Molecular Organization of Large Fragments in the Maize B Chromosome: Indication of a Novel Repeat

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ABSTRACT

The supernumerary B chromosome has no apparent effects on plant growth, and its molecular makeup is difficult to unravel, due to its high homology to the normal complement, which prevents conventional cloning. This difficulty was overcome previously by microdissecting the B chromosome under the microscope to result in 19 B clones, one of which is B specific and highly repetitive, dispersing over one-third of the B long arm and most regions of the centromeric knob. To gain insights into the molecular structure of the B chromosome, this sequence was used to screen a genomic library constructed from W22 carrying 16 B's. Five clones (>10 kb each) were isolated, and all were repetitive, showing homology with A chromosomes in Southern and FISH analyses. Two of them were further characterized and sequenced. Each is composed of several restriction fragments with variable degrees of repetitiveness. Some of these are B specific and others have variable degrees of homology with the A chromosomes. The order of each characteristic group is not contiguous; they intersperse within those of other groups. Sequence analysis reveals that their sequences (~26 kb) have no homology with any published gene other than sequences of transposable elements (retrotransposons and MITEs) and the B as well as the A centromeres. We uncovered a 1.6-kb CL-repeat sequence, seven units of which were present in the two clones in defective forms. Those repeats mostly arrange in tandem array in the B chromosome. Moreover, we detected transposition of a retrotransposon and a MITE element involved in the genesis of these two sequences.

THE maize B chromosome was originally identified by Kuwada (1915) and has been a subject of extensive cytogenetic studies ever since. Yet, little is known about its molecular structure and organization because of difficulty in cloning of B sequences, owing to high homology between the B and the standard complement (A chromosomes), which prevents access of conventional approaches. This hindrance was overcome by Cheng and Lin (2003). They used a micromanipulator to dissect pachytene B chromosomes out of a slide under a microscope, and the B fragments were amplified by degenerate oligonucleotide-primed PCR. Cloning of the resulting products resulted in 19 B clones, which hybridized with genomic DNA in a B-dosage-dependent manner and with the pachytene B chromosome by fluorescence *in situ* hybridization (FISH) analysis, corroborating their B origin. These clones are repetitive sequences, and all but 1 also hybridize with A chromosomes. Sequence comparison reveals that 12 of these sequences have homology with the knob repeat and flanking regions of several maize genes. Six have no homology with any documented sequence. The remaining one, pBPC51, is B specific and was mapped—by a series of systematic deletions (hypoploid of B-10L translocations)—to the distal heterochromatic region of the B long arm, a map position that was substantiated by FISH analysis. This sequence provides a unique opportunity for isolation of large B sequences by conventional protocols.

In this article, we took advantage of the unusual properties of this clone—its B-specific and repetitive nature—to screen large fragments of the B chromosome from a λ-library constructed from genomic DNA carrying 16 B chromosomes. Five clones were obtained, two of which were further sequenced and characterized. We found retrotransposons, a miniature inverted-repeat transposable element (MITE), CentC, and a 1.6-kb CL-repeat family, which is mostly arranged in tandem array in the two clones as well as in the B chromosome.

MATERIALS AND METHODS

Plant material: Two maize inbred lines (W22 and L289) were used in this study. The W22 plant bearing 16 B chromosomes (W22 + 16B) was used to construct a λ-library. L289 carrying 2 B chromosomes (L289 + 2B) was employed to prepare mitotic and meiotic chromosome spreads for FISH analysis, and the L289 containing 3 B's (L289 + 3B) and no B (L289 + 0B), respectively, were used for Southern analysis.

λ-library construction and screening: The maize genomic library was constructed by S.-F. Peng (unpublished results), following the instructions of the supplier, using λDASH II/

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY426742 and AY426743.

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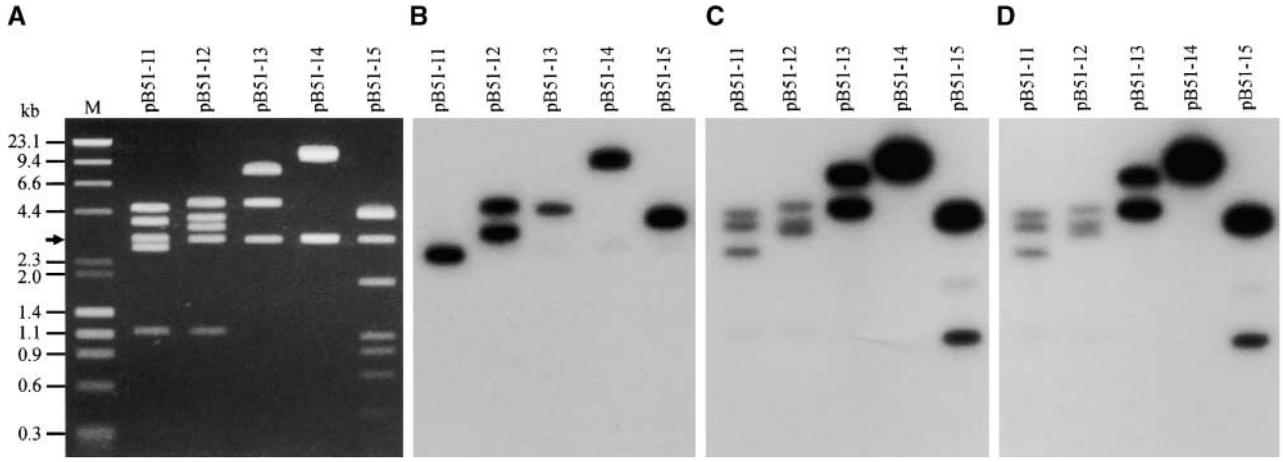


FIGURE 1.—Hybridization of the blot of five B clones with pBPC51 and L289 with and without B. Clones pB51-11, pB51-12, pB51-13, pB51-14, and pB51-15 were cut with *Not*I and *Xba*I, stained with ethidium bromide (A), and probed with pBPC51 (B), L289 + 3B (C), and L289 + 0B (D), respectively. M, size marker (λ*Hin*dIII digest mixed with ϕX174-*Hae*III digest). Molecular weights are shown on the left, and the arrow indicates the position of the plasmid vector.

*Eco*RI as vector (Stratagene, La Jolla, CA). Briefly, genomic DNA of W22 + 16B was partially digested with *Tsp*509I (New England Biolabs, Beverly, MA) and fractionated in sucrose gradient centrifugation at 154,000 × *g* for 24 hr. The 9- to 23-kb fraction was ligated to the arms of the λ-phage DASH/*Eco*RI, packaged (using Gigapack III Gold-11 packaging extract), used to infect host cells [XL1-Blue MRA (P2)], amplified, and plated at a low titer (200 plaques/100-mm plate on the same host cells). Plaques were lifted and hybridized with the B-specific clone pBPC51 (CHENG and LIN 2003) as the probe. Plaques with positive signals were selected as potential B-carrying candidates. The λDNA preparation was carried out as described by AUSUBEL *et al.* (1990).

Genomic DNA isolation and Southern analysis: Isolation of maize genomic DNA was previously described by LIN and CHOU (1997). Southern blot analysis followed the method of LIN *et al.* (1997). The B centromeric probe used in this study was the PCR product of a pZmBs-based sequence (pBC1; Y.-P. LIN, unpublished results). The pBC1 sequence was cloned from the PCR products of W22 + 16B using primers 5′-AAC TGCAGTTAGGGTCTATGGTTTGGGCCT-3′ and 5′-CGGG ATCCATCAAACGCCAAGCCCTG-3′. The clone pBC1, when used as a probe, produced no signal in Southern hybridization with L289 + 0B but clear signal with L289 + 1B, and its signal on pachytene spreads (carrying the B chromosome) is restricted to the B centromere and subtelomeric region of the B long arm, identical to that of pZmBs (ALFENITO and BIRCHLER 1993).

Polymerase chain reaction: Two primers were designed to amplify CL-repeat sequences from the five B clones and the B chromosome: BP3 (5′-GATTCTTGGTTATGGACAACA ATGC-3′) from the 3′ terminus of the CL repeat and BP5 (5′-GCACTACATATGGTTTAAGATAGC-3′) from the 5′ terminus. The polymerase chain reaction (PCR) was performed for 30 cycles with the following conditions: 94° for 30 sec, 55° for 30 sec, and 72° for 3 min.

Fluorescent *in situ* hybridization: Using DIG-11-dUTP, large B fragments and CL repeats (pCLa1 and PCR products of pB51-12) were labeled by nick translation of the DIG-nick translation labeling system (Roche). The pCLa1 was cloned from PCR products of L289 + 3B, amplified by BP3 and BP5 primers. Chromosome spreads of root tips and pachytene chromosomes were prepared as outlined by LIN (1977) and BURNHAM (1982), respectively. The procedures of *in situ* hybridization, signal detection, and image analysis followed the protocols previously published by CHENG and LIN (2003). The signal detection was performed with either anti-mouse IgG (most cases) or anti-rabbit IgG conjugated with FITC.

Sequence analysis: DNA sequences similar to the two B clones, pB51-12 and pB51-15, were searched in the GenBank database using BLASTN of the National Center for Biotechnology Information website. Multiple alignments were made using the program PILEUP of the Genetics Computer Group program (Wisconsin, version 10.0) and displayed using the BoxShade server (http://www.ch.embnet.org/software/BOX_form.html). The sequences have been submitted to GenBank with accession nos. AY426742 (pB51-12) and AY426743 (pB51-15).

