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Two Maize Genes Are Each Targeted Predominantly by Distinct Classes of *Mu* Elements

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Manuscript received May 1, 1993
Accepted for publication August 12, 1993

ABSTRACT

The Mutator transposable element system of maize has been used to isolate mutations at many different genes. Six different classes of *Mu* transposable elements have been identified. An important question is whether particular classes of *Mu* elements insert into different genes at equivalent frequencies. To begin to address this question, we used a small number of closely related Mutator plants to generate multiple independent mutations at two different genes. The overall mutation frequency was similar for the two genes. We then determined what types of *Mu* elements inserted into the genes. We found that each of the genes was preferentially targeted by a different class of *Mu* element, even when the two genes were mutated in the same plant. Possible explanations for these findings are discussed. These results have important implications for cloning *Mu*-tagged genes as other genes may also be resistant or susceptible to the insertion of particular classes of *Mu* elements.

MUTATOR stocks of maize have a 20–50-fold higher mutation frequency than non-Mutator stocks (ROBERTSON 1978). This high mutation frequency is caused by the movement of a heterogeneous family of transposable elements referred to as *Mu* elements. All *Mu* elements share similar ~200-bp terminal inverted repeats [reviewed in WALBOT (1991)]. Different *Mu* elements are grouped into classes according to the sequences internal to their inverted repeats. When elements share similar internal sequences, they are defined as belonging to the same class of *Mu* elements. Different classes of *Mu* elements have completely unrelated internal sequences.

A total of six classes of *Mu* elements have now been characterized [reviewed in CHANDLER and HARDEMAN (1992)]. The first class of *Mu* elements includes *Mu1*, the first *Mu* element identified (BARKER *et al.* 1984; BENNETZEN *et al.* 1984), *Mu2*, a 1.7-kb element also referred to as *Mu1.7* (TAYLOR and WALBOT 1987) and *Mu1-del*, a 1.0-kb element (HARDEMAN and CHANDLER 1989). The second and third classes of elements are defined by *Mu3* (CHEN *et al.* 1987; OISHI and FREELING 1987) and *Mu4* (TALBERT, PATTERSON and CHANDLER 1989). The fourth class of *Mu* elements includes *Mu6*, *Mu7* (V. CHANDLER, unpublished data; CHANDLER and HARDEMAN 1992), and *rcy:Mu7* (SCHNABLE, PETERSON and SAEDLER 1989). *Mu8* defines the fifth class of *Mu* elements (FLEENOR *et al.* 1990). The sixth class includes *Mu5* (TALBERT, PATTERSON and CHANDLER 1989), *MuA1* (QIN and ELLINGBOE 1990), *MuA2* (QIN, ROBERTSON and ELLINGBOE 1991), *dMuR*, *MuR1* (CHOMET *et al.* 1991) and *Mu9* (HERSHBERGER, WARREN and WALBOT 1991). *MuR1*, which is very similar if not identical to *MuA2* and *Mu9*, has been shown to be the genetically defined regulator of Mutator activity (CHOMET *et al.* 1991; QIN, ROBERTSON and ELLINGBOE 1991; HERSHBERGER, WARREN and WALBOT 1991). The regulatory element has been renamed *MuDR*, in honor of DON ROBERTSON who discovered this system.

Because of the high mutation frequency in Mutator stocks, and the ability to obtain mutants at many different loci, Mutator elements have been widely used as insertional tags for cloning genes. However, the heterogeneity of *Mu* elements can complicate the cloning of *Mu*-induced alleles since there is no simple genetic test to identify which class of elements is inserted in the gene of interest. The *Mu1* class of elements has been found most often in *Mu*-induced alleles. For example, of the 58 molecularly characterized *Mu*-induced alleles reviewed by WALBOT (1991), 44 (76%) contained an insert belonging to the *Mu1* class (28, *Mu1*; 12, *Mu1-del*; and 4, *Mu2*); 2 (3.5%) belonged to the *Mu3* class; 2 (3.5%) belonged to the *Mu7* class, 7 (12%) to the *Mu8* class; 2 (3.5%) to the *MuDR* class, and 1 is an unnamed *Mu* insertion. This high *Mu1* bias has been proposed to be caused by the average higher copy number and higher transposition frequencies of *Mu1* elements compared to other *Mu* element types in most Mutator stocks (BENNETZEN *et al.* 1993). A few mutant alleles isolated from Mutator stocks have contained non-*Mu* element insertions, including two belonging to the *Spm* family of elements (PATTERSON *et al.* 1991) and one whose identity is unknown (MCCARTY *et al.* 1989).

It must be emphasized that although *Mu1* has been

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the most frequently isolated insertion in *Mu*-induced mutations overall, the frequency at which it is found in different loci can vary greatly. There are four genes for which the nature of multiple Mutator-induced insertions has been determined. The *Mu1* class of elements are clearly found at a high frequency in *bz1*. For example, in one study, all 11 *Mu*-induced *bz1* mutants were found to contain a *Mu1* insertion (BROWN, ROBERTSON and BENNETZEN 1989). In another study, all 8 *Mu*-induced *bz1* mutants were found to contain only *Mu1* or *Mu1-del* insertions (HARDEMAN and CHANDLER 1989). Of 22 *Mu*-induced *bz1* mutants characterized (BROWN, ROBERTSON and BENNETZEN 1989; HARDEMAN and CHANDLER 1989; TAYLOR, CHANDLER and WALBOT 1986), only one contains a different class of element from *Mu1* (SCHNABLE, PETERSON and SAEDLER, 1989). In contrast, among four *Mu*-induced normal derivatives of the *Kn1-0* mutation, one was found to contain a *Mu1* insertion, one a *Mu8* insertion, one a *Mu7* insertion and the remaining derivative contained a non-*Mu* insertion (VEIT *et al.* 1990). *Mu1* was found more often in *adh1* and *bz2*, but additional elements have also been found at these genes. Of four reported *adh1* mutants, three contained *Mu1* elements and one contained *Mu3* (BENNETZEN *et al.* 1984; FREELING and BENNET 1985; CHEN *et al.* 1987). For *bz2*, three insertions were *Mu1* and one was a *MuDR* element (MCLAUGHLIN and WALBOT 1987; NASH, LUEHRSEN and WALBOT 1990; HERSHBERGER, WARREN and WALBOT 1991). The data collected so far could be significantly biased by the large number of *bz1* alleles examined relative to other genes, especially if *Mu1* has an increased specificity for *bz1* relative to other elements as discussed in BENNETZEN *et al.* (1993).

