The EMBO Journal vol.15 no.19 pp.5246–5255, 1996

Misdivision analysis of centromere structure in maize

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The size and organization of a representative plant centromere from the supernumerary B chromosome were determined using a repeated sequence specific to the centric region. Several derivatives of the B chromosome that suffered from misdivision of the centromere were analyzed for the content and organization of their B repeat. In all these derivatives, major rearrangements were detected. Some misdivisions produced a significant reduction in size of the B-specific cluster. These results demonstrate that the B repeat is part of the functional centromere, that it is spread throughout its length, and that plant centromeres are composed of repeat units that can be significantly changed in copy number without a change in function.
Keywords: B chromosome/centromere/meiosis/misdivision/*Zea mays*

Introduction

Equal partitioning of chromosomes is a crucial step of cell division in eukaryotes and requires special machinery for the duplicated DNA to be divided precisely between the two daughter cells. Proper segregation of chromosomes is insured by the interactions between the spindle fibers and the kinetochore, an integrated protein and nucleic acid complex that may also contain the motor necessary for chromatid movement towards the spindle poles during anaphase (Bloom, 1993; Pluta *et al.*, 1995). The kinetochore proteins assemble on a particular chromosomal domain, the centromere. This *cis*-acting DNA element is required for segregation of sister chromatids at the onset of anaphase during mitosis and meiosis II, and is also the point of attachment of chromosome homologs at the first meiotic division.

In some unicellular eukaryotes the structure of the centromere and the minimal functional elements have been precisely defined. A specific DNA segment only 125 bp in length is sufficient to provide segregation in the budding yeast *Saccharomyces cerevisiae* (Clarke and Carbon, 1985; Clarke, 1990). In contrast, the centromere regions from the fission yeast *Schizosaccharomyces pombe* contain blocks of repetitive, heterochromatin-like, untranscribed DNA sequences of 40–100 kb in size on each of the three *S.pombe* chromosomes (Clarke, 1990; Clarke *et al.*, 1993). Very little is known about the size and complexity of centromeres in the genomes of multicellular eukaryotes. In humans, the tandemly repeated α satellite or alphoid sequence is found in diverged forms at all centromeres. Monomers of 171 bp are present in tandem repeats organized further into chromosome-specific higher order units in arrays of 250–>4000 kb (Wevrick and Willard, 1989; Warburton and Willard, 1990; Willard, 1990). In *Drosophila*, the minichromosome *Dp1187* (Karpen and Spradling, 1990) contains centric heterochromatin of 1 Mb in size and contains three islands of complex DNA (single copy or middle repetitive DNA), interspersed with blocks of highly repetitive satellite DNA (Le *et al.*, 1995; Murphy and Karpen, 1995).

In plants, very little is known about the organization of centromeres. Recently, a centric repeated sequence has been recovered from a maize supernumerary or B chromosome (Alfenito and Birchler, 1993), which has allowed us to examine the structure of a plant centromere. This sequence was initially found in a search for those specific to the B chromosome. B chromosomes are highly heterochromatic, contain few or no active genes and are dispensable (Jones and Rees, 1982). Their maintenance in populations is due to an accumulation mechanism. During plant development, the maize B chromosome behaves exactly as any of the normal A chromosomes in all mitoses except one. During maturation of the pollen grain, the B chromosome frequently nondisjoins at the second microspore division, which results in one sperm cell with two B chromosomes and the other with none (Roman, 1947; Carlson and Chou, 1981). The sperm with the B chromosomes preferentially joins with the egg, as opposed to the polar nuclei in the process of double fertilization (Roman, 1948). Thus, despite the unusual properties of the B chromosome, it behaves in a similar manner to the A chromosome with regard to centromere activity and can serve as a model for the study of centromere structure and function.

A functional role was proposed for the B-specific repeat based on its localization at the centromere and a strong homology to the maize knob sequence (Alfenito and Birchler, 1993). Knobs are composed of a tandem repeat of 185 bp in length and behave as a centromere during meiosis in the presence of a variant of chromosome 10 (Rhoades and Vilkomerson, 1942; Rhoades, 1978; Peacock *et al.*, 1981). On the assumption that the B-specific sequence is one component of the centromere, we undertook a detailed study to determine the organization of the B repeat cluster. The chromosome-specific nature of the probe allows one to follow a single centromere in the karyotype using this system.

We find that the B-specific sequence is a degenerate tandem array that extends over >9 Mb of DNA. The functional region of the centromere is defined by breakpoints in a collection of misdivision derivatives. Misdivision of univalent centromeres occurs at meiosis and results in breaks within the centromere. As observed

frequently at anaphase I, a univalent will divide such that the two arms will often migrate to opposite poles and form new chromosomes. Misdivision has been described in early cytogenetic studies in *Fritillaria* (Darlington, 1939) and wheat (Sears, 1952). In maize, a number of misdivision derivatives have been described for the B chromosome (Carlson, 1970, 1978) and are available to study rearrangements in the centromere region. Pulsed-field gel electrophoresis analysis of those derivatives shows that following misdivision only a fraction of the B-specific cluster remains, suggesting that the B-specific sequence is present in the functional centromere and is spread over its length. This analysis reveals that a significant latitude of sequence organization occurs for centromeres and that they can be extensively subdivided but still retain function.

Results

The B-specific sequence is a degenerate tandem array

The initial cloning of B centromeric DNA yielded several fragments which had in common a sequence that hybridized specifically to the genomic DNA from maize lines containing B chromosomes (Alfenito and Birchler, 1993). When DNA from a line with several B chromosomes, digested with *Acc*I, was hybridized with the B-specific clone, an intense band of 1.4 kb was detected, along with other bands of lesser intensity. The 1.4 kb fragment represents the plurality of the B repeat cluster. It is organized in tandem, as suggested by the fact that digests with several restriction enzymes (*Acc*I, *Dpn*II, *Alu*I, *Hae*III), each recognizing only one site within the repeat, all produce a pattern with a major band at 1.4 kb. Multiple fragments of other molecular size were also detected, indicating degeneracy of the sequences.

