson 1996; Smit and Riggs 1996). The function of the acidic residue missing at position 325 might be carried out by the glutamate at position 327 in Cbh2p.

A *cbh2*Δ *abp1*Δ strain exhibits a severe growth defect: Previous studies showed that deletion of *cbh1*$^+$ results in no obvious growth defects, and deletion of *abp1*$^+$ gives a slow growth phenotype that is accentuated at extreme temperatures (Halverson *et al.* 1997; Baum and Clarke 2000). We found that deletion of the entire *cbh2*$^+$ open reading frame results in no deleterious effect on growth at 30° (Figure 2) or at any other temperature tested (18°, 25°, and 35°; data not shown). We also tested each of the pairwise double mutation combinations for growth at 30°. A *cbh2*Δ *cbh1*Δ strain was indistinguishable from wild type or from either single deletion strain (Figure 2). In contrast, a *cbh2*Δ *abp1*Δ strain showed a markedly slower growth rate than either single deletion strain, indicative of an enhancement of the slow-growth phenotype observed in the *abp1*Δ strain (Figure 2). This is similar to the phenotype previously described for the *cbh1*Δ *abp1*Δ strain (Figure 2; Baum and Clarke 2000), and suggests partial functional redundancy between Cbh2p and Abp1p. The triple deletion combination may be lethal, as we were unable to recover such a strain (data not shown).

To investigate the nature of the slow growth phenotype in the *cbh2*Δ *abp1*Δ strain, we compared fixed, DAPI-stained cells from this strain with wild type and the other single or double mutation combinations (Figure 3). The morphology and cell cycle distribution profiles of cells of *cbh2*Δ or *abp1*Δ single deletion strains were not significantly different from wild type, aside from a rare chain of three or four cells that failed to separate after nuclear division (Figure 3 and Table 2). Similar results were obtained with the *cbh2*Δ *cbh1*Δ strain (data not shown). In contrast, 12% of the *cbh2*Δ *abp1*Δ cells were multiseptated and often branched, resembling hyphae (Figure 3D and Table 2). These aberrant cells contained multiple nuclei and an occasional septated compartment lacking a nucleus. This suggests that the slow growth phenotype arises in part from a failure of some cells to separate after division, as has been described for the *cbh1*Δ *abp1*Δ strain (Baum and Clarke 2000).

A *cbh2*Δ *abp1*Δ strain exhibits mitotic defects: The analysis of DAPI-stained cells also revealed that among cells undergoing mitosis, the *cbh2*Δ *abp1*Δ strain showed a significant increase in aberrant segregation events when compared with either of the single deletion strains (Table 3). The most common defect observed was the asymmetric localization of two or more chromatin masses within ~8% of the cells, in contrast to normal divisions wherein the two chromatin masses of equal size move relatively symmetrically from the center of the cell (Figure 3E and Table 3). Mitotic *cbh2*Δ *abp1*Δ cells also showed an increased incidence of elongated chromatin masses (~4%; Figure 3F and Table 3), which may represent failure of chromosomes to separate or loss of chromosomes from the spindle, and an increased frequency of unequal distribution of chromatin (~3%; Table 3). These results indicate that the *cbh2*Δ *abp1*Δ strain has significant chromosome segregation defects, similar to those described in the *cbh1*Δ *abp1*Δ strain (Baum and Clarke 2000).

