Molecular Characterization of a Maize B Chromosome Centric Sequence

Mark R. Alfenito[1] and James A. Birchler[2]

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Manuscript received September 1, 1992
Accepted for publication June 14, 1993

ABSTRACT

Supernumerary chromosomes are widespread in the plant kingdom but little is known of their molecular nature or mechanism of origin. We report here the initial cloning of sequences from the maize B chromosome. Our analysis suggests that many sequences are highly repetitive and shared with the normal A chromosomes. However, all clones selected for B-specificity contain at least one copy of a particular repeat. Cytological mapping using B chromosome derivatives and *in situ* hybridization show that the B specific repeats are derived from the centric region of the chromosome. Sequence analysis of this repeat shows homology to motifs mapped to various plant and animal centromeres and to the maize neocentromere. A precise localization of these sequences among breakpoints within the B centromere and an homology to a facultative centromere, suggest a role for this sequence in centromere function.

SUPERNUMERARY B chromosomes have been documented in over a thousand plants and more than 260 animal species (for review, see JONES and REES 1982). Despite their frequency throughout the plant and animal kingdoms, they do not have any obvious genetic functions, yet are maintained in populations because they have acquired various accumulation mechanisms. Most species with B chromosomes only have them in some members of the population; therefore, supernumerary chromosomes are likely to be more widespread than cytological reports indicate. Moreover, because only those chromosomes that acquire accumulation mechanisms will be maintained, the process that leads to their formation must be even more common. Initially we were interested in analyzing, on the molecular level, the mechanisms that result in the inactivity of these chromosomes and that confer their unusual behavior during the life cycle. The selection for B-specific sequences resulted in the serendipitous cloning of material from the B chromosome centromere, the behavior of which is distinct from A chromosome centromeres.

The B chromosome of maize has been extensively characterized. It exerts an influence on the A chromosomes by increasing recombination frequencies (RHOADES 1968) and decreasing overall plant vigor at high copy number (RANDOLPH 1941). Despite the fact that B chromosomes appear to be genetically inert, they all increase their numbers via processes that maintain themselves in populations. For example, the maize B nondisjoins at the second pollen mitosis producing unequal partitioning of the replicated B's into the two sperm involved in double fertilization. This is followed by a preferential fertilization of the egg by the sperm containing the B chromosomes. Translocation analysis indicates that the portion of the B chromosome predominantly responsible for this fertilization behavior is at or near the centromere (CARLSON 1986). The nondisjunction property requires the B centromere and the distal euchromatic tip of the long arm to be in the same nucleus although not necessarily on the same chromosome (ROMAN 1947). Because of their nondisjunction property, B chromosomes translocated with A segments have become indispensable genetic tools for mapping genes, studying chromosomal mechanics, and producing dosage series in maize.

B chromosomes are usually highly heterochromatic, which is often associated with an abundance of high copy DNAs. Despite this, renaturation and thermal denaturation kinetics of DNA from maize containing B chromosomes show no detectably altered DNA base composition from the A chromosomes (CHILTON and MCCARTHY 1973). From the same study, analytical ultracentrifugation did not yield DNAs of different buoyant densities in corn with and without Bs. We have observed no detectable differences on ethidium stained gels in the discrete bands present between variously digested maize lines containing zero and multiple Bs, indicating the lack of highly repeated B-specific sequences. These observations suggest that the DNA composition of the B chromosome is, in general, similar to that of the A chromosomes.

Despite gross DNA similarities between A and B chromosomes, differences must exist, because of the distinct behavior of the B. The B chromosome has

[1] Present address: Department of Biological Sciences, Stanford University, Stanford, California 94305-5020.
[2] Present address: Division of Biological Sciences, University of Missouri, Tucker Hall, Columbia, Missouri 65211.

unusual properties as described above and has never been observed to synapse with A chromosomes, indicating the likelihood of at least some specific or diverged sequences. On this assumption, we developed a cloning strategy based on a differential screen that would recover specific clones from the B.

MATERIALS AND METHODS

Library construction and screening: Genomic maize DNA was isolated (CHEN, GREENBLATT and DELLAPORTA 1987) from the maize High Loss line +15 Bs. A partial *Sau*3AI digestion was performed. The DNA was fractionated on a 1.5–5 M NaCl gradient by centrifugation at $2.5 \times 10^5 g$ for 3 hr. Aliquots that contained DNAs of greater than 10 kb were used for the ligations to λ phage EMBL3 *Bam*HI arms. As a control, maize A DNA and A + B DNA was digested to completion with *Sau*3AI. Upon inspection of ethidium bromide stained agarose gels, no major detectable species were found to be greater than 10 kb. Therefore, few if any sequences should be selected against during library construction solely on the basis of their size. The bacterial host strain was KH803. Based on the cytological lengths of the A and B chromosomes (CARLSON 1977), 28% of the nuclear DNA should be from the B chromosome in a plant containing fifteen Bs. Therefore, a high percentage of clones should contain insertions from the B chromosome. λ Phage from the unamplified library were plated at a very low titre. Duplicate plaque lifts were performed (GRUNSTEIN and HOGNESS 1975). One set of filters was hybridized with oligolabeled (FEINBERG and VOGELSTEIN 1983) A DNA and the other set was hybridized with oligolabeled A + 15Bs DNA. Plaques that hybridized more strongly to the A + B probe were chosen as potential B clone candidates. This differential screen was carried out using two slightly different protocols. The first protocol involved denaturing and reannealing both total genomic probes to a C_ot of greater than 10 for 3 days in an attempt to eliminate highly repetitive sequences (HAKE and WALBOT 1980) before they were oligolabeled. The second protocol omitted the reannealing step thereby avoiding a bias against repetitive DNAs.

Southern blot analyses: Three micrograms of maize genomic DNA were digested with a restriction enzyme as per the manufacturer's recommendation (New England Biolabs) and then run on a 0.8% agarose gel in 1 × TAE (MANIATIS, FRITSCH and SAMBROOK 1982). All gels were viewed on a UV transilluminator in the presence of ethidium bromide to confirm the presence and complete digestion of the DNA samples. The gels were denatured and neutralized (MANIATIS, FRITSCH and SAMBROOK 1982) and blotted overnight to a nylon membrane. The DNA probes were oligo-labeled (FEINBERG and VOGELSTEIN 1983). DNA hybridizations and washes were done essentially by the method of RABINOW and BIRCHLER (1989).

