The Mutation *bronze-mutable 4 Derivative 6856* in Maize Is Caused by the Insertion of a Novel 6.7-Kilobase Pair Transposon in the Untranslated Leader Region of the *Bronze-1* Gene

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ABSTRACT

The *Ds*-controlled allele, *bz-m4 Derivative 6856* [*bz-m4 D6856*], is reported to have an altered temporal- and tissue-specific pattern of gene expression. We have cloned this allele and have characterized it at the molecular level. The mutation was caused by the insertion of a complex transposon-like structure 36 base pairs downstream from the *Bz* mRNA cap site. The insert is 6.7-kbp long. *Ds* elements, each approximately 2 kbp in length, are at both ends of the insert. The sequence between the *Ds* elements is a partial duplication of flanking sequences from the 3′ end of the *Bz* gene. These data suggest that *Ds* initially inserted near the 3′ end of the gene and mobilized adjacent sequences as it transposed.

BRONZE (*Bz*) is the structural gene for UDP glucose 3-*O*-flavonol glucosyltransferase (UFGT) (EC 2.4.1.91) which is required for the synthesis of the red and purple anthocyanin pigments in maize (LARSON and COE 1977; DOONER and NELSON 1977a). The *Ds* (*Dissociation*) transposable element-induced allele, *bz-m4 D6856*, is unusual because it alters the temporal and tissue-specific expression of the *Bz* gene product (DOONER and NELSON 1977a; DOONER 1981). In mutant seed, UFGT is detectable 14 days after pollination (DAP), and peaks by 22–26 DAP. In wild-type seeds, UFGT activity does not increase substantially until 26 DAP and reaches its highest level after black layer formation and seed maturation (DOONER and NELSON 1977b). Furthermore, UFGT, which is restricted to the aleurone layer of the wild-type kernels, is preferentially localized in the subaleurone endosperm in *bz-m4* kernels (DOONER 1981). Presumably these changes in timing and tissue-specificity of *Bz* expression are due to the insertion of a *Ds* transposable element near the 5′ end of the gene in *bz-m4 D6856*.

The origin of *bz-m4 D6856* is complex (Figure 1). MCCLINTOCK (1952) observed that a transposable element at one locus would "spread" to adjacent loci. In the maize line she was studying, *Ds*, in the presence of *Ac* (*Activator*), caused chromosome breaks immediately distal to the *shrunken* (*sh*) locus. From this stock, MCCLINTOCK isolated new mutable alleles of the flanking genes, *C-I* (*dominant colorless*) or *Bz*. The original *bz-m4* allele was isolated in that study (B. MCCLINTOCK, personal communication). This *bz-m4* line was stably recessive for the *shrunken* (*sh*) trait. Later MCCLINTOCK demonstrated that recombination between *sh* and *bz* in this stock was substantially reduced, indicating that the unstable *bz-m4* allele arose concommitantly with a deletion of chromosomal material in the interval between these loci (MCCLINTOCK 1965; DOONER 1981).

In the presence of *Ac*, the original *bz-m4* allele formed dicentric chromosomes at a high frequency. In a subsequent generation this *bz-m4* reverted to a *Bz′*[2] allele (B. MCCLINTOCK, personal communication). This was unstable, indicating that a *Ds* element was near or at the *Bz′* allele. Subsequently, again with *Ac* present, a gamete from a *Bz′-m* plant, culture #6771, mutated to an unusual dark bronze, recessive allele which also had a reduced frequency of dicentric formation. This allele was *bz-m4 D6856*. MCCLINTOCK isolated various revertants and changes of state from these several forms of *bz-m4*. Of the various *bz-m4* alleles, *bz-m4 D6856* is known to have an altered temporal- and tissue-specific gene expression. However, the regulation of the other alleles has not been investigated.

The product of the *Sh* gene, sucrose synthase (SS), is a major endosperm protein required for full levels of starch biosynthesis in the developing seed

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[2] Changes of state and germinal revertants to either purple or bronze phenotypes are designated prime (′) alleles. This indicates that the new form of the allele is not necessarily identical, at the molecular level, to its wild-type or mutable progenitor.

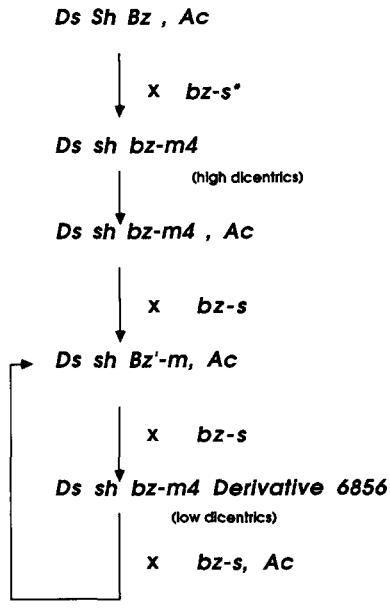

FIGURE 1.—The origins of the *bz-m4* allelic series. Each selection shown was crossed with a *bz-S* stock and the progeny were used for further selections. (Adapted from B. MCCLINTOCK, personal communication.)

(CHOUREY and NELSON 1976). Since a deletion between *sh* and *bz* occurred during the origin of *bz-m4*, GERATS and co-workers (1983) proposed that the regulatory region from the *Sh* gene was fused to and thus controlled the expression of the *bz-m4*. This hypothesis could be tested directly by determining whether the regulatory sequences of *Sh* abut *Bz* coding information in *bz-m4*.

We therefore cloned *bz-m4 D6856* and analyzed the molecular organization of the mutant as a first step toward understanding how this transposable element mutation alters control of the *Bz* gene. The mutant allele, *bz-m4 D6856*, has a novel 6.7-kbp insertion in the 5′ untranslated leader region of the *Bz* gene. The large size and position of insertion within the *bz-m4 D6856* allele suggest that the insertion interferes with wild-type *Bz* regulatory functions which direct normal transcription of the gene. The insert in *bz-m4 D6856* is unusual as compared to other maize transposable elements which have been examined at the DNA level. Structurally, the insert resembles a class II bacterial transposon (KLECKNER 1981), wherein two insertion sequences are in direct orientation with intervening DNA. The intervening sequences are actually a duplication of 3′ flanking sequences from the *bz* locus. The novel insertion in *bz-m4 Derivative 6856* is an example of the type of genomic rearrangements which are generated by the activity of transposable elements in maize.

MATERIALS AND METHODS

Strains: There are a series of unstable alleles at the *bz* locus designated *bz-m4* (MCCLINTOCK 1956). The pedigrees of these alleles are shown in Figure 1. The *bz-m4 D6856* allele was introduced into the W22 inbred line by J. KERMICLE at the University of Wisconsin and maintained in the absence of *Ac*, *i.e.*, under conditions where *Ds* mutations should remain stable. A nonmutant allele, designated *Bz-McC*, is believed to be a progenitor allele of the *bz*-mutable (*Ds*) alleles isolated by B. MCCLINTOCK; it is distinct from the *Bz* allele found in Brink's color converted W22 inbred (DOONER and NELSON 1979; DOONER 1981, 1986; FURTEK *et al.*1988; RALSTON, ENGLISH and DOONER 1988). The *Bz-McC* allele was introduced into the W22 inbred background. Lines homozygous for *bz-m4 D6856* and *Bz-McC* were obtained from H. DOONER, Advanced Genetics Sciences. H. DOONER also generously provided a purple revertant isolated from *bz-m4 D6856*; this allele, *Bz′-[m4 D6856]:1*, produced wild-type levels of UFGT (DOONER 1981).

