

# Characterization of a Maize Chromosome 4 Centromeric Sequence: Evidence for an Evolutionary Relationship With the B Chromosome Centromere

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## ABSTRACT

Previous work has identified sequences specific to the B chromosome that are a major component of the B centromere. To address the issue of the origin of the B and the evolution of centromere-localized sequences, DNA prepared from plants without B chromosomes was probed to seek evidence for related sequences. Clones were isolated from maize line B73 without B chromosomes by screening DNA at reduced stringency with a B centromeric probe. These clones were localized to maize centromere 4 using fluorescence *in situ* hybridization. They showed homology to a maize centromere-mapped sequence, to maize B chromosome centromere sequences, and to a portion of the unit repeat of knobs, which act as neocentromeres in maize. A representative copy was used to screen a BAC library to obtain these sequences in a larger context. Each of the six positive BACs obtained was analyzed to determine the nature of centromere 4-specific sequences present. Fifteen subclones of one BAC were sequenced and the organization of this chromosome 4-specific repeat was examined.

THE centromere is responsible for two key chromosomal functions in mitosis and meiosis. First, it is the point of sister chromatid attachment and the site of the regulation of their disjunction. Second, the centromere is the focus for the formation of the kinetochore, where microtubules connect to the chromosomes during anaphase in mitosis and meiosis. Several plant centromeric sequences have been reported. ALFENITO and BIRCHLER (1993) recovered a repetitive element that is spread throughout the maize B chromosome centromere (KASZAS and BIRCHLER 1996, 1998) and that is specific to that chromosome. A more general centromere sequence was found by JIANG *et al.* (1996), who recovered a repetitive element, pSau3A9, from sorghum that hybridizes to the centromeres of sorghum chromosomes as well as those of rice, wheat, maize, barley, rye, and oat (MILLER *et al.* 1998). Another centromeric sequence, CCS1 from the grass *Brachypodium sylvaticum*, hybridizes to the centromeres of wheat, barley, rye, and maize as well as the heterochromatin of rice chromosomes (ARAGON-ALCAIDE *et al.* 1996). PCR-based cloning was used to isolate two maize sequences homologous to CCS1: MCS1A and MCS1B. Using this information, two maize centromeric sequences, CentA and CentC, were recovered from a cosmid library of an oat-

maize chromosome 9 addition line (ANANIEV *et al.* 1998). CentA is a medium-copy-number dispersed retrotransposon. The previously identified maize centromeric sequences, MCS1A and MCS1B (ARAGON-ALCAIDE *et al.* 1996), are part of the long terminal repeat of the CentA element. CentC is a tandemly repeated element. The centromeres of rice were analyzed by DONG *et al.* (1998), who found that the repeats consisted of seven different units, some of which were common to other grasses and some of which were specific to the Bambusoideae subfamily or to *Oryza sativa*.

COPENHAVER *et al.* (1999) defined the DNA sequences delimiting the regions in which recombination is blocked surrounding the centromeres of *Arabidopsis thaliana*. These regions contain a segment of a 180-bp repeat array flanked by moderately repetitive DNA. The five centromeres physically mapped to DNA contigs between 550 and 1790 kb in length, not including the repetitive arrays of the 180-bp repeats. ROUND *et al.* (1997) gave a conservative estimate of the 180-bp repeat arrays as ~600 kb per chromosome. However, some chromosomes, for example, chromosome 1, have >1 Mb of this sequence. Detailed sequence analysis showed that the DNA surrounding the 180-bp repeat arrays are different for chromosomes 2 and 4 due to varying amounts of transposons and retrotransposons.

As noted above, ALFENITO and BIRCHLER (1993) isolated sequences from the maize B chromosome. Clones from a line containing 15 B chromosomes were selected on the basis of stronger hybridization to maize genomic DNA containing B chromosomes than to DNA without B chromosomes. Using *in situ* hybridization and analysis of 25 centromere misdivision events, the B chromosome

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sequences were found to occur throughout the functional domain of the B centromere (ALFENITO and BIRCHLER 1993; KASZAS and BIRCHLER 1996, 1998). Changes in the physical structure of the B repeat array are correlated with altered meiotic transmission (KASZAS and BIRCHLER 1998). The presence of centromere repeats specific to one chromosome raised questions about the nature of the sequence elements comprising centromeres in general.

The B-specific clone, pZmBs, was used in this study to select related sequences from a library of A chromosome DNA. By analyzing sequences from the A chromosomes that have homology to the B repeat, new information was gained on the origin of the B chromosome. The homologous sequences are detectable only at the primary constriction of chromosome 4, illustrating the diversity of sequences comprising centromeric regions and suggesting an evolutionary relationship with the B chromosome centromere.

## MATERIALS AND METHODS

Maize genomic DNA was prepared as described in GARDNER *et al.* (1993) except that fresh plant tissue was ground in liquid nitrogen rather than freeze dried. Southern blot analysis (SOUTHERN 1975) was carried out as described (SAMBROOK *et al.* 1989). Agarose gels (0.7%) were run in 0.5× TBE (0.045 M Tris-borate, 0.001 M EDTA) electrophoresis buffer. The B chromosome centromeric clone pZmBs (ALFENITO and BIRCHLER 1993) was used as a probe to select clones from a library prepared from the maize B73 inbred line (without B chromosomes; ZHANG and WALKER 1993) in GEM11 (Promega, Madison, WI) grown in *Escherichia coli* strain LE392. The insert of pZmBs was amplified by polymerase chain reaction (MULLIS *et al.* 1986) using T3 and T7 primers flanking the cloning site in Bluescript (Stratagene, La Jolla, CA) and then labeled by random priming using a DECAprime procedure (Ambion, Austin, TX). Autoradiographs were visualized using a Fuji image analyzer.  $\lambda$  clones were screened by hybridization with plaque lifts onto nylon membrane (SCHLEICHER and SCHUELL, Keene, NH) at low stringency [60°, 2× SSC, 2% sodium dodecyl sulfate (SDS)]. DNA sequences were obtained using an ABI PRISM 377 sequencer.

**Fluorescence *in situ* hybridization procedure:** Sequences were labeled with biotin-14-dATP using the BioNick labeling system (Life Technologies, Grand Island, NY). *In situ* hybridization was performed according to the Dako (Carpinteria, CA) gen point procedure with some modifications. Deparaffinization and pretreatment were not necessary due to the method used to prepare the slides (KATO 1998). Background quenching remained low with these modifications. Fluorescein isothiocyanate (FITC)-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 200–400 times was used as the fluorescent tag. It was applied to the slides and incubated at 37° for 30–60 min. Subsequently, the slides were immersed in PN buffer (Dako gen point) for 5 min and then in mounting medium with propidium iodide, which was used to stain the chromosomes. Root tip chromosomes were prepared using nitrous oxide treatment (KATO 1998). Slides were stored at room temperature for use in fluorescence *in situ* hybridization (FISH) experiments.