Chromosomal *in situ* analysis: Maize microsporocytes were harvested and fixed in 3 parts 95% ethanol to 1 part glacial acetic acid for 3 days. The samples were stored at −20° until ready for use. The *in situ* hybridizations were done as described by PHILLIPS and WANG (1982).

Sequence analysis: Nested deletions of approximately 300 bp were made using the Bethesda Research Laboratories *Exo*III and Mung Bean Nuclease Kit. All sequence analysis was done in the Bluescript (SK+) vector. T3 and T7 primers were used on double-stranded vectors using the Sequenase Version II Kit to generate sequence by the dideoxynucleotide method (SANGER, NICKLEN and COULSON 1977). Samples were run in 6 M urea 6% polyacrylamide gels in 1 × TBE (MANIATIS, FRITSCH and SAMBROOK 1982) at 2000V for greater than six hours. Gels were dried on a gel dryer and exposed at room temperature overnight.

Production of B-A aneuploids: The B chromosome nondisjoins at the second pollen mitosis, and the gamete that receives the B chromosome centromere preferentially fertilizes the egg. Therefore, with a translocation between an A and a B chromosome, it is possible to create a segmental aneuploid for the region brought into coupling with the B centromere. For example, the long arm of chromosome *10* has been translocated to the short arm of the B chromosome in *TB-10L18*. In this construct, 10L carries a phenotypic marker, *R-scm3*, which allows the tracking of the B centric chromosome. Therefore, when the B nondisjoins, the duplicated segments of 10L will either fertilize the egg or the polar nuclei. Because the 10L-BL chromosome carries the *R-scm3* marker, which pigments both the aleurone and the scutellum, it is possible to follow this chromosome to either of these tissues in an *r* background. Kernels that showed the *R-scm3* marker only in the embryo were chosen as being hyperploid for the 10L-BL chromosome. Conversely, kernels that showed *R-scm3* expression only in the endosperm, and no embryo color were chosen as 10L-BL hypoploid embryos.

For *TB-10Sc*, the progeny were analyzed from a cross in which the marker stock *y9* (10S) was crossed by males of *TB-10Sc* carrying *Y9*. Hyperploids were selected on the basis of *y9* endosperm (phenotypically light yellow). Seedlings were grown and hypoploids were selected on the basis of *y9* seedling color (pale green).

RESULTS

Isolation of B chromosome specific sequences: Two slightly different procedures were used to select phage containing B-enriched sequences. The first protocol involved a long renaturation of the genomic DNA before radiolabelling to eliminate repetitive DNAs (see MATERIALS AND METHODS). Of roughly 1000 phage screened by this method, one phage was recovered that hybridized more strongly to the A + B DNA probe than to the A DNA alone. This phage is referred to as EMBL3–8Bb.

The second protocol minimized a bias against highly repetitive DNAs by eliminating the reannealing step. This screen yielded 7 phage of 4000 screened that hybridized either more strongly or exclusively to the A + B DNA. All of these phage were then used as probes onto Southerns containing digested genomic DNA of a maize line with and without B chromosomes (Figure 1). Note that all of the phage share a portion of the hybridization pattern in common indicating that all eight phage likely share some sequence. Because phage EMBL3-8Bb was isolated first, the majority of the characterizations have been done on this clone. Similarities among the other phage will be discussed later.

Since EMBL3-8Bb yielded stronger hybridization to the maize line containing B chromosomes, this clone was considered to contain either sequences unique to or highly enriched on the B relative to the A chromosomes. Accordingly, portions of EMBL3-

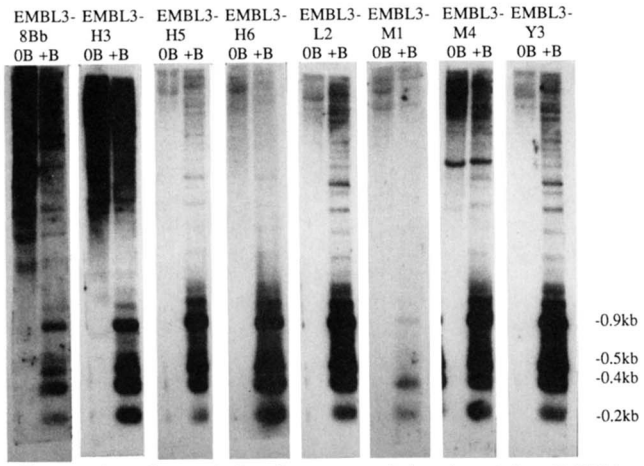

FIGURE 1.—Genomic Southerns containing A and A + B DNAs, each digested with *Xba*I and probed with one of the eight phage isolated from the two screens. Molecular weights of the major fragments are noted on the right.

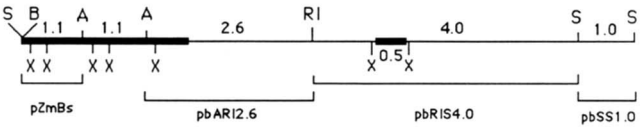

FIGURE 2.—Restriction map of EMBL3-8Bb. The bold lines represent regions of B-specific DNA and the thin lines represent A/B shared repetitive sequences. The exact position of the 0.5-kb *Xba*I, B-specific fragment contained within the 4-kb *Eco*RI-*Sal*I fragment is not known. The subclones generated from this phage are labeled below. A, *Acc*I; B, *Bam*HI; RI, *Eco*RI; S, *Sal*I; X, *Xba*I.

8Bb were subcloned to find the sequence(s) responsible for the stronger B hybridization. The B-specific subclone that was characterized in the greatest detail, is referred to as pZmBs (*Zea mays*, B-specific) and contains a ~1.1-kb *Bam*HI-*Acc*I fragment (Figure 2). When used to probe a Southern of maize DNA with and without B chromosomes, in two distinct genetic backgrounds (High Loss and Black Mexican Sweet), hybridization is detected only in the +B lanes, even after long exposures of the autoradiogram, indicating that pZmBs represents a sequence specific to the B.

