Sequences Required for Paramutation of the Maize *b* Gene Map to a Region Containing the Promoter and Upstream Sequences

Garth I. Patterson,[1] Kenneth M. Kubo, Terry Shroyer[2] and Vicki L. Chandler

Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403-1229

Manuscript received December 9, 1994
Accepted for publication May 15, 1995

ABSTRACT

The *b* gene encodes a transcriptional regulator of the maize anthocyanin biosynthetic pathway. Certain *b* alleles participate in paramutation, an allele-specific interaction that heritably alters transcription. The moderately transcribed *B'* allele heritably reduces the transcription of the highly transcribed *B-I* allele in a *B'/B-I* heterozygote, such that the *B-I* allele becomes *B'*. To identify the *cis*-acting sequences required for paramutation, we used *B'* or *B-I* alleles to isolate intragenic recombinants with *B-Peru*, an allele that is insensitive to paramutation and has distinct tissue-specific regulation. Physical mapping of the recombinant alleles showed that most of the crossovers were in a small region near the 5' end of the *b*-transcribed region. Analysis of the recombinant alleles revealed that the ability to cause and respond to paramutation and the control of tissue-specific expression both localize to the 5' region of the gene. The 3' boundary of these functions lies just upstream of the translation initiation codon. The 5' boundary has been estimated to be no more than 0.1 cM further upstream (1–150 kb). Thus, sequences critical for paramutation lie upstream of the *b* coding sequences and may include transcriptional regulatory sequences.

PARAMUTATION is defined as an allele-specific interaction that leads to a heritable change in one allele at a high frequency (BRINK 1973). A number of *trans*-inactivation phenomena in plants, where a transgene inactivates another transgene or an endogenous gene, have been described and recently compared to paramutation (MATZKE and MATZKE 1993; PATTERSON and CHANDLER 1995a). In maize, paramutation has been studied at three regulatory genes of the anthocyanin pigmentation pathway, *b*, *r*, and *pl* (for review see BRINK 1973; PATTERSON and CHANDLER 1995a). Anthocyanins are red to purple pigments derived from the phenylpropanoid pathway (DOONER *et al.* 1991). Our studies focus on *b*, which encodes a basic helix-loop-helix transcription factor that activates the transcription of the anthocyanin biosynthetic genes (CHANDLER *et al.* 1989; GOFF *et al.* 1990; RADICELLA *et al.* 1991). Paramutation of the *b* gene results when the *B'* allele (light pigment in sheath, culm, husk and tassel) and the *B-I* allele (dark pigment in the same tissues as *B'*) are heterozygous (COE 1966). When a *B'/B-I* heterozygote is crossed, 100% of the progeny receive *B'*, but both chromosomes segregate normally (COE 1966). Thus, an interaction between *B-I* and *B'* results in the alteration of *B-I* to *B'*, 100% of the time, and this directed heritable alteration is termed paramutation. The level of pigment produced by old *B'* (*B'* in the previous generation) and new *B'* (*B-I* in the previous generation) is similar (PATTERSON and CHANDLER 1995b), and the new *B'* is as equally capable as old *B'* in changing a *B-I* allele into *B'*, 100% of the time (COE 1966).

Some of the genetic requirements for paramutation are known. The ability to paramutate *B-I* is linked to *B'* (COE 1966). The interaction is allele-specific; most alleles of *b* have no effect on *B-I* and are not affected by *B'* (COE 1966; COE *et al.* 1988; PATTERSON and CHANDLER 1995a). It is clear that most alleles that do not undergo paramutation have numerous structural differences relative to *B-I* and *B'* (RADICELLA *et al.* 1992; D. HAWTHORNE, D. SELINGER, M. BEAUDET, G. PATTERSON and V. CHANDLER, unpublished data), making it difficult to determine which sequences are responsible for the inability to participate in paramutation. Mutants with transposable element insertions disrupting the coding region of *B-I* and *B'* are not defective for paramutation (COE 1966; T. SHROYER, G. PATTERSON and V. CHANDLER, unpublished data).

Analyses comparing the expression and structure of *B-I* to *B'* have revealed that the different pigment phenotypes of *B'* and *B-I* are caused by different rates of *b* transcription (PATTERSON *et al.* 1993). Transcription of the *b* gene in *B'/B'* or *B'/B-I* plants is ~10-fold lower than in *B-I/B-I* plants, resulting in less B protein, less transcription of the anthocyanin biosynthetic genes, and less pigment. Since paramutation results in transcriptional changes, the promoter and upstream regions of *B'* and *B-I* were sequenced and compared up

Corresponding author: Vicki L. Chandler, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229.
E-mail: chandler@molbio.uoregon.edu

[1] *Present address:* Department of Molecular Biology, Wellman 8, Massachusetts General Hospital, Boston, MA 02114.

[2] *Present address:* Department of Biochemistry, University of California, San Francisco, CA 94143.

to ~1500 bp upstream of the transcription start site; no sequence alterations were found. Extensive restriction mapping of the two alleles was performed, including the comparison of dozens of potential DNA methylation sites. The extent of methylation was the same, and no alteration in restriction sites or insertions or deletions were detected (PATTERSON *et al.* 1993).

The association of paramutation with changes in *b* transcription suggests transcriptional regulatory sequences may be involved in paramutation, but no direct evidence implicating these sequences has yet been reported. Fine structure mapping of *B'* and *B-I* was undertaken to determine where the ability to participate in paramutation maps relative to the region controlling transcription. Intragenic recombinants between these alleles and *B-Peru,* an allele of *b* that does not participate in paramutation, were isolated. Characterization of these recombinants revealed that the 5′ end of the *b* gene is a hotspot for recombination with rates of recombination dramatically lower in the more 3′ portions of the gene. Phenotypic analysis of the recombinants showed that *B'*, *B-I* and *B-Peru* expression in mature plant tissues is controlled by 5′ regulatory sequences, as was previously shown for seed-specific expression of *B-Peru* (RADICELLA *et al.* 1992). These intragenic recombinants were tested for participation in paramutation. The ability of *B'* to cause paramutation and the ability of *B-I* to respond to paramutation maps to the 5′ region of each allele. The implications of the correspondence between transcriptional control and participation in paramutation are discussed.

MATERIALS AND METHODS

Genetic stocks: All stocks have the *r-g:Stadler* allele of *r*, the *Pl-Rh* allele of *pl*, and fully functional dominant alleles for the anthocyanin biosynthetic genes (see COE *et al.* 1988 for review). Description of the different *b* alleles are summarized in Table 1 and details provided in RESULTS. The *B-I* allele was first found at a Nebraska agricultural fair (EMERSON 1921). Stocks containing the *B'*, *B-I*, *gl2 b wt*, *b'-v1* and *b'-v4* alleles were obtained from E. H. COE, JR. (University of Missouri). The *b* allele in the *gl2 b wt* stock was obtained from the Maize Coop and is of unknown origin. We have designated this *b* allele as *b-534*. This allele has a different restriction map from the *b-K55* allele described in COE (1966) and produces no detectable *b* RNA (G. PATTERSON and V. CHANDLER, unpublished data). *Glossy2* (*gl2*) and *white tip* (*wt*) are flanking morphological markers 19 and 11 map units, respectively from *b*. The *B-Peru* and *b-m1* stocks were obtained from M. G. NEUFFER (University of Missouri). The *b-Pm5* allele was isolated as previously described (PATTERSON *et al.* 1991). A *B-v* stock, which was isolated in an ultraviolet light mutagenesis of *B-I* (COE 1966), contains an ~5-kb insertion in exon 7 (T. SHROYER, G. PATTERSON and V. CHANDLER, unpublished results) and was obtained from W. SHERIDAN (University of North Dakota). The *B-v* allele, like *B-I*, undergoes paramutation (COE 1966). Thus, a *B'-v* stock was made by crossing *B-v* to *B'* to produce a *B'/B-v* heterozygote that was self-pollinated. The *B'-v/B'-v* progeny were identified by crossing to appropriate testers.

DNA materials: Maize DNA was isolated from leaves (DELLAPORTA *et al.* 1983) or from immature ears (RIVIN *et al.* 1982). Probes used for restriction mapping were as follows: 550b, a *Hin*dIII/*Bam*HI fragment of *b-W23* (+152 to +730); BIu4, a *Dde*I fragment of *B-I* (−411 to −8); 314, a *Bgl*II/*Sal*I fragment of *B-I* (−836 to −522); S/G700, a *Spe*I/*Bgl*II fragment of *B-I* (−1487 to −836); and B'v1.6, a *Bgl*II fragment of *B'-v* (~ −8000 to −6400). All numbering is relative to the *B-I* transcription start site at +1 (RADICELLA *et al.* 1992). The isolations of *B'*, *B-I*, *B-Peru* and *b-W23* genomic clones were described previously (CHANDLER *et al.* 1989; RADICELLA *et al.* 1992; PATTERSON *et al.* 1993). Extensive restriction mapping of *B'-v*, *B-I* and *B'* has detected no differences in the 5′ flanking region of these alleles (PATTERSON *et al.* 1993; G. PATTERSON, T. SHROYER and V. CHANDLER, unpublished data). These alleles all contain an ~9-kb *Bam*HI/*Eag*I fragment extending from ~ −8 kb to the *Bam*HI site located at +730. DNA from a *Bam*HI/*Eag*I digest of *B'-v* was size fractionated, and the ~9 kb fraction was cloned into lambda Zap Express (Stratagene). One plaque that hybridized to the S/G700 probe was found, and a plasmid (pB'v1) was excised from the phage vector according to the manufacturer's specifications. Restriction mapping verified that the insert in the plasmid had the same restriction map as the *B'-v* genomic DNA. Sequences upstream of the ~2-kb insertion in *B-Peru* (black bar in Figure 2) were cloned from the *b-Perum220* allele, a mutant derivative of *B-Peru* (PATTERSON *et al.* 1991). In *b-Perum220,* the 210 bp of sequences just upstream of the insertion are 96% identical to the region represented by the BIu4 probe from *B'* and *B-I* (HARRIS *et al.* 1994). Southern blots using the BIu4 probe confirmed *B-Peru* has these sequences upstream of the ~2-kb insertion.

DNA gel blot analysis: DNA gel blot analyses and hybridizations were performed as described (CHANDLER *et al.* 1989), except that MSI nylon (Fisher) was used. All DNA digests were done with a five- to 10-fold excess of enzyme according to manufacturer's specifications. Probes were labeled using random hexamer priming (FEINBERG and VOGELSTEIN 1983). Hybridizations were at 42° in 50% formamide, 1% sodium dodecyl sulfate (SDS), 6× SSC (20× SSC = 3 M sodium chloride, 0.3 M sodium citrate), 10% dextran sulfate; washes were at 58°, 0.2–0.3× SSC, 0.1% SDS.

All recombinant alleles were tested with at least two different restriction digests using the upstream probes (B'v1.6, Spe/G700 and BIu4 probes) (Figure 2). *B'* and *B-I* have identical restriction maps, but *B-Peru* fragments produced with *Bcl*I, *Hin*dIII/*Nru*I, *Bgl*II/*Ava*I, *Sac*I/*Nru*I and *Sac*I/*Ava*I digests are different from those produced by *B'* and *B-I* alleles. Similarly, sites at the 3′ end of the alleles were examined. Sites tested include two *Bcl*I sites (+4 and +7 kb relative to the transcription start site) that are unique to *B-Peru* and a *Bcl*I site unique to *B-I* and *B'* (+10 kb relative to the transcription start site).

PCR analysis: Allele-specific PCR was performed using oligonucleotides that hybridize differentially to a polymorphism 13 bp upstream of the initiator methionine codon (RADICELLA *et al.* 1992). *B-Peru* has a 3-bp insertion relative to *B-I* and *B'*. The oligonucleotides that distinguish the two sequences are D675 (*B-I*, *B'* specific), CACGCGCCTTACTAATCC, and GIP4 (*B-Peru* specific) CGCGCCTTACTAAtaaTCC. The insertion in *B-Peru* relative to *B-I* and *B'* is indicated by lower case letters in the GIP4 oligonucleotide. The oligonucleotide D675 has a 1-bp mismatch with the sequence of *B-I* in RADICELLA *et al.* (1992); however, the sequence of the oligonucleotide is likely to be correct as it matches the *B-I* cDNA sequence and amplifies *B-I* genomic DNA efficiently. Accordingly, a correction for the *B-I* sequence has been sent to Genbank. The oligonucleotides used to prime the other direction are GIP1, ATGCCTGCTTGCAT-

TGCAC (matches sequence in the first exon of *B'*, *B-I* and *B-Peru*) or GIP5, CTAACGTGGACACTCTGTC (matches sequence in *B-Peru* ~40 bp upstream of the transcription start site). The reactions were performed according to AUSUBEL *et al.* (1987) with the following modifications. One microgram of genomic DNA and 0.25 μg of each primer were used. The amplifications were performed at 3 mM $MgCl_2$. The reactions were given a hot start: all components except the Taq polymerase (Promega) were added, and the reactions were placed at 94°, while 2.5 units of enzyme were added to each tube. After enzyme addition, the following cycle was performed 30 times: 94°, 1.5'; 61°, 2'; 72°, 3'. Samples were cooled overnight at 4°, and one-fifth of the reaction was electrophoresed on a 1% agarose gel.

