Genetic Study of the Loss and Restoration of Mutator Transposon Activity in Maize: Evidence Against Dominant-Negative Regulator Associated With Loss of Activity

Jennifer Brown*† and Venkatesan Sundaresan†

*Genetics Program, State University of New York, Stony Brook, New York 11794, and †Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Manuscript received August 26, 1991
Accepted for publication December 30, 1991

ABSTRACT

The Mutator system of transposable elements is characterized by a family of transposons called Mu transposons that share common termini and are actively transposing in Robertson's Mutator (Mu) lines of maize. Mu lines lose transposition activity during propagation by either outcrossing or inbreeding. This loss of transposition activity, which can occur at non-Mendelian frequencies, is in the form of loss of forward transposition activity resulting in a decrease in the generation of new mutations, as well as the loss of mutability of Mu transposon induced mutations, and it has been correlated with hypermethylation of the Mu elements. Previous studies have concluded that restoration of Mutator transposon activity by crossing inactive lines back to active lines is incomplete or transient, and depends upon the sex of the inactive parent. Further, it has been proposed that the inactive system is dominant to the active system, with the dominance possibly mediated through a negative regulatory factor that is preferentially transmitted through the female. In this study, we have examined the frequencies of loss and restoration of Mu transposon activity using a Mu line carrying an insertion in the bronze1 locus. We find that transmission of Mu transposon activity to non-Mu plants can occur at high rates through males and females, but individual cases of decreased transmission through the male were observed. We also find that in crosses between inactive-Mu and active-Mu plants, reactivation was efficient as well as heritable, regardless of the sex of the inactive parent. Similar results were obtained whether the inactivation occurred in an outcross or a self. In all cases examined, loss of Mu transposon activity was correlated with hypermethylation of Mu elements, and reactivation was correlated with their demethylation. Our results indicate that an inactive Mu system does not exhibit dominance over an active Mu system. We conclude that contrary to current models, inactivation and its maintenance is not obligatorily associated with a dominant negative regulatory factor whether nuclear or cytoplasmic, and we propose a revised model to account for these and other observations.

ACTIVE transposable element systems can be highly mutagenic and may play an important role in the evolution of genomes, yet the many copies of transposons found in most genomes are usually quiescent [see McClintock (1965, 1984) and Fedoroff (1989) for reviews]. Transposable elements in maize have been studied since the pioneering work of McClintock (1950). Maize transposons can lose activity both during somatic growth of the plant and during gamete formation. Some losses result from changes in the DNA sequence, but more often, the DNA sequence is unchanged but methylated [Fedoroff (1989) for a review]. Transposon activation in maize can occur spontaneously, and also in response to natural and experimental stresses (McClintock 1984; Peschke, Phillips and Gengenbach 1987; Walbot 1988; Burr and Burr 1988; Hardeman and Chandler 1989).

This study concerns heritable changes in the activity of the (Mutator) Mu, transposons of maize. The Mu transposons were discovered in maize populations which had elevated mutation rates at many loci (Robertson 1978). These mutations are caused by a family of transposons present in many copies named Mu, which are characterized by common long terminal inverted-repeats and variable internal sequences [for review see Walbot (1991)]. Rare copies of Mu transposon sequences can also be found in the genomes of plants which have no known history of Mu activity (Talbert, Patterson and Chandler 1989). Mutable alleles which are controlled by Mu activity, due to insertion of a Mu element at the locus, can be used as genetic markers for Mu activity (Walbot 1984; Bennezen, Brown and Springer 1988; Freehill 1988; Robertson and Stinar 1989). Mu transposons lose activity in some progeny in each generation (Robertson 1986; Walbot 1986; Bennezen 1987). Loss of activity is accompanied by a drop in mutation rates, a lack of forward transposition, stabilization of previously mutable alleles, and disappearance of Mu ex-
trachromosomal circular DNA (above references; Sundaresan and Freeling 1987). The loss of Mu activity could arise from segregation of genetic factors (e.g., an autonomous Mu element) or from epigenetic silencing of the Mu system (Robertson 1986). A putative autonomous Mu element has been identified in stocks segregating 1:1 for Mu activity (Qin Robertson and Ellingboe 1991; Chomet et al. 1991). Inheritance of Mu activity frequently exhibits non-Mendelian segregation, indicative of epigenetic events that lead to losses of activity (Robertson 1986; Walbot 1986; Bennetzen, Brown and Springer 1988; Martienssen et al. 1989). In some of these cases, Mu can be reactivated by crosses to active Mu lines (Robertson 1986; Walbot 1986), but the activity losses which follow inbreeding are reportedly irreversible (Robertson 1983, 1986; Bennetzen 1987). It has been proposed that a dominant negative regulatory factor present in plants that have lost Mu activity inactivates the Mu system of active plants when they are crossed, and further that this repression of Mu activity is inherited as a maternal dominant trait (Walbot 1986; Bennetzen 1987).