To determine the extent of the tandem organization, partial digests were made with *Dpn*II. The rationale is that upon digestion with a dilution series of *Dpn*II of DNA from a genotype that contains two normal B centromeres in a reciprocal translocation (TB-9Sb) between the B chromosome and the short arm of chromosome 9, which carries a genetic marker, an electrophoretic ladder of bands should appear if the B repeat is organized in an exact tandem array. Alternatively, if nonhomologous interspersed sequences are present between each unit, then the size of the bands will not be an exact multiple of the monomer. In this case, no ladder will be seen. Complete digestion shows a complex pattern, with bands of <1 kb to up to 10 kb (Figure 1). The strongest signal corresponds to the most abundant monomer of ~1.4 kb in size. Upon dilution of *Dpn*II, new bands appear with a strong signal (Figure 1, arrows). Their size is an exact multiple of the basic unit (i.e. 2.8, 4.2, 5.6 kb). The progressive disappearance of the monomer corresponds to the appearance of fragments of higher molecular weight. The same dilution series was subjected to clamped homogeneous electric fields (CHEF) electrophoresis (separation in the range 1–20 kb) to detect higher size fragments that would represent five, six or more copies in tandem. No discrete band that would represent such multiples was found (data not shown). We conclude that the B repeat is arranged in tandem up to four units, with no interspersed sequence.

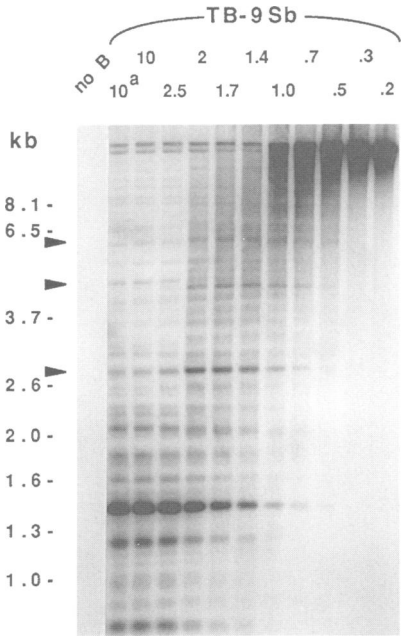

Fig. 1. Partial restriction digest of genomic DNA from the translocation TB-9Sb that carries two copies of the normal B centromere. A dilution series of *Dpn*II from 0.2 to 10.0 units, indicated above each lane, was chosen for the digest of 1.5 μg DNA for 40 min at 37°C. *Dpn*II recognizes one restriction site within the B-specific repeat. The first lane corresponds to a maize line with no B chromosomes. The Southern blot was probed with the B-specific sequence. Bands corresponding to multiples of the basic unit of 1.4 kb are indicated by arrows. [a]Digest with 10 units of *Dpn*II for 3 h.

There is, however, a considerable number of fragments of different size, illustrating the heterogeneity within the B repeat cluster. This result could occur because of an interspersion of other sequences among tandem arrays or by divergence of the sequences within the overall cluster.

Higher level organization of the B repeat cluster

As a control for the analysis of changes in centromere structure, normal B chromosomes, propagated independently for several decades, were examined on CHEF gels to determine the degree of polymorphism in the B-specific cluster. High molecular weight DNA was prepared from maize lines with normal B centromeres [Black Mexican Sweet (BMS)+B, High Loss+B, TB-9Sb hyperploid; see Materials and methods for description of the genotypes], digested with various restriction enzymes that rarely cut within the B-specific cluster and hybridized with the B-specific sequence probe (Figure 2). The first two of these lines contain unaltered B chromosomes.

A conventional Southern analysis of the B-specific cluster revealed several 6 bp recognition restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII) that did not generate fragments small enough to be resolved by this method. This result suggests that the B repeat cluster has a sequence composition atypical of maize DNA and that these enzymes could be used effectively to analyze larger fragments in this chromosomal region.

Restriction fragments generated by *Eco*RI are numerous and highly variable in size (in the range 20–>200 kb), as seen by CHEF analysis (Figure 2A). There are no *Eco*RI recognition sites in the canonical B-specific repeat, so these cleavages might represent interspersed sequences or

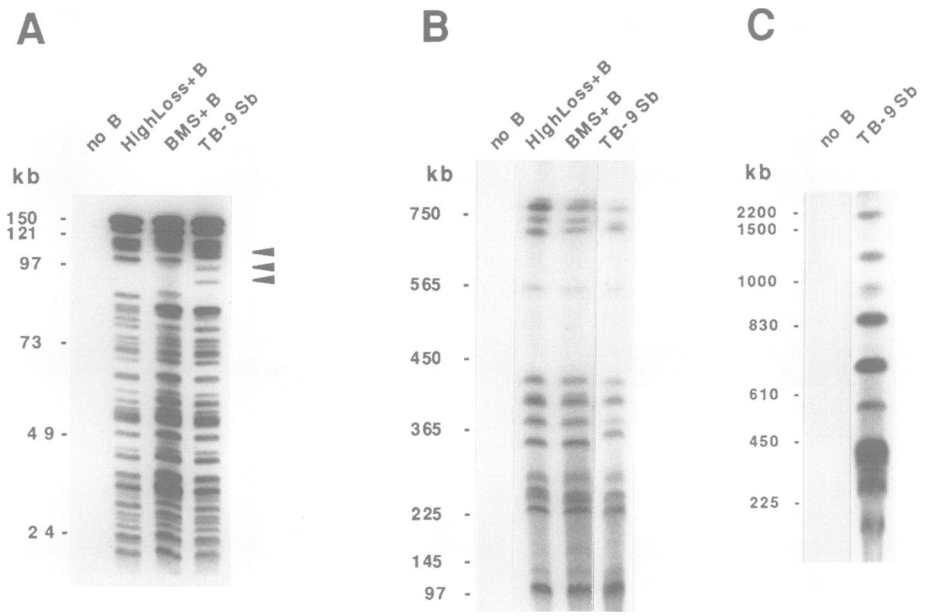

Fig. 2. Size and organization of the B centromeric region determined by a CHEF analysis. High molecular weight DNA from maize lines containing normal B chromosomes, as indicated, was digested with restriction endonucleases, subjected to CHEF electrophoresis, Southern transferred and hybridized to the B-specific sequence. CHEF conditions were as follows. (**A**) 1% agarose for 26 h at 200 V in 0.5× TBE with a ramped pulse time from 1.8 to 5.1 s. Three additional bands of 90, 100 and 110 kb unique to TB-9Sb are indicated by arrows. (**B**) 1% agarose for 26 h at 200 V in 0.5× TBE with a ramped pulse from 20 to 50 s. (**C**) 1% agarose for 21 h at 200 V in 0.5× TBE with a ramped pulse time from 50 to 90 s. Size estimates were made using *S.cerevisiae* chromosomes or λ DNA concatemers as size standards.

nucleotide substitutions in the repeats that created *Eco*RI sites. In addition, the intensity of the signal varies among the bands, indicating that the fragments are present in multiples and/or they are more or less densely populated by the B repeat. The restriction patterns for the BMS and High Loss lines and the translocation are almost identical. The B cluster was also examined with the 8 bp recognition enzyme *Pme*I (Figure 2B). Digested fragments are fewer than with the 6 bp recognition enzymes, but there is a large variation in size from 50 to >800 kb. Using different electrophoretic parameters (Figure 2B and C), the cluster of fragments >700 kb can be separated. Five fragments are detected that vary in size from 700 to >2000 kb.