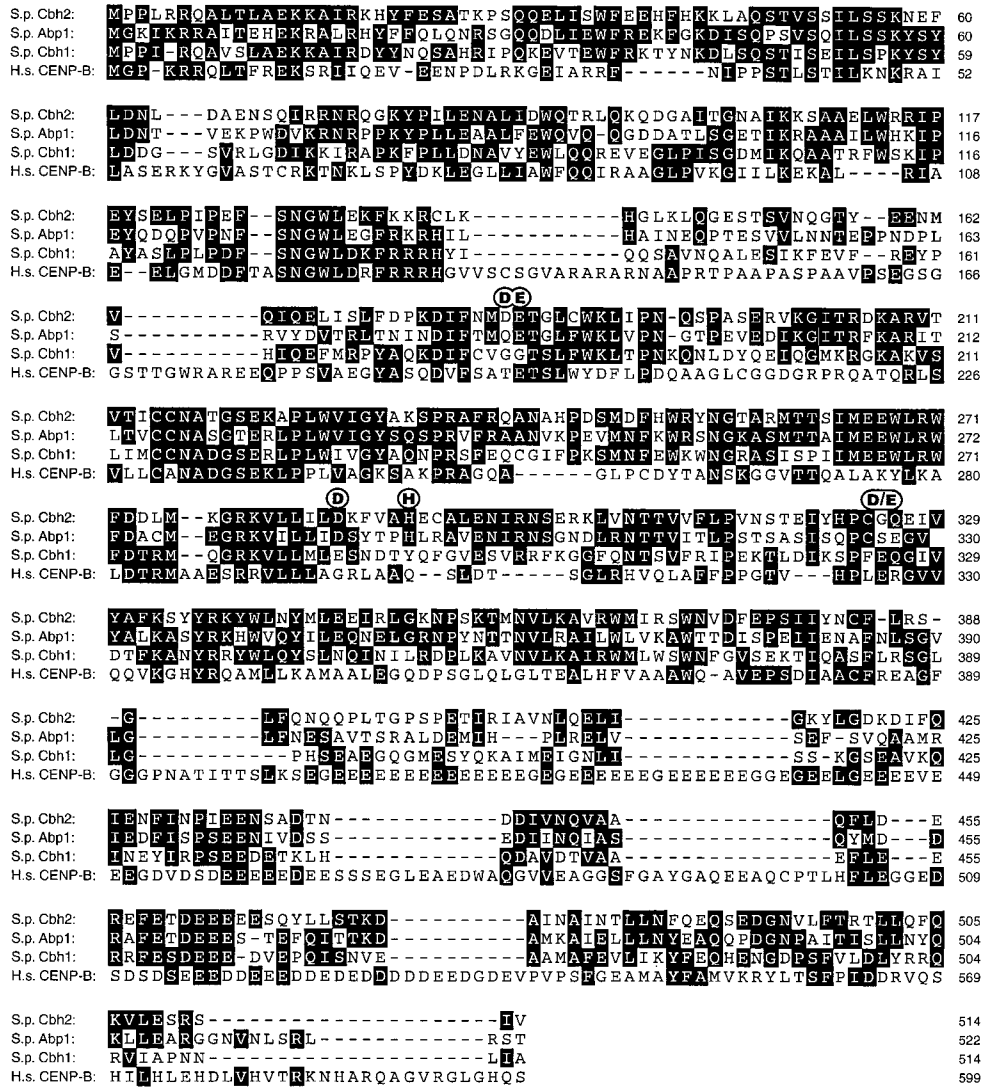

FIGURE 1.—Comparison of *S. pombe* CENP-B homologs with human CENP-B. Shaded boxes indicate amino acids identical in two or more proteins and dashes represent gaps. Only the residues found in CENP-B are boxed at positions where two pairs of proteins are identical. Residues that are invariant in IS*306*-Tc1 transposases and highly conserved in other *pogo* transposases are shown circled above the sequence alignment.

Both *cbh1*Δ and *abp1*Δ single deletion strains lose a 78-kb linear artificial minichromosome at moderately elevated frequencies, whereas the *cbh1*Δ *abp1*Δ strain was not testable due to its aberrant growth phenotype (HALVERSON *et al.* 1997; BAUM and CLARKE 2000). We constructed *cbh2*Δ, *cbh2*Δ *cbh1*Δ, and *cbh2*Δ *abp1*Δ strains bearing the same minichromosome and assessed them for minichromosome loss rate (Table 4). The *cbh2*Δ strain exhibited a moderate elevation (14-fold) in minichromosome loss when compared with a wild-type

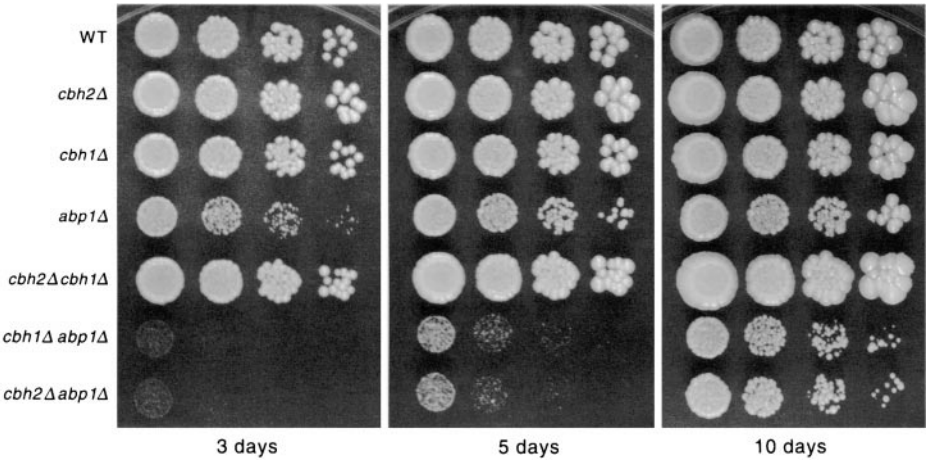

FIGURE 2.—Growth of *S. pombe* CENP-B homolog null deletion strains. Fivefold serial dilutions of log-phase cells from the following strains were spotted on yeast extract medium supplemented with adenine and leucine and incubated at 30° for the indicated time: Sp223 (WT), Spx34-6B (*cbh2*Δ), SBP072098-21B7 (*cbh1*Δ), SBP082996 (*abp1*Δ), Spx34-11A (*cbh2*Δ *cbh1*Δ), SBP072198-1C (*cbh1*Δ *abp1*Δ), and Spx33-5B (*cbh2*Δ *abp1*Δ).