We have used a mutable allele as a genetic marker for Mu transposon activity: bzMum9, which contains Mu1 inserted at the bronzel (bz1) locus (Brown, Robertson and Bennetzen 1989; Doseff, Martienssen and Sundaresan 1991). We have studied the rate of Mu activity loss following male vs. female inheritance, the reversibility of losses in Mu activity following outcrossing and selfing, and the effect of male vs. female inheritance on Mu reactivation. Also, we assay individual cases of Mu loss and Mu reactivation for changes in the methylation of Mu genomic DNA, to ask whether such changes are correlated with the mutability of the bzMum9 allele. Our results contradict some of the predictions of existing models, and we propose a revised model for the inheritance of changes in Mu activity.

MATERIALS AND METHODS

Genetic markers: kzMum9 (bronzel Mu induced mutable 9) is an allele of Bz1 (bronzel) which was isolated by Robertson (Brown, Robertson and Bennetzen 1989). In Mu-active plants its phenotype is expressed in the aleurone layer of maize endosperm as purple dots on a bronze background, and in plant vegetative tissues as purple stripes on a green or bronze background. The Bz1 gene encodes a flavonoid glucosyl-transferase essential to conversions of anthocyanin precursors to anthocyanins (purple and red pigments). Expression requires presence of the complementary color genes A, B, Bz2, C, Cz, Pr, R (Coe, Neuffer and Hossington 1988). Absence of Bz1 (null) results in bronze aleurone and bronze or green plant in plants which are Mu-active, kzMum9 confers a null bz phenotype. kzMum9 contains a Mu1 element inserted at the 3' intron exon junction of the single intron in Bz1 (Doseff, Martienssen and Sundaresan 1991).

sh (shrunken) mutant is defective in starch biosynthesis. The dry kernel is partially collapsed, due to incomplete filling of the endosperm (Coe, Neuffer and Hossington 1988), compared to a normal Sh kernel which is plump.

Genetic stocks: The bzMum9 stock was provided to us from D. S. Robertson (Iowa State University) as Sh bzMum9/sh bz, which was Mu-active. We propagated it by selfing, as well by outcrossing to a Mu bz tester (see below) and to a sh Bz1 tester obtained from the maize Co-op. When Mu activity is lost, bzMum9 loses its mutability, but is distinguished from the null bz allele of the tester by its linkage to Sh (2 map units distal to Bz1). The Sh bzMum9/sh Bz1 stock was generated by selecting plump kernels from the cross Sh bzMum9/sh bx × sh Bz/sh Bz. Homozygous Sh bzMum9 was obtained by selfing Sh bzMum9/sh Bz plants and selecting for the plump mutable bronze bzMum9 phenotype.

The non-Mu; Δ(sh bz) tester carrying a deletion of the Sh and Bz1 loci (sh bz X5) was generated by J. Mottinger (University of Rhode Island). These plants have no Mu activity, and only 0-2 copies of Mu DNA in the genome. We classify them non-Mu for our experiments. There is no recombination between sh and bz in this stock.

sh bz/Δ(sh bz); Mu-active plants were generated by crossing stocks known to be Mu active by their bzMum9 phenotype, to Δ(sh bz) non-Mu testers (Mu active; Sh bzMum9/sh bz × non-Mu Δ(sh bz)/Δ(sh bz)). Shrunken bronze kernels (sh bz) were selected from ears which show Mu activity (i.e., somatic mutability) in greater than 98% of the Sh bzMum9 segregants. These selections are likely to be Mu active; Δ(sh bz)/Δ(sh bz), as Mu loss is occurring at less than 2% on these ears.

Crossovers: For the study of Mu inactivation in outcrosses, plants heterozygous for sh Bz/Sh bzMum9 were first selfed to obtain bzMum9 homozygous identified as plump bronze mutable kernels. These bzMum9 homozygotes, selected from different selfed ears, were crossed as exact reciprocals to non-Mu; Δ(sh bz) homозygous testers, and the resulting ears were examined for the presence of plump stable (i.e., nonmutable) bronze kernels among mutable bronze progeny. Since the mutability of bzMum9 is dependent on Mu activity (Doseff, Martienssen and Sundaresan 1991), and bzMum9 is Sh linked, the stable bronze kernels are most likely Sh bzMum9/Δ(sh bz) and Mu-inactive.

For the study of Mu inactivation by selfing, the Sh bzMum9/sh Bz stock was selfed to obtain bzMum9 homozygotes, identified either as plum, mutable bronze or plum, stable bronze kernels. The Sh bz phenotype kernels are most likely Sh bzMum9 homozygous, and Mu-inactive. To test the heritability of the stable bronze phenotype, some bronze kernels were selected, grown, and testedcrossed to non-Mu; Δ(sh bz). Every kernel on the resulting ears was examined on a dissecting microscope for the presence of purple revertant sectors. Kernels with one or more purple sectors are classified as mutable, and thus Mu-active.

For the Mu reactivation crosses, Mu-inactive bzMum9 homozygotes derived from selfs of sh Bz/Sh bzMum9 were selected by their Sh bz phenotypes. These were grown and crossed reciprocally to Δ(sh bz)/sh bx Mu-active plants (Table 3, crosses 1 and 11-18), or were first selfed and then crossed reciprocally in the next generation to Δ(sh bz)/sh bx Mu-active plants (Table 3, crosses 2-10). The bzMum9 phenotype of each kernel on the resulting ears was scored and used to classify Mu activity. As a control for the heritability of the Mu-inactive phenotype, the second ears of the Mu-inactive parents were also reciprocally crossed by non-Mu; Δ(sh bz) testers, and the progeny kernels were examined for purple sectors. Individual cases of Mu-reactivation from each cross were subsequently testcrossed to Δ(sh bz), and...
the heritability of the Mu-reactivation was scored by looking for \(bzMum9\) mutability in the kernels.