**Bacterial artificial chromosome screening:** The Cent4 sequence contained in a Bluescript KS+ plasmid was sent to

Genome Systems (St. Louis) and used as a probe to screen a bacterial artificial chromosome (BAC) library prepared from B73 maize DNA partially digested with *Hind*III. Six positive BACs in the pBeloBAC11 vector were provided.

**BAC DNA preparation:** BAC DNA preparation was performed following the instructions in the Benchmarks protocol using the QIAGEN (Valencia, CA) maxipreps procedure (KIRSCHNER and STRATAKIS 1999).

**Restriction analysis of BACs:** Restriction enzyme digestions were carried out as described in SAMBROOK *et al.* (1989). Pulsed-field electrophoresis (contour-clamped homogeneous electric field) was performed for 12 hr with an initial pulse of 0.2 sec and a final pulse of 13 sec. Standard gels were run for 24–40 hr at 25 V to achieve maximum resolution.

**Radioactive labeling:** Cent4, CentC, and CentA sequences were released from their respective plasmids by restriction enzyme digestion. Digests were separated on low-melting-point agarose. The inserts were excised from the gel and diluted with ddH<sub>2</sub>O. The gel fragment was then labeled with the Decaprime II Ambion procedure. Blots were prehybridized at 42° for 4 hr and incubated with probe at 42° for 24 hr. Blots were washed in 0.2× SSC, 0.1% SDS a total of four times for 15 min each at 65°.

**Sequence analysis:** Sequences were compared to GenBank entries using BLAST software at the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>) and alignments were performed using LaserGene (Madison, WI) software. The 15 subclone sequences from BAC 22106 are the following GenBank accession numbers: B33, AF334166; B39, AF334167; B16, AF334168; B49, AF334169; B51, AF334170; B58, AF334171; B7, AF334172; C20, AF334173; C23, AF334174; C24, AF334175; C27, AF334176; C29, AF334177; C30, AF334178; S68, AF334179; and S94, AF334180.

## RESULTS

Under high-stringency conditions (60°, 0.2× SSC, 0.1% SDS), the B chromosome-specific sequence (pZmBs) does not hybridize to maize DNA without B chromosomes. However, we found that under low-stringency (60°, 2× SSC, 2% SDS), hybridization was detected to specific bands using Southern analysis. DNA from black Mexican sweet corn with no B chromosomes was cut with 19 different restriction enzymes. With several enzymes recognizing 4-bp sites, a pattern typical of repetitive sequences was detected upon hybridization with pZmBs, with the fragments at the highest representation being ~2.4, 1.9, and 1.4 kb in length (see Figure 1). However, most 6-bp recognition enzymes, including the two shown in Figure 1, did not release fragments in this size range. This banding pattern was similar to that seen when detecting B chromosome DNA with pZmBs under high-stringency conditions (ALFENITO and BIRCHLER 1993; KASZAS and BIRCHLER 1996). The failure of several restriction enzymes to release “plurality” sequences suggested a repetitive structure of considerable length in which no recognition sites were present.

**Isolation of B chromosome homologous clones:** Approximately 550,000  $\lambda$  clones were screened by hybridization with pZmBs, of which 31 showed homology. From these, 18 secondary positives were obtained. Six

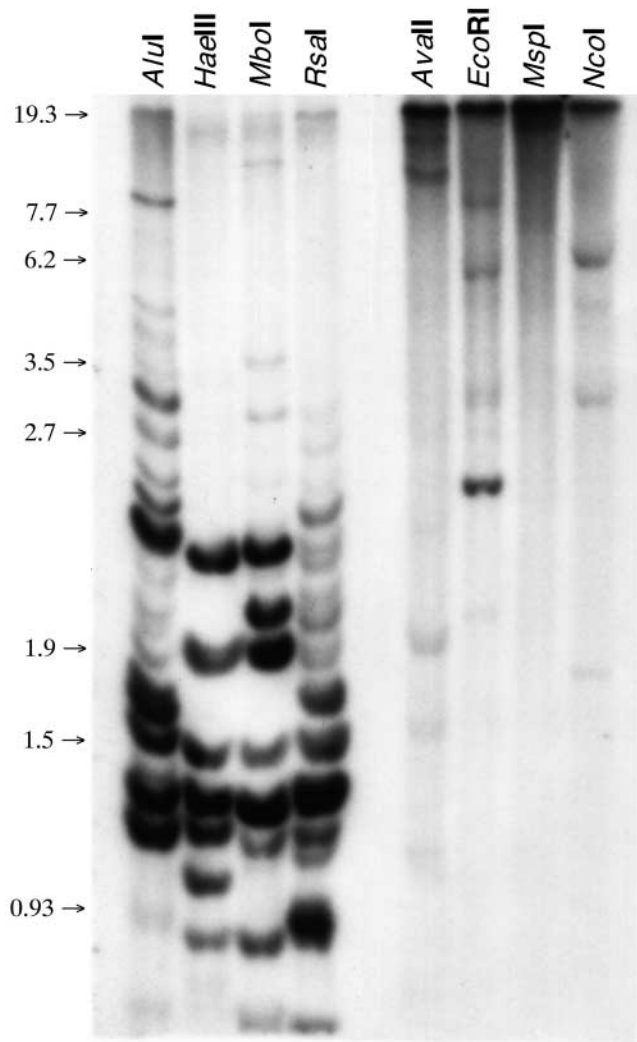


FIGURE 1.—Hybridization of B centromeric clone, pZmBs, to restricted DNA of black Mexican sweet corn without B chromosomes. Washes were performed at low stringency (60°, 2× SSC, and 2.0% SDS). Fragment sizes in kilobases (kb) are shown at the left.

of these were examined using Southern analysis and hybridization with pZmBs. Subclones from 2 of the phage (1 and 13) were selected for further analysis on the basis of strong hybridization to pZmBs.

The  $\lambda$  clones were cut with the enzymes *SacI*, *BamHI*, *EcoRI*, and *XbaI* to select restriction fragments that hybridized strongly to pZmBs. Digestion with *XbaI* is illustrated in Figure 2A. A 1498-bp *XbaI* restriction fragment of  $\lambda$  clone 1 with strong hybridization to pZmBs was subcloned and is referred to as 1Xb1498. A 741-bp *XbaI* fragment of  $\lambda$  clone 13 was subcloned and named 13Xb741. Figure 2B shows the hybridization pattern of a Southern blot made from the digestion using pZmBs as a probe. 1Xb1498 and 13Xb741 were sequenced (GenBank accession nos. AF242892 and AF242891, respectively) and compared (Figure 3).