Restriction mapping revealed that EMBL3-8Bb contains a tandem repeat of the B-specific sequence (Figure 2). Southern analysis revealed two other small restriction fragments homologous to this 1.1-kb sequence within the phage but that did not represent a complete 1.1-kb unit. The two members of the tandem repeat are not perfectly conserved resulting in loss or displacement of restriction sites. The remaining portions of EMBL3-8Bb are distinct from the B-specific repeat. When DNA fragments from the remaining regions (pbARI2.6, pbRIS4.0, and pbSS1.0) are used as probes onto Southerns containing A and A+B DNA's, hybridization indicative of highly repetitive sequences is observed in both the −B and +B lanes (not shown). This result suggests that these sequences are highly repetitive on the A chromosomes and have at least one or more copies on the B.

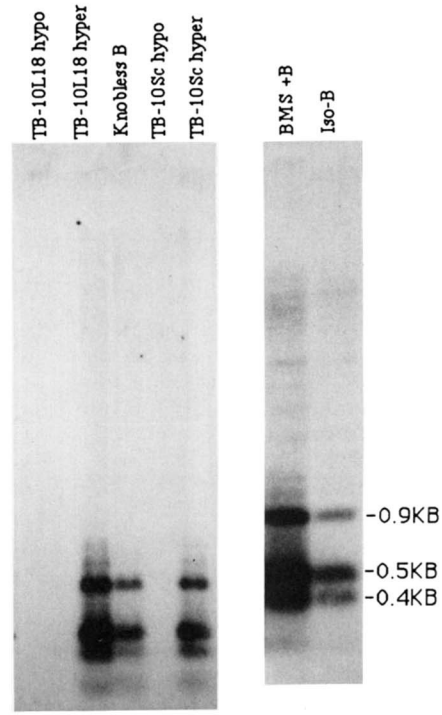

FIGURE 3.—Genomic Southern of DNAs digested with *Xba*I and probed with pZmBs. Lanes 1 and 2 contain *TB-10L18* hypoploid and hyperploid DNAs, respectively. Lane 3 is the knobless B chromosome (*TB-9Sb-1852*). Lanes 4 and 5 contain *TB-10Sc* hypoploid and hyperploid DNAs, respectively. Lane 6 represents a wild-type B chromosome from the Black Mexican Sweet line. Lane 7 contains a B long arm isochromosome.

The maize High Loss line +B's, from which the library was constructed, has been maintained separately from the −B line (RHOADES and DEMPSEY 1973). Therefore, it was tested whether the sequences contained within pZmBs might represent some polymorphism that has been fixed and amplified in the separate lines over subsequent generations. Southern analysis was performed on samples in which the B DNA content was varied while maintaining a uniform background (Figure 3). To do this, we used the B-A reciprocal translocation, *TB-10L18*, that has a breakpoint within the diminutive short arm of the B chromosome and in the proximal region of the long arm of chromosome *10* (10L) (LIN 1979) on which a genetic marker (*R-scm3*) resides (Figure 4). Because of the B chromosome's ability to nondisjoin in the second pollen mitosis, it is possible to create individuals with or without virtually the entire B chromosome using *TB-10L18*, and classify them in a segregating F_1 population by means of a genetic marker (see MATERIALS AND METHODS). If pZmBs is from the B chromosome, we would predict that it should hybridize only to the individuals that inherit the B chromosomes. The results of this test are shown in Figure 3. We can rule out the possibility that pZmBs is an amplified polymorphism because specific hybridization to pZmBs is noted only in the samples containing

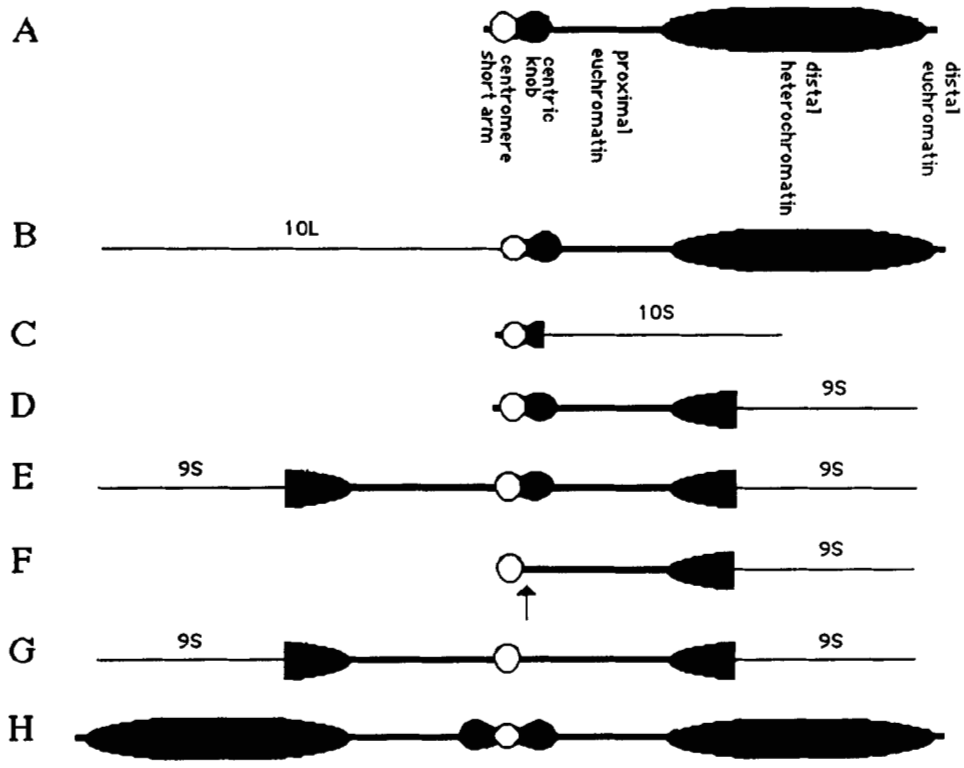

FIGURE 4.—(A) Diagram of a B chromosome showing diminutive short arm, centromere and long arm. The centric knob abutts the centromere. The balance of the chromosome consists of a large euchromatic and heterochromatic regions. (B) The B-centric fragment of *TB-10L18* shows how the long arm of chromosome *10* is joined to the short arm of the B. (C) The B-centric fragment of *TB-10Sc* shows how 10S bisects the centric knob. (D) Normal *TB-9Sb* B-A chromosome. The *TB-9Sb* translocation breaks in the distal heterochromatin of the B and carries 9S with it. (E) Pseudoisochromosome of *TB-9Sb*. Note asymmetry of the centric knob. (F) The *TB-9Sb-1852* chromosome is telocentric and lacks the short arm and the BL centric knob. Arrow denotes missing knob. (G) An isochromosome, *TB-9Sb-2820*, that was derived from a telocentric. It has no remaining centric heterochromatin. (H) An isochromosome of the entire B long arm.