**DNA analysis:** The methods used were essentially the same as described by Martienssen et al. (1990). A sample of 3–5 µg of DNA isolated from leaf tissue of maize plants at the seven leaf stage was digested with 10 to 20 units of restriction enzyme (HinfI, HpaI or Mspl) overnight, electrophoresed on 1% agarose gels, blotted onto nylon membranes and hybridized with \({}^{32}\text{P}\)-labeled probe. The Mu1 probe used was an internal 0.9-kb NotI-TthIII1 fragment, the same as described by Martienssen for \(bzMum9\) mutability in the kernels.

The methods used were essentially the same as described by Barker et al. (1984), its location indicated in Figure 3C. The Bz probe was the plasmid pD3MS9 (Scheiffelin et al. 1985) carrying the internal 630-bp MluI-SstI fragment of the Bz1 gene (obtained from Oliver Nelson, University of Wisconsin), and its location is diagramed in Figure 5C.

**RESULTS**

**Comparison of male and female inheritance of Mu transposon activity:** The Mu stock carrying the \(bzMum9\) allele transmits Mu activity at high rates and at non-Mendelian ratios, i.e. the ratios of active to inactive progeny are variable, and not consistent with segregation of a defined number of autonomous or positive regulatory elements. Using this stock we have examined whether loss of Mu transposon activity is more likely following male inheritance than female inheritance. Maize is monoecious and produces spatially separated male and female flowers on the same plant. In a reciprocal cross, a single Mu plant is used both as a male parent to pollinate the ear of a single tester plant, and as a female parent receiving pollen from the same tester. By performing reciprocal crosses of \(bzMum9\) plants to non-Mu; \(\Delta(sh\ bz)\) testes, we compared the frequencies of Mu loss among progeny of \(bzMum9\) plants when they were used as males, to those when the same plants were used as females. The \(bzMum9\) phenotype when Mu is active is small purple spots on bronze kernels, shown in Figure 1A. When Mu is inactive, \(bzMum9\) mutability is lost and the phenotype is a uniform bronze kernel, shown in Figure 1B. This mutation results from an insertion of a Mu1 transposon in the Bronze I gene (Brown, Robertson and Bennetzen 1989). In Table 1, the numbers of Mu-active mutable bronze and Mu-inactive, stable bronze progeny from each of the reciprocal crosses to \(\Delta(sh\ bz)\) are listed and compared. Results from the \(bzMum9\) parent used as a male are listed first, in part A, and those from the \(bzMum9\) parent used as a female are listed second, in part B, for comparison.

The data in Table 1 show Mu activity at \(bzMum9\) was lost more frequently when inherited from the male parent, in 15 out of 17 crosses, although the differences were usually small. On average 8.2% of the progeny of Mu males lost Mu activity (444 in 5440) while only 3.8% of the progeny of Mu females lost Mu activity (163 in 4290). These differences in male transmission vs. female transmission are statistically significant by the \(X^2\) test \((X^2 = 79, P < 0.01)\). Further, the variance in rates of loss of Mu activity between individual crosses is greater when Mu is inherited from the male \((SD = 10.8\%)\) compared to inheritance from the female \((5.6\\% SD)\). In two exceptions of the 17 sets of reciprocal crosses, Mu activity was lost at nearly identical frequencies following both male and female inheritance (cross 5) or slightly higher rates through the female (cross 6). In one case the difference between male and female Mu inheritance was large, with 42.5% loss through the male and only 12% through the female (cross 8). We conclude that Mu activity in our \(bzMum9\) line is lost more often when inherited from the male parent than from the female, at a small but significant rate, and also that Mu inheritance is more variable through the male.

**Reactivation of inactive Mu derived from outcrosses:** Individual cases of loss of Mu-activity were selected from the progeny of the outcrosses in Table 1, and the ability to reactivate Mu was tested. The plump, stable bronze kernels, which carry the inactive \(bzMum9\) allele, were grown and crossed reciprocally to Mu-active; \(\Delta(sh\ bz)/sh\ bz\) plants. The progeny were then screened for Mu activity using the mutability of \(bzMum9\) in the kernel and its linkage to \(Sh\) as genetic markers. Progeny which inherit the Mu-inactive plump, bronze phenotype are classified as Mu-inactive, while progeny which regain a mutable \(bzMum9\) phenotype are classified as Mu-active. The second ears of these plants were reciprocally crossed by the non-Mu;
\[ \Delta(sh \ bz) \] tester, to confirm that the inactive state was heritable.

The results of these reactivation crosses are shown in Table 2. Crossing the Mu-inactive plants to Mu-active testers restores Mu-activity in 97.1% of the progeny (1307 of 1346) when inactive Mu is inherited from the male (Table 2, part A), and similarly in 96.7% of the progeny (1232 of 1274) when inactive Mu is inherited from the female (Table 2, part B). These values do not differ from each other significantly \( (X^2 = 0.47, P = 0.3-0.5) \). In no case was spontaneous reactivation observed in the control crosses with the non-Mu; \( \Delta(sh \ bz) \) tester (data not shown). Thus the Mu-regulated mutable phenotype of \( bzMum9 \) can be restored by crosses to Mu-active plants. Further, the sex of the parent contributing the inactive Mu genome in our crosses did not significantly affect the outcome of reactivation experiments.