A map of 1Xb1498 showing regions of homology with

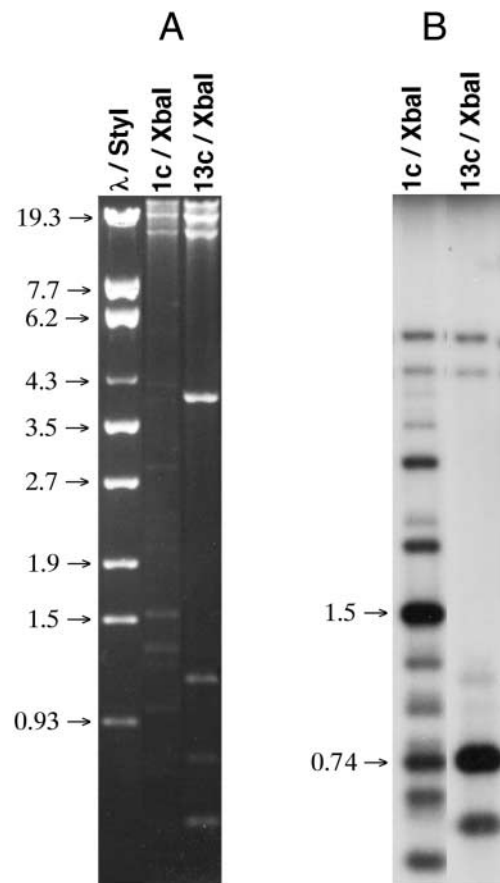


FIGURE 2.—Restriction analysis of B-specific homologous phage. (A) Ethidium bromide-stained gel of restriction digests of  $\lambda$  clones 1c and 13c cut with *XbaI*. Molecular weights are shown on the left. (B) Autoradiograph of Southern analysis of phage 1c and 13c digested with *XbaI* probed with B centromeric clone pZmBs and washed at low stringency.

13Xb741 and various sequences from GenBank is shown in Figure 4. These comparisons revealed some homology with telomeric sequences from tobacco, *Chlorella vulgaris*, *A. thaliana*, *Plasmodium*, wheat, and maize. In addition, homology was found with several other DNA sequences. First, most of 13Xb741 was represented in 1Xb1498 (from nucleotides 847 to 1498 as shown in Figure 5). Over the 88% of 13Xb741 that is homologous to 1Xb1498, there are only five base differences and one base gap between the clones (Figure 4), giving an overall similarity of 99.1%. Second, homology was found to maize clone pBF268 (GenBank accession no. S46927; BURR *et al.* 1992) in three areas: 848–1113 (28–295 in pBF268 accession), 1152–1269 (320–438 in accession), and 1276–1498 (52–274 in accession). pBF268 was selected using a subtelomeric sequence but genetically maps to the centromeric region of chromosomes 3, 4, and 6 (BURR *et al.* 1992). Overall similarity was 87.4%. Third, homology was found to the maize knob repeat clones (M32527; DENNIS and PEACOCK 1984) in two areas: 1304–1364 and 896–964 (58–118 and 54–122 in accession, respectively), with an overall similarity of



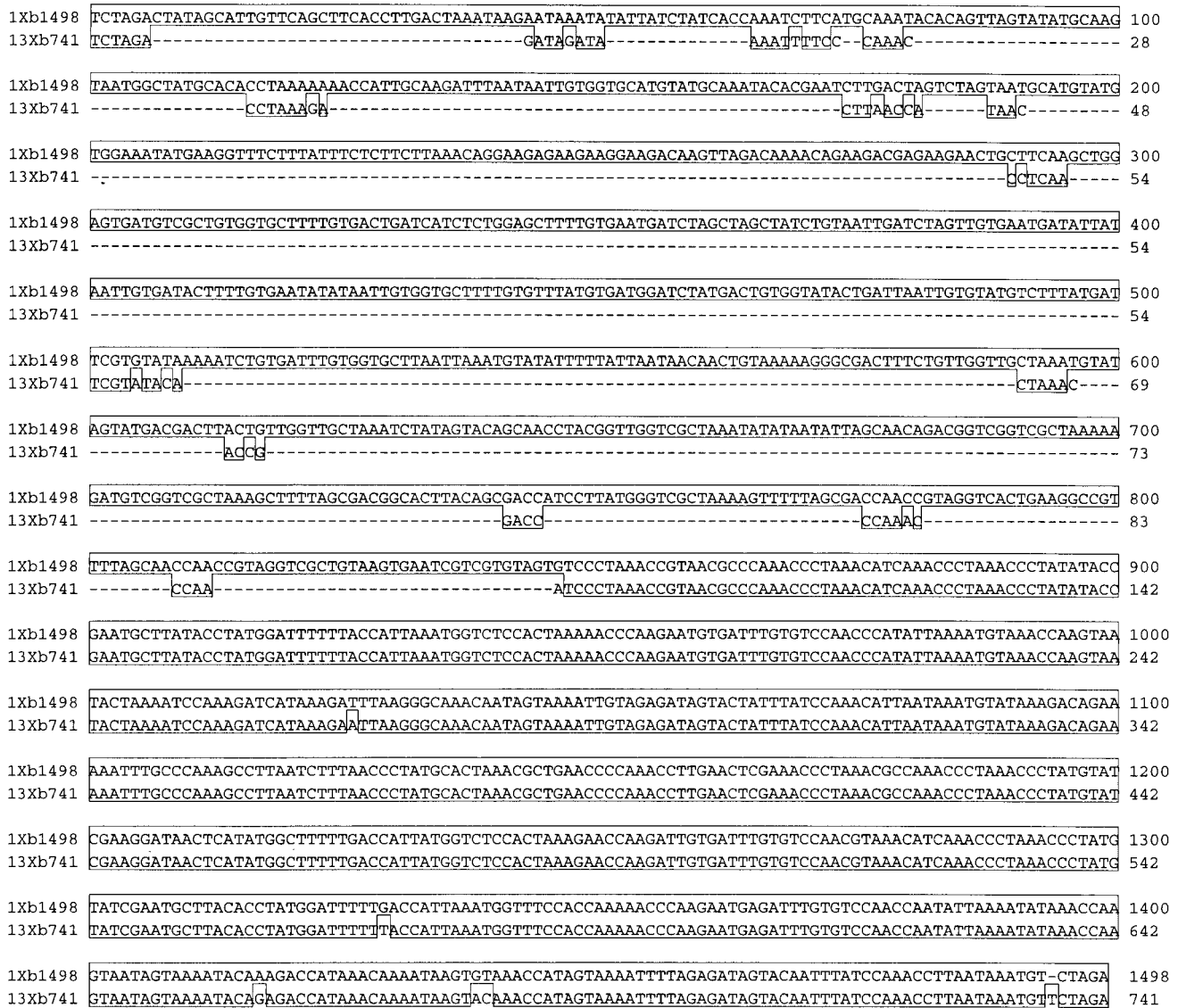


FIGURE 3.—DNA sequence of clones 1Xb1498 and 13Xb741. Sequence matching 1Xb1498 is boxed.

87.7%. Fourth, homology was found to the maize B centromere clones such as K5 (GenBank accession no. U62000). Three areas of similarity were found: 915–996, 1319–1400, and 1467–1498 (119–200, 119–200, and 745–776 in accession, respectively). Overall similarity was 86.7%. Last, homology was found to a maize MADS box gene, ZEMa (GenBank accession no. X91882). ZEMa is an alternatively spliced gene producing five different transcripts (MONTAG *et al.* 1995). The region of similarity was from nucleotide (nt) 317 to 847 (3717–4246 in accession) with 97.0% identity. This sequence lies outside the region of similarity with the B-specific and knob homologies and does not represent a complete gene copy of ZEMa.