Enzymes and reagents: Restriction endonucleases, Bal 31 nuclease, T4 DNA ligase, DNA polymerase I, Klenow fragment, M13 sequencing primers and cloning vectors were purchased from New England Biolabs, Bethesda Research Laboratory and Promega Biotech. Deoxy- and dideoxy-nucleotides were purchased from Pharmacia or Bethesda Research Laboratories. ^{35}S-dATP, ^{32}P-dCTP, ^{32}P-dATP and ^{32}P-dTTP were purchased from New England Nuclear or Amersham Corporations.

Oligonucleotide primers for DNA sequencing were synthesized using an Applied Biosystems Model 381A DNA Synthesizer and desalted by gel filtration chromatography.

Genomic blot hybridization analysis: Approximately 10 μg of maize genomic DNA were restricted for 3 hr with 20–50 units of enzyme, under conditions specified by the suppliers. The DNA was fractionated on 0.5–0.8% agarose gels in 50 mM Tris-borate buffer (pH 8.1), with 1 mM EDTA, at 45–60 V for 30–50 hr at 22°. After depurination, denaturation and neutralization of the gel, DNA fragments were transferred to nitrocellulose by overnight blotting (SOUTHERN 1975). Conditions for hybridization, washing and autoradiography were essentially those described by FEDOROFF, MAUVAIS and CHALEFF (1983).

Genomic cloning: DNA was isolated as described by SHURE, WESSLER and FEDOROFF (1983) from immature tassels of 7-week-old plants homozygous for the *bz-m4 D6856* allele. Genomic DNA was digested with *Sau3A* for varying lengths of time to generate a randomly sized population of molecules, and fractionated on 5–20% NaCl gradients. Fragments from ~13 to 20 kilobase pairs (kbp) in length were cloned into the *Bam*HI sites in lambda EMBL3 (FRISHAUF *et al.* 1983). Phage were plated on *Escherichia coli* strain K803 (WOOD 1966). Approximately 1.2 million recombinant phage (3–5 genomic equivalents) were screened using the plaque hybridization method of BENTON and DAVIS (1977). The probe, pMBzP1, contains a unique 0.85 kb *Pst*I fragment from the 3′ end of the transcribed region of *Bz-McC* allele (Figure 2) (FEDOROFF, FURTEK and NELSON 1984; FURTEK *et al.* 1988). A single phage, designated EMBL3[bzm4]#5, had strong homology to the *bz* locus probe and was isolated by the plaque purification method (MANIATIS, FRITSCH and SAMBROOK 1982).

An 18-kbp genomic clone of the *Shl* locus (pSh21.6) was isolated from a Black Mexican sweet corn library. Details of the library construction and screening are given elsewhere (MCCARTY, SHAW and HANNAH 1986). The 18-kbp *Bam*HI insert contains a 10.5-kbp *Eco*RI fragment which is identical at the restriction map level (L. C. HANNAH and SHAW, unpublished data) to the *Sh* clone p17.6 (SHELDON *et al.* 1983; ZACK, FERL and HANNAH 1986).

Subcloning: Phage and phage DNA were prepared essentially as described by ARBER *et al.* (1983). The phage insert

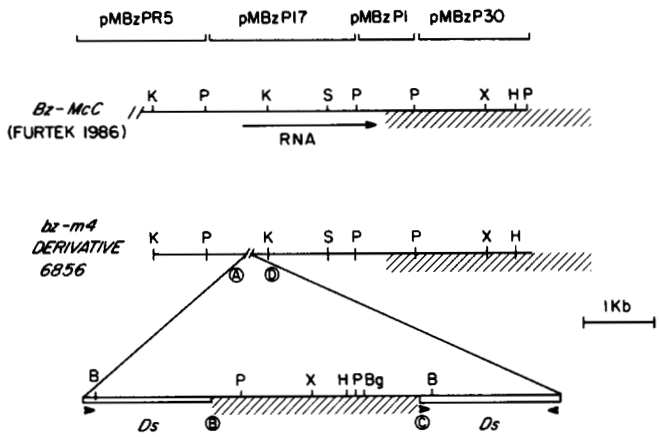

FIGURE 2.—Restriction maps of *Bz-McC* and *bz-m4 Derivative 6856*. Plasmid subclones of *Bz-McC*, which were used for mapping the *bz-m4* allele, are identified on the top line. Restriction maps of the two cloned alleles are shown below. The *Ds* elements and *bz* duplication were identified by hybridization. B = *Bam*HI; Bg = *Bgl*II; H = *Hin*dIII; K = *Kpn*I; P = *Pst*I; X = *Xho*I. The segment of *Bz-McC* which has been duplicated in *bz-m4 D6856* is underscored with hatch marks.

was subcloned as several restriction fragments into pUC vectors 8, 18 and 19. Plasmid stocks were maintained in *E. coli* JM83 (YANISCH-PERRON, VIEIRA and MESSING 1985). Plasmid DNA was prepared by a modification of the alkaline lysis technique (MANIATIS, FRITSCH and SAMBROOK 1982).

DNA sequencing: On the basis of fine-structure restriction mapping of plasmid clones, individual fragments (0.3–1.2 kbp) containing *Ds-bz* rearrangement breakpoints were subcloned into M13 vectors mp18 and mp19 (NORANDER, KEMPE and MESSING 1983; YANISCH-PERRON, VIEIRA and MESSING 1985). For convenient reference, these rearrangement-breakpoints are labelled alphabetically in Figure 2.

DNA sequences were determined by the dideoxynucleotide termination method (SANGER, NICKLEN and COULSEN 1977) with modifications described by BIGGIN, GIBSON and HONG 1983. The sequences at the A and B rearrangement-breakpoints were verified by analysis of at least two independent phage clones containing each segment. The sequence at the C rearrangement breakpoint was obtained from overlapping clones generated using *Bal* 31 exonuclease (PONCZ *et al.* 1982). Partial sequence of the D rearrangement breakpoint was obtained and then extended using synthetic oligonucleotide primers complementary to sequences within the *bz* insert in M13.

UFGT measurements: UFGT levels in immature seed were determined as described in KUHN and KLEIN 1987.

Northern analysis: Immature kernels, homozygous for *Bz-McC* or *bz-m4 D6856* alleles, were harvested at 32 and 26 DAP, respectively, frozen in liquid nitrogen and stored at −70°. Total RNA was prepared by the methods of CONE, BURR and BURR (1986) and enriched for poly(A)$^+$ RNA by passage through Hybond-mAP filters (Amersham Corporation, Arlington Heights, IL). Denatured RNAs were fractionated on a 1% formaldehyde gel buffered 1 × MOPS, stained with acridine orange and transferred to nitrocellulose (MANIATIS, FRITSCH and SAMBROOK 1982). Filters were hybridized at 42° with the probe pMBzP1 labeled by the random priming method Boehringer Mannheim Biochemicals, Indianapolis, Indiana).