RESULTS

Isolation of large B chromosome fragments: Using pBPC51 as a probe to screen ~2000 λ-clones from an amplified library, constructed from genomic DNA with 16 B chromosomes, nine positive phages were identified, five of which were further characterized. Following digestion with *Not*I, the inserts (>10 kb) were ligated into pBlueScript SK$^-$ and designated as pB51-11, pB51-12, pB51-13, pB51-14, and pB51-15, respectively. The *Not*I plus *Xba*I digests of these clones revealed one to six fragments of insert DNA (Figure 1A). When the blot was probed with pBPC51, one or two fragments of each clone displayed positive signals (Figure 1B). Hybridization of the same blot with genomic DNA of L289 + 3B as probe revealed signals on all insert fragments of pB51-11, pB51-12, pB51-13, and pB51-14 and on three of the six fragments of pB51-15 (Figure 1C). The remaining three fragments of pB51-15 without hybridization signal represented low-copy-number sequences in the L289 + 3B probe, which were not visible due to insufficient exposure under regular repetitive protocol. When probed with L289 + 0B, the same blot gave a similar hybridization pattern except the intensity of the largest

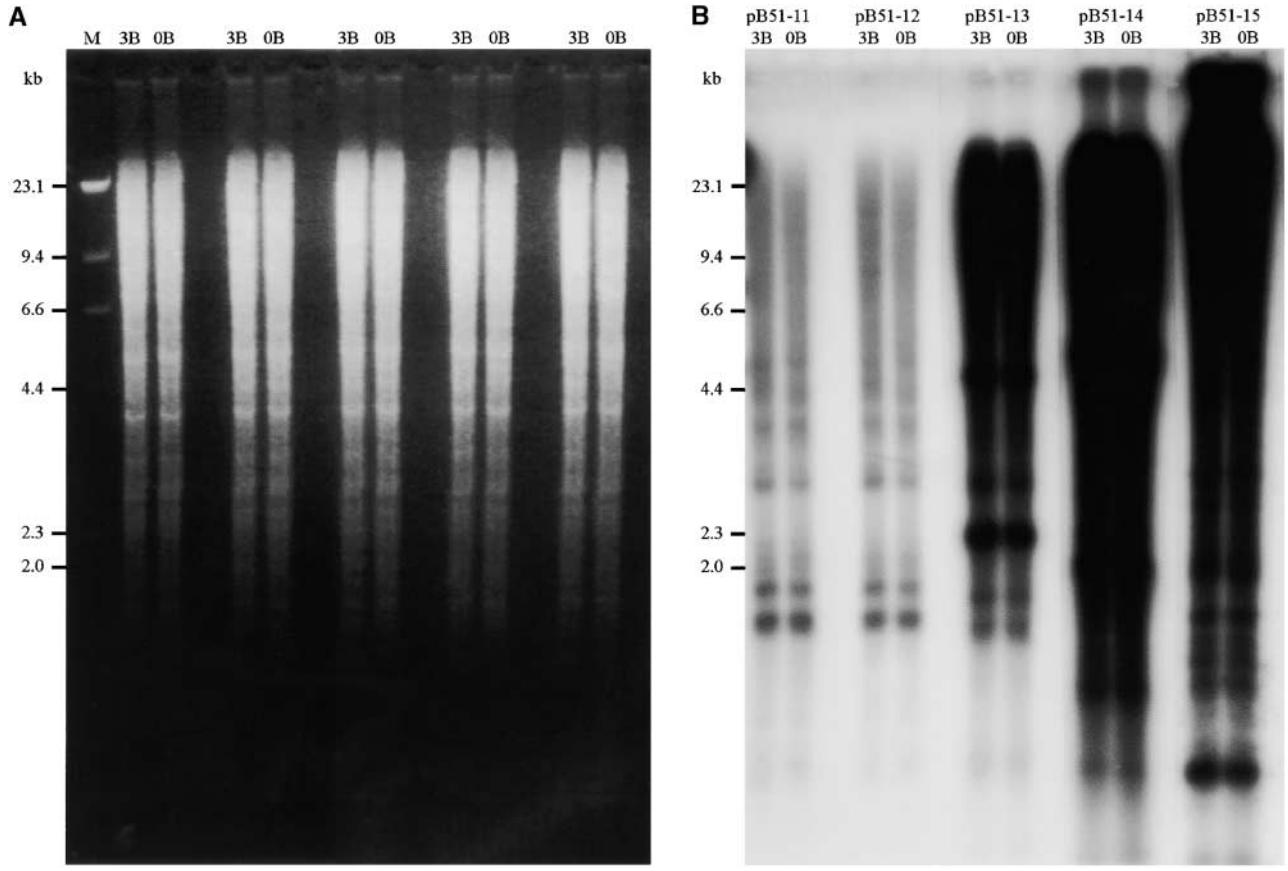


FIGURE 2.—Southern analysis of five B clones. Genomic L289 + 3B and L289 + 0B DNAs were digested with *Bam*HI (A) and probed by pB51-11, pB51-12, pB51-13, pB51-14, and pB51-15, respectively (B). Molecular weights are indicated on the left.

fragment of pB51-13 was slightly reduced (Figure 1D). Taken together, these five clones were proven to be derived from the B chromosome, containing repetitive sequences common to the A and B chromosomes.

Hybridization of the *Bam*HI digests of L289 + 3B and L289 + 0B with each of the five clones as probe resulted in a smeared pattern as well as discrete bands (Figure 2). The result indicates that the sequences of these five clones are highly or medium repetitive.

FISH localization of the five B clones: To analyze the B location of the five clones, pachytene B chromosome was hybridized with the inserts as probes in FISH analysis (Figure 3). By and large, FISH signals appeared in all regions of the B chromosome. Figure 3A shows hybridization of pB51-11, where major signals are present on four distal heterochromatic regions. The hybridization pattern of pB51-12 is similar to that of pB51-11 (Figure 3B). FISH signals of pB51-13 were more concentrated on the distal heterochromatic region H3 (Figure 3C), and those of pB51-14 dispersed more evenly over almost the entire B chromosome (Figure 3D). Clone pB51-15 hybridized strongly on the distal heterochromatic regions, but with the centromeric knob and the proximal euchromatic region in lesser degrees (Figure 3E). Thus, the data verified the B origin of these sequences.

To study the distribution of the B clones on the A chromosomes, the five B clones were also used to probe root-tip chromosomes. The results are shown in Figure 4. FISH signals of the five clones dispersed on all A chromosomes, with the signals of pB51-11 and pB51-12 being the weakest (Figure 4, A and B) and that of pB51-13 and pB51-14 the strongest (Figure 4, C and D). Particularly interesting is the last clone (pB51-15), whose signals concentrated mostly around the centromeric regions (Figure 4E), although some signals dispersed on one or both arms. These results indicate that the five B clones contain repetitive sequences homologous to all A chromosomes. As would be expected, FISH signals of the five B clones were also present on the B chromosome of root-tip cells. The signals of pB51-11 and pB51-12 on the B chromosome displayed greater intensity than those on the A chromosomes (Figure 4, A and B). The pattern of pB51-13 and pB51-14 signals on the B chromosome is similar to that of the A chromosomes (Figure 4, C and D). Although the signals of pB51-15 appeared densely in the vicinity of the A centromeres, it did not do so on the B centromere (Figure 3E and Figure 4E).

Southern analysis: Since pB51-12 contains the B exclusive sequence (pBPC51) and its hybridization patterns

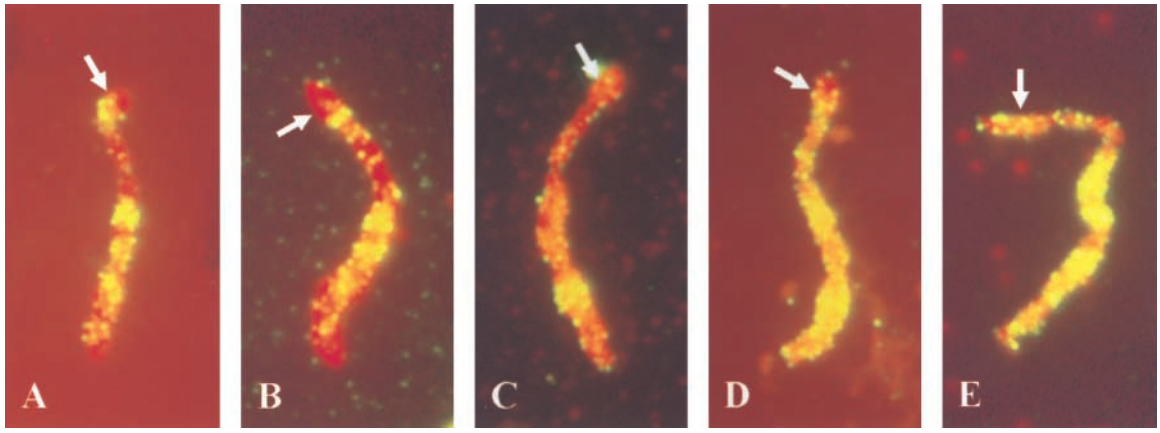


FIGURE 3.—FISH of pachytene B chromosome probed with five B clones. Chromosome stain is red, and hybridization signal is yellow. Arrows indicate the centromeric knob of the B chromosome. (A) pB51-11, (B) pB51-12, (C) pB51-13, (D) pB51-14, (E) pB51-15.

to genomic DNA with and without B are similar (Figure 2), it was expected to contain sequences unique to B and those in common with the A chromosomes. To determine its detailed molecular organization, the clone was digested with five enzymes (*Bam*HI, *Hin*dIII, *Pst*I, *Xba*I, and *Xho*I). The resulting 18 fragments were subcloned into pBlueScript, and each (from 5′ to 3′ end, pB51-12-1 to pB51-12-18, respectively) was used to probe the *Bam*HI and *Xba*I digests of L289 + 3B and L289 + 0B. The results are presented in Figure 5. On the basis of their signal patterns, the 18 subclones could be divided into three groups. The first group (pB51-12-1 and pB51-12-18) is a B-specific sequence, hybridizing with L289 + 3B but not with L289 + 0B. The second group, including 5 subclones, has two types of signals, either specific or not specific to the B chromosome.