It is not apparent why 1Xb1498 should show homology to the ZEMa gene. The similarity begins in the last exon (the terminal 130 bp) and extends into the nontranscribed 3' end. The fact that only a small region

of homology with ZEMa is within 1Xb1498 indicates that this segment of ZEMa has been separated from the complete gene. One explanation is that the gene fragment is a pseudogene that has been transposed to a centromeric location. Alternatively, it may represent a DNA rearrangement that occurred during the library construction.

**FISH localization of B homologous clone:** FISH localization of 13Xb741 to B73 maize chromosomes showed hybridization to the primary constrictions of two chromosomes in a mitotic metaphase spread. The chromosomes exhibiting hybridization were medium sized. Selected trisomic stocks were used to determine to which chromosome the hybridization localized. Using 13Xb741 as a FISH probe to root tip chromosome spreads of maize trisomic for chromosome 4 showed hybridization to the primary constriction of three chromosomes, indicating that related sequences are present at the chromo-

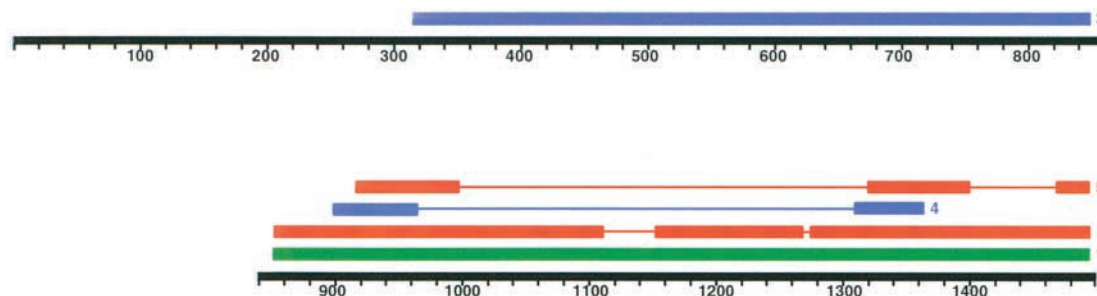


FIGURE 4.—Sequence features of 1Xb1498 and 13Xb741. Above the scale are shown homologies of 1Xb1498 to 13Xb741 and other sequences in GenBank. See text for detailed descriptions. 1, *Zea mays* 13Xb741; 2, *Z. mays* clone pBF268; 3, *Z. mays* ZEMa gene; 4, *Z. mays* knob repeat; 5, *Z. mays* B-specific centromere sequence.

some 4 centromeric region (Figure 5). This result is interesting in that previously reported maize centromeric sequences hybridize to all maize A chromosomes, albeit to varying extents (ANANIEV *et al.* 1998). Subsequently, we refer to the repeat unit as Cent4.

**Genomic Southern:** To gain a better understanding of the nature of Cent4, radioactively labeled probe was hybridized to genomic Southern blots of non-B-containing maize DNA. Using *DpnII*, a restriction enzyme that has a site within the Cent4 clone, several bands were detected (Figure 6). There is hybridization of Cent4 to plurality bands of ~600 bp, 1.2 kb, 1.8 kb, and 2.4 kb as well as to other less abundant or less homologous fragments. In addition, *RsaI* releases a plurality fragment of 350 bp, while *XbaI* digests show strong hybridization to 750-bp fragments. Both show hybridization to additional fragments. These patterns of hybridization are consistent with a degenerate repetitive nature in which many copies of the sequence form the plurality

but variation contributes to the presence of additional fragments of different molecular weights.

**BAC clone analysis:** The Cent4 sequence was used as a probe to screen a BAC library of maize B73 DNA (Genome Systems) prepared from plants without B chromosomes. The six positive BACs 22102–22107, from a total of 92,160 screened, were subjected to restriction analysis. The fragments were applied to standard as well as contour-clamped homogeneous electric field (CHEF) gel electrophoresis. These two procedures allowed for both small (100 bp to 10 kb) and large

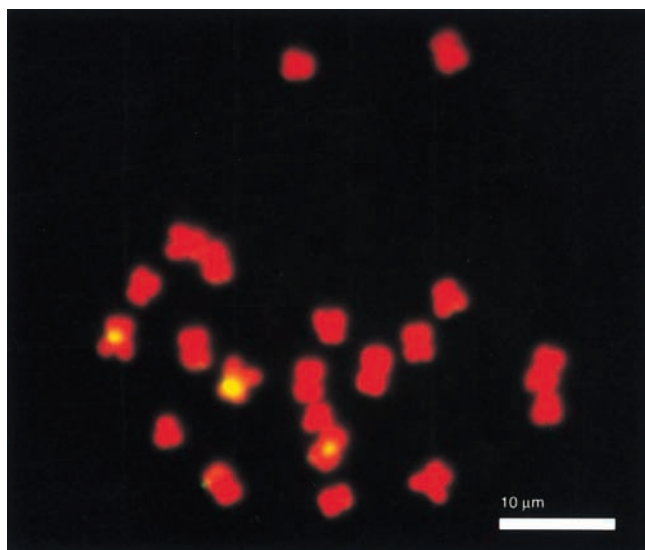


FIGURE 5.—Localization of 13Xb741 using FISH. 13Xb741 was labeled with FITC and used as a probe onto root tip chromosome spreads prepared from a plant that was trisomic for chromosome 4. Mitotic chromosome spreads were prepared as described in MATERIALS AND METHODS.

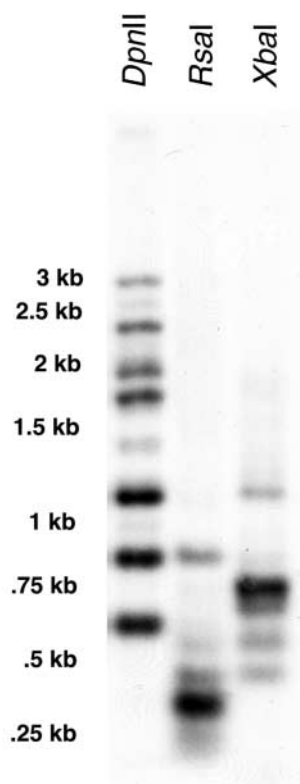


FIGURE 6.—Genomic organization of Cent4. Radioactively labeled Cent4 was hybridized to a Southern blot containing B73 maize DNA. No B chromosomes were present. Washes were made at high stringency as described in MATERIALS AND METHODS. DNA was restricted with *DpnII*, *RsaI*, and *XbaI*.