**Reactivation of inactive-Mu derived by selfing:**

Loss of Mu activity can also occur in the progeny of selfed or intercrossed Mu plants (Walbot, 1986; Robertson 1983). We tested the heritability of the Mu inactivation derived by selfing by looking for spontaneous reactivations in the next generation following crosses to non-Mu testers. \( sh \) Bz/\( sh \) bzMum9 individuals were selfed, and plump stable bronze kernels corresponding to the \( bzMum9 \) Mu-inactive homozygotes were selected, grown, and test crossed to non-Mu; \( sh \) bz testers. More than 99% of over \( 10^4 \) progeny examined visually were Mu-inactive, plump stable bronze kernels. This demonstrates that Mu inactivation following selfing is a heritable change. When spontaneous reactivation occurs, it is rare and occurs at less than 1% (i.e., 0 to 2 kernels in a ear of 300 kernels). A more detailed microscopic examination was undertaken for kernels from 7 different ears. We found spontaneous reactivation in 7 out of 1628 kernels examined (about 0.4%).

We then examined whether Mu inactivations derived by selfing Mu plants were reversible. Plump uniform bronze kernels homozygous for \( bzMum9 \) were planted, and the resulting plants were reciprocally crossed to active Mu; \( \Delta(sh \ bz) \) sh bz plants. In the progeny of these crosses, a \( Sh \) bzMum9 mutable phenotype in the kernel indicates that Mu is reactivated, while a \( Sh \) bz kernel phenotype indicates that Mu remains inactive. Results of these crosses are shown in Table 3. From 96 to 97% of the progeny have regained the \( bzMum9 \) phenotype and Mu activity, demonstrating that the Mu inactivations tested were reversible. Figure 2 shows an example of reactivated \( bzMum9 \) kernels (A), as well as the control Mu-inactive \( bzMum9 \), propagated by crossing to the non-Mu; \( sh \) bz tester (B). Occasionally in the progeny from these reactivation crosses, kernels with large revertant sectors were observed. An example of such a kernel with a large revertant sector covering one-eighth of the kernel is shown in Figure 2C. The size of this sector indicates restoration of \( bz \) mutability occurred early
in development, suggesting Mu activity was restored early, within the first two or three divisions of the fertilized endosperm nucleus. The frequency of such kernels with large sectors was rare (i.e., in about 0.25% of the total progeny). Kernels with large mutant sectors are also observed to arise at low rates in crosses of active bzMum9 plants to testers (DOSEFF, MARTIENSENS and SUNDARESAN 1991). These sectors appear to be due to excision events rather than suppression (DOSEFF, MARTIENSENS and SUNDARESAN 1991).

To distinguish whether Mu reactivation is affected by the sex of the Mu-inactive gametophyte in our crosses, Mu-inactive individuals were used both as males and as females in the crosses to Mu-active testers. The data is shown separately in Table 3. Part A shows results of crosses of Mu-inactive males by Mu-active tester females and part B shows results of the reciprocal cross, Mu-inactive females crossed to Mu-active tester males. Both types of cross give similar results: 97.5% (3498 of 3589) of the progeny of Mu-inactive males were reactivated by the female Mu-active tester, and 96.6% (4173 of 4318) of the progeny of Mu-inactive females were reactivated by the male Mu-active tester. While the differences between these sets of data are small (<1%), they are statistically significant ($X^2 = 4.6, P = 0.020.05$).

To test for the heritability of Mu activity following reactivation, plants grown from bzMum9 spotted kernels from the above reactivation crosses were tested to cross to non-Mu; D(sh bz) testes. The mutable phenotype was inheritable, giving on the average 50% Sh bzMum9 mutable, and 50% sh bz progeny. This demonstrates that the Mu reactivation scored in the kernel is accompanied by Mu reactivation in the embryo and is inheritable. Further, we find that these reactivated Mu lines remain heritably active for at least three successive generations of outcrossing, as indicated by the mutability of the bzMum9 allele as well as by the unmethylated state of the MuI elements (see below), i.e., their behavior is no indistinguishable from lines that have never lost Mu activity.

