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Robertson's Mutator system in maize: Studies on the regulation of activity and the prevalence of Mu1-homologous DNA sequences in diverse lines of maize

Roth, Bradley Allen, Ph.D.

Iowa State University, 1987
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Robertson's *Mutator* System in maize:
Studies on the regulation of activity and
the prevalence of *Mul*-homologous DNA sequences
in diverse lines of maize

by

Bradley Allen Roth

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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GENERAL INTRODUCTION

Robertson's Mutator system in maize was originally described in the late 1970s (Robertson, 1978a) as a system with the ability to induce new germinal mutations at an extraordinarily high frequency, approximately 50-fold above the background mutation rate. Previously, it had been shown by Rhoades (1936, 1938), McClintock (1946, 1956), Peterson (1953), and others that discrete units exist within the maize genome that are capable of "controlling" the expression of unlinked genes. These entities, termed controlling or transposable elements, are mobile genetic units, which can move from one chromosomal location to another within the maize genome. Upon transposition, an element may alter normal gene expression by inserting near or into a gene (McClintock, 1956). McClintock (1951) also demonstrated that loci containing controlling elements were associated with various chromosomal rearrangements such as breaks, inversions, deletions, and duplications. Transposable element systems have also been described and extensively studied in other prokaryotic and eukaryotic organisms (see reviews, Shapiro, 1983).

In a recent review, Lillis and Freeling (1986) divided known maize transposable element systems into four major classes: (1) controlling element systems, of which ten or more families have been identified (Fedoroff, 1983; Döring and
Starlinger, 1984; Pereira et al., 1985; Nevers et al., 1985), (2) a retroviral-like element called Bsl (Mottinger et al., 1984; Johns et al., 1985), (3) biologically uncharacterized insertions with structures similar to transposons (Nevers et al., 1985; McElflesh and Strommer, 1986), and (4) Mutator (Mu) (Robertson, 1978a).

Controlling element families have been shown to be composed of two types of elements; autonomous elements capable of encoding factors required for transposition (Dooner et al., 1986) and system regulation, and non-autonomous elements which are defective in these functions. When an autonomous element is present and active in a genetic background, transposition of non-autonomous elements may occur. In other words, a mutation induced by the non-autonomous element has the potential to become unstable (mutable) in the presence of the autonomous element. The Dt-al-m (Rhoades, 1936, 1938), Ac-Da (McClintock, 1946) and Spm (En-I) (McClintock, 1956; Peterson, 1953) systems are the best characterized controlling element systems on both the genetic and molecular levels. Both autonomous and non-autonomous elements have been cloned from the Ac-Da (Fedoroff et al., 1983) and Spm (En) systems (Pereira et al., 1985; Pereira et al., 1986). In general, the non-autonomous elements, Da and dSpm (I) were deletion derivatives of the autonomous elements Ac and Spm (En), respectively (Fedoroff et al., 1983; Döring et al., 1984; Pereira et al., 1986).
The transposable element named Bsl was genetically identified after infection of maize plants with barley stripe mosaic virus (BSMV) (Mottinger et al., 1984). Subsequent molecular characterization showed that the Bsl element had structural characteristics similar to previously cloned animal retroviruses (Johns et al., 1985).

Biologically uncharacterized insertions with structures similar to transposons have also been shown to occur in plants (see review, Nevers et al., 1985). McElfresh and Strommer (1986) described a procedure utilizing DNA denaturation and specific reassociation conditions to specifically select snapback DNAs for cloning. Some of the DNA sequences cloned were thought to be putative terminal repeated sequences from transposon-like structures.

Finally, a great deal of interest has centered recently on the Mutator system of maize. This review will focus on four aspects of the Mutator system of maize: (1) the genetics of the Mutator system; (2) molecular characterization of the transposable elements associated with the Mutator system called Mu elements; (3) the regulation of the Mutator system; and (4) the presence of endogenous Mu1-homologous DNA sequences in nonMutator lines, leading to a consideration of the possible origin of Mutator.
Genetic studies

Robertson's Mutator system was discovered during studies designed to locate \emph{y9} on chromosome 10 (Robertson, 1978a). F1 plants heterozygous for \emph{y9} and other marker genes were crossed as males to tester stocks or to standard lines (M14/W22 hybrid). Progeny seeds from these crosses were self-pollinated to check for the presence of \emph{y9}, then seeds from all selfed progeny ears were seedling tested. A 30-fold increase in the induction of new seedling mutants was observed in the \emph{y9} line compared to the control lines. Highly mutagenic stocks derived from this \emph{y9} line were termed Mutator lines by Robertson and the phenomenon of a high frequency of mutant induction was called Mutator activity. In further experiments, Robertson (1978a) found that the transmission of the Mutator activity did not follow simple Mendelian inheritance in that approximately 90% of the progeny derived by outcrossing a Mutator line showed Mutator activity. This led Robertson to speculate initially that three duplicate dominant genes might account for the high frequency of Mutator plants in the outcrosses; Mutator plants would then be expected to occur in frequencies of 75% or 50% in later outcross generations. However, high mutability (in about 90% of progeny) continued to be transmitted on outcrossing. Robertson (1978a, 1983) described the test shown in Figure 1 to determine the frequency of new mutations, and hence the Mutator activity of maize lines. He noted that approximately
Figure 1. Robertson's standard test for determining the mutation rate (Mutator activity) of Mutator and control lines
**Standard Mutator Test**

Female Parent  
Non-Mutator  
Parent  

2nd Ear (if available)  

\[ \times \]

Screen  
\[ \times \] Progeny  
for Mutants  

Male Parent  
Mutator  
Parent  

\[ \times \]

Screen  
\[ \times \] Progeny  
for Mutants  

Those \( F_1 \) crosses used  
in which neither parent  
segregated for a mutant  

A minimum of 50 \( F_1 \) seeds planted  

Each \( F_1 \) plant self-pollinated  
and the selfed ears screened  
for seedling mutants  

\[ \begin{array}{cccccccc}
\times & \times & \times & \times & \times & \times & \times & \times \\
* & * & * & * & * & * & * & * \\
\end{array} \]

\( * \) = Ears Segregating Mutants  

Mutation Frequency = \( \frac{\text{No. of } *}{N} \)
40% of the new mutants were unstable (mutable), which suggested to him that a controlling element system was involved in their induction. In order to test this, Robertson and Mascia (1981) determined if **Mutator** would substitute for the regulatory element in the following controlling element systems: *Dt-a-dt*, *Ac-Ds*, *En-I*, and *Fcu-r-cu*. No interaction was observed, and thus, no functional homology existed between **Mutator** and any of the controlling element receptors tested. When tested for **Mutator** activity, only *Ac-Ds* and *Fcu-r-cu* lines induced new mutations, but at a frequency much lower than that found in **Mutator** crosses. These tests demonstrated that if **Mutator** was a controlling element system, it was unrelated to these previously identified systems.

In further experiments to define the **Mutator** system, Robertson (1981a) tested two possible models of transmission of **Mutator** activity that could be responsible for the non-Mendelian patterns of inheritance. One model assumed that **Mutator** functioned as a single gene with segregation distortion (SD) activity, as had been described for the SD locus in *Drosophila* (Hartl and Hiraizium, 1976). If **Mutator** plants had SD activity, plants heterozygous for **Mutator** and a closely linked marker gene on the chromosome homologous to the one carrying **Mutator** should show a deficiency of the marker gene phenotype in a testcross. Testcrosses between **Mutator** lines
heterozygous for individual marker genes from each of the ten maize chromosomes and testers resulted in no deficiency in the segregation of the marker gene in any case, as did tests with a series of waxy marked translocations. Thus, no evidence of SD activity was found. The second model of transmission tested was cytoplasmic inheritance which plays a role in the transmission of the delta system (Minamori and Sugimoto, 1973) and with hybrid dysgenesis (Bregliano et al., 1980) in Drosophila. There was no evidence that Mutator was transmitted more effectively through the female parent compared to the male parent; thus, cytoplasmic transmission of Mutator was not occurring. More recently, Robertson (1985b) showed that the transmission of Mutator activity, as measured by the frequency of unselected mutations, was somewhat higher when Mutator plants were crossed as males. The basis for this is, at present, unknown.

Robertson has tested the interaction of several mutagens on the expression of Mutator activity. Ultraviolet light irradiation of mature pollen resulted in a synergistic mutagenic response. The synergistic response increased in a linear manner to reach a peak at 35 seconds and then decreased linearly to 45 seconds of irradiation (Robertson, 1982, 1984a). Gamma ray doses from 600 rads to 1,600 rads resulted in a reduced induction of new mutants in the Mutator stocks proportional to the dose of radiation applied (Robertson,
Tests were also conducted to determine what mutagenic interaction, if any, would occur when *Mutator* plants were treated with ethylmethanesulfonate (EMS). In the small populations tested, no apparent synergistic mutagenic effect was observed (Robertson, 1982).

In further studies, the distribution, induction, and stability of mutations induced in *Mutator* lines (so-called *Mutator* or *Mu*-induced mutations) were investigated. *Mutator* induces forward recessive mutations (Robertson, 1985b) with no specificity for susceptible loci (Robertson, 1978b). Putative *Mutator*-induced mutations have been mapped to all chromosome arms except 6S, 8S, and 8L (all of which contain few genetically mapped genes) by testing the mutants with A-B translocation stocks or by allelism tests (Robertson, 1978b; Donald Robertson, Iowa State University, Ames, Iowa, personal communication). The pattern of distribution of putative *Mutator*-induced mutants suggested that the system was a general rather than specific mutator system. *Mutator* activity and the induction of mutants at different specific loci, varied however (Robertson, 1985b). Of nine loci tested, the mutation frequencies ranged from $7.54 \times 10^{-5}$ to $4.0 \times 10^{-6}$. Similarly, Walbot and co-workers (1986) tested the frequency of the induction of mutants in the anthocyanin pathway loci. They also observed differential susceptibility of these loci to mutation in the *Mutator* background.
Germinal stability of Mutator-induced mutants has been studied at the gll, gl8, bt2, wx, adh1-S3034, and al loci. Little or no germinal reversion was seen for a gll and two gl8 mutants (Robertson, 1984b). However, a high reversion rate of 2.9 x 10^-3 was found for a bt2 mutant (Robertson, 1984b), and predicted for a mutable waxy allele by analysis of the pollen by staining (Sackitey and Robertson, 1984). Reversion of the Mu-induced mutant, adh1-S3034, occurred at a frequency of approximately 10^-5 revertants per pollen grain (Freeling et al., 1982). Germinal revertants of Mu-induced al mutants to A1 have also been recovered (Donald Robertson, Iowa State University, Ames, Iowa, personal communication). Somatic reversion (mutability), occurring in 40% of seedling mutants, was observed in loci of the anthocyanin pathway. Three a1, two a2, and eight bz1 Mu-induced mutable mutants have been described (Robertson, 1985b). Walbot (1986) has also described somatic mutability in Mu-induced bz1 and bz2 alleles. Studies of these mutants will be discussed later in relation to the regulation of the Mutator system.

