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A Maize Homolog of Mammalian CENPC Is a Constitutive Component of the Inner Kinetochore

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Genes for three maize homologs (*CenpcA*, *CenpcB*, and *CenpcC*) of the conserved kinetochore assembly protein known as centromere protein C (CENPC) have been identified. The C-terminal portion of maize CENPC shares similarity with mammalian CENPC and its yeast homolog Mif2p over a 23–amino acid region known as region I. Immunolocalization experiments combined with three-dimensional light microscopy demonstrated that CENPC is a component of the kinetochore throughout interphase, mitosis, and meiosis. It is shown that sister kinetochore separation occurs in two discrete phases during meiosis. A partial separation of sister kinetochores occurs in prometaphase I, and a complete separation occurs in prometaphase II. CENPC is absent on structures known as neocentromeres that, in maize, demonstrate poleward movement but lack other important features of centromeres/kinetochores. CENPC and a previously identified centromeric DNA sequence interact closely but do not strictly colocalize on meiotic chromosomes. These and other data indicate that CENPC occupies an inner domain of the maize kinetochore.

INTRODUCTION

Centromeres and their associated organelles, known as kinetochores, are responsible for key aspects of chromosome movement, sister chromatid cohesion, and cell cycle regulation (Allshire, 1997; Choo, 1997a; Nicklas, 1997). In animals, the varied functions of the centromere/kinetochore complex can be at least partially attributed to well-defined ultrastructural subdomains. For example, the microtubule-based motor protein CENPE (for centromere protein E) is found in the outer kinetochore plate, a protein thought to sense tension at the kinetochore (recognized by the 3F3/2 antibody) is localized to the interzonal region, and the kinetochore assembly proteins CENPA and CENPC are localized to the inner kinetochore plate (Campbell and Gorbsky, 1995; Pluta et al., 1995; Cooke et al., 1997; Warburton et al., 1997). In contrast, plant kinetochores are poorly characterized. Plant kinetochores lack the plate structure characteristic of animal kinetochores (Baskin and Cande, 1990), and the biochemical makeup of the plant kinetochore is essentially unknown (Richards and Dawe, 1998). Little is known overall about the evolutionary conservation of higher eukaryotic kinetochores or, more specifically, how plant kinetochores generate and regulate chromosome movement.

Among the almost 40 kinetochore proteins identified in mammals and fungi, only a handful are thought to be evolutionarily conserved and essential for kinetochore function (Choo, 1997a). One such kinetochore protein is mammalian CENPC. Its yeast homolog is Mif2p. The similarity between CENPC and Mif2p is significant but limited to two small blocks of amino acids (Brown, 1995; Meluh and Koshland, 1995). Region I consists of 23 amino acids with 43% identity to human CENPC, and region II consists of 52 amino acids with 29% identity to human CENPC (these regions have also been called block 2 and block 3; Brown, 1995). Within each region is a known *mif2* temperature-sensitive mutation, suggesting that the conserved regions represent functional domains (Brown et al., 1993; Brown, 1995). Both CENPC and Mif2p have definite DNA binding characteristics, although no specific DNA binding site has been demonstrated (Sugimoto et al., 1994; Yang et al., 1996; Meluh and Koshland, 1997; Sugimoto et al., 1997). Perhaps the most striking homology is in the phenotypes of *cenpc* and *mif2* mutants, both of which display mitotic delay and aberrant chromosome segregation (Brown et al., 1993; Fukagawa and Brown, 1997; Kalitsis et al., 1998).

The necessity of CENPC for chromosome segregation is further illustrated by studies of structurally aberrant chromosomes with additional or novel centromeres. Many stable

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translocations that contain one inactive and one active centromere exist in humans. Whereas CENPB may be present at either the active or inactive centromeres, CENPC is consistently present only at the active centromere (e.g., Sullivan and Schwartz, 1995). CENPC is also present on human chromosome fragments known as marker chromosomes (Choo, 1997b). A number of marker chromosomes have lost their natural centromeres and are transmitted by "neocentromeres," which are centromeres that have arisen in novel positions and lack most or all of the DNA sequences found at normal centromeres. Despite their unusual structure and position, all human neocentromeres analyzed to date contain CENPC (Depinet et al., 1997; du Sart et al., 1997).

Although the exact function of CENPC remains unknown, it is likely that CENPC functions early in the cell cycle during kinetochore assembly. Injected anti-CENPC antibodies delay metaphase for several hours when injected into interphase cells but have no significant effect when injected after the initiation of prophase (Tomkiel et al., 1994). Similarly, CENPC mRNA and protein levels are highest during the G_1 phase of the cell cycle and drop off during S and subsequent phases (Knehr et al., 1996). Also consistent with a role in initiating assembly (and its DNA binding role) is the fact that CENPC localizes close to the centromeric DNA at the inner kinetochore plate (Saitoh et al., 1992). CENPC may contribute to centromere specification by establishing kinetochore boundaries and/or mediating the assembly of additional kinetochore proteins (Tomkiel et al., 1994).

Here, we report the identification and characterization of the maize CENPC protein and demonstrate immunocytochemically that it is a constitutive kinetochore protein. The CENPC antibodies provide a novel marker for addressing basic questions about maize kinetochore structure and function at all stages of the mitotic and meiotic cell cycles. In one set of studies, we used maize CENPC antibodies to provide a comprehensive, three-dimensional description of meiotic sister kinetochore separation during meiosis. The data suggest that kinetochore separation during meiosis is a biphasic process that is initiated in metaphase I and is completed in prometaphase II. We also used immunolocalization to determine whether CENPC is present at neocentromeres. In maize, neocentromeres are observed only in the presence of abnormal chromosome 10 and are composed of heterochromatic knobs that move rapidly poleward by interacting tangentially with spindle microtubules (Rhoades, 1952; Peacock et al., 1981; Yu et al., 1997). Our data support the previous suggestion that neocentromeres are specialized for poleward movement and may differ significantly from the more complex kinetochores (Yu et al., 1997). Finally, we conducted high-resolution light microscopy to determine whether maize CENPC localizes to an inner domain of the kinetochore. As a marker for the inner kinetochore, we used the cereal centromeric DNA element identified by Jiang and co-workers (1996). The data indicate that as in animals, there is a close interaction between CENPC and the centromere.