**Correlation with methylation of MuI elements:** It has been shown by others that active Mu sequences in the genome are hypomethylated and that inactivation of Mu is accompanied by hypermethylation of Mu DNA (CHANDLER and WALBOT 1986; BENNETZEN 1987). To confirm whether Mu-genomic DNA is differentially methylated in genomes of Mu-active and Mu-inactive plants derived in our crosses, we assayed the DNAs with methylation-sensitive restriction endonucleases. Hinfl cleaves 5' GANTC 3', found once in each Mu IR, but is inhibited by 5-methylcytosine in the DNA sequence (COLASANTI and SUNDARESAN 1991). We examined the methylation states of MuI (a 1.4-kb element) and Mu 1.7 (a 1.7-kb element) in the inactive bzMum9 plants derived from selves, as well as inactive bzMum9 plants derived from outcrosses. These Mu elements are usually present in multiple copies in Mu genomes. The inactive bzMum9 plants

**TABLE 3**

**Restoration of mutability to inactive bzMum9 homozygotes derived by selfing, by crossing to active Mu; sh bz/Δ(sh bz) plants**

<table>
<thead>
<tr>
<th>Cross No.</th>
<th>Stable</th>
<th>Mutable</th>
<th>Stable</th>
<th>Mutable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sk μbzMum9</td>
<td>Sk μbzMum9</td>
<td>Sk μbzMum9</td>
<td>Sk μbzMum9</td>
</tr>
<tr>
<td></td>
<td>A. Mu-active Δ X Mu-inactive Δ progeny</td>
<td>B. Mu-inactive Δ X Mu-active Δ progeny</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>284</td>
<td>0</td>
<td>385</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>92</td>
<td>1</td>
<td>455</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>350</td>
<td>0</td>
<td>184</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>164</td>
<td>1</td>
<td>249</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>71</td>
<td>13</td>
<td>275</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>119</td>
<td>36</td>
<td>450</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>103</td>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>168</td>
<td>13</td>
<td>492</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>291</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>312</td>
<td>4</td>
<td>138</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>245</td>
<td>2</td>
<td>107</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>223</td>
<td>18</td>
<td>192</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>153</td>
<td>6</td>
<td>125</td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>149</td>
<td>11</td>
<td>288</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>182</td>
<td>4</td>
<td>165</td>
</tr>
<tr>
<td>16</td>
<td>9</td>
<td>195</td>
<td>7</td>
<td>147</td>
</tr>
<tr>
<td>17</td>
<td>8</td>
<td>207</td>
<td>12</td>
<td>151</td>
</tr>
<tr>
<td>18</td>
<td>8</td>
<td>190</td>
<td>15</td>
<td>221</td>
</tr>
</tbody>
</table>

Summary: 91 | 3498 | 145 | 4173

Reactivation rate: 97.5% | 96.6%
were grown from stable bronze kernels from the crosses described earlier. Active bzMum9 plants, grown from mutable bronze kernels selected from the same ear as the inactives, were used for comparison. Genomic DNAs were prepared identically in parallel and to test the methylation of Mu sequences they were assayed by digestion, Southern blotting, and hybridization with Mu1 internal sequences which are homologous to both Mu1 and Mu1.7.

Examples of these studies are shown in Figure 3. Cleavage by HinfI, is expected to generate a 1.3-kb Mu1 fragment and a 1.6-kb Mu1.7 fragment (see diagram in Figure 3C). Genomic DNAs from the Mu-active plants derived from selfs (Figure 3A) and outcrosses (Figure 3B) were digested with HinfI and probed with a Mu1 internal probe, shown in Figure 3A, lanes 2 and 4, and Figure 3B, lane 1. The Mu1 probe hybridizes prominently at 1.3 and 1.6 kb, indicating complete digestion of the genomic copies of Mu1 and Mu1.7 DNA. However, when genomic DNAs of the Mu-inactive sibling plants were digested with HinfI and probed with Mu1, many hybridizing bands larger than 1.3 and 1.6 kb appear (Figure 3A, lanes 1 and 3; Figure 3B, lanes 2 and 3). This is indicative of partial digestion, and the size fragments released probably vary with the position of the next available HinfI site in the flanking genomic DNA. Partial digestion probably results from the inhibition of HinfI by DNA methylation at the Mu termini in the Mu-inactive genomes, indicating hypermethylation of Mu1 and Mu1.7 elements relative to their Mu-active siblings. These results have been reproduced identically on over 40 sets of sibling pairs from related bzMum9 families, and are also consistent with previous findings by others (Chandler and Walbot 1986; Walbot and Warren 1988; Bennettzen, Brown and Springer 1988). The hypermethylation observed for the Mu1 elements in general, was also observed when a Mu1 element at a single locus was examined, i.e., the Mu1 element at the bz1 locus in bzMum9. A correlation between inactivation and hypermethylation of the HinfI sites of this element could be detected by using a Bz-specific probe, shown in Figure 5B, lanes 1 and 2.

We have also assayed for methylation of the internal Mu1 sequences at HpaII/MspI sites. The recognition sequence for these enzymes, CCGG, occurs 8 times in Mu1 (Barker et al. 1984), and 13 times in Mu1.7 (Taylor and Walbot 1987). Both enzymes are methylation sensitive: HpaII will not digest CCGG, while MspI will not digest 5mCCGG (Nelson and McClelland 1988). To excise Mu elements from flanking genomic DNA, we digested with TthIII-I, a DNA-methylation insensitive enzyme which cleaves in the Mu1 terminal inverted repeat (Barker et al. 1984). TthIII-I generates 1.1-kb Mu1 and 1.5-kb Mu1.7 fragments. Figure 4, lanes 1–4 show TthIII-I digested DNAs from Mu-active and Mu-inactive bzMum9 plants hybridized to the Mu1 internal probe. Each DNA sample is digested to completion, demonstrated by discrete hybridization to Mu1 at 1.1 and 1.5 kb. Subsequently, a sample of each TthIII-I digested genomic DNA was redigested with methylation sensitive HpaII and hybridized to the Mu1 probe, shown in