B chromosomes. Also, pZmBs does not hybridize to sequences on 10L because the same results are observed when other B-A translocations involving other chromosome arms, such as 10S, are used (see below). The hybridization pattern for *TB-10L18* hyperploid (Figure 3) matches the hybridization pattern observed for the normal B chromosome (Figure 3).

Analysis of other clones: The other seven phage were all subsequently shown by Southern analysis to contain sequences homologous to pZmBs, which accounts for their similar hybridization patterns (above). When used for Southern analysis to genomic DNA from A and A + B stocks, it can be seen in Figure 1 that all seven phage also contain varying amounts of A/B shared repetitive DNA's, especially in EMBL3-8Bb, EMBL3-H3, and EMBL3-M4. These genomic blots also demonstrate the similar hybridization patterns observed when the phage are compared. Assuming that all DNAs from the B chromosome are equally clonable, and considering that 8/8 phage contain a representative of the same sequence, it is reasonable to conclude that pZmBs contains representatives of a major repetitive B-specific sequence.

B-Specific cluster maps to the centric region of the B: Using several B-A translocations with breakpoints along the length of the B (*TB-10L18*, *TB-10Sc*, *TB-1La*, *TB-10L19*, *TB-4Lb* and *TB-9Sd*) (BECKETT 1991), it was determined by comparison of hypoploids and hyperploids, that EMBL3-8Bb was most likely derived from the B centric region. As mentioned above, there is no hybridization to *TB-10L18* hypoploids, which have the tip of the B short arm present on chromosome *10*. Of the others listed above, the most proximally broken B long arm translocation is *TB-10Sc* (Figures 3 and 4C). It divides the centric knob, placing the majority of the long arm of the B onto chromosome *10*. The reciprocal chromosome contains the B short arm, the B centromere, part of the centric knob, and a portion of 10S. Hybridization with the B-specific probe was detected in Southerns only in the lane containing the B-centric fragment (*TB-10Sc* hyperploid) and not in the lane containing the B fragment from the middle of the centric knob to the end of the long arm (*TB-10Sc* hypoploid) (Figure 3). However, the B-centric chromosome of *TB-10Sc* carries not only the B centromere but also the proximal half of the centric knob.

Therefore, to resolve if the B-specific cluster indeed maps to the centromere or is contained within the centric knob, three derivatives involving "misdivision of the centromere" were examined. The molecular basis of "misdivision" is not known, but the work of DARLINGTON (1939) on Fritillaria and SEARS (1952) on wheat suggests that it results from breakage of univalent chromosomes within the centromere due to attachment of the meiotic spindle from both poles. This results in the two different replicated arms of a chromosome going to opposite poles. The fact that the different arms have been observed migrating to opposite poles during misdivision indicates that the centromere has been divided during the process and that both subdivisions can function. If the two broken

ends join, an isochromosome is produced, but if they do not, telocentrics result.

CARLSON AND CHOU (1981) described a pseudoisochromosome of the *B-9* chromosome of *TB-9Sb*. This new chromosome has the normal B-9S arm on one side of the centromere but a partially deleted arm, in particular missing the centric knob and part of the proximal euchromatin, on the other side (Figure 4). This original isochromosome often undergoes misdivision of the centromere yielding each of the respective arms as telocentric chromosomes. The fact that both arms of the pseudoisochromosome can be recovered is consistent with misdivision involving a break within the centromere to produce two functional chromosomes. *TB-9Sb-1852* is one such telocentric that is derived from the arm missing the centric knob (CARLSON and CHOU 1981; CARLSON and ROSEMAN 1992) (Figure 4). As alluded to above, this chromosome was derived by the following sequence of events. First there was a misdivision that caused an elimination of the B short arm. One of the two long arms suffered a partial deletion before joining with the other to form the original isochromosome. This was followed by a break within the centromere to produce a telocentric chromosome that was consequently deleted for both the short arm and the centric knob. The B-specific probe, pZmBs, hybridizes to DNA prepared from plants carrying this chromosome (Figure 3). This result, when combined with that of *TB-10Sc*, can eliminate all of the sequences from a point within the centromere to the tip of the long arm as possible sites for the localization of the B-specific cluster.

The second derivative of the B chromosome formed by a misdivision event is an isochromosome containing two long arms (see Figure 4). This chromosome was found by R. W. STAUB and L. E. HILL and kindly made available to us. This chromosome is presumed to have been formed by a break within the centromere followed by the two broken, replicated, long arms joining to form the isochromosome. The resulting chromosome is deleted for a region from a point within the centromere to the tip of the short arm. The B-specific probe hybridizes to DNA from plants carrying this chromosome (Figure 3). This result can eliminate all of the sequences from a point within the centromere to the tip of the short arm as possible sites for the localization of the B-specific cluster.

The results of the cytological localization can be summarized as follows. The analysis of hypoploids of *TB-10L18* and of *TB-10Sc* indicate that there are no copies of the B-specific sequence in the chromosomal regions that extend from the respective translocation breakpoints to the tips of the short and long arms. In contrast, the B-specific sequences are represented in the *TB-9Sb-1852* chromosome. This derivative has

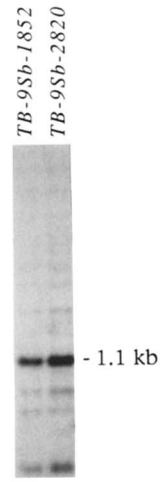

FIGURE 5.—Genomic Southern of DNA digested with *Acc*I and probed with pZmBs. Left, *TB-9Sb-1852*; right, *TB-9SB-2820*.

resulted from two misdivisions of the centromere. Misdivision must occur within the functional region of the centromere because both products are recoverable. This fact also suggests that the centromere is a repetitive structure. The two misdivisions have eliminated the short arm and a portion of the long arm beyond the breakpoint in *TB-10Sc*. The presence of the B-specific sequence in the *TB-9Sb-1852* chromosome but absent in the hypoploids of *TB-10L18* and *TB-10Sc* suggests, by a process of elimination, that these sequences are a component of the centromere.