In addition to insertional mutants, transposable element systems have been shown to induce deletions, duplications, inversions, and chromosome breaks. Insertion-sequence-like (IS) elements in Drosophila were shown to be associated with deletion mutants (Green, 1977). These types of rearrangements have been observed in maize with the dissociation component of
the \textit{Ac-Da} system (McClintock, 1951), and recently, have been associated with the \textbf{Mutator} system. Taylor and Walbot (1985) reported a 74 base pair (bp) deletion associated with a Mu-induced \textit{Adhl} allele, \textit{adhl-S3034a}. Alleman suggested that Mu-induced deletions were responsible for the appearance of seeds with sectors of recessive tissue when \textbf{Mutator} plants with aleurone and endosperm alleles were pollinated on to tester stocks (Mary Alleman, University of Wisconsin, Madison, Wisconsin, personal communication). Robertson and Stinard (1987) accumulated genetic evidence that showed that twelve putative Mu-induced \textit{vg2} mutants were the result of deletions (terminal and subterminal) of varying length. They hypothesized that a portion of the stable mutants generated by the \textbf{Mutator} system may therefore be the result of Mu-associated deletions.

\textbf{Molecular studies}

A mutable mutant allele of \textit{Adhl-S}, \textit{adhl-S3034}, was recovered from a genetic background containing Robertson's \textbf{Mutator} by crossing allyl alcohol-treated pollen grains with an \textit{adhl} tester plant (Strommer et al., 1982). The mutant exhibited only 40\% of the normal level of \textit{Adhl} RNA expression compared to the progenitor allele. Southern hybridization experiments using an \textit{Adhl} probe to detect progenitor and mutant \textit{Adhl} DNA sequences, revealed the presence of a DNA insertion
approximately 1.5 kilobase pairs (kb) in size at the mutant locus. This insertion was subsequently cloned (Bennetzen et al., 1984), found to be nearly 1.4 kb in length, and named Mill. The Mill insertion was localized to intron-1, 73 base pairs (bp) downstream from the 5-prime exon/intron junction in the *Adh₁-S* gene by sequencing the borders. A 9 bp direct duplication of the intron was associated with the ends of the 1.4 kb insertion. It was hypothesized (Bennetzen et al., 1984) that the Mill insertion affected *Adh₁* message levels by depressing RNA processing or transcription. Barker and co-workers (1984) later sequenced the Mill element. The element, shown in Figure 2, was 1367 bp in length, flanked by 9 bp direct repeats of host DNA at the insertion site, and had terminal inverted repeats of 213 bp and 215 bp which shared 95% homology. Two direct repeats of 104 bp with 96% homology were present in the internal portion of the element. Four open reading frames (ORFs) were also found, two on each DNA strand. The GC content of the entire element and the central 60 bp region of the element were approximately 70% and 26%, respectively.

Multiple copies (10 to 70 per diploid genome) of elements similar in size and structure to Mill (referred to as Mill-like elements) were found in *Mutator* plants (Barker et al., 1984; Bennetzen, 1984), whereas non-*Mutator* lines had none or only a few copies of the Mill-like elements (Alleman and Freeling,
Figure 2. Restriction maps of the Mul and Mul.7 transposable elements

Restriction endonuclease target sites are indicated by the vertical lines: H, HinfI; T, Tth111I; Al, Avai; A2, AvaII; Tq, TaqI; M, MluI; BE, BstEII; Bg, BglII; BN, BstNI; Sm, SmaI; Ss, SatII. The elements contain no sites for the restriction endonucleases BglI, BamHI, BglII, EcoRI, HindIII, KpnI, PstI, PvuII, Sau3A, or XbaI.
1986; Bennetzen, 1984). This was shown by cleaving genomic DNA of Mutator plants with restriction endonucleases, such as TthI or HinI, each of which cuts once within each terminal repeated sequence of Mut. Thus, all elements similar in size and structure to Mut, were reduced to a single-sized fragment which was detected by Southern blot hybridization to a Mut DNA probe. However, smaller and larger sequences homologous to Mut have also been observed by several groups (Taylor et al., 1986; Luther Talbot, University of Oregon, Eugene, Oregon, personal communication; Joseph M. Anderson and David Morris, Iowa State University, Ames, Iowa, personal communication; Brad Roth, unpublished results). A Mut-homologous sequence, 300-400 bp larger than Mut, was detected by hybridization in some Mutator lines (Bennetzen, 1984). The larger element, named Mut.7, also shown in Figure 2, contained 380 bp of unique sequence DNA inserted into a Mut-like element. It also showed a few restriction site polymorphisms as well as some small deletions relative to Mut (Taylor et al., 1986). A smaller Mut element called MutS was cloned from a Mutator-induced bzl mutant allele, bzl-Mut6; it appeared to be a simple deletion derivative of Mut (Joseph M. Anderson Iowa State University, Ames, Iowa, personal communication). In addition, another element, Mut3, was cloned and characterized from another mutant Adh allele, adh-3F124, which arose in a Mutator background (Karen Oishi, University of Arizona, Tucson, personal communication). The Mut3 element was
400 bp larger than Mu1, and contained Mu1-homologous terminal inverted repeats and one large open reading frame, the internal portion being quite unrelated to other Mu elements.

The patterns of inheritance of Mu elements may be followed by Southern hybridization experiments. Alleman and Freeling (1986) and Bennetzen and co-workers (1987) showed that Mu elements were capable of transposition. Southern hybridization analyses revealed that the Mu1 hybridization profiles changed in progeny plants relative to the parent plant when genomic DNAs of each were digested with restriction endonucleases that have no target sites within Mu1. Generally, about one half of the bands seen in progeny plants were not present in the parent suggesting that the Mu elements present in the Mutator line transposed to new genomic locations. Bennetzen et al. (1987) and Alleman and Freeling (1986) have pointed out that, because outcross progeny retain roughly one half of the parental bands in addition to novel (transposition) bands, transposition was most likely associated with the replication of Mu1-like elements. This is similar to the transposition of the bacterial elements Tn3 (Gill et al., 1978) and bacteriophage Mu (Toussaint and Resibois, 1983).

The implication that Mu transposable elements are involved in Mutator activity has been further substantiated by the isolation of Mu1-homologous DNA sequences from Mutator-induced mutable alleles. To date, Mu1-like element insertions have
been shown to be associated with the following mutations: adhl-S3034, the original Mut isolate (Bennetzen et al., 1984); al-Mum2 (O'Reilly et al., 1985); bzl-mul (Taylor et al., 1986); and shl (Ben and Francis Burr, Brookhaven National Laboratory, Brookhaven, New York, personal communication). A Mul.7 element has also been isolated from the bzl-mu2 allele (Taylor et al., 1986).

Sundaresan and Freeling (1987) have recently observed extrachromosomal forms of Mul and Mul.7 in active Mutator lines. These closed, circular elements were isolated from immature male flowers and were present in about 1 copy per 5 to 20 cells. Mu elements in this structural form were not found in inactive Mutator lines (i.e., no new mutations being induced). The investigators suggested that these extrachromosomal elements may therefore be transposition intermediates.

Regulation of Mutator and Mutator activity

We consider that the regulation of Mutator and Mutator activity involves those events or characteristics which maintain the active state of the Mutator system in a particular Mutator maize line. Regulation studies have included measures of Mutator activity, relationships between germinal and somatic mutability, the timing of Mutator activity, loss of Mutator activity, and the role of Mu element DNA modification in the
regulation of transposition and somatic excision. The
definitions of terms used in this section are given in Table 1.

Measuring Mutator activity

Several criteria are currently utilized to measure Mutator
activity.

(1) A standard method to measure Mutator activity was
originally described by Robertson (1978a). This was discussed
previously and shown in Figure 1. This method has proved to be
quite cumbersome and labor intensive. However, no more
efficient method is available to test for the presence of a
Mutator system in maize line because Mutator lines are defined
by their ability to induce new germinal mutants (Donald
Robertson, Iowa State University, Ames, Iowa, personal
communication).

(2) Germinal Mutator activity has been shown to be
correlated with the transposition of Mu elements to new
locations as shown by hybridization profiles of Southern blots
of genomic digests (Alleman and Freeling, 1986; Bennetzen et
al., 1987). Although experiments of this kind can provide a
reliable method to confirm the presence of Mutator activity,
these analyses are in fact more labor intensive and costly
compared to Robertson's standard test.

(3) The appearance of somatic mutability has also been
used to predict the presence of a germinally active Mutator
system (Chandler and Walbot, 1986; Walbot, 1986; Bennetzen, 1987). Some controversy exists concerning this point, however, because Robertson (1985f, 1986a) found that germinal activity and somatic mutability were not necessarily well correlated. Although the loss of somatic mutability seemed to be correlated with the loss of germinal activity, the converse was not always true (Robertson, 1985h). In some situations, somatic mutability was observed in lines which had no appreciable germinal activity. Furthermore, molecular evidence has associated germinal activity with a replicative transposition of Mul-like elements (Alleman and Freeling, 1986; Bennetzen et al., 1987), whereas somatic mutability was probably caused by the excision of a Mul-like element from a mutant allele (Robertson, 1986a). Taken together, these observations would suggest that one must be careful when using somatic mutability exclusively to predict the presence of a germinally active Mutator system.