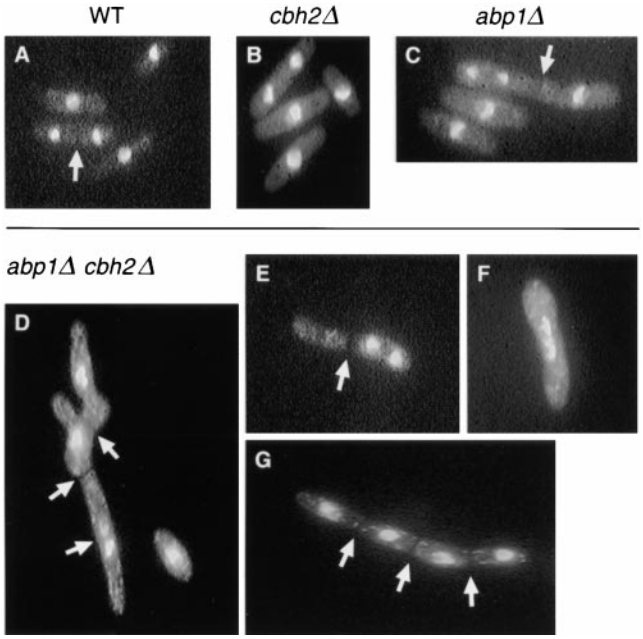

FIGURE 3.—*cbh2*Δ *abp1*Δ strains exhibit aberrant morphology and mitotic defects. Fixed DAPI-stained cells were examined by fluorescence microscopy at 120× magnification. Arrowheads indicate cell septa. (A and B) Examples of the predominantly normal cells from the wild-type control strain (Sp223) and the *cbh2*Δ strain (Spx34-6A). The *abp1*Δ strain (SBP082996) shows occasional defects such as the multinucleate cell in C. In contrast, cells from the *cbh2*Δ *abp1*Δ strain (Spx33-5B) were frequently multinucleate and branched (D), showed asymmetric localization of chromatin (E), stretched chromatin (F), or were multinucleate but not branched (G). The nucleus in the lower left corner of D may be "cut" (*c*ell *u*ntimely *t*orn) by the beginning of septum formation.

strain, and the *cbh2*Δ *cbh1*Δ double deletion combination did not dramatically increase the minichromosome loss rate over that of the *cbh2*Δ or *cbh1*Δ single deletion strains. In contrast, the *cbh2*Δ *abp1*Δ double deletion combination yielded a 68-fold increase in minichromosome loss rate. This elevated rate is likely to be an underestimate, since minichromosome loss events in the 12% of cells that are multiseptated (Table 2) would not be detected by the half-sectoring assay due to the presence of multiple nuclei. This suggests that the functional redundancy between Cbh2p and Abp1p involves cellular processes that affect chromosome segregation.

The phenotypic consequences of mutations that cause chromosome segregation defects are sometimes exacerbated by the presence of drugs that alter the mitotic spindle. We tested whether the single and double deletion strains exhibit altered sensitivity to the microtubule destabilizing drug thiabendazole (TBZ; Figure 4). Among the three single deletion strains, only *abp1*Δ showed a significant increase in sensitivity. The *cbh2*Δ *cbh1*Δ double deletion strain was not significantly hypersensitive to TBZ. In contrast, both the *cbh2*Δ *abp1*Δ and *cbh1*Δ *abp1*Δ double deletion strains exhibited hypersensitivity to TBZ, consistent with the increased frequency of mitotic errors in these strains. Under the conditions used, a *mad2*Δ control strain, which is defective in the spindle checkpoint (HE *et al.* 1997), exhibited only moderate TBZ sensitivity.

Cbh2p localizes to the nucleus: To determine the subcellular distribution of Cbh2p, we conducted indirect immunofluorescence assays. We constructed a strain containing an HA epitope-tagged version of Cbh2p expressed from the repressible *nmt* promoter on a plasmid. Cells grown under either repressing or nonrepressing conditions exhibited a similar punctate pattern when stained for Cbh2p; the more intense staining obtained under nonrepressing conditions is shown in Figure 5. The Cbh2p foci were almost exclusively contained within the nucleus and ranged in number from 1 to 4 distinguishable spots (mean = 2.4). This distribution pattern is consistent with a single spot on each native chromosome plus the minichromsome present in this strain, in an asynchronous culture (FUNABIKI *et al.* 1993). In general, interphase cells exhibited a more limited Cbh2p distribution that was located at the nuclear periphery, consistent with a centromere-specific distribution (Figure 5; FUNABIKI *et al.* 1993; SAITOH *et al.* 1997).

Cbh2p is present in centromeric heterochromatin: To

TABLE 2

Abnormal morphology in *cbh2*Δ *abp1*Δ strains

Strain	% branched or multiseptated	% interphase	% mitotic	Total no. of cells scored
WT	<0.5	25	75	202
*cbh2*Δ	2	26	72	148
*abp1*Δ	1.3	26	73	156
*cbh2*Δ *abp1*Δ	12	18	70	184

Fixed, DAPI-stained cells from strains Sp223 (WT), Spx34-6A (*cbh2*Δ), SBP082996 (*abp1*Δ), and Spx33-5 (*cbh2*Δ *abp1*Δ) were examined by fluorescence microscopy. Morphologically normal cells exhibiting a clearly defined sickle-shaped nucleus characteristic of interphase were scored as interphase; all other normal cells were classed as mitotic. The percentage of cells in each category is given and the total number of cells scored is shown in the last column.