Further misdivision of the centromere in the telocentrics described above can occasionally produce new isochromosomes. *TB-9Sb-2820* (see Figure 4) is an isochromosome derived from a knobless telocentric, *TB-9Sb-1854* (CARLSON and CHOU 1981) of similar structure to *TB-9Sb-1852*. The metacentric nature of this chromosome permits better spreading of the region surrounding the centromere, which allows a more definitive test for any remaining heterochromatin. There appears to be none in this chromosome, which is consistent with its derivation (W. CARLSON, personal communication). Figure 5 shows a Southern hybridization with the B-specific probe comparing *TB-9Sb-2820* and *TB-9Sb-1852*, illustrating the presence of these sequences in both chromosomes.

In addition to the above localization of the B clone, chromosomal *in situ* hybridization using a tritiated B-specific probe, pZmBs, was performed by the method of PHILLIPS and WANG (1982). Chromosomes from two genetic stocks, High Loss +B's and *TB-10L18* hyperploid for the B centromere and the long arm, were used as substrates for the hybridization. The large arrowhead in Figure 6A points to the hybridization over the acrocentric centromere region of the B chromosome. In many cells, there is possibly a minor silver grain deposition at the junction of the distal heterochromatin and distal euchromatin. The nature of this deposition is uncertain because, on the basis of

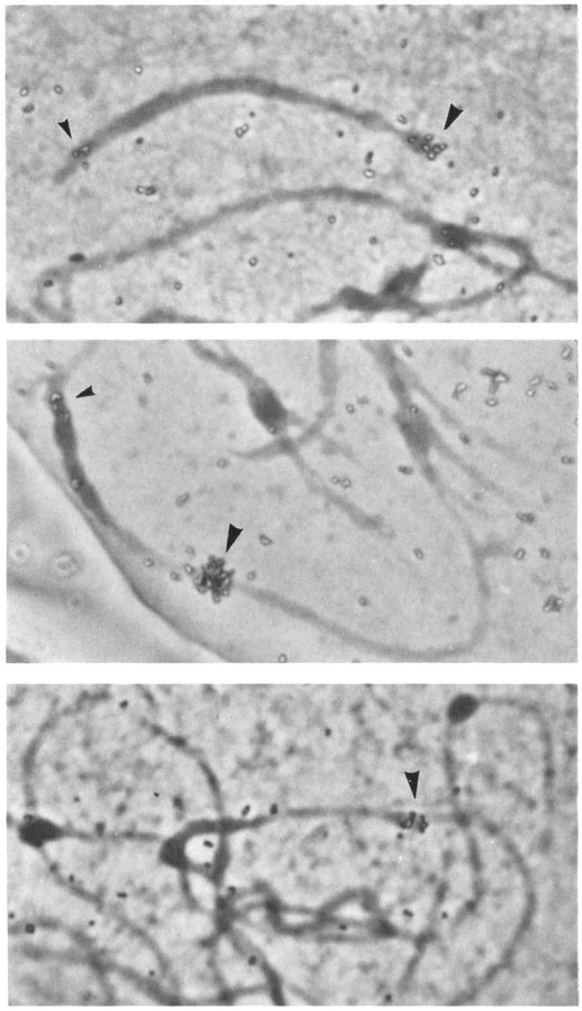

FIGURE 6.—*In situ* analysis of chromosomes in pachynema. Large arrows mark silver grain deposition over centromeres. Small arrows mark possible minor hybridization sites. In all figures, the probe is pZmBs. (A) Bivalent of a B chromosome (1500×). (B) The compound BL-10L chromosome of *TB-10L18* is shown with hybridization over the center of the chromosome. The pycnotic B long arm is on the left with the euchromatic 10L on the right. Again, a minor region of hybridization is possible at the distal tip of the B (1500×). (C) *TB-10L18* showing the "bracket" hybridization to the stretched centromere (1500×).

Southern hybridizations, no homology was detected in this region of the chromosome when separated from the centromere region in the *TB-10Sc* translocation (Figure 3). The potentially reduced stringency of the *in situ* procedure might permit hybridization between this region of the chromosome and some sequence within the probe that is not detectable by Southerns. (Note that the hybridization conditions for the Southerns and the *in situ* were distinct.)

TB-10L18 was also hybridized to the B-specific sequence (Figure 6B). In this genetic construct, hybridization appears over the centromere of the chromosome containing 10L on one arm and the long arm of the B (BL) on the other. The relatively long BL-10L chromosome sometimes stretches during the smear process. In some of these cells, brackets of hybridization can be seen at the centromere (Figure 6C) suggesting that the B-specific sequence may not be continuous through the region or that the density of DNA in this region is below the level of detectable hybridization. Alternatively, the centromere might have been stretched during preparation and the brackets are an artifact of the smear procedure.

```
  +1    GGATCCTACA GATAAGGGGA TGTTTCCCTA ACTCAAAAAC CTAAGCCATA
 +51    ACTAGTCCCT AACCCATAAA CACTAAACCA TAAACCCTAA TCCCTGAACC
+101    ATAACCCTAA ACCATAAATC CTAAAACCCA AACTCTAAAA GTCTAGAGAT
+151    AGAAAAAACA TGTCCCAAGC CCTAAACATT AACATCAAAC GCCAAGCCCT
                                                    AGCCAT
+201    GAAC--CCAA AGCCCTAAGT TTTGAAGGCT GACGC--ATG GACTTTTTAC
       GAACGACCAT TTTCAATAAT ACCGAAGGCT AACACCTATG GATTTTTGAC
+251    CATTAAATGG TCTCCACCAA GAAACAAAAG AATGTGATTT GCGTCCAATC
       CAAGAAATGG TCTCCACC-A GAAATCCAAG AATGTGATCT ATGGC-----
+301    CATAGTAAAA TTTAACCCAA ACAATTGCAA ACATCCAGAC AACATAAACG
       ---------- ---AA-GGAA ACATATG
+351    CTATAGGACA GGGCCAAACA AGTATTGTTT ACAGTCTGGC AATGAGCAAA
+401    TTGTCTGTAA TTTCTTTTTA TATGTTTTGT AAAAAATATC ATTAGTCCCA
+451    TACTTGTTTT TTGAGTGGCC ATAAACTTTC ATTGATGTCC ATAACCAATA
+501    AACATTGAAA TAACACTAAA TATCTTTTAA GATAACAAGG CCTGACCAGT
+551    ATTGTTTAAT AAGCTAGCAA TGAGCAAATT GTAATGAAAA TTGGAATGAA
+601    ATTTCAATTT ATCTAGACCA TAAGGGATGT CTAGAGATAA GAAAATTTTG
+651    GCCCTAATTC AAAAACCTAA GCCATAACCC TAAATTCAAA AACCTAAGGC
+701    CATAACCTAA ACCATGAACC CCAAACACTT AACACAAGAC CCTAAACCTT
+751    GAACCCTAAA GCCCTAAATC ATAAACCCTA ATCCTGAACC ATAACCCTAA
+801    ATCATAAACC ATAAGCCCTA AAGGCCCAAA CCATAGACCC TAAAGGGCTT
+851    TGTACTAAAG GGAACACCTA TGGATTTTTT GCCTCCTGTC CATATAAATG
+901    TCTACACATA AGAAAATGTT TGTCTAACTC AAAAACCTAA GACATAACCC
+951    AAACACTTAG TACCAGTCCC TAACCCATAA ACCCTAAACC ATAAACCTTG
+1001   ATCCTGTGAC CTGCAG
```