RESULTS

Comparison of the molecular structures of *bz-m4 D6856* and the wild-type *Bz-McC* alleles: The restriction map of the recombinant insert in EMBL3 (bzm4 #5) is shown in Figure 2. Homologies to *Bz* sequences were determined by Southern hybridizations with various *Bz*-specific probes (FURTEK *et al.*1988) (pMBzP30 is identical to pAc127P1 in FEDOROFF, FURTEK and NELSON 1984). The positions of Bz-McC subclones, which were used as probes, are shown above the restriction map of the wild-type allele. Representative data, supporting the alignments and homologies between the two clones are summarized below.

(1) EMBL3[bzm4]#5 DNA was digested with the restriction enzyme *Pst*I and probed with pMBzP17 DNA. This probe hybridized to two bands from the *bz-m4* clone: a 2.9-kbp fragment and a 4.3-kbp fragment (data not shown). These data indicate the *Ds* transposon is inserted in the pMBzP17 segment; this was verified by further mapping and sequence analyses (shown below). (2) The pMBzP1 probe hybridized to two bands from *Pst*I digested EMBL3[bzm4]#5 DNA: 0.85-kbp fragment, which corresponds to the *Pst*I fragment at the 3′ end of the transcript in the *Bz-McC* allele; and a 2.9-kbp fragment. The 2.9-kbp *Pst*I fragment, which also hybridized to the probe pMBzP17, spans one end of the *Ds* insertion and includes a partial duplication of the flanking sequences near the 3′ end of *Bz* gene (see Figure 7B below). (3) The probe pMBzP30 hybridized to 4.8-kbp and 15.4-kbp *Bam*HI fragments from the *bz-m4* clone. The 4.8-kbp fragment is within the *Ds* insertion. The larger *Bam*HI fragment includes 6.2 kbp beginning in the right most *Ds* element, and 9.2 kbp from the right arm of the phage vector. The probe pMBzP30 also hybridized to a 1.6-kbp doublet in a *Pst*I digest of the *bz-m4* clone. (4) The probe pMBzPR5 hybridized to a single 1-kbp band in a *Pst*I/*Bam*HI double restriction digestion of the *bz-m4* clone. The *Bam*HI restriction site is in the polylinker at the end of the long arm of the lambda vector and marks one end of the recombinant insert in the phage.

Structure of the *Ds*-transposon in *bz-m4 derivative 6856*: The positions of *Ds* elements within *bz-m4* clone were also determined by Southern hybridization methods. A 2.6-kbp *Ds* clone, isolated from the genomic clone of the *sh-m5933* allele (DÖRING, TILLMAN and STARLINGER 1984), hybridized to two bands from a *Pst*I digest of the *bz-m4* clone DNA: A 2.9-kbp fragment and 4.3-kbp fragment (data not shown). These are the fragments which also hybridized to pMBzP17. These *Pst*I fragments are not contiguous (Figure 2), therefore the data indicate that there are two *Ds* elements within the *bz-m4 D6856* allele. The size of the *Ds*-containing insertion in *bz-m4* was determined as follows: (1) The combined size of these two

Ds hybridizing fragments (2.9 + 4.3 kbp) plus that of the *Pst*I fragment between them (1.6 kbp) is 8.8 kbp. (2) The size of the *Pst*I insert in the subclone pMBzP17 is 2.07 kbp. Therefore the size of entire insertion in *bz-m4 D6856* is the difference between (1) and (2) which is approximately 6.7 kbp. More extensive restriction mapping and Southern hybridizations, as well as sequence analyses confirmed these results.

The 2.6-kbp *Ds* clone, which was used as a probe, is part of the double *Ds* structure described by DÖRING, TILLMAN and STARLINGER (1984) and belongs to the class of *Ds* elements which are deletion derivatives of *Ac* (DÖRING and STARLINGER 1984). The *Ds* probe hybridized strongly to *bz-m4* clone DNA, even after high stringency washes (0.1 × SSC, 1 hr, 65°). This result suggests that the *Ds* elements in *bz-m4 D6856* are also in the class of *Ds* elements which are deletion derivatives of *Ac* elements. This was confirmed by partial sequence analyses of the *Ds* elements from the *bz-m4* clone (see below).

There is a *Bam*HI restriction site 0.18 kbp from one end of the unit *Ds* element described by DÖRING, TILLMAN and STARLINGER (1984). This was used as a marker initially to localize the rearrangement/breakpoints (Figure 2 labeled A and C) between the *Ds* elements and *Bz* DNA in the *bz-m4* clone. The positions of the other rearrangement/breakpoints (B and D) were tentatively assigned by restriction mapping and Southern hybridizations and confirmed by sequence analyses.

The 6.7-kbp insertion in the *bz-m4 D6856* allele is inserted ~300 bp upstream from a *Kpn*I site near what corresponds to the mRNA cap site of *Bz-McC* (FURTEK *et al.* 1988). The 6.7-kbp insertion has a structure similar to the class II type bacterial transposon (as defined in KLECKNER 1981): two *Ds* elements, 2 kbp in length, are at either end of the insert in direct orientation with respect to each other. These *Ds* elements flank a 2.75-kbp segment of DNA which is actually a duplication of *Bz* sequences flanking the 3′ end of the locus.

Genomic mapping: The restriction map of the *bz-m4 D6856* clone is essentially consistent with hybridization patterns observed on genomic Southern blots. Additional restriction sites, outside the boundaries of the *bz-m4* clone and *Bz-McC* subclones shown in Figure 2, were utilized in the genomic mapping experiments. Representative data are shown in Figure 3. The hybridization patterns of DNA from the *Bz-McC* allele and that from *bz-m4 D6856* are shown in adjacent lanes. (1) A 6.4-kbp *Bgl*II fragment begins 3 kbp upstream from the translation start site of *Bz-McC* and extends to a position approximately 2 kbp downstream of the polyadenylation sites (DEAN *et al.* 1986; FURTEK *et al.* 1988; RALSTON, ENGLISH and DOONER 1988). The pMBzP1 probe hybridized to a single 6.4-kbp *Bgl*II fragment from *Bz-McC* DNA (lane A) but hybridized strongly to a ~6.8 kbp *Bgl*II fragment and faintly to a ~6.6-kbp *Bgl*II fragment in the mutant. Assuming that the size estimates for the restriction map of the cloned DNA are more accurate, these data confirm that the insertion in *bz-m4* is 6.7 kbp. The difference in hybridization intensity between the *Bgl*II fragments can be explained by a difference in the length of the hybridizing regions in the two bands. (2) Comparison of the two genomic DNAs by restriction with *Hin*dIII (lanes E and F) and double digests of *Hin*dIII/*Kpn*I (lanes G and H) were in agreement with maps of the cloned DNAs.

