Cloning and Characterization of Maize B Chromosome Sequences Derived From Microdissection

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ABSTRACT

Isolation of sequences from the maize B chromosome is always hampered by its high homology with the normal complements. In this study, this handicap was overcome by cloning the sequences from the pachytene B chromosomes dissected out of a slide by a micromanipulator followed by degenerate oligonucleotide-primed PCR. The isolated sequences were found to hybridize with genomic DNA in a B-dosage-dependent manner and with the pachytene B chromosome by fluorescence *in situ* hybridization (FISH), corroborating their B origin. A total of 19 B sequences were isolated, all of which are repetitive and, with one exception, are homologous to the A chromosome(s). Three sequences have strong homology to maize sequences that include two knob repeats and one *zein* gene (noncoding region), and 10 others are homologous to the noncoding region of *Adh1*, *Bz1*, *Gag*, *Zein*, and B centromere to a lesser degree. Six sequences have no homology to any gene. In addition to FISH, the B-specific sequence and a partially B-specific one were also mapped, by seven newly characterized TB-10L translocations, to a similar location on the central portion of the distal heterochromatic region, spreading over a region of about one-third of the B chromosome.

SUPERNUMERARY B chromosome of maize is an extra or nonessential chromosome, and its origin is still not clear. In the form of B-A translocations, it has been widely used for gene deletion mapping (Roman and Ullstrup 1951; Beckett 1978) and for gene dosage analysis (Birchler 1979; Lin 1982). It differs from the normal complements (A chromosomes) in several aspects: it is mitotically telocentric and highly heterochromatic, and it has no detectable genetic effects on the plant, except with high numbers (Randolph 1941). In addition, it enhances recombination on the A chromosomes (Rhoades 1968) and undergoes nondisjunction at the second pollen mitosis (Randolph 1941; Roman 1947). Yet, B and A are not molecularly divergent.

Relatively few articles on B sequence have been published, and all indicated high homology between B and A chromosomes. Chilton and McCarthy (1973) performed a thermal kinetics study on maize DNA with and without B and found no detectable altered base composition between the two samples. They also analyzed the same DNA by ultracentrifugation and observed the same buoyant densities. Alfenito and Birchler (1993) screened 5000 phages from a λ-library, constructed from a line carrying 15 B's, by differential hybridization, using genomic DNA with and without B as probes. Only 8 phages hybridized more strongly to the B-carrying DNA, and all were subsequently shown to be a portion of the same sequence. By *in situ* hybridization and B-A translocations, they mapped the sequence to the centric region of the B chromosome. Page *et al.* (2001) observed recently that this sequence has homology to the fourth centromere. Stark *et al.* (1996) compared the digestion pattern of DNA with and without B, and no obvious difference was observed. They did *in situ* hybridization of mitotic B chromosomes with labeled genomic DNA without B and demonstrated the presence of hybridization signals almost the entire length of the B chromosome. They also constructed three partial genomic libraries from the 0.5- to 3.0-kb fractions of B-containing DNA digested with three different enzymes and screened the libraries by comparing the hybridization pattern between genomic probes with and without B chromosomes. No B-specific clone was found, suggesting again similar DNA composition between B and A chromosomes.

In this article, a different approach was adopted. We dissected the B chromosome from microsporocytes and obtained 19 B sequences, 18 of which share homology with the A chromosomes. Thus, our results confirm the previous conclusions of similarity between B and A chromosomes. In addition, by fluorescence *in situ* hybridization (FISH) analysis, we mapped all sequences, 2 of which were also localized by B-10L translocations.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. BH814952–814955 and BH814957–814977.

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MATERIALS AND METHODS

Plant material: Three inbred lines (L289, W22, and B73) were used in this study. Of three types of L289 plants, one carrying 2 B's (L289 + 2B) was used to prepare the pachytene chromosomes for microdissection as well as FISH analysis, and the two others containing 1 B (L289 + 1B) and no B (L289 + 0B), respectively, were the positive and negative controls in mapping studies. Three W22 plants bearing no B (W22 + 0B), 6 B's (W22 + 6B), and 16 B's (W22 + 16B), respectively, were used to characterize all isolated sequences. B73 was used as a positive control of degenerate oligonucleotide-primed PCR (DOP-PCR) and as the maternal parent for production of hyperploid and hypoploid plants. Seven B-10L translocations (TB-10L7, TB-10L16, TB-10L20, TB-10L26, TB-10L32, TB-10L36, and TB-10L38) isolated by Lin (1972) were used to generate hyperploids and hypoploids. They were carried by W22 and have a breakpoint on the B long arm (BL) and the second breakpoint on the long arm of chromosome 10 (10L; Lin 1974).

Synthesis of plants hyperploid or hypoploid for the long arm of chromosome 10: Hyperploid and hypoploid plants were produced from crossing W22 carrying the B-10L translocation as staminate parents to B73. A B-10L translocation contains two different chromosomes, 10-B and B-10. The former carries the 10th centromere and the distal portion of the BL; the latter bears the B centromere and the distal portion of the 10L. When B-10 undergoes nondisjunction at the second pollen mitosis, two sperm will be produced: one with 10-B B-10 B-10 (hyperploid) and the other with 10-B (hypoploid). The B-10 can be followed by the expression of *R-scm* (colored aleurone and scutellum) located on the 10L portion of B-10. A hyperploid kernel has colored scutellum but colorless aleurone, and a hypoploid one has colorless scutellum but colored aleurone.

Preparation of pachytene chromosomes: The slide was prepared by following the conventional protocol (Burnham 1982). Briefly, L289 + 2B was grown in the field, and microsporocytes undergoing meiosis I were fixed in ethanol-glacial acetic acid (3/1) overnight before being stored in 70% ethanol at −20°. A single anther of a floret was placed into a drop of propionic carmine on a slide. The anther was cut transversely, and microsporocytes were gently squeezed out by a needle. After the anther walls were discarded, a coverslip was applied. The slide containing isolated B's in most cells at the appropriate stage was frozen in liquid nitrogen. The coverslip was removed with a razor blade, and the slide was air-dried for microdissection.

Isolation, amplification, and cloning of B DNA collected from pachytene B chromosomes: Pachytene B chromosomes were isolated with a microneedle driven by a mechanical micromanipulator (Leitz) under an inverted microscope (IX70; Olympus, Lake Success, NY). The microneedle was prepared as follows: a borosilicate glass rod with an external diameter of 1.0 mm was pulled on a micropipette puller (P-80; Shutter Instrument) to form a microneedle with a tip diameter of 2–5 μm, which was shaped on a microforge (MF-83; Narishige, Greenvale, NY). The B fragments scraped off from a cell were treated with a 0.1-μl drop of proteinase K buffer [0.5 mg/ml proteinase K in 1× Takara (Berkeley, CA) PCR buffer], which was transferred to the reaction mixture of DOP-PCR for amplification. The B DNA was amplified by the method outlined by Telenius *et al.* (1992). PCR mixture (final volume 50 μl) included 0.1 μl dissected DNA, 1× PCR buffer (Takara), 0.7 μm degenerate oligonucleotide primer (6-MW 5′-CCG ACT CGA GNN NNN NAT GTG G-3′), 0.2 mm dNTP, and 2.5 units *Taq* DNA polymerase (Takara). After initial denaturation (94°, 10 min), 5 low-stringency cycles were run before a transition time of 3 min at 72°. This was followed by 25 high-stringency cycles and a final extension at 72° for 10 min. The low-stringency cycle was performed as follows: 94° for 1 min, 30° for 1.5 min, and 72° for 3 min, and the high-stringency cycle was performed as follows: 94° for 1 min, 55° for 1 min, and 72° for 1.5 min. To increase the amount of PCR product, DNA (2 μl) from DOP-PCR was used as template for further amplification with 25 high-stringency cycles. The second-round PCR products were ligated into a linearized vector (pBluescript), transformed to competent cells (*Escherichia coli*, DH5α) and plated onto Luria broth (LB)/Amp/X-gal/isopropyl thiogalactoside plates for selection of white colonies, which were transferred to a fresh ampicillin-containing plate and numbered individually.