(4) A phenomenon that may be used to predict the absence of Mutator activity, is the covalent DNA modification of restriction endonuclease target sites within Mu elements, which has been correlated with the loss of somatic mutability (Chandler and Walbot, 1986; Bennetzen, 1987). Modification of Mu elements was also observed in Mutator plants that had lost germinal Mutator activity due to inbreeding (David Morris, Iowa
State University, Ames, Iowa, personal communication). Modification will be discussed at length later in this section.

(5) The Mu element copy number may also determine Mutator activity (Robertson et al., 1985; Walbot, 1984). Active Mutator lines contain between 10 to 70 copies of Mu elements per diploid genome (Bennetzen, 1984; Alleman and Freeling, 1986; Bennetzen et al., 1987). Alleman and Freeling (1986) showed that Mu element copy number was maintained in outcross progeny plants which had Mutator activity. However, a doubling of Mu element copy number was observed upon self-pollination of an active Mutator plant. Plants that have lost Mutator activity by outcrossing have also been analyzed for Mu element copy number. These plants had fewer than ten copies of Mu elements per genome (Bennetzen et al., 1987; Mary Jane Skogen-Hagenson, Phillips Petroleum Co., Bartlesville, Oklahoma, personal communication). It is uncertain whether the loss of element copies accompanied, or was the causative factor in, the loss of Mutator activity from these plants. Walbot and Warren (1987) carried out extensive studies on the regulation of Mu element copy number in maize lines with either an active or inactive Mutator system, as measured by somatic mutability (although, as previously discussed, somatic mutability is not necessarily a reliable measure for Mutator activity). They demonstrated that the same average number of Mu elements were
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<td>Germinal Mutator activity</td>
<td>the increased frequency of new germinal mutants generated by the Mutator system measured by Robertson's standard seedling test</td>
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<td>Outcross-Mu-loss</td>
<td>loss of germinal Mutator activity in approximately 10% of the outcross progeny</td>
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<tr>
<td>Inbred-Mu-loss</td>
<td>loss of Mutator activity in Mutator lines that have been inbred for Mutator sequences</td>
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<tr>
<td>Somatic mutability</td>
<td>the ability to form wild type sectors on a mutant background in somatic tissue</td>
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<td>Mutable mutant</td>
<td>a Mutator-induced mutant that exhibits somatic mutability; approximately 40% of all mutants induced in the Mutator system are mutable</td>
</tr>
<tr>
<td>Stable mutant</td>
<td>a somatically stable Mutator-induced mutant</td>
</tr>
<tr>
<td>Element modification</td>
<td>the covalent DNA modification of restriction endonuclease target sites within Mu elements, shown to be correlated with the loss of germinal activity and/or somatic mutability</td>
</tr>
<tr>
<td>Copy number</td>
<td>the number of copies of Mu elements present in a maize line, expressed as number of copies per genome</td>
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transmitted through both male and female parents; however, there was wide absolute variation of the copy number of elements in individual progeny. Bennetzen has also noted that Mu element copy number generally was not a good criterion for predicting either Mutator activity (Bennetzen et al., 1987) or somatic mutability (Bennetzen, 1985).

(6) A final criterion which may be used to predict Mutator activity is based on the presence of circular Mul-homologous DNA in putative Mutator lines. It has been shown that a 100% correlation exists between the presence of circular Mu element DNAs with an active Mutator system (Sundaresan and Freeling, 1987). None have been seen with inactivated systems.

Timing of Mutator activity

Robertson measured the timing of Mu activity in the following ways: (1) positive allelism tests of phenotypically similar male-transmitted mutants demonstrated that mutants were induced premeiotically (Robertson, 1980); (2) sectors of seeds heterozygous for the same mutant on ear maps confirmed premeiotic induction and showed that the mutations occurred very late in development (Robertson, 1980); (3) the induction of somatic sectors in Mu plants heterozygous for plant and endosperm marker genes did not occur, thus confirming the hypothesis that Mutator activity did not occur throughout maize ontogeny (Robertson, 1980); (4) mutants that occurred in less
than Mendelian ratios, suggested that Mutator was active early in development at a time before cell lineages that determine the ear and tassel bifurcate (Robertson, 1986d); (5) discordant endosperm mutants suggested that activity might also occur during the gametophyte stage of development (Robertson, 1985d, 1986d); and (6) gamma ray and ultraviolet light treatment of mature pollen tentatively established the induction of mutants during the first cell divisions after fertilization (Robertson, 1986d). Studies on the timing of Mutator activity also demonstrated that it was triggered developmentally, rather than chronologically (Robertson, 1980). Robertson also showed that Mutator was most active in the germ line tissue very late in development (Robertson, 1980, 1981b). As discussed earlier, mutagenic events associated with the Mutator system, are presumably the result of transposition of the Mu elements (Alleman and Freeling, 1986; Bennetzen et al., 1987). Thus, these studies on the timing of Mutator activity provide information concerning the time during development when Mu elements may actually transpose.

Loss of Mutator activity

In the original description of the Mutator system, Robertson (1978a) demonstrated that Mutator activity was occasionally lost when Mutator plants were outcrossed. A later study of 563 outcrosses suggested that loss could be expected
in approximately 10% of the outcross progeny (Robertson, 1985g). The loss of Mutator activity in this manner was subsequently termed, "outcross-Mu-loss" (Robertson, 1986b). Outcross-Mu-loss lines do not regain Mutator activity either by continued outcrossing or inbreeding these loss lines (Donald Robertson, Iowa State University, Ames, Iowa, personal communication). However, germinal activity was observed in the progeny plants of outcross-Mu-loss lines crossed with active Mutator lines (Robertson, 1986b). This showed that outcross-Mu-loss was not due to a cellular environment hostile to the normal functioning (i.e., transposition) of Mu elements from the active Mutator parent. Somatic mutability has also been shown to be lost upon outcrossing (Bennetzen, 1985; Walbot, 1986). As with germinal activity restoration, somatic mutability has been restored in these lines by outcrossing the somatically stable mutant line to a line with an active Mutator system (Robertson et al., 1985; Walbot, 1986).

Mutator activity may also be lost by inbreeding Mutator lines for several generations (Robertson, 1983, 1986b). These lines were called inbred-Mu-loss lines (Robertson, 1986b). Initial experiments showed that the F1 (called Mu², i.e., two putative doses of Mutator) progeny which resulted from crossing two different Mutator lines (previously maintained by outcrossing) had a 1.5-fold increase in Mutator activity over that of the parents. If two Mu² plants were crossed, the
progeny plants ($\text{Mu}^4$) show no further increase in germinal activity. As this inbreeding regime was continued, germinal activity decreased rapidly and was lost entirely in $\text{Mu}^{16}$ plants. **Mutator** activity was not restored when the experiment was continued (up to the sixth generation of inbreeding) (Donald Robertson, Iowa State University, Ames, Iowa, personal communication). Robertson (1987) repeated these studies and found, for the most part, similar results. Robertson (1983) initially hypothesized that loss of **Mutator** activity due to inbreeding occurred because of an increased copy number of $\text{Mu}$ elements. However, as discussed earlier, several groups showed that although $\text{Mu}$ element copy number doubles after the first inbreeding (or selfing) generation, it then leveled off as inbreeding continued (Alleman and Freeling, 1986; Bennetzen et al., 1987; David Morris, Iowa State University, Ames, Iowa, personal communication). Southern blot hybridization analyses showed that loss of **Mutator** activity in these lines was accompanied by loss of $\text{Mu}$ element transposition (Bennetzen et al., 1987). In later experiments by Robertson (1986), inbred-$\text{Mu}$-loss lines were crossed to lines with an active **Mutator** system. The resulting progeny showed complete or nearly complete inactivation of the incoming active $\text{Mu}$ system contributed by the $\text{Mu}$ parent. If $\text{Mu}$ element copy number was the only determinant for an active **Mutator** system, then simply outcrossing an inactivated line should have restored **Mutator**
activity. However, once inactivation was established, inbred-Mu-loss lines did not recover Mutator activity even upon repeated outcrossing. Loss of Mutator activity was passed on to most of the progeny of crosses between inbred-Mu-loss and active Mu lines (Robertson, 1986b). In fact, it appeared that the inheritance of inbred-Mu-loss and of Mutator activity were similar.

Mutator activity was also shown to be lost when active Mutator systems were transferred into various inbred maize lines (Robertson, 1986c). Mutator plants were crossed with inbred lines B73, Mo17, A632, and B76, and the progeny then subsequently backcrossed five times. Progeny of each backcross generation were tested for the retention of Mutator activity, which occurred rarely in these crosses. The generation in which loss occurred varied from inbred to inbred; however, Mu activity was generally lost by the fifth generation of backcrossing except for two series of crosses with A632. A decrease in the copy number of Mu elements was observed when Mutator activity was lost but no modification of the Mu elements was evident (David Morris, Iowa State University, Ames, Iowa, personal communication). Similar results were obtained by Bennetzen and coworkers (1987), who reported loss of Mutator activity in two or fewer generations when Mutator was transferred to inbred lines A188, B73, P8, Q66. He also reported that the following inbred lines could be used to
maintain Mutator and Mutator activity: A632, Ms71, Oh43, H95, H99. He suggested that hybrid lines could also be used to maintain Mutator stocks (e.g., Robertson's maintenance of Mutator in Q60 and B70 hybrid lines). Mutator activity has been maintained, and Mu elements have been built up to high copy number in Freeling's 1S2P inbred line (Strommer et al., 1982).