TABLE 3

Mitotic defects are elevated in a *cbh2*Δ *abp1*Δ strain

Strain[a]	% normal	% unequal	% stretched	% asymmetric	% cut	Total no. of cells scored
WT	99.5	0.5	<0.5	<0.5	<0.5	203
*cbh2*Δ	99.3	0.7	<0.7	<0.7	<0.7	145
*abp1*Δ	92.2	<0.6	2.6	4.5	0.6	154
*cbh2*Δ *abp1*Δ	83.5	2.9	4.1	8.2	1.2	170

Fixed DAPI-stained cells were examined for the frequency of mitotic defects shown in Figure 3. Unequal, cells containing segregated chromatin of unequal intensity; stretched, cells with a longer chromatin mass than is observed in wild type; asymmetric, cells in which two or more chromatin masses were observed at one end of the cell (with or without a septum). The percentage of cells in each category is given and the total number of cells scored is shown in the last column.

[a] Strains are as described in Table 2.

test whether the observed Cbh2p distribution is localized to centromeric heterochromatin, we used a chromatin immunoprecipitation (ChIP) assay, which allows detection of both direct and indirect associations between proteins and specific DNA sequences *in vivo* (Orlando and Paro 1993; Hecht *et al.* 1996; Orlando *et al.* 1997). Briefly, formaldehyde crosslinking was used to "fix" protein-DNA and protein-protein interactions *in vivo*, and then isolated chromatin was sheared to a mean fragment size of ~850 bp. The resulting material was used in a standard immunoprecipitation reaction, the formaldehyde crosslinks were reversed, and DNA was purified for use in PCR reactions to test for the presence of specific sequences of interest.

*cbh2*Δ cells containing an N-terminal GST-fused version of Cbh2p under *nmt* promoter control were grown under repressing conditions and subjected to ChIP. Primers corresponding to centromeric sequences and to noncentromeric control sequences were used in PCR reactions with the anti-GST-precipitated material (Figure 6A). Reactions testing for the unique centromeric central core (c2-10/11), where the kinetochore is likely assembled (Polizzi and Clarke 1991), and the CAR, which is present in two copies, clearly indicated enrichment in the immunoprecipitated (+Ab) material (Figure 6B). In contrast, only very weak amplification was observed in three reactions targeting sequences within the high-copy centromeric K repeat, which together with the central core is sufficient to confer partial centromere activity on a plasmid (Baum *et al.* 1994). Since even the mock immunoprecipitation (−Ab) reactions sometimes produced weak amplification products, enrichment values were calculated and compared to those obtained from a control strain (Sp223) that lacks GST and thus indicates the level of nonspecific immunoprecipitation (Figure 6B). Only enrichment values greater than threefold above the corresponding control reactions were considered to be significant to ensure a strict standard for a clearly positive result. However, it should be noted that by any standard a negative result may arise from trivial factors such as lack of accesibility of the epitope and thus should be considered with caution. By these criteria, the results for the K repeat reactions were negative, as were those for the three single-copy noncentromeric control genes *top2, leu1*, and *hyp*, and the three different *ars* sequences (Figure 6B). Similar

TABLE 4

Loss of a linear minichromosome in *cbh2*Δ and double mutant strains

Strain	Relevant genotype	Total colonies assayed	Chromosome loss per division[a]	Fold increase
Spx34-10	WT	16,719	$5.7\ (\pm 1.8) \times 10^{-4}$	(1)
Spx34-6A	*cbh2*Δ	30,819	$7.8\ (\pm 3.1) \times 10^{-3}$	14
Spx34-13	*cbh1*Δ	2,357	8.9×10^{-3}	16
SBP082996	*abp1*Δ	13,049	$3.2\ (\pm 0.33) \times 10^{-3}$	6
Spx34-7A	*cbh1*Δ *cbh2*Δ	15,301	$1.2\ (\pm 0.49) \times 10^{-2}$	21
Spx33-5B	*cbh2*Δ *abp1*Δ	3,256	$3.9\ (\pm 0.97) \times 10^{-2}$	68
Spx40-E9[b]	*mis6-302*	10,536	4.9×10^{-3}	9
Spx47-1[b]	*mis6-302 cbh2*Δ	4,313	5.6×10^{-3}	10

[a] Results are given as mean frequency of half-sectored colonies. Standard error is shown in parentheses for values derived from three or more independent platings.

[b] *mis6-302* strains were tested at a low semipermissive temperature (23°).

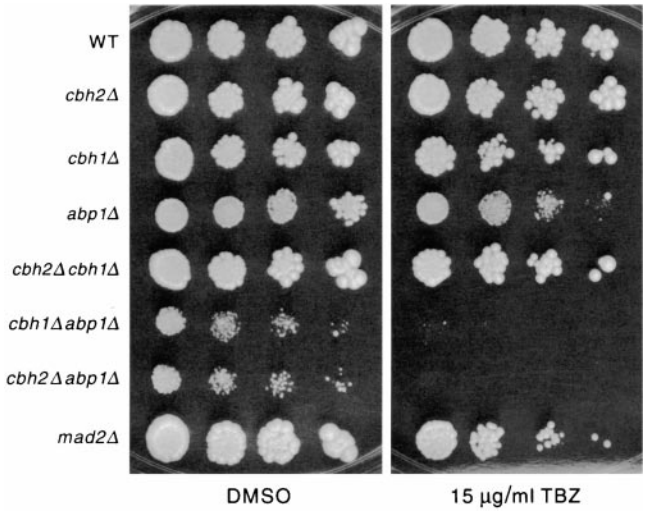

FIGURE 4.—Thiabendazole (TBZ) sensitivity of *S. pombe* CENP-B homolog null deletion strains. Fivefold serial dilutions of log-phase cells were spotted on yeast extract medium containing appropriate supplements and the indicated concentration of TBZ suspended in DMSO, or DMSO alone, and incubated at 30° for 7 days. Strains are as indicated in Figure 2 and Table 1.