Characterization of PCR-generated clones: The plasmid DNA of a selected colony was prepared by a rapid screening protocol. Each numbered clone was first inoculated into 500 μl LB with ampicillin in a 1.5-ml tube by a sterile toothpick and incubated at 37° overnight. Bacterial cells were spun down, and the supernatant was removed. The pellet was mixed with 20 μl 5× loading dye and resuspended by vortexing. The cells were lysed with equal volume of phenol/chloroform (1/1), vortexed for 1 min, and centrifuged at 12,000 rpm for 10 min. The upper aqueous phase, containing plasmid DNA, was loaded on a gel for electrophoresis, blotted onto a nylon membrane, and probed with genomic (W22 + 16B) DNA. Clones giving positive hybridization signal were recultured and their plasmid DNA was then prepared by alkaline miniprep method (Sambrook *et al.* 1989). The insert DNA was amplified using M13 forward/reverse primers and used to probe the genomic DNA for characterization of the nature of cloned sequences.

Genomic DNA isolation and Southern hybridization: Plant DNA was isolated by the method of Saghai-Maroof *et al.* (1984) with modifications by Lin and Chou (1997). The procedure of blotting, probing, hybridization, and washing was done by the method outlined by Lin *et al.* (1997).

Fluorescence *in situ* hybridization of pachytene B chromosome: Identification of microsporocytes containing pachytene chromosomes was performed according to Phillips and Wang (1982), and preparation of FISH slides followed protocols of J. Lamb, A. Kato and J. Birchler (personal communication) and Kaszás *et al.* (2002), both with modifications. Briefly, immature microsporocytes of L289 + 2B were fixed in ethanol-glacial acetic acid (3/1) overnight, and an anther was squashed in propionic carmine to determine the development stage of microsporocytes. When the appropriate stage was found, the remaining two anthers were treated with 2% Onozuka R10 cellulase (Yakult Honsha) and 5% pectinase (Serva) in 4 mm citric acid/6 mm sodium citrate buffer (pH 4.8) at 37° for 3 hr. The resulting protoplasts were suspended in ethanol-acetic acid (1/1), dropped onto a slide, and dried slowly.

Probe preparation, hybridization, and signal detection followed essentially the method of Lee *et al.* (1999). Probe was prepared from an isolated sequence labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis) by PCR amplification using M13 forward/reverse primers, purified through a G-50 column, precipitated in ethanol, and resuspended in TE (10 mm Tris-Cl, 1 mm EDTA, pH 8.0). Hybridization mixture (50% formamide, 2× SSC, 10% dextran sulfate, 0.1% SDS, and 5 ng/μl probe DNA) was dropped onto the slide prepared above and a coverslip was applied. The slide was heated at 80° for 2 min on a hot plate and incubated at 37° overnight in a humid chamber.

After hybridization, the slide was washed with 20% formamide in 2× SSC at 45° for 10 min, with 2× SSC at 42° for 10 min, and with 2× SSC at room temperature three times, 5 min each. The hybridization signal was detected with mono-

TABLE 1

Twenty-five microdissection sequences and accession numbers in GenBank

Clone no.	Accession no.
pBPC1	BH814972
pBPC2	BH814957
pBPC3	BH814970
pBPC4	BH814954
pBPC5	BH814965
pBPC6	BH814961
pBPC7	BH814959
pBPC8	BH814955
pBPC14	BH814962
pBPC15	BH814966
pBPC16	BH814958
pBPC21	BH814976
pBPC23	BH814975
pBPC28	BH814974
pBPC29	BH814971
pBPC31	BH814969
pBPC32	BH814964
pBPC42	BH814963
pBPC43	BH814960
pBPC45	BH814968
pBPC48	BH814953
pBPC49	BH814973
pBPC50	BH814952
pBPC51	BH814977
pBPC52	BH814967

clonal anti-digoxin (Sigma, St. Louis), amplified with FITC-conjugated anti-mouse IgG (Sigma), counterstained with 1 μg/ml propidium iodide, and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). It was visualized under an Olympus BH2 fluorescent microscope equipped with appropriate filter sets. The image was captured with a cooled charge-coupled device camera (Penguin 150CL; Pixera) and processed in Photoshop (Adobe, San Jose, CA).

Sequence analysis: Sequence comparison was performed using BLAST software of the National Center for Biotechnology Information (NCBI) website. Twenty-five sequences were registered in the genome survey sequences database (dbGSS) of GenBank. Their accession numbers are listed in Table 1.

RESULTS

Isolation of B sequence: A bivalent B chromosome was removed directly from a pachytene slide by a micromanipulator (see MATERIALS AND METHODS). It was distinguished by its large heterochromatic block and a large knob at one end (Figure 1A; LIN 1979). Monitored under microscope, an isolated B chromosome was scraped off from the cell (Figure 1B). The resulting fragments, amassed to a pile and adhered statically onto the needle tip, were transferred into a drop of proteinase K buffer placed on a separate slide. A total of 10 B chromosomes were collected, and the B-containing buffer was added to the reaction mixture for amplification.

Figure 2A displays the gel electrophoresis of the first PCR products (5 μl). The dissected B fragments yielded products of 0.2–4.5 kb (Figure 2A, lane 3), and the positive control (genomic DNA of B73 as template) yielded products of 0.2–6.0 kb (Figure 2A, lane 4). Unexpectedly, the negative controls (no template DNA) produced a substantial amount of products (Figure 2A, lanes 2 and 5). To determine if DNA in the negative controls was amplified from maize DNA, the gel was blotted and probed with genomic (W22 + 16B) DNA (Figure 2B). Hybridization signal was present in the products of B DNA and B73 (Figure 2B, lanes 3 and 4), but not in that of the negative controls (Figure 2B, lanes 2 and 5). The signal of the B DNA is much weaker than that of B73; thus, the B DNA was amplified along with contaminant DNA. The products of the second PCR (Figure 2C) have a similar hybridization pattern (Figure 2D), but the signal intensity of the B products is enhanced (Figure 2D, lane 3). Furthermore, to clarify whether the unexpected products are the result of inadequate DOP-PCR protocols, products of the second PCR (Figure 2E) were probed by the contaminated products (Figure 2E, lane 2). No hybridization was observed in the products of B73 DNA (Figure 2F, lane 4) but very strong signal was observed in that of the negative controls (Figure 2F, lanes 2 and 5) and the B DNA sample (Figure 2F, lane 3), suggesting a negative correlation (see DISCUSSION for implications).

Southern analysis: A library was constructed from the B PCR products. A total of 1671 clones were screened for the presence of B sequences. Probed with labeled genomic (W22 + 16B) DNA, 40 positive clones were identified. Their inserts were used to probe genomic DNA of (W22 + 0B), (W22 + 6B), and (W22 + 16B) digested with three enzymes (*Pst*I, *Eco*RI, and *Sac*I). Out of 40 positive clones, 25 produced hybridization signals, and the remainder did not. Southern analyses of the 25 clones are listed in Table 2. Sixteen clones have an increase in signal intensity paralleled with an increase in B numbers, 8 others do not, and 1 is B specific. Among those clones with B-dosage response, 4 have an increased intensity in all signals. For example, the signal of pBPC4 is smeared in the *Sac*I-digested DNA (Figure 3, A1 and A2, lanes 7–9), and in discrete bands in two other digests (Figure 3, A1 and A2, lanes 1–6). All of these signals show high correlation with the B number. Six others (pBPC2, pBPC6, pBPC7, pBPC14, pBPC16, and pBPC43) have two types of signals: one is responsive to the B dosage (discrete bands) and the other is not (smeared portion; data not shown). The last 6 clones are also B-dosage responsive, but different from the clones described above in one aspect. For example, pBPC45 has two signals in all three DNA digests: one major and one minor signal in *Pst*I and *Eco*RI digests (Figure 3, B1 and B2, lanes 1–6) and two major bands in the *Sac*I digest (Figure 3, B1 and B2, lanes 7–9). All minor signals have similar intensity, but major bands in (W22 + 6B) are stronger than those in (W22 + 0B).