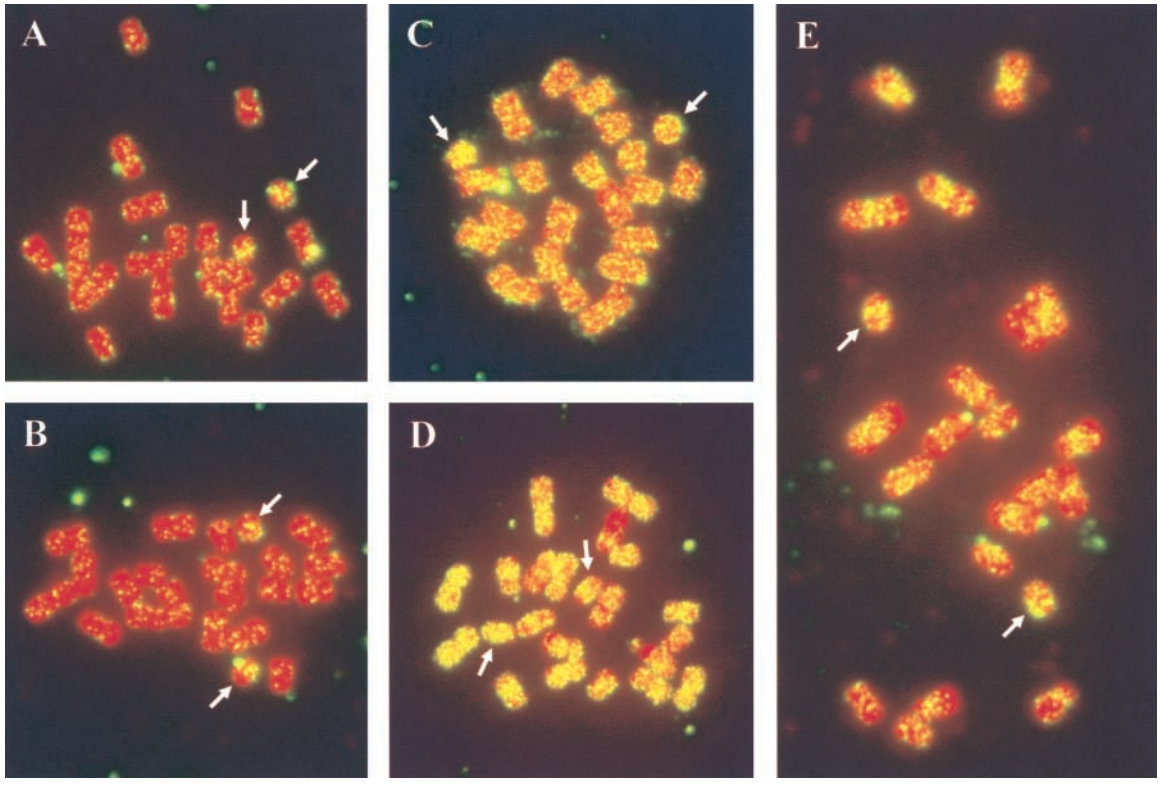


FIGURE 4.—FISH of root-tip chromosomes at metaphase probed with five B clones. Five clones, labeled with FITC, were used to probe the chromosome spreads of root tips of the L289 + 2B seedlings. Arrows indicate B chromosomes. (A) pB51-11, (B) pB51-12, (C) pB51-13, (D) pB51-14, (E) pB51-15.

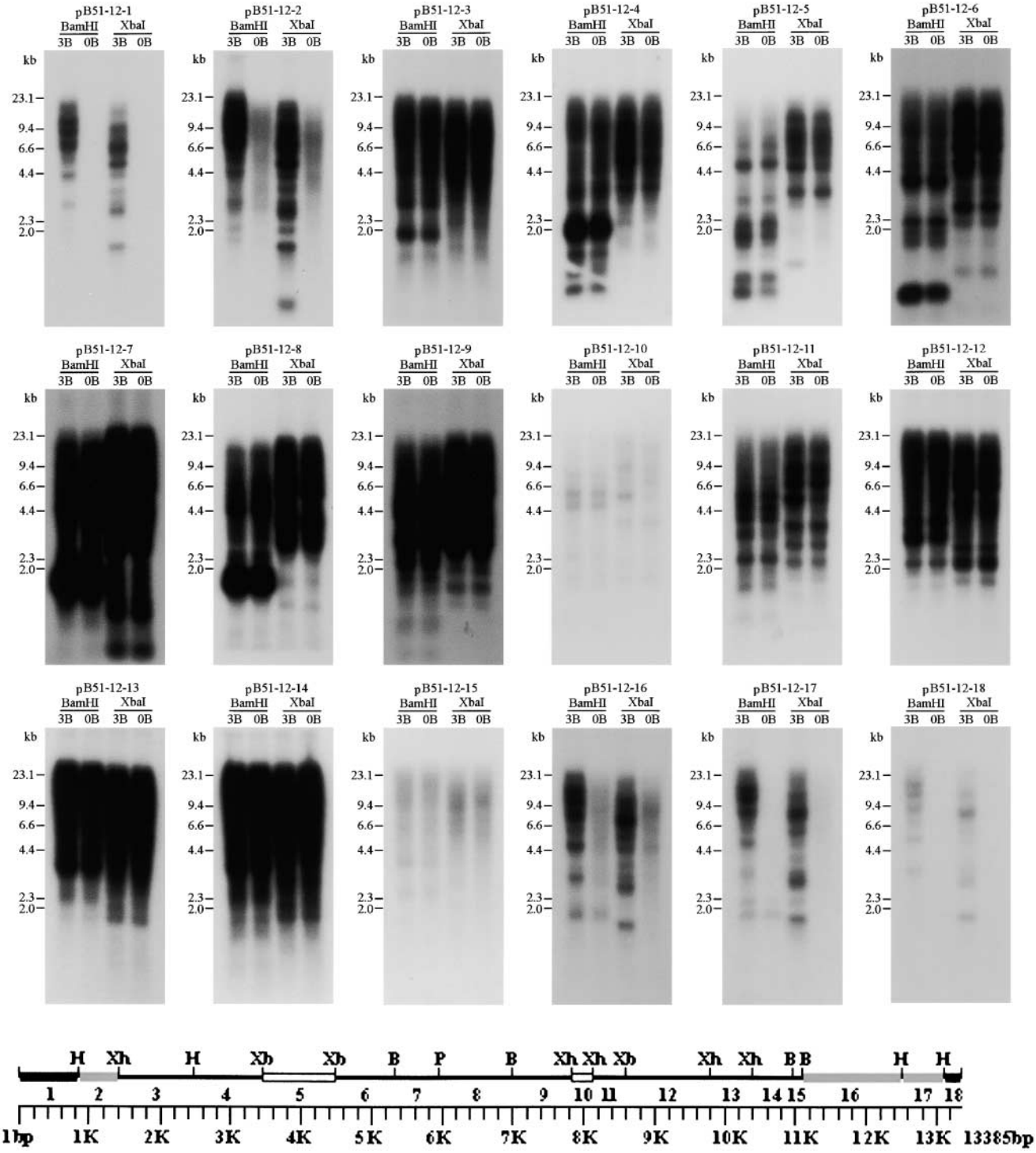


FIGURE 5.—(Top) Southern analysis of 18 fragments derived from pB51-12. Autoradiograms show hybridization of 18 subclones of pB51-12 as probes to *Bam*HI and *Xba*I digests of L289 + 3B and L289 + 0B. The size marker is indicated on the left. (Bottom) The results of Southern analysis. Solid bar, B-specific sequences; shaded bar, the partial B-specific sequence; open bar, one signal specific or more intense in B-carrying DNA; thick line, no response to the B dosage. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; Xb, *Xba*I; Xh, *Xho*I.

Clones pB51-12-2, pB51-12-16, and pB51-12-17 show strong hybridizations to L289 + 3B but weak ones to L289 + 0B, and 2 others display multiple signals, one of which is present in the *Xba*I digest of L289 + 3B but either not present (pB51-12-5) or weakly present (pB51-12-10) in that of L289 + 0B. The remaining 11 subclones formed the third group, which are of high copy number and show a similar hybridization pattern as well as inten-

sity between DNAs with and without the B chromosome. It is notable that fragments of each group were not contiguous—they interspersed between fragments of other groups. For example, while the two B-specific fragments occupied the two termini, two fragments of the second group were inserted in two different locations within the third group.

The same analysis was applied to pB51-15, and results are shown in Figure 6. Fourteen subclones (from 5′ to 3′ end, pB51-15-1 to pB51-15-14, respectively), generated by cloning the 14 restriction fragments resulting from digestion of pB51-15 by the same five enzymes, were used to probe the *Bam*HI and *Xba*I digests of L289 + 3B and L289 + 0B, and none of them displayed a signal pattern indicative of a B-exclusive nature. Seven of them yielded B-specific as well as not-B-specific signals; signals of pB51-15-3, pB51-15-4, pB51-15-5, pB51-15-6, and pB51-15-13 in L289 + 3B were stronger than those in L289 + 0B. The two other subclones displayed multiple bands, and each had a single signal either B specific (pB51-15-14) or much more intense (pB51-15-10) in the *Bam*HI and *Xba*I digests of L289 + 3B, respectively. The remaining seven subclones hybridized equally between DNAs with and without the B chromosome.