Several hypotheses could explain the variation in the frequency with which different *Mu* elements are found inserted at different loci. First, it is possible that different *Mu* elements are more or less active in different Mutator stocks. The experiments mentioned above were performed in different laboratories, with the Mutator stocks in different genetic backgrounds. Second, different *Mu* elements may be targeted to different genes, perhaps due to a specific sequence found in the gene or a difference in the chromatin structure surrounding the gene. Third, as most investigators screen for mutable alleles, various *Mu* element types may be under- or overrepresented. For example, as proposed by BENNETZEN *et al.* (1993), an element that could serve as an efficient intron might insert without causing a detectable phenotype. Finally, if *Mu* elements move preferentially to linked locations, then the location of the gene, with respect to the *Mu* elements linked to it in the progenitor stock, would affect which *Mu* element would insert into that gene. Although *Ac* elements are known to preferentially move to linked locations [reviewed in FEDOROFF (1989)], this remains an open question for *Mu* elements. These results raise several important questions. Are some genes better targets than other genes for *Mu* elements in general? Are particular genes preferred targets for certain classes of *Mu* elements?

To begin to address these questions, we used a small number of Mutator plants from similar backgrounds, to generate multiple independent mutations at two different genes, *bz1* and *sh1*. We then determined whether insertions were present in these mutant alleles and if so, what type. Since the same stocks were used to isolate both sets of mutations, any difference in the types of insertions found in the two genes should not be due to major differences in genetic background. Furthermore, as the *bz1* and *sh1* genes reside two map units apart on chromosome 9, both would be linked to the same *Mu* elements in the progenitor stock. Finally, by isolating multiple mutations at two different genes, we could compare the overall mutation frequency of these genes and determine what classes of *Mu* elements are inserted into the two genes.

MATERIALS AND METHODS

Stocks: The purple aleurone *Bz1 Sh1* Mutator stock was obtained from D. ROBERTSON, Iowa State University, and details of its stock construction were previously described (HARDEMAN and CHANDLER 1989). The tester stock containing the recessive *bz1 sh1* alleles is a W23/K55 hybrid and was obtained from M. G. NEUFFER, University of Missouri. The maize nomenclature used in this manuscript is according to the latest maize nomenclature committee recommendations. The name and symbol of a gene locus is represented in lower case italics (*bronze1*, *bz1*). When a mutant allele is recessive, it is designated by a lower case italicized symbol followed by a hyphen and allele designation (*bz1-A326*). Dominant alleles are designated with the first letter of the symbol capitalized (*Bz1*).

Isolation of mutations: The isolation and initial characterization of the *bz1* mutants was previously described as mutations isolated from a high copy number *Mu1* stock (HARDEMAN and CHANDLER 1989). A total of 650 *Mu*; *Bz1 Sh1/Bz1 Sh1* plants were crossed as female by *bz1 sh1/bz1 sh1* tester pollen, resulting in 587 ears that were scored for mutants. A total of ~76,300 kernels were scored, estimated by weighing the kernels, and dividing this number by the average weight of the kernels, as determined by weighing several samples of 100 kernels.

DNA samples: The *Mu1* plasmid, pMJ9, was obtained from J. BENNETZEN (BENNETZEN *et al.* 1984) and the *Mu1* internal fragment used as a probe, A/B5, is the 650-bp *Ava*I/*Bst*NI fragment from pMJ9 (CHANDLER, RIVIN and WALBOT 1986). The *Mu2* plasmid, p*Mu1.7*, was obtained from L. TAYLOR, and the *Mu2*-specific probe was prepared as described (TAYLOR, CHANDLER and WALBOT 1986). The *Mu3* plasmid, pKO121, was obtained from K. OISHI and the *Mu3* internal fragment used as a probe is the ~1.0-kb *Xba*I/*Hind*III fragment from pKO121 (OISHI and FREELING 1987). The *Mu4* internal fragment used as a probe was the ~650-bp *Eco*RI/*Tth*III-1 fragment from p6R6-680 (TALBERT, PATTERSON and CHANDLER 1989). The *Mu6* and *Mu7* internal fragments used were the ~360-bp *Eco*RI/*Ava*I fragment of *Mu6* and the ~160-bp *Eco*RI/*Bst*XI fragment of

Mu7 (V. CHANDLER, unpublished results). The *Mu8* plasmid, p713, was obtained from S. WESSLER and the *Mu8* internal fragment used as a probe was the ~730-bp *PstI/SalI* fragment from p713 (FLEENOR *et al.* 1990). The *MuDR*-specific probes were the Mu* probe (~1.3-kb *EcoRI/BamHI* fragment of *dMuR*) and the ~700-bp *BamHI/HindIII* fragment of *dMuR* (CHOMET *et al.* 1991). The *bz1* probe was a *PstI* subclone obtained from D. FURTEK and O. NELSON (FEDOROFF, FURTEK and NELSON 1984; FURTEK 1986). Two of the *sh1* probes, indicated in Figure 7, were prepared from the *sh1* plasmid p17.6, obtained from C. HANNAH (SHELDON *et al.* 1983) as follows: *sh1*-B is the ~1.2-kb *BglII* fragment, and *sh1*-C is the ~1.8-kb *SacI* fragment. The *sh1*-A probe is an ~800-bp *PstI/SacI* fragment of the *sh1* gene prepared from an ~8-kb *PstI* fragment of the *sh1-A83* allele (CHOMET *et al.* 1991).

Southern blot analysis: Maize DNA was isolated from leaf tissue as described by DELLAPORTA, WOOD and HICKS (1983), and digested with restriction enzymes for 2 hr (conditions according to suppliers). Approximately 3 μg of each maize DNA sample were loaded per lane, electrophoresed, and blotted as previously described (CHANDLER, RIVIN and WALBOT 1986), except that MSI nylon membrane (Fisher) was used. All restriction fragments used as hybridization probes were purified from low melting-point agarose (FMC) gels and labeled by random hexamer priming (FEINBERG and VOGELSTEIN 1983).