Somatic mutability, as discussed earlier, has been used as a measure of Mutator activity, or, more specifically, as a measure of the excision of a Mu element from a particular locus (sometimes called the reporter allele). The inheritance of somatic mutability of Mutator-induced mutable mutants of the anthocyanin pathway has been studied by several groups (Walbot, 1986; Robertson, 1985e, 1985h, Bennetzen, 1985). The presence of high somatic mutability (as measured by many somatic reversion events) in bz2-mul was shown to be a labile condition (Walbot, 1986), with a tendency to become weakly mutable (few somatic reversion events) to stable (no somatic reversion events) during either outcrossing to standard lines or upon self-pollination (Walbot, 1986). Walbot showed that the inactive state (stable) of bz2-mul was usually permanently maintained with only rare reactivation of somatic mutability in subsequent crosses to standard lines (Walbot, 1986). Robertson has also shown that self and sibling crosses between plants from somatically stable kernels of al-Mum2, as well as
outcrosses to \textit{al sh2}, do not reactivate somatic mutability of this locus (Donald Robertson, Iowa State University, Ames, Iowa, personal communication). Walbot (1986) observed a strong maternal effect on the inactivation of somatic mutability on \textit{bz2-mul}, and on the efficient reactivation of cryptic \textit{bz2-mul} alleles when crossed to an active \textit{Mutator} line. In general, this showed that the mutability state of the female parent determined the mutability state of the progeny. Other groups have not seen this phenomenon in such a clear cut manner (Jeffrey Bennetzen, Purdue University, West Lafayette, Indiana, personal communication; Michael Freeling, University of California, Berkeley, California, personal communication). However, Robertson and coworkers (1985) and Bennetzen (1985) restored somatic mutability in stable \textit{al-Mum1} and \textit{al-Mum3}, and \textit{bz-Mum4} derivatives of the mutable alleles, respectively, by crossing with active \textit{Mutator} lines. Although the condition of somatic mutability was labile in \textit{Mutator}-induced mutants, generally the "more mutable" the parental kernel was, the greater chance that its progeny kernels would be so as well. Conversely, if a parental kernel showed low somatic mutability or was stable, the progeny kernels were only as mutable as the parental kernels (Bennetzen, 1985; Donald Robertson, Iowa State University, Ames, Iowa, personal communication).
Mu element modification and regulation of Mutator activity

Regulation of transposable element activity by DNA methylation has been observed with IS10, an insertion sequence in E. coli (Roberts et al., 1985) and in the Ac-Ds system of maize (Schwartz and Dennis, 1986; Chomet et al., 1987). Chandler and Walbot (1986) demonstrated, by Southern blot hybridization, that the loss of somatic mutability in a Mutator-induced aleurone gene, bz2-mul, was correlated with an inhibition of digestion of Mu elements by the restriction enzymes HinfI, MluI and BglII. Plants were identified in which this so-called modification of all Mu elements occurred within one generation. Other plants contained both modified and unmodified Mu elements which suggested that modification could also occur in a progressive manner over several generations. The experimenters hypothesized that DNA modification of Mu elements resulted in nonfunctional elements, incapable of somatic excision (Chandler and Walbot, 1986).

Generally, in active Mutator plants, the Mu elements are undermodified compared to total maize DNA (Bennetzen, 1987). It was shown that intercrossing Mutator lines lead, again, to a hypermodification of Mu elements in plants which had lost Mutator activity and where Mu element transposition no longer occurred (David Morris, Iowa State University, Ames, Iowa, personal communication; Jeffrey Bennetzen, Purdue University, West Lafayette, Indiana, personal communication). Bennetzen
concluded that modification of Mu elements was due most likely to methylation of cytosine residues at the 5-prime position in the DNA sequences 5'-CG-3' and 5'-CNG-3' because restriction endonucleases sensitive to these types of methylation, such as EcoRI, MspI and HpaII, were not capable of digesting Mu elements present in the inactive lines. In fact, some sites for potential C-methylation in Mu elements showed a higher degree of methylation than others (Bennetzen, 1987). The modified state of Mu elements, like the loss of Mutator activity, was stable and heritable upon outcrossing. Inactive Mutator lines with modified Mu elements showed a maternal dominance for inactivating and modifying an incoming active Mutator system in subsequent crosses. Although Mutator activity may be lost through outcrossing (Robertson, 1978a, 1986b), no modification of Mu elements was observed in these lines (Bennetzen, 1987; David Morris, Iowa State University, Ames, Iowa, personal communication).

Endogenous Mu-like homologous DNA sequences in nonMutator lines and possible origins of the Mutator system

The prevalence of Mu-like homologous DNA sequences in various lines of maize has been investigated by several groups (Bennetzen, 1984; Chandler et al., 1986). Bennetzen (1984) reported that Mu-like elements were present in multiple copies only in lines derived from Robertson's original Mutator stock.
Several cornbelt inbred lines and three exotic teosinte lines were included in these analyses and showed no hybridization to a Mul probe. Chandler and coworkers (1986) later showed that Mul-homologous DNA sequences were, in fact, present in one to three copies per haploid genome in all cornbelt inbred lines tested (W22, W23, Ky21, A188, B73, and B37), as well as in Wilber's Knobless Flint and Black Mexican Sweet. Approximately forty copies of sequences homologous to the Mul termini were also shown to be present in these lines. Restriction endonuclease mapping suggested that some of the Mul-homologous sequences in these lines were very similar in structure to Mul. However, the lines tested showed no genetic evidence for the high mutagenic activity associated with Mutator lines.

Many maize lines have also been shown to contain DNA sequences homologous to the transposable elements, Ac and Spm (Fedoroff et al., 1983; O'Reilly et al., 1985). However, like the maize lines with Mul-homologous sequences, most lines containing Ac and Spm sequences, showed no genetic activity characteristic of these systems most lines showed no genetic activity characteristic of these systems.

Obviously, a crucial question which arises is: are the Mu-homologous sequences present in these lines capable of giving rise to active Mu elements, the progenitors of an already active system, or are they merely relics of previously active systems? It was hypothesized that the creation of
stable inbred lines by maize breeders over many generations by selfing and/or sibling crossing, may have selected for the loss of active transposable element systems by element modification, dilution of element copy number, or segregation of regulatory factors (Chandler et al., 1986). In a species such as maize, with a high level of inbreeding (natural and artificial), active transposable element systems are exposed to selection almost immediately due to the induction of recessive lethal or sterile mutations (Charlesworth and Langley, 1986).

McClintock (1984) hypothesized that environmental and genetic stresses, which have been correlated with increased mutability in plants, may be responsible for activating previously silent transposable elements in maize. This was exemplified by McClintock's (1946) discovery of the Ac-Ds system. She concluded that a chromosome breakage event induced the activation of the Ac-Ds system (McClintock, 1984). In any particular maize line, dormant elements with the potential for becoming transposable, as well as other repetitive DNAs, may be activated to transpose in response to stress or challenges that are beyond measurement. For example, treatments which invoke DNA repair pathways may be responsible for transposable element activation. It is also possible that previously dormant elements may become transiently demethylated, and thus, activated (Chandler et al., 1986).
In the research for my doctoral dissertation, I have addressed several questions concerning Robertson's Mutator transposable element system of maize. The research reported here involves the genetic and molecular analyses of an inbred lineage of the Mutator-induced mutable Al mutant, al-Mum2, and of the prevalence of "mutator" activity and Mul-homologous DNA sequences in widely diverse lines of maize. I report the inheritance of the expression of somatic mutability in an inbred lineage of al-Mum2. I also report on the characterization of the Mu elements present in the al-Mum2 lineage, describing element copy number, state of modification, and their effects on somatic and germinal Mutator activity. Finally, I report on the prevalence of "mutator" activity and Mul-homologous DNA sequences in widely diverse (geographically) nonMutator lines of maize. Because of the variety of topics covered in my research, I have chosen to present my results as a series of separate papers, or sections.

Section I reports on the inheritance of expression of somatic mutability in an inbred lineage of al-Mum2. This section contains three major areas: initial genetic crosses, crosses to characterize loss of somatic mutability, and statistical analyses. Isolation of the al-Mum2 allele, initial genetic crosses, and tests for germinal Mutator activity, were
carried out by the second author, D. S. Robertson. My contribution as senior author entailed all crosses made to characterize the loss of somatic mutability in this line, the statistical analyses carried out on these crosses, with the advice of the second author, D. W. Morris, and the interpretation of all data.

Section II presents the results of hybridization analyses to characterize the Mu elements present in the al-Mum2 inbred lineage. My contribution as senior author involved all molecular analyses (carried out in the laboratory of D. W. Morris), and interpretation of the molecular data. This section reports on the modification of Mu elements in the al-Mum2 lineage and its relation to somatic mutability and germinal activity.

Section III culminates my doctoral research. In this section, I report on the prevalence of "mutator" activity and Mul-homologous DNA sequences in widely diverse nonMutator lines of maize. A large number of nonMutator lines were screened by the second author, D. S. Robertson, for the presence of "mutator" activity. Only two lines and their derivatives were found to exhibit a "mutator" activity. However, hybridization analyses revealed the presence of Mul-homologous DNA sequences in most lines screened. We consider the possible origin of Mutator in the light of these results.
My dissertation research was completed under the supervision of Dr. David W. Morris who provided laboratory space and supplies, but more importantly; gave encouragement, guidance, and friendship.
SECTION I. THE EFFECT OF INBREEDING ON SOMATIC MUTABILITY AND GERMINAL ACTIVITY IN A MUTATOR-INDUCED ALEURONE MUTANT OF ZEA MAYS L.
The effect of inbreeding on somatic mutability and germinal activity in a Mutator-induced aleurone mutant of Zea Mays L.