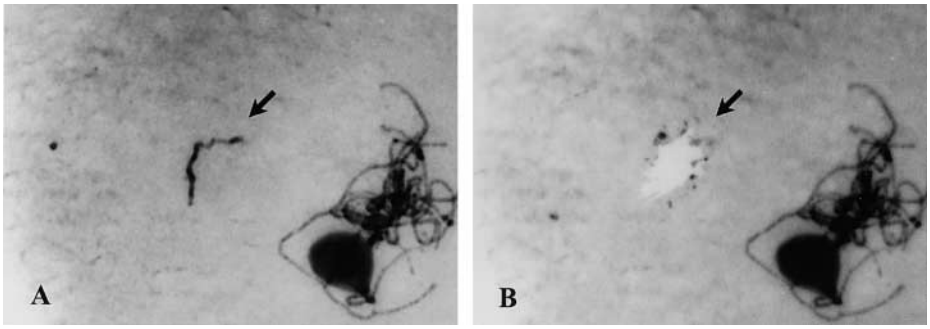

FIGURE 1.—Isolation of an individual B chromosome by micromanipulator. (A) B bivalent (arrow) at the pachytene stage. (B) The B bivalent in A (arrow) was removed from the cell.

Yet, the intensity between (W22 + 6B) and (W22 + 16B) is alike.

Clone pBPC31 is a representative sequence that does not have the B-dosage response. It has smeared signals in the *Pst*I digest (Figure 3, C1 and C2, lanes 1–3), smeared signals plus one discrete band in the *Eco*RI digest (Figure 3, C1 and C2, lanes 4–6), and two discrete bands in the *Sac*I digest (Figure 3, C1 and C2, lanes 7–9). The signal pattern and intensity are identical in samples with and without B chromosome, implying abundance of the sequence in A chromosomes. A particularly interesting clone (pBPC51) is a B-specific sequence. Its hybridization signal is smeared in all three enzyme digests, absent in (W22 + 0B), but present in (W22 + 6B) and (W22 + 16B) (Figure 3, D1 and D2). Moreover, it also has B-dosage response; its signal is more intense in (W22 + 16B) than in (W22 + 6B).

Signal complexity of the 25 sequences varies considerably. On the basis of the signal pattern expressed in three enzyme digests, those sequences were grouped into three types: a smeared signal (type I; Figure 3D2), a smeared signal in addition to discrete bands (type II; Figure 3, A2 and C2), and only discrete bands (type III; Figure 3B2). Among 25 clones, 4 belong to type I, 14 to type II, and 7 to type III (Table 2).

FISH analysis: To firmly establish that the isolated 25 sequences originated from the B chromosome, we used fluorescence *in situ* hybridization to analyze the pachytene B chromosome, which is composed of—from proximal to distal—a short arm (BS), a centromere, a centromeric knob (CK), a proximal euchromatic region (PE), four distal heterochromatic regions (H1–4), and a distal euchromatic tip (DE) as shown in Figures 5A and 7. Using these sequences as probes, the hybridization signal was detected in all but 6 sequences as listed in Table 3, indicative of B origin. Absence of hybridization associated with the exceptional sequences indicates that they were either not from the B chromosome or from B but not detectable by the FISH system of this study. The first view agrees with the results of sequence comparison detailed in DISCUSSION. The remaining 19 sequences that hybridized with the B chromosome displayed eight different signal patterns. Five patterns are uniquely expressed by a single sequence, two others each by 2 sequences, and the last one by 10 sequences (Table 3). The unique signal patterns were individually represented by pBPC16 with signal on the entire B chromosome, by pBPC7 on all B regions but DE, by pBPC48 on all regions except BS and CK, by pBPC51 on all regions but PE and DE (Figure 4A), and by pBPC50 on CK and H1–4 (Figure 4B). The signal pattern, expressed by 2 sequences, had signals on either CK and H4 (pBPC23 and pBPC21; Figure 4C) or all B regions except BS and DE (pBPC4 and pBPC14). The last pattern expressed by 10 sequences (*e.g.*, pBPC28; Figure 4D), displayed signals on the entire B chromosome except BS.

Sequence analysis: Nineteen B clones were sequenced and analyzed by Blast Two sequences (TATUSOVA and MADDEN 1999). The B sequences average 345 bp (184–743 bp) long (Table 4) and are 57% A-T rich (47–69%). Of the 19 sequences, 1 has its entire sequence in common with and embodied in another sequence: pBPC21 [nucleotide (nt) 1–215] corresponds to nt 396–181 and nt 216–1 of pBPC23 (92% identity). In addition, two pairs of sequences are partially homologous to each other: nt 59–371 of pBPC28 matches nt 98–403 of pBPC43 (81% identity) and nt 1–246 of pBPC1 matches nt 7–252 of pBPC49 (93% identity). The remaining 13 sequences exhibit no homology to any other sequence.

In view of the fact that previously isolated B sequences in maize (PEACOCK *et al.* 1981; ALFENITO and BIRCHLER 1993) and rye (SANDERY *et al.* 1990; BLUNDEN *et al.* 1993) are of tandem repetitive nature, it is interesting to see if similar structure exists in the isolated sequences. Using Tandem Repeats Finder (BENSON 1999), we found that only 1 of 19 B sequences contains short tandem repeats; pBPC7 has repeats of 3 different sequences: 2 of 18 bp (3.3 and 1.9 copies, respectively) and 1 of 15 bp (2.3 copies). The remaining sequences bear no tandem repeats.

The B sequences were compared with sequences in GenBank (Table 4). Six clones do not show homology to any published sequence. The entire sequence of pBPC48 is homologous to two noncoding regions of maize 22-kD *zein* gene (90% identity). Nine clones have a fraction of sequence homologous to the noncoding regions in the GenBank entries of several maize genes, including *zein*, *bz1*, and *adh1*. Three clones homologous