There are few banding pattern changes between the lines with several B chromosomes and a line with two copies of the TB-9Sb translocation. Changes that have occurred since the creation of B–A translocations ~50 years ago are minor. Compared with the complex restriction pattern in a normal B (Figure 2A), three additional bands of 90, 100 and 110 kb are unique to TB-9Sb (Figure 2A, arrows). No major changes can be detected among the three lines after a *Pme*I digest (Figure 2B). These results indicate that there is little polymorphism among the B chromosomes in laboratory collections of maize, which allows a comparative study of misdivision derivatives presented below.

A CHEF analysis with other rare cutters was not as revealing. Some enzymes generated several discrete bands of ≤1 Mb (*Mlu*I, *Nru*I, *Sfi*I, *Sma*I, *Pvu*II), along with very large fragments of >2 Mb. These enzymes are methylation sensitive. Plant DNA is usually highly methylated at CG and CNG sites (Gruenbaum *et al.*, 1981), especially in transcriptionally inactive regions. Control hybridizations, indicating complete restriction digests, were made with single-copy genes that show unique restriction patterns (data not shown).

This region was analyzed further by double digests with *Pme*I and *Eco*RI. The complexity of the restriction pattern was clarified by using 2-D CHEF electrophoresis. High molecular weight DNA from TB-9Sb was digested with *Pme*I and subjected to CHEF (first dimension). The restricted *Pme*I fragments were then digested with *Eco*RI (second dimension), which generated smaller fragments in the range 20–200 kb. Thus we can build an inventory of *Eco*RI fragments for each *Pme*I fragment. There is a large variability in size among the *Eco*RI fragments (Figure 3A). In general, the sum of the *Eco*RI fragments approaches the size of the respective *Pme*I fragment. Therefore there are not multiple identical *Eco*RI fragments in most *Pme*I fragments. This result also indicates that there is a minimum of interspersed sequences without homology to the B-specific repeat. Interestingly, three *Pme*I fragments (350, 370 and 400 kb) generate *Eco*RI fragments of 55 kb (see arrow).

Misdivision causes rearrangements within the centromere

Misdivision refers to the observation that at meiosis a univalent chromosome divides such that various members of the replicated arms go to opposite poles (Figure 4). Telocentric chromosomes and isochromosomes are often the stable products of misdivision (Figure 4). An isochromosome will be produced if the broken chromosome fuses with itself. Thus, it consists of two identical arms attached to one another via the centromeric region. If there is no such repair, a telocentric chromosome is formed, presumably by healing via the addition of telomeric sequences to the broken end (McClintock, 1941, 1942). Although misdivision causes breaks within functional domains, it generates fragments that are still able to attach to mitotic spindle fibers. This fact suggests that the centromere is a repetitive structure, portions of which

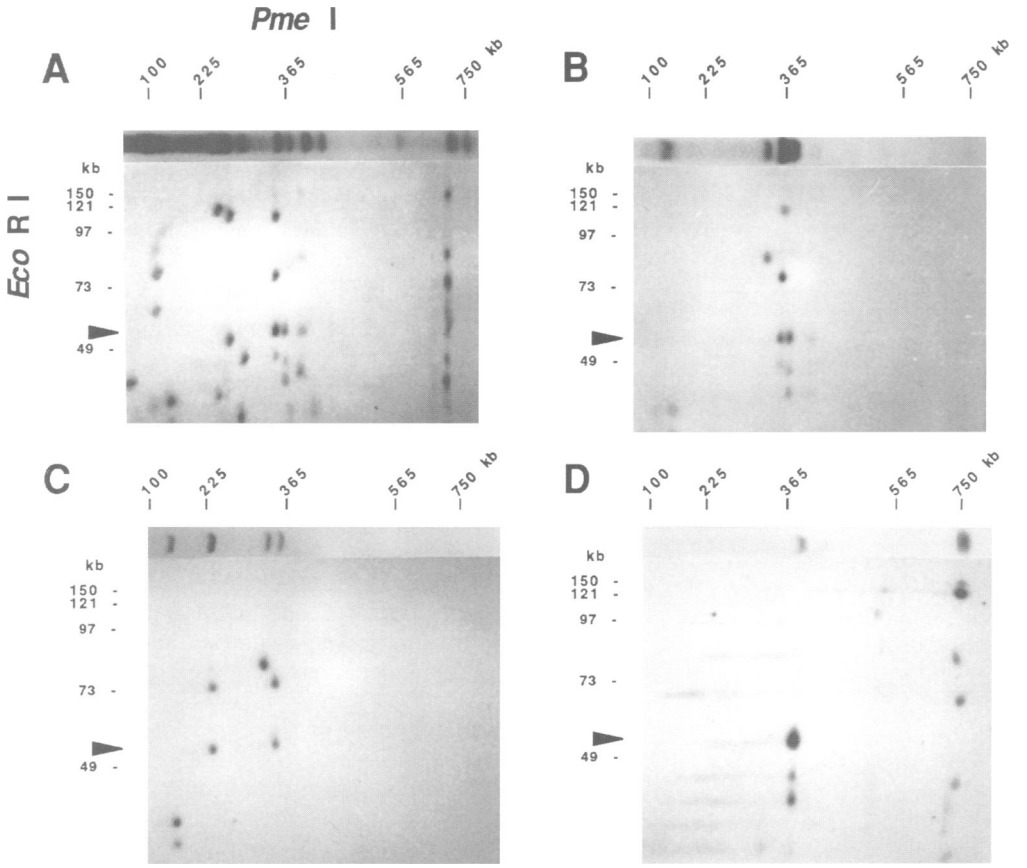

Fig. 3. A 2-D analysis of *Pme*I fragments in the B-specific cluster of TB-9Sb and several misdivision derivatives. *Pme*I digests of high molecular weight DNA from TB-9Sb and selected derivatives were subjected to redigestion with *Eco*RI and run on CHEF electrophoresis. The Southern blotted gel was hybridized to the B-specific sequence. First dimension conditions: 1% agarose for 26 h at 200 V in 0.5× TBE with a ramped pulse from 20 to 50 s. Second dimension conditions: 1% agarose for 26 h at 200 V in 0.5× TBE with a ramped pulse time from 1.8 to 5.1 s. (**A**) TB-9Sb. (**B**) Telo2-1(–). (**C**) Ring4-1(–). (**D**) Ring1(+). A 55 kb fragment found in each genotype is indicated by an arrow. Size estimates were made using *S.cerevisiae* chromosomes or λ DNA as size standards.

can still function. Early observations on the formation of ring chromosomes led to cytological evidence for this conclusion (McClintock, 1932).