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Robertson's Mutator maize lines contain a highly mutagenic transposable element system. Mutator induces new germinal mutations at a frequency 50-fold above the background level. Approximately 40% of the mutants induced are somatically unstable (mutable). It was previously shown that inbreeding Mutator lines resulted in the loss of germinal Mutator activity. Thus, we produced an inbred lineage (by selfing and sibling crosses) of the Mutator-induced mutable mutant, al-Mum2, to test an hypothesis that somatic mutability, like germinal activity, would be lost after three to four generations of inbreeding. On the contrary, a high level of somatic mutability was maintained through seven generations of inbreeding. Germinal activity was also maintained in some plants through the sixth inbred generation. We hypothesize that a functional change in Mutator regulation occurred in this line. Some loss of somatic mutability was observed after the fifth generation of inbreeding. The events that led up to this loss were not dependent on the direction of the cross performed. However, further inactivation of somatic mutability in subsequent crosses was dependent on the level of mutability in the female parent.
INTRODUCTION

Nearly a decade ago, Robertson described a stock of maize which induced a high frequency of recessive mutations at a number of different loci (Robertson 1978, 1985a). This line of maize was subsequently named Mutator. Mutator was shown to be transmitted in a non-Mendelian manner as a dominant trait to approximately 90% of the outcross progeny when Mutator plants were crossed either as males or females to non-Mutator standard lines (Robertson 1978). Robertson also showed that about 40% of these mutants were unstable (mutable) which strongly suggested that transposable elements were involved in their induction. This was substantiated when an unstable mutant allele of Adh1, adh1-S3034, isolated from a Mutator background, was found to contain a DNA insertion 1.4 kilobase pairs (kb) in size in the first intron of the gene (Strommer et al. 1982; Bennetzen et al. 1984). The DNA insertion, named Mül, was 1367 base pairs (bp) long and had some of the structural characteristics of previously cloned transposable elements, such as terminal inverted repeats. In addition, it had caused a 9 base pair (bp) duplication of the host DNA at its site of insertion (Barker et al., 1984). Mül elements have been implicated in Mutator activity because they are present in high copy numbers only in Mutator lines (Bennetzen, 1984), and they, and
structurally related elements, called Mul.7 elements (Taylor et al., 1986), have been found to be associated with most Mutator-induced mutant alleles (for a recent review see Lillis and Freeling, 1986; Döring and Starlinger, 1986). Alleman and Freeling (1986), and Bennetzen and coworkers (1987) also demonstrated transposition of Mul elements by comparative Southern hybridization analyses of parent and progeny DNAs.

Mutator activity, as measured by the frequency of induction of new germinal mutations, may be lost either by outcrossing or inbreeding Mutator stocks (Robertson 1983, 1986). Loss of Mutator activity occurred in approximately 10% of outcross progeny (referred to as outcross-Mu-loss) (Robertson 1985b, 1986). Some outcross-Mu-loss stocks were restored to Mutator activity if an active Mutator system was introduced by crossing (Robertson, 1986). Loss of activity was also seen to occur after three to four generations of intercrossing Mutator stocks (inbred-Mu-loss) (Robertson 1983, 1986). Inbred-Mu-loss lines did not regain Mutator activity even after three successive generations of outcrossing. Furthermore, when inbred-Mu-loss stocks were crossed with active Mutator systems, no reactivation occurred (Robertson, 1986), and the incoming active Mutator system itself was inactivated.
Studies have also been carried out to determine if the somatic mutability exhibited by some Mutator-induced mutant alleles can be lost under similar conditions. Robertson and coworkers (1985), for example, demonstrated that stable derivatives of al mutable lines can be obtained by outcrossing to al tester lines. In the stable derivatives, loss of mutability was accompanied by the loss of germinal Mutator activity. In some cases, mutability could be restored simply by crossing to an active Mutator line. Walbot (1986) has described the loss of somatic mutability at bz2-mul. She found that mutability could be partially or completely lost either during outcrossing to tester lines or upon self-pollination. In this line, loss of mutability was, for the most part, permanent and could not be restored. In addition, Walbot noted a strong female effect on the inheritance of somatic mutability, when she found that, in most instances, highly mutable seeds used as the female parent produced highly mutable progeny ears, and female parental seeds of low mutability produced ears of low mutability.

In this paper, we describe genetic studies involving a Mutator-induced A1 allele, al-Mum2 (Robertson et al., 1985; O'Reilly et al., 1985). This mutant exhibits colorless to pale purple seeds with small purple spots in the aleurone, where the ability to synthesize aleurone pigments has been
restored, presumably by Mu element excision from the mutant locus. A lineage of this allele was produced by interbreeding and selfing with the assumption that inbreeding would result in the loss of somatic mutability. Somatic mutability in the \textit{a1-Mum2} lineage was retained through the seventh inbred generation, but loss of somatic mutability occurred in some of the progeny of the fifth intercross generation. Germinal activity was also retained through six generations of inbreeding. We describe the crosses that resulted in the loss of somatic mutability, the progeny of subsequent crosses involving plants from seeds with reduced mutability, and a putative female effect on the loss of somatic mutability in reciprocal crosses of plants from low mutable seeds with those from highly mutable seeds. We also demonstrate that germinal activity was not always correlated with somatic mutability in this \textit{a1-Mum2} lineage.
MATERIALS AND METHODS

Maize stocks

During the summer of 1980, three independent, putative Mutator-induced mutable mutants were produced at the A1 locus by applying pollen of a Mutator line (A1 Sh2/A1 Sh2) to the silks of a1 sh2/a1 sh2 tester plants (sh2 is a mutant allele of Sh2, used here as a contamination marker). Seeds of one a1 mutable mutant, designated a1-Mum2, were pale purple and had small spots of fully colored cells indicative of somatic reversion events presumably caused by the excision of the Mu element from the locus resulting in the restoration of normal A1 gene function. The original a1-Mum2 mutant kernel was selfed, and outcrossed to a1 sh2 to confirm that an a1 mutant had been produced. Plants from the seeds of selfed progeny ears were then outcrossed to a fully colored purple standard line and assayed for Mutator activity by Robertson’s standard test (Robertson 1978, 1983). The selfing and intercrossing regime shown in Figure 1 was then established with this material and included successive selfing and/or sibling crosses. Highly mutable seeds were planted in each case through the 1985-86 crosses.

Crosses during the summer of 1986 involved material that first exhibited a significant loss of somatic mutability. Unless otherwise indicated, the highly mutable seeds used in
Figure 1. Pedigree and inbreeding regime of al-Mum2 Class 4 stocks.

The original isolate, 80-1223-2, was selfed and in the following generation outcrossed to a purple standard line. Subsequent selfing or sibling crosses are indicated. X = al-Mum2 sibling crosses (or outcrosses to purple standard lines as indicated), x = self pollination.
Pl Mu x al sh2

80-1223-2
(original isolate)

Purple stand. x 81-82-5504

82-2153

82-83-2581

83-8207-1

84-1119

85-6151

81-82-5503 x Purple stand.

82-2154

82-83-3582

83-8207-2

84-1120

85-5151

 Different ears than the '85 - '86 ears

85-86 (-5558 x -5559) → 86 (-607, -609, -821)
85-86 (-5558 x -6558) → 86 (-819)
85-86 (-5559 x -5558) → 86 (-821)
85-86 (-5559 x -6558) → 86 (-617)
85-86 (-5559 x -6559) → 86 (-611, -807)
85-86 (-6558 x -6559) → 86 (-613, -813)
85-86 (-6559 x -6558) → 86 (-615, -619, -815)

86 (-8109, -9110)
86 (-9109, -9110)
86 (-809)
86 (-811)
86 (-817)
86 (-623)
86 (-823)

Duplicate planting of Class 4 highly mutable line, 85-86-6558
the 1986 crosses were sibling kernels to the parents of the 1985-86 family 6558, which was a putative homozygous, highly mutable stock.

Classification and quantification of al-Mum2 somatic mutability

Seeds from the progeny ears from the 1985-86 and 1986 crosses were classified according to the scale shown in Figure 2: Class 1, stable; Class 2, low mutability; Class 3, medium mutability; Class 4, high mutability; Class 5, purple. Figure 3 illustrates an ear with all Class 4 kernels (top), an ear segregating for Class 4 through Class 1 kernels (middle), and an ear segregating for Class 2 and Class 1 kernels (bottom).

The Class average for each ear was calculated by multiplying the total seed count of each class represented on the ear by the number of that particular class, adding the weighted totals, and dividing by the total number of seeds on the ear. The Class average was calculated in a similar manner for the population of seeds within the families from exact reciprocal crosses made in the summer of 1986 (e.g., seven ears from 609/809 crosses and seven ears from 809/609 crosses).
Figure 2. Classification scale for \(a1\text{-Mum2}\) and stable alleles

From crosses involving \(a1\text{-Mum2}\), the range of somatic mutability between \(a1\) stable (Class 1, top kernel), and \(A1\) stable (purple, Class 5, bottom kernel) includes: Class 2 (first row of three kernels), Class 3 (second row), and Class 4 (third row) kernels.
Figure 3. Examples of progeny ears from \textit{al-Mum2} crosses

Ears which contain only Class 4 kernels (top), Class 1, 2, 3, and 4 kernels (middle), and Class 2 and Class 1 stable kernels (bottom) are shown.
Statistical analysis

Ordered classification frequency analyses (Snedecor and Cochran, 1980) were performed on the 1986 reciprocal cross families to determine if significant differences in somatic mutability existed within populations of seeds in reciprocal cross families, and on each individual reciprocal cross to test for differences between progeny ears.
RESULTS

The al-Mum2 interbred lineage was produced to investigate the conditions which lead to the inactivation, or reduction, of somatic mutability. Figure 1 shows the pedigree of this lineage which generated the material used in the 1985, 1985-86, and 1986 crosses.

Sixteen ears were produced as a result of intercrossing families 5151 and 6151 in 1985. Seven ears consisted entirely of Class 4 seeds; six had predominantly Class 4 seeds with a few Class 2 seeds; one had predominantly Class 4 seeds with a few stable seeds (Class 1); and two ears ranged from Class 4 to Class 2, and Class 4 to Class 1, respectively.

The lines used in the 1985 crosses had undergone 5 generations of inbreeding (either selfing or sibling crossing, see Figure 1), producing lines with the equivalent of 32 putative "doses" of Mutator. Compared to a previous inbreeding program described by Robertson (1983), the inbreeding in this experiment was more intense because it involved three generations of selfing. Nevertheless, somatic mutability and germinal activity were maintained throughout the inbreeding regime.

In the 1985-86 winter nursery, seeds from four of the ears from the 85-6151 x 85-5151 crosses were sown (as
families 5558, 5559, 6558, 6559) and the intercrossing continued. The results from these are shown in Table 1. Although all the seeds planted were from ears that had only, or predominantly, Class 4 kernels, intercrosses between families 5558, 5559, and 6559 showed a marked loss of mutability. On the other hand, intercrosses between plants in family 6558 with families 5558, 5559, and 6559 gave ears consisting of all, or mostly, Class 4 seeds. In cases where exact reciprocal crosses were made, the Class average of the progeny was similar, whatever the direction of the cross.

