Molecular Analysis of Multiple *Mutator*-Derived Alleles of the *Bronze* Locus of Maize

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ABSTRACT

Very few mutations derived from *Mutator* maize lines have been studied at the molecular level. The variety of *Mu* elements that can induce mutations, the relative frequency of mutant induction by insertion of a given class of *Mu* elements or by a *Mu*-induced genomic rearrangement, a possible intragenic insertion site specificity, and the molecular nature of reversion events are all unknown in the *Mutator* system. To address these questions, we have isolated several partially or fully inactivated *bronze* alleles from *Mutator* maize lines and structurally characterized them by gel blot hybridization of genomic DNA. The mutations were induced in three parental *Bronze* alleles which differ by polymorphisms flanking the coding region. Each of the 14 inactivated *bronze* mutants characterized was found to contain an insert which cross-hybridized with the transposable element *Mu1*. Detailed maps of 11 of these alleles revealed a 1.4-kb insert with restriction sites characteristic of *Mu1*. These *Mu1* insertions were found dispersed throughout both of the *Bronze* exons and in either orientation relative to *Bronze* transcription. Stable and somatically unstable (mutable) mutant alleles differed with respect to the covalent modification of restriction sites within the inserted *Mu1* element. Several germinal revertants of one mutable *bronze* allele, *bzMum4*, were isolated. These all were associated with excision of the *Mu1* element from the affected locus.

A uniquely high frequency and low specificity of *de novo* mutant induction is the definitional characteristic of the *Mutator* transposable element system (ROBERTSON 1978, 1985; ROBERTSON and MASCIA 1981; BENNETZEN *et al.* 1987a). The numerous mutations isolated from *Mutator* stocks, particularly those alleles which show phenotypic reversion events in somatic sectors (*i.e.*, mutability), have proven useful for studies of *Mutator* regulation (ROBERTSON 1981; BENNETZEN 1985; WALBOT, CHANDLER and TAYLOR 1985; CHANDLER and WALBOT 1986; WALBOT 1986; BENNETZEN, BROWN and SPRINGER 1988). *Mutator*-associated mutations have been isolated in genes which determine such phenomena as disease resistance, seed dormancy, embryo development, seed starch and/or protein deposition, and the synthesis of anthocyanin, carotenoid or chlorophyll pigments (ROBERTSON 1978, 1981, 1985; BENNETZEN 1985; WALBOT, CHANDLER and TAYLOR 1985; WALBOT, BRIGGS and CHANDLER 1986; BENNETZEN, BROWN and SPRINGER 1988; BENNETZEN *et al.* 1988). In order to understand these important physiological processes, several laboratories have set out to molecularly clone *Mutator*-derived alleles by the process of transposon tagging, with some initial success (O'REILLY *et al.* 1985; MCLAUGHLIN and WALBOT 1987; MCCARTY and CARSON, 1988). Implicit in these tagging experiments is the assumption that the transposable element most closely associated with *Mutator* activity, *Mu1* (BENNETZEN 1984; BENNETZEN *et al.* 1984, 1987b; ALLEMAN and FREELING 1986), will be inserted at the mutated locus. This general expectation has been borne out by the observation that *Mu1*, or a 350 bp larger relative of *Mu1* called *Mu1.7* (TAYLOR and WALBOT 1987), has been found inserted in each of seven independent mutations at *A1*, *Adh1*, *Bronze*, and *Bronze2* (STROMMER *et al.* 1982; BENNETZEN *et al.* 1984; O'REILLY *et al.* 1985; TAYLOR, CHANDLER and WALBOT 1986; MCLAUGHLIN and WALBOT 1987). However, all maize lines contain numerous sequences homologous only to the ends of *Mu1* (CHANDLER, RIVIN and WALBOT 1986). Two of these elements, *Mu3* and *Mu8*, have been cloned as insertions in *Mutator*-derived *Adh1* (OISHI and FREELING 1988) and *Waxy* alleles (VARAGONA, FLEENOR and WESSLER 1988), respectively. These results demonstrate that other classes of elements can be involved in mutant induction in a *Mutator* background.