The study of misdivision through an analysis of a collection of derivatives of the B chromosome addressed the precise localization of the B-specific repeat within the centromeric region. TB-9Sb is the progenitor chromosome. It is a reciprocal translocation between part of the B chromosome and the short arm of chromosome 9, the two components being referred to as B-9 and 9-B. The first derivative recovered was a pseudoisochromosome (also referred to as pseudoiso) involving the B-9 chromosome of the translocation (Carlson and Chou, 1981). In one arm this new chromosome has all the features of the normal B-9. In the other arm, there is a break in the euchromatic portion of the B long arm. Therefore this new arm has a deletion for the proximal part of the long arm which includes the knob and a portion of the centromere. Many telocentrics and isochromosomes were derived from this pseudoiso via misdivision. In one class the adjacent knob is missing; in the other class the knob is present (Carlson, 1978, 1986, 1988). For presentation purposes we codified these derivatives according to their chromosomal constitution. This classification includes the type of chromosome (telo, iso or ring), followed by the number of misdivisions that created the derivative, and also in parentheses the presence (+) or absence (–) of the centric knob. Different

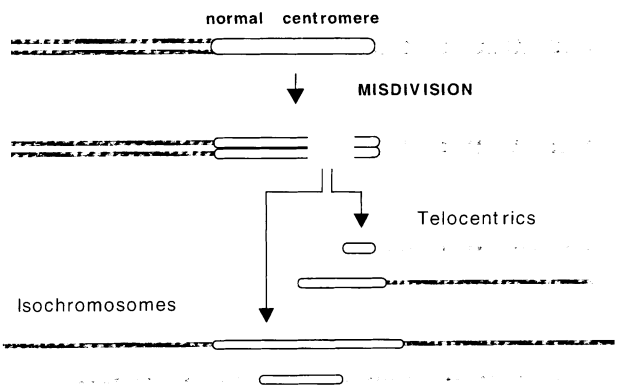

Fig. 4. Misdivision of the centromere. At meiosis, the centromere of a univalent chromosome may undergo fission such that one replicated arm goes to one pole and the other arm goes to the opposite pole. If the broken chromosome repairs, an isochromosome will be produced that is a mirror image of the broken centromere. If it does not repair, a telocentric is formed, presumably by healing via the addition of telomeric sequences.

isolates with the same chromosome constitution and products of the same number of misdivisions are numbered. For example, a knobless telocentric derived directly from misdivision of the pseudoiso will be called telo2(–), and two ring chromosomes derived from iso3(–) will be referred to as ring4-1(–) and ring4-2(–). These derivatives are displayed diagrammatically in Figure 5.

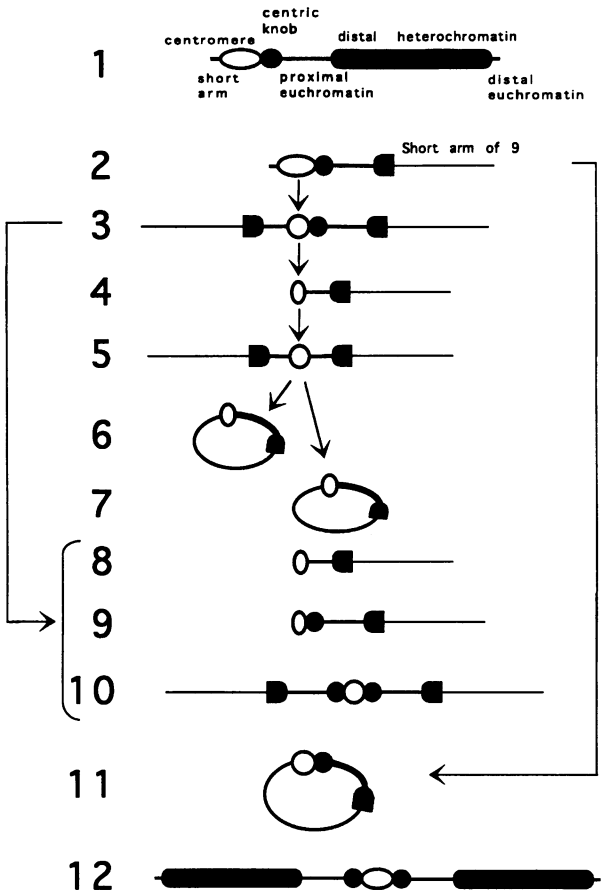

Fig. 5. Schematic representation of a normal maize B chromosome, a translocation between the B chromosome and chromosome 9, and a collection of misdivision derivatives of the B centromere. Line 1: normal B chromosome; the cytological landmarks are noted. Line 2: a translocation (TB-9Sb) between the B chromosome, including the centromere and part of the short arm of chromosome 9. Telocentrics and isochromosomes can be generated via misdivision of the centromere of the translocation TB-9Sb. Line 3: the first misdivision generated a pseudoisochromosome. The broken centromere became attached to the euchromatic region adjacent to the knob. This arm suffered from a partial deletion, including the centric knob and part of the proximal euchromatin. This original pseudoisochromosome often undergoes misdivision. A number of isochromosomes and telocentrics were formed, some of which do or do not contain the centric knob. Line 4: telocentric chromosome derived from the pseudoisochromosome and referred to as telo2-1(–). Line 5: isochromosome or iso3(–) derived by misdivision of telo2-1(–). Line 6: a ring chromosome derived from iso3(–) (line 5), and referred to as ring4-1(–). Line 7: ring4-2(–) derived from iso3(–) (line 5). Line 8: telo2-2(–), derived from (3). (9) Telo2(+), also derived from (3). (10) Iso2(+), derived from the pseudoisochromosome in (3). Line 11: a ring chromosome referred to as ring1(+) formed after misdivision of the normal TB-9Sb. Line 12: isochromosome of the entire B long arm, formed after misdivision of the normal B chromosome.

We can follow the succession of rearrangements caused by misdivision, beginning with TB-9Sb, followed by the pseudoiso, then telo2-1(–), iso3(–) and finally two ring chromosomes, ring4-1(–) and ring4-2(–) (Figure 5, lines 2–7). Other isochromosomes and telocentrics derived from the pseudoiso provide additional cases of misdivision for study (Figure 5, lines 8–10). In addition, an iso-B long arm chromosome (or iso-BL) was derived independently from the normal B chromosome by misdivision of the centromere of a univalent B (Figure 5, line 12). The iso-BL no longer has any portion of the minute short arm, but is duplicated for the long arm.