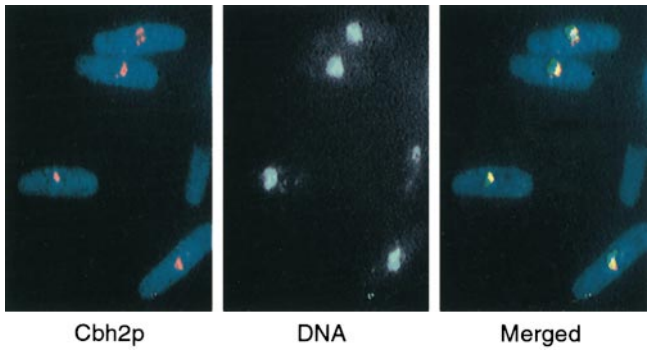

FIGURE 5.—Immunolocalization of Cbh2p. HA-tagged Cbh2p expressed from an *nmt* promoter on a plasmid in strain SBP32590 was visualized by indirect immunofluorescence microscopy at 120× magnification. False-color images of Cbh2p-HA detected by a fluorescein-conjugated secondary antibody (red), total DNA detected by DAPI staining (green), and the overlapping signal (yellow) are shown.

results were obtained from at least two independent PCR assays of two independent chromatin isolations. This pattern contrasts with previous results using the same assay with Cbh1p, which was observed to give at least a weak positive signal at each region tested (BAUM and CLARKE 2000). Thus it appears that Cbh2p is specifically associated with centromeric heterochromatin in and around the unique centromeric central core region.

Another fission yeast protein, Mis6p, is thought to localize exclusively to the central core region of centromeric heterochromatin (SAITOH *et al.* 1997). *mis-6*$^+$ is an essential gene, and a strain containing the conditional allele *mis6-302* exhibits altered chromatin at the central core and severe chromosome segregation defects when grown at a semipermissive temperature (TAKAHASHI *et al.* 1994; SAITOH *et al.* 1997). We tested whether *cbh2*$^+$ and *mis-6*$^+$ interact by assessing minichromosome loss in a *mis6-302 cbh2*Δ strain grown at a semipermissive temperature (Table 4). No significant difference was observed between the double mutant strain and a *mis6-302* strain, nor was any enhancement of the *mis6-302* temperature-sensitive growth phenotype observed (data not shown), indicating that *cbh2*$^+$ and *mis-6*$^+$ do not exhibit any obvious genetic interaction.

Chromatin localization of Abp1p: Since phenotypic analyses suggested that both Cbh1p and Cbh2p exhibit some degree of functional redundancy with Abp1p (this work and BAUM and CLARKE 2000), and since Abp1p binds tightly to centromeric central core and repeat sequences *in vitro* (HALVERSON *et al.* 1997; LEE *et al.* 1997; BAUM and CLARKE 2000), we asked if the *in vivo* chromatin distribution of Abp1p overlaps with that of either Cbh1p or Cbh2p. *abp1*Δ cells expressing an N-terminal GST-fused version of Abp1p under *nmt* promotor control were subjected to the ChIP assay as described above. Under these conditions, the Abp1p-GST fusion complemented the *abp1*Δ slow-growth phenotype (data not shown). GST-precipitated extracts gave weakly positive signals for both the central core, which is known to contain a strong *in vitro* binding site for Abp1p (BAUM and CLARKE 2000), and the core-associated repeat sequences (Figure 6B). Although these signals were weak, they were reproducibly stronger than those from the mock immunoprecipitation (−Ab) reaction in a minimum of two independent PCR reactions on two independent chromatin isolations and gave enrichment values five- to sixfold greater than those from the Sp223 control reactions (Figure 6B). This result is consistent with the observation that Abp1p binds to central core sequences *in vitro* (HALVERSON *et al.* 1997; BAUM and CLARKE 2000) and with the hypothesis that Abp1p and Cbh2p play redundant roles in the formation of centromeric heterochromatin. Abp1p also showed weak but significant enrichment (three- to fivefold over the control) for the single-copy gene sequences used as controls. The presence of Abp1p at numerous genomic sites is in agreement with its high abundance in chromatin (BAUM and CLARKE 2000). The multicopy K repeat reactions were reproducibly negative by the criterion for significance used here, which was not expected, given the presence of sites to which Abp1p binds *in vitro* that are within 850 bp of the HI and M primers (NGAN and CLARKE 1997). This may reflect a limitation of the ChIP assay, as it is seems likely that in formaldehyde-fixed heterochromatin, some DNA binding proteins would be masked by the presence of other proteins and thus would be less accessible to antibody precipitation.