Insertion site specificity is the rule, rather than the exception, for all transposable element systems investigated (CALOS and MILLER 1980; GREENBLATT 1984; MORI *et al.* 1988). *Mutator* is unusual in its ability to induce mutations at all loci investigated, and at

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roughly similar frequencies (ROBERTSON 1985). The high mutagenic rate compared to the low copy number (10–70) of *Mu1* transposable elements in *Mutator* lines led to the prediction that *Mu* elements would have either a high transposition frequency, a gene specific insertion preference, or both (BENNETZEN 1984). The latter prediction has been substantiated by subsequent studies (ALLEMAN and FREELING 1986; BENNETZEN *et al.* 1987b; BENNETZEN, BROWN and SPRINGER 1988). The initial observation that three independent *Mu1* insertions of *Adh1* were all in the same intron (STROMMER *et al.* 1982; BENNETZEN *et al.* 1984), and that all seven independent *Mu1* or *Mu1.7* insertions in the *A1*, *Adh1*, *Bronze* or *Bronze2* loci have been in the same orientation relative to transcription of the mutated allele (BENNETZEN *et al.* 1984; O'REILLY *et al.* 1985; TAYLOR, CHANDLER and WALBOT 1986; MCLAUGHLIN and WALBOT 1987), suggested that *Mu1* may exhibit some microspecificity for insertion within a given gene.

This report describes the results of gel blot hybridization analysis of 14 independent *bronze* mutations isolated from *Mutator* maize lines. We determine the nature, position and orientation of the element inserted in *Bronze* in 11 of these mutant alleles. In addition, we present gel blot hybridization data which indicate that germinal reversion of one *Mutator*-derived *bronze* mutation, *bzMum4*, is associated with excision of the *Mu1* element.

MATERIALS AND METHODS

Isolation of *bronze* mutations from *Mutator* maize lines: The *bronze* mutations *bzMum1-bzMum8*, *bzMus1-bzMus3*, and *bzMus5-bzMus10* were isolated in a maize plot containing detasseled plants of the genotype *c sh bz wx/c sh bz wx* and purple *Mutator* plants of the genotype *C Sh Bz Wx/C Sh Bz Wx*. The 334,392 kernels from the detasseled plants were screened for bronze or mutable bronze kernels. Mutants *bzMum9-bzMum11* were identified on ears derived from self-pollination of distantly related purple *Mutator* stocks that segregated a 3:1 ratio of purple to bronze or bronze mutable kernels.

Isolation of germinal phenotypic revertants of *bzMum4*: Plants hemizygous for bronze were constructed by crossing to a *bronze-shrunken* deletion line (BENNETZEN 1985). Hemizygous *bzMum4* stocks were self-pollinated or crossed to siblings or the *bronze-shrunken* deletion line. To minimize the possibility of contaminating pollen, hand pollinations were performed in a plot isolated both spatially and temporally from any other maize lines. Plants derived from putative revertant (*i.e.*, purple) kernels were self-pollinated or outcrossed to the *bronze-shrunken* deletion line to confirm the revertant phenotype.

Hybridization experiments: Maize genomic DNA was prepared according to the method of SHURE, WESSLER and FEDOROFF (1983). Restriction enzymes were purchased from Bethesda Research Labs, New England Biolabs or Boehringer Mannheim Biochemicals and used in a two-fold excess under conditions specified by the manufacturer. Genomic DNA (6–8 μg) was digested with the appropriate restriction enzyme(s) and resolved on 0.7% agarose gels. *Hin*dIII digested lambda DNA was used as a molecular weight marker. The DNA was transferred to a nitrocellulose or nylon (Micron Separations, Inc.) filter which was hybridized and washed as described previously (BENNETZEN 1984). Radiolabeled DNA was prepared using the hexamer labeling procedure (FEINBERG and VOGELSTEIN 1983). Unincorporated nucleotides were removed by Sephadex G-75 gel filtration chromatography. Plasmids pMBZP1 and pMBZPR5, containing the cloned *Bronze* gene (FEDOROFF, FURTEK and NELSON 1984), were provided by Drs. D. FURTEK and O. NELSON. Probe P1 is an 842-bp *Pst*I fragment and probe PR5 is a 1.8-kb *Pst*I/*Eco*RI fragment of the cloned *Bronze* allele (FURTEK 1986; see Figure 3). The purified 1.0-kb *Tth*111I fragment of *Mu1* was used as a probe to detect genomic *Mu1* elements (BARKER *et al.* 1984; BENNETZEN 1984).