RESULTS

Identification and Characterization of CENPC

In previous sequence analyses of human, mouse, and sheep CENPC as well as yeast Mif2p, a conserved 23–amino acid sequence known as region I has been identified (Brown, 1995; Meluh and Koshland, 1995). This short peptide sequence was used in a BLAST (Atschul et al., 1990) search of an expressed sequence tag (EST) database containing 200,000 sequences (compiled by Pioneer Hi-Bred). Two partial cDNAs were initially identified that differed in length by 5 bp. The longest cDNA (CTSBB17) was 725 bp long. As shown in Figure 1, the CTSBB17 cDNA detected an ~2.7-kb transcript on a maize RNA gel blot.

To identify additional 5′ *Cenpc* sequence, the CTSBB17 cDNA was used as a probe to screen two different cDNA libraries. One cDNA was isolated from each library, with the longer one (CENPCcDNA4) being 1315 bp in length. An additional six ESTs were subsequently identified by using the CENPCcDNA4 DNA sequence to once again search the EST database. The longest cDNA identified in this way was CDCAG84, which is 1611 bp long. Table 1 describes each cDNA with respect to its length and the source of the mRNA

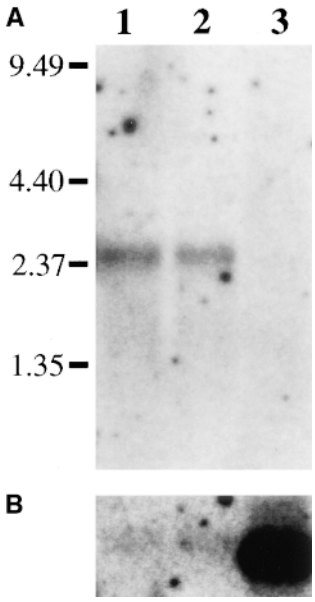

Figure 1. RNA Gel Blot Analysis of the *Cenpc* Transcript.

Lane 1 contains young tassel poly(A)$^+$ RNA; lane 2 contains young seedling poly(A)$^+$ RNA; and lane 3 contains mature pollen poly(A)$^+$ RNA. Approximate RNA sizes in kilobases are indicated at left.
(A) Probed with the CTSBB17 cDNA.
(B) The loading control. The blot in **(A)** was reprobed with a maize actin clone.

Table 1. *Cenpc* cDNAs

cDNA	Locus	Length (kb)	Tissue Source
CTSBB17	*CenpcA*	0.7	Young tassels; 0.1 to 1.4 cm
CHSTM64	*CenpcA*	0.7	Seedlings; recovering from heat shock
CENPCcDNA3	*CenpcA*	0.8	Shoot meristems and leaf primordia from 3- to 12-day-old plants
CENPCcDNA4	*CenpcA*	1.3	Leaves from 2-week-old plants
CNDAC35	*CenpcA*	1.5	Germinating seeds; 2 and 3 days old
CDPER72	*CenpcB*	0.2	Embryos; 13 days after pollination
CSCAD58	*CenpcB*	0.4	Cultured shoots
CZABB82	*CenpcB*	1.5	Anthers; prophase I
CDCAG84	*CenpcB*	1.6	Embryos; 15 days after pollination
CXMAD86	*CenpcC*	1.4	Anthers; prophase I

used for its synthesis. As can be seen, *Cenpc* cDNAs were identified in a wide variety of tissues, ranging from young embryos to prophase I–staged anthers. In addition, our RNA gel blot analysis indicated that the 2.7-kb band exists in young seedling and tassel tissue (Figure 1A, lanes 1 and 2) but not in mature pollen (Figure 1A, lane 3). Together, these data suggest that *Cenpc* mRNA is expressed in most maize tissues but may not be expressed in all cells or all stages of the cell cycle.

Among the collection of 10 cDNAs were representatives from three different loci: *CenpcA* (five cDNAs), *CenpcB* (four cDNAs), and *CenpcC* (one cDNA) (Table 1). A comparison of the three genes is shown in Figure 2. *CenpcA* and *CenpcB* are 83% identical, with single-nucleotide changes as well as small insertion/deletion polymorphisms throughout the available sequence. In one *CenpcB* cDNA (CZABB82), there is a 36-bp deletion relative to *CenpcA*; in the other (CDCAG84), the 36-bp deletion is replaced with a 129-bp insertion. The insertion is AT rich and bordered by close matches to known monocot splice site consensus sequences, suggesting that it is an unspliced intron (AG↓GCATGT at the 5′ end and TGCAG↓GA at the 3′ end; Filipowicz et al., 1994). A cytosine in the putative 5′ splice junction does not fit the consensus and may have caused the aberrant splicing. The insertion contains an in-frame stop codon, suggesting that the translated product is not functional. *CenpcA* and *CenpcC* are 99.9% identical except in the C-terminal coding sequence and 3′ untranslated region: here, the two sequences diverge completely (discussed below). The predicted amino acid sequences for the partial cDNAs indicate 82% similarity and 78% identity between CenpcA and CenpcB, 80% similarity and 76% identity between CenpcB and CenpcC, and 96% similarity and 95% identity between CenpcA and CenpcC. The fact that the three *Cenpc* genes have distinctive 3′ untranslated sequences (with ⩽70% sequence identity) allowed us to generate gene-specific probes (Figure 2A). As shown in Figure 2C, each of the probes produced a different hybridization pattern when used on maize genomic DNA blots. *CenpcB* is a single-copy gene, whereas there are at least two copies each of *CenpcA* and *CenpcC.*

The BLAST algorithm (Atschul et al., 1990) was used to search the public databases for proteins similar to the C-terminal