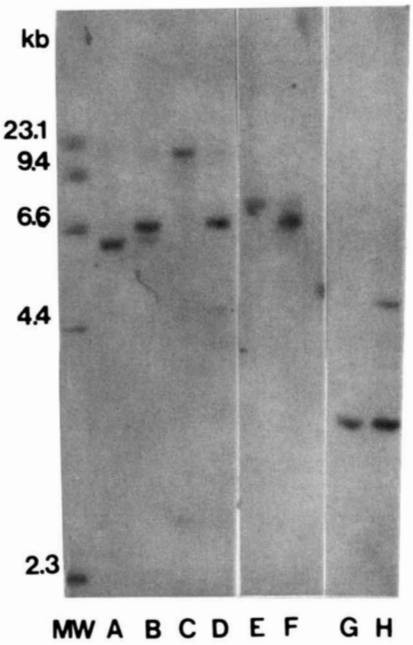

FIGURE 3.—Genomic Southern mapping of the *bz-m4 Derivative 6856* allele. Wild-type *Bz-McC* DNA and *bz-m4 Derivative 6856* DNA are in adjacent lanes. Lanes A and B, *Bgl*II digests; lanes C and D, *Bam*HI digests; lanes E and F, *Hin*dIII digests; lanes G and H, *Kpn*I and *Hin*dIII digests. The blot was probed with labeled pMBzP1 DNA (specific activity > 2 × 10^8 cpm/μg DNA).

In the *Bam*HI digests, the probe pMBzP1 hybridizes to a 16-kbp fragment of *Bz-McC* DNA (lane C) and two fragments of the *bz-m4* DNA (lane D): a 4.8-kbp fragment from within the transposon-like structure and a 6.7-kbp fragment. However, the predicted size of the second *Bam*HI fragment [derived from the genomic restriction map of the wild-type *Bz-McC* allele (FEDOROFF, FURTEK and NELSON 1984)] is 9.3 kbp. This result indicates that there must be an additional restriction polymorphism in the *bz-m4 D6856* allele somewhere beyond the right end of the EMBL3-[bzm4]#5 clone.

Excision of the 6.7-kbp transposon-like element: Genomic Southern hybridizations of *Kpn*I/*Bgl*II double digests of DNA from *Bz-McC*, *bz-m4 D6856* and a phenotypically wild-type revertant, *Bz′[m4 D6856]:1*, are shown in Figure 4. The pMBzP1 probe hybridized

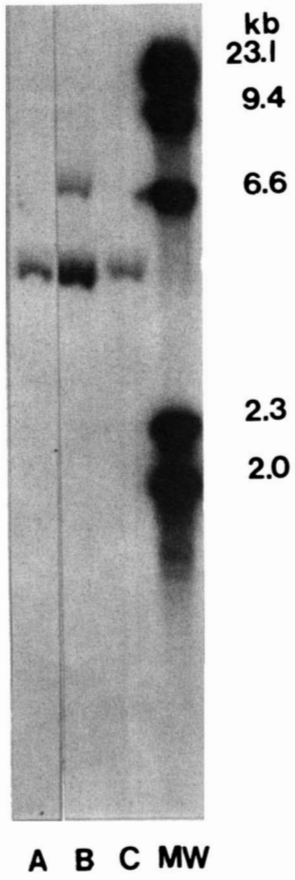

FIGURE 4.—Reversion of *bz-m4 Derivative 6856*. Genomic southern hybridization of *Bz-McC* (lane A), *bz-m4 Derivative 6856* (lane B) and *Bz'[m4 Derivative 6856]:1* (lane C) DNA *Kpn*I/*Bgl*II restriction digests. The plasmid pMBzP1 was used as a probe (specific activity $> 2 \times 10^8$ cpm/μg DNA).

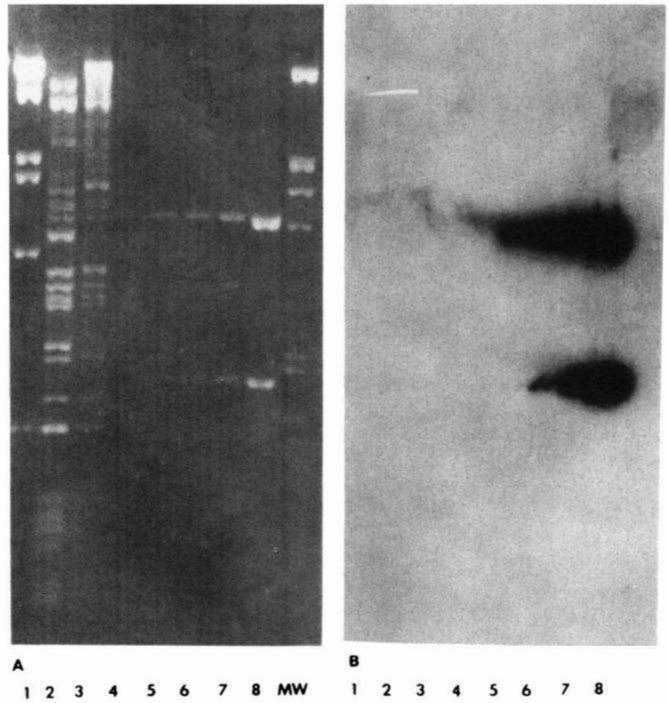

FIGURE 5.—Lack of homology between *sh* regulatory sequences and EMBL3[bzm4]#5 DNA. A, An ethidium-stained gel of EMBL3[bzm4]#5 and pSh2.9 DNA. EMBL3[bzm4]#5 DNA was digested with restriction endonucleases *Bam*HI and *Xho*I (lane 1), *Bam*HI and *Pst*I (lane 2), and *Pst*I (lane 3). Lane 4 is blank. Lanes 8 through 5 are a 20-fold dilution series of pSh 2.9 DNA which has been cut with *Pst*I. This restriction enzyme cuts once within the *sb* insert and also in the polylinker. Therefore both of the resulting fragments contain *sh* sequences. B, An autoradiogram of the Southern blot from A, probed with pSh2.9 (specific activity $> 8 \times 10^7$ cpm/μg DNA).

to 5.5-kbp and 3.6-kbp fragments of *bz-m4 D6856* DNA. The larger fragment contains part of the transposon-like *Ds* insertion. However, the probe hybridized to a single 3.6-kbp fragment in both the *Bz-McC* (lane A) and *Bz'[m4 D6856]:1* (lane C) DNAs. These results and more extensive genomic mapping (M. DOWE and A. S. KLEIN, manuscript in preparation) demonstrate that the duplication in the transposon-like structure (in the 5.5-kbp fragment) has been excised in the purple revertant and did not reinsert nearby on this chromatid.

***bz-m4 D6856* lacks *Sh* upstream sequences:** The pSh21.6 probe did not hybridize to the *bz-m4* clone (data not shown). A subclone, pSh 2.9, was constructed which contains 2.9 kbp of putative regulatory sequences for *Sh*. It extends from 2.9 kbp upstream of *Sh* to an *Eco*RI site 33 bp 5′ to the *Sh* CAAT box sequence. This *Sh* subclone was used to probe a Southern blot of *bz-m4* clone DNA (Figure 5). The ethidium bromide stained gel is shown in Figure 5A. Lanes 2 and 3 are digests of EMBL3[bzm4]#5 DNA. Lanes 5 thru 8 represent a 20-fold dilution series of pSh 2.9 DNA; lane 8 has the highest concentration of plasmid. The plasmid was cut with the restriction enzyme *Pst*I. There is a *Pst*I site within the *Sh* insert. Therefore restriction with *Pst*I produces two fragments (3.7 and 1.8 kbp) both of which contain *Sh* and vector sequences. The entire plasmid was nick translated and used to probe the blot. The autoradiogram of the Southern blot is shown in Figure 5B. There was no homology between the *Sh* probe and the *bz-m4 D6856* clone.