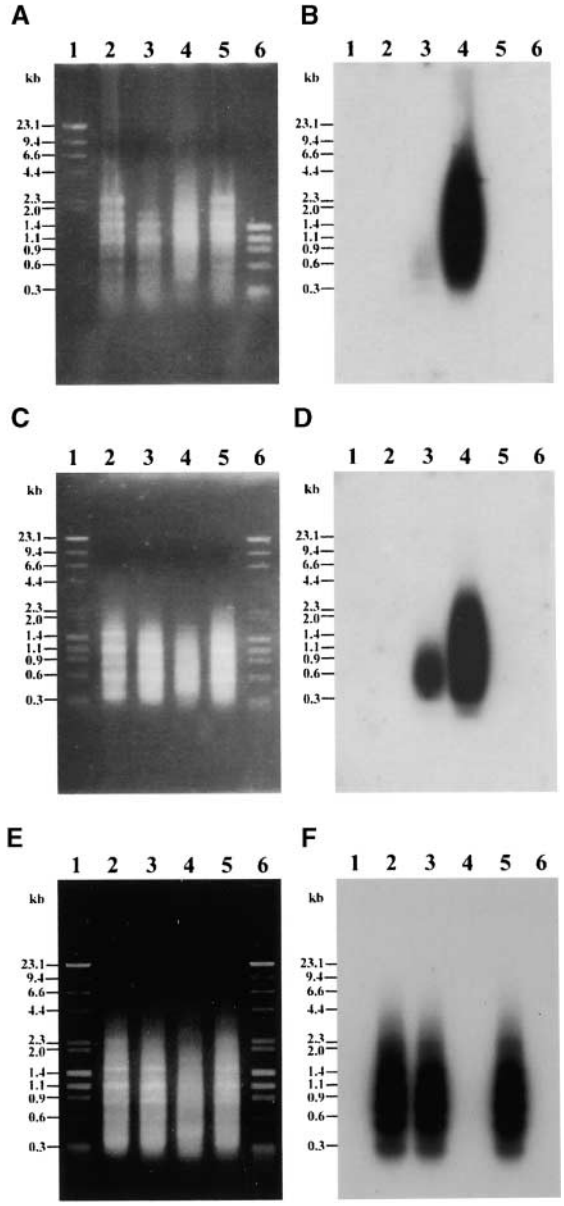

FIGURE 2.—Characterization of products from first and second PCRs. (A) Gel electrophoresis of the products of the first PCR. (B) Autoradiogram of the first PCR products probed with genomic (W22 + 16B) DNA. (C) Gel electrophoresis of the products of the second PCR. (D) Autoradiogram of the second PCR products probed with genomic (W22 + 16B) DNA. (E) Gel electrophoresis of the products of the second PCR. (F) Autoradiogram of the second PCR products probed with the products of the negative control. Lane 1, size marker (λ-*Hin*dIII digest or with Φx174-*Hae*III digest); lane 2, negative control (no template); lane 3, dissected B fragments as template; lane 4, positive control [B73 genomic DNA (110 ng) as template]; lane 5, negative control (no template); lane 6, size marker (Φx174-*Hae*III digest or with λ-*Hin*dIII digest).

to *bz1* are sequences sharing homology with the LTR region of retroelement.

Of particular interest are sequences related to maize knobs and the B centromere. Two sequences (pBPC23 and pBPC21) show homology to a maize 180-bp knob-specific repeat clone (GenBank accession no. M32522; DENNIS and PEACOCK 1984). pBPC23 is 399 bp in length, homologous to three regions of the knob sequence: nt 1–30, 35–214, and 215–394 (147–176, 1–180, and 1–180, respectively, in M32522, average identity 96%). The second sequence, pBPC21, is 215 bp in length and its nt 3–182 correspond to knob region 180–1 in M32522 (95% identity). PEACOCK *et al.* (1981) observed that the repeated sequence is specific to knobs on A chromosomes and the B-centromeric knob, which was verified by our FISH analysis (Figure 4C; other data not shown). Accordingly, these two sequences may have derived from the centromeric knob of the B chromosome. A third sequence (pBPC51) has 329 bp and is B specific. A portion of the sequence is present in seven documented B-centromeric sequences (90% identity). Thirty-three base pairs (nt 293–325) of pBPC51 correspond to nt 485–517 of K7 (accession no. U62002), to nt 361–393 of K5 (U62000), to nt 417–449 of K2 (U621988), to nt 485–517 of K11 (U61997), to nt 485–517 of K10

TABLE 2

Southern analysis of 25 sequences isolated by microdissection of the pachytene B chromosome

Clone no.	B-dosage response	Signal complexity[e]
pBPC4	+[a]	II
pBPC8	+	II
pBPC48	+	II
pBPC50	+	III
pBPC2	(+)[b]	II
pBPC6	(+)	II
pBPC7	(+)	II
pBPC14	(+)	II
pBPC16	(+)	II
pBPC43	(+)	II
pBPC45	<+>[c]	III
pBPC5	<+>	III
pBPC15	<+>	III
pBPC32	<+>	III
pBPC42	<+>	III
pBPC52	<+>	III
pBPC31	−[d]	II
pBPC1	−	II
pBPC3	−	II
pBPC21	−	I
pBPC23	−	I
pBPC28	−	I
pBPC29	−	II
pBPC49	−	II
pBPC51	B specific	I

[a] Positive B-dosage response.
[b] Only a portion of signals are B-dosage responsive.
[c] B-dosage responsive clone with similar signal intensity between (W22 + 6B) and (W22 + 16B).
[d] No response to B dosage.
[e] I, smeared signal; II, smeared signal plus discrete bands; III, only discrete bands.

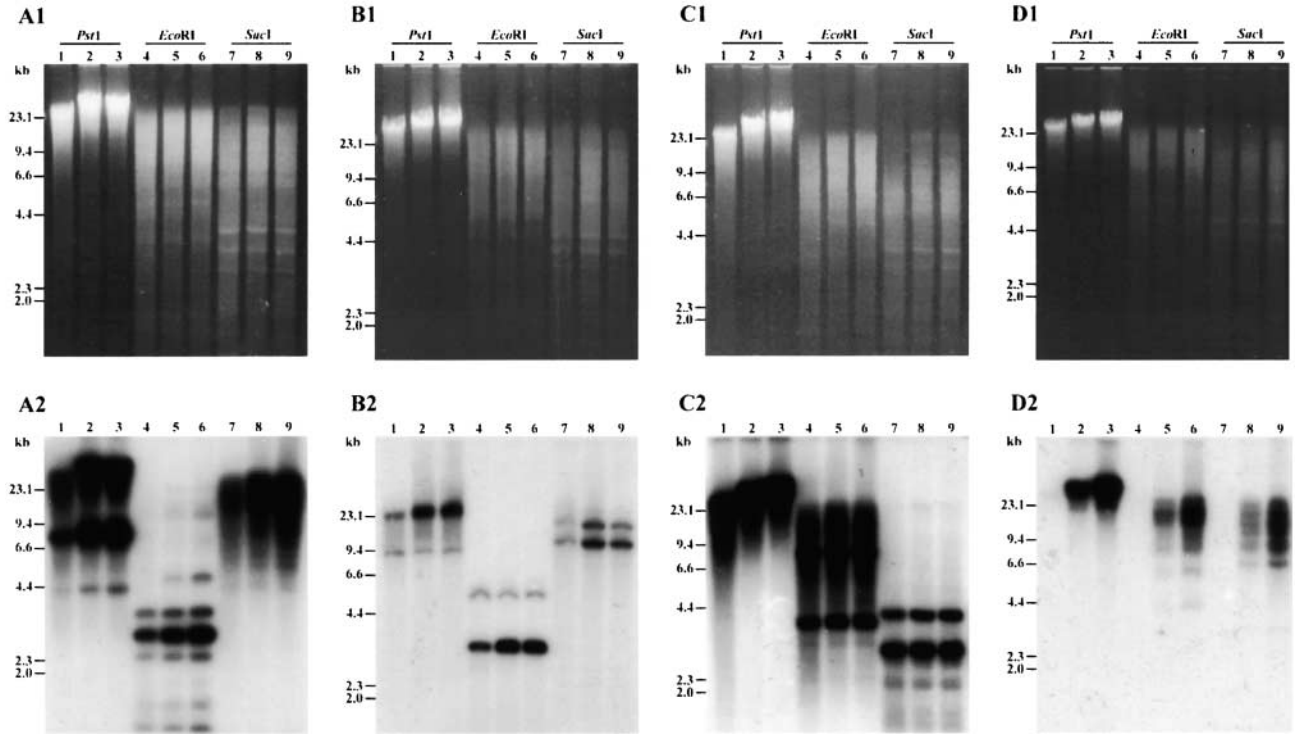

FIGURE 3.—Southern blot analysis of four cloned sequences. Genomic DNA (W22 + 0B, lanes 1, 4, and 7; W22 + 6B, lanes 2, 5, and 8; and W22 + 16B, lanes 3, 6, and 9) was digested with *Pst*I, *Eco*RI, and *Sac*I (A1, B1, C1, and D1) and probed with clones pBPC4 (A2), pBPC45 (B2), pBPC31 (C2), and pBPC51 (D2). The molecular weights are shown on the left of the ethidium-bromide-stained gels (top) and the corresponding Southern blots (bottom).