RESULTS

Isolation and mapping of *Mutator*-associated *bronze* mutations: Seventeen *bronze* mutations were isolated in a screen of 334,392 maize kernels. Three additional *bronze* mutant alleles (*bzMum9*, *bzMum10*, *bzMum11*) were obtained from self-pollinated ears of different outcross progeny in a purple *Mutator* background. Eleven of these mutant alleles were mutable, exhibiting the small revertant sectors characteristic of *Mutator*-induced mutations (ROBERTSON 1981; WALBOT, BRIGGS and CHANDLER 1986). These were termed "*Mum*" alleles. One of these mutants, *bzMum7*, yielded pale purple kernels with dark purple spots. Nine of the *bronze* inactivations isolated were somatically stable (no revertant sectors) and were termed "*Mus*" alleles. Mutations *bzMus1-bzMus3*, *bzMus5-bzMus7* and *bzMus9-bzMus10* could be activated to a low frequency of mutability by crossing to an active *Mutator* stock, while *bzMus8* was not amenable to such a *trans*-activation. We decided to prepare detailed restriction maps of *bzMum1-bzMum11*, *bzMus1*, *bzMus2* and *bzMus8*. Each of these mutants and their parental *Bronze* alleles were mapped by genomic gel blot hybridizations using the 1.8-kb *Pst*I/*Eco*RI fragment of *Bronze* (PR5) as a probe for the 5′ end of the *Bronze* locus and the 842-bp *Pst*I fragment of *Bronze* (P1) as a probe for the 3′ end of the *Bronze* locus (FURTEK 1986; RALSTON, ENGLISH and DOONER 1988). The results indicated that 11 of the mutants were induced in two different parental *Bronze* alleles, each of which is distinct from the first *Bronze* alleles to be cloned and sequenced (FEDOROFF, FURTEK and NELSON 1984; FURTEK 1986; RALSTON, ENGLISH and DOONER 1988). Almost all restriction sites within the transcribed region of *Bronze* were conserved. Despite extensive mapping of all the *Bronze* alleles from the different inbreds known to have contributed to the stocks from which these mutant alleles were selected, we could not identify a progenitor allele for *bzMum6*, *bzMus8* and *bzMum10*. Mapping of these alleles indicated, in each case, an insertion at the *Bronze* locus which cross-hybridized with the central *Tth*111I frag-