Figure 3.—Southern blots showing examples of methylation of HinfI sites in the Mu1 and Mu1.7 elements of inactive Mu plants. A, HinfI digests of genomic DNA from Mu-inactive plants derived from selfs (lanes 1 and 3) and sibling active plants (lanes 2 and 4). B, HinfI digests of genomic DNA from a Mu-active plant (lane 1) and sibling Mu-inactive plants (lanes 2 and 3) from an outcross to a non-Mu deltahbz tester. C, Restriction map of Mu1 and the related Mu1.7 element, showing the location of the probe used, and the restriction sites for HinfI (H), TthIII-I (T) and HpaII/MspI (unlabeled). Restriction sites are indicated by vertical lines above the element. The cross-hatched region of Mu1.7 is not present in the Mu1 element.

Figure 4.—Methylation of internal HpaII and MspI sites in the Mu1 elements of inactive Mu plants. The DNAs used here were from the same plants as in Figure 3A. Lanes 1, 5, 9, Mu-active plant; lanes 2, 6, 10, sibling Mu-inactive plant from selfed ear. Lanes 3, 7, 11, Mu-active plant; lanes 4, 8, 12, sibling Mu-inactive plant from selfed ear. Lanes 1–4 were digests with TthIII-I alone. Lanes 5–12 were double digests with TthIII-I and either HpaII (lanes 5–8) or MspI (lanes 9–12). The blot was probed with the same Mu1-specific probe as in Figure 3.
Figure 4, lanes 5–8. The HpaII/MspI digestion is expected to generate a fragment of 360 bp and several small fragments of less than 150 bp (Figure 3C). The Mu inactive samples in lanes 6 and 8 contain two fragments hybridizing to Mu at approximately 360 and 700 bp, while the Mu-active samples, in lanes 5 and 7, contain only the 360 bp fragment (the smaller HpaII digested fragments are not visible). This indicates that the Mu-inactive DNA samples are only partially digested by HpaII relative to their Mu-active siblings DNAs. Digestion by MspI gives results that are essentially the same as with HpaII. Mu-inactive DNAs, shown in lanes 10 and 12, hybridize at 360 bp and 700 bp while the Mu-active samples in lanes 9 and 11 hybridize only at 360 bp (again, the smaller fragments are not visible). We conclude that sequences internal to Mu1 termini in the Mu-inactive genomes are hypermethylated at the C\(^{5m}\)CGG and 5mCCGG positions, becoming resistant to HpaII and MspI digestion, but that the same sites are unmethylated in the Mu-active genome. These results on inactive plants derived from selfing are in agreement with previous observations with Mu-inactive plants derived from inbreeding (BENNETZEN 1987).

The methylation of genomic Mu DNA was also tested following Mu reactivation. Mu-inactive plants derived from selfs were crossed both to Mu-active; sh bz testers and to non-Mu; sh bz testers as described earlier (Figure 2). Six sibling progeny from each cross were assayed by HinfI, probed with Mu1 and compared. The results are shown in Figure 5A. The Mu1 elements in the genomic DNAs of Mu-inactive progeny from test crossed Mu-inactive plants remain resistant to HinfI digestion (lanes 1–6 of Figure 5A) giving many higher molecular weight fragments. In contrast, the Mu1 elements in genomic DNAs of reactivated Mu progeny derived from reactivation crosses are completely digested by HinfI, and show hybridizing bands only at 1.3 and 1.6 kb when probed with Mu1 (lanes 7–12 of Figure 5A). The methylation at HinfI sites in genomic Mu DNA is therefore reversed when the Mu transposons are reactivated. Similar results were obtained for the internal HpaII and MspI sites, i.e., the methylation of these sites was also completely reversed upon reactivation (not shown). Further, hypermethylation of the Mu1 element at bronze in bz Mum9 was also found to be reversed upon reactivation (Figure 5B, lane 3), consistent with the results observed for the Mu1 elements overall.

DISCUSSION

We have used a mutable allele bz Mum9 to monitor heritability of Mu transposon activity in a line exhibiting non-Mendelian transmission of Mu activity. The results from our study can be summarized as follows. (1) Mu activity was transmitted at high rates through both the male and female (Table 1). The rate of transmission through the female was both slightly higher and less variable than through the male. This difference was small in most cases, but when a plant is losing Mu activity at a high rate, the difference between male and female transmission can be substantial (Table 1, cross 8). (2) Mu activity lost through outcrossing was restored efficiently by crossing to active Mu plants (Table 2). The efficiency of reactivation was not significantly affected by the sex of the inactive parent. (3) Inactive Mu derived by selfing was also reactivated efficiently by crossing to active Mu plants (Table 3). In this case reactivation was slightly more efficient when the active Mu plants were the female in the reactivation cross. The reactivated state was heritable through at least three generations of outcrossing. (4) Hypermethylation of Mu1 elements occurred in Mu-inactive plants, regardless of whether they are derived by outcrossing or by selfing. In both cases, this hypermethylation could be completely reversed by crossing to active Mu plants, whether they were crossed as males or as females.