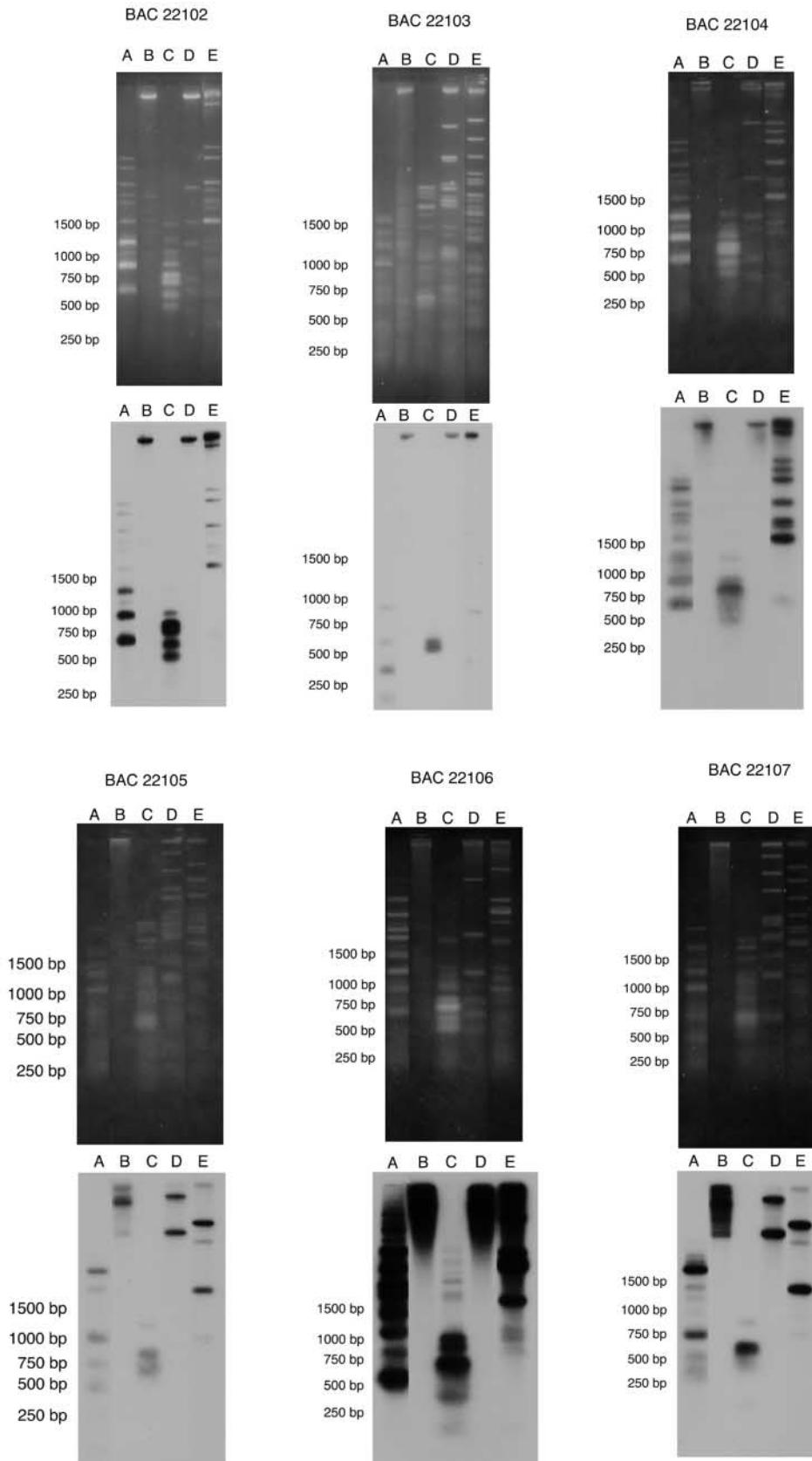


FIGURE 7.—Southern analysis of Cent4-containing BACs. BACs were digested with *DpnI*, *AluI*, *HaeIII*, *HhaI*, and *MspI* (Lanes A–E, respectively) and subjected to Southern analysis. The molecular weights are shown on the left of the ethidium bromide-stained gel (top) and Southern blots (bottom), respectively. The Southern blots were washed at high stringency. Film was exposed for 12 hr.