Sequence analysis: The sequences of pB51-12 were compared with those deposited in the GenBank database. Homology of pB51-12 (13,385 bp) to various sequences from GenBank is summarized in Figure 7. First, pBPC51 (GenBank accession no. BH814977, nt 1–324) was present in two terminal regions of pB51-12 (nt 791–1116 and nt 12,461–12,783, respectively), having an overall similarity of 90%. Second, three regions (nt 1141–1263, nt 11,490–11,598, and nt 12,808–13,101, respectively) were homologous to the B centromere clones, exemplified by B4a with 81–89% identity. Similarity of these regions to B4a included four segments with an overall similarity of 83%: nt 1141–1263, nt 11,490–11,535, nt 11,525–11,598, and nt 12,808–13,101 (nt 361–481, nt 362–407, nt 578–653, and nt 361–653 in GenBank accession no. U61992, respectively). Finally, nine regions (total 4766 bp) had homology to a maize genomic clone (226 kb) carrying *bz1* in the short arm of chromosome 9 (nt 16,447–19,891, GenBank accession no. AF391808). Four of these (nt 4598–5158, nt 8410–9075, nt 9103–9626, and nt 9627–10,626) were homologous to the coding region of *RIRE2* gag/pol (nt 19,332–19,891, nt 19,529–18,873, nt 18,848–18,325, and nt 18,303–17,303 in accession, respectively) and the remainder (nt 1315–1637, nt 1669–2481, nt 10,658–11,002, nt 11,010–11,243, and nt 11,261–11,473) to its upstream sequences (nt 17,626–17,303, nt 17,264–16,453, nt 17,264–16,991, nt 16,688–16,921, and nt 16,659–16,447 in accession, respectively).

Clone pB51-15 was sequenced and analyzed with the same program. The clone had a sequence of 12,955 bp, and the result is presented in Figure 8. There were four regions (nt 3232–3562, nt 4018–4064, nt 4696–5022, and nt 6232–6399) in common with pBPC51 (nt 1–329, nt 21–67, nt 1–324, and nt 1–167 in GenBank accession no. BH814977, respectively; 89% identity), five regions (nt 2297–2384, nt 3518–3558, nt 3636–3804, nt 4983–5022, and nt 5067–5345) with the K11 clone of maize B centromere (nt 577–662, nt 477–517, nt 485–651, nt 477–516, and nt 381–662 in GenBank accession no. U61997, respectively; 84% identity), and seven regions (nt 256–331, nt 458–641, nt 731–2044, nt 3–43, nt 256–337, nt 518–1099, and nt 1580–2043) with the LTRs of the maize gypsy/Ty3-type retrotransposon *Tekay* (nt 1662–1737, nt 1858–2047, nt 2136–3442, nt 10,533–10,573, nt 10,803–10,884, nt 11,062–11,638, and nt 11,657–12,118 in GenBank accession no. AF050455, respectively; 89% identity). Also observed was a region (nt 2043–2275) having 91% identity with the maize clone *mPIF381* (nt 239–6 in GenBank accession no. AF416324; Zhang *et al.* 2001), a MITE element.

Five regions (totaling ~2.5 kb) showed similarity to the maize centromeric bacterial artificial chromosome (BAC) clone 15C5 (GenBank accession no. AC116033; Nagaki *et al.* 2003). The first region (nt 6571–7644) was composed of approximately seven maize centromeric repeats (CentC-156a, GenBank accession no. AF078922; Ananiev *et al.* 1998) that are homologous to three tracts of CentC (tracts D, E, and F) in BAC 15C5 (nt 23,487–30,931, nt 61,798–63,812, and nt 73,475–75,071 in accession, respectively; 88% identity). This region is followed closely by three regions (nt 7642–9051, nt 9067–9125, and nt 9327–9435) correspondent to subclone ZMAB-C91 (nt 88,984–87,575, nt 87,458–87,400, and nt 87,194–87,086 in accession, respectively; 88% identity) in reverse polarity. The last region (nt 1368–1440) is a small fragment with sequence homology to a 5′ LTR region (nt 76,888–76,962 in accession; 86% identity), located downstream of CentC tract F.

Sequences that are unique to the B chromosome are of special interest for studying the molecular structure of the B chromosome. Accordingly, particular effort was made to examine each of the two regions found on pB51-12 and pB51-15 that do not hybridize to genomic DNA lacking a B chromosome. On pB51-12 there were two subclones (pB51-12-1 and pB51-12-2) at the 5′ terminus and three (pB51-12-16, pB51-12-17, and pB51-12-18) at the 3′ terminus (Figure 5). On pB51-15 four subclones from the internal region (pB51-15-3, pB51-15-4, pB51-15-5, and pB51-15-6) and one subclone located near the 3′ terminus (pB51-15-13) had B-specific sequences (Figure 6). In terms of length, the B-specific region associated with pB51-12 is 3.6 kb and that related to pB51-15 is 5 kb. To determine whether the four regions were different segments of a long B-specific region or a repeat unit that appears more than four times in the two clones, the 5′ terminal fragment of pB51-12 (pB51-12-1; 904 bp), the B-specific fragment based on Southern analysis, was used to compare with other regions by the Blast Two program (Tatusova

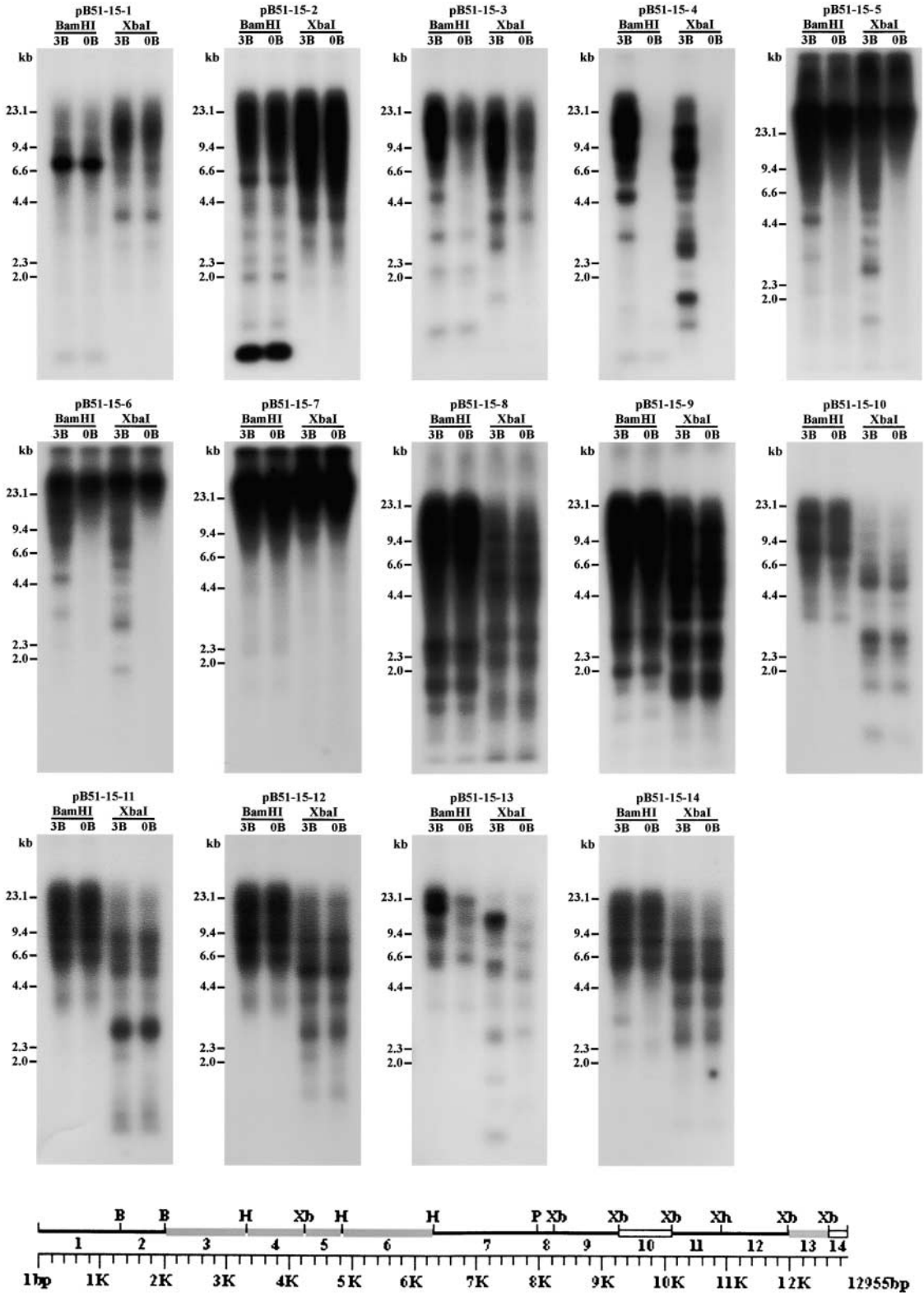


FIGURE 6.—(Top) Southern analysis of 14 fragments derived from pB51-15. Autoradiograms show hybridization of 14 subclones of pB51-15 as probes to *Bam*HI and *Xba*I digests of L289 + 3B and L289 + 0B. The size marker is indicated on the left. (Bottom) The results of Southern analysis. Shaded bar, the partial B-specific sequence; open bar, one signal specific or more intense in B-carrying DNA; thick line, no response to the B dosage. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; Xb, *Xba*I; Xh, *Xho*I.

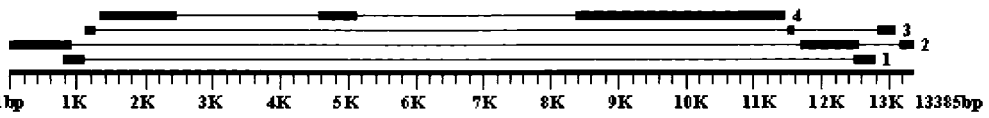


FIGURE 7.—Functional features of pB51-12. Homology of pB51-12 to pBPC51, pB51-12-1, and other sequences in GenBank is represented by solid bars. 1, pBPC51; 2, pB51-12-1; 3, maize B centromere sequence (B4a); 4, maize 9S *bz1* genomic clone.

and MADDEN 1999). Clone pB51-12 had two matched regions—one (nt 1–904) at the 5′ terminus and the other (nt 11,682–12,571 and nt 13,185–13,361; Figure 7) at the 3′ terminus—and pB51-15 had one (nt 2459–3345, nt 3888–4809, and nt 5418–6345; Figure 8), suggesting existence of a single repeat unit, designated as CL repeat, which appeared in triplet in each clone.