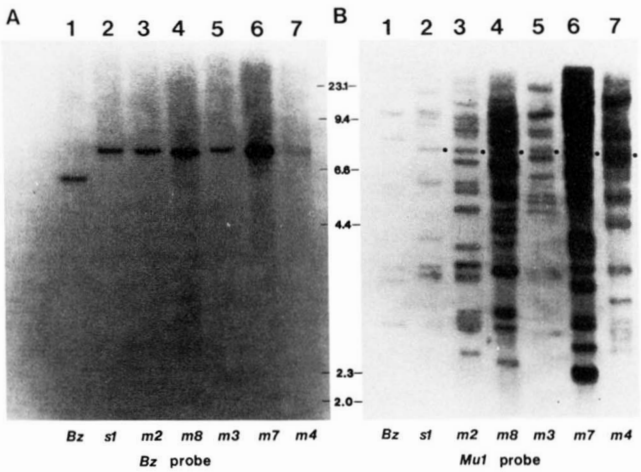

FIGURE 1.—Presence of 1.4-kb insertions in several Mutator-derived bronze mutations. A filter replica of agarose gel resolved DNA digested with *Bgl*II was hybridized to labeled (A) P1 *Bronze* DNA or (B) *Mu1* DNA. Lane 1, Progenitor *Bronze* allele; 2, *bzMus1*; 3, *bzMum2*, 4, *bzMum8*; 5, *bzMum3*; 6, *bzMum7*; 7, *bzMum4*. The size of the bands in A are 7.5 kb in lanes 2–7 and 6.1 kb in lane 1. The closed circles in B mark the *Mu1* bands that comigrate with the mutant *bronze* alleles in A. The migration positions and sizes (in kb) of molecular weight standards are indicated.

ment of *Mu1* (data not shown). Since the progenitor that had contributed these alleles was not found in our search, we did not analyze these mutations any further.

Gel blot analysis of genomic DNA from parental and mutant lines, digested with a restriction enzyme that cleaves outside of *Bronze* and *Mu1*, indicated that the bands which hybridized to a *Bronze* probe in the mutant lines were about 1.4 kb larger than the band which hybridized in the progenitor line (Figure 1A). Rehybridization of this and analogous filters with a probe homologous to the central region of *Mu1* revealed the presence of multiple bands in all of the mutant genomes, but with only one band common to all of the mutant alleles. This band comigrated with the *Bronze* restriction fragment (Figure 1B).

Location and orientation of the insertions: The 1376-bp *Mu1* element specifies a single *Bst*EII site at bp 735 and a unique *Not*I site at bp 315 (BARKER *et al.* 1984). Localization of the *Bst*EII and *Not*I sites in a *bzMu* allele defines the location and orientation of the *Mu1* insert. However, *Bz1* also contains single *Bst*EII and *Not*I sites 47 bp apart within the coding region (FURTEK 1986; RALSTON, ENGLISH and DOONER 1988). As insertions were found on both sides of the parental *Bst*EII and *Not*I sites, two different *Bronze* probes were employed for the analysis. One probe, P1, was used to map insertions 3′ (relative to the direction of *Bronze* transcription (DOONER *et al.* 1985)) to the parental *Bst*EII and *Not*I sites while probe PR5 was used for those insertions 5′ to the *Bronze Bst*EII and *Not*I sites. Since the *Bst*EII and *Not*I sites within the parental *Bronze* allele are only separated by 47 bp, they are readily distinguished from the sites in *Mu1* (Figure 2). The location and orientation of *bzMum1*, *bzMum3*, *bzMum4*, *bzMum5*, *bzMum7*, *bzMum8*, *bzMum9* and *bzMum11* were determined by digesting genomic DNA with *Bgl*II and *Bst*EII and, separately, with *Bgl*II and *Not*I (*e.g.*, Figure 3). However, *Not*I is a methylation sensitive enzyme and only partially digests the *Mu1* element in *bzMus1*, *bzMus2* and *bzMum2*. We confirmed the *Mu1* orientation inferred from *Not*I partial digestion for *bzMus1* by using the methylation requiring enzyme *Apy*I (RAZIN *et al.* 1980). There are three *Apy*I sites in *Mu1* at positions 1016, 1074 and 1080 (CHANDLER, TALBERT and RAYMOND 1988). When the internal *Not*I sites are methylated, then the internal *Apy*I sites are also generally methylated (BENNETZEN 1987; BENNETZEN, BROWN and SPRINGER 1988). Although there are *Bronze Apy*I sites between the *Bgl*II site and the *Apy*I sites contained in the *bzMus1 Mu1*-like element, these sites are in actively transcribed regions of the genome and are not methylated (BENNETZEN, BROWN and SPRINGER 1988). Therefore, a comparison of the *Bgl*II/*Bst*EII and *Bgl*II/*Apy*I bands of the *bzMus1* allele detected by gel blot hybridization indicated the orientation of this internally methylated *Mu1* element (data not shown).