RESULTS

Isolation of mutants: A maize stock of Mutator parentage that contained a typical number of *Mu1*-like elements (~20–60), was used to isolate mutations in *bz1* and *sh1*. The *bz1* and *sh1* genes are located 2 map units apart on chromosome *9*. Ears from the Mutator plants were crossed by pollen from *bz1 sh1* tester plants as described in MATERIALS AND METHODS. The F_1 progeny from these crosses were screened for *bz1* and *sh1* mutations by examining the phenotype of the kernels. The *bz1* gene encodes UDPglucose: flavonol 3-*O*-glucosyl transferase, an enzyme involved in anthocyanin biosynthesis (LARSON and COE 1977; DOONER and NELSON 1977; DOONER 1983). The *sh1* gene encodes sucrose synthase, an enzyme that catalyzes the interconversion of sucrose and UDP-glucose plus fructose (CHOUREY and NELSON 1976). If no mutation occurred, the F_1 kernels would be plump with purple aleurones. Stable *bz1* mutant kernels would be plump with bronze aleurones, and unstable *bz1* mutant kernels would be plump and have bronze aleurones with purple revertant sectors. Stable or unstable mutations at *sh1* would result in shrunken purple kernels, as small revertant sectors do not visibly change the phenotype.

A total of 11 putative *bz1* mutations (10 with an unstable and 1 with a stable phenotype), and a total of 18 putative *sh1* mutations were isolated. The putative mutants were planted and crossed by a *bz1 sh1* homozygous tester stock to determine if the mutations were heritable. Four of the unstable *bz1* mutants survived and transmitted the mutant phenotype to their progeny. Of the remaining seven putative mutants, four could not be tested because no progeny were produced, two produced wild-type progeny, and one was not transmissible as only the *bz1 sh1* tester chromosome was inherited by progeny. Eight *sh1* mutants survived and transmitted the mutant phenotype to their progeny. Of the remaining 10 putative mutants, 5 could not be tested as no progeny were produced and 5 produced only wild-type progeny. Table 1 summarizes these data. The estimated frequency of mutations recovered at *bz1* and *sh1* was 8×10^{-5} and 1×10^{-4}, respectively (Table 1). The transmission of wild-type progeny by several putative mutants could be due to nonconcordance between the embryo and endosperm caused by *Mu* element instability or by mistaken scoring.

TABLE 1

Number of *bz1* and *sh1* mutants isolated

Loci	Mutants		Estimated mutation Frequency[b]
	Isolated	Transmitted[a]	
bz1	11	4	8×10^{-5}
sh1	18	8	1×10^{-4}

A total of 587 ears containing ~76,300 kernels were scored for bronze or shrunken kernels.

[a] Of the putative *bz1* mutants isolated, four either did not germinate or failed to produce progeny, two produced only wild-type progeny, and one, which was originally reported as having transmitted a mutant phenotype (HARDEMAN and CHANDLER 1989), upon further outcrossing transmitted only the chromosome containing the *bz1 sh1* tester alleles to progeny. Of the putative *sh1* mutants isolated, five either did not germinate or failed to produce progeny and five produced only wild-type progeny.

[b] The mutation frequencies were estimated as follows:

$$\frac{\text{no. mutants}}{\text{no. putative mutants tested}} \times \text{total putative mutants} \times \frac{1}{\text{total kernels scored}}.$$

All of the mutations were recovered as single mutant kernels on otherwise non-mutant ears. Two of the Mutator plants produced both a *bz1* and a *sh1* mutation. The *bz1-A326* mutant and the *sh1-A344* mutant were derived from different ears of the same Mutator plant. Similarly, the *bz1-A328* mutant and the *sh1-A345* mutant were derived from different ears of the same Mutator plant.

Molecular analysis of the *bz1* mutants: Two of the *bz1* mutations (*bz1-A48* and *bz1-A52*) were previously examined using Southern blot analyses and both appeared to contain *Mu1*-related insertions (HARDEMAN and CHANDLER 1989). This conclusion was based on the size of the elements, ~1.4-kb, and the presence of a single *BstEII* and a single *NotI* restriction enzyme site. Although several known *Mu* elements (*Mu1*, *Mu5* and *Mu8*) are ~1.4 kb, only *Mu1* contains both *BstEII* and *NotI* restriction enzyme sites. In addition, we found that the unique *bz1*-hybridizing sequences in these two alleles also hybridize to a *Mu1* internal probe. Thus, the insertions in the *bz1-A48* and *bz1-*

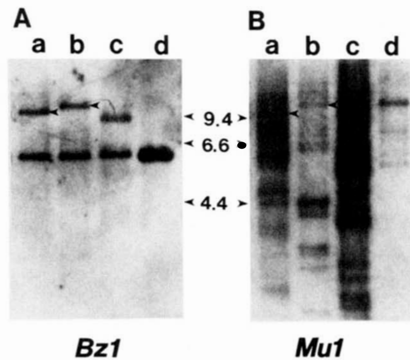

FIGURE 1.—DNA blot analysis of *bz1* mutants *bz1-A326* and *bz1-A328*. (A) Southern blot of leaf DNA samples (~3 μg) digested with *Bgl*II and hybridized with the *bz1* probe P1, indicated in Figure 2. Lane a, *bz1-A326/bz1*; lane b, *bz1-A328/bz1*; lane c, *Bz1/bz1* (wild-type sibling of *bz1-A326*); lane d, *bz1* tester. (B) The same blot hybridized with the *Mu1* internal probe. The arrowheads indicate the altered *bz1* restriction fragment observed in each mutant relative to the progenitor *Bz1* fragment. The migration of *Hin*dIII digested λ DNA, used as a size standard, is indicated in kilobase pairs (kb).

A52 mutants are most likely *Mu1*-related elements (HARDEMAN and CHANDLER 1989).

To determine what type of insertions were responsible for the unstable mutant phenotype of the two remaining *bz1* mutations, *bz1-A326* and *bz1-A328*, DNA was prepared from progeny of the mutants and examined by Southern analyses using *bz1* and *Mu1* probes. An example of one such Southern blot hybridized with the *bz1* probe is shown in Figure 1A. The *bz1-A326* mutant contains a unique ~10.3-kb *Bgl*II restriction fragment (lane a) that is ~1.4 kb larger than the ~8.9-kb *Bgl*II restriction fragment in the progenitor allele (lane c). Further experiments revealed that the insertion in *bz1-A326* contained both *Bst*EII and *Not*I restriction enzyme sites (data not shown). In addition, the *bz1*-hybridizing fragment from *bz1-A326* is the same size as a fragment that hybridizes with the *Mu1*-internal probe (compare lane a in Figure 1, A and B), suggesting that these two fragments may be the same. As there are a large number of *Mu1*-hybridizing fragments in this stock, it is possible that the *Mu1*-hybridizing fragment is merely comigrating with the *bz1*-hybridizing fragment in this mutant. However, the combination of the presence of *Mu1*-diagnostic restriction sites and the comigration of a fragment hybridizing to *bz1* and *Mu1* probes suggest that the insertion in *bz1-A326* is a *Mu1*-related element.