The collection of misdivision products also includes a ring chromosome derived directly and independently from the normal B-9 chromosome of TB-9Sb (Figure 5, line 11; Carlson, 1973). Ring chromosomes can be formed from a tertiary trisomic (Carlson, 1973; Ghidoni, 1973). Such an aneuploid consists of the normal diploid complement 9/9 and the portion of 9 carried on the B-9. At meiosis, the B-9, which behaves as a univalent, apparently suffers a misdivision of the centromere. Ring chromosomes are generated when the end of 9S and the broken B centromere fuse.

All these aberrations would be expected to have rearrangements of the centromere sequences because of the divisive nature of the misdivision phenomenon. In a telocentric, the centromere would probably be reduced in size (Figure 4). In an isochromosome, it would be unchanged, reduced or perhaps increased compared with the progenitor. If centromeres consist of degenerate repetitive structures, one might expect a lesser complexity in structure in a telocentric or in an isochromosome relative to its progenitor.

Misdivision of the centromere reduces the size and complexity of the B-specific cluster

As an initial step, we attempted to detect differences between the various derivatives of TB-9Sb by CHEF analysis. In a conventional Southern analysis, several restriction enzymes were found not to fragment the B-specific cluster. Upon CHEF gel separation, it was revealed that these enzymes produced fragments ranging from 20 to >200 kb. After digestion with one such enzyme (*Eco*RI) and hybridization with the B-specific sequence, a significant reduction in complexity with every misdivision derivative was found, illustrated by fewer restriction fragments (Figure 6A). The number of fragments homologous to the B-specific sequence is reduced by the first misdivision that generates the pseudoiso (Figure 6A, lanes 2 and 3). Most fragments remaining in the pseudoiso are also seen in the normal TB-9Sb. The missing fragments are presumably part of the centromere that is closer to the short arm, which was deleted upon misdivision.

The next series of breaks leading to the formation of several isochromosomes and telocentrics causes changes that are less extensive than the one resulting from the first misdivision. These derivatives have a variety of restriction patterns that are all unique for the respective products analyzed (Figure 6A, lanes 4 and 8–10), although several fragments remain present in all derivatives. The effect of a third misdivision associated with the formation of an isochromosome from telo2-1(–) was analyzed with the derivative iso3(–) (Figure 6A, lane 5). This chromosome and its progenitor have an almost identical restriction pattern. They only differ in that the isochromosome has additional fragments, one of ~60 kb, and two others >120 kb. Other digests with *Hin*dIII and *Bgl*II (data not shown) failed to detect any differences between these derivatives. A fourth misdivision simplifies the restriction pattern further. Ring4-1(–) and ring4-2(–) retain some fragments in common with the parental isochromosome (Figure 6A, lanes 6 and 7). Ring4-1(–) shows additional

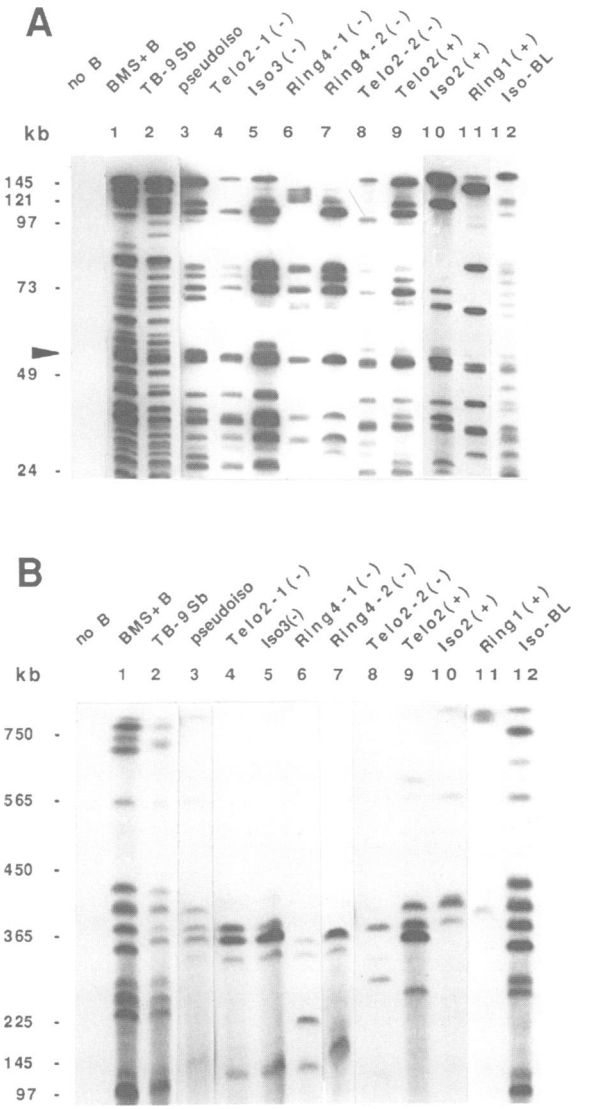

Fig. 6. Size of the B-specific region in several misdivision derivatives analyzed by CHEF. High molecular weight DNA was digested with the restriction enzyme *Eco*RI, subjected to CHEF electrophoresis, Southern blotted and hybridized to the B-specific sequence. CHEF conditions were as follows. (**A**) 1% agarose for 26 h at 200 V in 0.5× TBE with a ramped pulse time from 1.8 to 5.1 s. The genotypes are: BMS+B, TB-9Sb hyperploid, pseudoisochromosome, telo2-1(–), iso3(–), ring4-1(–), ring4-2(–), telo2-2(–), telo2(+), iso2(+), ring1(+) and isoBL chromosome. A 55 kb fragment in common with all derivatives is indicated by an arrow. (**B**) 1% agarose for 26 h at 200 V in 0.5× TBE with a ramped pulse from 20 to 50 s. *Pme*I was used for the digests of the same genotypes as for *Eco*RI. The numbers below the genotypes refer to the original descriptions of the derivatives in Figure 5. Lanes corresponding to the various derivatives were assembled from different Southern transfers. All derivatives were analyzed along with TB-9Sb in the same gel as a reference genotype for band sizing. Size estimates were made using *S.cerevisiae* chromosomes or λ DNA as size standards.

fragments of 80 and 130 kb. Ring4-2(–) displays a loss of only a few fragments and the addition of an extra 45 kb band.