In the 1986 summer nursery, intercrosses between plants from four additional 1985 ears were made. The results from these crosses, shown in Table 2, were similar to those obtained in the previous crosses (which were made with plants from sibling ears). As before, two types of families could be discerned: those that produced ears with only, or predominantly, Class 4 seeds (called high lines), and those that gave ears with predominantly Class 2 and Class 1 seeds (designated low lines). In crosses between high and low mutability lines, highly mutable seeds prevailed in the progeny ears whether the high line was used as the male or female parent.

One of the 1985 ears, 85-6151-8/5151-3T had 237 Class 4, 64 Class 3, and 28 Class 2 seeds. To investigate the inheritance of the Class 2 phenotype from this cross, eight
Table 1. Results of intercrossing plants from al-Mum2 Class 4 stocks in the 1985-86 winter nursery

<table>
<thead>
<tr>
<th>85-86</th>
<th>Family/ Plant #</th>
<th>Mutability Classification(^a)</th>
<th>Class Average</th>
<th>Intercross Ear</th>
<th>Exact Reciprocal(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family/ (d' parent)</td>
<td>Plant #</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6558-1</td>
<td>6559-2</td>
<td>4.00(^c)</td>
<td>-d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6558-2</td>
<td>6559-6</td>
<td>4.00(^c)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6558-4</td>
<td>5559-4T</td>
<td>4.00(^c)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6558-5</td>
<td>5558-5</td>
<td>4.00(^c)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6558-6</td>
<td>5559-5</td>
<td>3.98(^c)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6558-7</td>
<td>6559-5</td>
<td>4.00(^c)</td>
<td>4.00(^c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6558-8</td>
<td>6559-3</td>
<td>3.99(^c)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6558-8T</td>
<td>5559-2</td>
<td>4.00(^c)</td>
<td>4.00(^c)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Classification for 6558 parent ear: 230 Class 4 seeds)

| 6559-1 | 5558-7 | 2.02 | 2.09 |
| 6559-2 | 5559-8 | 2.03 | - |
| 6559-3 | 5559-1 | 2.19 | - |
| 6559-6 | 6559-5 | 3.99\(^c\) | - |
| 6559-7 | 6559-8 | 3.95\(^c\) | - |
| 6559-8 | 5559-9 | 1.98 | - |
| 6559-9 | 5559-9 | 1.84 | 1.47 |

(Classification for 6559 parent ear: 310 Class 4, 1 Class 3, 1 Class 2 seeds)

| 5558-1 | 6558-1 | 3.89\(^c\) | - |
| 5558-2 | 5559-6 | 1.91 | 1.73 |
| 5558-3 | 6558-2 | 4.00\(^c\) | - |
| 5558-4 | 6558-4 | 3.93\(^c\) | - |
| 5558-5 | 5559-7 | 1.98 | - |
| 5558-6 | 5559-3 | 2.04 | 2.35 |

(Classification for 5558 parent ear: 234 Class 4, 1 Class 3 seeds)

| 5559-1 | 6559-7 | 2.56 | - |
| 5559-4T | 5558-4 | 2.17 | - |
| 5559-7 | 5558-3 | 2.02 | - |
| 5559-8 | 5559-1 | 1.97 | - |

(Classification for 5559 parent ear: 292 Class 4 seeds)

\(^a\)Mutability scale as in Materials and Methods.
\(^b\)Exact reciprocal crosses are recorded only once.
\(^c\)Crosses involving family 6558.
\(^d\)No test.
Table 2. Results of intercrossing plants from *al-Mum2* stocks (seeds) from sibling ears of material from Table 1 in the 1986 summer nursery

<table>
<thead>
<tr>
<th>1986 Family/ Plant #</th>
<th>Mutability Classification&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Class Average</th>
<th>Intercross Ear</th>
<th>Exact Reciprocal&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family/ Plant #</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family/ Plant #</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Classification for 8109 parent ear: 188 Class 4 seeds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8109-1 9109-3</td>
<td>1.95</td>
<td>1.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8109-3 9109-1</td>
<td>1.39</td>
<td>1.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8109-5 9109-2</td>
<td>1.97</td>
<td>1.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8109-6 9109-2</td>
<td>1.62</td>
<td>-c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Classification for 8110 parent ear: 182 Class 4 seeds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8110-1 9110-5</td>
<td>4.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8110-2 9110-1</td>
<td>4.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8110-5 9109-4</td>
<td>3.96</td>
<td>3.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8110-6 9110-10</td>
<td>4.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8110-7 9110-7</td>
<td>3.95</td>
<td>4.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8110-8 9110-2</td>
<td>4.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Results of all crosses with family 9109 are recorded in exact reciprocal column: (Classification for 9109 parent ear: 223 Class 4, 2 Class 2 seeds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Classification for 9110 parent ear: 132 Class 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9110-3 8110-8</td>
<td>3.98</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9110-4 8109-4</td>
<td>4.00 (nubbin)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9110-5 8110-6</td>
<td>4.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9110-6 8110-1</td>
<td>4.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9110-10 8110-3</td>
<td>4.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9110-11 8109-6</td>
<td>4.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Mutability scale as in Materials and Methods.  
<sup>b</sup>Exact reciprocal crosses are recorded only once.  
<sup>c</sup>No test.
plants from Class 2 seeds were either selfed and outcrossed to \textit{al sh2}, or reciprocally crossed to \textit{al sh2}. Few mutable seeds were observed on any of the progeny ears, as shown in Table 3. Thus, the level of mutability exhibited by the progeny seeds was significantly less than that of the seeds sown to produce the parent plants.

During the summer of 1986, crosses were made to investigate the inheritance of somatic mutability in exact reciprocal crosses between various mutable classes of seeds. Seeds resulting from crosses between the 1985-86 families, 5558, 5559, 6558, and 6559 (described in Table 4), and 1985 Class 4 seeds were planted. The latter seeds were the same ear as those planted in family 6558 (designated as high/high). All these plants were reciprocally crossed and the results are shown in Tables 5 through 8. The results show the Class average of seeds on individual ears derived from each cross. The range of Classes observed on each ear is recorded within parentheses. In some cases, individual plants were outcrossed as males to \textit{al sh2} tester plants to determine the mutability potential when crossed as a male. Ordered classification frequency analysis statistical tests were used to compare the Class averages of ears from exact reciprocal crosses. Finally, this test was used to compare the Class averages from the total population of kernels from all the ears in the families involved in reciprocal crosses.
Table 3. Results of selfing and reciprocal crosses of plants from *al-Mum*2 Class 2 seeds (high/high) in the 1986 summer nursery

<table>
<thead>
<tr>
<th>1986</th>
<th>Family &amp; Plant#</th>
<th>Class Planted</th>
<th>Mutability Classification</th>
<th>Class Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Selfed Ear</td>
<td><em>a1sh2</em> (o)</td>
</tr>
<tr>
<td>1986</td>
<td>Mutability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8113-1</td>
<td>2</td>
<td></td>
<td>1.01</td>
<td>1.00</td>
</tr>
<tr>
<td>8113-2</td>
<td>2</td>
<td></td>
<td>-</td>
<td>1.00</td>
</tr>
<tr>
<td>8113-3</td>
<td>2</td>
<td></td>
<td>1.03</td>
<td>-</td>
</tr>
<tr>
<td>8113-4</td>
<td>2</td>
<td></td>
<td>1.03</td>
<td>-</td>
</tr>
<tr>
<td>8113-5</td>
<td>2</td>
<td></td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>8113-6</td>
<td>2</td>
<td></td>
<td>1.01</td>
<td>1.00</td>
</tr>
<tr>
<td>8113-9</td>
<td>2</td>
<td></td>
<td>-</td>
<td>1.00</td>
</tr>
<tr>
<td>8113-10</td>
<td>2</td>
<td></td>
<td>1.04</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4. Origin of al-Mum2 material used in crosses in the 1986 summer nursery

<table>
<thead>
<tr>
<th>FEMALE PARENT</th>
<th>85-86</th>
<th>5558</th>
<th>5559</th>
<th>6558</th>
<th>6559</th>
</tr>
</thead>
<tbody>
<tr>
<td>5558</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5559</td>
</tr>
<tr>
<td>Predominantly Class 2 Low/Low</td>
<td>86-621 (2) ^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5559</td>
<td>Predominantly Class 4 High/Low</td>
<td></td>
<td>Predominantly Class 2 Low/Low</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Predominantly Class 2 Low/Low</td>
<td>86-607, 609, 821 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6558</td>
<td>Predominantly Class 4 High/Low</td>
<td>Predominantly Class 2 Low/Low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Predominantly Class 2 Low/High</td>
<td>86-819 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6559</td>
<td>Predominantly Class 2 Low/High</td>
<td>Predominantly Class 4 Low/High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Predominantly Class 2 Low/Low</td>
<td>86-611, 807 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a The number in () is the Class of seed planted in the 1986 families indicated.
When plants from Class 2 seeds from a low/low line (607 and 807 and 621 and 821) were crossed reciprocally, the progeny gave ears which were mostly stable and contained only a few Class 2 seeds (Table 5). One exception was the ear produced by cross 86-621-6 x 86-821-5; the progeny of this ear had a Class average of 1.66 when 86-621-6 was used as the female parent. This value was significantly higher than that calculated for the ear produced in the reciprocal cross. In reciprocal crosses of the 607 and 807 families, the Class averages of the progeny ears were 1.014 (607/807) and 1.016 (807/607), and not significantly different. The reciprocal crosses of families 621 and 821, however, were analyzed in two ways; with and without the exceptional ear 86-621-6/821-5 (and its reciprocal, 86-821-5/621-6). Including the exceptional ear, a significant difference was observed between the Class averages of reciprocal ears; this was not so if the exceptional ear (and its reciprocal) was omitted from the analysis. Outcrossoes of plants in these families to al sh2 gave ears with a Class average similar to the al-Mum2 parent. These results showed that crossing plants from Class 2 seeds caused a further reduction in mutability rather than generating progeny with (the same, or) a higher Class average.