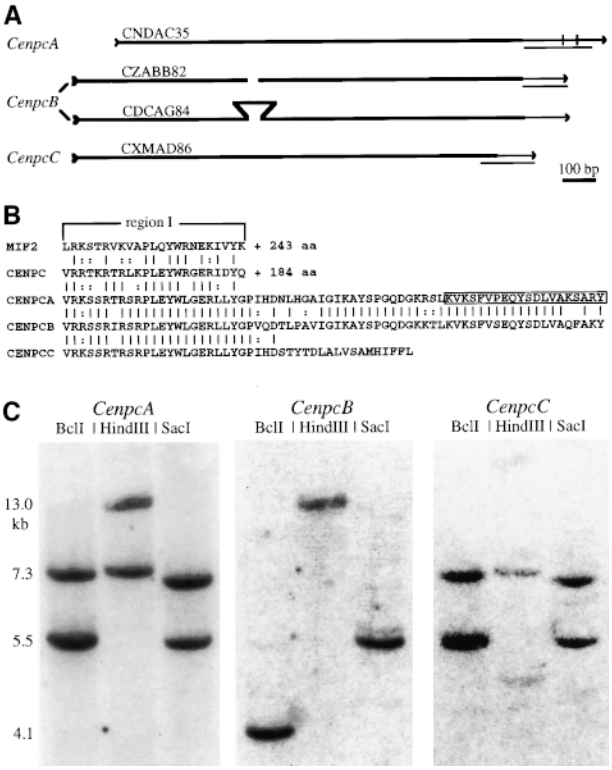

Figure 2. Comparison of *CenpcA*, *CenpcB*, and *CenpcC.*

(A) Gross structure of four partial cDNAs. Two clones of *CenpcB* are shown that differ by a 129-bp insertion. The coding sequence is shown with boldface lines. Vertical lines in the 3′ untranslated region of *CenpcA* identify two other polyadenylation sites identified among the available *CenpcA* cDNAs. Lines beneath the 3′ untranslated region sequences indicate the positions of gene-specific probes used in **(C)**.

(B) Predicted amino acid (aa) sequence of the conserved region I of Mif2p and human CENPC compared with the homologous regions of CENPCA, CENPCB, and CENPCC. The C-terminal 20 amino acids of CENPCA (boxed) were used to generate anti-CENPC antibodies. The amino acids connected by colons have similar properties; those connected by vertical lines are identical.

(C) DNA gel blot analysis of maize genomic DNA from the inbred B73. Three sets of identical restriction digests (BclI, HindIII, and SacI) were electrophoresed on the same gel, blotted together, and then separately probed with the gene-specific probes indicated in **(A)**. Numbers at left indicate molecular length markers in kilobases.

419 amino acids of *CENPCA* (encoded by CNDAC35). The top four matches were to the human, sheep, mouse, and chicken CENPC proteins, respectively. The yeast Mif2p protein was the ninth-highest scoring match. Further alignments of CENPCA with human CENPC and yeast Mif2p identified significant similarity only in the region previously identified as region I. We did not detect any similarity to the region II sequence, which lies ⩾128 amino acids downstream of region I in CENPC and Mif2p (Brown, 1995; Meluh and Koshland, 1995). The sequence distal to region I may not be required for CENPC function at all, because CENPCA/B and CENPCC diverge completely three amino acids downstream of region I (Figure 2B). The possibility remains that a sequence homologous to region II is encoded by the uncharacterized 5′ portion of *Cenpc.*

To prepare antibodies against the CENPC protein product, we injected a multiple antigenic peptide (MAP) derived from the C-terminal 20 amino acids of the predicted CENPCA protein into rabbits (see Figure 2B). The resulting antibodies were affinity purified against the CENPCA MAP and used on protein blots. As shown in Figure 3, anti–CENPCA MAP antibodies recognized the bacterially expressed protein product from a thioredoxin–CENPCA fusion protein as well as a 129-kD protein in maize tissue extracts (the ~39-kD CENPCA fusion protein was constructed with the partial cDNA CTSBB17). The 129-kD product was most abundant in extracts from young ear tissue but was also observed in extracts from tassel and root tips (data not shown). Because there is no similarity between the C-terminal 20 amino acids of CENPCA and CENPCC (Figure 2B), these antibodies are presumed not to identify CENPCC. The anti–CENPCA MAP antibodies may or may not identify CENPCB, which differs from CENPCA by three of the 20 C-terminal amino acids (Figure 2B). We refer to the antibodies as "anti-CENPC" antibodies, with the understanding that the antiserum probably only identifies a subset of the total CENPC proteins.

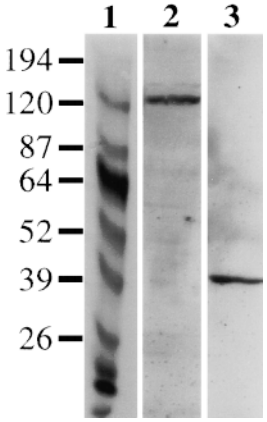

Figure 3. Protein Gel Blot Analysis Using Affinity-Purified Anti-CENPC Antibodies.

Lane 1 contains size standards; lane 2 contains protein extract from young maize ear tissue; and lane 3 contains the bacterially expressed thioredoxin–CENPCA fusion protein (from pThioCTSBB17; see Methods). All lanes are from the same gel. Approximate sizes in kilodaltons are indicated at left.

The 129-kD apparent molecular mass of maize CENPC is between the apparent molecular masses of human CENPC (140 kD) and Mif2p (94 kD) (Saitoh et al., 1992; Meluh and Koshland, 1997). It is interesting that for both human CENPC and Mif2p, the apparent molecular masses are significantly greater than the predicted molecular masses, which are 107 and 62.5 kD, respectively. A similar discrepancy is likely to exist for maize CENPC, because the mRNA is only ~2.7 kb. Indeed, our data suggest that the C-terminal portion of maize CENPC migrates aberrantly in SDS gels. The predicted molecular mass of the thioredoxin-partial CENPC fusion protein is 32.4 kD, yet the observed molecular mass was ~39 kD (Figure 3, lane 3; not shown is the thioredoxin moiety alone that migrates at its predicted molecular mass of 15 kD).

Immunolocalization of CENPC to Interphase and Mitotic Kinetochores

Three-dimensional light microscopy and affinity-purified anti-CENPC antibodies were used to study the subcellular localization of CENPC. CENPC staining was observed in all cells of the maize root tip, as illustrated in Figure 4. In interphase cells (Figure 4A), the average number of spots per nucleus was 16 ± 3, with a low of 10 and a high of 21 ($n = 27$). These quantitative data are consistent with the fact that $2n = 20$ in maize; the fact that only 16 spots were observed on average can be explained as a result of random centromere–centromere associations, which have been observed at early prophase in maize meiocytes (e.g., Gillies, 1981). By analyzing the nuclei for 4′,6-diamidino-2-phenylindole (DAPI) staining intensity, we also were able to differentiate the cells in G_1 from those in G_2 (see Methods). Although CENPC staining was apparent in all cells, the staining in the G_2 phase was generally more intense than in G_1, suggesting that CENPC is gradually incorporated into kinetochores during interphase.