FIGURE 7.—Sequence of pZmBs. The region of the knob sequence that shows homology to the B-specific sequence is shown underlined. The consensus helix-loop-helix binding sites for each sequence are enclosed in boxes.

Sequence analysis: The clone pZmBs was sequenced (Figure 7). A feature of one part is that it contains the nucleotides CCCTAAA or some variant (*e.g.*, CCCTAA or CTAAA) spaced at approximately every 15 bp. Using the DNA sequence program of MOUNT and CONRAD (1986), we determined that the longest open reading frame contains 35 codons. However, since CCCTAAA does not consistently fall into frame, it most likely does not represent a coding sequence. The other distinct B-specific sequence of pZmBs is roughly 71% A/T rich.

The FASTA program (PEARSON and LIPMAN 1988) was used to compare pZmBs in both orientations to sequences held at GenBank. It was determined that the CCCTAAA regions of pZmBs were similar to: *Plasmodium falciparum* telomere (55% over nucleotides (nt) 13–1029), *Arabidopsis thaliana* telomere (69% over nt 27–251), *Plasmodium berghei* telomere (54% over nt 27–1046), and *Homo sapiens* telomere (59% over nt 19–362). Southern analysis indicates

that the Arabidopsis telomere probe hybridizes with maize (RICHARDS and AUSUBEL 1988) suggesting a similar telomere sequence. Despite such homologies, the B-specific sequence differs from that of telomeres in several ways. All telomeres sequenced to date consist of short, tandem, nearly identical repeats as a result of the mechanism of action of telomerase (BLACKBURN 1991). While the sequence CCCTAAA appears frequently in pZmBs, the B sequence is neither tandem nor identical. Moreover, the clone pZmBs hybridizes to the centromere region and not the telomeres.

While it is unlikely that a chromosome would have its own unique telomere on only one of its arms, we tested whether the sequences are associated with the tip of the short arm, which is difficult to observe cytologically as a distinct entity from the centromere. As noted above, we probed a B isochromosome (R. W. STAUB and L. E. HILL, personal communication) that is a derivative deleted for the B short arm and duplicated for the entire long arm (Figure 4), and have observed a hybridization pattern indistinguishable from that of normal Bs (Figure 4A). Similarly, *TB10L-18* is broken in the short arm of the B, and hybridization is only detected in plants euploid and hyperploid for the B centromere. Hypoploids, that contain only the translocated copy of the B short arm, show no hybridization to pZmBs. Therefore, we conclude that the sequence does not represent the telomere or subtelomeric region of the short arm of the B.

Another significant homology involves the maize knob (PEACOCK *et al.* 1981), a 180-bp tandem repeat found in most maize lines, that showed a 72% fit over a 90-bp stretch corresponding to nt 195–284 (Figure 7). Normally, knobs are an apparently inert sequence that have little effect on the maize genome. However, in the presence of an unusually large block of heterochromatin on the long arm of chromosome *10* (*K10*), all knobs throughout the genome are used preferentially as spindle attachment sites during meiosis (RHOADES 1952), *i.e.*, they become neocentromeres. This neocentromere formation leads to the preferential recovery of knobbed chromosomes during megasporogenesis. It is not unreasonable that neocentromeres would have similar sequence motifs to normal centromeres since they can act as a facultative substitute. The sequence homology between the knob and the B-specific repeat, coupled with its cytological location, might suggest a centromeric role for the B repeat sequence.

DISCUSSION

Using two distinct differential screen protocols, we have cloned eight phage containing DNAs from the B chromosome of maize. Based on the cytological criteria from B-A translocations, misdivision derivatives, and *in situ* hybridization, the B-specific cluster appears to be derived from the centromere region.

While the B chromosome behaves normally throughout the sporophyte (ALFENITO and BIRCHLER 1990), it functions uniquely in the second pollen mitosis of the gametophyte, leading to the expectation that the B chromosome might contain distinct sequences. This unique mitotic behavior is, at least in part, determined by the centromere. In all B-A translocations induced to date, the B centromere-containing portion undergoes nondisjunction. The cytologically defined centromere is most precisely located between the short arm breakpoint of *TB-10L18* and the long arm breakpoint of *TB-10Sc*, which breaks in the middle of the centric knob. In addition to the centromere, also required in the same nucleus for full nondisjunction levels are the adjacent centric knob, the distal euchromatic tip and some lesser sites (ROMAN 1947; CARLSON and CHOU 1981; CARLSON 1988). For example, when the proximal heterochromatin (centric knob) is deleted, the chromosome disjoins properly at the second microspore division (CARLSON and CHOU 1981). This observation could perhaps argue, that, in fact, it is the knob adjacent to the centromere, that is responsible for nondisjunction. However, with *TB-10Sc*, which divides the knob, the B centromere-containing chromosome nondisjoins but the reciprocal chromosome does not (BECKETT 1975). Therefore, it appears that the centromere, as well as other sequences, are required for the nondisjunction property. It is interesting to note in this regard that the B centromeric region, which behaves differently from the A centromeres, coincidently contains a major B-specific sequence cluster.