Abp1p was initially identified by its ability to bind *in*

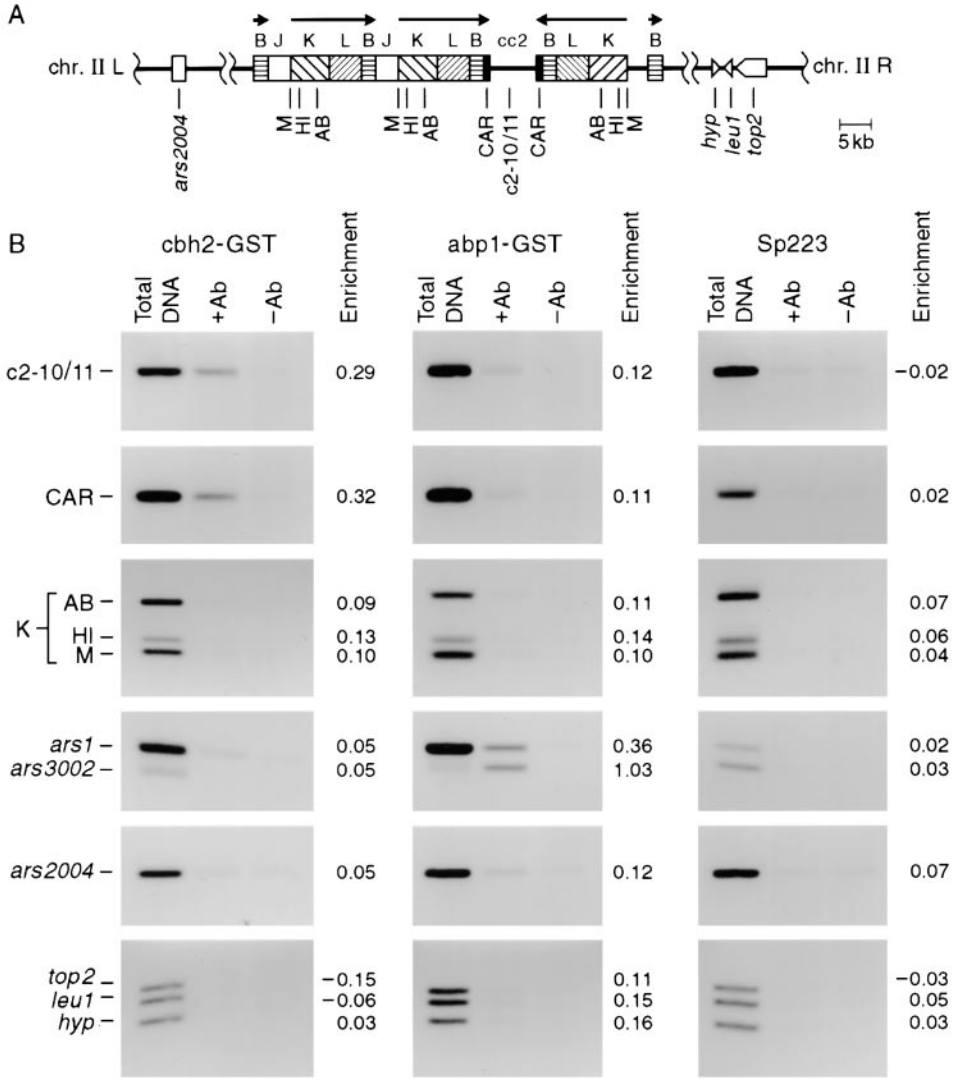

FIGURE 6.—Chromatin immunoprecipitation of Cbh2p and Abp1p. GST-tagged Cbh2p and Abp1p were each expressed from an *nmt* promoter on a plasmid in strains JIY49 and SBP082996, respectively. (A) Map of *S. pombe* centromere II and surrounding sequences, indicating regions tested for chromatin localization of GST-fused Cbh2p and Abp1p. Fragment c2-10/11 is unique to the *cen2* central core (cc2), and fragment CAR, within the *cen2* core-associated repeat, is present in two copies (solid boxes). Fragments AB, HI, and M are within the centromeric K repeat, which in addition to the three copies shown in *cen2* is present in 14–15 additional copies in the other two centromeres. The *ars2004* fragment is located at least 90 kb from *cen2*. The single-copy genes *top2*, *leu1*, and the hypothetical open reading frame *hyp* [SBPC1A4.01 (accession no. AL031174)] are located >300 kb from *cen2*. Arrows above the line indicate location and orientation of the repeated centromeric sequences. (B) Results of PCR analysis of ChIP material. Reverse images of ethidium bromide-stained gels are shown. Primer sets are indicated at left; the *ars1* (318 bp) and *ars3002* (285 bp) fragments are located on chromosomes 1 and 3, respectively. Total DNA is the product of the ChIP procedure prior to the immunoprecipitation step, and +Ab and −Ab indicate actual and mock (beads only) immunoprecipitations, respectively. Enrichment is calculated by subtracting the −Ab signal from the +Ab signal and dividing by the total DNA signal as determined by densitometry. Enrichment is a relative measure, because total DNA samples were diluted 150-fold relative to the +Ab and −Ab reactions prior to PCR. Strain Sp223 does not express a GST protein and thus serves as a control for nonspecific protein precipitation by the antibody.

vitro to a region from *ars3002*, which led to its designation as *A*RS *b*inding *p*rotein 1 (MURAKAMI *et al.* 1996). We used the ChIP assay to determine if Abp1p interacts *in vivo* with *ars3002*, *ars2004*, or *ars1*, all of which are known to function as replication origins on the chromosome (DUBEY *et al.* 1994; OKUNO *et al.* 1997; GOMEZ and ANTEQUERA 1999). Abp1p gave strong positive signals with *ars3002* and *ars1*, indicating that it is present at these replication origins *in vivo* (Figure 6B). The *ars2004* sequences were not significantly enriched in this experiment when compared to controls. Thus Abp1p does interact with at least two *ars* regions *in vivo*.