The *bz1-A328* mutant contains a unique *Bgl*II restriction fragment of ~11.3 kb (lane b) that is ~2.4 kb larger than the progenitor allele (lane c). This finding is consistent with an insertion of ~2.4 kb that contains no *Bgl*II sites. Digests with two other restriction enzymes were also consistent with an insert size of ~2.4 kb (data not shown). As can be seen by comparing lane b in Figure 1, A and B, the *bz1* hybridizing fragment in this mutant also appears to be the same size as a fragment that hybridizes with the *Mu1*-internal probe. However, with this digest, a *Mu1*-homologous restriction fragment of approximately the same size is also observed in the *bz1* tester stock (Figure 1B, lane d). To determine if the *bz1*-homologous fragment in *bz1-A328* also hybridizes with *Mu1* probes additional digests were done. A *Sac*I fragment from *bz1-A328* hybridized to both *bz1* and *Mu1* probes and a similar sized fragment was not observed in the *bz1* tester (data not shown). Thus, the insertion in *bz1-A328* appears to hybridize with *Mu1* probes.

As the insertion in *bz1-A328* appeared to be related to, but larger than *Mu1*, we were interested in determining whether the insertion was related to *Mu2*, a *Mu* element that is closely related to *Mu1*, but contains an additional 385 bp of novel DNA. Therefore, we rehybridized the blot in Figure 1 with a fragment unique to *Mu2* and found that the insertion in *bz1-A328* also appears to hybridize to the *Mu2*-specific probe (data not shown). The *Mu2*-specific probe did not hybridize with the *Mu1*-hybridizing fragment in the *bz1* tester.

The *Mu2* elements studied previously contain one *Bst*EII site and no *Not*I sites (TAYLOR and WALBOT 1987). Therefore we tested whether the insertion in *bz1-A328* contained these sites and found that the insertion contains a *Not*I site but is not digested by the *Bst*EII restriction enzyme, the opposite of what had been found with the *Mu2* element (TAYLOR and WALBOT 1987). Interestingly, the *Mu* element *rcy:Mu7*, an ~2.2-kb element that shares no internal sequence identity with *Mu2*, contains one *Not*I site and no *Bst*EII site. To determine if the insertion in *bz1-A328* might be related to the *rcy:Mu7* element, a blot similar to the one shown in Figure 1 was hybridized to fragments from *Mu6* and *Mu7*, both of which are closely related to *rcy:Mu7*. The insertion in *bz1-A328* did not hybridize to either the *Mu6* or *Mu7* fragments, suggesting the insertion is not related to *rcy:Mu7*.

These data suggest that the insertion in *bz1-A328* belongs to the *Mu1* class of elements. However, its precise relationship to this class is unclear, as it appears to be larger than *Mu2*, the largest known member of this class, and it has a different restriction map with *Not*I and *Bst*EII than either *Mu1* or *Mu2*.

The data presented above suggest that all four of the insertions in the *bz1* mutants belong to the *Mu1* class of *Mu* elements. The approximate locations of the insertions in the *bz1-A326* and *bz1-A328* alleles, as well as the insertions in the two previously described alleles, are shown in Figure 2.

Molecular analysis of the *sh1* mutants: To determine whether insertions were present in the *sh1* mutants, blot analyses were performed on DNA from progeny of each of the eight *sh1* mutants and from the progenitor *Sh1* allele, using several restriction

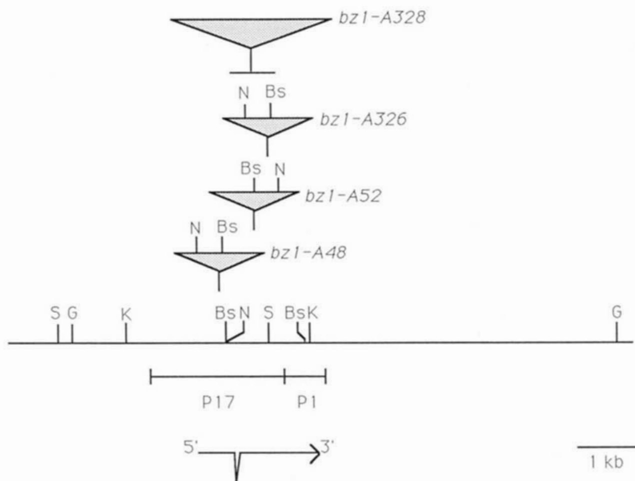

FIGURE 2.—Restriction map of *Bz1* progenitor allele and the approximate location of the insertions in the *bz1* mutants. The restriction enzyme sites are abbreviated as follows: G, *Bgl*II; K, *Kpn*I; Bs, *Bst*EII; N, *Not*I; S, *Sac*I. The *bz1* probes P1 and P17, used in mapping the insertion sites, are indicated below the map (FURTEK 1986). The location of the *bz1* transcript is indicated by the arrow with the intron shown (RALSTON, ENGLISH and DOONER 1988). The position of each insertion was determined by performing single and double digestions of maize DNA using enzymes that cut at the indicated sites.

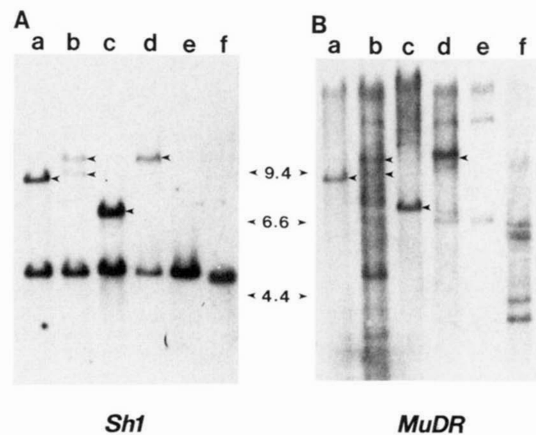

FIGURE 3.—DNA blot analysis of *sh1* mutants; *sh1-A83*, *sh1-A85*, *sh1-A344* and *sh1-A345*. (A) Southern blot of leaf DNA samples (~3 μg) digested with *Pst*I and hybridized with the *sh1*-B probe indicated in Fig. 7. Lane a, *sh1-A83/Sh1*; lane b, *sh1-A85/sh1*; lane c, *sh1-A344/sh1*; lane d, *sh1-A345/sh1*; lane e, *sh1* tester; lane f, *Sh1/sh1* (wild-type sibling of *sh1-A83*). (B) Same blot hybridized with the *MuDR* internal probe, Mu* (MATERIALS AND METHODS). The arrowheads point to the altered restriction fragments observed in each mutant relative to the progenitor *Sh1* fragment. The migration of *Hin*dIII digested λ DNA, used as a size marker, is indicated in kilobase pairs (kb).