Isolation of intragenic recombinants: The nomenclature for the recombinant alleles is as follows: the 5' (left) and the 3' (right) region of each recombinant allele is separated by the symbol, ||. For example, *BP*||*BI* has the 5' region of *B-Peru* and the 3' region of *B-I*. Different recombinant alleles are numbered (*e.g.*, *BP*||*BI-3*). To isolate *BP*||*B'* and *BP*||*BI* recombinants, (*gl2 b-m1 wt*)/(*Gl2 B' Wt*) and (*gl2 b-m1 wt*)/(*Gl2 B-I Wt*) plants were detasseled and open pollinated in an isolation plot with pollen from *gl2 b-534 wt* plants, and progeny were scored for seed color. *b-m1* is a *Ds1* insertion in *B-Peru* (CLARK *et al.* 1990). *Glossy2* (*gl2*) and *white tip* (*wt*) are flanking morphological markers 19 and 11 map units, respectively from *b*. All purple seeds, the phenotype expected for recombinants, were planted, self-pollinated and outcrossed to testers. As one control for contamination, the self-pollinated ears were scored for recessive markers not present in either parent, *e.g.*, sugary kernels, yellow endosperm kernels, or red cob and pericarp, which probably have *su1*, *Y1* and *P-rr*, respectively (COE *et al.* 1988). These were discarded.

To isolate *B'*||*BP* recombinants, *b'-v1* and *b'-v4* alleles were used in combination with *B-Peru*. *b'-v1* and *b'-v4* are derivatives of *B-v* and *B'-v* that are indistinguishable from *B'* in their ability to paramutate *B-I* (COE 1966). However, *b'-v/b'-v* plants are green because they contain a lesion in the coding region that prevents activation of the anthocyanin pathway (Table 1) (T. SHROYER, G. PATTERSON and V. CHANDLER, unpublished data). Specifically, (*Gl2 b'-v1 Wt*)/(*gl2 B-Peru wt*) and (*Gl2 b'-v4 Wt*)/(*gl2 B-Peru wt*) plants were detasseled and open pollinated with pollen from *gl2 b-534 wt* plants. The progeny were separated by seed color, and only the white seed were planted to exclude the *B-Peru/b-534* (purple seed) parental class of progeny. The white seed contains one parental class, *b'-v/b-534*, and the rarer desired recombinant, *B'*||*BP/b-534*. Recombinants were scored in two ways. For some seed, seedlings were scored for glossy and white-tipped leaves, and only the plants of the phenotype expected for recombinants with associated crossing over (nonglossy and white-tipped leaves) were allowed to grow to maturity (experiment 2, Table 2). Another group of seedlings was allowed to grow to maturity without prescreening for recombinant flanking markers (experiment 1, Table 2). These mature plants were scored for color, self-pollinated and outcrossed to testers. Plants resulting from fertilization by contaminant pollen were identified and discarded. Because the two independent *b'-v* alleles gave equivalent frequencies of intragenic recombinants and are phenotypically identical, the recombinants produced were grouped for determining recombination frequencies.

To isolate a *BI*||*BP* recombinant, we used *B-I* and *b-Perum5* (*b-Pm5*). *b-Pm5* has a *Spm-w* insertion in the *B-Peru* allele ~700 bp 3' of the transcription start in intron 2 (Table 1) (PATTERSON *et al.* 1991). Specifically, a (*gl2 b-Pm5 wt*)/(*Gl2 B-I Wt*) stock was hand-pollinated with *gl2 b-534 wt* pollen. The spotted seed (*b-Pm5/b-534*) were culled and the colorless seed (*B-I/b-534* or *BI*||*bPm5/b-534*) planted, and the resulting seedlings scored for glossy and white tip markers. The nonglossy white-tipped plants, indicative of a crossover between *B-I* and *wt*, were grown to maturity and scored for plant color. One plant with dark stripes, the phenotype expected for a recombination event between the *B-I* promoter and the *Spm-w* element in *B-Peru*, was identified.

The number of individuals scored was calculated as (number of ears scored) × (average number of seed per ear). The average number of seed per ear was determined by counting the seed on 15 randomly selected ears and calculating the mean. The calculation of number of recombinants in the open-pollinated experiments required some estimation because of contamination. The identification of contaminants required the production of viable progeny, since the most common indicator of contamination (sugary kernels) is a recessive trait. The putative recombinants that did not produce viable seed might have been either genuine recombinants or contaminants. To correct for this, we calculated the contamination frequency in the plants that could be scored and used this number to estimate the total number of recombinants isolated. The following equation gives the value used for recombination frequency: $[A + B(A/(A+C))] \times 2/$(number of individuals scored), where A is the number of authentic recombinants (putative recombinants with none of the genetic markers that indicate contamination), B is the number of putative recombinants that did not give any progeny, and C is the number of contaminants (putative recombinants that had one or more of the genetic markers that indicate contamination). For example, in the isolation of *BP*||*B'* recombinants, 106 purple seed were found in the 2.2×10^5 seed scored. All of these seed were planted: 29 were the result of contamination, 65 showed no indication of contamination, and 24 did not produce seed. The calculation of recombination frequency is as follows: $2[65 + 24(65/(65+29))]/220{,}000 = 7.4 \times 10^{-4}$.

Confidence intervals in mapping: The upper limit of the confidence intervals for the experiment in Figure 1 was calculated as the following binomial: the probability of 0 recombinants in 3058 trials, $p^0(1-p)^{3058} < 0.05$, where p is the map distance between the markers. The upper limit for the experiment of COE (1966) was calculated similarly.

Visual scoring of anthocyanin pigment: A 1–10 scoring scheme was defined for tassel and leaf sheath tissue. All plants scored as *B'* were 6 or less on the tassel scale and 8 or less on the sheath scale; all plants scored as *B-I* were 9–10 on the tassel and sheath scales. In the tassel: 1, light purple only in the glume bar; 2, medium purple only in the glume bar; 3, intense purple in the glume bar only; 4, intense purple in the glume bar with color extending less than half the length of the glume; 5, intense purple in the glume gar with purple extending approximately halfway up the face of the glume; 6, intense purple glume bar with color extending more than half the length of the glume; 7, tassel glumes are purple except at tips; 8, glumes are totally purple with light pigment on tassel branches; 9, intense pigment on all glumes with medium pigment on tassel branches; 10, intense pigment in all parts of the tassel. In cases in which different branches on the same plant had different scores, an average was taken. In no case were different regions on a tassel different by more than 2 units on this scale. In the sheath: 1, no purple; 2, purple barely detectable; 3, medium purple at base of sheath with color extending less than halfway up the sheath before becoming undetectable; 4, intense purple at base of sheath with color extending less than half the length of the sheath; 5, intense purple at base with color extending about half the length of the sheath before becoming undetectable; 6, intense purple at base with color extending more than half the length

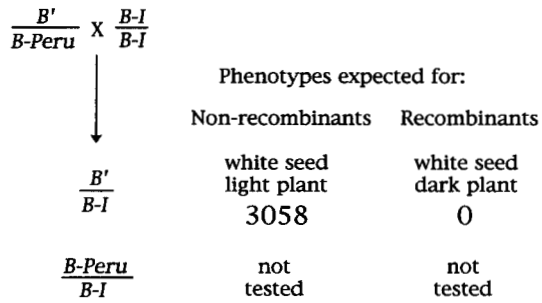

FIGURE 1.—Sequences required for paramutation are tightly linked to *B'*. The crosses performed to screen for recombination between *B-Peru* and *B'* are shown with the predicted seed and plant phenotypes for nonrecombinants and recombinants. A total of 3058 white seeds were planted; all produced light plants. The purple seed were not screened for recombinants because spontaneous paramutation would produce the same phenotype as that expected for recombinants (see text).

of the sheath; 7, intense purple at base of sheath, decreasing to faint color at the top; 8, intense purple at base of sheath, decreasing to medium color at top; 9, intense purple throughout sheath, with green barely detectable; 10, intense purple throughout the sheath, no green showing. In rare cases in which different sheaths on the plant gave different scores, an average was taken. In no case did different sheaths on the same plant differ by more than one unit.

RESULTS

The ability to paramutate *B-I* maps to *B'*: COE (1966) demonstrated that the ability of *B'* to paramutate *B-I* maps in the vicinity of *B'* on chromosome 2 (COE 1959, 1966). He combined *B'* (light purple plant, paramutates *B-I*) with *b-K55* (green plant, unable to paramutate *B-I*) in a heterozygote. When the heterozygote was crossed to *B-I*, the ability to paramutate was always transmitted with the light color of *B'*. Thus, the ability to paramutate is linked to *B'*. Since only 44 *B'/B-I* progeny were tested, these data are consistent with the ability to paramutate being as far as 6 map units from *B'* (see MATERIALS AND METHODS for calculations).

To address whether a separate, but closely linked, gene causes paramutation, additional recombination mapping experiments were performed. The experiment was similar to that of COE (1966), except that the paramutation-insensitive *B-Peru* allele was used instead of the paramutation-insensitive *b-K55* allele. *b-K55* is recessive to *B'* and *B-I:* therefore, to determine the genotype of a particular plant, COE had to cross to testers and score the color of progeny families (COE 1966). The *B-Peru* allele does not paramutate *B-I* and is not sensitive to *B'* (COE *et al.* 1988) but is dominant for pigment production in the aleurone layer of the seed (STYLES *et al.* 1973; PATTERSON *et al.* 1991). Since *B'* and *B-I* do not produce aleurone pigment, the *B'/B-I* and *B-Peru/B-I* progeny can be distinguished by aleurone color, enabling much larger numbers of plants to be more easily scored.

B'/B-Peru plants were crossed with *B-I/B-I* plants (Figure 1), and the *B'/B-I* progeny, identified by lack of aleurone pigment, were planted and scored for plant color. Nonrecombinant *B'/B-I* plants would be light because *B'* efficiently paramutates *B-I*. If a recombination event separated the sequences controlling the tissue-specific pigmentation pattern of *B'* (colorless aleurone) from closely linked sequences causing paramutation, rare seeds with colorless aleurone (*B'/B-I*) that produced dark purple plants would be observed, because the recombinant *B'* would not alter *B-I*.

Several thousand seeds with colorless aleurones (genotype *B'/B-I*) were planted and scored. None of the 3058 *B'/B-I* plants had a dark phenotype that would indicate the isolation of a *B'* recombinant that had lost the ability to paramutate *B-I* (Figure 1). Thus, the ability to cause paramutation either resides within the *B'* allele or is very tightly linked. The *B-Peru/B-I* siblings were not screened for the reciprocal recombinant, a *B-Peru* allele that had become paramutagenic, because the *B-I/B-I* parent will transmit *B'* to some of the progeny due to spontaneous paramutation (COE 1966), which is seen in our stocks at a frequency of 1–10%. This experiment demonstrates that the sequences required for the ability to paramutate *B-I* and the sequences required for the tissue-specific pigmentation pattern of *B'* are tightly linked. Given that no recombinants were seen in 3058 progeny, there is a >0.95 probability that the ability to paramutate is <0.1 cM from *B'* sequences conferring tissue specificity (see MATERIALS AND METHODS for calculation).

The interpretation of these experiments is complicated by the fact that the structure of the *B-Peru* allele is different from that of *B'* in the sequences immediately upstream of the transcription start site. Sequencing, hybridization and restriction mapping with *B-Peru*, *B'* and *B-I* genomic and cDNA clones indicates that the transcribed region of *B-Peru* is 98% identical to that of *B'* and *B-I*, which are indistinguishable from each other (RADICELLA *et al.* 1991, 1992; PATTERSON *et al.* 1993). However, the *B-Peru* sequences immediately upstream of the transcription start site are unrelated to *B'* and *B-I* (comparing 1980 and 1487 bp upstream of the *B-Peru* and *B'*, *B-I* transcription start sites, respectively). If no sequence similarity is available for homologous recombination upstream of the transcription start, then even sequences that are physically very far away might be genetically close. However, when the *B-I* and *B-Peru* alleles are heterozygous with each other, recombination between flanking morphological markers and *b* gives typical map distances as that observed with other *b* alleles. Thus, *B-Peru* and *B-I* map to the same location relative to other chromosome 2 markers, suggesting that the local lack of homology is not dramatically altering recombination.