One independent misdivision, namely the ring1(+), reduces the size of the B-specific cluster by a very large amount (Figure 6, lane 11). The restriction pattern is very simple, although the chromosome has undergone only one misdivision. Another independent misdivision event represented by the iso-BL chromosome led to substantial changes when compared with the normal B chromosome (Figure 6B, lanes 1 and 12). All these derivatives (10 in total) have a distinct restriction pattern, but one fragment of ~55 kb is common to all.

Digests with *Pme*I also show that there is a tremendous reduction in the number of B-specific fragments after the first misdivision (Figure 6B). Subsequent misdivisions also reduce the B-specific cluster to a simplified structure, as illustrated by fewer remaining fragments in every derivative (Figure 6B, lanes 2–11). In particular, the rings derived from iso3(–) [ring4-1(–) and ring4-2(–)] are reduced to a region that consists of only four and three fragments, which sum up to 970 and 920 kb, respectively. These two derivatives have the smallest B-specific cluster found in this group of chromosomes.

Double digests with *Pme*I and *Eco*RI provide additional information on the differences between these chromosomes. For the telo2-1(–), few *Pme*I fragments remain, and two of four are identical in composition to those of TB-9Sb, as indicated by the second dimension *Eco*RI pattern (Figure 3B). The two *Pme*I fragments of 350 and 370 kb have in common a 55 kb fragment (arrow). An *Eco*RI fragment of this size is also detected in the ring1(+) and in the ring4-1(–) double digests (Figure 3C and D, respectively). The single *Eco*RI digests reveal that a fragment of this size is the only one in common for all the derivatives (Figure 6A, arrows).

To test whether the 55 kb *Eco*RI fragment present in these chromosomes is identical, double digests with *Eco*RI and *Dpn*II were performed on ring1(+) and ring4-1(–). The centromere structures of both rings are different, and they originated from independent events. All *Eco*RI fragments share in common the 1.4 kb *Dpn*II sequence, along with several other size variants (Figure 7A and B). The 55 kb *Eco*RI fragment of ring1(+) differs in composition from the one in ring4-1(–). A 7.5 kb *Dpn*II sequence is present in the 55 kb *Eco*RI fragment in ring1(+) but not in ring4-1(–) (Figure 7A, arrow). In addition, a 1.6 kb sequence is present in ring4-1(–) but not in ring1(+) (Figure 7B, arrow). Each *Eco*RI fragment contains the 1.4 kb monomer (Figure 7A and B). Variants of the 1.4 kb sequence are also shared by several *Eco*RI fragments. Results from the *Eco*RI/*Dpn*II double digests suggest that a substantial fraction of the centromere is similar in sequence organization.

The amount of the B-specific repeat in misdivision derivatives

An estimation of the relative copy number of the B repeat by dot-blot analysis was performed for all the derivatives (Table I). Equal amounts of DNA from each genotype were transferred to a nylon filter, and then hybridized with a probe from the B repeat or from the alcohol dehydrogenase 1 (*Adh1*) gene as a loading control. The level of hybridization was measured by phosphorimagery. Ring chromosomes are frequently lost in somatic tissue because of sister chromatid exchange during mitosis (McClintock, 1938). Therefore the values for these chromosomes were corrected so as to account for the loss in the tissues used to prepare the DNA. The amount of hybridization of the Shrunken 1 (*Sh1*) sequence (which is located on 9S) with the ring or its progenitor chromosome was quantitated. The values obtained were compared for

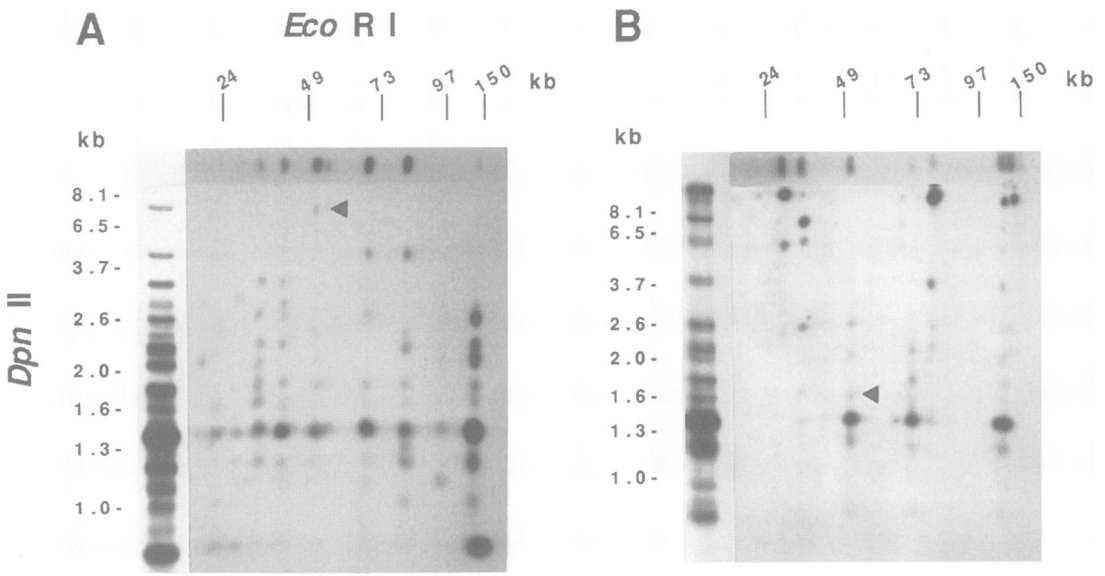

Fig. 7. A 2-D analysis of *Eco*RI fragments in the B-specific cluster of ring1(+) and ring4-1(–). *Eco*RI digests of high molecular weight DNA from ring1(+) and ring4-1(–) were subjected to redigestion with *Dpn*II and run on standard electrophoresis gels. The Southern blotted gel was hybridized to the B-specific sequence. First dimension conditions: 1% agarose for 26 h at 200 V in 0.5× TBE with a ramped pulse from 1.8 to 5.1 s. Second dimension conditions: 1.2% agarose for 24 h at 30 V in 1× TBE. (**A**) Ring1(+). (**B**) Ring4-1(–). Size estimates were made using λ DNA as the size standard.

equivalence between the two chromosomes, and the ratio B-specific/*Adh1* adjusted accordingly. As expected, misdivision leads to a drastic reduction in the copy number of the B repeat, as indicated by changes in the hybridization ratio of B-specific/*Adh1* (Table I). The values correlate well with each misdivision event and the estimated size of the centromere, as judged by the sum of all *Pme*I restriction fragments (Table I). The first misdivision that formed the pseudoiso reduced the B-specific copy number by half. The second misdivision reduced the centromere further by half of the remaining sequences [see telo2-1(–)], whereas the third misdivision to create the mirror image isochromosome [iso3(–)] almost doubled the copy number. The simplest explanation is that during misdivision the break occurred near the telomere end of the centromere. This observation explains the lack of restriction fragment differences between the latter two chromosomes for the B-specific region (Figure 6A and B, lanes 4 and 5), and also the near identical value of the restriction fragment complexity (Table I). The fourth misdivision reduced the copy number by about two times for ring4-1(–) and by more than three times for ring4-2(–).