Site of the *Ds*-transposon insertion in *bz-m4 D6856*: The exact site of the 6.7-kbp insertion in the *bz* locus was determined by DNA sequencing. The insertion is 36 bases downstream of the major mRNA cap site in the untranslated leader of the wild-type *Bz-McC* allele (Figure 6). The characteristics 11-bp inverted terminal repeats of *Ds* elements are found at the ends of the insert as is the 8-bp duplication of the target sequence.

Sequence analyses at the four rearrangement breakpoints of *bz-m4 D6856*: The *bz-m4* clone has four *Ds/bz* rearrangement breakpoints: Two *bz/Ds* junctions at the ends of the 6.7-kbp transposon-like element and two internal *Ds/bz* junctions at either end of the duplicated *bz* sequence. The four rearrangement breakpoints are labeled alphabetically in

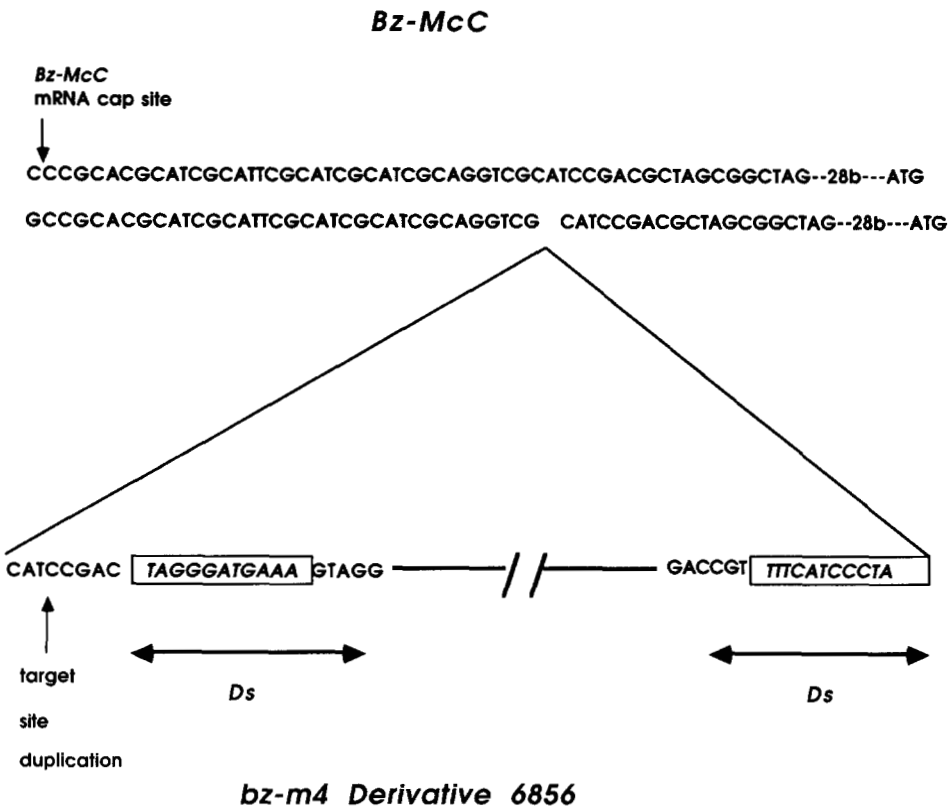

FIGURE 6.—Sequence at the insertion site of the *Ds* composite transposon in *bz-m4 Derivative 6856*. This corresponds to junctions A and D in Figure 2.

Figure 2. While the entire clone of *bz-m4 D6856* has not been sequenced, more extensive sequence comparisons to *Bz-McC* and the 2040 bp unit *Ds* element (DÖRING, TILLMAN and STARLINGER 1984) are given in Figure 7.

Junction A: In Figure 7A the sequence of the *bz-m4 D6856* allele, at the left-most rearrangement breakpoint, is compared to that of the *Bz-McC* allele (FURTEK *et al.* 1988) and the unit *Ds* element (DÖRING, TILLMAN and STARLINGER 1984). Arbitrary numbers were assigned to the base positions of the *bz-m4* allele because we have not determined the precise size of the insertion in *bz-m4* (*e.g.*, to the exact base pair). There were two polymorphisms in this *bz-m4* sequence relative to that of the *Bz-McC* allele. There is a three base insertion (CGG), immediately upstream from the TATAA box (at positions −36 to −34 of the wild-type allele), in the *Bz-McC* allele relative to the sequence of *bz-m4 D6856*. A 3-bp insertion could be the relic of a visitation by a transposable element such as *Spm* at that site (SAEDLER and NEVERS 1985; ZACK, FERL and HANNAH 1986). The sequence of an independently isolated *Bz-McC* allele (RALSTON, ENGLISH and DOONER 1988) is identical to the *bz-m4* clone at this position. There is also a 2-base change [GC—→ CG] in the *bz-m4 D6856* sequence 1 bp before the wild-type cap site. Since the promoter for *bz-m4 D6856* has not yet been identified, it is not clear whether these sequence differences would have any impact on the transcription of the mutant allele.

The *Bz/Ds* rearrangement breakpoint is marked in bold type in Figure 7A. Eight bases [CATCCGAC] of *bz* sequence at the junction are also found at the other end of the 6.7-kbp insertion (junction D, Figure 6) and represent the target site duplication caused by the *Ac/Ds* family of transposable elements upon integration. The *Ds* sequences at junction A are identical to the published sequence for the 2040-bp unit *Ds* element for at least 175 bp (DÖRING, TILLMAN and STARLINGER 1984). The insertion site of the *Ds*-transposon within *bz-m4 D6856* is adjacent to two 10 bp direct repeats [CGCATCGCAT; shadowed]. There is a 6-bp repeat [TCGCAT], which is also present 4 times in this region. DÖRING and STARLINGER (1984) have noted that the insertion sites of *Ac* and *Ds* elements are frequently characterized by the presence of short (6–10 bp) direct duplications of DNA sequence. This region of the *bz* locus has a high density of closely spaced direct repeats (FURTEK *et al.* 1988).