To compare the structure of *B-Peru* and *B'* further upstream, additional *B'* sequences were cloned (MATERIALS AND METHODS), and restriction maps of *B-Peru*, *B-I* and *B'* were generated. The probes used were de-

TABLE 1

Alleles of *b* used in this study

b allele	Derivation	Structural features	Phenotype	Reference
B-I	Natural	NA[a]	Dark purple plant, colorless seed	EMERSON (1921)
B'	Paramutant derivative of *B-I*	Indistinguishable from *B-I*	Light purple plant, colorless seed	COE (1966)
B-v	Insertion derivative of *B-I*	5-kbp insertion in exon 7	Dark purple sectors in plant	COE (1966)
B'-v	Paramutant derivative of *B-v*	Indistinguishable from *B-v*	Light purple sectors in plant	COE (1966)
b'-v1	Paramutant excision derivative of *B-v*	Loss of 5-kbp insertion in exon 7	Green plant, colorless seed	COE (1966)
b'-v4	Paramutant excision derivative of *B'-v*	Loss of 5-kbp insertion in exon 7	Green plant, colorless seed	COE (1966)
B-Peru	Natural	NA	Purple seed, weak purple plant	STYLES *et al.* (1973)
b-Pm5	Insertion derivative of *B-Peru*	*Spm-w* insertion in intron 2	Spotted purple seed	PATTERSON *et al.* (1991)
b-m1	Insertion derivative of *B-Peru*	*Ds1* insertion in exon 5	Colorless seed, green plant	CLARK *et al.* (1990)
b-534	Lab stock, obtained from Maize Coop	Polymorphic to *B-I* and *B-Peru*	Green plant, colorless seed	PATTERSON *et al.* (1991)

[a] NA, not applicable.

rived from *B'* and the related alleles *B-I* and *B'-v*. *B-v* and *B'-v* contain a transposable element insertion in exon 7 of *B-I* and *B'*-like alleles, respectively (Table 1) (T. SHROYER, G. PATTERSON and V. CHANDLER, unpublished data). The transposable element has no effect on paramutation (COE 1966). Sequencing and extensive restriction mapping revealed no differences between the upstream sequences of *B'*, *B'-v* and *B-I* (PATTERSON *et al.* 1993) (data not shown). Figure 2 shows the restriction maps of *B-Peru* and of *B'*, *B-I* (this nomenclature is used to indicate that the structure of both alleles is the same). All of the indicated probes from the upstream region of *B'*, *B-I* also hybridize to *B-Peru* at the high stringency conditions used. Probes derived from the region between B'v1.6 and S/G700 recognize sequences that are repeated >10 times in the maize genome, so could not be used to determine if this region is also linked to *B-Peru*. However, the restriction map of the region between these two probes in *B'*, *B-I* is not similar to the restriction map of *B-Peru* in the same region (Figure 2) (data not shown). These results demonstrate that *B-Peru* does have extensive sequence similarity to *B'*, *B-I* upstream of the transcription start site, but it has additional sequences as well. Thus, re-

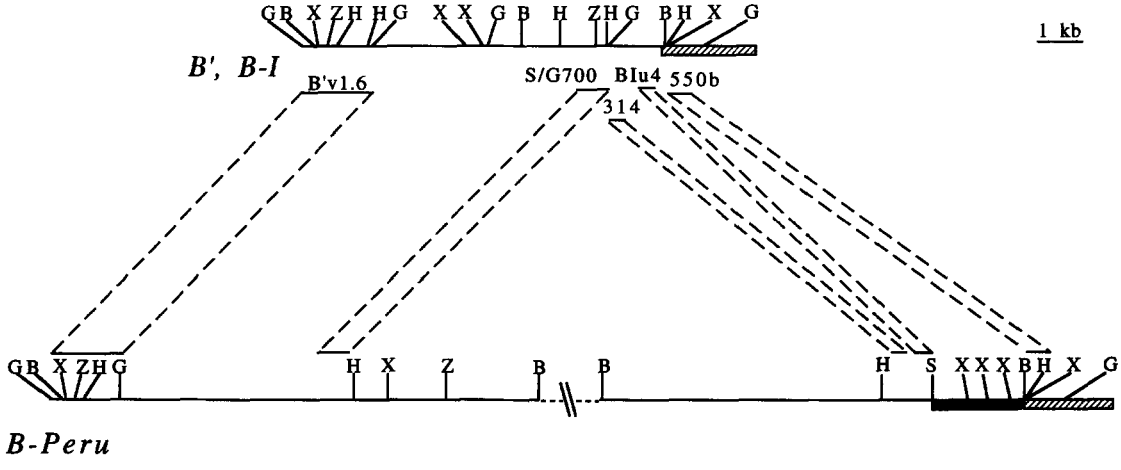

FIGURE 2.—Restriction map of *B-Peru* and *B'*, *B-I*. The *B'*, *B-I* map is a composite of *B'*, *B-I* and *B'-v*, which are indistinguishable in every region tested with the exception of the transposable-element insertion in the coding region of *B'-v* downstream of the region shown (PATTERSON *et al.* 1993) (data not shown). ■ in *B-Peru* indicates an insertion of ~2 kb that has no sequence identity with *B'*, *B-I*. ▩ indicate the beginning of the transcribed regions of *B'*, *B-I* and *B-Peru*, which are ~98% identical. The location of the DNA fragments used as probes is exactly as shown on the *B'*, *B-I* map. In *B-Peru* the S/G700 probe hybridizes to the *Bgl*II/*Hin*dIII fragment shown, and the 314 probe hybridizes to the *Hin*dIII/*Spe*I fragment shown, but the location of hybridization was not precisely mapped. Dashes between the *Bcl*I sites in *B-Peru* indicates an uncertain length of DNA separating the fragments that hybridize with the BIu4 and 314 probes from the fragments that hybridize with the B'v1.6 and S/G700 probes. G, *Bgl*II; B, *Bcl*I; X, *Xba*I; Z, *Xho*I; H, *Hin*dIII; S, *Spe*I.

combination could, in principle, occur using this homology that exists upstream of the transcription start of the two alleles. While the possibility of a very tightly linked gene causing paramutation cannot be eliminated by these data, the failure to detect a recombination event separating sequences required for paramutation from *B′* regulatory sequences supports the hypothesis that the sequences involved in paramutation are a part of *B′*.

Fine structure mapping of sequences necessary for paramutation and tissue-specific expression: The data in Figure 1 show that the ability to cause paramutation resides very near or within *B′*. Paramutation affects *b* transcription (PATTERSON *et al.* 1993), raising an important question: Does the capacity of *B′* to paramutate *B-I* and the capacity of *B-I* to respond to *B′* map to the same location as the sequences that control tissue-specific expression? *B-Peru,* which differs in tissue-specific expression and does not participate in paramutation, was used to isolate recombinant alleles between *B-Peru* and *B′* or *B-I.* These intragenic recombinants were then tested for their tissue-specific expression and their ability to paramutate *B-I* or to respond to *B′*.

Isolation of recombinants: The strategy to isolate intragenic recombinants takes advantage of the tissue-specific patterns of pigmentation in *B-Peru, B-I* and *B′* plants. *B-Peru* produces pigmentation in the aleurone layer of the seed, but *B′* and *B-I* do not (STYLES *et al.* 1973). Transient transformation assays showed that this difference in seed expression is controlled by ~2 kb of sequences that contain the promoter, upstream flanking sequences, and part of the untranslated leader of *B-Peru* and *B-I* (RADICELLA *et al.* 1992). The 5′ sequence of *B-Peru* used in the transformation experiments includes all of the 2-kb insertion shown in Figure 2, which has no sequence identity with *B-I* (RADICELLA *et al.* 1992). In contrast to the upstream sequences, the coding regions of *B-Peru* and *B-I* are ~98% identical, and the regions downstream of the coding region have 85–90% sequence identity for several kb (RADICELLA *et al.* 1991, 1992; D. BROWN and V. CHANDLER, unpublished data).

The first strategy was to isolate an intragenic recombinant between *b-m1,* a mutant derivative of *B-Peru,* and *B′* (Figure 3A). *b-m1* was isolated from a strain with active *Ac/Ds* transposable elements (CLARK *et al.* 1990), and sequence analysis indicates that it contains a *Ds1* element in the coding region, ~1800 bp downstream from the transcription start site (J. CLARK and V. CHANDLER, unpublished data). *b-m1* does not produce pigment in the aleurone due to the *Ds1* element, which can excise only in the presence of an *Ac* element (FEDOROFF 1989). As there is no active *Ac* present in this experiment, the *b-m1* allele behaves as a stable recessive, producing colorless seeds and plants. *B′* also does not produce pigment in the aleurone, because it does not have the necessary regulatory sequences. If an intragenic recombination event combines the *B-Peru* upstream regulatory sequences with the *B′* coding sequence that lacks a *Ds* element, the result should be an allele that can produce pigment in the aleurone. Figure 3A schematically shows the expected result if the recombination event is associated with crossing over of flanking markers. The recombinants isolated in this manner have the 5′ flanking sequences of *B-Peru* combined with the 3′ flanking sequences of *B′* and a transcribed region that is a hybrid of the two alleles. These recombinants were designated *BP\|\|B′*. Recombinants between *b-m1* and *B-I* were isolated in a similar manner and were designated *BP\|\|BI.*

A similar strategy was used to isolate reciprocal recombinants (Figure 3, B and C). In one case *B-Peru* and *b′-v* alleles were used. *b′-v,* which produces green plants lacking anthocyanins, is fully able to paramutate *B-I* (COE 1966). Two *b′-v* alleles, *b′-v1* and *b′-v4,* are independent paramutant derivatives of the *B-v* or *B′-v* allele (COE 1966), which contain a transposable element insertion in exon 7 (Table 1) (T. SHROYER, G. PATTERSON and V. CHANDLER, unpublished data). Restriction mapping indicates that the element has excised from *b′-v1* and *b′-v4* (data not shown). These alleles produce green plants with no anthocyanins. Presumably, the green phenotype is the result of excision of the element, producing a mutation at the excision site. An intragenic recombination event that combines the functional *B-Peru* coding sequences with the *b′-v* upstream regulatory sequences should result in an allele that produces a colorless seed and a light purple plant (Figure 3B). Recombinants isolated in this manner have the 5′ flanking sequences of *B′* combined with the 3′ flanking sequences of *B-Peru* and a transcribed region that is a hybrid of the two. These recombinants are designated *B′\|\|BP.*

The final recombinant was isolated between *B-I* and *b-Perum5* (*b-Pm5*). *b-Pm5* is a mutant allele of *B-Peru* that contains an *Spm-w* transposable element inserted into the second intron (PATTERSON *et al.* 1991). In the presence of a *Spm-s* element elsewhere in the genome, this allele produces frequent spotting in the aleurone and faintly pigmented plant sectors. A recombinant that combines the regulatory sequences of *B-I* with the coding region of *b-Pm5,* which contains the transposable element, should produce an allele that makes colorless aleurone with intensely pigmented plant sectors.

Recombinants were isolated by crossing plants heterozygous for the alleles of interest to a *gl2 b-534 wt* tester. Recombinants were identified by plant and seed color as shown in Figure 3 and as explained in detail in MATERIALS AND METHODS. The frequency at which intragenic recombinants were isolated was $\sim 1 \times 10^{-3}$ and did not differ significantly from experiment to experiment (Table 2). The frequency of recombination between *B′* and *b-m1* was similar to that between *B-I* and *b-m1*. The flanking markers of the intragenic re-

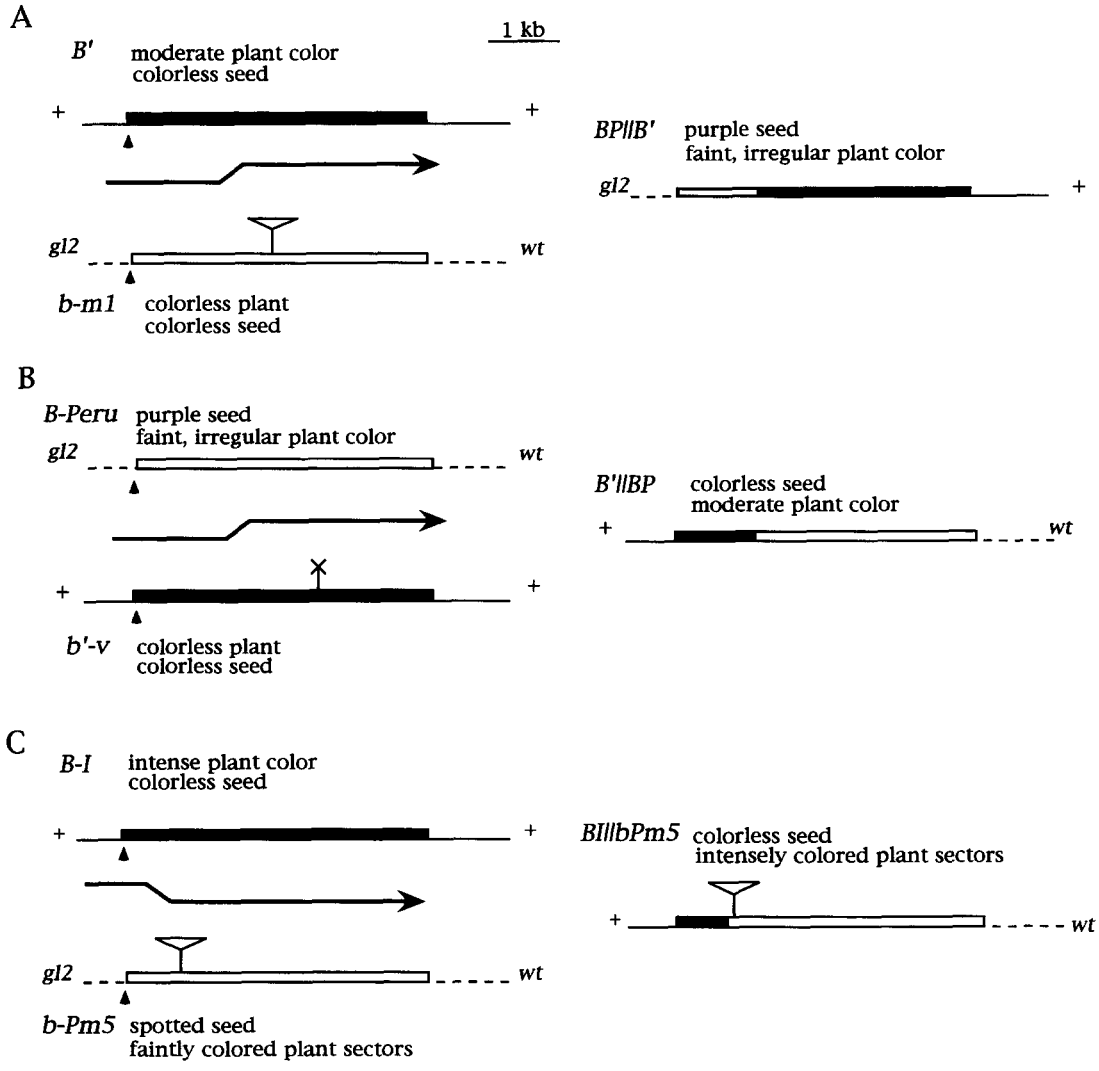

FIGURE 3.—Schematic of intragenic recombination. The boxes represent the transcribed regions of *B-I* and *B-Peru*. The vertical arrows indicate the junction between highly conserved and unrelated sequences. The lesion or insertions that create the mutant phenotype of *b'-v*, *b-m1* and *b-Pm5* are shown as an X or triangles, respectively. In all cases seed color refers to color in the aleurone layer of the seed.