Isolation and analysis of germinal revertants of *bzMum4*: We isolated several putative germinal revertants of *bzMum4* at a frequency of 1 in 12,000. One revertant was discovered as a 3-kernel ear sector on an otherwise fully bronze and bronze mutable ear. Results of self-pollination and test crosses of this and several other putative revertant plants were consistent with these being true phenotypic reversion events, as were molecular analyses employing flanking restriction fragment length polymorphism markers. The genomic restriction maps of these revertants indicated excision of the *Mu1*-like element (Figure 4 and data not shown).

DISCUSSION

We have isolated bronze mutations from a *Mutator* stock at a rate of about 1/20,000. This frequency, and the fact that just under half of these mutant alleles were somatically unstable, is in agreement with the standard mutagenic properties of an active *Mutator* stock (ROBERTSON 1978). Since all but one of the nine stable *bronze* alleles identified in this screen could be induced to somatic mutability by crossing to an active *Mutator* stock, it appears that *Mutator* does not commonly cause viable deletions encompassing the *Bronze* locus as an initial mutagenic event.

Our molecular analyses indicate that the factor responsible for most mutations in *Mutator* plants is *Mu1* or a *Mu1*-like element. This is important for gene tagging experiments since the transposon is required

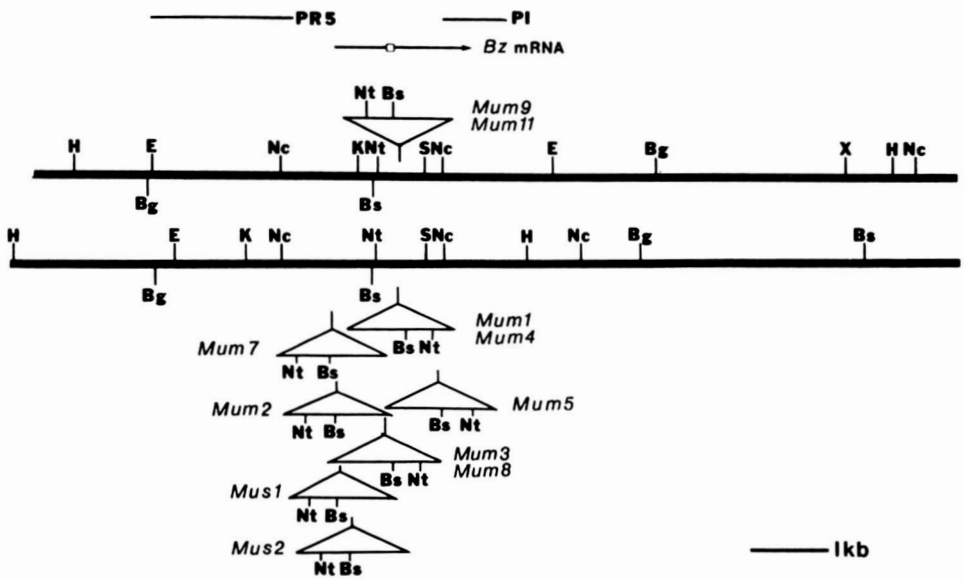

FIGURE 2.—Restriction maps of *Bronze* alleles and *Mu1* insertions. Abbreviations are: Bg, *Bgl*II; Bs, *Bst*EII; H, *Hin*dIII; K, *Kpn*I; Nc, *Nco*I; Nt, *Not*I; E, *Eco*RI; S, *Sst*I; X, *Xba*I. The relative positions of the probes used (P1 and PR5) and of the *Bronze* mRNA [determined for the *Bronze* gene isolated by FEDOROFF and coworkers (1984)] are indicated. The position and extent of the *Bronze* intron is represented by an open box.