(U61996), to nt 486–518 of B7b (U61995), and to nt 397–429 of B3 (U61991). Since only 33 out 329 bp are identical to the published B-centromeric sequences, whether pBPC51 originated from the B centromere is a matter of speculation.

Using Open Reading Frame Finder (NCBI), the longest open reading frames (ORFs) range from 22 to 124 codons (Table 4). Disregarding sequences homologous to knob and B-centromeric sequences, the longest ORF (pBPC7) covers 115 codons matching with maize *zein* protein. The second longest ORF (pBPC8) includes 107 codons, which have no similarity to any gene in GenBank. The next ORFs in this order have 100 and 94 codons, respectively, and both are not comparable to any documented protein. The remaining ORFs are shorter than 72 codons. Whether or not these ORFs are valid cannot be determined until the regions upstream and downstream of these sequences are available.

Mapping B sequences by B-10L translocations: Seven B-10L translocations (TB-10L7, TB-10L16, TB-10L20, TB-10L26, TB-10L32, TB-10L36, and TB-10L38) with a breakpoint located on the BL were used to map the B sequences. The breakpoint of TB-10L7 and TB-10L16 is located in the PE region (Figure 5, B and C), and five others break in the distal heterochromatic regions: TB-10L38 at the junction of H1 and H2 (Figure 5D), TB-10L26 and TB-10L32 in H2 close to the H2-H3 junction (Figure 5, E and F), and TB-10L20 as well as TB-10L36 in H3 near the H3-H4 junction (Figure 5, G and H). Of these, the breakpoint of TB-10L20 is proximal to that of TB-10L36. The map positions of these breakpoints were summarized in Figure 7. Results of this study are consistent with the previous observation by LIN (1978) on the breakage of TB-10L7, TB-10L20, TB-10L36, and TB-10L38.

These translocations were used to map the B sequences. Feasibility of such mapping depends on whether a sequence conforms to one of the two following requirements: it is either B specific or not B specific but its signal in +1B DNA is distinguishable from that in +0B DNA. Out of 19 B sequences, 17 do not meet either requirement; they are not B specific. And 8 of these hybridize equally among W22 + 0B, W22 + 6B, L289 + 0B, and L289 + 1B. Nine others have their signal intensity in L289 + 1B indistinguishable from that in L289 + 0B, although their signals are more intense in W22 + 6B than in W22 + 0B (Figure 3A2). Of the remaining two sequences, pBPC51 is B specific. And pBPC50 is not B specific but some of its signals are B specific in L289 + 1B digested with *Bam*HI (Figure 6B, lane 1) and with *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, and *Sac*I (data not shown). Thus, only these two sequences could be mapped by translocations.

The rationale of mapping is as follows: the 10-B of each translocation carries the portion of BL distal to the breakpoint, and the B-10 bears the proximal portion. A

TABLE 3

FISH analysis of 25 sequences isolated by microdissection of the pachytene B chromosome

Clone no.	FISH	Position[c]
pBPC16	+[a]	BS, CK, PE, H1, 2, 3, 4, DE
pBPC7	+	BS, CK, PE, H1, 2, 3, 4
pBPC48	+	PE, H1, 2, 3, 4, DE
pBPC51	+	BS, CK, H1, 2, 3, 4
pBPC50	+	CK, H1, 2, 3, 4
pBPC23	+	CK, H4
pBPC21	+	CK, H4
pBPC4	+	CK, PE, H1, 2, 3, 4
pBPC14	+	CK, PE, H1, 2, 3, 4
pBPC28	+	CK, PE, H1, 2, 3, 4, DE
pBPC1	+	CK, PE, H1, 2, 3, 4, DE
pBPC2	+	CK, PE, H1, 2, 3, 4, DE
pBPC3	+	CK, PE, H1, 2, 3, 4, DE
pBPC6	+	CK, PE, H1, 2, 3, 4, DE
pBPC8	+	CK, PE, H1, 2, 3, 4, DE
pBPC29	+	CK, PE, H1, 2, 3, 4, DE
pBPC31	+	CK, PE, H1, 2, 3, 4, DE
pBPC43	+	CK, PE, H1, 2, 3, 4, DE
pBPC49	+	CK, PE, H1, 2, 3, 4, DE
pBPC5	−[b]	
pBPC15	−	
pBPC32	−	
pBPC42	−	
pBPC45	−	
pBPC52	−	

[a] Detectable hybridization signals on the B chromosome.
[b] No hybridization signals on the B chromosome.
[c] Position of B signals: BS, short arm; CK, centromeric knob; PE, proximal euchromatic region; H1, 2, 3, 4, distal heterochromatic regions; DE, distal euchromatic tip.

hypoploid progeny contains a 10-B but no B-10. The hyperploid plant, on the other hand, carries a 10-B and two B-10's (see MATERIALS AND METHODS). When a B signal is present in the hypoploid DNA, it must be located distal to the breakpoint, that is, on the 10-B. On the other hand, when the B signal is absent in the hypoploid DNA, it should be located proximal to the breakpoint; that is, it is on the B-10. Independent of the break position, the hyperploid DNA should have the signal.

Figure 6A shows the result of mapping the first sequence (pBPC51). The hybridization signal is present in L289 + 1B but not in L289 + 0B (Figure 6A, lanes 1 and 2), indicative of sequence unique to the B. It is also present in both hyperploid and hypoploid of TB-10L7, TB-10L16, and TB-10L38 (Figure 6A, lanes 3–8). Two of the remaining translocations, TB-10L20 and TB-10L36, possess signal in the hyperploid but not in the hypoploid (Figure 6A, lanes 13–16). TB-10L26 is different from the five aforementioned translocations, in that its hypoploid has eight discrete bands but it lacks the intense smeared background and a 2.0-kb band (Figure 6A, lane 10). TB-10L32 has a signal pattern comparable to that of TB-10L26, but its hypoploid deletes a 2.8-kb band, which, apparently, is located between the two breakpoints (Figure 6A, lane 12). Results imply that pBPC51 is a repetitive sequence spreading in a region from TB-10L38 to TB-10L20, and the last two translocations break in the middle of the region with TB-10L26 proximal to TB-10L32 (Figure 7).

Figure 6B illustrates results of mapping pBPC50 to the similar location. Hybridization signal appears in both L289 + 1B and L289 + 0B with the exception of three bands (2.3, 5.3, and 6.6 kb). The 2.3- and 6.6-kb bands are present in the former, but deleted in the latter, and the third one is present in the DNA of both, but its intensity is much (at least fivefold) stronger in the former than in the latter (Figure 6B, lanes 1 and 2). The sequence was mapped on the basis of these polymorphic signals. The 5.3-kb band is present in the hyperploid and hypoploid of seven translocations, but reduced in the hypoploids of TB-10L20 and TB-10L36 (Figure 6B, lanes 14 and 16). The pattern of the 2.3-kb band in the seven translocations is identical to that of