TABLE 2

Summary of the insertions found in the *sh1* mutants

Allele name	Size if insert (kb)	Class of *Mu* elements[a]
sh1-A83	~4.0	*MuDR*
sh1-A85	~4.0	*MuDR*
sh1-A89	~3.7	*MuDR*
sh1-A95	~1.4	*Mu1*
sh1-A337	~3.0	Unknown
sh1-A340	~1.4	*Mu1*
sh1-A344[b]	~2.2	*MuDR*
sh1-A345[c]	~5.0	*MuDR*

[a] The determination of the class of *Mu* elements inserted in these alleles is described in the text.
[b] The *sh1-A344* mutant was derived from the same Mutator parent as the *bz1-A326* mutant.
[c] The *sh1-A345* mutant was derived from the same Mutator parent as the *bz1-A328* mutant.

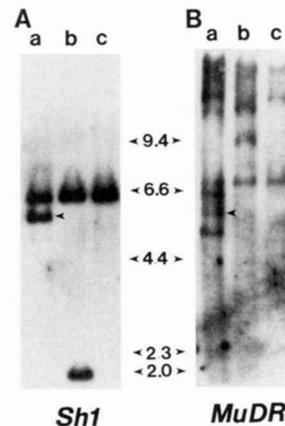

FIGURE 4.—DNA blot analysis of *sh1* mutant *sh1-A89*. (A) Southern blot on leaf DNA samples (~3 μg) digested with *Pst*I and hybridized with the *sh1*-C probe indicated in Figure 7. Lane a, *sh1-A89/sh1*; lane b, *Sh1/sh1* (wild-type sibling of *sh1-A89*); lane c, *sh1* tester. (B). Same blot hybridized with the *Bam*HI/*Hin*dIII internal sequence of *MuDR*. The arrows point to the altered restriction fragment observed in *sh1-A89* relative to the progenitor *Sh1* fragment. The migration of *Hin*dIII digested lambda DNA, used as a size marker, is indicated in kilobase pairs (kb).

enzymes. A summary of the *sh1* mutant alleles, the size of the insertion in each allele, and the potential class of *Mu* insertion is in Table 2. Examples of data supporting our conclusions are in Figures 3–6, and a summary of the insertion sites is in Figure 7. Briefly summarized, five of the insertions belonged to the *MuDR* class and two of the insertions belonged to the *Mu1* class of elements. We were unable to determine the identity of one of the insertions.

As stated, our data suggest that five of the *sh1* mutants contained *MuDR*-related insertions ranging in size from 2.2 to 5.0 kb. An example of a DNA blot containing DNA from progeny of four of these *sh1* mutants; *sh1-A83*, *sh1-A85*, *sh1-A344* and *sh1-A345* is shown in Figure 3A. Each of the plants from which DNA was prepared was heterozygous, containing the mutant allele and the tester *sh1* allele. The DNA was digested with *Pst*I and the blot was hybridized with the *sh1*-B probe. The size of the *Pst*I fragments of the progenitor *Sh1* allele is ~5 kb (Figure 3A, lane f); this is approximately the same size as the *sh1* tester allele (lane e). The *sh1-A83* and *sh1-A85* alleles, contain an ~9-kb fragment in addition to the ~5-kb *sh1* tester fragment (Figure 3A, lanes a and b), consistent with insertions in both the *sh1-A83* and *sh1-A85* allele of ~4.0 kb that contain no *Pst*I sites. The *sh1-A85* allele has an additional fragment of ~10 kb, that other digests have shown is due to incomplete digestion of

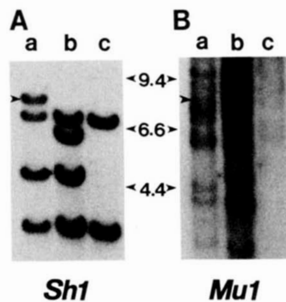

FIGURE 5.—DNA blot analysis of the *sh1* mutant *sh1-A95*. (A) Southern blot of leaf DNA samples (~3 μg) digested with *Bgl*II and hybridized with the *sh1*-C probe indicated in Figure 7. Lane a, *sh1-A95/sh1*; lane b, *Sh1/sh1* (wild-type sibling of *sh1-A95*); lane c, *sh1* tester. (B). Same blot hybridized with the *Mu1* internal probe. The arrowheads point to the altered restriction fragment observed in *sh1-A95* relative to the fragment from the progenitor *Sh1* allele. The migration of *Hin*dIII-digested λ DNA, used as a size marker, is indicated in kilobase pairs (kb).

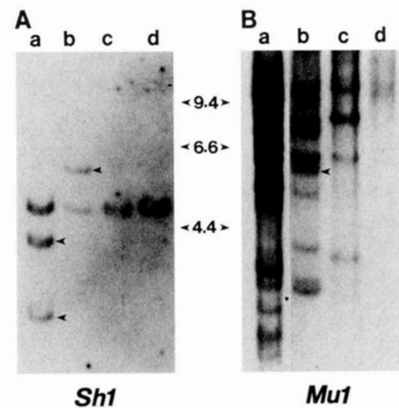

FIGURE 6.—DNA blot analysis of *sh1* mutants *sh1-A337* and *sh1-A340*. (A) Southern blot on leaf DNA samples (~3 μg) digested with *Pst*I and hybridized with the *sh1*-B probe indicated in Figure 7. Lane a, *sh1-A337/sh1*; lane b, *sh1-A340/sh1*; lane c, *Sh1/sh1* (wild-type sibling of *sh1-A337*); lane d, *sh1* tester. (B). The same blot hybridized with the *Mu1* internal fragment. The arrowheads point to the altered restriction fragment observed in the *sh1-A337* and *sh1-A340* alleles relative to the fragment from the progenitor *Sh1* allele. The fragment in *sh1-A340* also hybridizes to the *Mu1* internal probe, while the fragments in *sh1-A337* do not. The migration of *Hin*dIII digested lambda DNA, used as a size marker, is indicated in kilobase pairs (kb).

the *Pst*I site in the *sh1-A85* allele. The *sh1-A344* allele contains an ~7.2-kb fragment (Figure 3A, lane c), consistent with an ~2.2-kb insert that does not contain a *Pst*I site. The *sh1-A345* allele contains an ~10-kb fragment (Figure 3A, lane d), consistent with an insertion of ~5.0 kb that does not contain a *Pst*I site. Two other restriction enzymes (or combinations of enzymes) have been used to analyze these *sh1* mutations. The results for all four alleles and with all enzymes tested were consistent with the size of the inserts indicated by the *Pst*I digests. In addition, DNA samples from the original mutant plants, digested with one restriction enzyme, were analyzed by DNA blot analyses and the indicated insertion sizes were the same as in those of the progeny (data not shown).