The sequence analysis indicates there is homology to telomeres in many plant and animal systems but the sequences in the B chromosome are not arranged in a pattern typical of telomeres. Telomere-degenerate sequences have been found at the centromeres in many vertebrate species by *in situ* analysis (MEYNE *et al.* 1990) and in *A. thaliana* by genetic mapping (RICHARDS, GOODMAN, and AUSUBEL 1991). Thus, in such diverse organisms as vertebrates, dicots and monocots, there appears to be a related sequence present at the centromeres.

There is also homology over a portion of the sequence to the maize neocentromere (knob). Because the knob can facultatively function as a centromere, sequence homology or similar structural motifs to bonafide centromeres might be expected, suggesting a role of the B-specific repeat in centromere functions. Spaced 3' to the region of homology, there is present in both, a consensus helix-loop-helix binding site (CANNTG), that is a requisite component of yeast centromeres (CAI and DAVIS 1990). Eighteen individ-

ual repeats of the knob repeat have been sequenced from maize, teosinte, and Tripsacum (DENNIS and PEACOCK 1984). The invariant nucleotides among the eighteen sequences surround the helix-loop-helix consensus site as well as some of the regions of homology to the B-specific repeat. This conservation implies a functional constraint on the evolution of these portions of the nucleotide sequence. Other than the above mentioned feature, we found no significant homology with centromere sequences from yeast (for review see CLARKE 1990; CLARKE *et al.* 1986). We note, however, that GRADY *et al.* (1992) found that an oligonucleotide patterned after yeast centromere sequences was present at the centromeres of humans. This oligonucleotide also hybridizes to maize DNA.

The juxtaposition of a normal centromere and a knob on the B chromosome is an unusual arrangement in maize. The neocentromere sequence isolated from the A chromosomes shows weak labeling to the B knob but not to centromeres of A or B chromosomes (PEACOCK *et al.* 1981). Likewise, the B-specific repeat only labels the B chromosome centromere in *in situ* hybridizations (and Southern transfers), showing no labeling of A centromeres or neocentromeres, despite the partial homology to the latter detected by sequence comparisons. The neocentromere organization is a tandem repeat of 180 bp without interspersion of CCCTAAA motifs, which appears to be typical of normal centromere regions in several organisms.

We hypothesize that the B-specific cluster is at least one of the functional components of the maize B chromosome centromere, based on its cytological localization and its homology to a facultative centromere. Among higher eukaryotes, the maize neocentromere repeat was the first cloned sequence for which centromere function is known. The neocentromere is composed of a tandem array of a 180-bp sequence. Human sequences have been cloned that have been implicated as components of centromeres. One sequence, the alpha satellite, is located at centromere regions and produces anaphase bridges when introduced into African green monkey cells (HAAF, WARBURTON and WILLARD 1992). The alphoid sequence contains a helix-loop-helix binding site (MASUMOTO *et al.* 1989) that associates with the CENP-B protein component of human centromeres (PLUTA *et al.* 1990). When antibody staining for CENP-B is combined with *in situ* hybridization using a CCCTAA probe in Chinese hampster cells, the kinetochore shows a repetitive pattern of antibody binding that is flanked by regions of CCCTAA (ZINKOWSKI, MEYNE and BRINKLEY 1991). Another repeat termed, sn5, also localizes to the centromeres (JOHNSON *et al.* 1992). Yet a third sequence has been isolated that exhibits evolutionary conservation, localizes to centromeric regions, and shows sequence similarity to yeast centromeres (GRADY *et al.* 1992). The relationship of these to each other is not yet clear. Neither is the overall centromere structure. Because of the misdivision products that are available for the B centromere, it can be argued that the localization of the B-specific cluster is within the region that functions as a centromere—an argument corroborated by an homology to the neocentromere. Further study will be required to determine the nature of the B-specific sequences and other types of centromeric repeats as well as the general organization of the centromere.

We would like to thank MARCUS RHOADES and ELLEN DEMPSEY for supplying us with the High Loss line, RICK STAUB for the iso-B chromosome, and WAYNE CARLSON for *TB-9Sb-1852* and *TB-9Sb-2820*. The *in situ* analysis would not have been possible without the expert assistance, time and facilities of RON PHILLIPS and SUZANNE LIVINGSTON. We thank JIEHUA YE for technical assistance. MIKE CUMMINGS is gratefully acknowledged for his patient assistance with the computer analysis.

LITERATURE CITED

ALFENITO, M. R., and J. A. BIRCHLER, 1990 Studies on B chromosome stability during development. Maydica **35:** 359–366.

BECKETT J. B., 1975 Genetic breakpoints of the B-A translocations of maize. Maize Genet. Coop Nsltr. **49:** 130–134.

BECKETT, J. B., 1991 Cytogenetic, genetic and plant breeding applications of B-A translocations in maize, pp. 493–529 in *Chromosome Engineering in Plants: Genetics Breeding and Evolution,* edited by P. K. GUPTA and T. TSUCHIYA. Elsevier, New York.

BLACKBURN, E. H., 1991 Structure and function of telomeres. Nature **350:** 569–573.

CAI, M., and R. W. DAVIS, 1990 Yeast centromere binding protein CBF1, of the helix-loop-helix protein family, is required for chromosome stability and methionine prototrophy. Cell **61:** 437–446.

CARLSON, W. R., 1977 The cytogenetics of corn, pp. 225–304, in *Corn and Corn Improvement,* edited by G. F. SPRAGUE. American Society of Agronomy, Madison, Wisc.

CARLSON, W. R., 1986 The B chromosome of maize. CRC Crit. Rev. Plant Sci. **3:** 201–226.

CARLSON, W. R., 1988 B chromosomes as a model system for nondisjunction, pp. 199–207 in *Aneuploidy, Part B: Introduction and Test Systems.* Alan R. Liss, New York.