Strong CENPC staining was also observed in the centromeric regions during mitotic prophase (Figure 4B), metaphase (Figure 4C), and anaphase (Figure 4D) chromosomes. As expected for a kinetochore protein, CENPC staining coincided with the ends of thick bundles of microtubules known as kinetochore fibers (Figures 4C and 4D).

Immunolocalization of CENPC to Meiotic Kinetochores but Not Neocentromeres

Our meiotic localization data are shown in Figure 5. We achieved the best CENPC staining in meiotic cells by using a rapid fixation method that involves extruding microsporo-

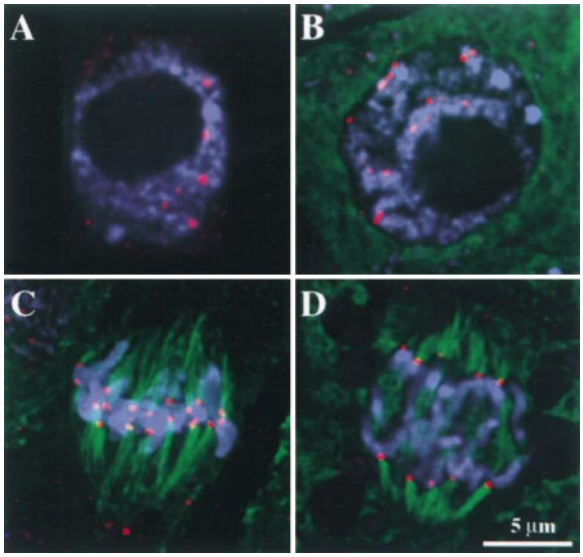

Figure 4. Immunolocalization of CENPC during Interphase and Mitosis.

Images are single optical sections. CENPC staining is in red, tubulin is in green, and DNA is in purple.
(A) Interphase. DNA content analysis (see Methods) indicated that this cell is in G_1 phase (the staining in this cell was brighter than that of the average G_1 cell).
(B) Mitotic prophase.
(C) Mitotic metaphase.
(D) Mitotic anaphase.
Note that in **(C)** and **(D)**, CENPC staining coincides with the ends of thick (green) microtubule fibers known as kinetochore fibers. Bar in **(D)** = 5 μm for **(A)** to **(D)**.

cytes directly into fixative (Staiger and Cande, 1990). The earliest stage at which meiocytes could be extruded intact from the anther was during pachytene (characterized by the synapsis of homologous chromosomes), when staining was localized to ~10 spherical regions ~1 μm in diameter (Figure 5A). Computational chromosome straightening (Dawe et al., 1994) revealed the expected centromeric localization of CENPC (Figure 5B). As the homologous chromosomes separated during diplotene and diakinesis, the homologous centromeric regions also separated to produce 20 smaller spots (Figure 5C). Sister kinetochores were not observed until prometaphase, when a subset of the kinetochores appeared to be composed of two subunits (Figure 5D). The partial separation of sister kinetochores was also apparent during metaphase I and anaphase I (Figures 5E and 5F). Although we were able to resolve the sister kinetochores on many chromosomes in prometaphase through anaphase, we were never able to resolve all the sister kinetochores. It is possible that many of the sister kinetochores went undetected due to the relatively poor axial resolution of three-dimensional light microscopy (i.e., from optical section to optical section; Inoué, 1995).

In telophase I and interkinesis-staged nuclei (the interphase following meiosis I), 10 kinetochores were usually identifiable in each nucleus (Figure 5G). The sister kinetochores were incompletely separated, as they were during prometaphase I through anaphase I. As shown in Figure 5H, a more pronounced separation of sister kinetochores was observed during prophase II. Even at prophase II, however, the sister kinetochores often appeared to be connected by a thin thread of CENPC-positive material (Figure 5H, inset). During prometaphase II, the kinetochores were fully separated and oriented to opposite surfaces of the chromosomes (Figure 5I). During anaphase II, the kinetochores disjoined; after telophase and cytokinesis, four microspores with 10 kinetochores each were produced (data not shown).

Additional immunolocalization experiments were performed on meiocytes from plants that displayed neocentromere activity. Neocentromeres are knobs (see Figure 5B) that are mobilized on the spindle by one or more genes on abnormal chromosome 10 (Dawe and Cande, 1996). During anaphase II, the neocentromeres can be observed stretching chromosome arms by as much as 4 μm along the spindle axis (Yu et al., 1997). As shown in Figure 6, CENPC staining was absent on neocentromeres at anaphase II, although strong staining remained at kinetochores.

Double Labeling of CENPC and the Sau3a9 Cereal Centromere Repeat

Mammalian CENPC is localized to the inner kinetochore plate, immediately adjacent to the centromeric heterochromatin (Saitoh et al., 1992). We used a probe to the conserved Sau3a9 cereal centromere repeat (Jiang et al., 1996) to determine whether maize CENPC has a similar sublocalization. Polychromatic fluorescent beads were used as internal standards to accurately align the CENPC (fluorescein isothiocyanate channel) and Sau3a9 (rhodamine channel) images. As illustrated in Figures 7A and 7B, CENPC and Sau3a9 staining were closely associated but not strictly colocalized during meiosis. We anticipated that the CENPC staining would lie on the poleward side of the centromere, especially when the kinetochores were under tension during metaphase I and II. However, only at metaphase II was there a significant tendency ($P = 0.002$) for CENPC staining to lie on the poleward side of the centromeric staining (Figure 7C). At metaphase I, there was no consistent localization pattern within the spindle axis, indicating that there are distinct structural differences between the centromere/kinetochore complexes of metaphase I and metaphase II.