In a related series of experiments, Class 4 seeds derived from high/low and low/high (see Table 4) crosses, or 1985
Table 5. Results of intercrossing plants from *al-Mum2* Class 2 lines in the 1986 summer nursery

<table>
<thead>
<tr>
<th>1986 Family/ Plant #</th>
<th>Mutability Classification</th>
<th>( t_{calc} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Family &amp; Plant #</td>
<td>600 Family Ears</td>
</tr>
<tr>
<td>Cross: Class 2 from low/low ( \times ) Class 2 from low/low.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>607-1 (2) 807-2 (2)</td>
<td>1.04 (2-1) ( b )</td>
<td>1.01 (2-1) ( b )</td>
</tr>
<tr>
<td>607-2 (2) 807-7 (2)</td>
<td>1.01 (2-1) ( b )</td>
<td>1.02 (2-1) ( b )</td>
</tr>
<tr>
<td>607-3 (2) 807-8 (2)</td>
<td>1.01 (2-1) ( b )</td>
<td>1.01 (2-1) ( b )</td>
</tr>
<tr>
<td>607-4 (2) 807-6 (2)</td>
<td>1.00 (2-1) ( b )</td>
<td>1.01 (2-1) ( b )</td>
</tr>
<tr>
<td>609-4 (2) 807-5 (2)</td>
<td>1.00 (2-1) ( b )</td>
<td>1.01 (2-1) ( b )</td>
</tr>
</tbody>
</table>

Population analysis: 607/807 Class avg = 1.014
(n = 1292, range 2-1)
807/607 Class avg = 1.016
(n = 1613, range 2-1)

Hypothesis: \( \mu_1 = \mu_2 \), no reciprocal difference
\( t_{calc} = 0.40, t_{\alpha, 0.05} = 1.96 \)
therefore, accept hypothesis

Cross: Class 2 from low/low \( \times \) Class 2 from low/low

| Cross: Class 2 from low/low \( \times \) Class 2 from low/low |
|---------------------|---------------------|---------------------|
| 621-1 (2) 821-3 (2) | 1.01 (2-1) \( b \)  | 1.01 (2-1) \( b \)  | 1.01 (2-1) \( b \)  | 0.00 |
| 621-3 (2) 821-2 (2) | 1.06 (2-1) \( b \)  | 1.18 (2-1) \( b \)  | 1.06 (2-1) \( b \)  | 0.00 |
| 621-6 (2) 821-5 (2) | 1.66 (2-1) \( b \)  | 1.08 (2-1) \( b \)  | 10.71 |

Population analysis: 621/821 Class avg = 1.133
(n = 748, range 2-1)
(excluding 621-8/821-5) = 1.015 (n = 400)
821/621 Class avg = 1.044
(n = 659, range 2-1)
(excluding 821-5/621-6) = 1.012 (n = 347)

Hypothesis: \( \mu_1 = \mu_2 \), no reciprocal difference
Including 621-6/821-5: \( t_{calc} = 13.65, t_{\alpha, 0.05} = 1.96 \)
therefore, reject hypothesis
Excluding 621-6/821-5: \( t_{calc} = 0.177, t_{\alpha, 0.05} = 1.96 \)
therefore, accept hypothesis

\( a \)Class of seed planted.
\( b \)Class range of ear.
Class 4 seeds from high/high were planted in the 1986 summer nursery to determine whether reciprocal crosses between two families of each Class 4 type would produce progeny ears with similar levels of somatic mutability. Reciprocal crosses between plants from Class 4 seeds from two high/low lines (families 613 and 813) produced stable and low mutable seeds (Table 6). Although significant statistical differences existed within individual reciprocal crosses, there was no overall significant difference between the Class averages of seeds from all the 613 and 813 progeny ears. Similarly, reciprocal crosses between plants from Class 4 seeds from two low/high lines (families 615 and 815) also produced seeds which were stable and of low mutability. Again, no reciprocal differences between Class averages was seen for seed populations of these crosses, although two individual reciprocal crosses (615-2 x 815-7 and 615-4 x 815-1) resulted in significant differences in the Class averages of the progeny ears. Outcrosses of the plants from high/low and low/high Class 4 seeds as males to al sh2 testers resulted in progeny ears with lower Class averages compared to the reciprocal cross with the same male parent. This was true for all outcrosses to al sh2 except the cross involving 615-1 as the male parent. The Class average produced by this outcross was 1.88 compared to 1.43 of the 815-3/615-1 cross, where 615-1 was the male parent.
Table 6. Results of intercrossing plants from *al-Mum* Class 4 lines in the 1986 summer nursery

<table>
<thead>
<tr>
<th>Family/ Plant #</th>
<th>600 Family Ears (individual)</th>
<th>800 Family Ears (individual)</th>
<th>( t_{\text{calc}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d'parent)</td>
<td>Class Avg xaleh2 Class Avg xaleh2 crosses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>1986</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cross: Class 4 from high/low x Class 4 from high/low

| 613-1 (4) | 813-3 (4) | 1.93 (2-1) | 1.25 (3-1) | 1.83 (3-1) | 1.72 (3-1) | -2.43  |
| 613-3 (4) | 813-1 (4) | 2.00 (3-1) | 1.79 (3-1) | 2.29 (3-1) | 1.24 (3-1) | 10.19 |
| 613-6 (4) | 813-8 (4) | 1.74 (2-1) | 1.51 (3-1) | 1.31 (3-1) | 1.05 (2-1) | -4.73 |
| 613-7 (4) | 813-10 (4) | 1.04 (2-1) | 1.37 (2-1) | 4.92 |
| 613-9 (4) | 813-6 (4) | 1.93 (3-1) | 1.40 (2-1) | 1.89 (2-1) | 1.57 (3-1) | -0.91 |

Population analysis: 613/813 Class avg = 1.754
(n = 1693, range 3-1)
813/613 Class avg = 1.755
(n = 1122, range 3-1)

Hypothesis: \( \mu_1 = \mu_2 \), no reciprocal difference

\[ t_{\text{calc}} = 0.05, \quad t_{\text{crit}} = 1.96 \]

therefore, accept hypothesis

Cross: Class 4 from low/high x Class 4 from low/high

| 615-1 (4) | 815-3 (4) | 1.58 (2-1) | 1.88 (3-1) | 1.43 (2-1) | 1.13 (2-1) | -1.54 |
| 615-2 (4) | 815-7 (4) | 1.88 (2-1) | 1.12 (4-1) | 1.37 (2-1) | 1.09 (2-1) | -7.34 |
| 615-3 (4) | 815-4 (4) | 1.98 (2-1) | 1.89 (2-1) | -1.68 |
| 615-4 (4) | 815-1 (4) | 1.48 (2-1) | 1.14 (2-1) | 1.14 (2-1) | -2.09 |
| 615-6 (4) | 815-8 (4) | 1.54 (2-1) | 1.94 (2-1) |                      |

Population analysis: 615/815 Class avg = 1.668
(n = 1303, range 2-1)
815/615 Class avg = 1.635
(n = 967, range 3-1)

Hypothesis: \( \mu_1 = \mu_2 \), no reciprocal difference

\[ t_{\text{calc}} = 1.57, \quad t_{\text{crit}} = 1.96 \]

therefore, accept hypothesis

Cross: Class 4 (high/high) x Class 4 (high/high)

| 623-4 (4) | 823-7 (4) | 3.98 (4-3) | 3.66 (4-2) | 3.95 (4-1) | 3.50 (4-1) | 0.74 |
| 623-5 (4) | 823-1 (4) | 4.00 (4)   | 3.37 (4-1) | 4.00 (4)   | 3.66 (4-1) | 0.00 |
| 623-7 (4) | 823-2 (4) | 3.96 (4-2) | 3.26 (4-1) | 4.83 |
| 623-1 (4) | 823-6 (4) | 3.88 (4-1) | 4.00 (4)   | (self)     |
| 623-9 (4) | self     | 4.00 (4)   |                      |
| 623-4 (4) | 823-3 (4) |                      | 3.88 (4-1) | 2.52 (4-1) |
| 623-6 (4) | 823-4 (4) | 4.00 (4)   |                      |

\( ^a \) Class of seed planted.

\( ^b \) Class range on ear.
The results obtained from the continued inbreeding of plants from *al-Mum* Class 4 seeds (*high/high*) are also shown in Table 6. Although some reduction in somatic mutability was observed in some of the crosses, the majority of the seeds produced retained high mutability. Plants that were selfed gave similar results.

Plants from Class 2 seeds from either a *low/low* line (609, 611) or a *low/high* line (617, 617.1) were crossed with plants from Class 4 seeds (*high/high* line) to determine if any difference in the Class average would occur in the progeny ears of reciprocal crosses. Reciprocal crosses involving families 609 and 809 showed, without exception, a significant difference in Class averages (Table 7). When the plants from Class 2 seeds were used as females, they invariably generated ears which had a Class average significantly less than if they were used as males. The Class averages of the population of seeds in the 609 family was 2.04, and in the 809 family, 3.07.