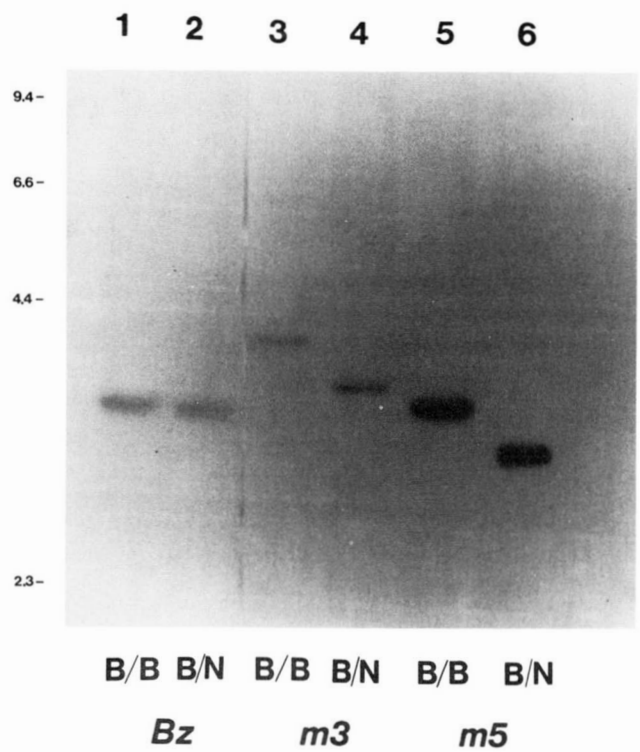

FIGURE 3.—*Mu1* insertion position and orientation in *bzMum3* and *bzMum5*. A filter replica of agarose gel resolved DNA digested with *Bgl*II and *Bst*EII [lanes 1, 3 and 5 (B/B)] or *Bgl*II and *Not*I [lanes 2, 4 and 6 (B/N)] was hybridized to labeled P1 DNA. Lanes 1 and 2, Progenitor *Bronze* allele; lanes 3 and 4, *bzMum3*; lanes 5 and 6, *bzMum5*. The migration positions and sizes (in kb) of molecular weight markers are indicated.

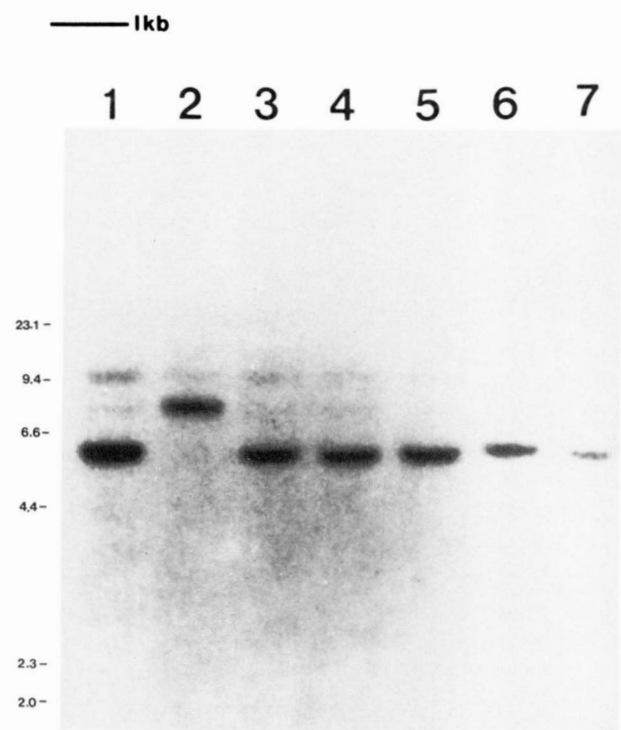

FIGURE 4.—Excision of the *Mu1* element from *bzMum4* in revertant plants. A filter replica of agarose gel resolved DNA digested with *Bgl*II and *Eco*RI was hybridized with labelled P1 *Bronze* DNA. Lane 1, Progenitor *Bronze* allele of *bzMum4*; 2, *bzMum4*; 3, revertant 1; 4, revertant 2; 5, revertant 3A; 6, revertant 3B; 7, revertant 4. Revertants 3A and 3B represent DNA derived from two of the "three-kernel-sector" revertants. The relative migration positions and sizes (in kb) of molecular weight standards are indicated. The bands in lanes 6 and 7 are less intense due to inefficient filter binding or hybridization of these DNAs. The faint bands visible in lanes 1–4 are due to a second region of the maize genome that weakly cross-hybridizes with any *Bronze* probe.