DISCUSSION

CENPC is a conserved protein that is required for kinetochore function. It and the histonelike protein CENPA are

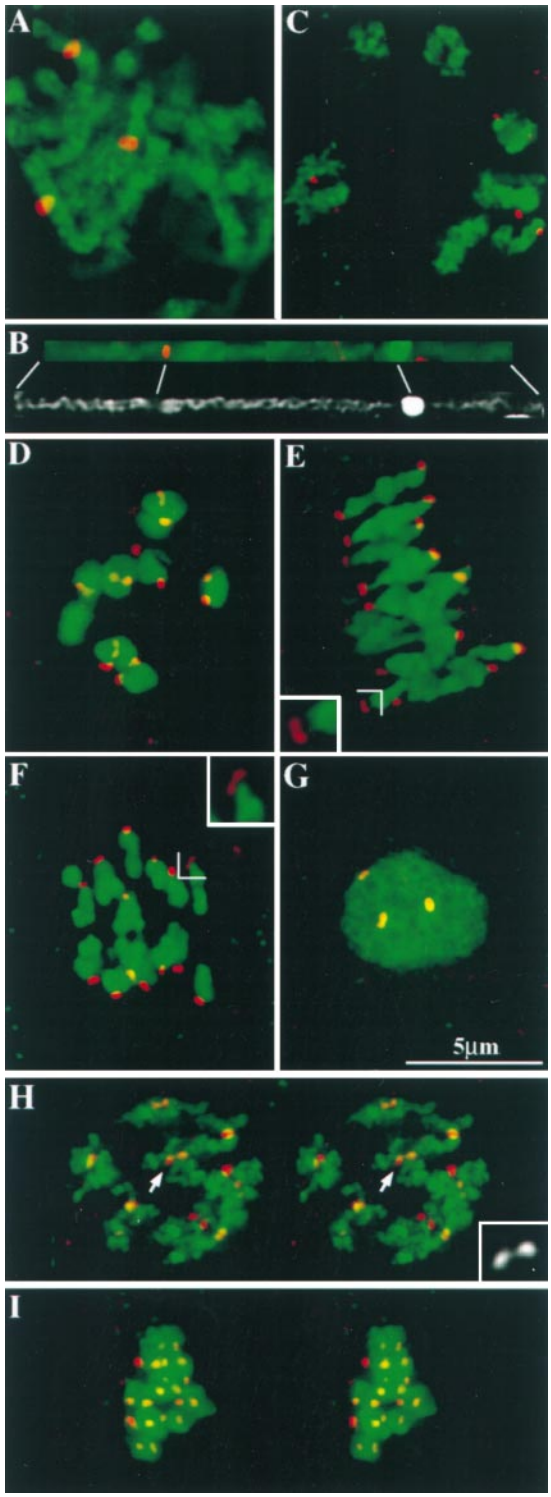

Figure 5. Immunolocalization of CENPC during Meiosis.

Images in **(A)** to **(G)** are single optical sections. CENPC staining is in red; DNA staining is in green. The signal appears yellow when red (CENPC) and green (DNA) overlap.

thought to be involved in defining kinetochore boundaries or otherwise assembling the kinetochore (Tomkiel et al., 1994). As a first step toward understanding the mechanisms that govern kinetochore formation in plants, we have identified the genes for three maize homologs of CENPC (CENPCA, CENPCB, and CENPCC) by their similarity to a conserved portion of CENPC and Mif2p known as region I (Brown, 1995; Meluh and Koshland, 1995; Figure 2). DNA gel blot analysis with gene-specific probes suggests that there are at least five different *Cenpc* genes in maize. Antibodies generated against CENPC localized specifically to the kinetochore region of chromosomes, providing strong evidence that maize CENPC, human CENPC, and Mif2p are functionally homologous proteins. The broad evolutionary conservation of CENPC suggests that CENPC homologs will be discovered in other plants as well.

Our expression data (Figure 1) and the tissue sources from which cDNAs were isolated (Table 1) indicate that *Cenpc* is expressed in a wide variety of vegetative and reproductive cell types. We were unable to detect *Cenpc* mRNA in pollen, however, suggesting that *Cenpc* mRNA levels may be downregulated in some cell types or at some stages of the cell cycle. At the subcellular level, our immunolocalization data support the idea that the CENPC protein is present at kinetochores throughout mitosis and meiosis (Figures 4 and 5) as well as during interphase, occurring not only in mitotic G_1, S, and G_2 but also during meiotic interkinesis (Figure 5G) and the tetrad stages (data not shown). Overall, these data support the interpretation that CENPC is a constitutive kinetochore protein (Knehr et al., 1996) and that the kinetochore is present at centromeres at all stages of the cell cycle (Choo, 1997a).

(A) Pachytene.
(B) Chromosome 7 straightened from a pachytene cell stained for CENPC, as in **(A)**. The straightened chromosome is compared with a "standard" chromosome 7 (below) that was a part of a previous study of the maize pachytene karyotype (Dawe et al., 1992). Note that the red CENPC staining corresponds to the nonstaining gap that characterizes maize centromeres. The brightly stained cytological feature on the chromosomes is a knob.
(C) Diakinesis.
(D) Early prometaphase I.
(E) Late prometaphase and early metaphase I. The enlargement (inset) illustrates that sister kinetochores can often be resolved from each other at this stage.
(F) Anaphase I. The enlargement (inset), as shown in **(E)**, illustrates the partial separation of sister kinetochores typical of this stage.
(G) Interkinesis (interphase between meiosis I and II).
(H) Prophase II, shown as a stereopair. Note the nearly complete separation of sister kinetochores. At this stage, there often remains a thin thread of CENPC-positive material that connects the two kinetochores (inset). Arrows indicate the region shown in the inset.
(I) Prometaphase II, shown as a stereopair. Note the complete separation of sister kinetochores.
Bar in **(G)** = 5 μm for **(A)** to **(I)**.