Other chromosomes derived from the pseudoiso show values that are consistent with the nature of their centromere. Relative to the pseudoiso, the telo2-2(–) centromere is reduced drastically in size and the iso2(+) centromere is slightly smaller. The exception is telo2(+), in which the ratio is almost twice as large. This rearrangement led to an increase in the amount of B repeat cluster relative to the progenitor pseudoiso. Because a 350 kb band is stronger in intensity relative to other bands in the same lane and to the same fragment in the pseudoiso, all the observations on this derivative can be reconciled by postulating that a fraction of the B repeat has been duplicated (Figure 6B). A complex rearrangement might have been generated during its formation or an intermediate isochromosome could have occurred before recognition of the telocentric, which root tip chromosome analysis confirms. Finally, the ratio for ring1(+) is three times smaller than for the normal centromere, which correlates well with the loss of restriction fragments in this derivative.

Table I. Quantitation of the B-specific repeat in several misdivision derivatives and comparison with an estimate of the size of the B-specific cluster

Genotype	Ratio (B-specific/*Adh1*)	No. of misdivision events	Restriction fragment complexity (kb)
TB-9Sb	90.0	0	9000
Pseudoisochromosome	30.3 ± 3.1	1	4170
Telo2-1(–)	13.6 ± 1.0	2	1970
Iso3(–)	25.7 ± 3.1	3	1970
Ring4-1(–)	16.3 ± 6.0	4	970
Ring4-2(–)	7.9 ± 2.3	4	920
Telo2-2(–)	17.8 ± 1.2	2	1100
Telo2(+)	65.4 ± 9.3	2	3550
Iso2(+)	38.7 ± 1.1	2	3340
Ring1(+)	19.5 ± 0.3	1	2400

Discussion

Organization of the B repeat in a normal B centromere

The combination of restriction enzymes that rarely cut within the B repeat cluster, the use of a molecular probe that hybridizes only to the centric region of the B chromosome and the availability of a collection of modified B chromosomes led to an understanding of the overall organization of the B repeat cluster and its localization within the functional region of the centromere. We were able to confirm the organization of the B repeat in degenerate tandem arrays (Alfenito and Birchler, 1993). Tandem repeats are arranged up to four units and are interspersed with related sequences that vary in length.

This heterogeneity of fragment length and content is reflected in digests with *Eco*RI (Figure 2A). The long-range organization of the B repeat cluster was further defined after digest with the enzyme *Pme*I (Figure 2B). This analysis also revealed fragments ranging from <50 to ~2200 kb. The degeneracy of the B repeat is reminiscent of the organization of α satellite sequences in human centromeres. For example, the analysis of human chromosome 17 (Warburton and Willard, 1990) shows that α satellite arrays are characterized by the presence of localized homogeneous domains which consist of particular sequence units and higher order repeat length variants. Such domains can extend in length up to several hundred kb.

The sum of all fragments for TB-9Sb totals ~9 Mb, which is a minimum estimate of the size of the B-specific cluster. This value is larger than the human Y centromere, whose size and sequence composition have been determined (Cooper *et al.*, 1993; Tyler-Smith *et al.*, 1993). The Y centromere is contained within <1.5 Mb of DNA, which includes a large array of α satellite sequences and other adjacent repeats. The maize value of 9 Mb potentially underestimates the size of the B-specific region if several of the B-specific fragments are repeated several times leading to comigration of the fragments from different sites within the cluster. The *Pme*I/*Eco*RI double digests, however, suggest that such organization is minimal (Figure 3). The diverse lengths of the *Eco*RI fragments, corresponding to each of those generated with *Pme*I, verify the heterogeneity of the latter. The *Eco*RI/*Dpn*II double digests illustrate the extensive representation of the B-specific sequence in the *Eco*RI fragments.

The B-specific repeat is spread throughout the length of the centromere

A Southern analysis of several misdivision derivatives of the B chromosome reveals that centromeric breaks take place in the B-specific cluster (Figure 6). All of the derivatives have rearrangements, but each pattern is unique. These findings strongly suggest that the B repeat cluster is located within the functional region of the centromere.

Misdivision can reduce the size of the centromeric region. One misdivision of the normal centromere in TB-9Sb formed the pseudoiso (Figure 6A and B, lane 3), which possesses a striking simplification of the restriction pattern. This pseudoiso apparently results from the fusion of one broken centromere to a euchromatic region of the B chromosome. Its structure is therefore similar to that found in the centromere of a telocentric. By examining the change in the number of restriction fragments, we can conclude that the B repeat cluster in the pseudoiso has been reduced by more than half relative to the normal TB-9Sb cluster. In addition, the relative copy number of the B repeat and the size of the B repeat cluster have been reduced by half or more (Table I).

As all of these derivatives differ one from another, the misdivisions must have occurred at various places within the centromere. The most extreme case involves ring1(+). Its restriction pattern is one of the most simplified of all the derivatives (Figure 6B, lane 11), although it is the product of only one misdivision. The break undoubtedly took place at an off-center site within the centromere.

Misdivision of the pseudoiso generated a variety of telocentrics and isochromosomes. Despite the reduction in complexity of the cluster in each, an *Eco*RI fragment of ~55 kb is present in the normal centromere and in all derivatives (Figure 6A). Furthermore, a fragment of this molecular weight is contained within different *Pme*I fragments, as observed by a 2-D analysis of three TB-9Sb derivatives (Figure 3). This fragment probably corresponds to a region that is reiterated within the B-specific cluster because a fragment of this size persists after all misdivisions, thus potentially reflecting a requirement for function. A comparison of *Pme*I fragments of ring1(+) with ring4-1(–) or ring4-2(–) shows none in common. This result is consistent with their origins, and suggests that different portions of the centromere can still function. Moreover, the 55 kb *Eco*RI fragment present in ring1(+) and ring4-1(–) differs in composition, as revealed by *Eco*RI/*Dpn*II double digests (Figure 7). This result proved heterogeneity between the 55 kb fragments and demonstrated no apparent fragment in common between these two small but functional centromeres.