as a hybridization probe for the isolation of a tagged gene. *Mu1* has been employed successfully as a tag in the molecular cloning of the maize genes *A1* (O'REILLY *et al.* 1985), *Bz2* (MCLAUGHLIN and WALBOT 1987) and *Vp1* (MCCARTY and CARSON 1988). In our analyses, no *Mu1.7* element insertions were observed at *Bronze*, although a *Mu1.7* element has been found inserted in one *bronze* allele from another *Mutator* line (TAYLOR, CHANDLER and WALBOT 1986). This is not surprising, since the number of *Mu1* elements is quite large compared to the number of *Mu1.7* elements in most *Mutator* maize lines (BENNETZEN 1984; ALLEMAN and FREELING 1986; TAYLOR and WALBOT 1987; BENNETZEN *et al.* 1987b) including the

stocks from which we derived these *bronze* alleles (data not shown). Other classes of *Mu* elements, with termini like those of *Mu1* and *Mu1.7* but with nonhomologous internal sequences, are found at combined copy numbers of over forty per diploid genome in all maize lines examined (CHANDLER *et al.* 1988; CHANDLER, RIVIN and WALBOT 1986; J. L. BENNETZEN, unpublished observation), but were not associated with any of the *bzMu* alleles that we analyzed. This demonstrates that these elements, abundant in both *Mutator* and standard maize stocks, transpose rarely into *Bronze* in response to the *Mutator* transposase. Since transposition by a replicative mechanism, like that observed for *Mu1* (ALLEMAN and FREELING 1986; BENNETZEN *et al.* 1987b), leads to an increase in genomic element copy number, this may explain the consistent amplification of *Mu1* and the rare amplification of other *Mu* elements in *Mutator* stocks. Since these classes of *Mu* elements differ primarily in their internal sequences, the preferential transposition of *Mu1* could be due to an enhanced recognition of *Mu1* termini mediated by the internal regions of the element. Alternatively, *Mu* elements other than *Mu1* or *Mu1.7* may not show the gene-specific insertion preference associated with *Mu1/Mu1.7* (BENNETZEN, BROWN and SPRINGER 1988) or may be trapped in a form (*e.g.*, modified) or chromosomal location that is unavailable to transposase.

Mu1 insertions were found in at least six distinct sites in a 2-kb region of the *Bronze* locus. This demonstrates a lack of strong *Mu1* insertion specificity and may partly explain why *Mutator* induces mutations at similar frequencies in many genes (ROBERTSON 1985). This property may give *Mutator* an advantage over other systems for gene tagging since most transposable elements show significant insertion site preferences (CALOS and MILLER 1980; GREENBLATT 1984; MORI *et al.* 1988). The pairs of mutant alleles *bzMum1/bzMum4*, *bzMum9/bzMum11*, and *bzMum3/bzMum8* were indistinguishable at our level of detection (less than 50 bp). This may be either an indication of some degree of intragenic *Mu1* insertion specificity or it may be interpreted as evidence that these pairs of alleles were not independently derived. The alleles *bzMum9* and *bzMum11* arose in different fields and in unrelated *Mutator* stocks and, hence, must be due to independent events. However, *bzMum1/bzMum4* and *bzMum3/bzMum8* were identified in the same wind-pollinated isolation plot. Since *Mutator* commonly induces mutations in the ear prior to meiosis (ROBERTSON 1980, 1981), it seems likely that a tassel sector containing several mutant pollen derived from the same premeiotic event might also occur. Detailed sequence analysis of genomic clones of *bzMum1*, *bzMum4*, *bzMum9*, *bzMum11*, *bzMum3* and *bzMum8* will be required to absolutely determine the similarity of insertion sites in these six alleles.