Characterization of CL repeat: To determine the 5′ and 3′ ends as well as the full length of this repeat unit (the CL repeat), sequences of the three matched regions (mentioned above) with their flanking sequences were aligned by the GCG program PILEUP, and the result is shown in Figure 9. Seven regions (four in pB51-12 and three others in pB51-15) were found to be associated with the CL repeat. For convenience of discussion, the four regions of pB51-12 (nt 1–1295, nt 1296–1314, nt 11,474–12,962, and nt 12,963–13,385) were designated as BR12-1, BR12-2, BR12-3, and BR12-4, respectively, and the three regions of pB51-15 (nt 2276–3662, nt 3663–5193, and nt 5194–6570) as BR15-1, BR15-2, and BR15-3, respectively. Particularly interesting is the finding that the last base of the 3′ terminus of BR12-1 was in direct contact with the first base of the 5′ terminus of BR12-2 (Figure 10). Furthermore, the former matched perfectly with the 3′ terminus of BR12-3 and the latter with the 5′ terminus of BR12-4, BR15-2, and BR15-3 (Figure 9), suggesting that the contact point between BR12-1 and BR12-2 is the junction of two CL repeats (a similar junction occurred between BR12-3 and BR12-4; Figure 10). In other words, the 3′ terminus of BR12-1 represents the 3′ end of the CL repeat, and the 5′ terminus of BR12-2 the 5′ end. Accordingly, the sequence extending from the 3′ to the 5′ end, 1552 bp, is the length of the CL repeat. In addition, since four junctions (two in pB51-12 as described above and two distorted ones in pB51-15, carrying deletions of 75 and 5 bp at the 3′ end, respectively) of the seven repeat regions possessed a junction structure similar to that of BR12-1 and BR12-2 (Figure 16), most CL repeats in the chromosome appeared to be in the structure of tandem array.

To determine if the proposed CL repeat is valid, pBPC51 was used to probe the *Hin*dIII—cutting singly in the repeat (Figure 9)—digests of the genomic DNA of L289 + 3B and L289 + 0B. According to the estimation above, a predominant 1.6-kb signal would be expected, if the CL repeat is present in tandem array in the B chromosome. Results confirmed this expectation. A major signal of this size and several minor ones were shown only in L289 + 3B (Figure 11), indicating that the genomic distribution of the B-specific sequence is mostly in the form of tandem repeats and that each unit is ~1.6 kb. The minor signals with variable intensity independent of molecular size might arise from either incomplete or isolated CL repeats. This result was further confirmed by PCR analysis, using primers derived from two end sequences of the CL repeat (primers BP3 and BP5, Figure 9). A major 1.6-kb product was amplified from DNAs of L289 + 3B but not of L289 + 0B (Figure 12A, lanes 7 and 8). Also produced from the former were two smaller (0.8 and 1.0 kb) smeared products. Likewise, the 1.6-kb product was produced when pB51-12 and pB51-15 were used as templates, apparently from BR12-3 and BR15-2, respectively (Figure 12A, lanes 3 and 6). However, no product appeared in the reactions of other B clones (pB51-11, pB51-13, and pB51-14; Figure12A, lanes 2, 4, and 5, respectively), suggesting that the CL repeats in these clones were missing one of the two primer-binding sites.

To clarify whether the two smaller products (0.8 and 1.0 kb; Figure 12A, lane 7) were amplified from different forms of the CL repeat in the B chromosome, the gel of PCR products (Figure 12A) was blotted and probed with pBPC51. The hybridization signals were evident

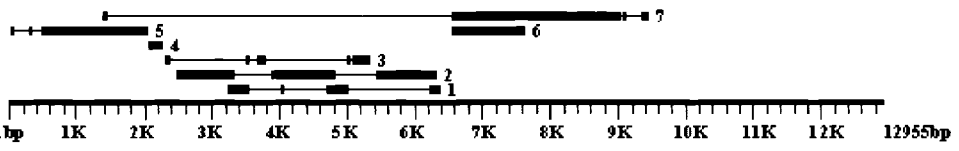


FIGURE 8.—Functional features of pB51-15. Homology of pB51-15 to pBPC51, pB51-12-1, and other sequences in GenBank is shown by solid bars. 1, pBPC51; 2, pB51-12-1; 3, maize B centromere sequence (K11); 4, maize *mPIF*; 5, maize gypsy/Ty3-type retrotransposons *Tekay*; 6, maize centromeric repeat CentC; 7, maize BAC clone 15C5.

not only in the 1.6-kb product, but also in the 0.8 and 1.0 kb as well as in the smeared products (Figure 12B). The 0.8-kb signal was much weaker than the 1.0-kb one (Figure 12B, lane 7), although it had more products, suggesting that it deleted most of the pBPC51 sequence. This explanation conforms to the result of the same analysis using the product of pB51-12 as probe, where the signal intensity is proportional to the amount of PCR products (Figure 13A). Since the product of pB51-12 was amplified from BR12-3, which is a defective CL repeat deficient in the 5′ terminal 14 bp and two internal regions (box III and box IV, Figure 9), the 0.8- and 1.0-kb products of L289 + 3B should derive from the truncated versions of the CL repeat. The smeared products represented the CL-repeat-carrying sequences amplified from sequences that had the binding site of either BP3 or BP5 and a second binding site located in upstream or downstream regions of the CL repeat.

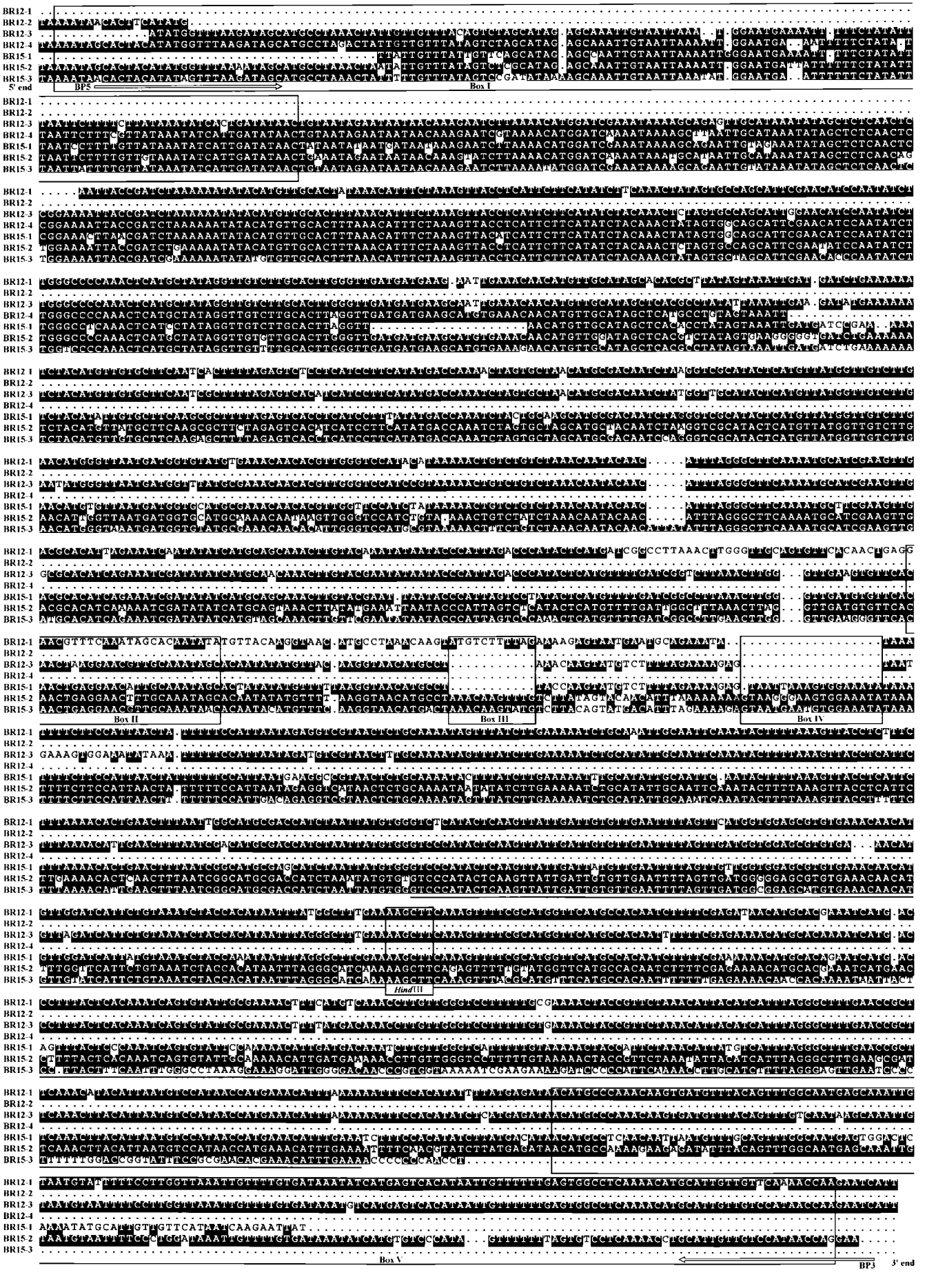
In addition to the 1.6-kb product, a second product was produced from pB51-12 and pB51-15, although it was not visible in the gel stained with ethidium bromide (Figure 12A, lanes 3 and 6). This product was revealed in Southern hybridization of the gel with the pBPC51 probe, where an intense 1.6-kb signal and a weaker 0.8-kb one were visible (Figure 12B, lanes 3 and 6). The 0.8-kb fragment resulted from a second binding site of primer BP5 located in box II (Figure 9), which had a 58% similarity to BP5. The last five nucleotides of the 3′ end of BP5 had a perfect match with this site. The product amplified from BP5 binding to this site and BP3 is expected to be 738 bp in pB51-12 and 768 bp in pB51-15.