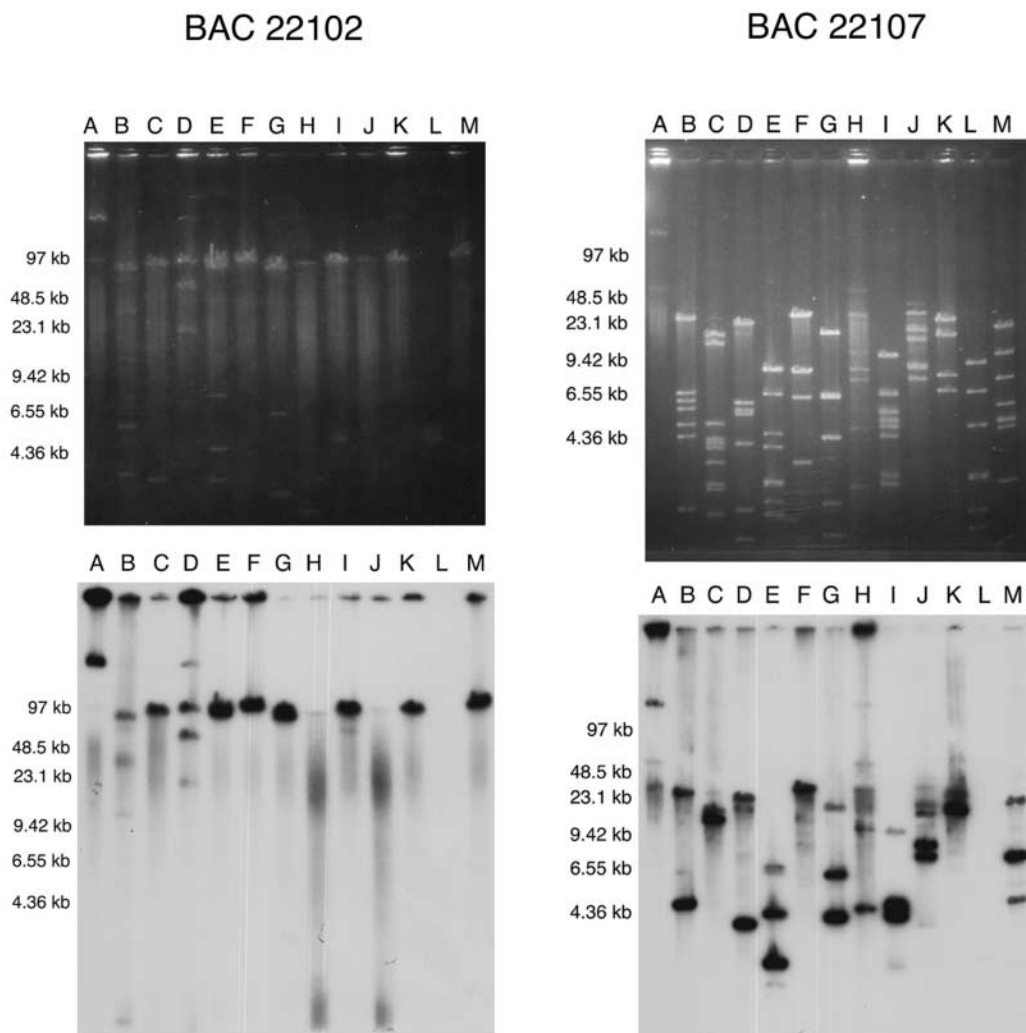


FIGURE 8.—CHEF analysis of representative BACs. BACs were digested with restriction enzymes and subjected to electrophoresis on a 1% agarose CHEF gel for 12 hr at 200 V with an initial pulse of 0.2 sec and a final pulse of 13 sec. The molecular weights are shown to the left of the ethidium bromide-stained gel (top) and Southern blots (bottom), respectively. Lane A is undigested BAC. Lanes B–M are digested with *EcoRI*, *EcoRV*, *BglII*, *BstXI*, *Clal*, *HindIII*, *KpnI*, *NcoI*, *SacII*, *SfiI*, *XbaI*, and *XhoI*. *XbaI* (lane L) has recognition sites within Cent4. Therefore, with 22102 the insert has been completely digested and has migrated off the gel. The other enzymes do not recognize sequences within Cent4 and produce few fragments. Together these observations suggest a high representation of Cent4 within 22102. With 22107, the fragments not digested with *XbaI* do not contain Cent4 as evidenced by comparing the ethidium-stained gels with the Southern analysis.

(2–150 kb) size fragments to be evaluated. Both standard and CHEF gels were Southern blotted (SAMBROOK *et al.* 1989) and hybridized to radioactively labeled Cent4.

In Figure 7, Southern blots demonstrate that Cent4 is present in all six BACs with variation in pattern among them. In BAC 22102, for example, *DpnI* releases several fragments of low molecular weight. *HaeIII* releases four major fragments that show strong hybridization to Cent4, indicating that the majority of Cent4 repeats contain *HaeIII* sites. *AhuI* and *HhaI* digestion does not result in low-molecular-weight fragments, suggesting that they do not recognize any sites within the majority of Cent4 units. *MspI* digestion results in several fragments of diverse size.

A comparison of the hybridization patterns with the Cent4 probe among the six BACs demonstrated similar but not identical patterns among 22102, 22104, and 22106. BACs 22105 and 22107 exhibit related hybridization patterns between them but differ from the aforementioned three. BAC 22103 is the most distinctive of the group. Despite the similarity of hybridization patterns with Cent4, the ethidium bromide-stained patterns illustrate the diversity of the six BACs. A comparison of the ethidium-stained gels and the hybridization patterns illustrates considerable representation of sequences in the BACs with no homology to Cent4.

CHEF gel analysis was also applied to the set of BACs. The BAC insert size was estimated by digesting all six with *NotI*, which releases each insert as a single linear

**TABLE 1**  
**Cent4-containing BACs**

BAC	Insert size (kb)	Amount of non-Cent4 DNA (kb)	Estimate of Cent4 DNA (kb)
22102	123	10	113
22103	80	55	25
22104	140	12	128
22105	120	100	20
22106	110	15	95
22107	60	39.5	20.5
Total	633	131.5	501.5

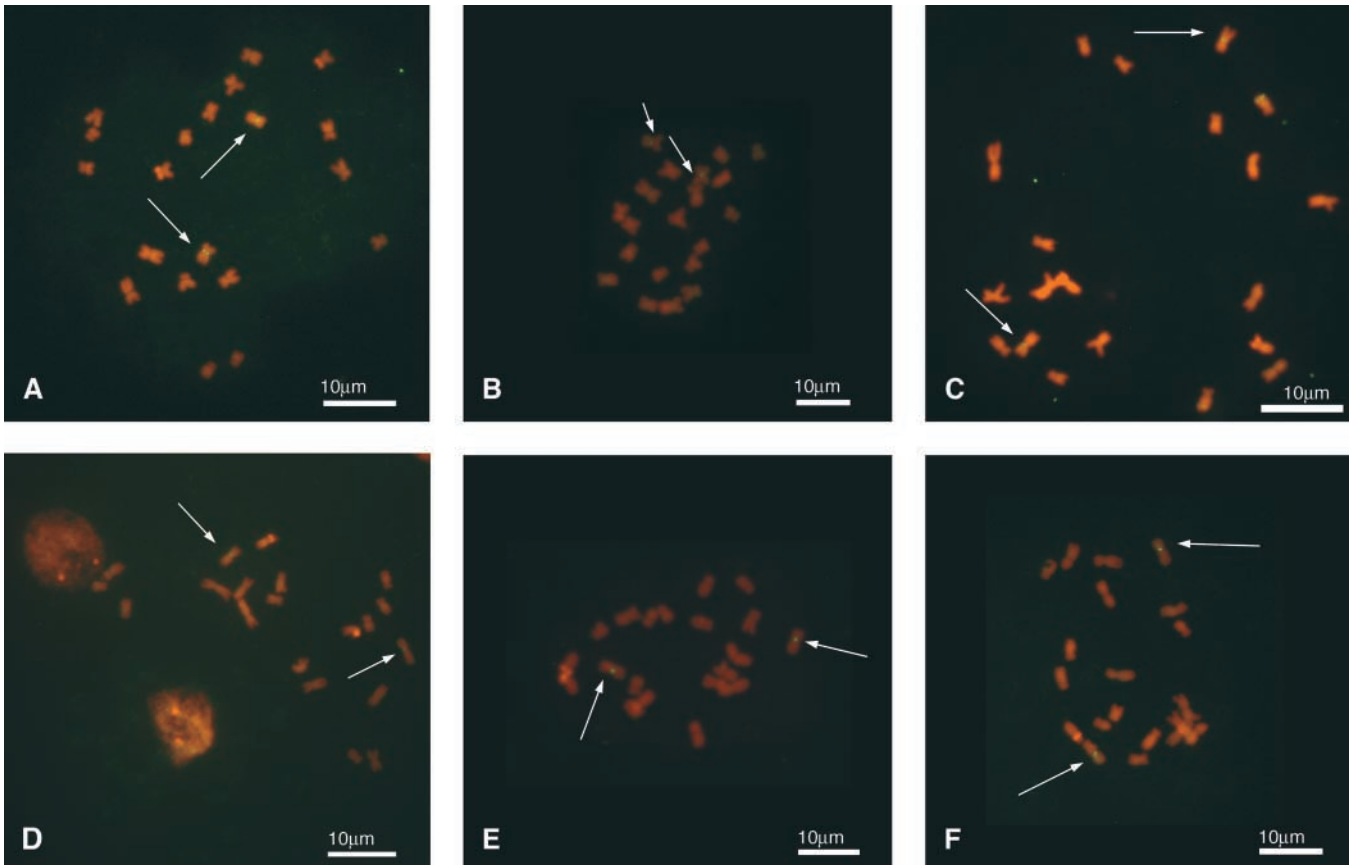
fragment. Size estimates are listed in Table 1. To examine the organization of Cent4-containing fragments, the BACs were digested with 6-bp recognition enzymes. BACs 22102, 22104, and 22106 appear to be the most repetitive in nature, judging by the small number of fragments released by the enzymes tested. BACs 22103, 22105, and 22107 appear to contain more complex DNA that introduces restriction sites, compared to the others. Representative blots are shown in Figure 8.