All *Mu1* insertions in the *bronze* alleles analyzed were very near or within the two *Bronze* exons. *Bronze* expression at 1% of wild-type levels yields a kernel that is fully purple in color (SCHIEFELBEIN 1987). Hence, many *Mu1* insertions that partially inactivated *Bronze* would have gone undetected in our mutant screen. Insertions of *Mu1* in the first intron of *Adh1*, for instance, led to about 40% of the normal level of *Adh1* expression (STROMMER *et al.* 1982; BENNETZEN *et al.* 1984). Mutation *bzMum7* is our only partial inactivation allele of *Bronze* and also contains our most 5′ *Mu1* insertion. This insertion may be in the promoter or transcript leader. Most of the other insertions, which are clearly or probably in exons, yield a null, somatically mutable phenotype. This indicates that, in these alleles, *Mu1* does not serve as an intron (KIM *et al.* 1987; WESSLER, BARAN and VARAGONA 1987) to allow efficient reconstruction of a functional *Bronze* mRNA. These data also demonstrate that the presumed somatic excision of *Mu1* from several different coding regions of *Bronze* can yield a somewhat functional protein. The two stable *bzMus* alleles analyzed in detail were both found to have internal modification of sites within *Mu1*. The lack of somatic reversion in these stable alleles, then, is probably an outcome of an inability to excise *Mu1* rather than a low likelihood that such an excision would yield a functional *Bronze* allele.

The first six *Mutator*-derived mutations analyzed were found to contain *Mu1* or *Mu1.7* insertions in the same orientation relative to transcription of the affected locus (BENNETZEN *et al.* 1984; O'REILLY *et al.* 1985; TAYLOR, CHANDLER and WALBOT 1986; MCLAUGHLIN and WALBOT 1987). Of the eleven *Mutator*-derived *bronze* mutants we have studied in detail, six contain *Mu1* insertions in the same orientation as previously described in the six cases analyzed at *A1*, *Adh1*, *Bronze*, and *Bronze2*. The other five *bronze* alleles in our study, however, contained a *Mu1* insertion in the opposite orientation. Hence, although *Mu1* may have an orientation bias for insertion into some maize genes, this bias is not absolute.

We have isolated several germinal revertants of *bzMum4* at a frequency of about 10^{-4}. One of these revertants was found as a three-kernel ear sector and, hence, is unlikely to be due to a pollen contamination. This three-kernel-sector revertant was correlated with excision of the *Mu1* element from *bronze*. This indicates that a *Mu1* element can excise from DNA at a reasonable frequency and that these events can occur premeiotically, as do many *Mutator*-induced insertion events (ROBERTSON 1980). Since *Mu1* appears to transpose primarily via a replicative, nonexcisive mechanism, it is not clear whether an excised *Mu1*

element would be lost or would reinsert into chromosomal DNA. An excised *Mu1* might be one source of the circular *Mu1*-related elements detected by SUNDARESAN and FREELING (1987) in the developing tassel of active *Mutator* stocks.

Mu1 insertion results in a 9-bp duplication of host DNA (BENNETZEN *et al.* 1984). Exact excision of the transposable element would maintain the reading frame if insertion occurred in an exon and so may not have a deleterious effect on protein function. In addition, 1% of the normal level of *Bronze* enzymatic activity yields a wild-type phenotype (SCHIEFELBEIN 1987). Thus, an in-frame excision of *Mu1* could lead routinely to phenotypic reversion at *Bronze*. Current work is underway to sequence the appropriate regions of *bzMum4* revertants in order to determine whether an insertion "legacy" has been left behind by the departed element (SACHS *et al.* 1983). In this regard, the *bzMum4* "revertant" identified as a three kernel sector does produce a kernel with a pale purple phenotype.

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