CARLSON, W. R., and T. S. CHOU, 1981 B chromosome nondisjunction in corn: control by factors near the centromere. Genetics **97:** 379–389.

CARLSON, W. R., and R. R. ROSEMAN, 1992 A new property of the maize B chromosome. Genetics **131:** 211–223.

CHEN, J., I. M. GREENBLATT and S. DELLAPORTA, 1987 Transposition of *Ac* from the *P* locus of maize into unreplicated chromosomal sites. Genetics **117:** 109–116.

CHILTON, M.-D., and B. J. MCCARTHY, 1973 DNA from maize with and without B chromosomes: a comparative study. Genetics **74:** 605–614.

CLARKE, L., 1990 Centromeres of budding and fission yeasts. Trends Genet. **6:** 150–154.

CLARKE, L., H. AMSTUTZ, B. FISHEL and J. CARBON, 1986 Analysis of centromeric DNA in the fission yeast *Schizosaccharomyces pombe.* Proc. Natl. Acad. Sci. USA **83:** 8253–8257.

DARLINGTON, C. D., 1939 Misdivision and the genetics of the centromere. J. Genet. **37:** 341–364.

DENNIS, E. S., and W. J. PEACOCK, 1984 Knob heterochromatin homology in maize and its relatives. J. Mol. Evol. **20:** 341–350.

FEINBERG, A. P., and B. VOGELSTEIN, 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13.

GRADY, D. L., R. L. RATLIFF, D. L. ROBINSON, E. C. MCCANLIES, J. MEYNE and R. K. MOYZIS, 1992 Highly conserved repetitive DNA sequences are present at human centromeres. Proc. Natl. Acad. Sci. **89:** 1695–1699.

GRUNSTEIN, M., and D. HOGNESS, 1975 Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. **72:** 3961–3965.

HAAF, T., P. E. WARBURTON and H. F. WILLARD, 1992 Integration of human alpha-satellite DNA into simian chromosomes: centromere protein binding and disruption of normal chromosome segregation. Cell **70:** 681–696.

HAKE, S., and V. WALBOT, 1980 The genome of *Zea mays*, its organization and homology to related grasses. Chromosoma **79:** 251–270.

JOHNSON, D. H., P. M. KROISEL, H. J. KLAPPER and W. ROSENKRANZ, 1992 Microdissection of a human marker chromosome reveals its origin and a new family of centromeric repetitive DNA. Hum. Mol. Genet. **1:** 741–747.

JONES, R. N., and H. REES, 1982 *B Chromosomes.* Academic Press, New York.

LIN, B.-Y., 1979 Two new B-10 translocations involved in the control of nondisjunction of the B chromosome in maize. Genetics **92:** 931–945.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MASUMOTO, H., H. MASUKATA, Y. MURO, N. NOZAKI and T. OKAZAKI, 1989 A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromere satellite. J. Cell Biol. **109:** 1963–1973.

MEYNE, J., R. J. BAKER, H. H. HOBART, T. C. HSU, O. A. RYDER, O. G. WARD, J. E. WILEY, D. H. WURSTER-HILL, T. L. YATES and R. K. MOYZIS, 1990 Distribution of nontelomeric sites of the (TTTAGGG) in telomeric sequence on vertebrate chromosomes. Chromosoma **99:** 3–10.

MOUNT, D. W., and B. CONRAD, 198 Improved programs for DNA and protein sequence analysis on the IBM personal computer and other computer systems. Nucleic Acids Res. **14:** 443–454.

PEACOCK, W. J., E. S. DENNIS, M. M. RHOADES and A. PRYOR, 1981 Highly repeated DNA sequence limited to knob heterochromatin in maize. Proc. Natl. Acad. Sci. **78:** 4490–4494.

PEARSON, W. R., and D. J. LIPMAN, 1988 Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:** 2444–2448.

PHILLIPS, R. L., and A. S. WANG, 1982 *In situ* hybridization with maize meiotic cells, pp. 121–122 in *Maize for Biological Research*, edited by W. F. SHERIDAN. Plant Molecular Biology Association, Charlottesville, Va.

PLUTA, A. F., N. SAITOH, I. GOLDBERG and W. C. EARNSHAW, 1992 Identification of a subdomain of CENP-B that is necessary and sufficient for localization to the human centromere. J. Cell Biol. **116:** 1081–1093.

RABINOW, L. J., and J. A. BIRCHLER, 1989 A dosage-sensitive modifier of retrotransposon-induced alleles of the Drosophila *white* locus. EMBO J. **8:** 879–889.

RANDOLPH, L. F., 1941 Genetic characteristics of the B-chromosomes in maize. Genetics **26:** 608–631.

RICHARDS, E. J., and F. M. AUSUBEL, 1988 Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. Cell **53:** 127–136.

RICHARDS, E. J., H. M. GOODMAN and F. M. AUSUBEL, 1991 The centromere region of *Arabidopsis thaliana* chromosome 1 contains telomere-similar sequences. Nucleic Acids Res. **19:** 3351–3357.

RHOADES, M. M., 1952 Preferential segregation in maize, pp. 66–80 in *Heterosis*, edited by J. W. GOWEN. Iowa State Press, Ames.

RHOADES, M. M., 1968 Studies on the cytological basis of crossing over, pp. 229–241 in *Replication and Recombination of Genetic Material*, edited by W. J. PEACOCK and R. D. BROCK. Australian Academy of Science, Canberra.

RHOADES, M. M., and E. DEMPSEY, 1973 Chromatin elimination induced by the B chromosome of maize. I. Mechanism of loss and the pattern of endosperm variegation. J. Hered. **64:** 13–18.

ROMAN, H., 1947 Mitotic nondisjunction in the case of interchanges involving the B-type chromosome in maize. Genetics **32:** 391–409.

SANGER, F., S. NICKLEN and A. R. COULSON, 1977 DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.

SEARS, E. R., 1952 Misdivision of univalents in common wheat. Chromosoma **4:** 535–550.

ZINKOWSKI, R. P., J. MEYNE and B. R. BRINKLEY, 1991 The centromere-kinetochore complex: a repeat subunit model. J. Cell Biol. **113:** 1091–1110.

Communicating editor: W. F. SHERIDAN