To determine the distribution of the CL repeat in the B chromosome, the CL-repeat clone (pCLa1; see MATERIALS AND METHODS) and PCR products, amplified by BP3 and BP5 from pB51-12 (Figure 12A, lane 3), were used as probe to hybridize the pachytene B chromosome. As shown in Figure 14, FISH signals of both probes appeared strongly on the distal heterochromatic regions H1, H2, and H3 and weakly on the centromeric knob. This result agrees with that of a similar hybridization using pBPC51 as probe (CHENG and LIN 2003; Figure 4A), except that signals of the centromeric knob were relatively weaker. This signal reduction may result from lower homology of the repeat sequence (1223/1552) other than that of pBPC51 with the centromeric knob.

To gain further insight into the nature of the CL-repeat PCR products, the *Bam*HI and *Xba*I digests of L289 + 3B and L289 + 0B were probed by the PCR products of pB51-12, pB51-15, and L289 + 3B, primed by BP3 and BP5 (Figure 15). The product of pB51-12 hybridized with L289 + 3B but not with L289 + 0B, indicative of a B exclusive repeat (Figure 15A). Hybridization pattern of the same digests with the product of pB51-15 is identical with that of pB51-12 except for the appearance of slight, smeared signals associated with L289 + 0B (Figure 15B). These exceptional signals must be the results of the presence of two short stretches of sequences (Figure 9, box III and box IV), which were homologous to the A chromosomes and deleted from pB51-12. PCR products using L289 + 3B as a template yielded basically the same pattern as above but with three additional signals (0.4, 0.5, and 0.9 kb) in the *Xba*I digest of L289 + 3B (Figure 15C). These three signals represented either the repeat sequences of the B chromosome not present in pB51-12 and pB51-15 or the sequences unrelated to the two clones that were amplified by BP3 and BP5 primers.

In view of sequence similarity between the CL repeat and the B centromeric sequences, these two possibilities were further characterized. According to sequence analysis, two terminal regions of the CL repeat had homology with B centromere sequences, including the sequence of BP3 and BP5 (Figure 9, box I and box V). To look into the possibility that the B centromere sequences were amplified by the primers of the CL repeat, the PCR products of L289 + 3B were probed by the B centromere sequence (see MATERIALS AND METHODS). Two intense (0.8 and 1.0 kb) and one very weak (1.6 kb) signal were observed after prolonged exposure (Figure 13B), suggesting the presence of an appreciable portion of B centromere sequences in the 0.8- and 1.0-kb products. The weak 1.6-kb signal reflected the small proportion of the CL-repeat sequence capable of annealing with the B centromere probe. This result is consistent with the fact that the size of the three additional signals of the *Xba*I digest of L289 + 3B (Figure 15C) corresponds to the three B-specific signals (0.4, 0.5, and 0.9 kb) noted by ALFENITO and BIRCHLER (1993) in the *Xba*I digests of the B-carrying DNA after being probed by pZmBs. These data, taken together, indicate that the B centromere sequences were amplified by the primers of CL repeat and that most products happened to be the same size as the truncated CL repeats.

All of the CL repeats present in the two clones were not complete units; each unit deleted either one (3′ or 5′) of the two ends or both (Figures 9 and 16). Of the three regions matched with pB51-12-1, two were associated with pB51-12 and one with pB51-15. The 5′ matched region of pB51-12 carried two units: the upstream unit (BR12-1; 1295 bp) missed the 5′ end and the downstream unit (BR12-2; 19 bp) missed the 3′ end. The 3′ end of BR12-1 was closely followed by the 5′ end of BR12-2, forming a junction of two tandem repeats as mentioned above (Figure 10). The second matched region, located in the 3′ terminus of pB51-12, also contained two units (BR12-3 and BR12-4) and had the same structure as that of the 5′ matched region (Figure 10). BR12-3 and BR12-4 had 1495 and 423 bp, respectively. The last matched region, situated in pB51-15, was composed of three repeat units: BR15-1, BR15-2, and BR15-3 in close association. BR15-1, the upstream unit (1387 bp), was deficient in both the 3′ and 5′ ends, and BR15-2 (1531


BR12-1
BR12-2
BR12-3
BR12-4
BR15-1
BR15-2
BR15-3
5' end
BP5
Box I
Box II
Box III
Box IV
Box V
HindIII
BP3
3' end

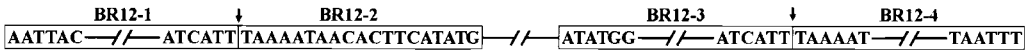


FIGURE 10.—Tandem CL repeats in pB51-12. Four CL repeats (BR12-1, BR12-2, BR12-3, and BR12-4) are boxed, and arrows indicate the junction between two repeats.

bp) contained only the 5′ end. The last unit in the row, BR15-3 (1377 bp), likewise deleted the 3′ end of the repeat unit.

Insertion of sequences into the CL repeat: Sequence of pB51-12 is a complex structure, composed of CL repeats, portions of 9S *bz* clone (containing retrotransposon *RIRE2*), and two stretches of unknown element(s). As shown in Figure 16, the CL repeats are located at the 3′ (BR12-1 and BR12-2) and 5′ (BR12-3 and BR12-4) termini. The remaining elements lie in the central region delimited by BR12-2 and BR12-3. Particularly striking is the finding that the last five nucleotides of the 3′ end of BR12-2 are identical to the first five nucleotides of the 5′ end of BR12-3, forming a pair of direct repeats flanking the central region (Figures 9 and 17A), which has a 6-bp inverted repeat (IR) at its two termini (Figure 17A). Moreover, the sequence of the 3′ end of BR12-2, the 5-bp repeat mentioned above, and the sequence following the same 5-bp repeat of the 5′ end of BR12-3 is contiguous in the CL-repeat sequence (Figures 9 and 17A). It appears that BR12-2 and BR12-3 were originally two consecutive components of the same CL repeat and were separated by an insertion (Figure 17A). The inserted element is most likely a mobile element—a retrotransposon, MITE, or transposable element—since the two 5-bp direct repeats at the 3′ and 5′ ends of BR12-2 and BR12-3, respectively, may be its target sequences, and the 6-bp IR flanked by the direct repeats were the residual IR termini. To our knowledge, only three mobile elements have a 5-bp target site duplication (TSD): *RIRE2*, *Dasheng*, and *RIRE8*. Two additional facts suggest that this mobile element may be a member of *RIRE2* family. First, the direct repeat fits the consensus TSD sequence of 194 *RIRE2* elements: A (44%) T(40%) A(39%) T(21%) G(37%) as documented by JIANG *et al.* (2002). Second, the 5′ terminal three nucleotides (TGT) of the central region and the 3′ terminal five nucleotides (CGACA) are identical to the 5′ and 3′ termini of *RIRE2* LTR, respectively. In addition, the central region carried two coding sequences of the gag/pol region of *RIRE2*, although not in direct contact with the 3′ and 5′ termini (Figure 16).

The sequence structure of pB51-15 is more complicated. From the 5′ to the 3′ terminus, it contains retrotransposon, MITE, CL repeats, a centromeric BAC, and an unknown element (Figure 16). Three CL repeats (BR15-1, BR15-2, and BR15-3) are located in the central region of the clone. The region upstream of these CL repeats is a MITE element (*mPIF*; ZHANG *et al.* 2001). The first three nucleotides (TTA) of the 5′ end of BR15-1 are identical with the TSD sequence of *mPIF* (Figures 9 and 17B), suggesting insertion of the element. But the 3′ terminus of *mPIF* was lost by invasion of an LTR sequence of a retrotransposon (*Tekay*) and so was its associated 3′ target sequence. It looks likely that the *mPIF* first inserted into a CL repeat and then was disrupted by *Tekay* (Figure 17B). Also disrupted was the downstream of the CL repeats, where the 3′ terminus of BR15-3 was contiguous with a portion of maize centromeric BAC 15C5 (NAGAKI *et al.* 2003). This portion is composed of two components: a CentC tract followed by clone ZMABC91 in reverse polarity (Figure 16). No apparent signal of transposition event is evident. Following ZMABC91 is a 3.5-kb unknown sequence. It looks as though BR15-3 was invaded by part of BAC 15C5, including a portion of a CentC tract and its downstream sequence, which, since then, underwent complex rearrangement before recruiting the downstream unknown sequence (Figure 17B). The exact events leading to the current structure remain to be studied.

DISCUSSION

Molecular composition and organization of the B chromosome has been a challenge to maize geneticists, because it resists cloning by conventional protocols, owing to its repetitive nature and high homology to the A chromosomes. In our previous study (CHENG and LIN 2003), this problem was circumvented by cloning sequences from pachytene B chromosomes by microdissection. Nineteen B sequences were previously obtained, one (pBPC51) of which is B specific and highly repetitive in Southern analysis under regular exposure

FIGURE 9.—Multiple alignments of seven CL-repeat regions in pB51-12 and pB51-15. Sequences of BR12-1, BR12-2, BR12-3, BR12-4, BR15-1, BR15-2, and BR15-3 were aligned by using the GCG program PILEUP and visualized using BOXSHADE. Conserved nucleotides are indicated by white letters on black background. Sequences associated with pBPC51 are underlined, and those with B centromeric sequences are in box I and box V. The sequence of BR15-2 deleted in BR12-1, BR12-2, BR12-3, and BR12-4 is represented by box III and box IV. The positions of the CL-repeat primers, BP3 and BP5, are indicated by open arrows, and box II indicates the low homologous sequences of BP5.