As previously reported, the *sh1-A83* allele has been cloned, and the ~4.0-kb insert shown to be in the same class of elements as *MuDR* (CHOMET *et al.* 1991). Restriction mapping of this allele was consistent with the hypothesis that the insertion in *sh1-A83* lacks ~1 kb relative to the full length *MuDR* element. Given the similar size range, we were interested in determining whether any of the other insertions in the *sh1* alleles were related to *MuDR*. Therefore we removed the *sh1* probe from the blot shown in Figure 3A and rehybridized with Mu*, a *MuDR* internal probe (Figure 3B). As can be seen by comparing Figure 3, A and B, the fragments that hybridize to the *sh1* probe in the *sh1-A83*, *sh1-A85*, *sh1-A344* and *sh1-A345* alleles (lanes a–d) also hybridize to the *MuDR* probe. This experiment has been repeated with DNA digested with *Pst*I in combination with two other restriction enzymes and in all cases the fragments from the *sh1* alleles hybridized to both the *sh1* and the *MuDR* probes (data not shown). These data are consistent with the hypothesis that the insertions in the *sh1-A85*, *sh1-A344* and *sh1-A345* alleles are all members of the *MuDR* class, as is the insertion in the *sh1-A83* allele (CHOMET *et al.* 1991).

MuDR has unique *Bgl*II, *Eco*RI and *Bcl*I sites characteristic for this class of element. To determine whether these restriction enzyme sites were present in the *MuDR*-related elements in the *sh1* mutants, double digests using *Pst*I and each of the three restriction enzymes listed above were carried out on DNA samples from plants with each of the three alleles. These digests were then analyzed on DNA blots hybridized with either the *sh1*-A or *sh1*-B probes shown in Figure 7. The ~4-kb insert in the *sh1-A85* allele contains a *Bcl*I site and an *Eco*RI site, but does not contain a *Bgl*II site. The ~2.2-kb insert in the *sh1-A344* allele does not contain a *Bgl*II, *Eco*RI or *Bcl*I site. The ~5-kb insert in the *sh1-A345* allele has a *Bcl*I site, an *Eco*RI site, and a *Bgl*II site. Assuming these inserts are closely related to *MuDR*, we used the position of these three restriction sites in *MuDR*, and the location of these restriction sites in the insertions to map the approximate location of the insertions in the *sh1* gene (Figure 7).

The fifth *sh1* mutation that contains a *MuDR* related insertion is *sh1-A89*. Figure 4 illustrates a DNA blot containing *Pst*I digested DNA from progeny of the *sh1-A89* mutant that is heterozygous for the *sh1-A89* allele and the *sh1* tester allele. The blot was hybridized with the *sh1*-C probe, shown in Figure 7. The progenitor *Sh1* fragment is 1.9 kb (lane b) and the fragment in the *sh1-A89* allele is 5.9 kb (lane a), suggesting the *sh1-A89* allele contains an ~4-kb insertion without a *Pst*I site. To determine whether the insertion was related to *MuDR*, the *sh1* probe was removed from the blot in Figure 4A and the blot was rehybridized with a *MuDR* probe, as shown in Figure 4B. As can be seen by comparing lane a in Figure 4, A and B, a

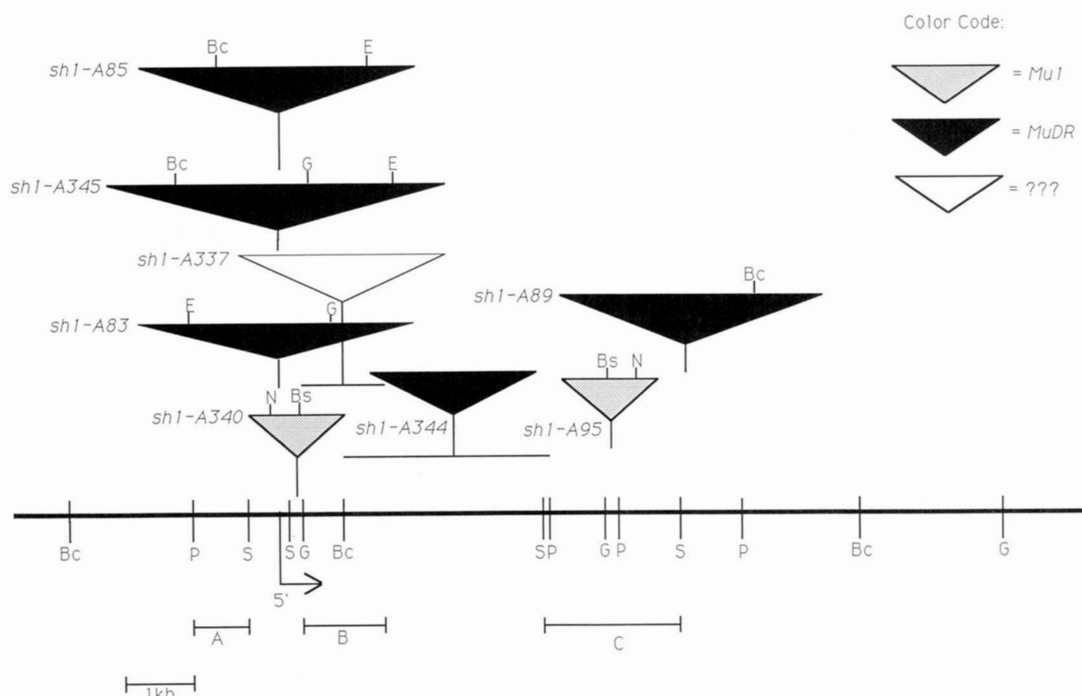

FIGURE 7.—Restriction map of the *Sh1* progenitor allele and the approximate locations of the insertions of the *sh1* mutants. The restriction enzyme sites are abbreviated as follows: B, *Bam*HI; Bc, *Bcl*I; Bs, *Bst*EII; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; N, *Not*I; S, *Sac*I; X, *Xba*I. The *sh1* probes used in Figures 3–6 are indicated below the map. Additional probes that are not shown were also used in mapping these alleles. The location of the start of the *sh1* transcript is indicated as an arrow marked 5′ (WERR *et al.* 1985). The positions of the insertions were determined by single and double digestions. The fragments that contain the insertions in *sh1-A337* and *sh1-A344* are shown as lines.