combinants between *b-m1* and *B-I* or *B'* were primarily *gl2* and *Wt* (Table 3), indicating that most of the recombination events were associated with crossing over. Three recombinants had parental flanking markers (*Gl2, Wt*), but physical mapping (see below) suggests that these alleles were derived by a crossover in the *b* gene, combined with a second crossover in the 19 map unit *gl2-b* interval. None of the 133 recombinant alleles shown in Table 3 had the structure that would be expected for a gene conversion event. The fact that the recombinants contained the *gl2 Wt* marker configuration demonstrates that transcription terminates closest to *wt*, which is centromere proximal to *b*. Thus, the *b* gene is transcribed in the direction of the centromere.

Structure of recombinant alleles: The recombinants were physically mapped to determine what part of the allele had *B-Peru* sequence and what part had *B'* or *B-I* sequence. Initially, the alleles were mapped using several restriction fragment length polymorphisms (RFLPs) upstream and downstream of the transcribed region. The 22 *B'*||*BP* recombinants, the *BI*||*bPm5* recombinant, 10 *BP*||*B'* and three *BP*||*BI* recombinants were analyzed in this way (MATERIALS AND METHODS). All of the *B'*||*BP* and *BI*||*bPm5* recombinants had the 5′ *B'* and *B-I* sites and all of the *BP*||*B'* and *BP*||*BI* recombinants had the 5′ *B-Peru* sites (data not shown). The 22 *B'*||*BP* alleles and the *BI*||*bPm5* allele had the 3′ *B-Peru* sites, and all 10 *BP*||*B'* alleles and all three *BP*||*BI* alleles tested had the 3′ *B'* and *B-I* sites (data not shown). Thus, all of the recombinants examined had the structures expected for a crossover event within the *b* transcription unit.

Three of the recombinant alleles shown in Table 3 had parental flanking genetic markers. A gene conversion event within the *b* locus could produce a recombinant allele with nonrecombinant flanking markers, as

TABLE 2

Frequency of recombinant allele isolation

Recombinant	No. scored	No. of recombinants[a]	Frequency[b]
BP||B′	2.2×10^5	65	7.4×10^{-4}
BP||BI	1.8×10^5	63	9.2×10^{-4}
B′||BP			
Experiment 1[c]	7×10^3	5	9.2×10^{-4}[d]
Experiment 2	4.1×10^4	17	
BI||bPm5	4.6×10^3	1	4×10^{-4}

[a] The numbers scored for *BP\|\|B′* and *BP\|\|BI* were estimated by counting a random sample of 15 ears and multiplying the average by the total number of ears scored. For *B′\|\|BP* and *BI\|\|bPm5* the exact number of plants scored is indicated.

[b] For *BP\|\|B′* and *BP\|\|BI*, the recombination frequency was estimated as described in MATERIALS AND METHODS. Otherwise, the recombination frequency is 2 × (number of recombinants)/(number scored).

[c] Experiments 1 and 2 differ slightly in method of scoring, as described in MATERIALS AND METHODS.

[d] This frequency represents a weighted average of experiments 1 and 2.

TABLE 3

Flanking markers of intragenic recombinants

Parental genotype	Markers on recombinant chromosomes: Parental *Gl2 Wt*	Parental *gl2 wt*	Recombinant *gl2 Wt*	Recombinant *Gl2 wt*
gl2 b-m1 wt / *Gl2 B-I Wt*	2[a]	0	61	0
gl2 b-m1 wt / *Gl2 B′ Wt*	1[b]	0	64	0
Gl2 b′-v Wt / *gl2 B-Peru wt*	0	0	0	5[c]

[a] One of these plants died as a seedling; the other recombinant chromosome was physically mapped as described in RESULTS and represents a crossover within the *b* gene in combination with a second crossover in the 19 map unit *gl2-b* interval.

[b] This plant died as a seedling; thus it was not possible to determine the nature of the recombination event that produced it.

[c] Only the recombinants from experiment 1 (see Table 2) are included in this number, as these recombinants were isolated without prescreening for recombinant flanking markers.

could a double crossover, one within the *b* gene, the second in the *glossy2* to *b* interval (19 map units). Two of the plants with recombinant alleles died as seedlings before DNA isolation. The remaining recombinant allele (designated *BP\|\|BI-3*), was analyzed for RFLPs. This recombinant allele had the *B-Peru* sites in the 5′ region and *B′*, *B-I* sites in the 3′ region, indicating a crossover occurred within *b*. Thus, with the possible exception of the two alleles that were lost, all of the recombinant alleles appear to have derived from crossover events rather than gene conversion events without associated crossing over.

Testing of sites closer to the transcription start site allowed the point of crossover to be further localized. Figure 4 shows polymorphisms near the transcription start site that were used to determine the recombinants' structure. The coordinates are relative to the start of transcription of *B-I* and *B′* (+1), which is the same for both alleles (RADICELLA *et al.* 1992; PATTERSON 1993). A single bp change at +823 changes an *Ava*I site in *B-I* and *B′* into an *Nru*I site in *B-Peru*. These sites were examined in the recombinants with examples shown in Figure 5, A and B. One *B′\|\|BP* recombinant, *B′\|\|BP-20* yielded fragments that migrated at the same size as the *b′-v*, *B′*, *B-I* fragments, indicating that this recombinant has an *Ava*I site but no *Nru*I site at +823 (lane 4, Figure 5, A and B). The presence of *b′-v* sequence at this position suggests the crossover occurred downstream. The other 21 *B′\|\|BP* recombinants produced fragments that comigrated with *B-Peru* indicating that these recombinants have an *Nru*I site at +823 but no *Ava*I site (see one example in lane 3, Figure 5, A and B). Since these recombinants are like *B-Peru* at this polymorphism, the crossover events occurred upstream. DNA from *BP\|\|B′* and *BP\|\|BI* plants always yielded the fragments that comigrated with the *B′*, *B-I* fragments, indicating that all of these alleles have an *Ava*I site at +823. This pattern is consistent with all the crossovers occurring upstream. It was not necessary to examine *BI\|\|bPm5* using these digests, because the insertion is upstream of the *Nru*I/*Ava*I polymorphism. The sectored phenotype of the recombinant allele indicates that the crossover occurred upstream of this insertion, transfering it to *B-I*. Restriction mapping of the recombinant allele verified that *BI\|\|bPm5* has the *Spm-w* insertion at a similar location to the *b-Perum5* allele (data not shown).

A *Dde*I site at +203 that is found in *B-Peru*, but not in *B′*, *B-I* or *b′-v*, was also examined in the recombinant alleles (Figure 5C) to further localize the crossover point. Figure 5C shows two recombinants, *BP\|\|B′-14* and *BP\|\|BI-1*, that like 12 of the 13 tested *BP\|\|B′* and *BP\|\|BI* alleles produce a *Dde*I fragment typical of *B-Peru*, indicating these recombinant alleles have the *B-Peru* sequence at this site. This result places the crossover between +203 and +823 in these alleles. The *BP\|\|BI-2* allele is an exception for this class in that it produces the *B-I Dde*I fragment (data not shown), placing the site of crossover in this allele between the heterology between *B-Peru* and *B-I* at +43 and +203. None of the 22 *B′\|\|BP* or the *BI\|\|bPm5* alleles produced the *B-Peru Dde*I fragment (Figure 5C). This result indicates that all of these alleles have the *B′*, *B-I* sequence at this site, placing the crossover between +203 and +823.

To further localize the crossover point, a polymorphic site at +454 just upstream of the initiation codon for *B-Peru* and *B-I* at position +475 was examined using

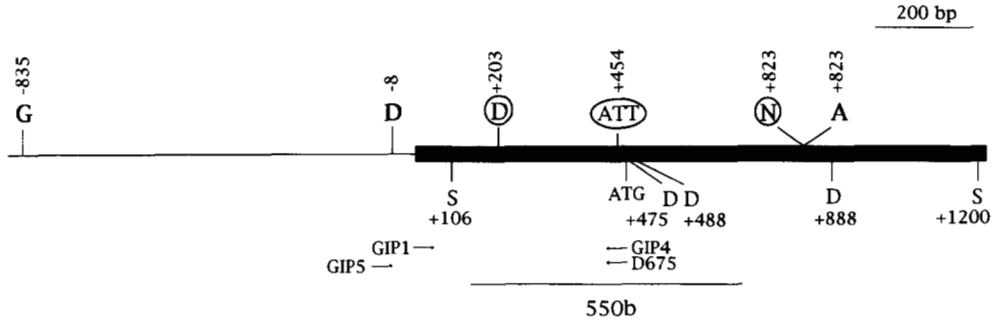

FIGURE 4.—Polymorphisms used to distinguish the *B-I* and *B′* sequence from the *B-Peru* sequence. The sites above the line are unique to *B-Peru* (circled letters) or *B′* and *B-I* (bold letters). The sites below the line are found in all three alleles. The location of the primers used in the PCR analysis are shown as arrows below the line. The box represents the region in which *B-Peru* is very similar to *B′* and *B-I* (~98% DNA sequence identity). The location of hybridization by the 550-bp probe is indicated. All coordinates are relative to the start of *B-I* and *B′* transcription at +1 (RADICELLA *et al.* 1992). G, *Bgl*II; D, *Dde*I; S, *Sac*I; N, *Nru*I; A, *Ava*I; ATT, 3-bp insertion in *B-Peru* relative to *B′* and *B-I;* ATG, initiator codon.

allele-specfic PCR. *B-Peru* has a 3-bp insertion relative to *B′* and *B-I* (shown by ATT in Figure 4). DNA oligonucleotides that matched the *B-Peru* or *B′*, *B-I* sequence across this insertion were used in PCR reactions with DNA heterozygous for recombinant alleles and *B-I* (MATERIALS AND METHODS). In the case of *B′*||*BP* and *BI*||*bPm5*, the upstream oligonucleotide (GIP1, see Figure 4) was from a region near the start of transcription in which *B′*, *B-I* and *B-Peru* have identical sequence. When DNA from a *b′-v* plant is amplified with the *B′*, *B-I*-specific oligonucleotide (D675, see Figure 4), amplification is robust, as indicted by a 400-bp fragment (lane 12, Figure 6). This 400-bp fragment is the specific amplification product, as it is the expected size, and hybridizes to the 550-bp probe (data not shown). The higher molecular weight fragments are nonspecific as they do not hybridize to the 550-bp probe and are only seen in a subset of the reactions. When the *B-Peru* specific oligonucleotide (GIP4, see Figure 4) is used with *b′-v* DNA, amplification is weak or not detectable (lane 11, Figure 6) (data not shown). Conversely, DNA from *B-Peru* amplifies well only with the *B-Peru* specific oligonucleotide (compare lanes 13 and 14, Figure 6). Some of the *B′*||*BP* recombinant alleles amplify well with the *B-Peru* specific oligonucleotide, indicating that these alleles have the *B-Peru* sequence at this site (Figure 6). Other alleles do not amplify with the *B-Peru* oligonucleotides (Figure 6). The amplification of the *B-I* allele in these heterozygous plants serves as an internal control for the amplification reaction. Similar reactions were performed for a subset of *BP*||*B′* and *BP*||*BI* alleles (data not shown). In these reactions, the upstream oligonucleotide was the *B-Peru* specific oligonucleotide GIP5 (Figure 4). These results establish that the physical crossover point for all the recombinants lies within the transcribed *b* region. A summary of the number of recombination events that occurred within each interval for each type of recombinant is shown in Figure 7.