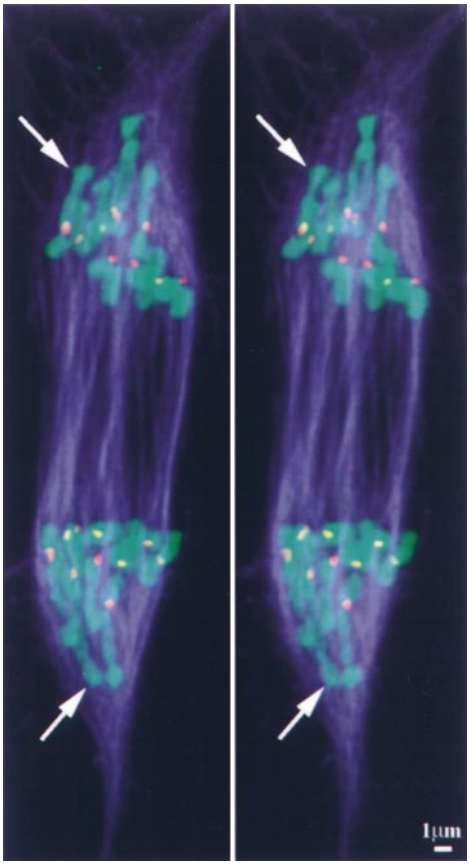

Figure 6. Demonstration That CENPC Does Not Localize to Neocentromeres.

An anaphase II cell from a plant homozygous for abnormal chromosome 10 is illustrated as a stereopair. Neocentromeres extend toward the spindle poles (arrows). CENPC staining is in red, tubulin is in purple, and DNA is in green. The signal appears yellow when red (CENPC) and green (DNA) overlap.

Immunolocalization of CENPC allowed the direct visualization of sister kinetochores during meiosis in maize. During meiosis I, the sister kinetochores appeared to be fused until late prometaphase, when a bipolar spindle was established. Between the stages of prometaphase I and interkinesis, the sister kinetochores could be resolved from each other but remained closely associated (Figures 5D to 5G). At the onset of prophase II, the kinetochores began a full separation that was completed in prometaphase II (Figures 5H and 5I). These results closely parallel observations from a variety of other higher plants (Lima-de-Faria, 1956; Stack, 1975; Suzuki et al., 1997). As noted earlier, the apparent fusion of sister kinetochores before meiosis I spindle formation provides a cytological mechanism for ensuring that the two kinetochores segregate to the same pole (Darlington, 1937; Goldstein, 1981; Dawe, 1998). The molecular mechanisms governing meiotic sister kinetochore cohesion are not understood, but they may involve proteins, such as mammalian Cor1, that bind to inner centromeric regions early during prophase I (Moens and Spyropoulos, 1995).

In animals, CENPC is observed on all active kinetochores, including neocentromeres that are formed at novel locations (Depinet et al., 1997; du Sart et al., 1997). Neocentromeres in maize are capable of rapid poleward movement but differ in several respects from animal neocentromeres as well as normal maize kinetochores (Yu et al., 1997). The simple 180-bp knob repeat that makes up the bulk of maize neocentromeres (Dennis and Peacock, 1984; Ananiev et al., 1998) is similar in size to the mammalian α satellite, which is sufficient to organize a functional centromere/kinetochore complex on human microchromosomes (Harrington et al., 1997). The knob repeat also has significant similarity to a clone that localizes to the centromeric region of the maize B chromosome (Alfenito and Birchler, 1993). On the other hand, maize neocentromeres are probably incapable of active participation in chromosome alignment and move poleward in an apparently unregulated fashion (Yu et al., 1997). Furthermore, maize neocentromeres have been observed only in the presence of abnormal chromosome 10 and its associated meiotic drive system (Rhoades and Dempsey, 1985; Dawe and Cande, 1996). The neocentromeres of maize are probably a simplified class of centromeres/kinetochores with limited capabilities (Yu et al., 1997).

Consistent with the idea that neocentromeres differ from centromeres, we show here that neocentromeres lack CENPC staining (Figure 6). These data indicate that a significant portion of the CENPC proteins that typically interact with maize kinetochores do not interact with neocentromeres. However, because we have only characterized three members of the *Cenpc* gene family and our antibodies were generated against a synthetic peptide unique to CENPCA, we cannot rule out the possibility that neocentromeres interact with a class of divergent CENPC or CENPC-like proteins.

By double labeling meiotic metaphase cells with anti-CENPC antibodies and the conserved Sau3a9 cereal centromere repeat, we show that CENPC and the centromeric DNA are closely associated (Figure 7). It is not known whether the Sau3a9 repeat is required for centromere function, but it is localized to the primary constriction in sorghum, maize, wheat, rice, barley, rye, and oats (Jiang et al., 1996). The Sau3a9 signal coincides with the ends of spindle microtubules in maize (Yu et al., 1997), further suggesting that the sequence lies within the region containing the functional centromeric DNA. Our analysis of CENPC and Sau3a9 staining relative to the spindle poles (Figure 7C) indicates that in metaphase II, CENPC tends to lie in a poleward position relative to the centromere. These data are consistent with the observation that mammalian CENPC is a component of the inner kinetochore plate (Saitoh et al., 1992). In contrast, a poleward orientation of CENPC relative to the Sau3a9 repeat was not observed during metaphase I. We do not understand why metaphase I and II differ in this

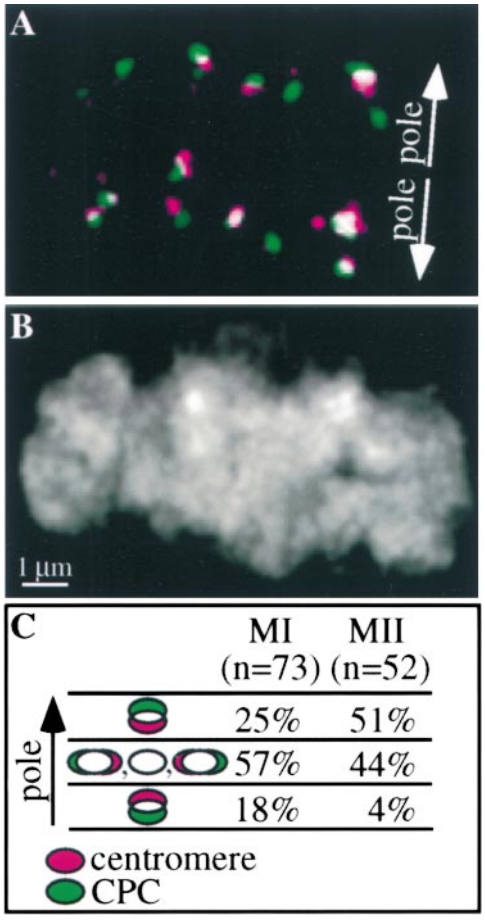

Figure 7. Demonstration That CENPC and the Sau3a9 Centromeric DNA Repeat Interact Closely.