fragment that hybridizes to the *sh1* probe in *sh1-A89* also hybridizes to the *MuDR* fragment. This experiment has been repeated with two separate combinations of restriction enzymes and in all cases the fragment in *sh1-A89* hybridizes to both the *sh1* and *MuDR* probes. Therefore the insertion in *sh1-A89* is likely to be a member of the *MuDR* class of *Mu* elements. Other restriction digests of progeny of the *sh1-A89* allele have also suggested that the insert in this allele is ~4 kb, however two different restriction digests of DNA from the original mutant plant indicated that the insert was ~5 kb (data not shown). Further Southern blot analysis using DNA from progeny of *sh1-A89* revealed that the insertion in *sh1-A89* is not cut with *Eco*RI or *Bgl*II, even though analysis of DNA from the original mutant plant demonstrated that the insertion in *sh1-A89* did contain an *Eco*RI site and a *Bgl*II site (data not shown). As instability of the *MuDR* class has been reported (CHOMET *et al.* 1991), we hypothesize that a deletion occurred in the insertion in the *sh1-A89* allele and the deleted element was transmitted to the progeny we analyzed. Assuming the insertion in *sh1-A89* to be related to *MuDR*, its location in the *sh1* gene was determined as shown in Figure 7.

Two of the *sh1* mutations appear to contain *Mu1* related insertions. Figure 5A shows a DNA blot containing *Bgl*II-digested DNA from progeny of the *sh1-A95* mutant, which is heterozygous for the *sh1-A95* allele and the tester *sh1* allele. This blot was hybridized to the *sh1*-C probe. The progenitor *Sh1* allele is ~6 kb (lane b) and the *sh1-A95* allele is ~7.4 kb (lane a), suggesting an insertion of ~1.4 kb. Figure 6 illustrates DNA blot analysis of *Pst*I digested DNA from progeny of the *sh1-A340* mutant which is heterozygous for the *sh1-A340* allele and the tester *sh1* allele. This blot was hybridized to the *sh1*-B probe. The progenitor *Sh1* allele (lane c) and the tester *sh1* allele (lane d) are both ~5 kb. The *sh1-A340* allele contains an ~6.4-kb *Pst*I fragment (lane b), suggesting that the insert in *sh1-A340* is ~1.4 kb. The size of the inserts in *sh1-A95* and *sh1-A340* have been confirmed by two separate restriction digests of DNA from the original mutant plants. Further restriction mapping of progeny of the *sh1-A95* and *sh1-A340* mutants revealed that both of the insertions contain a *Bst*EII site and a *Not*I site characteristic of *Mu1*-related elements. The *sh1* probe was removed from each blot and each blot was hybridized with a *Mu1*-internal probe (Figures 5B and 6B). As can be seen by comparing lane a in Figure 5, A and B, and lane b in Figure 6, A and B, a *Sh1* hybridizing band in the two mutants also hybridizes to the *Mu1*-internal probe. Assuming the insertions in the two *sh1* alleles are related to *Mu1*, their positions in *sh1* are shown in Figure 7.

The *sh1-A337* allele contains two fragments that hybridize to the *sh1*-B probe, (Figure 6A, lane a), an ~4.3-kb fragment and an ~2.7-kb fragment, suggesting the insertion contains at least one *Pst*I site. Southern blot analyses of DNA of plants containing the *sh1-A337* allele digested with either *Sac*I, *Bgl*II, or *Bcl*I

produced single fragments indicative of an insertion in *sh1-A337* of ~3.0 kb (data not shown). Taken together, these data suggest that the insert in *sh1-A337* is at least ~3.0 kb and contains at least two *Pst*I sites as the size of the two *Pst*I fragments from this allele together are only ~2 kb larger than the ~5.0-kb *Pst*I fragment of the *Sh1* allele. To determine if this insertion might be related to a *Mu* element, we removed the *sh1*-B probe from blots and successively reprobed with internal fragments specific for each class of *Mu* element. None of the *Mu*-internal fragments tested hybridized to the insertion in *sh1-A337*. As the classes of *Mu* elements can be quite heterogeneous, it is still possible that this insertion belongs to one of the previously identified *Mu* classes. It is also possible that the insertion represents a novel *Mu* element class, or a non-*Mu* insertion.

DISCUSSION

A small number (650) of Mutator plants were used to isolate mutations at both *bz1* and *sh1*. From this screen, 4 *bz1* and 8 *sh1* mutations were isolated and all 12 alleles contained insertions, 11 of which appear to be related to *Mu* elements. The *bz1* and *sh1* genes were mutated at similar frequencies, suggesting that both genes are equally receptive to *Mu* element insertions. However, the two genes appeared to be targeted by different classes of *Mu* elements.

All four insertions into *bz1* are related to the *Mu1* class of elements. The insertions in three of the *bz1* mutations are the size of *Mu1* and contain both a *Bst*EII site and a *Not*I site, diagnostic of *Mu1* elements. Furthermore, the restriction fragment that hybridized to *bz1* sequences in each mutant was the same size as a restriction fragment that hybridized to an internal probe from *Mu1*. These findings strongly suggest that these three insertions are *Mu1*-like elements. The fourth *bz1* mutation contains an additional ~2.4 kb of DNA relative to the progenitor allele and the restriction fragment that hybridized to *bz1* sequences in this mutant also hybridized to an internal probe from *Mu1* and a unique probe from the *Mu2* element (a member of the *Mu1* class of elements) suggesting that this insertion is also related to the *Mu1* class of *Mu* elements.

Using similar criteria, two of the *sh1* mutants also appear to contain *Mu1*-like insertions. Of the six remaining *sh1* mutations, five appear to contain insertions that belong to the *MuDR* class of *Mu* elements. This conclusion is based on the finding that in three different restriction digests, the *sh1*-hybridizing sequences in these alleles also hybridize to an internal *MuDR* probe. In addition, four of these inserts have restriction sites found in *MuDR* and one of these four inserts has been cloned and found to contain a *MuDR*-like insert (CHOMET *et al.* 1991). The final *sh1* mutation contains an ~3.0 kb insert that does not hybridize to internal sequences of any of the known *Mu* elements. It is possible that this insert is a novel *Mu* element, or it might belong to a family of elements distinct from *Mu*. Therefore, five out of eight *sh1* mutations appear to contain *MuDR* related insertions, two appear to contain *Mu1* related insertions, and one contains an insertion whose identity is unknown.