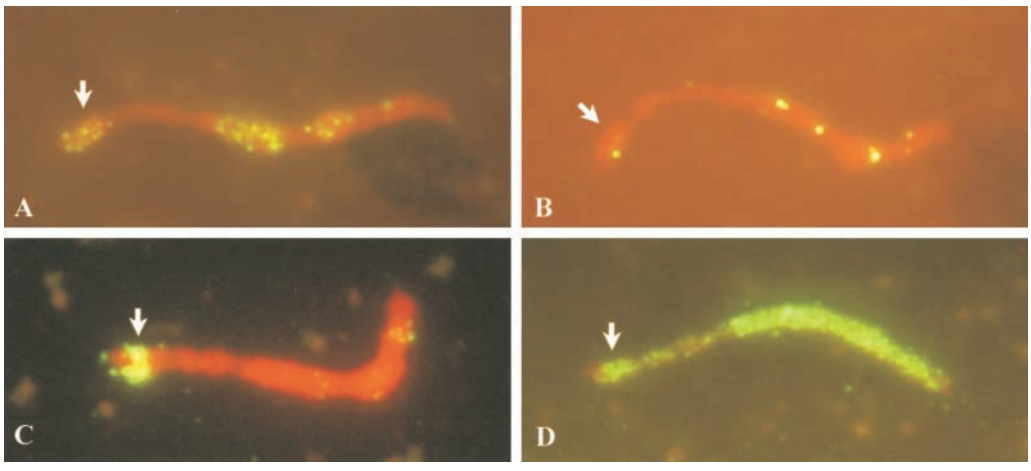

FIGURE 4.—FISH analysis of four B sequences. Pachytene B chromosome was probed with pBPC51, pBPC50, pBPC21, and pBPC28. Chromosome stain is red and B signal is yellowish. Arrows mark the centromeric knob of B chromosome. The hybridization signals of pBPC51 (A) appear on BS, CK, and H1–4; pBPC50 (B) on regions similar to A but in smaller areas; pBPC21 (C) on CK and H4; and pBPC28 (D) over the entire B chromosome except BS (see Figures 5A and 7 for various B regions).

TABLE 4

Sequence analysis of 19 B sequences

Clone no.	Size (bp)	Nucleotide comparisons			
		Sequence in GenBank	X[a]	Y[b]	Z[c]
pBPC1	248	Maize clone ZMMBBb_0138B04	5–238	94	40
		Maize *bz1* genomic region	1–246	92	
pBPC2	239	Maize 22-kD α *zein* gene cluster	12–33	100	22
			Also 163–239	84	
pBPC3	292	Maize alcohol dehydrogenase 1 gene	202–252	98	64
			Also 1–184	87	
pBPC4	301	None	—	—	58
pBPC6	743	None	—	—	44
pBPC7	396	Maize 22-kD α *zein* gene cluster	2–312	93	115
pBPC8	349	None	—	—	107
pBPC14	620	None	—	—	94
pBPC16	345	Maize *bz1* genomic region	167–196	96	27
			Also 133–224	88	
pBPC21	215	Maize 180-bp knob-specific repeat	3–182	95	69
pBPC23	399	Maize 180-bp knob-specific repeat	35–214	95	124
			Also 215–394	98	
pBPC28	373	Maize 19-kD *zein* protein	1–109	93	54
pBPC29	354	None	—	—	100
pBPC31	184	Maize retroelement PREM-2 *gag* gene	3–181	94	28
		Maize 22-kD α *zein* gene cluster	9–184	93	
pBPC43	411	Maize 19-kD *zein* protein	98–147	96	72
pBPC48	244	Maize 22-kD α *zein* gene cluster	1–244	90	68
pBPC49	254	Maize *bz1* genomic region	11–254	97	34
pBPC50	262	None	—	—	32
pBPC51	329	Maize B centromere sequence	293–325	90	44

[a] The portion of a B sequence in terms of the nucleotide position that is the most homologous to the GenBank sequence.
[b] Identity of X between B and GenBank sequences.
[c] The longest possible open reading frame.

the 5.3-kb band except for TB-10L26, TB-10L32, TB-10L20, and TB-10L36, where the 2.3-kb band is absent in the hypoploids (Figure 6B, lanes 10, 12, 14, and 16). The last B signal (6.6 kb) has a signal pattern similar to that of the 2.3-kb band, although its intensity is reduced in the hypoploids of TB-10L26 and TB-10L32 (Figure 6B, lanes 10 and 12). These results place pBPC50 in two regions of B chromosome: one between TB-10L38 and TB-10L26 and the other between TB-10L32 and TB-10L20 (Figure 7). In addition, since the other hybridization signals are present in L289 + 0B, they are also located in A chromosomes, but their exact location is not clear.

DISCUSSION

Nineteen B sequences were obtained from microdissection of pachytene B chromosomes and amplified with DOP-PCR, and all of them are repetitive. Of these sequences, 18 can hybridize with A chromosomes, and the remaining 1 is B specific. Sequence analysis reveals that 13 B sequences have homology to several published genes or sequences. By using FISH and B-10L translocations, these sequences were mapped to various B regions.

Unexpected products appeared in the PCR amplification. These products were observed in the first PCR with and without B DNA template. The reaction without B DNA generated more products than that with B DNA (Figure 2A, lanes 2, 3, and 5). For the products of the latter, only a minor fraction hybridized with maize DNA (Figure 2B, lane 3), but for the products of the former, no hybridization was observed (Figure 2B, lanes 2 and 5). This result is not an artifact of PCR because the PCR products of genomic (B73) DNA included in the same assay hybridized strongly with maize DNA (Figure 2, A and B, lane 4) as would be expected. Apparently, the unexpected products result from DNA contamination of an unknown source. They were favorably amplified when their concentration was higher than that of the B DNA template. This view was supported by the observation that the PCR products of B73 DNA failed to hybridize with the contaminated products (negative control) as probes (Figure 2F, lane 4) but that of negative controls and B DNA did so intensively (Figure 2F, lanes 2, 3, and 5). Excessive B73 DNA templates in the