The *b* gene contains a recombination hotspot: The recombination in *b* can be estimated at 22 kb/cM by dividing the distance between the *B-I/B-Peru* sequence divergence and the insertion sites by the recombination frequencies in Table 2. It is clear that the rate of recombination in the *b* gene is high relative to the average rate of recombination in the maize genome, which can be estimated to be 1460 kb/cM by dividing the size of the maize genome in kb by its size in cM (CIVARDI *et al.* 1994). The overall rate of recombination in *b* is similar to that previously estimated for *bz1* (14 kb/cM) (DOONER *et al.* 1985) and *a1* (12–25 kb/cM) (BROWN and SUNDARESEN 1991). However, our studies show that the recombination rate is not uniform within the *b* gene (Figure 7). Recombinants between the *Dde*I site and the PCR polymorphism are seen at frequencies of 4 and 8 kb/cM and similarily between the PCR polymorphism and the *Nru*I/*Ava*I polymorphism at rates of 15 and 8 kb/cM. In contrast, recombination rates between the *Nru*I/*Ava*I polymorphism and the mutant lesions in *b-m1* and *b′-v* were significantly lower at ~400 kb/cM. Thus, not only is *b* more recombinogenic than the maize genome as a whole, but the 5′ end of *b* is more recombinogenic than sequences further downstream in the coding region of *b*.

Pigmentation phenotype of recombinant alleles: The *B-Peru* allele produces a different phenotype from *B-I* and *B′* in a number of tissues. In the aleurone, *B-Peru* produces intense purple color, while *B-I* and *B′* produce none. In many tissues of the mature plant (*e.g.,* leaf sheath, tassel, and husk) *B-I* is intensely purple, *B′* is moderately purple, and *B-Peru* produces only very faint spotty color (RADICELLA *et al.* 1992; PATTERSON *et al.* 1993). Analysis of gene expression in transient transformation assays shows that the 5′ region of *B-Peru,* in which no sequence homology is seen with *B-I,* controls aleurone expression (RADICELLA *et al.* 1992). However, the transient assay could not be reliably used to identify the sequences that controlled differential expression in tassel, husk, and sheath. The availability of the recombinant alleles provides a means to map sequences that control expression in specific plant tissues.

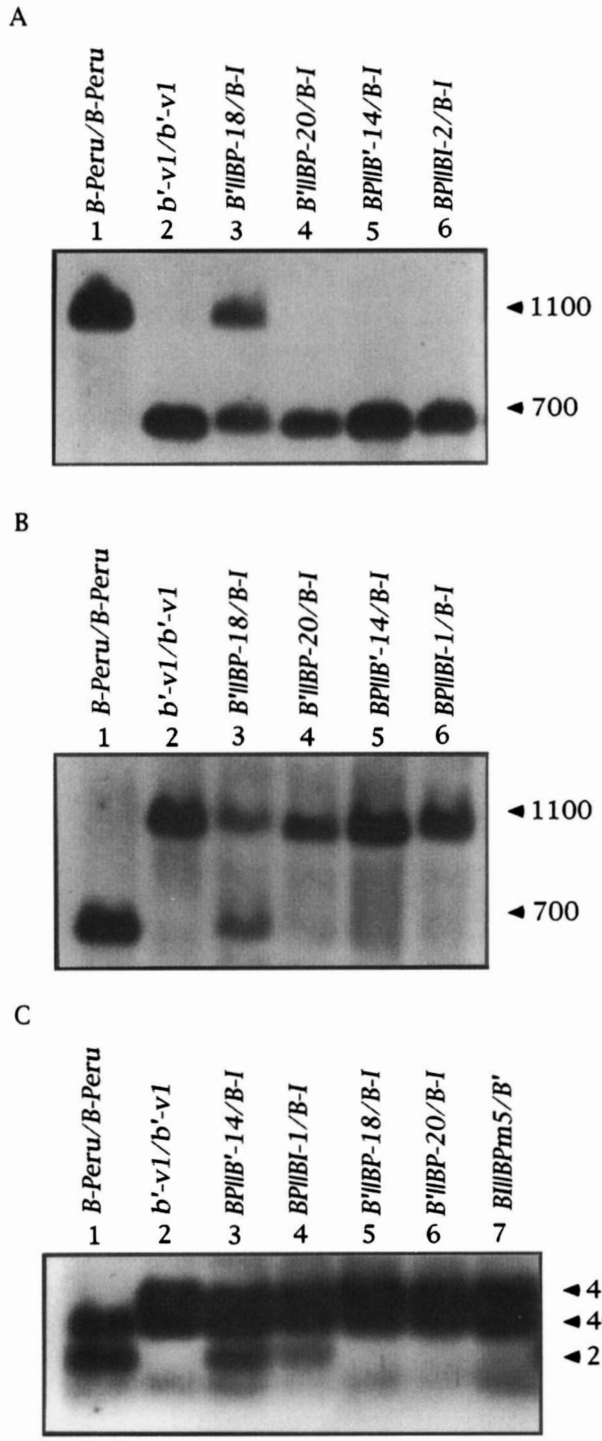

FIGURE 5.—Restriction analysis of recombinant alleles. Approximately 4 μg of maize DNA samples were digested with restriction enzymes, separated by electrophoresis, and blotted. The blots were probed with the 550-bp probe shown in Figure 4. This probe also hybridizes to the homologous *r* gene (CHANDLER *et al.* 1989), and the *r* cross-hybridizing band was trimmed out of the prints. The *b* genotype of the plants from which the DNA was isolated is shown above each lane. The size of the fragments seen was estimated by comparison to a *Hin*dIII / *Eco*RI digested lambda DNA ladder and is shown to the right. (A) *Sac*I / *Ava*I digest. (B) *Sac*I / *Nru*I digest. A *Sac*I digest produces a 1100-bp fragment recognized by the 550-bp probe for both *B-Peru* and *B-I*, *B'* alleles due to *Sac*I sites at +106 and +1200 (Figure 4). When *Nru*I or *Ava*I cut at the +823 site, a 700-bp fragment is produced. (C) *Dde*I digest. When *B-Peru* DNA is cut with *Dde*I and probed with the 550-bp probe, fragments of 270 and 400 bp are produced (lane 1). When *b'-v* is cut with *Dde*I, fragments of 480 and 400 bp are produced (lane 2). The 400-bp fragment is due to cutting at *Dde*I sites at +488 and +888, which are common to both types of alleles (Figure 4). The 270-bp fragment in *B-Peru* is due to cutting at +203. *b'-v* has no site at +203; instead, a 480-bp fragment is produced by cutting at −8 and +488.

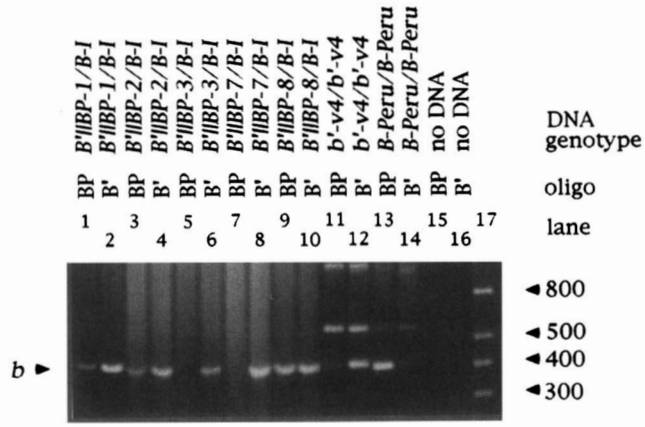

FIGURE 6.—PCR analysis of recombinant alleles. The oligonucleotides used in the PCR reactions (MATERIALS AND METHODS) match either the *B-Peru* genomic sequence (*BP* refers to oligonucleotides GIP1 and GIP4) or the *B'* and *B-I* genomic sequence (*B'* refers to oligonucleotides GIP1 and D675). The genotype of the genomic DNA used in each reaction is shown. The arrow marked with *b* indicates the fragment that hybridizes to the 550-bp probe shown in Figure 4; the other fragments seen in the gel do not hybridize to this probe. The sizes of the DNA fragments in the ladder in lane 17 are shown.

The *BP*| |*B'* and *BP*| |*BI* recombinant alleles produce the same pattern of pigment as *B-Peru* in all seed and mature plant tissues. The recombinants were isolated as *BP*| |*B'* / *b-534* or *BP*| |*BI*/*b-534* seeds, which had intense purple aleurones. Plants grown from these seed had very weak, spotty color in the sheath, husk and tassel, similar to *B-Peru*, but unlike *B'* or *B-I*. All classes of *BP*| |*B'* and *BP*| |*BI* recombinants shown in Figure 7 have similar aleurone, sheath, and tassel color phenotypes, indicating that upstream sequences confer this differential phenotype. Figure 8, A and B show a comparison of *B-Peru* and one recombinant allele, *BP*| |*B'-6*. A *BP*| |*B'-6*/*B-Peru* plant was crossed by a green plant with recessive *b-534*/*b-534* alleles, and the seed were planted and genotyped by RFLP analysis. The *BP*| |*B'*/*b-534* plants could be distinguished from the *B-Peru*/*b-534* sibs on the basis of RFLPs but not by phenotype.

The *B'*| |*BP* recombinants were identified as exceptional light purple plants (*B'*| |*BP*/*b-534*) among green siblings (*b'-v*/*b-534*). All of the purple-pigmented plants had moderate color typical of *B'*, with approximately equal anthocyanin intensity. To provide a direct comparison to *B'*, a *B'*| |*BP*/*B'* plant was crossed to *b-534*/*b-534*. The *B'*/*b-534* and *B'*| |*BP*/*b-534* siblings

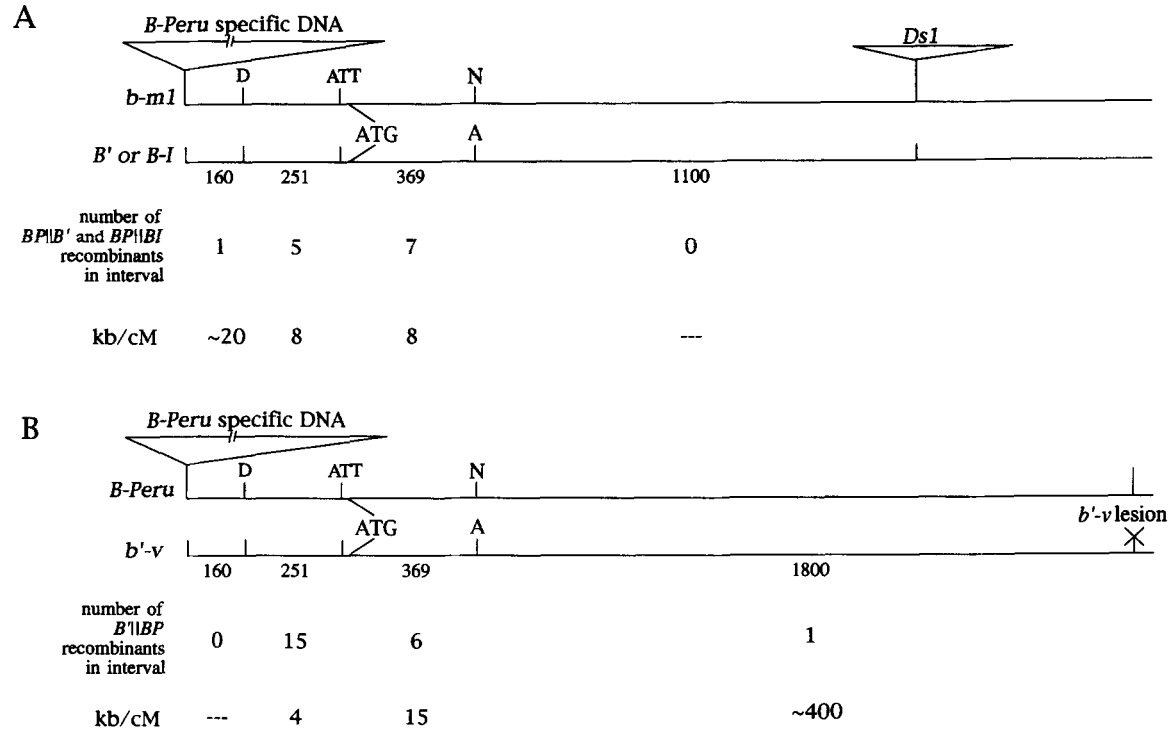

FIGURE 7.—Recombination frequency in different intervals of the *b* gene. The number of recombinants in each interval are shown. The drawings are to scale, except that the insertion at the 5′ end of *B-Peru* is shown smaller than its actual size (2 kb). The numbers under each interval indicate the number of bp in that interval. The ATG indicates the translation initiation codon. kb/cM is calculated as follows: (number of recombinants in the interval/total number of recombinants tested for crossover point) × (number of kb in the interval/recombination frequency from Table 2). (A) Recombinants between *b-m1* and *B′* or *B-I*. (B) Recombinants between *b′-v* and *B-Peru*.

were distinguished by RFLP analysis. The two genotypes produced indistinguishable phenotypes (Figure 8C).

The *BI*| |*bPm5* allele confers intense anthocyanin pigment in mature plant tissues, similar to *B-I*. This allele contains a *Spm-w* transposable element, and so instead of producing uniform intense color, like *B-I*, it produces sectors of intense color on a green background (Figure 8D). Stable revertants of the *BI*| |*bPm5* allele, which have lost the element, produce color indistinguishable from *B-I* (Figure 8D).