Our observations of heritable changes in Mu activity conflict with some of the expectations from published models. Following a discussion of differences in existing data and problems arising with current models, we present a simple model which is consistent with all reported data.

In one model, it has been proposed that the Mu system negatively regulates itself, by encoding or inducing a dominant-acting factor which both inactivates and methylates Mu DNA sequences (BENNETZEN 1987). The methylated Mu is reportedly progressively dominant to the active unmethylated Mu in the developing plant, causing a gradual, inevitable shutdown (BENNETZEN, BROWN and SPRINGER 1988). A second model predicts that Mu loss following inbreeding will be associated with Mu transposon methylation, and that neither the methylation nor the inactivity will be reversible (ROBERTSON 1986), i.e., that Mu inactivity is dominant once established. In both these models, it is proposed that loss of Mu activity from outcrossing will not be associated with hypermethylation of Mu elements. A third model also proposes that inactive, methylated Mu is dominant, but specifically when inherited from the female ("maternal effect," WALBOT 1986). In this model, inactive methylated Mu arises from outcrossing as well as inbreeding.

Our results show that the Mu inactivation which occurs in progeny of selfed Mu plants is correlated with Mu DNA hypermethylation, concordant with the predictions of ROBERTSON (1986), yet they can be efficiently reactivated by subsequently crossing to active-Mu plants. Since all the progeny of the reactivation cross had unmethylated Mu1 elements, this methylation must be completely reversible on crossing to
FIGURE 5.—Reactivation is correlated with loss of methylation of \textit{Mu1} and \textit{Mu1.7} elements. \textbf{A}, DNA from reactivated progeny of an inactive-\textit{Mu} plant derived from a self, crossed to an active \textit{Mu} plant as shown in Figure 2A, or inactive progeny from a cross to a non-\textit{Mu} tester as shown in Figure 2B, was digested with \textit{Hin}I and probed with a \textit{Mu1}-specific probe. Lanes 7–12, reactivated progeny; lanes 1–6, inactive progeny. \textbf{B}, Methylation of the \textit{Hin}I sites of the \textit{Mu1} insertion at the bronze locus in bzMum9 in an active \textit{Mu} plant (lane 1) and inactive sibling (lane 2) from a selfed ear; and re-activated progeny of the inactive plant (lane 3). The DNAs were digested with \textit{Hin}I, and the blot was probed with the \textit{Bz} probe. DNAs were from the same inactive and active plants that were used in the blot shown in Figure 3A (lanes 3 and 4). \textbf{C}, Restriction map of the \textit{Bz1} gene with the \textit{Mu1} insertion in bzMum9. The \textit{Bz1} intron is shown as an open box. Numbers indicate the positions of restriction sites relative to the start of transcription. The \textit{Mu1} insertion (at position 711) is shown above the \textit{Bz1} gene. The \textit{Bz1} probe used in (B) covered nucleotide numbers 472–1101. Only the \textit{Hin}I sites in the \textit{Bz1} gene that flank the \textit{Mu1} insertion are shown. H = \textit{Hin}I, T = \textit{Tth}III-I, N = \textit{Not}I.

active \textit{Mu} lines, and further, the inactive methylated \textit{Mu} elements appear to have no inhibitory effect on the \textit{Mu} elements of the \textit{Mu}-active genome. On the contrary, the inactive bzMum9 \textit{Mu1} insertion was reactivated to mutability by the cross to \textit{Mu}-active plants, and this reactivation may be almost immediate (i.e., within the first two or three divisions after fertilization, Figure 2C). Therefore the inactivation which follows selfing is completely reversible. The study by Roberton (1986) gave a different result: \textit{Mu}-inactivations following several generations of inbreeding were found to be irreversible, and the inactivation of \textit{Mu} in that study may therefore be quite different from that in our selfing experiments. A second difference in the study by Roberton is that we have used somatic mutability to assay \textit{Mu} activity, rather than frequency of germinal mutation. It has been suggested that the two activities are not necessarily regulated in the same manner (Roberton and Stinard 1989). \textit{Mu} inactivation following outcrossing in our study did not seem to differ from inactivation following selfing: in both cases inactivation is correlated with hypermethylation of genomic \textit{Mu} elements, is reversible, and the reactivation is accompanied by demethylation of the genomic \textit{Mu1} elements. The ability of inactive \textit{Mu} to be efficiently reactivated in our lines cannot be explained by a transient or unstable nature of the inactivation or reactivation that we observe. First, when the inactive \textit{Mu} plants are propagated by outcrossing to non-\textit{Mu}; Δ(\textit{sh} \textit{bz}) testers, they remain inactive in subsequent generations. Second, following reactivation by crossing to active \textit{Mu}; Δ(\textit{sh} \textit{bz}) plants, \textit{Mu} activity is heritable for at least three successive generations of outcrossing to non-\textit{Mu} testers.