A model for the organization of the maize B centromere

The analysis of all these rearranged centromeres suggests that the centromere is a repeated structure that can be split into smaller fragments which are still able to function correctly. Cytological observations of maize meiocytes by McClintock (1932) suggested the repetitive structure of the centromere. Cases of ring chromosomes derived from normal linear chromosomes have been reported, indicating that the centromere can be divided and portions of it retain the ability to form a functional kinetochore. More recently, Zinkowski *et al.* (1991) observed that multiple fragments, resulting from detached or stretched kinetochores derived from mammalian and plant cells, still progress through mitosis. Their results provide evidence for a subunit organization within the kinetochore. Our molecular data confirm that the centromere can be rearranged rather significantly and different portions will still function.

The absence of the B repeat from the normal set of maize chromosomes suggests that either other short sequences interspersed within the B centromere are required for function in concert with the B repeat, or there is latitude for sequence composition involved in centromere formation. In the latter case, a single maize cell could utilize at least three forms of diverged centromere sequence (A, B and neocentromeres) which would be recognized by the same centromere factors that tether the microtubule machinery to the centromere DNA.

The reduction in size of the centromere with retention of function suggests that artificial chromosomes could be constructed using portions of the centromere. In unicellular organisms, artificial chromosomes have contributed significantly to genetic analysis. The estimated size of the smallest centromeres found in our study approaches a length that might be feasible to manipulate when assembling artificial chromosomes in this multicellular organism. Such constructs would have many uses in the study of chromosome behavior and in genetic engineering.

Materials and methods

Maize stocks

The BMS and High Loss lines were maintained with and without B chromosomes. Each B chromosome-containing line carried several copies

of the B chromosome. TB-9Sb is a reciprocal translocation between part of the B chromosome and the short arm of chromosome 9. In this study, the translocation stock TB-9Sb was used as a hyperploid, so that it contained two B centromeres. A hyperploid is a segmental aneuploid for the translocation B-9, and its constitution is 9 9-B B-9 B-9. Misdivision derivatives of TB-9Sb were generated and characterized by W.Carlson (Carlson, 1969, 1970, 1973, 1986; Carlson and Chou, 1981). The chromosome configuration of the derivatives was as follows. The TB-9Sb pseudoisochromosome (or pseudoiso), the telocentric TB-9Sb 1856 or telo2(+) and the isochromosomes TB-9Sb 2820 or iso3(–), TB-9Sb 5132 or iso2(+) are all heterozygous for the B–A translocation. The telocentrics TB-9Sb 1854 or telo2-1(–) and TB-9Sb 1852 or telo2-2(–) are homozygous for the B–A translocation. Ring1(+) was derived from a tertiary trisomic, and was maintained as a heterozygous 9 9-B B-9.

The other derivatives, ring4-1(–) and ring4-2(–), were produced by misdivision of the isochromosome iso3(–). In the constitution 9 9-B 9-B-9, this isochromosome can behave as a univalent during meiosis. The short arm of 9 on iso3(–) carries two markers, the *C1* and *Sh1* genes. Dominant alleles give colored and plump kernels, respectively; the recessive phenotypes are colorless and shrunken. Iso3(–) was crossed as a male onto a recessive tester line. Misdivisions that break the centromere in meiosis can be recognized in this cross because a bridge–breakage–fusion cycle (McClintock, 1939) is initiated. This cycle will continue in endosperm tissue and create a mosaic phenotype for *C1* and *Sh1*. In the embryo tissue, the broken end 'heals' and will be stabilized (McClintock, 1939). In the next generation, telocentric derivatives return to a nonmosaic phenotype, whereas ring chromosomes, because of their inherent instability, will again exhibit a mosaic pattern. The two rings were recovered and tested for heritability in this way. Their structure was confirmed by the cytological analysis of root tip mitotic cells.

Southern blot analysis

Genomic DNA was prepared as described by Chen *et al.* (1987). Restriction digests were conducted according to the manufacturer's recommendations. Electrophoresis was conducted in 1× TBE in a 1% agarose gel (Maniatis *et al.*, 1982). High molecular weight genomic DNA was prepared from protoplasts of young maize tissue, as described by Edwards *et al.* (1992). After digestion with cellulase and pectinase, protoplasts were embedded into 1.2% low melting point agarose at a final concentration of $3–8\times10^7$ cells/ml, and then treated with solutions containing 1% sodium *N*-lauroylsarcosine, 1 mg/ml proteinase K and 0.5× EDTA, pH 8.0, to release the DNA from the cells. The agarose-embedded DNA was subjected to electrophoresis to remove sheared fragments of <3 Mb. Restriction digests were performed at the appropriate temperatures. Pulsed-field gel electrophoresis was conducted on a CHEF-DRII apparatus (Bio-Rad) in 0.5× TBE. Pulsed-field gels were stained with ethidium bromide and irradiated at 60 mJ/cm^2 for 2 min, prior to transfer of the DNA to nylon filters. The dot-blot analysis was performed as follows. Undigested DNA was transferred onto a nylon filter and hybridized with a radioactive probe made from the *Adh1* (Schwartz, 1966; Gerlach *et al.*, 1982) gene as a loading control, and then to a probe corresponding to the B-specific sequence. For quantitating the B-specific sequence, relative densities of bands produced by Southern hybridization with the *Adh1* gene and the *Sh1* gene (Sheldon *et al.*, 1983) were obtained using a phosphorimager (Fuji). Calculations were corrected for the chromosomal copy number of the B centromere in each of the derivatives. An arc sin transformation was applied for all hybridization values (ratio B-specific/*Adh1* in Table I) that were expressed initially as a fraction of TB-9Sb. A mean ± SD value was calculated from four replicates, except for telo2-1(–) and iso3(–) whose values are based on three determinations.

Radioactive probes

Southern blots were probed with radiolabeled DNA from the appropriate DNA sequences subcloned into the Bluescript vector. DNA probes were synthesized by oligolabeling (Feinberg and Vogelstein, 1983). For the B-specific probe, hybridization was performed in conditions of 42°C with 5× SSC and 50% formamide, and washed at 68°C with 0.2× SSC and 0.1% SDS. For the *Adh1* and *Sh1* probes, hybridization was performed at 42°C in 5× SSC with 50% formamide, and washed at 50°C with 0.1× SSC and 0.1% SDS.

Acknowledgements

We are grateful to W.R.Carlson for providing several of the TB-9Sb derivative stocks and for sharing additional information on their structure and behavior. R.Staub supplied the isoBL chromosome. We thank Chris Carson, Doug Davis, Mei Guo and Mike Wanous for critical comments on the manuscript. This work was supported by a USDA grant to J.A.B.

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Received on May 8, 1996; revised on July 1, 1996