For each type of recombinant allele (*B′*| |*BP*, *BI*| |*bPm5*, *BP*| |*B′*, *BP*| |*BI*) recombinants were isolated in which the recombination event occurred upstream of the PCR polymorphism (see Figure 7), which is upstream of the translation initiation codon of the *b* gene. Thus, the entire protein coding region and 3′ flanking sequence of the three alleles are interchangeable and do not contribute to differences in tissue-specific expression. Differences upstream of the PCR polymorphism, including the first intron, the untranslated leader and sequences upstream of the transcription start site, are candidates for sequences responsible for the difference in *B′*, *B-I* and *B-Peru* expression.

***B′*| |*B-Peru* recombinants paramutate *B-I*:** If the *B′*| |*BP* recombinant allele can paramutate *B-I*, a *B′*| |*BP*/*B-I* heterozygote should be light purple and only transmit light purple progeny; if it cannot, the heterozygote should be dark purple and both light and dark progeny should segregate. The *B′*| |*BP* alleles were initially isolated as heterozygotes with the *b-534* allele, which does not participate in paramutation (data not shown). The first step to test the ability of this allele to paramutate *B-I* was to cross *B′*| |*BP*/*b-534* heterozygotes to *B-I*/*B-I* and score the progeny for pigment intensity. Plants were scored for anthocyanin intensity and genotyped by RFLP. The *B′*| |*BP*/*B-I* plants were always light purple, typical of a *B′*/*B-I* heterozygote, whereas the control *b-534*/*B-I* siblings were intensely pigmented (Figure 9A). A number of plants of each genotype were visually scored for pigment intensity on a scale of 1 (lightest) to 10 (darkest), described in MATERIALS AND METHODS. *B′*| |*BP*/*B-I* individuals were consistently much less pigmented than *b-534*/*B-I* plants (Table 4). To determine whether the recombinant allele is equivalent to *B′* in causing paramutation, siblings containing each allele were compared. A *B′*| |*BP*/*B′* plant was crossed to *B-I*/*B-I*, and the two types of progeny were identified by RFLP analysis and visually scored for color phenotype. The *B′*| |*BP*/*B-I* plants were phenotypically indistinguishable from the *B′*/*B-I* plants (Figure 9A). Thus, *B′*| |*BP* and *B′* are equivalent in the ability to alter *B-I* pigment in the heterozygote.

To be classified as paramutation, the effect of *B′*| |*BP* on *B-I* must be heritable. To examine heritability,

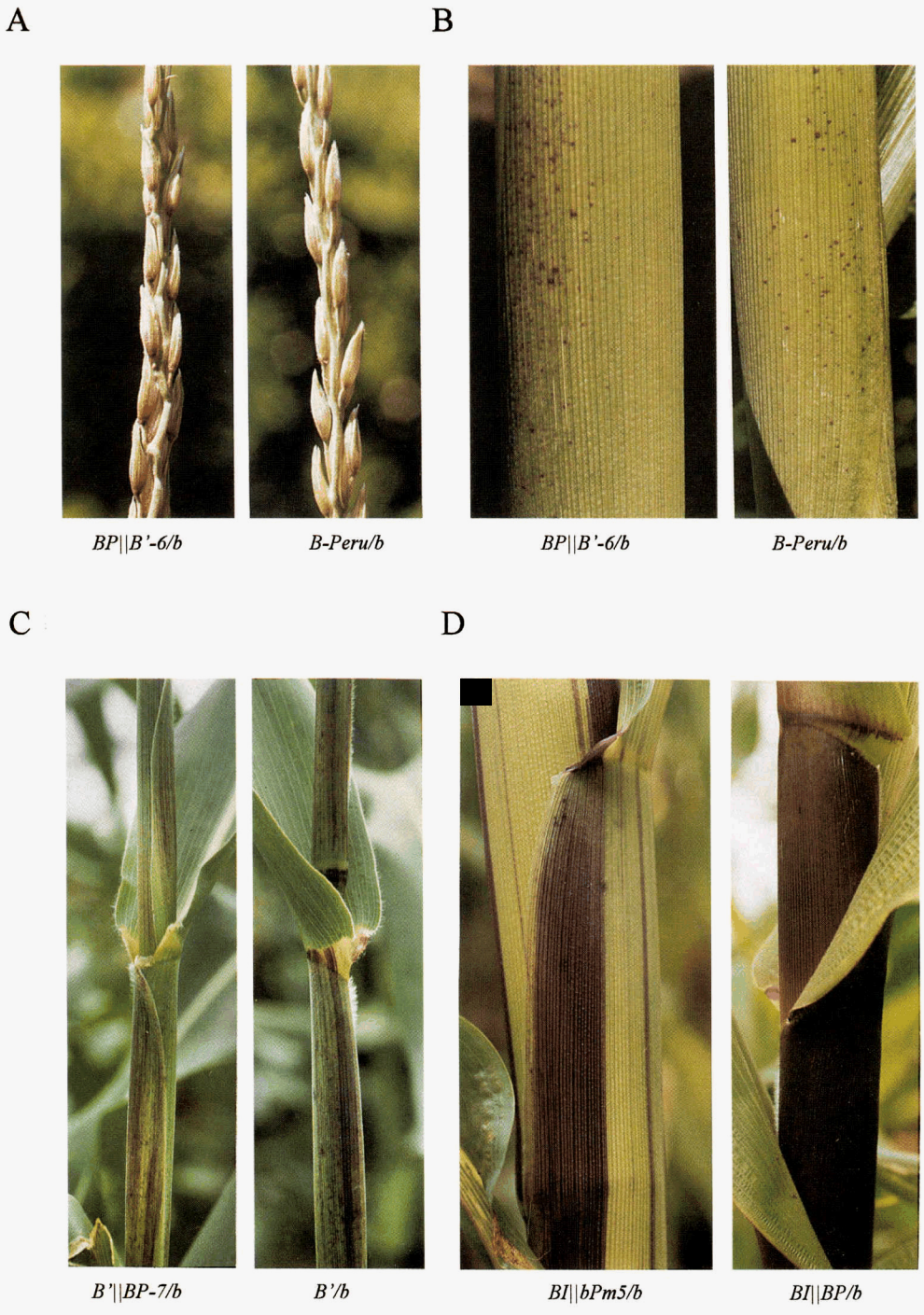

FIGURE 8.—Pigmentation phenotypes of recombinant alleles. Plants were photographed at maturity. Genotypes of plants are as shown. The recessive *b* allele is *b-534* (Table 1).

B'||BP/B-I and *b-534/B-I* siblings were self-pollinated, and the progeny were scored for color. If *B'||BP* does not heritably alter *B-I*, one-quarter of the *B'||BP/B-I* self progeny should be *B-I/B-I* and intensely colored. Several families were examined for each of the *B'||BP/B-I* recombinants, and all of the progeny were light purple (395 plants examined, all with tassel color scores <6 and sheath color scores <8). None of the progeny had the dark purple color typical of *B-I*, as was seen in the progeny of the *b-534/B-I* self control. Eleven percent of the progeny receiving the *B-I* chromosome from the *b-534/B-I* parent had the *B'* phenotype. These are likely to represent spontaneous changes of *B-I* to *B'* (COE 1966), as this is a typical spontaneous frequency. This experiment demonstrates that *B'||BP* heritably alters *B-I*, just as *B'* does, 100% of the time.

***BP||B'* alleles do not paramutate *B-I*:** *BP||B'* recombinant alleles were isolated as heterozygotes with *b-534*, an allele that does not cause or respond to paramutation. Heterozygotes representing 47 independently

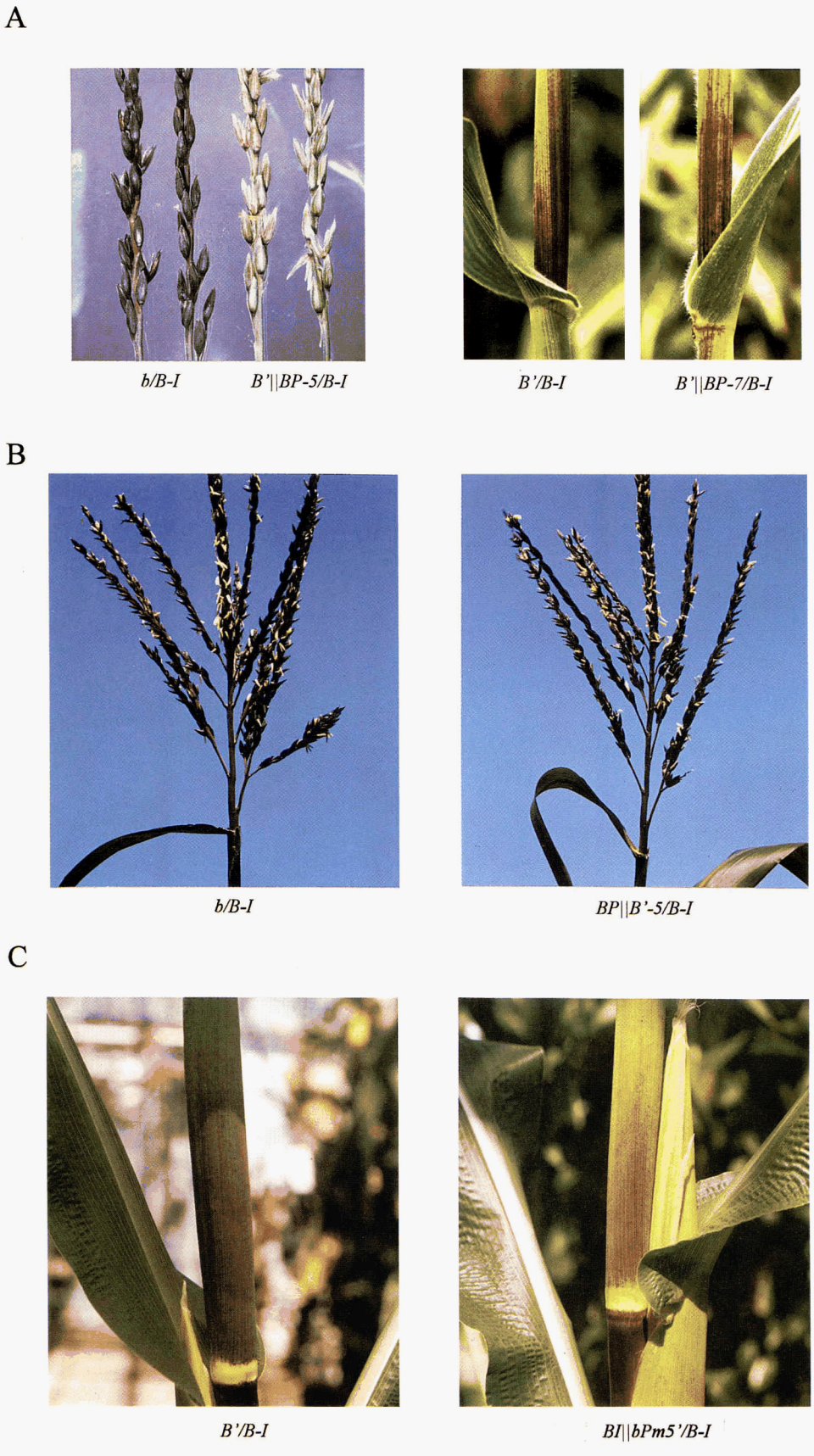

FIGURE 9.—Paramutation phenotypes of recombinant alleles. Plants were photographed at maturity. Genotypes of plants are as shown. The recessive *b* allele is *b-534* (Table 1).

TABLE 4

B'* \| \| *BP* recombinants paramutate *B-I

Genotype	No. of plants	Average color score	
		Sheath	Tassel
B' | | *BP-1/B-I*	5	5.6 ± 0.5	4.8 ± 1.2
b-534/B-I	3	9.7 ± 0.7	8.7 ± 0.7
B' | | *BP-2/B-I*	4	5.0 ± 0.7	3.0 ± 0.7
b-534/B-I	4	9.8 ± 0.4	7.5 ± 1.1
B' | | *BP-3/B-I*	4	7.0 ± 1.2	5.2 ± 1.5
b-534/B-I	3	9.0 ± 0.0	8.3 ± 1.2
B' | | *BP-4/B-I*	6	6.8 ± 1.1	5.5 ± 0.8
b-534/B-I	2	10 ± 0.0	9.5 ± 0.5
B' | | *BP-5/B-I*	4	5.0 ± 0.7	3.8 ± 0.8
b-534/B-I	4	9.2 ± 0.4	9.0 ± 0.0
B' | | *BP-6/B-I*	4	4.5 ± 1.7	3.5 ± 0.5
b-534/B-I	4	10 ± 0.0	8.5 ± 1.1

Plants of the genotypes indicated were visually scored for pigment and assigned a number that corresponded to the intensity of pigment in the organ indicated (1 is the lightest, 10 the darkest; see MATERIALS AND METHODS for complete description). The mean ± SE is shown. Scoring was done blind in that the genotype of the *b* locus was not known at the time of scoring.