**FISH localization of BACs:** All six BACs were labeled

for use as FISH probes. Each BAC hybridizes to one pair of centromeres (Figure 9). In some cases, interstitial signals were found, which are believed to be knob sites, because the knob unit is homologous to Cent4. A double label hybridization, using Cent4 and knob probes, confirmed their coincident hybridization (not shown). The differences in hybridization to knobs among the BACs may be due to either a specific sequence within some of the BACs or a variant of Cent4 that contains a greater amount of knob homologous sequence. All six BACs used as FISH probes onto trisomic 4 stocks resulted in three centromeric signals.

One rationale for using all six BACs as FISH probes was to determine if potential non-Cent4 sequences in the BACs would hybridize to other sites within the genome. If there were other repetitive centromeric sequences within the BACs that were not specific to chromosome 4, hybridization to all of the maize chromosome centromeres might occur if the sequence is sufficiently represented in the probe. For all six BACs, it is apparent that the only major centromeric DNA present, which is detectable via FISH, is specific to chromosome 4.

**CentC hybridization:** To determine if previously characterized centromere sequences were present within the BACs, radioactively labeled CentC and CentA were used



**FIGURE 9.**—FISH of BAC clones. BACs 22102–22107 were used as FISH probes onto maize mitotic chromosome spreads. Arrows indicate the primary constrictions of the labeled pair of chromosomes. A, 22102; B, 22103; C, 22104; D, 22105; E, 22106; and F, 22107.



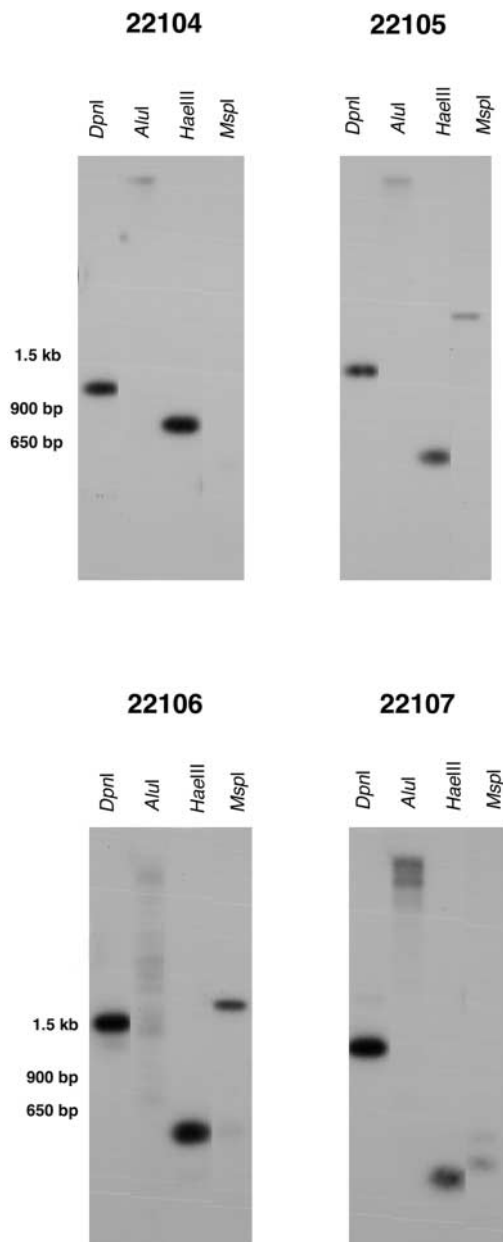


FIGURE 10.—CentC is present on Cent4-containing BACs. BACs were digested with various restriction enzymes, subjected to Southern analysis, and hybridized to a radioactive CentC probe. Molecular markers are shown to the left. Restriction enzymes used are shown at the top.

as probes onto Southern blots. Figure 10 shows that there is hybridization of CentC to BACs 22104, 22105, 22106, and 22107. No hybridization was detected with either BAC 22102 or 22103. These results show that there is proximity between CentC and Cent4 within these DNA fragments. CentA hybridization was not detected in any of the six BACs. The low representation of CentC in the BACs is consistent with the failure of the BACs to label other centromeres in FISH as described above.

To compare the localization of Cent4 to CentC, a double label FISH was performed. Under higher stringencies, CentC shows varying signal strengths among different centromere pairs. Figure 11 illustrates that one chromosome pair where CentC shows very weak hybridization coincides with the one to which Cent4 hybridizes.

**Sequencing of Cent4 copies:** To investigate further the organization of the Cent4 repeat, we subcloned BAC 22106 using the Invitrogen Topo blunt end cloning procedure. One hundred positive subclones were screened with a Cent4 probe onto Southern blots containing the isolated plasmids. It was possible to sequence both strands of 15 subclones in duplicate. GenBank accession numbers are given in MATERIALS AND METHODS. A consensus among the 15 subclones did not emerge, as variation among them was too large. However, certain sequence elements were contained within all copies. Telomere homologous sequences were scattered throughout all 15 subclones. Telomere repeats have been reported within the B-specific centromere repeat (ALFENITO and BIRCHLER 1993), as well as being mapped to A centromeric regions (BURR *et al.* 1992; GARDINER *et al.* 1996). Telomeric-like repeats have also been found at centromeric regions in Arabidopsis (RICHARDS *et al.* 1991) and tomato (PRESTING *et al.* 1996). All 15 subclones contained sequence homology to the knob and B repeats. While this homology is present in all subclones, the extent of the homology is highly variable among different copies. A related unit of ~480 bp in length can be found in most. This repeat unit is present up to three times in larger fragments. The repeat is found in tandem in subclones B39 and C20, while in other subclones the arrangement is variable. A schematic of subclone B39 is shown in Figure 12 with notation of homology to other subclones as well as the areas of knob homology, B repeat homology, and regions containing the 480-bp unit.

## DISCUSSION

We identified sequences from the A chromosomes of maize with homology to the B-specific centromere repeat. Two copies were analyzed in detail. Most of the sequence of 13Xb741 (89–741) (Cent4) is also present in 1Xb1498 (847–1498). The fact that this sequence block was found in two clones from different contexts and also showed a high level of conservation with maize clone pBF268 (see Figure 5) suggests that this sequence may be repeated multiple times in the maize genome.