Previously, only two *MuDR* related elements, *Mu9* and the *MuDR* related insertion at *sh1-A83*, had been isolated as insertions in a gene (CHOMET *et al.* 1991; HERSHBERGER, WARREN and WALBOT 1991). It is interesting that the *MuDR* related insertions we isolated at *sh1* are very heterogeneous. The insertion at *sh1-A345* is approximately the same size (~5 kb) as *MuDR* and has the three unique restriction sites found in *MuDR*. Insertions at the two alleles *sh1-A83* and *sh1-A85* are both ~4 kb and therefore appear to be deleted for a region of *MuDR*. However, these insertions differ with respect to several restriction sites found in *MuDR*, suggesting they are missing different regions of *MuDR*. The original insertion at *sh1-A89* appears to have been unstable, as demonstrated by our finding that DNA from the original mutant contained an ~5-kb insertion that was digested by both *Eco*RI and *Bgl*II, whereas the progeny of this mutant contained an ~3.7-kb insertion that was not digested by either *Eco*RI or *Bgl*II. Finally, the insertion at *sh1-A344* is only ~2.2 kb and therefore appears to be missing much of the *MuDR* sequence.

It is interesting that even in the small data set examined, we have observed a difference in the class of *Mu* element that inserted most often into the *bz1* and *sh1* genes. All of the *Mu* elements in the *bz1* mutants were *Mu1*-related (100%). In contrast, the *Mu1* class of elements accounted for only two (25%) of the insertions at *sh1*. The class of *Mu* elements found inserted most often in the *sh1* mutants is *MuDR* (62.5%).

When our data is combined with other published studies, it is clear that either the *bz1* gene is particularly susceptible to the insertion of *Mu1* elements, or resistant to the insertion of other classes of elements (reviewed in BENNETZEN *et al.* 1993). Of 26 independent alleles, 25 contained elements of the *Mu1* class (TAYLOR, CHANDLER and WALBOT 1986; BROWN, ROBERTSON and BENNETZEN 1989; HARDEMAN and CHANDLER 1989; this study), whereas only 1 contained a different class of *Mu* element (SCHNABLE, PETERSON and SAEDLER 1989). We would predict that as more *sh1* alleles are characterized a *MuDR* preference for *sh1* will be observed, as suggested by our data.

All the mutations examined in this study were derived from the same Mutator stock. Therefore, the simple hypothesis that different *Mu* elements are active in different Mutator stocks cannot explain why the two genes were targeted predominantly by distinct classes of *Mu* elements. Furthermore, the finding that

in two independent cases a single Mutator plant gave rise to a *bz1* mutation containing a *Mu1*-related element and a *sh1* mutation with a *MuDR*-related element, clearly demonstrates that different *Mu* elements can be active in the same Mutator plant.

Another hypothesis is that *Mu* elements transpose to linked sites and a difference in the class of *Mu* element linked to each gene cause the two genes to be mutated predominantly by different classes of elements. To invoke this hypothesis, one must invoke that *Mu* elements move preferentially to very tightly linked locations, as *bz1* and *sh1* are only two map units apart. Since there is no conclusive evidence that *Mu* elements move to linked locations [reviewed in CHANDLER and HARDEMAN (1992) and BENNETZEN *et al.* (1993)], we believe that the difference in the types of insertions found most often at the two genes is unlikely to be due to a difference in the *Mu* elements linked to the two genes.

A hypothesis that could account for our findings is that each class of *Mu* element has specific preferences for certain genes. The classes of *Mu* elements contain distinct features that could contribute to differences in targeting. One obvious difference between different classes of *Mu* elements is that they do not share the same internal sequences. It is possible that the differences in targeting are due to the differences in internal sequences among the various classes of *Mu* elements. One potential mechanism is suggested by studies with Drosophila *P* elements. *P* element derivatives containing a region of upstream regulatory DNA from the *engrailed* (*en*) gene insert at a very high frequency near the endogenous *en* gene and also at genes with similar expression patterns. The selective insertion of *P* elements did not occur at specific DNA sites. The authors speculated that the insertion specificity might be the result of a protein bound to the *en* fragment within the *P* elements bringing the *P* elements to a particular location in the genome via protein-protein or protein-DNA interactions (HAMA, ALI and KORNBERG 1990; KASSIS *et al.* 1992).

Another difference in the classes of *Mu* elements resides in their termini. Although all *Mu* elements contain similar *Mu*-termini, they are not all identical [reviewed in WALBOT (1991)]. For example, the termini of the *MuDR* and *Mu1* classes of elements are only ~85% identical (QIN and ELLINGBOE 1990). It is not known which sequences in the termini are necessary for transposition. Theoretically, some termini might require greater amounts of transposase or additional host factors to transpose. If this is the case, the timing of transposition could differ among the various classes of *Mu* elements. This could have an effect on which genes are targeted by a particular class of *Mu* element. For example, particular genes may be in an accessible chromatin structure for limited times only.

Mu elements have been found inserted within introns, exons, promoters or 5′ leaders [reviewed in BENNETZEN *et al.* (1993)]. Prior to our study, there were only three genes for which a large number of insertions had been analyzed, *adh1*, *bz1* and *kn1*. For all three genes, there did appear to be a bias in that a majority of the mutants contained insertions into particular regions of the genes [reviewed in BENNETZEN *et al.* (1993)]. Similarly, five of the eight *sh1* mutants we isolated contained insertions near the 5′ end of the gene. This potential intragenic specificity could be caused by element preferences, hot spots within the genes for insertions, or bias introduced by screening for particular phenotypes [reviewed by BENNETZEN *et al.* (1993)]. Since different *Mu* elements have different DNA sequences, how these sequences interact with the adjacent gene regulatory sequences could influence the frequency with which a mutant phenotype is generated, and the type of element found most often in a particular gene.

These results have important implications for cloning *Mu*-tagged genes as other genes may also be resistant or susceptible to particular classes of *Mu* elements. It remains to be determined what factors affect the target sites of the various classes of *Mu* elements. Transposition assays in which the *Mu* elements could be altered *in vitro* and then reintroduced into plants would facilitate testing the hypotheses discussed.

We thank S. BELCHER and J. KORTE for technical assistance, and G. PATTERSON, K. KUBO, A. BARKAN, H. FOSS and M. SAINZ for critical reading of the manuscript. This work was supported by the National Institutes of Health (GM 35871) to V.L.C. K.J.H. was supported by a Predoctoral National Institute of Health Training Grant (GM07413).

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Communicating editor: W. F. SHERIDAN