Junction B: This *Ds/Bz* junction is at one end of the duplicated 3′ flanking sequences from *Bz-McC* (Figure 2). The *Ds* element is truncated, missing 121 bp of its terminus including the 11 bp inverted repeat (Figure 7B). At the *Ds/Bz* junction, there is an extra G base; this may represent an insertion or a relic of the deletion event which removed the terminus of the *Ds* element. The *Bz* sequence in the duplication begins at a position equivalent to 401 bp downstream from

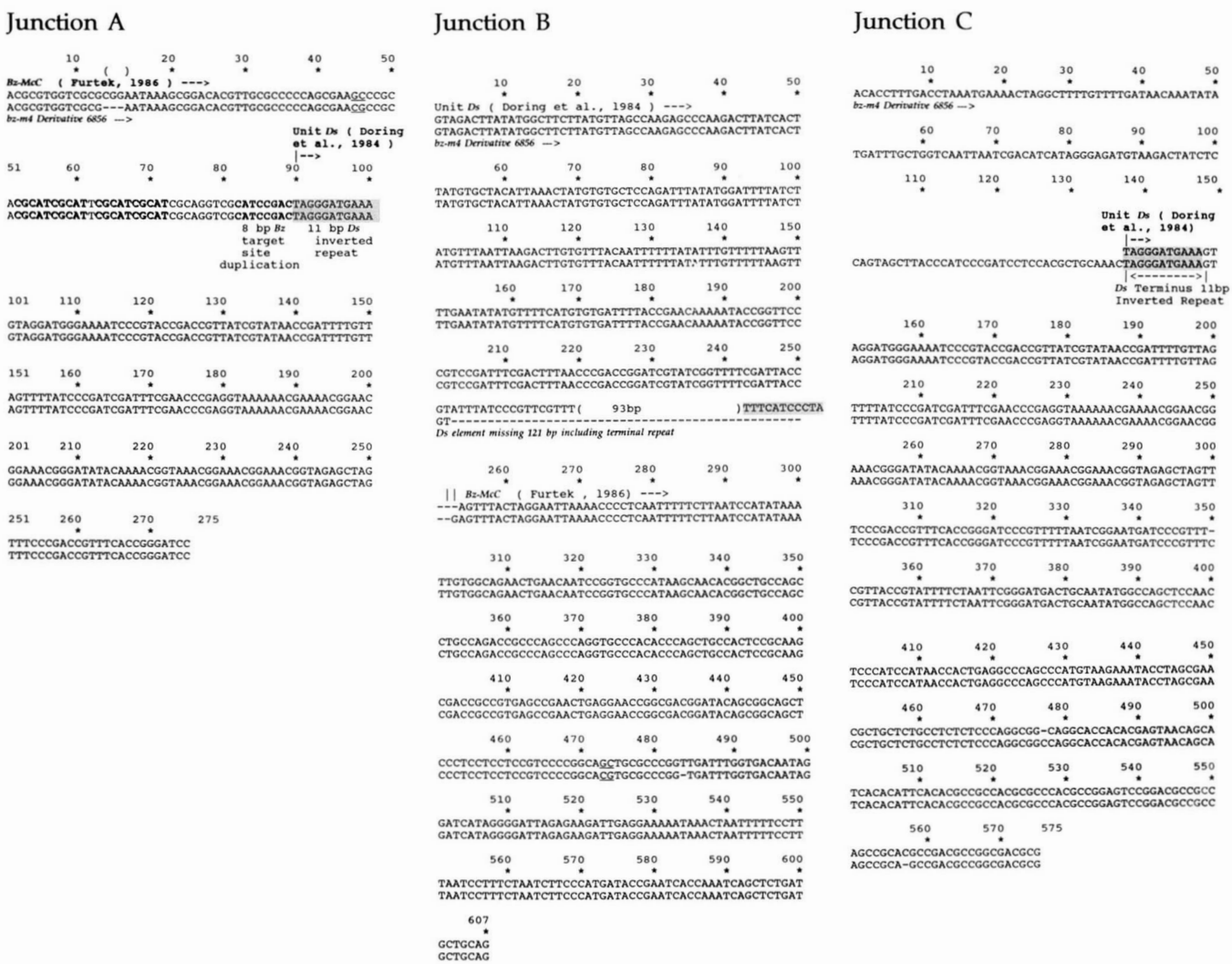

FIGURE 7.—Sequences at *Ds/bz* junctions A, B, and C in *bz-m4 Derivative 6856*.

the UGA termination codon. This is 3′ to the five polyadenylation sites which have been identified for wild-type *Bz* alleles (DEAN *et al.* 1986; FURTEK *et al.* 1988). There are two other small sequence polymorphisms which distinguish the duplication from the wild-type allele; these are marked in the Figure 7B.

Junction C: The sequence at the *bz/Ds* junction at the end of the duplicated segment is shown in Figure 7C. The restriction maps of the *bz-m4* clone and the *Bz-McC* clone correspond for this segment (up to the beginning of the *Ds* element). However, there are no sequence data available for the wild-type *Bz-McC* allele in this region (which would correspond to a position ~3.1 kbp downstream from the UGA stop codon of the gene). The complete 11-bp inverted repeat of the *Ds* element is present at junction C. Sequencing was extended approximately 440 bases into the *Ds* element to a *Mlu*I restriction site. There were several other single base changes at junction C in the *Ds* as compared to published sequence for the 2040-bp unit *Ds* element (DÖRING, TILLMAN and STARLINGER 1984).

Junction D: This is the *Ds/bz* junction at the other end of the 6.7-kbp insert. The sequence at the actual rearrangement breakpoint is shown as part of Figure 6 (discussed above). A 658-bp *Acc*I/*Kpn*I subclone of EMBL3[bzm4]#5 spanning this junction, was sequenced. The 341 bases of *Ds* element in this *Acc*I/*Kpn*I fragment are identical to the corresponding sequence of the unit *Ds* (DÖRING, TILLMAN and STARLINGER 1984). The remainder of this fragment (bases 342–658) is identical in sequence to that of the *Bz-McC* allele (FURTEK *et al.* 1988).

Expression of *bz-m4 D6856*: The expression of the *bz-m4 D6856* allele was examined by enzyme activity and Northern analyses. Previous work of DOONER and NELSON (1977a) showed that the maximum expression of the *bz-m4* allele in developing kernels occurred between 22 and 26 DAP. Expression of the wild-type allele increased substantially from 26 to 32 DAP.

UFGT levels were measured for the same seed lots as were used for poly(A)$^+$ mRNA isolation. UFGT was threefold less abundant in the *bz-m4 D6856* ker-

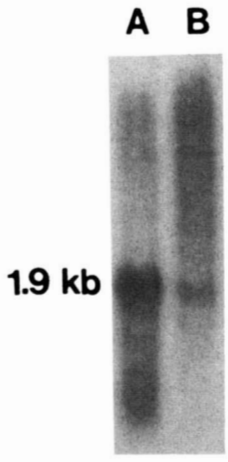

FIGURE 8.—Northern analysis of *bzm4 D6856* transcripts. Lane A, *Bz-McC* mRNA from 32 DAP immature kernels. Lane B, *bz-m4 D6856* mRNA from 26 DAP kernels.

nels at 26 DAP as compared to the 32 DAP *Bz-McC* kernels (respectively 42 nmol isoquercitrin produced/hr/endosperm equivalent *vs.* 126 nmol isoquercitrin produced/hr/endosperm equivalent). The probe pMBzP1 hybridized to a single transcript from both *BzMcC* RNA and *bzm4D6856* poly(A)$^+$ RNA (Figure 8). The *bz-m4* transcript was approximately 3–5-fold less abundant than the wild-type transcripts. The level of *bz-m4* mRNA was roughly proportional to the level of enzyme activity as compared to those of the wild-type allele. Both mutant and wild-type transcripts were approximately 1.9 kb in length.