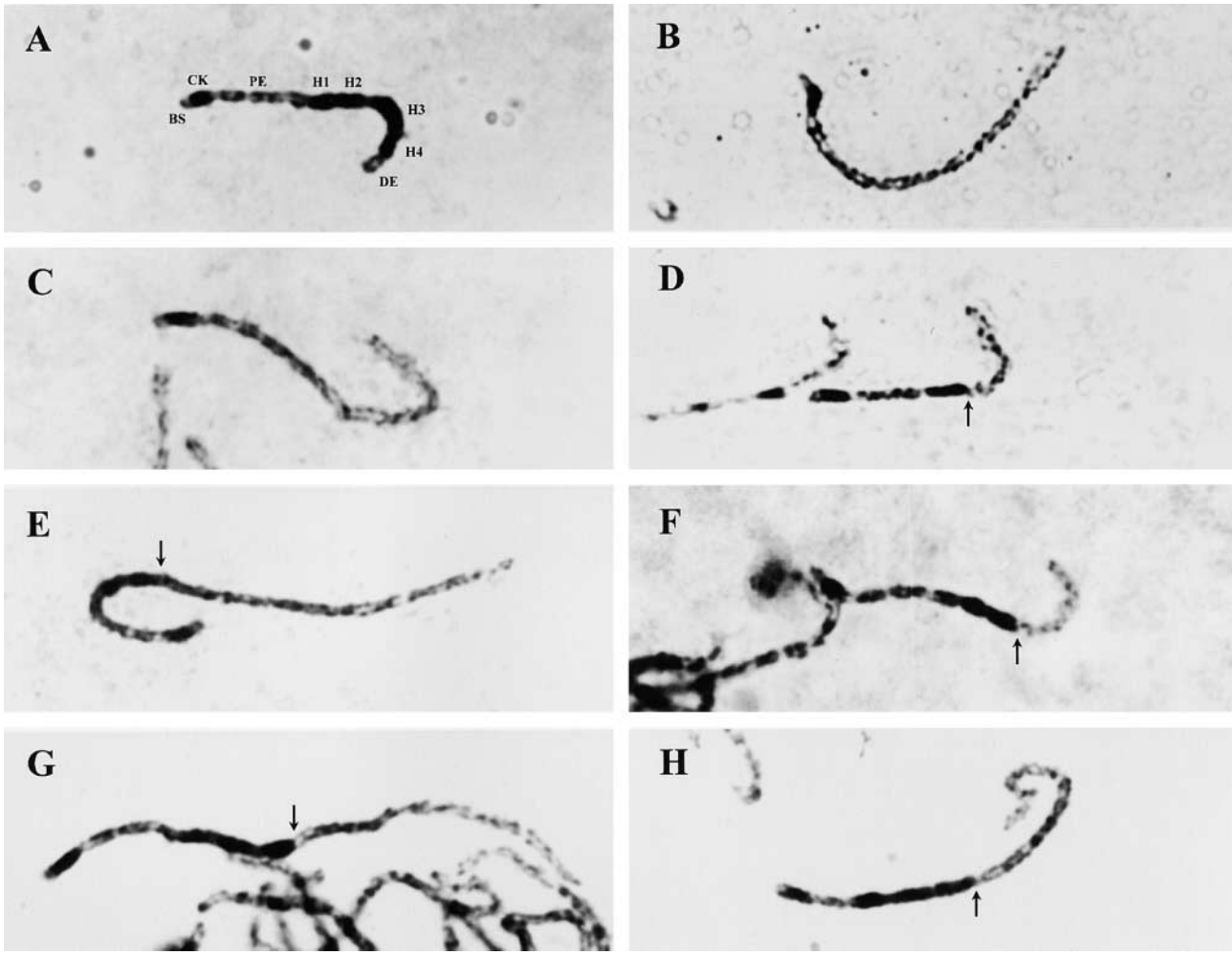

Figure 5.—Pachytene structure of the B chromosome and seven B-10L translocations. (A) B chromosome. (B) TB-10L7. (C) TB-10L16. (D) TB-10L38. (E) TB-10L26. (F) TB-10L32. (G) TB-10L20. (H) TB-10L36. The breakpoint of TB-10L7 and TB-10L16 could not be determined, since the PE region is cytologically indistinguishable from the euchromatic region of the long arm of chromosome 10. Arrow marks the breakpoint of a translocation.

first reaction suppressed amplification of the contaminated DNA. When B73 DNA was not present, the contaminated DNA was predominantly amplified. Similar results were documented by Stein *et al.* (1998). They isolated the short arm of maize pachytene chromosome 6 by microdissection and amplified with linker-adaptor-mediated PCR, which produced substantial products without DNA template. The products did not hybridize with maize DNA. They explained that enzyme solution containing bacterial DNA might be the source of contamination. Yet, contamination in other reagents may also be the source.

Most B DNA is in common with DNA of the A chromosomes. This is evidence that 18 B sequences hybridized with DNA of both B and A chromosomes (Table 2), indicating that most B sequences have counterparts in the A chromosomes. This observation conforms to earlier reports. Peacock *et al.* (1981) isolated a 185-bp repeat that, by *in situ* hybridization, probed specifically to knobs of B and A chromosomes. Viotti *et al.* (1985) identified a separate repetitive sequence and also mapped it to the knobs of both B and A chromosomes. Some copies of the sequence were located in the A euchromatic region and others were located in the distal heterochromatic block of the BL. Recently, Alfenito and Birchler (1993) isolated eight B-centromeric sequences, each about 10 kb in size. One sequence (EMBL3-8Bb, 9.8 kb) was analyzed in great detail. It consists of two subsequences, one (~6.4 kb) of which shares homology with A chromosome(s) and the other (2.9 plus 0.5 kb) is B specific. Stark *et al.* (1996) probed the mitotic metaphase B chromosome with labeled genomic DNA without B and noted that the hybridization signal spread over the entire length of the B chromosome. Similar situations also occur in other plant species. Sandery *et al.* (1991) isolated a rye B sequence by microdissection and microcloning. This sequence hybridized with both B and A chromosomes. Houben *et al.* (1996) also used microdissection to isolate the rye B chromosome and amplified it by DOP-PCR. The

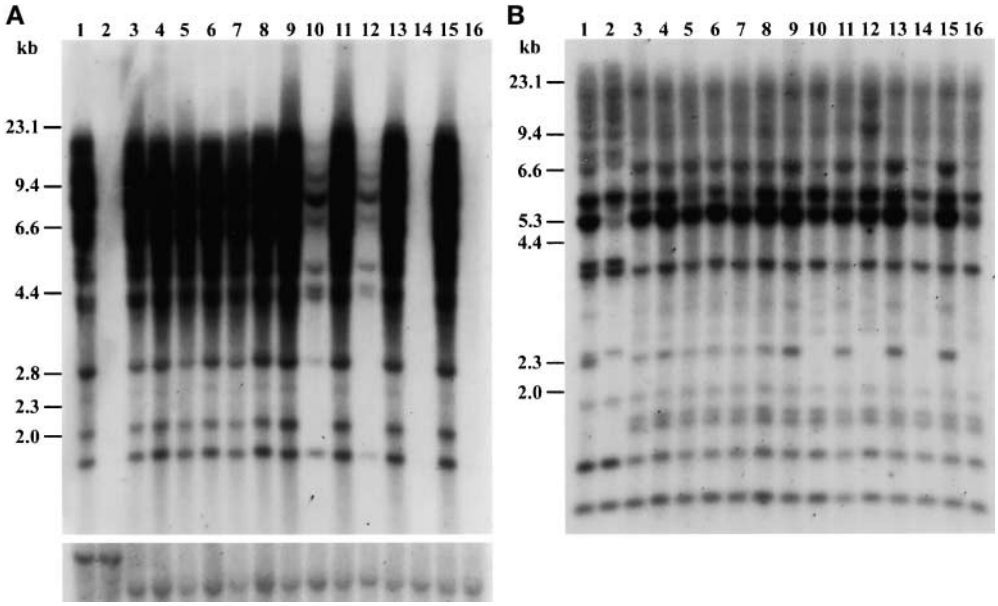

FIGURE 6.—Mapping two B sequences by seven B-10L translocations. DNA was digested with *Bam*HI and probed with pBPC51 (A) and pBPC50 (B). Signal of pBPC51 and the 6.6-kb band of pBPC50 were absent in the hypoploids of TB-10L20 and TB-10L36, reduced in that of TB-10L26 and TB-10L32, and present in that of other translocations. The 5.3-kb band of pBPC50 was reduced in the hypoploids of TB-10L20 and TB-10L36, and the 2.3-kb band was deficient in the hypoploids of TB-10L26, TB-10L32, TB-10L20, and TB-10L36. Lane 1, L289 + 1B; lane 2, L289 + 0B; lane 3, hyperploid of TB-10L7; lane 4, hypoploid of TB-10L7; lane 5, hyperploid of TB-10L16; lane 6, hypoploid of TB-10L16; lane 7, hyperploid of TB-10L38; lane 8, hypoploid of TB-10L38; lane 9, hyperploid of TB-10L26; lane 10, hypoploid of TB-10L26; lane 11, hyperploid of TB-10L32; lane 12, hypoploid of TB-10L32; lane 13, hyperploid of TB-10L20; lane 14, hypoploid of TB-10L20; lane 15, hyperploid of TB-10L36; lane 16, hypoploid of TB-10L36. Bottom panel of A is the internal control for DNA quantity. It was prepared by stripping the pBPC51 probe from the blot (top) before hybridizing with *umc133* (chromosome 6). Size markers are indicated on the left.

B-PCR products showed hybridization in both samples with and without B chromosomes. JAMILENA *et al.* (1995) isolated the B chromosome of *Crepis capillaris* by microdissection and amplified it by DOP-PCR, the products of which hybridized with the A chromosomes. A B-specific library was constructed from the products and 100 clones were analyzed, none of which is B specific. Taken together the results suggest that the sequences in B and A chromosomes are very much alike.