Cent4 possesses six copies of the common telomeric motif CCCTAAA, which may account for the similarity to telomeric sequences. Within Cent4, the areas of homology with the knob repeat, the B centromere sequence, and pBF268 overlapped. The two regions of highest similarity among these clones are the areas that correspond to the “knob-homologous region” in the B

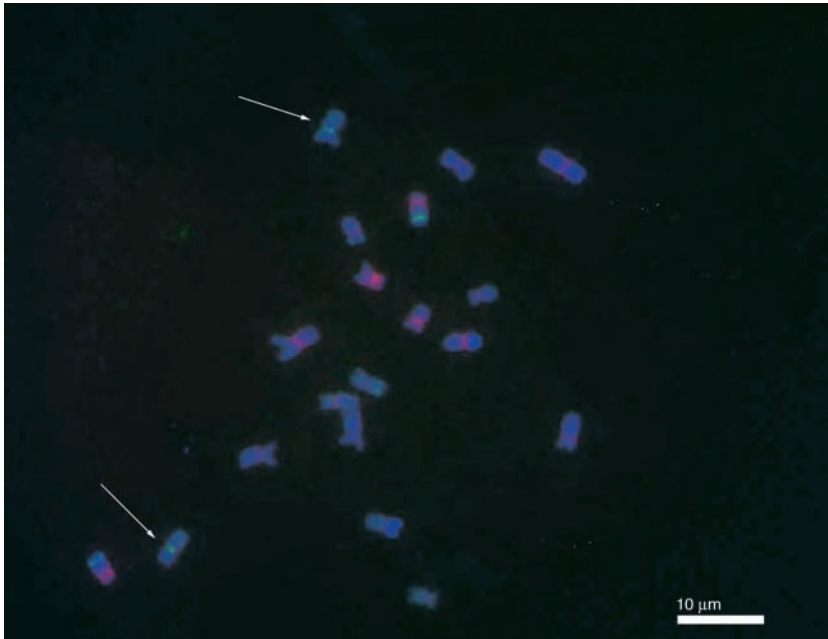


FIGURE 11.—Double label FISH with Cent4 and CentC. Cent4 was labeled with FITC and CentC was labeled with rhodamine. Mitotic chromosomes were stained with 4,6-diamidino-2-phenylindole (DAPI). The arrows indicate the centromeric region of chromosome 4.

centromere clones. This is the interval among the B centromere clones that shares the most conservation and is named for its homology to a 113-bp stretch in the maize knob sequence. The finding that Cent4 has regions of homology to the maize knob repeat is significant, because the knob acts as a neocentromere in certain genetic backgrounds (RHOADES and VILKOMERSON 1942; PEACOCK *et al.* 1981). However, neocentromeres in maize do not function precisely the same as normal centromeres (YU *et al.* 1997; DAWE *et al.* 1999). Nevertheless, the coincident location of these sequence elements at sites involved in chromosome movement is suggestive of a common role that could be either structural or functional.

The knob repeat is capable of binding to elements that facilitate chromosome movement during meiosis but that are not capable of forming a kinetochore (DAWE *et al.* 1999). It is possible that a combination of

knob-like repeats together with other repetitive elements, as we see in Cent4, is capable of forming a kinetochore.

It has been observed that CentC and CentA (ANANIEV *et al.* 1998) hybridize to all of the maize centromeres. The evidence showing the proximity of CentC to Cent4 in some BAC clones argues that Cent4 is present in the same chromosomal region as CentC. However, the weak hybridization of CentC to the chromosome 4 centromeric region suggests that CentC is present in smaller quantities in chromosome 4 than it is in the remainder of the centromeres.

#### Evolutionary relationship of the B repeat and Cent4:

The origin of B chromosomes has remained a mystery since their description in the early part of the past century. The original discovery of a specific repeat in the centromere of the B chromosome (ALFENITO and BIRCHLER 1993) raised further questions about its origin

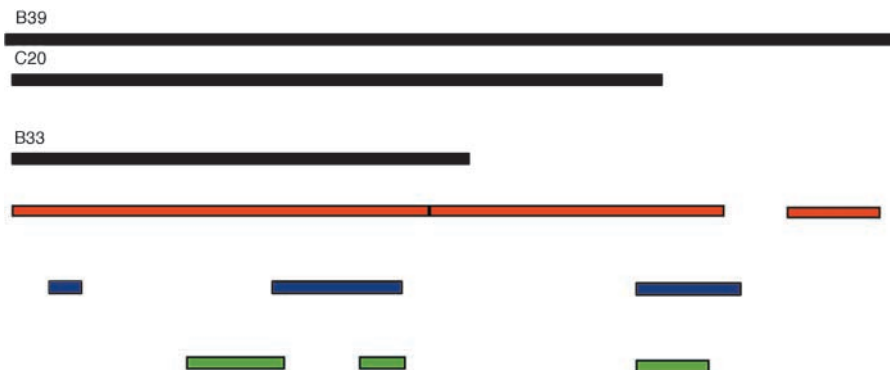


FIGURE 12.—Comparison of Cent4 containing subclones. A schematic shows the homology of three subclones B39, C20, and B33. B39 is shown in its entirety along with its homologies to the other two, the 480-bp Cent4 repeat (red), the B repeat (blue), and the knob repeat (green). B39 contains two 480-bp repeats in tandem. The second 480-bp repeat has a small 160-bp interspersed sequence within it that contains some B repeat homology. C20 contains 99% sequence identity from base pairs 30–710 in B39. B33 contains 99% sequence identity from base pairs 30–510 in B39. Black represents the subclone.

and centromere structure in that other centromeric sequences are represented to varying degrees on all chromosomes (JIANG *et al.* 1996; ANANIEV *et al.* 1998). Here we report that the primary constriction of chromosome 4 has a major representation of a repeat that is related to the B-specific centromere sequence. This finding also suggests an evolutionary relationship between chromosome 4 and the B, at least at the centromere, with the two sharing a common progenitor. The diverged nature of the two sequences, however, suggests a distant relationship.

One possibility for the origin of the B chromosome is that it might represent a degenerate homeologue of chromosome 4. The maize genome carries duplications of the majority of genes, indicating an ancient polyploidy nature that has evolved again toward diploidy (HELENTJARIS *et al.* 1988; GAUT and DOEBLEY 1997). One might suggest that one member of a homeologous set could evolve into a B chromosome with less consequences for the organism than the degeneration of a chromosome in a strictly diploid species. If this were the case, then one would predict that at least some genes on chromosome 4 would not be duplicated elsewhere in the present maize chromosomes. This does not appear to be the case at the resolution available (WILSON *et al.* 1999) and indicates that a different scenario for the origin of the B must be postulated.

Alternative hypotheses for the origin of the B include the possibility that it represents detritus from a wide hybrid cross with a related species. Following fertilization, all chromosomes of the donor species would have been lost with the exception of the progenitor to the B and the remaining maize chromosomes doubled to restore the diploid state. In this case, the progenitor to the B and chromosome 4 centromeres would share a common ancestor.

Last, it is possible that the B chromosome is a conglomerate of heterochromatinized sequences from several chromosomes. The presence of a chromosomal knob adjacent to the centromere of the B chromosome might suggest such a scenario. Knobs adjacent to centromeres are not found in any karyotype of maize or its relatives (BUCKLER *et al.* 1999). If this is the case, chromosome 4 is the most likely donor of the B centromere.

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