The images in **(A)** and **(B)** were derived from a projection of three optical sections covering a depth of 0.4 μm. The three-color data from a metaphase II cell are displayed as two separate images.
(A) Double labeling of CENPC (green) and Sau3a9 (red), with regions of overlap between CENPC and Sau3a9 appearing white.
(B) Chromosomes from **(A)**.
(C) The combined data from five metaphase I (MI) and four metaphase II (MII) cells are presented, where *n* is the number of centromeres per kinetochores scored. Illustrated here is that at metaphase II, but not at metaphase I, there is a significant tendency for the CENPC (CPC) staining to be on the poleward side of the centromere staining. Wherever CENPC staining was detected, Sau3a9 staining was also observed. Sau3a9 staining appears to be absent at some CENPC-positive areas because of the scaling that was necessary to accommodate intense Sau3a9 staining at other centromeres and the fact that only a portion of the three-dimensional data set is included in this projection.
Bar in **(B)** = 1 μm for **(A)** and **(B)**.

respect, but it is possible that the close interaction of sister kinetochores at metaphase I obscures the relative orientations of the Sau3a9 repeat and CENPC.

In addition to Sau3a9 and CENPC, the maize centromere/kinetochore complex contains MAD2, a homolog of the yeast cell cycle checkpoint protein Mad2p (Yu et al., 1999). Unlike the overlapping domains of CENPC and the centromere, CENPC and MAD2 occupy distinctly different domains in the maize kinetochore. The available data provide strong evidence that the plant kinetochore, like the mammalian kinetochore (Rieder, 1982), has a layered substructure: immediately adjacent to the centromere is a domain that contains CENPC (Figure 7), and over the CENPC domain is a domain that contains MAD2 (Yu et al., 1999). These results are surprising in light of the traditional description of the plant kinetochore as a proteinaceous "ball" embedded in a "cup" of chromatin (Baskin and Cande, 1990). Our data suggest that the apparently uniform kinetochore ultrastructure belies an array of distinct biochemical subdomains.

A variety of evidence suggests that the protein components of plant kinetochores are conserved (Richards and Dawe, 1998). Aside from the demonstrated conservation of CENPC and MAD2 (Yu et al., 1999), the gene for a putative tension-sensing kinetochore protein called ZW10 is conserved (Starr et al., 1997), and there are several examples of antisera cross-reacting with the kinetochores in both plants and animals (Mole-Bajer et al., 1990; Binarva et al., 1993; Houben et al., 1995). In contrast, the known conserved centromeric DNA elements are confined to the grasses (Aragon-Alcaide et al., 1996; Jiang et al., 1996; Dong et al., 1998), with little or no conservation among the centromeric sequences of other plants, animals, or fungi (Karpen and Allshire, 1997; Richards and Dawe, 1998). One way to better understand the centromere/kinetochore complex may be to use conserved protein components to identify and study the functional centromeric DNA sequences. For instance, in humans, immunocytochemistry with anti-CENPC and anti-CENPA antibodies combined with high-resolution in situ hybridization was used to identify the functional limits of the centromere (du Sart et al., 1997). In yeast, in vivo cross-linking followed by immunoprecipitation demonstrated that Mif2p interacts with CDEIII, one of three functional sequences in the yeast centromere (Meluh and Koshland, 1997). The identification of a CENPC homolog in maize should now make it possible to conduct such experiments in higher plants.

METHODS

Identification of *Cenpc* cDNAs

The amino acid sequence of the conserved region I identified in centromere protein CENPC and Mif2p (Meluh and Koshland, 1995) was used as a query against a large expressed sequence tag (EST) data-

base at Pioneer Hi-Bred (Johnston, IA). Two partial cDNAs were recovered from this search that were essentially identical in sequence (one with five extra nucleotides at the 5′ end and the other with nine extra nucleotides at the 3′ end). A third cDNA, CENPCcDNA3, was isolated from a λ library prepared from young shoots of the maize inbred W64A (using CTSBB17 as a probe). The W64A cDNA library was generously provided by Stephen Moose (Dekalb Genetics, Mystic, CT). A fourth cDNA, CENPCcDNA4, was isolated from a λ library prepared from 2-week-old leaves of the maize inbred B73. The B73 cDNA library was generously provided by Alice Barkan (University of Oregon, Eugene). CENPCcDNA4 was subsequently used as a query to identify six additional *Cenpc* ESTs in the Pioneer Hi-Bred database (Table 1). Sequencing was performed at the University of Georgia Molecular Instrumentation Facility. All cDNAs except CENPCcDNA3, CDPER72, and CSCAD58 were fully sequenced. Except in the case of *CenpcB*, as noted in the text, the sequences of cDNAs from the same gene were at least 99% identical (some polymorphism is expected because the cDNAs were obtained from different inbreds). GenBank accession numbers AF129857, AF129858, and AF129859 can be used to access the sequences for *CenpcA* (CNDAC35), *CenpcB* (CZABB82), and *CenpcC* (CXMAD86), respectively.

RNA and DNA Gel Blot Analyses

mRNA from maize tissues was extracted in Trizol reagent (Life Technologies, Grand Island, NY) and purified using the PolyATract mRNA Isolation System (Promega). Approximately 0.33 μg of young tassel (~5 cm in length), 0.14 μg of 2-week-old seedling (roots excluded), and ~2.0 μg of mature pollen poly(A)$^+$ RNA was electrophoresed on a formaldehyde gel (Sambrook et al., 1989). RNA gel blot hybridization and washing were performed according to the Neverfail RNA gel blot hybridization protocol (http://www.nwfsc.noaa.gov/protocols/northernblot.html), which is similar to standard procedures (Sambrook et al., 1989). The *Cenpc* probe for RNA gel blot hybridization was the CTSBB17 cDNA. As a control, the RNA gel blot was stripped and reprobed with the insert from a plasmid (pMAc1) containing a maize actin gene (Shaw et al., 1983). DNA gel blotting was performed by standard methods (Warren and Hershberger, 1994), except that the data were collected using BioMax film and intensifying screens (Fisher, Pittsburgh, PA). Gene-specific probes were generated from cDNAs by using polymerase chain reaction primers specific to the 3′ untranslated regions of each gene.