TABLE 5

BI* \| *bPm5* recombinants paramutate *B-I* after exposure to *B'*, but *BP* \| *BI* and *BP* \| *B'* do not paramutate *B-I

	Phenotype[a]	
	Light (*B'* phenotype)	Dark (*B-I* phenotype)
BI | *bPm5'/B-I*	25[b]	0
B'/B-I	26[b]	0
BP | *BI/B-I*	4[c]	73
B'/B-I	74	0
BP | *B'/B-I*	11[c]	292
B'/B-I	296	0

[a] All plants scored as *B'* in phenotype were ≤6 on the tassel color scale and ≤8 on the sheath scale. All plants scored as *B-I* in phenotype were 9–10 for tassel and sheath color scores. Scoring was done blind in that the genotype of the *b* locus was not known at the time of scoring.

[b] The sheath and tassel color scores (mean score ± SE) of the 25 *BI* \| *bPm5'/B-I* plants were 2.84 ± 0.14 and 3.04 ± 0.22, respectively. These are very similar to the scores of the 26 *B'/B-I* plants, 3.12 ± 0.13 and 3.88 ± 0.22, for sheath and tassel, respectively.

[c] These plants are most likely due to spontaneous paramutation of *B-I* in the *B-I/B-I* parent, rather than paramutation by *B-Peru* (see text).

isolated recombinants were crossed to *B-I/B-I* to test for paramutation. The fact that *BP* \| *B'* colors the aleurone, and *B-I* and *b-534* do not, enabled the separation of the two genotypes by seed color. The two types of seed were planted and the plant phenotypes were compared. Since *b-534* is incapable of paramutating *B-I*, the *b-534/B-I* progeny are expected to have dark purple pigment; if the *BP* \| *B'/B-I* sibs are lighter, then the recombinant allele is capable of altering *B-I* pigment. The *BP* \| *B'* recombinant had no effect on *B-I* pigmentation. All of the plants had a dark purple phenotype similar to that produced by *B-I* (Figure 9B). The two genotypes were scored visually for pigment intensity using the scale of 1 (lightest) to 10 (darkest), described in MATERIALS AND METHODS. The tassel color score of 664 *BP* \| *B'/B-I* plants was 9.77 ± 0.05, indistinguishable from that of the 642 *b-534/B-I* plants, 9.71 ± 0.05. The sheath color scores for both classes were 9–10, indicative of *B-I* phenotype.

The above experiments indicate that the presence of the 3′ region of *B'* causes no detectable paramutation in the F_1. Although *BP* \| *B'* does not affect *B-I* pigmentation in the *BP* \| *B'/B-I* heterozygote, *BP* \| *B'* could produce a heritable change observed in subsequent generations, as seen with *r* paramutation in maize. In this case, inducing *r* alleles have only a subtle effect on pigmentation produced by the sensitive *r* alleles in the F_1 heterozygote (MCWHIRTER and BRINK 1963) but do heritably alter the sensitive *r* alleles such that they have reduced pigment in the next generation. To test whether a similar phenomenon was occurring, the *BP* \| *B'/B-I* heterozygotes and the *b-534/B-I* siblings were self-pollinated, and the progeny visually scored for color (MATERIALS AND METHODS). Over 400 progeny were scored, representing 11 independent recombinants. The *B-I*-containing progeny of *BP* \| *B'/B-I* were indistinguishable from those of *b-534/B-I*. Over 90% of the *B-I*-containing progeny of each parent were dark (tassel and sheath color scores = 9–10). A few of the progeny were light (tassel color scores <6 and sheath color scores <8), presumably due to spontaneous changes of *B-I* to *B'*. The fact that the numbers of light progeny of *BP* \| *B'/B-I* were similar to the number of light progeny of *b-534/B-I*, and that the *B-I*-containing progeny were equally dark, shows that *BP* \| *B'* is equivalent to *b-534* in its inability to cause paramutation.

***BI* \| *bPm5* responds to paramutation by *B'*:** When *B-I* is heterozygous with *B'*, it acquires the ability to paramutate a naive *B-I* allele (*B-I* not previously exposed to *B'*). Thus, when *B'/B-I* is crossed with *B-I/B-I*, all progeny are light purple (COE 1966; PATTERSON and CHANDLER 1995b). To determine whether *BI* \| *bPm5* could be altered by *B'* in this manner, progeny from *BI* \| *bPm5/B'* × *B-I/B-I* crosses were scored for anthocyanin pigment phenotype and their genotype determined by RFLP analysis. Both classes of progeny were light purple with no obvious differences in pigment phenotype (Table 5; Figure 9C). Thus, when *BI* \| *bPm5* is exposed to *B'*, it is paramutated, becoming fully capable of altering *B-I*. The paramutated form is

designated *BI*| |*bPm5′*. In contrast, *BI*| |*bPm5/B-I* individuals are intensely pigmented and *B-I* is transmitted normally from these heterozygotes (data not shown). These results demonstrate that *BI*| |*bPm5* behaves like *B-I* in its ability to respond to paramutation by *B′*.

***BP*| |*BI* alleles are insensitive to paramutation by *B′*:** To determine whether the complementary recombinant allele, *BP*| |*BI*, can also be altered by *B′*, *BP*| |*BI/B′* heterozygotes were constructed. The purple seed color produced by *BP*| |*BI* was unaltered in the presence of *B′*, both in the F_1 and in progeny (data not shown), indicating that *BP*| |*BI* is not changed by *B′*. We also addressed whether the *BP*| |*BI* recombinant allele could be altered by *B′*, such that it could paramutate *B-I* in subsequent generations. *BP*| |*BI/B′* plants were crossed to *B-I/B-I*, the purple seed (*BP*| |*BI/B-I*) were planted, and the resulting plants scored for anthocyanin pigment levels. Most of the *BP*| |*BI/B-I* progeny were dark (tassel and sheath color scores = 9–10), indicating that *BP*| |*BI* is not able to paramutate *B-I* (Table 5), even after exposure to *B′*. A few of the progeny were light, but again the frequency is similar to the rate at which *B-I* spontaneously changes to *B′* (Coe 1966). Six of the *BP*| |*B′* recombinants were tested in a similar experiment, and these alleles also do not gain the ability to paramutate after being exposed to *B′* (Table 5).

DISCUSSION

Two types of recombination experiments were carried out to determine where the sequences required for *b* paramutation reside relative to the *b* coding region. First, over 3000 plants were scored in an attempt to identify a recombinant allele in which the ability to paramutate was separated from the sequences controlling *B′* tissue-specific expression (Figure 1). No separation was found, demonstrating that sequences mediating paramutation are no more than 0.1 cM from *B′*. Second, intragenic recombinants were used to determine if sequences required for paramutation were within the *b* coding region or flanking sequences. *B′*| |*BP* intragenic recombinants, which have 5′ sequences of *B′* and coding and 3′ flanking sequences of *B-Peru*, efficiently paramutate *B-I*. *BP*| |*B′* intragenic recombinants, which have 5′ sequences of *B-Peru* and coding and 3′ flanking sequences of *B′*, do not paramutate *B-I*. In several cases, recombination between alleles occurred upstream of the translation initiation codon. This result forms a 3′ boundary for where sequences required for paramutation must map. The finding that sequences mediating paramutation are <0.1 cM from *B′* provides an estimate for the 5′ boundary for where these sequences map. Within *b*, 0.1 cM can be <1 kb and as large as 40 kb (Figure 7). Between maize genes the distance has been estimated to be 1460 kb/cM and measured at 1560 kb/cM (Civardi *et al.* 1994), which would give a value of ~150 kb for 0.1 cM. Taken together, these experiments demonstrate that the unique ability of *B′* to paramutate *B-I* resides within the 5′ end of the allele or in very closely linked sequences upstream of the gene. Similarly, the ability of *B-I* to respond to *B′* also maps to the 5′ region. The *BI*| |*bPm5* intragenic recombinant is just as sensitive to paramutation as *B-I* alleles, and *BP*| |*BI* intragenic recombinants are not affected by *B′*. Again, there are examples where the recombination event occurred upstream of the translation initiation codon. These results further indicate that the B protein is not functionally different in alleles that participate in paramutation and alleles that do not.

Sequences that mediate the phenotypic differences between *B′* and *B-I* (the ability to respond to and cause paramutation and the different transcription rates) are located upstream of the translation initiation codon. The *BI*| |*bPm5* recombinant allele is like *B-I* with respect to its ability to activate the anthocyanin pathway and with respect to its ability to respond to paramutation. Similarly, the *B′*| |*BP* allele is like *B′*. Since these two alleles share the coding and downstream regions of *B-Peru*, it must be the upstream region that causes *BI*| |*bPm5* and *B′*| |*BP* to be like *B-I* and *B′*, respectively. The observation that the protein coding region does not contribute to differences between *B-I* and *B′* is consistent with previous studies that demonstrated that the B protein encoded by *B-I* and *B′* is functionally equivalent in activating the anthocyanin pathway (Patterson *et al.* 1993). Additional fine-structure mapping will be required to determine whether the sequences required to cause paramutation are the same as the sequences required to respond to paramutation.

We have examined the 5′ region for differences that might account for paramutation. Extensive analyses of methylation, restriction sites and DNA sequence have not revealed any differences between *B-I* and *B′* (Patterson *et al.* 1993). This includes comparing dozens of restriction sites in the 5′ flanking region and sequencing ~2000 bp of sequence upstream of the translation initiation codon in each allele. Our recombination results suggest that if there is a DNA change that is associated with paramutation and altered transcription, it will be located further upstream.

In all of the recombination experiments we have not observed significantly different recombination frequencies for *B-I* and *B′*. The rate of intragenic recombination between *B-I* and *b-m1* is the same as the rate between *B′* and *b-m1*. Similarly, *B′* and *B-I* map to the same chromosome 2 location relative to flanking markers. Thus, the difference in transcription between *B′* and *B-I*, and any gene sequence or structure differences that underlie the change in transcription, do not affect recombination frequencies. This suggests that no large scale chromosomal rearrangements undetectable by

DNA blot analyses, such as inversions that include the *b* coding region, are mediating paramutation.

Sequences controlling tissue-specific gene expression in *B-I, B′* and *B-Peru* also map to the 5′ end of each allele. DNA from *B-Peru* containing the promoter and untranslated leader (2.1 kb) is sufficient for expression in the aleurone in transient transformation assays, whereas a 3-kb DNA fragment from the same region of *B-I* does not promote aleurone expression (RADICELLA *et al.* 1992; D. BROWN and V. CHANDLER, unpublished data). The fact that *B′*||*BP* recombinant alleles produce a pigmentation pattern in the mature plant that is indistinguishable from that of *B′* indicates that sequences upstream of the initiator methionine codon in *B′* control its tissue-specific expression in all plant tissues. Similarly, the plant phenotype of *BP*||*B′* recombinants is like that of *B-Peru;* therefore, the 5′ end of *B-Peru* (sequences upstream of the translation initiation codon) controls its tissue-specific expression in all plant tissues.

It is clear from the analysis of transcription of *b* and transcription of the genes it regulates (PATTERSON *et al.* 1993), and from the genetic mapping presented here, that *b* paramutation works by altering transcription of *b,* not by altering the stability of the *b* mRNA or by changing the B protein. These results are consistent with the possibility that paramutation is mediated by transcriptional regulatory sequences in *B′* and *B-I.* A number of gene inactivation phenomena have been described in plants (FLAVELL 1994). In these phenomena, a gene introduced via transformation (transgene) causes the inactivation of a second transgene or a homologous endogenous gene (reviewed in MATZKE and MATZKE 1993; FLAVELL 1994). Mechanisms of transgene inactivation are not known. However, some of these transgene inactivation phenomena have been shown to act at a posttranscriptional level and thus might not be mechanistically similar to paramutation.

The maize *r* gene, which encodes a functionally equivalent protein to *b* (STYLES *et al.* 1973; GOFF *et al.* 1990), also undergoes paramutation. Interestingly, the location of sequences that control paramutation is different at *b* and *r*. KERMICLE (1974) reviewed early attempts to map the position of sequences in the *R-st* allele that causes paramutation. *R-st* is a complex locus with four linked *r* genes (reviewed in BRINK 1973; W. EGGLESTON, M. ALLEMAN and J. L. KERMICLE, personal communication). Recent experiments have demonstrated that multiple regions are required for *r* paramutation and these are distributed throughout the repeated regions. Interestingly, no single *r* region is required, but there is a strong correlation between the number of *r* repeats and the strength of paramutation (J. L. KERMICLE, W. EGGLESTON and M. ALLEMAN, personal communication).

Our experiments demonstrate that paramutation of *b* does not have the composite nature of *r* paramutation. All *b* alleles examined to date consist of only one genic region (PATTERSON *et al.* 1991, 1993). Our recombination analysis does not indicate that *b* paramutation can be lost in a stepwise fashion; *BP*||*B′* is completely unable to paramutate *B-I,* and *B′*||*BP* efficiently paramutates *B-I.* The composite structure of *R-st* leads to a high degree of instability. Approximately 1 progeny in 1000 of a *R-st/R-st* parent are full color revertants (ASHMAN 1960), some of which have lost the ability to cause paramutation (MCWHIRTHER and BRINK 1962). These losses are associated with unequal crossing over between *r* gene repeats in *R-st* (KERMICLE 1970; W. EGGLESTON, M. ALLEMAN and J. L. KERMICLE, personal communication). In contrast, *B′* is very stable as no derivatives incapable of paramutation have been observed in $\sim 10^5$ plants screened (PATTERSON 1993; K. KUBO, G. PATTERSON and V. CHANDLER, unpublished data).