DISCUSSION

The *Ds* mutation in *bz-m4 D6856* alters both the temporal and tissue-specific expression of the *bz* locus in the developing maize kernel. To understand how these changes in gene expression are controlled, we have isolated the mutant allele and have characterized its structure. The mutation is caused by a 6.7-kbp insert into the *Bz-McC* mRNA leader sequence. The insertion is similar to a class II type bacterial transposon (as defined in KLECKNER 1981): two *Ds* elements, 2 kbp in length, flank a 2.75-kbp segment of DNA. This intervening DNA is actually a duplication of 3′ flanking sequences from the *Bz* locus. The duplication begins at a position just past the multiple polyadenylation site for *Bz* (DEAN *et al.* 1988; FURTEK *et al.* 1988; RALSTON, ENGLISH and DOONER 1988). The two *Ds* elements have identical restriction maps, with the exception that in one of the *Ds* elements, there is a 121-bp deletion which includes the *Ds* inverted terminal repeat.

Origin of the transposon-like insert in *bz-m4 D6856*: The physical structure of the transposon-like insertion in *bz-m4 D6856* and the fact that the segment between the *Ds* elements is actually a duplication of the 3′ flanking sequences from the end *Bz* gene have further implications with respect to the origin of the *bz-m4* alleles. These observations suggest, as part of a working model, that a single 2-kbp *Ds* element was inserted near the 3′ end of the *Bz* gene in the original mutant allele (Figure 1). In support of this hypothesis, preliminary data from restriction digests of genomic DNA, obtained from one of the progenitor *bz-m4* alleles, indicate that the pMBzP1 probe hybridizes to a single fragment.

A combination of transposition and recombination may be invoked to account for subsequent generation of the complex transposon in *bz-m4 D6856*. One possible sequence of events is illustrated in Figure 9: (1) The 2-kbp *Ds* element excised from the original *bz-m4* allele, causing the allele on this chromatid to revert to a *Bz′-m* form. This *Ds* inserted into an unreplicated portion of the chromosome. This sequence of events is similar to that which GREENBLATT and BRINK (1962) proposed to account for two sectors arising from the transposition of the transposable element *Mp* from the P^{vv} allele (*variegated pericarp*). (2) Excision and transposition were followed immediately by an unequal crossover between *Ds* elements on sister chromatids. These events generated a partial duplication of the 3′ end of the *Bz* locus, flanked by 2-kbp *Ds* elements. Deletion of the (inner) terminal inverted repeat of one of the flanking *Ds* may have occurred during an abortive transposition. Deletions adjacent to and including part of a transposable element have been characterized in other systems (DOONER 1986; TAYLOR and WALBOT 1985; MARTIN, MACKAY and CARPENTER 1988). Finally this complex transposon was excised and inserted in the *Bz* mRNA leader sequence region generating the allele *bz-m4 D6856*. Aspects of this model are being tested.

Another complex *Ds* insertion has been observed at the *sh* locus. The *Ds*-mutation *sh-m5933* (COURAGE-TEBBE *et al.* 1983; DÖRING and STARLINGER 1984) has a 30-kbp insert at the locus which destroys gene function. The insert also has a composite transposon-like structure; in this instance a 4-kbp "double" *Ds* is at one end of the insertion and a 3-kbp *Ds* is at the other end. The 3-kbp *Ds* element is actually an internal deletion of the "double" *Ds* structure (COURAGE-TEBBE *et al.* 1983). The two *Ds* elements at either end of the insertion are in inverted orientation with respect to one another. The approximately 23 kbp of DNA, between the *Ds* elements, is of unknown origin but from within the maize genome. The ends of this sequence are partially duplicated (just inside the *Ds* elements). A second copy of part of this 30-kbp transposon and adjacent *sh* sequences are also found on chromosome 9S in the *sh-m5933* stock.

Reversion of *bz-m4 D6856*: The transposon-like structure in *bz-m4 D6856* effectively has three *Ac/Ds* termini which could act as targets for the putative *Ac*

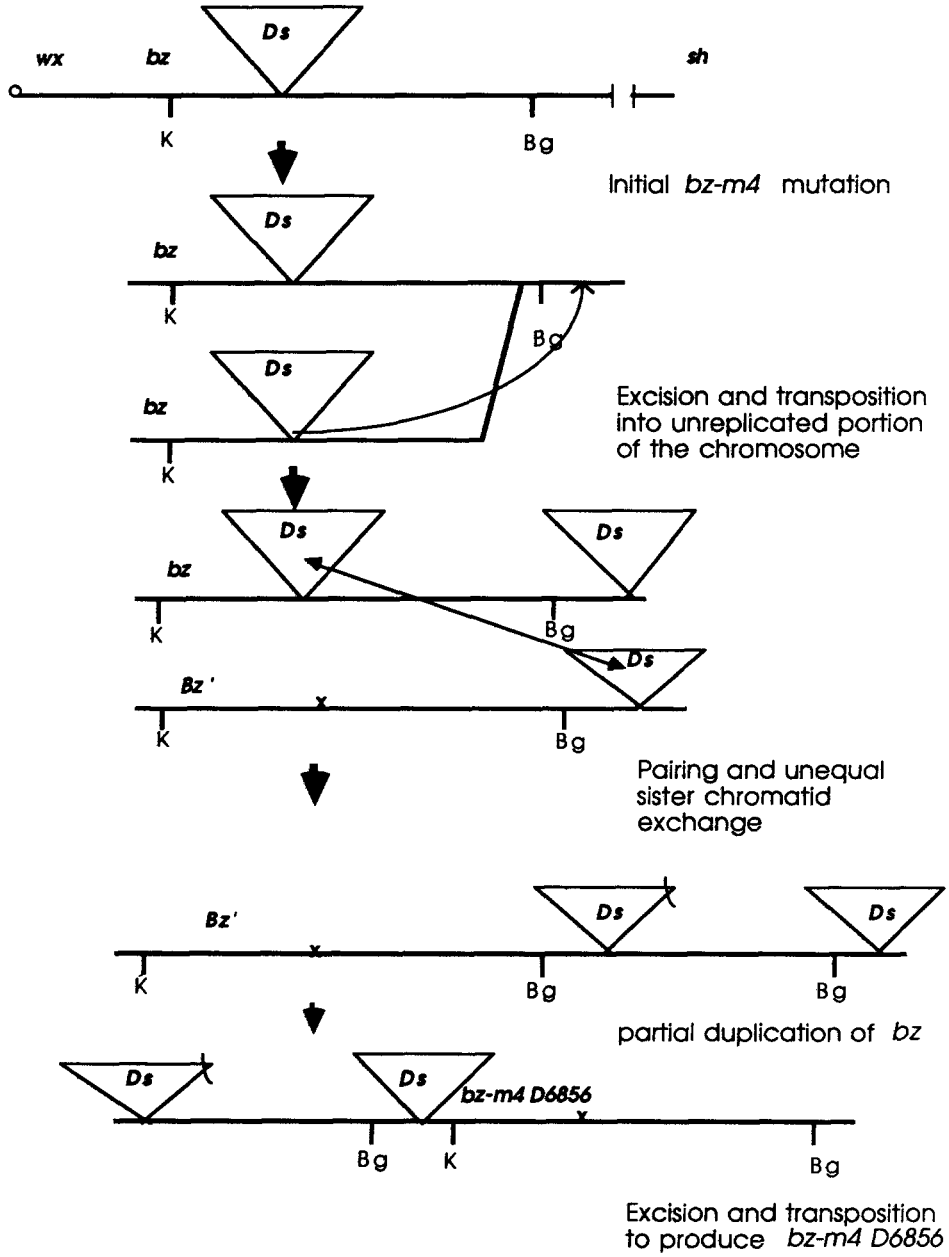

FIGURE 9.—A model for the formation of the complex transposon in *bz-m4 D6856*. The *Bg*III (=Bg) restriction site is in 3′ flanking sequences for *Bz* and *Kpn*I (=K) is within the transcriptional unit for *Bz*.