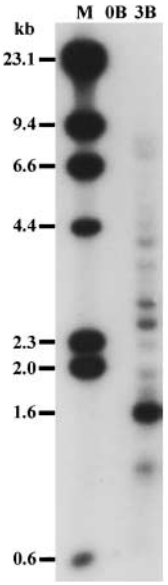


Figure 11.—Genomic organization of the CL repeat. Radioactively labeled pBPC51 hybridized the *Hin*dIII digests of L289 + 0B and L289 + 3B. M, size marker (λ*Hin*dIII digest). Molecular weights are shown on the left.

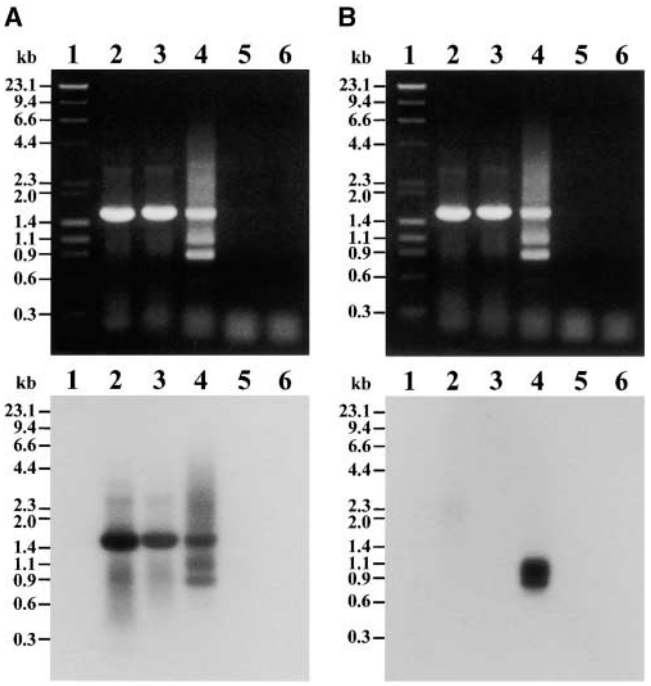


Figure 13.—Southern analysis of PCR products from CL repeat by BP3 and BP5 primers. The CL-repeat products of pB51-12 (lane 2), pB51-15 (lane 3), L289 + 3B (lane 4), L289 + 0B (lane 5), and no DNA (lane 6) were probed with the PCR product of pB51-12 (A) and the B centromere sequence (B). Lane 1, size marker (λ*Hin*dIII digest mixed with ϕX174-*Hae*III digest). Molecular weights are shown on the left.

time. In this study, we used this clone to isolate large B sequences for further characterization of the molecular structure.

Clone pBPC51 provides a unique approach for studying the maize B chromosome. Two previously published maize B-specific sequences (pZmBs, Alfenito and Birchler 1993; pBGBM18.2, Stark *et al.* 1996) are repetitive, and one (pZmBs) is located in the B centromere. Using pZmBs can isolate only sequences associated with this chromosome region. In other words, a conventional approach is suitable for studying the B centromere, but it is not effective for other regions of the B arms. This constraint was removed by pBPC51, which, as mentioned above, is B exclusive and present in high copy numbers, dispersing over a region of about one-third of the B long arm and most regions of the centromeric knob (Cheng and Lin 2003). The possibility of the presence of low copies, undetectable by FISH analysis, in the remaining B regions cannot be excluded. Accordingly, pBPC51 provides an opportunity to clone sequences associated with these B regions. It could also be used to isolate a large number of long B sequences to form a B-exclusive sublibrary, which would be invaluable for gaining insight into the genomic structure of the chromosome.

In this report, pBPC51 was used to screen a library,

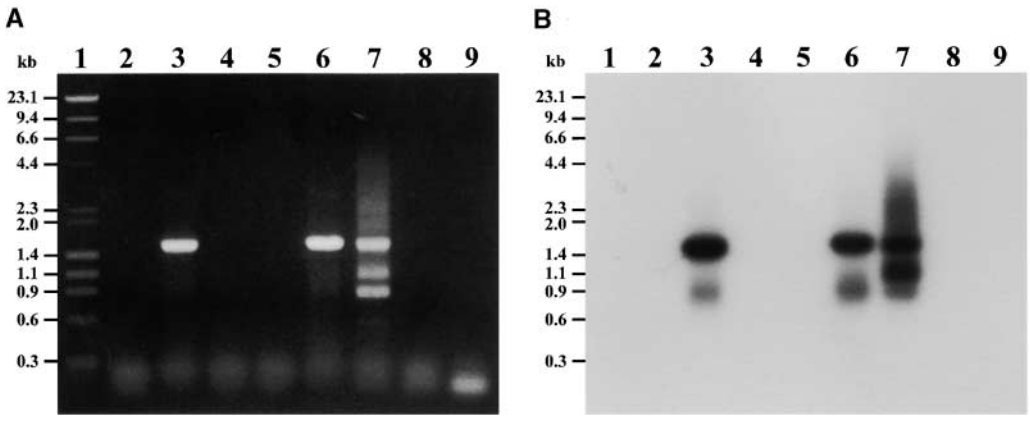


Figure 12.—PCR amplification of CL repeat. (A) Gel electrophoresis of the PCR products amplified, using BP5 and BP3 as primers, from the following DNAs: pB51-11 (lane 2), pB51-12 (lane 3), pB51-13 (lane 4), pB51-14 (lane 5), pB51-15 (lane 6), L289 + 3B (lane 7), L289 + 0B (lane 8), and no DNA (lane 9). Lane 1, size marker (λ*Hin*dIII digest mixed with ϕX174-*Hae*III digest). (B) Autoradiogram of the products probed with pBPC51. Molecular weights are shown on the left.

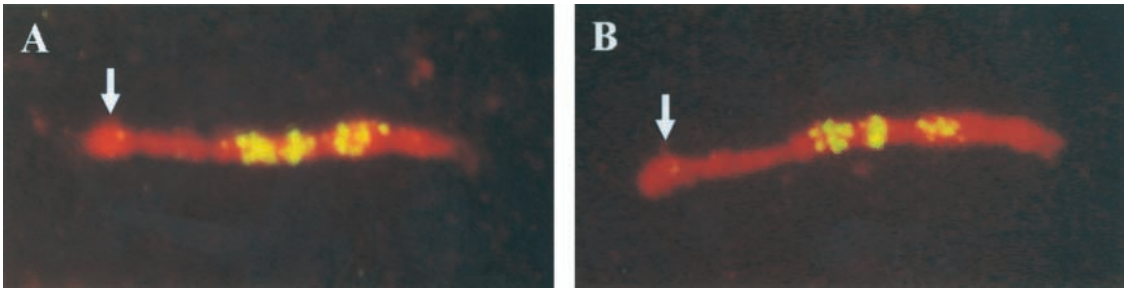


FIGURE 14.—FISH signal of CL repeat on the pachytene B chromosome. The CL-repeat insert (A) of pCLa1 and the PCR products (B) amplified from pB51-12 by BP3 and BP5 primers were used as probes to hybridize the pachytene B chromosome. FISH signals appeared intensely in the distal heterochromatic region, but weakly in the centromeric knob. The arrow indicates the centromeric knob of B chromosome.

constructed from DNA carrying 16 B's, resulting in five B sequences (>10 kb each), which are repetitive and homologous to the A chromosomes in Southern and FISH analyses. Two of them were further characterized and sequenced. Each is composed of B-specific sequence intermingled with those in common with A chromosomes. Both have no similarity to any published gene other than transposable elements (retrotransposons and MITEs) and the B and A centromeres. And both carry a novel CL repeat.

Sequence characteristics of five large B inserts confirm the previous report that most B sequences are repetitive and share homology with the A chromosomes. This is evident in Southern analysis where each, as a probe, generated smeared signals in the 0B DNA as well as in the 3B DNA, and the signal intensity is unrelated to the B number (Figure 2). Also evident are the FISH signals of these clones, which dispersed over all root-tip A chromosomes (Figure 4) and most regions of the pachytene B chromosome (Figure 3). These observations are consistent with earlier reports. PEACOCK *et al.* (1981) and VIOTTI *et al.* (1985) isolated a sequence present in multiple copies in B and A knobs. PAGE *et al.* (2001) found a B centromere sequence hybridizing with multiple restriction fragments of genomic (0B) DNA under low stringent conditions. STARK *et al.* (1996) noted that FISH signals of genomic DNA without B spread over the entire length of the mitotic metaphase B and A chromosomes. HSU *et al.* (2003) observed multiple Southern signals of TR-1 sequence in genomic (0B) DNA and its FISH signals in A and B knobs. CHENG and LIN (2003) isolated 19 B sequences from the pachytene B chromosome by microdissection; all are repetitive, and 18 hybridized with genomic (0B) DNAs in Southern analysis and with A (data not shown) and B chromosomes in FISH analysis.