Analysis of the recombinant *b* alleles indicates that recombination at the *b* locus has some interesting features. The *b* gene undergoes a high frequency of genetic recombination, as do other plant genes (NELSON 1968; FREELING 1978; DOONER 1986; BROWN and SUNDARESEN 1991; MOURAD *et al.* 1994). The overall rate of recombination as a function of physical distance in *b* (4×10^{-2} cM/kb) is comparable to that seen for the maize genes *bz1* (7×10^{-2} cM/kb) (DOONER 1986; DOONER *et al.* 1985) and *a1* (6×10^{-2} cM/kb) (BROWN and SUNDARESEN 1991), and the Arabidopsis gene *CSR1* (MOURAD *et al.* 1994). However, the rate of recombination varies widely across the *b* gene. Recombination is 100-fold higher ($\sim 1 \times 10^{-1}$ cM/kb) upstream of the *Nru*I/*Ava*I polymorphism at +823 relative to downstream ($\sim 1 \times 10^{-3}$ cM/kb). The recombination rate estimated for the intergenic region between *a1* and *sh2* ($\sim 7 \times 10^{-4}$ cM/kb) (CIVARDI *et al.* 1994) is similar to that seen in the downstream region of *b*. It has been suggested that essentially all recombination in maize occurs within genes (DOONER 1993). Our data are consistent with the idea that maize genes have hotspots for recombination in addition to gene sequences that are no more recombinagenic than intergenic regions. Analysis of intragenic recombination with *R-st* revealed that *r* is similar to *b* in that recombination is not uniform within the coding region. Interestingly, analysis of intragenic recombinants with *R-st* have revealed that, in contrast to *b,* recombination events occurred predominantly (70 of 72 examined) at the 3′ ends of the *r* genes (W. EGGLESTON, M. ALLEMAN and J. L. KERMICLE, personal communication). The variation in recombination within the *b* gene is reminiscent of recombination gradients that have been seen in a number of fungal genes, in which recombination varies up to 10-fold at different locations within the genes (NICOLAS and PETES 1994).

The regions of fungal genes in which recombination is highest are thought to be near points where genetic recombination is initiated (NICOLAS and PETES 1994).

It is possible that there is a hotspot at *b* due to recombination initiating near the 5′ end of the *b* gene, potentially because it is near the promoter. In yeast, double-strand DNA breaks, which initiate most meiotic recombination, have been observed at or near many potential transcriptional promoters (WU and LICHTEN 1994). Alternatively, the low recombination of the 3′ region of *b* may be due to a suppressive effect of specific sequences in these regions. The presence of insertions in the alleles do not explain the different recombination frequencies throughout *b*. It is unlikely that the *Ds1* insertion near the 3′ end is mediating the low recombination in the 3′ region, because recombination frequencies between *B-Peru* and *b′-v,* which does not have the 3′ insertion, are as low as those between *B-I, B′* and *b-m1,* which has the 3′ *Ds1* insertion (Figure 7). The one recombinant isolated from *B-I* and *b-Pm5* was isolated from a small number of plants, suggesting a high recombination rate, even though the *b-Pm5* allele has an ~8-kb insertion near the site of recombination. In addition, *B-Peru* has a 2-kb insertion at the 5′ end (relative to *B′* and *B-I*) (Figures 2 and 7) but nonetheless has a high rate of recombination nearby. If the 2-kb insertion in *B-Peru* acts as a recombination enhancer, we would expect to see recombination highest next to the insertion with a gradient of decreasing recombination. This is not observed (Figure 7).

The recombinants isolated in this study have recombinant flanking physical and genetic markers indicative of a crossover. The fact that at least 131 of the 133 recombinants (Table 3) were associated with crossing over is unusual, since intragenic recombination with and without associated crossing over are often similar in frequency (DOONER 1986; reviewed in FOSS *et al.* 1993). However, there have been two previous reports that recombination between alleles that contain *Ds* insertions and point mutations tend to be associated with crossing over most of the time (DOONER 1986; DOONER and KERMICLE 1986). As 110 of our recombinants involved the *Ds*-containing *b-m1* allele, our results provide additional data that strengthens this observation.

An understanding of the mechanism of paramutation and related gene-inactivation phenomena will require the elucidation of both *cis*- and *trans*-acting genetic requirements for the process, along with an understanding of the mechanism of action of the genetically identified factors. The genetic mapping of *cis*-acting sequences described in this paper represents a first step toward identifying these important sequences. This approach, together with the identification and characterization of mutants defective in paramutation, will enhance our understanding of the mechanism of allelic interactions and gene inactivation.

We are grateful to SUSAN BELCHER for her assistance with the field experiments and to E. H. COE, JR., M. G. NEUFFER and W. SHERIDAN for providing genetic stocks. We thank MARY ALLEMAN, JAY HOLLICK, TRUDEE TARKOWSKI, BRYAN PICKETT, MANUEL SAINZ, DAVE SELINGER and WIM VAN HEECKEREN for comments on the manuscript. This work was supported by grants from National Institutes of Health (NIH) (GM-35791) and American Cancer Society (NP-875) to V.L.C. G.I.P. and K.M.K. were supported by a NIH predoctoral training grant (GM-07413) and a National Science Foundation Plant Postdoctoral Fellowship, respectively.

LITERATURE CITED

ASHMAN, R. B., 1960 Stippled aleurone in maize. Genetics **45:** 19–34.

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. S. SEIDMAN *et al.,* 1987 *Current Protocols in Molecular Biology.* Greene/Wiley, New York.

BRINK, R. A., 1973 Paramutation. Annu. Rev. Genet. **7:** 129–152.

BROWN, J., and V. SUNDARESEN, 1991 A recombination hotspot in the maize *a1* intragenic region. Theor. Appl. Genet. **81:** 185–188.

CHANDLER, V. L., J. P. RADICELLA, T. P. ROBBINS, J. CHEN and D. TURKS, 1989 Two regulatory genes of the maize anthocyanin pathway are homologous: isolation of *b* using *r* genomic sequences. Plant Cell **1:** 1175–1183.

CIVARDI, L., Y. XIA, K. J. EDWARDS, P. S. SCHNABLE and B. J. NIKOLAU, 1994 The relationship between genetic and physical distances in the cloned *a1-sh2* interval of the *Zea mays* L. genome. Proc. Natl. Acad. Sci. USA **91:** 8268–8272.

CLARK, J. K., V. L. CHANDLER and M. G. NEUFFER, 1990 Characterization of two *Ds* mutants of *B-Peru.* Maize Genetics Cooperation Newsletter **64:** 59–60.

COE, Jr., E. H., 1959 A regular and continuing conversion-type phenomenon at the *b* locus in maize. Proc. Natl. Acad. Sci. USA **45:** 828–832.

COE, Jr., E. H., 1966 The properties, origin and mechanism of conversion-type inheritance at the *b* locus in maize. Genetics **53:** 1035–1063.

COE, E. H., M. G. NEUFFER and D. A. HOISINGTON, 1988 The genetics of corn, pp. 83–258 in *Corn and Corn Improvement,* edited by G. F. SPRAGUE and J. W. DUDLEY. American Society of Agronomy, Madison, WI.

DELLAPORTA, S. L., J. WOOD and J. B. HICKS, 1983 A plant DNA mini preparation. Plant Mol. Biol. Rep. **1:** 19–21.

DOONER, H. K., 1986 Genetic fine structure of the *bronze* locus in maize. Genetics **113:** 1021–1036.

DOONER, H. K., 1993 Genetic fine structure from testcross progeny analysis, pp. 303–306 in *The Maize Handbook,* edited by M. FREELING and V. WALBOT. Springer-Verlag, New York.

DOONER, H. K., and J. L. KERMICLE, 1986 The transposable element *Ds* affects the pattern of intragenic recombination at the *bz* and *r* loci in maize. Genetics **113:** 135–143.

DOONER, H. K., E. WECK, S. ADAMS, E. RALSTON, M. FAVREAU *et al.,* 1985 A molecular genetic analysis of insertions in the *bronze* locus of maize. Mol. Gen. Genet. **200:** 240–246.

DOONER, H. K., T. P. ROBBINS and R. A. JORGENSEN 1991 Genetic and developmental control of anthocyanin biosynthesis. Annu. Rev. Genetics **25:** 173–199.

EMERSON, R. A., 1921 The genetic relations of plant color in maize. Cornell Univ. Agric. Exp. Stn. Mem. **39:** 9–10.

FEDOROFF, N. V., 1989 Maize transposable elements, pp. 375–411 in *Mobile DNA,* edited by D. BERG and M. HOWE. American Society for Microbiology, Washington, DC.

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

FLAVELL, R. B., 1994 Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA **91:** 3490–3496.

FOSS, E., R. LANDE, F. W. STAHL and C. M. STEINBERG, 1993 Chiasma interference as a function of genetic distance. Genetics **133:** 681–691.

FREELING, M., 1978 Allelic variation at the level of intragenic recombination. Genetics **89:** 211–224.

GOFF, S. A., T. M. KLEIN, B. A. ROTH, M. E. FROMM, K. C. CONE *et al.,* 1990 Transactivation of anthocyanin biosynthetic genes following transfer of *b* regulatory genes into maize tissues. EMBO J. **9:** 2517–2522.

HARRIS, L. J., K. CURRIE and V. L. CHANDLER, 1994 Large tandem duplication associated with a *Mu2* insertion in *Zea mays B-Peru* gene. Plant Mol. Biol. **25:** 817–828.

KERMICLE, J. L., 1970 Somatic and meiotic instability of *R*-stippled, an aleurone spotting factor in maize. Genetics **64:** 247–258.

KERMICLE, J. L., 1974 Organization of paramutational components of the *R* locus in maize. Brookhaven Symposia Biol. **25:** 262–280.

MATZKE, M., and A. J. M. MATZKE, 1993 Genomic imprinting in plants: Parental effects and trans-inactivation phenomena. Annu. Rev. Plant Physiol./Plant Mol. Biol. **44:** 53–76.

MCWHIRTER, K. S., and R. A. BRINK, 1962 Continuous variation in level of paramutation at the *R* locus in maize. Genetics **47:** 1053–1074.

MCWHIRTER, K. S., and R. A. BRINK, 1963 Paramutation in maize during endosperm development. Genetics **48:** 189–203.

MOURAD, G., G. HAUGHN and J. KING, 1994 Intragenic recombination in the *CSR1* locus of Arabidopsis. Mol. Gen. Genet. **243:** 178–184.

NELSON, O. E., 1968 The *waxy* locus in maize. II. The location of the controlling element alleles. Genetics **60:** 507–524.

NICOLAS, A., and T. D. PETES, 1994 Polarity of meiotic gene conversion in fungi: contrasting views. Experientia **50:** 242–252.

PATTERSON, G. I., 1993 Paramutation: a directed, meiotically heritable change in gene expression. Ph.D. Thesis, University of Oregon, Eugene.

PATTERSON, G. I., and V. L. CHANDLER 1995a Paramutation in maize and related allelic interactions, pp. 121–141 in *Gene Silencing in Higher Plants and Related Phenomena in Other Eukaryotes,* edited by P. MEYER, an edition of *Current Topics in Microbiology and Immunology,* Vol. 197, Springer, Berlin.

PATTERSON, G. I., and V. L. CHANDLER, 1995b Timing of *b* paramutation. Maydica **40:** 35–41.

PATTERSON, G. I., L. J. HARRIS, V. WALBOT and V. L. CHANDLER, 1991 Genetic analysis of *B-Peru,* a regulatory gene in maize. Genetics **126:** 205–220.

PATTERSON, G. I., C. J. THORPE and V. L. CHANDLER, 1993 Paramutation, an allelic interaction, is associated with a stable and heritable reduction of transcription of the maize *b* regulatory gene. Genetics **135:** 881–894.

RADICELLA, J. P., D. TURKS and V. L. CHANDLER, 1991 Cloning and nucleotide sequence of a cDNA encoding *B-Peru,* a regulatory protein of the anthocyanin pathway in maize. Plant Mol. Biol. **17:** 127–130.

RADICELLA, J. P., D. BROWN, L. A. TOLAR and V. L. CHANDLER, 1992 Allelic diversity of the maize *b* regulatory gene: different leader and promoter sequences of two *b* alleles determine distinct tissue specificities of anthocyanin production. Genes Dev. **6:** 2152–2164.

RIVIN, C., E. ZIMMER and V. WALBOT, 1982 Isolation of DNA and DNA recombinants from maize, pp. 161–164 in *Maize for Biological Research,* edited by W. F. SHERIDAN. University of North Dakota Press, Grand Forks, ND.

STYLES, E. D., O. CESKA and K.-T. SEAH, 1973 Developmental differences in action of *R* and *B* alleles in maize. Can. J. Genet. Cytol. **15:** 59–72.

WU, T.-C., and M. LICHTEN, 1994 Meiosis-induced double-strand break sites determined by yeast chromatin structure. Science **263:** 515–518.

Communicating editor: J. A. BIRCHLER