Protein Gel Blots

Maize protein extracts were prepared by grinding tissues in liquid nitrogen. Five milliliters of protein extraction buffer (20 mM Tris-HCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 200 mM NaCl, pH 8.0) was added, and the samples were spun down to remove cellular debri. Proteins were separated by SDS-PAGE (Sambrook et al., 1989) and transferred to nitrocellulose membranes. Immunodetection was performed using the ECL protein gel blotting kit (Amersham, Arlington Heights, IL). Molecular weights were estimated using size standards from Life Technologies. In Figure 3, the CENPC bands were scanned by computer from the exposed film, and the colored protein size standards were scanned from the blot itself.

Bacterially expressed CENPC was used as a positive control on all protein gel blots. An EcoRI-XbaI fragment carrying the coding sequence from CTSBB17 was inserted into pThioHisA (Invitrogen, Carlsbad, CA) such that the truncated CENPC product was situated at the C terminus of a thioredoxin–CENPC fusion protein. This construct was called pThioCTSBB17. The pThioCTSBB17 protein product was partially purified with a ProBond nickel column (Invitrogen) before being used on protein gel blots.

Generation and Purification of Anti-CENPC Antibodies

A CENPC-specific multiple antigenic peptide (MAP) was prepared by the University of Georgia Molecular Instrumentation Facility by using a multiple peptide synthesizer (model 350 MPS; Advanced Chemtech, Louisville, KY). The CENPCA MAP consisted of the C-terminal 20 amino acids (KVKSFV-PEQYSDLVAKSARY) of CENPCA bound to an oligolysine core. Two rabbits were inoculated with the CENPCA MAP by the University of Georgia polyclonal antibody production service. The anti–CENPCA MAP antibodies in crude rabbit serum recognized the CENPCA MAP as well as the ~39-kD pThioCTSBB17 protein product on gel blots. Neither the CENPCA MAP nor the pThioCTSBB17 protein product was recognized by preimmune serum.

Anti-CENPC antibodies were affinity purified from crude rabbit serum using an Ultralink immobilization kit (Pierce, Rockford, IL). One milligram of CENPCA MAP was coupled to the resin in Mops buffer, and the antibodies were eluted with 0.1 M sodium citrate, pH 2.5. Antibody purification was confirmed using protein gel blots of the pThioCTSBB17 protein product. Neither the bacterially expressed fusion protein nor maize CENPC was recognized when the affinity-purified serum was immunodepleted with an excess of CENPCA MAP before protein gel blotting.

Immunocytochemistry

Fresh microsporocytes from the maize inbred line W23 were extruded from anthers into PHEMS plus 4% paraformaldehyde (Staiger and Cande, 1990) and allowed to fix for 10 min before being spun down onto cover slips (Dawe and Cande, 1996). A stock that was homozygous for abnormal chromosome 10 (in a nonspecific genetic background) was used to determine whether CENPC localized to neocentromeres. Mitotic cells were prepared from W23 seedlings grown in a moist chamber at 26°C. Root tips ~1 cm long were fixed for 30 min in PHEMS plus 4% paraformaldehyde, washed in PBS, and quickly frozen in PolyFreeze (Polysciences, Warrington, PA) with liquid nitrogen. Sections ~10 μm thick were cut on a cryostat at −20°C and transferred to polylysine-L-lysine–coated slides. Affinity-purified anti-CENPC antibodies and a mouse monoclonal antibody to tubulin (Asai et al., 1982) in antibody dilution buffer (Yu et al., 1997) were applied to the cells for 3 hr. The monoclonal antibody to α-tubulin was generously provided by David Asai (Purdue University, Lafayette, IN). The cells were rinsed in PBS, and a 1:30 dilution of fluoroscein isothiocyanate–conjugated goat anti–rabbit antibody (Jackson ImmunoResearch, West Grove, PA) was applied for an additional 3 hr. The cells were again rinsed, stained with 4′,6-diamidino-2-phenylindole (DAPI; 0.1 μg mL^{-1}), and observed by using a SA3.1 multidimensional light microscope system (model SA3.1; Applied Precision, Inc., Issaquah, WA) (see Yu et al., 1997). Kinetochores were not detected either by the crude rabbit preimmune serum or by purified IgG derived from the preimmune serum.

Chromosome Straightening

Pachytene chromosomes were computationally straightened from three-dimensional data sets as previously described (Dawe et al., 1994). The two straightened chromosomes 7 (Figure 5B) are from the KYS inbred, which is known for its exceptional pachytene cytology (Dawe et al., 1992).

Determining DNA Content by DAPI Staining Intensity

Twenty-three CENPC-positive mitotic interphase nuclei were analyzed quantitatively for the intensity of DNA staining. DNA content was estimated by first identifying an intensity threshold in each nucleus that excluded background cytoplasmic as well as nucleolar staining. The threshold value was used to create a "mask" that was then used to computationally remove the background pixels from the starting image (a similar method was used by Dawe et al., 1994). The sum of the gray level values within the nucleus was used to infer the DNA content of the cell. Seven nuclei were readily categorized as G_2 by their similarity in staining intensity to three early prophase cells. Another group of six nuclei stained with roughly half the intensity of the G_2 cells was categorized as G_1, whereas a third group of seven nuclei appeared to have the intermediate DNA staining indicative of S phase.

Combined in Situ Hybridization and Immunocytochemistry

The cereal centromere repeat (Jiang et al., 1996), cloned from sorghum by polymerase chain reaction (Yu et al., 1997), was used to detect maize centromeres. Combined in situ hybridization and immunocytochemical localization was performed as previously described (Yu et al., 1997). During these experiments, we noticed a slight ($\sim$0.1 μm) shift in the alignment of the fluoroscein isothiocyanate and rhodamine channels on our microscope system. To correct for this shift, we included 0.5-μm beads that fluoresce in both the fluoroscein isothiocyanate and rhodamine channels (Polysciences) at a concentration of 10^{-3} beads per mL in the cell suspension before spinning the cells onto coverslips. Cells with a bead close to but not over the chromosomal regions of interest were chosen for data collection. The fluoroscein isothiocyanate and rhodamine components of the data set were then manually shifted to properly align the two images of the bead as well as the remainder of the data set.

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