The major B-specific sequences published to date are tandem repeats in plant species. Three maize B-specific sequences reported previously—pZmBs (ALFENITO and BIRCHLER 1993), pBGBM18.2 (STARK *et al.* 1996), and pBPC51 (CHENG and LIN 2003)—are repetitive sequences. The first sequences gave strong signals in the centromeric region—also weak signals in the subtelomeric region of the B long arm—in *in situ* hybridization

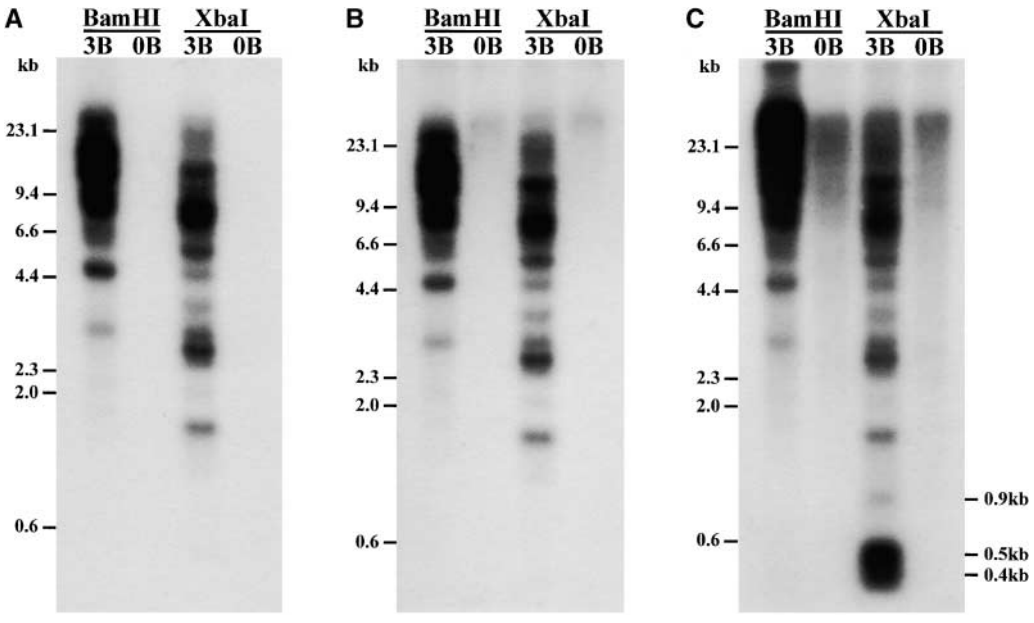


FIGURE 15.—Genomic Southern of PCR products of CL repeat by BP3 and BP5 primers. The *Bam*HI and *Xba*I digests of L289 + 3B and L289 + 0B were probed with PCR products derived from pB51-12 (A), pB51-15 (B), and L289 + 3B (C). Molecular weights are shown on the left or both sides.

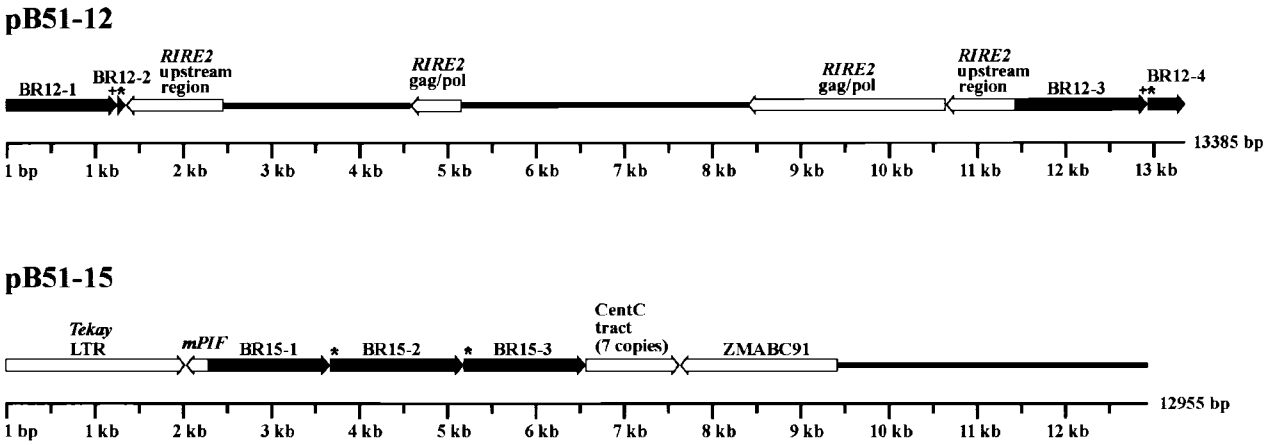


FIGURE 16.—Sequence organization of pB51-12 and pB51-15. CL repeats are indicated by solid arrows, and the other elements by open arrows. A thick line represents unknown sequences. The + and * represent the 3′ end and the 5′ end, respectively, of a CL-repeat unit.

and expressed signals of tandem array in Southern analysis. The pBPC51, dispersing over one-third of the B chromosome, was proven in this study to be a component of the CL repeats, most of which were also arranged tandemly in Southern analysis (Figure 11). Likewise, the tandem repeat was noted in B chromosomes of other plants. Two B clones, D1100 and E3900, were identified in rye by SANDERY *et al.* (1990) and BLUNDEN *et al.* (1993), respectively. Both were found to be units of tandem repeats in the same subtelomeric knob region. In *Brachycome dichromosomatica*, a 176-bp B-specific repeat (pBd49) isolated by JOHN *et al.* (1991) had a similar structure, and it is located in the centromeric region of the B chromosome by *in situ* hybridization (LEACH *et al.* 1995).

Three regions within the two clones did not have homology with any sequence in the GenBank database. Two (2 and 3 kb) of the regions are in the central region of pB51-12, and the third one (~3.5 kb) is at the 3′ terminus of pB51-15 (Figure 16). Six subclones of pB51-

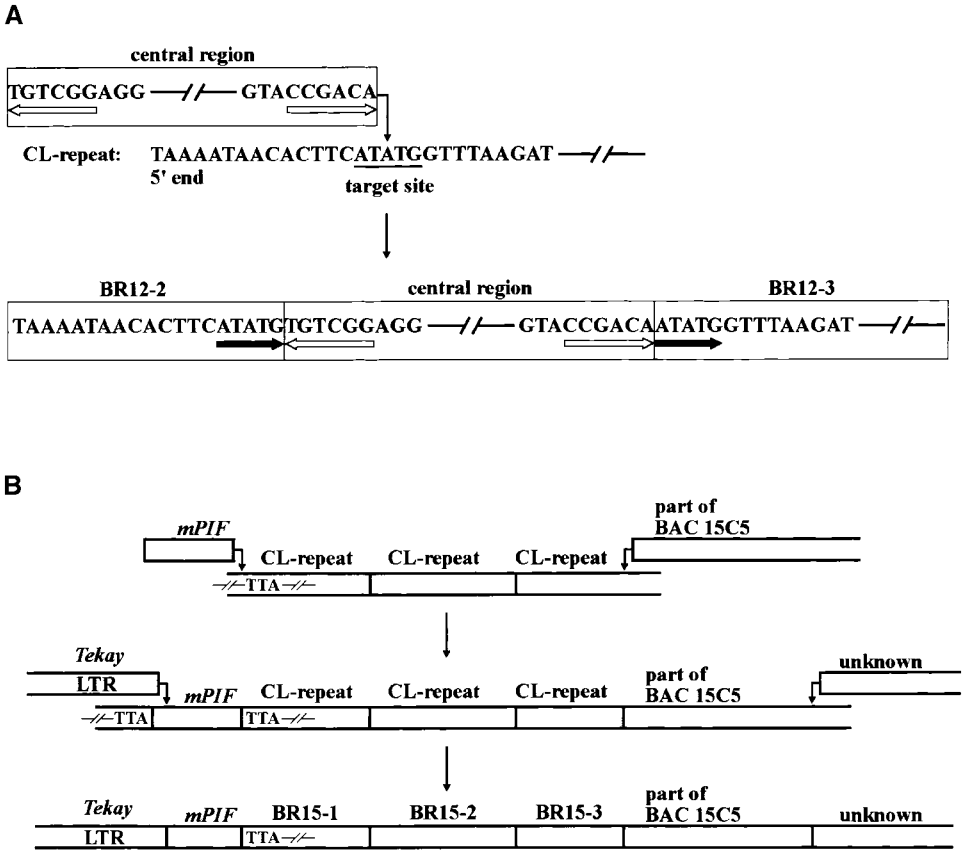


FIGURE 17.—Models for the origin of insertion in pB51-12 and pB51-15. (A) Structure of insertion in pB51-12. The positions of the direct repeats are indicated by solid arrows and the terminal inverted repeats of the central region by open arrows. The central region inserted at the 5-bp target site to result in two direct target repeats. (B) Sequential transpositions in pB51-15. The *mPIF* first invaded the 5′ end of the CL-repeat array before being disrupted by *Tekay*, and part of BAC 15C5 cut in at the 3′ side before recruiting the unknown sequence.

12 (pB51-12-4, pB51-12-5, pB51-12-7, pB51-12-8, pB51-12-9, and pB51-12-10) and four of pB51-15 (pB51-15-11, pB51-15-12, pB51-15-13, and pB51-15-14) contain exclusively sequences of these regions, and all, when used as probes in Southern analysis, show a highly repetitive signal pattern in DNAs with and without B's (Figures 5 and 6), suggesting that these novel sequences might derive from degenerated mobile elements, which remain to be discovered.

Two popular views of the origin of the B chromosome have been promoted: A chromosomes of the current host or that of closely related species (Battaglia 1964; Jones and Rees 1982). Recent data in maize are in agreement with the first view. Ward (1979) suggested 25 years ago a direct origin of the B chromosome from the K10 chromosome on the basis of his cytological comparison of the two chromosomes. Later, Page *et al.* (2001) implied that chromosome 4 was the donor of the B centromere on the basis of their observation of the homology between the centromeres of the B and fourth chromosomes. Recently, Cheng and Lin (2003) noted 10 microdissected B sequences having homology to A sequences, located in chromosomes 1, 4, 7, and 9. Consistent with this conclusion are the results of this study. Sequences of a genomic 9S clone and a centromeric BAC were borne in pB51-12 and pB51-15 clones. The exact process as to how those A sequences were transferred to the B chromosome is not clear, but results of this study indicate that transposition played an important role. Two transposable elements left clear footprints in the two clones sequenced in this study, indicating insertion of two A sequences into the CL repeat by way of transposition of a RNA retrotransposon (*RIRE2*) and a DNA MITE element (*mPIF*). Mechanisms associated with the introduction of other invading sequences into these clones remain to be explored. Other than transposition, Nassif *et al.* (1994) and Hsia and Schnable (1996) proposed synthesis-dependent strand annealing and abortive gap repair, respectively, as possible alternatives.

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