Interactions among CENP-B homologs: Mammalian CENP-B acts as a dimer, and both fission yeast Abp1p and Cbh1p migrate in gel filtration or sedimentation experiments at a size consistent with dimer formation (LEE *et al.* 1997; NGAN and CLARKE 1997). The results of the ChIP assay described above indicating that the fission yeast CENP-B homologs have some overlap in their *in vivo* chromatin distribution raises the possibility that these proteins may in some instances form heterodimers. To test this possibility, we constructed plasmids to test for homodimer and heterodimer formation in a budding yeast two-hybrid assay (JAMES *et al.* 1996). Experiments with Cbh1p showed neither self interaction nor interactions with either Cbh2p or Abp1p (data not shown). In contrast, both Cbh2p and Abp1p were capable of self interaction in the two-hybrid assay, but heterologous interactions were not detected (Figure 7). The negative result in the heterologous interaction test was verified by swapping the GAL4 DNA binding domain and transcription activation domain fusions in these plasmids (data not shown). Thus the co-localization to some regions of centromeric heterochromatin

FIGURE 7.—Two-hybrid test for protein-protein interactions. Left, the identity of the *GAL4* binding domain fusion (top) and the *GAL4* transcription activation domain fusion (bottom) tested in each sector. Strains of PJ69-4A bearing the indicated plasmids were spread on selective media lacking histidine or adenine and incubated at 30° for 3 days. Growth results from transcription of *GAL2-ADE2* and *GAL1-HIS3* via recruitment of the transcription activation domain fusion to the promotor via protein-protein interactions with the DNA binding domain fusion.

is most likely the result of Cbh2p and Abp1p binding independently, probably as homodimers.

DISCUSSION

We have presented the initial characterization of a third fission yeast homolog of CENP-B, Cbh2p. Like its mammalian counterpart, Cbh2p appears to localize exclusively to centromeric chromatin, yet it is dispensable for growth. The only defect observed in the *cbh2*Δ strain was a moderately elevated frequency of minichromosome loss. However, in the absence of another homolog of CENP-B, Abp1p, Cbh2p appears to be critical for growth and for chromosome segregation, as the double deletion strain exhibited severe defects in these processes. Since Abp1p also localizes to centromeric chromatin, the simplest explanation for this result is that Cbh2p and Abp1p play redundant roles in the establishment of a functional centromere. *cbh2*$^+$, however, is not functionally redundant with *cbh1*$^+$ (encoding another CENP-B homolog in fission yeast) as deletion of both genes results in little alteration in growth or minichromosome loss rate relative to single deletion strains, and deletion of either one is sufficient to cause severe growth defects in combination with *abp1*Δ. These results can be interpreted in terms of two parallel pathways leading to proper chromosome segregation, one of which involves both Cbh2p and Cbh1p, and the other involving Abp1p. However, this pathway model may be an oversimplification, since these proteins exhibit partially overlapping localization patterns and since chromatin proteins can participate in multiple pathways. For example, both Cbh1p and Abp1p clearly localize to noncentromeric as well as centromeric chromatin, suggesting that part of the growth defect observed in the *cbh1*Δ *abp1*Δ double mutant strain might be indicative of a noncentromeric function for these two proteins. Thus it appears that in fission yeast a family of CENP-B-like proteins has evolved to perform a complex set of partially overlapping functions in both centromeric and noncentromeric chromatin.

Cbh2p shows sequence conservation with Abp1p, Cbh1p, and CENP-B over its entire length (Figure 1). Because this conservation is extensive in the N terminus, which contains the DNA-binding domain of CENP-B, and because Abp1p and Cbh1p both are known to bind directly to DNA, it is likely that Cbh2p binds directly to DNA as well. Several *in vitro* centromere-DNA binding activities from fission yeast chromatin extracts are not dependent on Abp1p or Cbh1p (Lee *et al.* 1997; Ngan and Clarke 1997). The sequence homology between the three fission yeast homologues and CENP-B is less dramatic at the C terminus, the location of the CENP-B dimerization domain (Choo 1997). Nonetheless, Abp1p and Cbh1p fractionate at sizes consistent with multimer formation in gel-filtration or sedimentation experiments (Halverson *et al.* 1997; Lee *et al.* 1997), and both Abp1p and Cbh2p homodimerized in the budding yeast two-hybrid assay described here. Thus it appears likely that all three fission yeast homologs exhibit the DNA binding function, and at least two of the three exhibit the dimerization function of CENP-B, which suggests that these proteins are involved in the packaging of DNA into chromatin.