transposase. Several purple *Bz′* alleles, including *Bz′[m4 D6856]:1*, reverted with the excision of the duplication within the transposon-like structure (M. DOWE and A. S. KLEIN, manuscript in preparation). One of the interesting features of the *bz-m4 D6856* allele is that not only does it revert to a *Bz′* (purple) phenotype at high frequency, but in later generations some of these *Bz′* alleles mutate to a recessive *bz′-m* phenotype (B. MCCLINTOCK personal communication) (Figure 1). Similar phenomena have been observed by BRINK and WILLIAMS (1973).

Deletion of *sh* in the origin of *bz-m4 D6856*: The first *bz-m4* allele arose concomitantly with a deletion of part of the region between *sh* and *bz* (MCCLINTOCK 1965). Furthermore, *bz-m4* stocks are deleted for a substantial portion of the *sh* coding region (BURR and BURR 1982; SHELDON *et al.* 1983). These facts and the similarities in the temporal and tissue-specific nature of expression of *Sh* and of *bz-m4 Derivative 6856* led GERATS and co-workers (1983) to propose that the regulatory regions of *sh* had been fused to the *bz* locus in *bz-m4*. DOONER and co-workers (1985) and DOONER (1986) have demonstrated that the direction of transcription of *Bz* is from the centromere outward. DOONER suggested that this would place the *sh* promoter and upstream regulatory sequences on the 3′ end of the *bz* locus in the a putative *sh-bz* fusion product. On the other hand, transposable elements have been shown to mobilize chromosomal segments and also to invert the orientation of these segments (reviewed in KLECKNER 1981; B. MCCLINTOCK personal communication). We have investigated the fusion hypothesis, first proposed by GERATS *et al.* (1983), by hybridizing cloned DNA of the *Sh* locus to blots

containing the cloned *bz-m4 D6856* allele. There is no cross-hybridization between (1) a large (18 kbp) genomic clone of *Sh* and the *bz-m4* clone (data not shown) and (2) the upstream regulatory regions of *Sh* (pSh2.9) and the *bz-m4* clone. Any *Sh* sequences on chromosome *9* adjacent to *bz-m4 D6856* must lie outside the boundaries of the EMBL3[bzm4]#5 and some distance [>8.2 kbp 5′ or >2.0 kbp 3′] from the putative transcription unit in *bz-m4 D6856*.

The *Sh* and *Bz* loci on chromosome *9S* are separated by 2 map units. The size of the intrachromosomal region between *sh* and *bz* in *bz-m4 Derivative 6856* has been estimated to be 0.2 map unit (DOONER 1981). DOONER (1986) has determined that the *bz* locus is a hotspot for recombination in the maize genome; in the vicinity of *Bz* the approximate physical size of a map (recombinational) unit is 14 kbp. If this region of enhanced recombination extends distal to *bz*, then any remnants of *sh* sequences (*e.g.*, 3′ *sh* flanking DNA) in *bz-m4 D6856* could be within a few kbp of the 3′ end of the putative transcriptional unit in *bz-m4 D6856*. The end of the deletion breakpoint with respect to the *sh* locus has not been determined.

Control of gene expression in *bz-m4 D6856*: The point of insertion of the *Ds*-transposon in *bz-m4 D6856* corresponds to the +36 position of the *Bz-McC* transcript (Figure 6), within the 5′ untranslated leader region of the gene (FURTEK *et al.* 1988). The insertion therefore places the wild-type *Bz* promoter nearly 7 kbp upstream from the putative transcriptional unit in *bz-m4 Derivative 6856*. The mutant allele is clearly functional in certain tissues in the plant (DOONER and NELSON 1977b; DOONER 1981; KUHN and KLEIN 1987); therefore it must be transcribed and translated to produce UFGT enzyme.

The size and position of the *Ds*-transposon within the allele pose a problem in predicting how *bz-m4 D6856* is transcribed. At least two simple hypotheses are possible. Transcription of *bz-m4 D6856* could initiate at the major wild-type *Bz* promoter and extend through the entire transposon-like structure and through the *Bz* locus. The transposon would later be excised from the mRNA. Several *Mu1* insertions at the *alcohol dehydrogenase 1* locus (BENNETZEN *et al.* 1984; ROWLAND and STROMMER 1985, VAYDA and FREELING 1986), a *Ds1* element inserted at the same locus (SUTTON *et al.* 1984), and *Ds* and *Ac* insertions at the *waxy* locus (WESSLER, BARON and VARAGONA 1987) are spliced from pre-mRNAs for those genes. These insertions have variable effects on the level of transcription and/or expression of the genes in question. Alternatively the *bz-m4 D6856* transcript could initiate from another promoter within the 6.7-kbp insert. This new promoter could be within one of the *Ds* elements and would confer the altered pattern of tissue-specificity on the mutant allele. A TATA-like element (TATTTA) is found 29 bp upstream from the right most inverted terminal repeat in the *Ds*-transposon in *bz-m4 D6856*. If this served as a promoter element the resulting transcript from *bz-m4 D6856* would be approximately 1.9 kb.

In Northern analyses of poly(A^+) RNA from immature kernels of *bz-m4 D6856* only a single transcript of approximately 1.9 kb hybridized to the *Bz* probe. The fact that the mRNA from *bz-m4 D6856* is similar in size to that of the *Bz-McC* mRNA does not allow us to distinguish between a splicing mechanism or an alternative promoter for expression of the mutant allele, although these results favor the promoter hypothesis.

The molecular analysis of the structure of the *Ds*-controlled *bz-m4 D6856* allele has provided insights regarding the control of expression of this unusual temporal and tissue-specific regulatory mutation. The mutant allele resulted from the insertion of a novel 6.7-kbp composite transposon in the 5′ end of the locus, between the wild-type mRNA cap site and the start of translation of the gene product. Promoter and regulatory sequences from the deleted *sh* locus have not been fused to the *bz* transcriptional unit. Understanding how the insertion of the *Ds* flanked transposon qualitatively alters gene expression awaits identification of the promoter for *bz-m4 D6856* transcription. Significantly, the structure of the *Ds*-transposon in *bz-m4* illustrates at a molecular level how transposable elements facilitate duplication of a chromosomal segment and mobilization of this DNA to new sites in the genome.

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Note added in proof: These sequence data will apear in the EMBL/GENBANK/DDBJ Nucleotide Sequence Databases under the accession numbers X12951, 12970, and X12969, bronze.

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