In previous reports, the failure to completely re-activate \textit{Mu} in certain crosses led to the conclusion that inactive methylated \textit{Mu} was dominant (Walbot 1986; Bennetzen 1987). Since our data suggest that inactive \textit{Mu} following selfing or outcrossing is not dominant, the failure to reactivate \textit{Mu} in other experiments...
Loss and Restoration of Mu Activity

In reactivation crosses, Mu-inactive plants are crossed to Mu-active plants, and progeny are then scored for Mu activity. Therefore, a critical variable in reactivation crosses may be the Mu activity in the “active” parent. Our Mu-active sh bz/Δ(sh bz) plants are carefully selected as follows: shrunken bronze segregants [sh bz/Δ(sh bz)] are selected from ears segregating Sh bz Mum9/Δ(sh bz) which both show high levels of bzMum9 mutability (indicative of Mu activity) and are not losing Mu activity at all, or only at a very low rate (<2%) on the sh bz Mum9/Δ(sh bz) kernels. If, in the other studies, the Mu-active lines used are themselves losing Mu activity at significant rates, then they will not be completely effective in the reactivation crosses.

A second point clarified by our results is that the model that Mu activity is inhibited by a dominant cytoplasmic factor inherited from Mu inactive females (Walbot 1986) is not generalizable. Mu elements of Mu-inactive bzMum9 females are readily reactivated following crosses to Mu-active; sh bz males. Previous reports had stated that Mu-inactive females could not be reactivated following crosses to Mu-active males (Walbot 1986; Bennetzen 1987). Since our results rule out the possibility of a dominant factor inhibiting Mu activity, we suggest an alternative explanation. It is possible that in cases where reactivation of inactive females was significantly more efficient than reactivation of inactive males, the males used in the reactivation cross were themselves losing Mu activity and were therefore unable to reactivate Mu efficiently. Our results scoring Mu loss in reciprocal crosses to non-Mu testers show that loss of Mu activity is more likely to occur through male transmission than through female transmission (Table 1). While the average difference observed is relatively small (8.2% through the male vs. 3.8% through the female), the difference is significant, (P < 0.01), and in a plant that is losing Mu activity at a high rate, the difference in transmission can be quite substantial (for example cross 8 in Table 1: 43% loss through the male vs. a 12% loss through the female). Furthermore, in our own reactivation crosses (Table 3), using Mu-inactives from selfs crossed to Mu-active plants, restoration of mutability was slightly more efficient when the active Mu plant was the female, as we might expect. Martienssen et al. (1990) have shown that in their Mu lines, epigenetic Mu inactivity (correlated with hypermethylation of Mu elements) occurs progressively in the developing plant. This progressive inactivation means that the tassel, which develops from the plant apex, is more likely to produce methylated inactive Mu gametophytes than the ear, which develops from a lower bud. Thus, an increased rate of loss of Mu through the male may cause a sex bias in Mu inherit-

ance. Our data is consistent with these predictions (Martienssen et al. 1990).

In our model, regardless of the nature of the events that lead to inactivation, inactive Mu plants derived from selves or outcrosses are not different in their subsequent genetic behavior from non-Mu plants (i.e., plants that are not derived from Mutator lineages). The outcome of reactivation crosses therefore depends upon the behavior of the active Mu parent that is used for reactivation. Our results show that an inactive Mu system is neither dominant nor codominant over active Mu, and therefore supports a model in which inactivation is primarily due to the loss of a factor or factors required for maintenance of activity either due to segregation, or due to epigenetic silencing of the gene encoding the factor(s). Such a factor might bind to Mu sequences and catalyze Mu transposition activity, as well as protect Mu sequences from host methylases, either directly or indirectly by changes in the chromatin structure as suggested by the observations that the elements in Mu-active plants are hypomethylated at sites assayed by restriction enzymes (Chandler and Walbot 1986; Bennetzen 1987). The factor may be encoded by the putative autonomous Mu element (Qin, Robertson and El-lingboe 1991; Chomet et al. 1991). Nuclear proteins that bind specifically to the termini of the Mu elements have also been identified, though their functions have not been defined (Zhao and Sundaresan 1991). Random, or regulated loss of this factor either during mitotic divisions of the developing plant (Martienssen et al. 1990) or during meiotic division (Robertson 1986), might both inactivate Mu transposition and lead to hypermethylation of Mu elements. Crossing inactive Mu lines to active Mu lines would reintroduce the factor, which then causes the quiescent Mu elements to actively transpose and also protect Mu elements from methylation during the following replications in the developing embryo.

It remains possible that there may be negative modifiers of Mu activity in some of the genetic backgrounds used by other researchers. Genetic modifiers of other transposon systems have been previously described (McClintock 1958; Cuypers et al. 1988). It is possible that if such modifiers of Mu exist, they could lead to more frequent inactivation when present in Mu-active stocks. The inactive Mu lines derived from such crosses would then carry these modifiers, and could therefore result in higher rates of inactivation of Mu when crossed back to Mu-active plants. Our study cannot rule out the presence of modifiers in some backgrounds that could influence the outcome of crosses involving both Mu-active and Mu-inactive plants, but it does rule out models in which a dominant negative regulatory factor is obligatorily associated with inactivation of the Mu system or with
the subsequent maintenance of inactivity.

We thank D. S. Robertson for the bzMum9 stock, Rob Mortenssen for criticism, V. Chandler and D. S. Robertson for comments on the manuscript, and Sadie Arana for preparation of the manuscript. This research was supported by grants from the National Science Foundation (DCB-8702318), Pioneer Hi-Bred International and the Pew Foundation.

LITERATURE CITED


Communicating editor: W. F. Sheridan