Most B sequences shared by the B and A chromosomes may be of retroelement or other related DNA origin. Fourteen B sequences of this study showed either absence of or partial B-dosage response (Table 2). Two of these, pBPC21 and pBPC23, are knob-specific sequences (Table 4), and 12 others had FISH signal in almost all B regions (Table 3) and A chromosomes (data not shown). Such wide B distribution and no B-dosage response are consistent with the nature of widely dispersed retroelements (or other related DNA). This notion is supported by the finding that 3 of the above sequences are highly homologous to the LTR region and 1 other is highly homologous to the *gag* gene of maize retroelement. The remaining 8 sequences either are in the noncoding regions of known genes or show no identity to any published sequence. It is possible that they are representatives of mobile inverted-repeat elements (BUREAU and WESSLER 1994) or other related sequences.

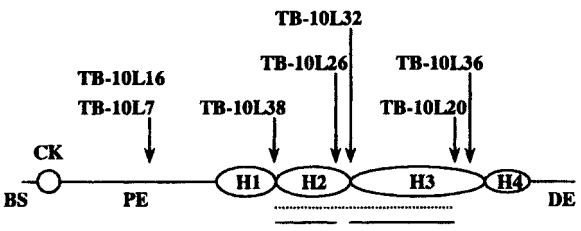

FIGURE 7.—Map position of pBPC50 (—) and pBPC51 (···) in relation to the breakpoints of seven B-10L translocations.

Yet, not all B sequences are homologous to A chromosomes; few are B specific. One (pBPC51) of 19 B sequences isolated in the current study is B specific (Table 2). It was mapped to the BL region between the breakpoints of TB-10L38 and TB-10L20, covering about one-third of the B chromosome (Figure 7). FISH analysis confirmed this map location (Figure 4A). Another clone (pBPC50), partially B specific, is located in the same regions, but its distribution in the regions is discontinuous (Figures 4B and 7). These are the first maize B sequences mapped to the BL and to a large chromosome region. Two other maize sequences specific to the B chromosome have been published before. The first sequence (pZmBs) was obtained by ALFENITO and BIRCHLER (1993) and mapped to the B centromere. The second one (pBGBM18.2) was isolated by STARK *et al.* (1996) from the PCR products of a B-specific randomly amplified polymorphic DNA marker, and its position on the B chromosome was not determined. In rye, two B-specific sequences, λE1100 and λE3900, were cloned from two partial libraries made from the *Dra*I and *Eco*RI digests, respectively, of DNA carrying B's. The former was from a 1.1-kb band (SANDERY *et al.* 1990), and the latter was from a 3.9-kb band (BLUNDEN *et al.* 1993). Both were mapped to the distal region of the B

TABLE 5

Sequence analysis of six non-B sequences isolated by microdissection

Clone no.	Size (bp)	Nucleotide comparisons		
		Sequence in GenBank	X[a]	Y (%)[b]
pBPC45	497	Maize complete chloroplast genome	1–497	95
pBPC5	868	*Janthinobacterium lividum* 16S ribosomal RNA gene	288–768	98
		Maize complete chloroplast genome	392–764	77
pBPC15	1143	*J. lividum* 16S ribosomal RNA gene	288–1143	99
		Maize complete chloroplast genome	392–946	79
pBPC32	688	*Burkholderia pseudomallei* 23S rRNA gene	1–491	93
		Maize complete chloroplast genome	3–97	94
pBPC42	489	*B. vietnamiensis* 23S rRNA gene	1–489	96
		Maize complete chloroplast genome	81–175	94
pBPC52	193	Bacterium 16S ribosomal RNA gene	1–193	99
		Maize complete chloroplast genome	112–193	85

[a] The portion of a non-B sequence in terms of the nucleotide position that is the most homologous to the GenBank sequence.
[b] Identity of X with the GenBank sequence.

long arm by *in situ* hybridization (Blunden *et al.* 1993). In *Brachycome dichromosomatica*, a 176-bp tandem repeat sequence (pBd49) likewise unique to the B chromosome was isolated by subtractive DNA hybridization, but its position has not been determined (John *et al.* 1991).

The location of B sequence detected by FISH analysis agrees with that mapped by B-10L translocations with minor exceptions. The FISH signal of pBPC51 was located on all B regions but PE and DE (Figure 4A; Table 3). Accordingly, the B signal in Southern analysis is expected to appear in the hypoploid of TB-10L36 carrying H4 and DE (Figure 7), but the result is contrary to this expectation. The B signal was in the hyperploid but not in hypoploid of TB-10L36 (Figure 6A, lanes 15 and 16). A similar minor discrepancy was observed in comparison of the FISH map (Figure 4A) with the B-A translocation map (Figure 7). In the first map, pBPC51 was located in H1 in addition to other regions, but in the second map, it was not in this region. Such a difference was noted previously by Alfenito and Birchler (1993). They mapped a B-specific sequence to the centromere by using hypoploids of TB-10L18 and TB-10Sc, but *in situ* hybridization located it to the centromere as well as the junction of H4 and DE. They attributed such variation to lower stringency associated with *in situ* protocols that promote hybridization of part of the probe to the H4-DE junction, which is not detectable by Southern analysis. In this study, the major stringency variation between the two analyses is the wash conditions, which is high stringency (0.1× SSC, 0.1% SDS) in Southern blotting but low stringency (2× SSC, 0% SDS) in FISH analysis. Also different is the hybridization temperature, which is 42° in the former but 37° in the latter.

Of the 25 sequences isolated in this study, 6 resulted obviously from contamination during microdissection or PCR amplification. One (pBPC45) of the 6 sequences has its entire sequence homologous with maize chloroplast genome sequences (95% identity) and the remaining 5 were homologous with prokaryotic ribosomal RNA genes (>93% identity) as well as chloroplast sequences (77–94% identity; Table 5). These sequences were used to probe the pachytene B chromosome in FISH analysis, and none hybridized with either B (Table 3) or A chromosome (data not shown), indicating a non-B, non-A origin. Contamination of the chloroplast sequence might arise from chloroplast DNA present either on the pachytene B chromosome or in regions nearby on the slide. Yet, it is questionable that the source of contamination would be the same for 5 other sequences. Alternatively, the contamination may originate from PCR reagents.

Contrary to the FISH analysis, all six sequences hybridized strongly with maize genomic DNA; all expressed strong signals of multiple bands (Figure 3B2; Table 2). That these sequences hybridized in Southern analysis with the genomic DNA but did not hybridize in FISH analysis with chromosomes A or B implies the contamination of organelle DNA during extraction of genomic DNA. This explanation is understandable for pBPC45, as plant genomic DNA extracted by conventional protocols carries a substantial amount of chloroplast DNA (Zhang *et al.* 2002). The same explanation can be extrapolated to the other five sequences, because parts of these sequences are also homologous to maize chloroplast sequences (Table 5). Consistent with this view is the fact that the same signal intensity was detected between 6B and 16B DNA (Figure 3B2). Yet, why the signal of 0B DNA is consistently less intense than that of 6B DNA is still unclear.

Results of this study shed some light on the possible origin of the B chromosome. Of the 19 B sequences,

13 were homologous to the noncoding region of maize genes, including *bz1*, *adh1*, a 22-kD *zein* gene, and a 19-kD *zein* gene, located on chromosomes 9, 1, 4, and 7, respectively. In addition, 15 sequences (Table 3) showed FISH signals in most B regions and also displayed broad signals in 20 A chromosomes (data not shown). Five major knobs showing FISH signals of pBPC21 are located on 5 different chromosomes (data not shown) as noted in the Black Mexican maize by Peacock *et al.* (1981). Accordingly, the data are in agreement with the view that the B chromosome may evolve from multiple A chromosomes of maize, as proposed by Page *et al.* (2001). They uncovered a centromere sequence of chromosome 4 that is homologous to the B centromere. In consideration of the finding in conjunction with the cytological distribution of knobs, they suggested, as one possibility, "that the B-chromosome is a conglomerate of heterochromatinized sequences from several chromosomes" (p. 301).

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