Taken together, the co-localization of Cbh2p and Abp1p to central core chromatin, the mitotic defects in the double deletion strain, and the likely conservation of the biochemical functions required for packaging chromatin suggest that these two proteins cooperate to establish proper chromatin structure at the centromere. It is not clear, however, how disruption of centromeric chromatin alone could lead to the aberrant cell morphology observed in the *cbh2*Δ *abp1*Δ strain. This unusual morphology, in which some cells apparently proceed through mitosis and form a septum, but fail to separate completely, might be the result of alterations in cell cycle regulation, such that positive signaling of the cell separation machinery is disrupted. Previous work demonstrated that Cbh1p exhibits a similar relationship to Abp1p as Cbh2p: the *cbh1*Δ strain has no defect aside from a moderate elevation in minichromosome loss, while the absence of both proteins leads to profound defects that are similar to the defects in the *cbh2*Δ *abp1*Δ strain described here (Baum and Clarke 2000). Since both Cbh1p and Abp1p may localize to euchromatic regions as well as to centromeric chromatin, this result was explained by proposing a redundant role for these proteins in establishment of chromatin configurations needed for both centromere function and for proper expression of some hypothetical gene(s), the disruption of which leads directly or indirectly to the observed morphological phenotype (Baum and Clarke 2000). In the case of Cbh2p, this explanation is less appealing, because of its apparent centromere specific-

ity. However, it should be noted that both the indirect immunofluorescence and the ChIP assays, which suggest centromere-specific binding of Cbh2p, cannot completely rule out the possibility that Cbh2p is present in some euchromatin as well. Thus it is possible that the phenotype of the *cbh2*Δ *abp1*Δ strain, too, is the result of disruption of both a centromere function and some aspect of euchromatin function.

*abp1*Δ strains exhibit a slow-growth phenotype that is more severe than would be expected solely on the basis of the mitotic defects observed in these strains (Halverson *et al.* 1997; Baum and Clarke 2000; this work). Since both Abp1p and Cbh1p are present at replication origins, as revealed by *in vitro* binding studies (Murakami *et al.* 1996; Lee *et al.* 1997) and the ChIP assay described above (Baum and Clarke 2000), it is possible that these proteins play a role in DNA replication. However, FACS analysis of cells from *abp1*Δ, *cbh1*Δ, and *abp1*Δ *cbh1*Δ strains did not reveal a significant replication block, and a subtle role for Abp1p in DNA replication would not easily explain the magnitude of the slow growth phenotype in the *abp1*Δ strain (Halverson *et al.* 1997; Baum and Clarke 2000). A small replication defect could possibly result in the mitotic errors (minichromosome loss and stretched or unequal segregation of bulk chromatin) that were observed at low frequency in the *abp1*Δ strain and at higher frequency in the *cbh2*Δ *abp1*Δ and *cbh1*Δ *abp1*Δ double mutant strains. For example, the occasional failure to complete replication of a chromosome, if not detected or repaired, could result in aberrant segregation. The simplest hypothesis, however, is that the mitotic defect in the *cbh2*Δ *abp1*Δ strain is the result of a centromere defect, rather than a replication problem, since Cbh2p did not show unambiguously positive signals for the replication origins in the ChIP assay, whereas both proteins were detected in centromeric heterochromatin. Also, the enhanced sensitivity to the microtubule-destabilizing drug thiabendazole in both *cbh2*Δ *abp1*Δ and *cbh1*Δ *abp1*Δ strains is most easily explained by a synergy between a centromere defect and a disruption of the mitotic spindle by the drug.

The sequence conservation between CENP-B family members and transposases from the *pogo* superfamily has led to the hypothesis that CENP-B (and related proteins) may retain transposase-like nicking activities that in turn might influence the evolution and maintenance of repetitive centromeric DNA through recombinational mechanisms (Kipling and Warburton 1997). Since Cbh2p is the only fission yeast CENP-B family member to have retained most, or possibly all, of the D,D35E motif thought to define the catalytic site of *pogo* superfamily transposases, it will be of interest to determine whether Cbh2p possesses any DNA nicking activities. The observation that Cbh2p appears to bind only to the unique central core and adjacent core-associated repeat sequences, which are present in only one and two copies, respectively, suggests that Cbh2p might not play a role in maintenance of the more highly repetitive centromeric sequences that surround the central core, such as the K repeat. Variation in the number of these more highly repeated sequences at fission yeast centromeres has been observed in closely related lab strains, and that variation was attributed to homologous recombination mechanisms (Steiner *et al.* 1993). A direct test of the effect of mutations in the fission yeast CENP-B family members on repeat stability would therefore be complicated by the possibility that mutation of these genes could alter centromeric chromatin structure, which in turn could alter homologous recombination frequency independent of any putative transposase activity.

In fission yeast, the three CENP-B family members described to date have apparently evolved to perform separate, but partially overlapping, functions that contribute to chromosome segregation fidelity. Each of the three proteins has a somewhat different localization pattern, and the null mutant strains have distinguishable phenotypes. The presence of multiple functionally redundant CENP-B family members in fission yeast implies that there may be additional homologs in mammalian systems that are at least partially functionally redundant with CENP-B. However, none of the mammalian CENP-B-related proteins described in the literature to date, aside from the canonical CENP-B, have characteristics consistent with a centromere function, nor is the degree of sequence conservation between those proteins and CENP-B nearly as high as that observed among the fission yeast family members ($\geq$40% identity). Furthermore, a BLAST search of the nearly complete public human genome database, including unfinished sequences, did not reveal any new candidate genes with sequence identity to CENP-B >30% (data not shown). As the public human genome database is updated, additional CENP-B family members may be identified that warrant testing for functional redundancy with CENP-B. Alternatively, the evolution of regional centromeres may have occurred such that the divergence between fission yeast and mammals took place prior to the development of multiple and functionally redundant CENP-B family members in yeast.

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