Similar results were observed in crosses involving plants of families 611 and 811. In the population of kernels produced by families 611 and 811, an overall higher Class average was observed when the Class 4 plant was used as a female compared to the lower Class average generated when the Class 2 plant was used as a female. Class averages of 2.30 and 3.37 in the 611 and 811 families, respectively, were
Table 7. Results of reciprocally crossing plants from Class 2 seeds with plants from Class 4 seeds (high/high)

<table>
<thead>
<tr>
<th>1986</th>
<th>Family/ Plant #</th>
<th>600 Family Ears</th>
<th>800 Family Ears</th>
<th>t_{calc}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Family &amp; Plant # (parent)</td>
<td>Class Avg</td>
<td>xalsh2</td>
<td>Class Avg</td>
</tr>
<tr>
<td>Cross: Class 2 from low/low x Class 4 (high/high).</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>609-1 (2)</td>
<td>809-6 (4)</td>
<td>1.14 (4-1)</td>
<td>2.41 (4-1)</td>
<td>15.29</td>
</tr>
<tr>
<td>609-2 (2)</td>
<td>809-4 (4)</td>
<td>1.06 (2-1)</td>
<td>1.02 (2-1)</td>
<td>1.25 (2-1)</td>
</tr>
<tr>
<td>609-3 (2)</td>
<td>809-3 (4)</td>
<td>2.62 (4-1)</td>
<td>3.86 (4-1)</td>
<td>18.79</td>
</tr>
<tr>
<td>609-5 (2)</td>
<td>811-7 (4)</td>
<td>2.35 (4-1)</td>
<td>3.97 (4-2)</td>
<td>19.88</td>
</tr>
<tr>
<td>609-6 (2)</td>
<td>809-7 (4)</td>
<td>3.46 (4-1)</td>
<td>3.91 (4-1)</td>
<td>3.72 (4-1)</td>
</tr>
<tr>
<td>609-7 (2)</td>
<td>809-8 (4)</td>
<td>1.51 (4-1)</td>
<td>3.53 (4-1)</td>
<td>18.04</td>
</tr>
<tr>
<td>609-8 (2)</td>
<td>809-5 (4)</td>
<td>2.07 (4-1)</td>
<td>2.43 (4-1)</td>
<td>4.24</td>
</tr>
</tbody>
</table>

Population analysis: 609/809 Class avg = 2.036
(n = 2109, range 4-1)
809/609 Class avg = 3.074
(n = 2098, range 4-1)

Hypothesis: \( \mu_1 = \mu_2 \), no reciprocal difference
\( t_{calc} = 28.05, \ t_{\alpha,0.05} = 1.96 \)
therefore, reject hypothesis
### Cross: Class 2 from low/low x Class 4 (high/high)

<table>
<thead>
<tr>
<th>Population analysis:</th>
<th>611/811 Class avg = 2.302</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 1077, range 4-1)</td>
<td>811/611 Class avg = 3.370</td>
</tr>
<tr>
<td>(n = 2480, range 4-1)</td>
<td>Hypothesis: $\mu_1 = \mu_2$, no reciprocal difference</td>
</tr>
<tr>
<td>$t_{calc} = 34.93$, $t_{0.05} = 1.96$</td>
<td>therefore, reject hypothesis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cross: Class 2 from low/high x Class 4 (high/high)</th>
<th>617/817 Class avg = 2.804</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 975, range 4-1)</td>
<td>817/617 Class avg = 3.805</td>
</tr>
<tr>
<td>(n = 1090, range 4-1)</td>
<td>Hypothesis: $\mu_1 = \mu_2$, no reciprocal difference</td>
</tr>
<tr>
<td>$t_{calc} = 25.02$, $t_{0.05} = 1.96$</td>
<td>therefore, reject hypothesis</td>
</tr>
</tbody>
</table>

---

*Class of seed planted.  
*Class range on ear.
slightly higher than those of the 609 and 809 families. An exception to these results was the progeny ear from the 611-4 x 811-9 cross. A higher Class average was observed when the plant from the Class 2 seed (611-4) was used as the female parent.

Another type of Class 2 by Class 4 cross involved plants from Class 2 seeds from a low/high (family 617) crossed with plants from Class 4 seeds (high/high, family 817). As in previous experiments, significant differences in the mutability of seeds resulting from reciprocal crosses were observed in both individual crosses and over the populations of kernels produced by families 617 and 817. The Class averages of the population of kernels were 2.80 when 617 plants were crossed as females and 3.81 when 817 plants were crossed as females. These values were significantly higher than the Class averages of similar crosses made previously (609 and 809, and 611 and 811 reciprocal crosses).

Two additional crosses performed during the summer of 1986 involved plants from Class 1 stable seeds sown as 619-1 and 819.1-1. When these plants were reciprocally crossed, the progeny ears produced mostly stable seeds with less than 1% Class 2 seeds (Table 8). When the tiller of 619-1, 619-1T, was reciprocally crossed with a plant from a Class 4 seed (high/high), a significant difference was observed between the Class averages of the progeny ears. Higher kernel
Table 8. Results of crosses involving plants from Class 1 seeds

<table>
<thead>
<tr>
<th>Cross: Class 1 from high/low x Class 1 from low/high</th>
</tr>
</thead>
<tbody>
<tr>
<td>619-1 (1) 819.1-1(1) 1.01 (2-1) 1.01(2-1) 1.01(2-1) 0.00</td>
</tr>
<tr>
<td>623-2 (4) 819.1-1(1) 3.88 (4-1) 1.02(2-1) 3.85 (4-1) 3.48(4-1) 136.67</td>
</tr>
</tbody>
</table>

Hypothesis: $\mu_1 = \mu_2$, no reciprocal difference

- $619-1/819.1-1$: $n = 194$
- $819.1-1/619-1$: $n = 284$

$^{t}_{calc} = 0.00$, $t^{\infty, 0.05} = 1.96$

therefore, accept hypothesis

Cross: Class 1 from high/low x Class 4 (high/high)

<table>
<thead>
<tr>
<th>Cross: Class 1 from high/low x Class 4 (high/high)</th>
</tr>
</thead>
<tbody>
<tr>
<td>619-1T (1) 823-5 (4) 2.21 (4-1) 1.02(2-1) 3.85 (4-1) 3.48(4-1) 136.67</td>
</tr>
</tbody>
</table>

Hypothesis: $\mu_1 = \mu_2$, no reciprocal difference

- $619-1T/823-5$: $n = 42$
- $823-5/619-1T$: $n = 269$

$^{t}_{calc} = 136.67$, $t^{\infty, 0.05} = 1.96$

therefore, reject hypothesis

---

^aClass of seed planted.

^bClass range of ear.
mutability was observed when the plant from the Class 4 seed was used as a female, but lower kernel mutability was observed when the tiller of the plant from the Class 1 seed was used as a female, similar to the results obtained in the progeny of Class 2 x Class 4 crosses.

Finally, experiments were carried out to determine the germinal Mutator activity of plants used in the al-Mum2 inbred lineage by Robertson's standard test (Robertson, 1978, 1983). The levels of germinal Mutator activity and somatic mutability are shown in Table 9 for individual plants used in the al-Mum2 lineage. Mutator activity was retained in some plants through the sixth generation of inbreeding. Germinal activity was observed in plants 83-8207-2 and 84-1120-5, on the right side of the pedigree shown in Figure 1. However, loss of germinal Mutator activity occurred in the 83-8207-1 progeny even though somatic mutability was maintained. This demonstrated that somatic mutability and germinal activity were not concomitantly lost in the progeny of this plant.

Evidence bearing on the relationship of somatic mutability and germinal activity in the al-Mum2 inbred lineage was also obtained by the analysis of the 1985-86 progeny (Table 9). In families which demonstrated low somatic mutability (85-86-5558, -5559, and -6559), a wide range of germinal activity was present in the plants tested. In one plant, 85-86-5558-1, however, no germinal activity was
<table>
<thead>
<tr>
<th>Plant#</th>
<th>#Generations</th>
<th>Inbreeding</th>
<th>Level of al Somatic Mutability</th>
<th>Germinal Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>82-2154-5</td>
<td>-</td>
<td>5</td>
<td>21.7</td>
<td></td>
</tr>
<tr>
<td>82-83-3582-6</td>
<td>1</td>
<td>4</td>
<td>8.33</td>
<td></td>
</tr>
<tr>
<td>83-8207-1</td>
<td>2</td>
<td>4</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>8207-2</td>
<td>2</td>
<td>4</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>8207-3</td>
<td>2</td>
<td>4</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>8207-4</td>
<td>2</td>
<td>4</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>84-1120-4</td>
<td>3</td>
<td>4</td>
<td>3.03</td>
<td></td>
</tr>
<tr>
<td>85-6151-8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5151-3T</td>
<td></td>
<td>4</td>
<td>3.64</td>
<td>NT&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>85-86-5558-1</td>
<td>5</td>
<td>1.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>5558-3</td>
<td>5</td>
<td>1.85</td>
<td>27.27</td>
<td></td>
</tr>
<tr>
<td>85-86-5559-2</td>
<td>5</td>
<td>1.42</td>
<td>4.55</td>
<td></td>
</tr>
<tr>
<td>5559-8</td>
<td>5</td>
<td>1.42</td>
<td>8.70</td>
<td></td>
</tr>
<tr>
<td>85-86-6558-2</td>
<td>5</td>
<td>3.35</td>
<td>17.95</td>
<td></td>
</tr>
<tr>
<td>6558-4</td>
<td>5</td>
<td>3.71</td>
<td>19.15</td>
<td></td>
</tr>
<tr>
<td>6558-5</td>
<td>5</td>
<td>3.64</td>
<td>9.30</td>
<td></td>
</tr>
<tr>
<td>6558-6</td>
<td>5</td>
<td>3.64</td>
<td>9.09</td>
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</tr>
<tr>
<td>6558-7</td>
<td>5</td>
<td>3.62</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>6558-8</td>
<td>5</td>
<td>3.53</td>
<td>17.50</td>
<td></td>
</tr>
<tr>
<td>85-86-6559-5</td>
<td>5</td>
<td>2.03</td>
<td>2.27</td>
<td></td>
</tr>
<tr>
<td>6559-6</td>
<td>5</td>
<td>2.21</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>6559-7</td>
<td>5</td>
<td>2.28</td>
<td>18.60</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>No test, no outcross ears available

<sup>b</sup>Class average of estimated seed counts of approx. 50 outcross ears.
detected. Similarly, when plants which maintained high somatic mutability were tested for germinal activity, a wide range of values was again obtained, with one plant, 85-86-6558-7, again having no activity. The loss of germinal activity in this plant may have been due, however, to the outcross-Mu-loss phenomenon previously described by Robertson (1986). Plants must be outcrossed and subsequently self-pollinated to measure Mutator activity. These results show that there was no correlation between the level of somatic mutability, and the degree of germinal Mutator activity in this line. Equally important, loss of somatic mutability was not necessarily accompanied by the loss of germinal activity.
DISCUSSION

We have produced an inbred lineage of a \textit{Mutator}-induced, mutable allele of \textit{Al}, \textit{al-Mum2} (Robertson \textit{et al.}, 1985; O'Reilly \textit{et al.}, 1985), which has retained a high level of somatic mutability through seven generations of inbreeding, and germinal activity through six inbred generations. Robertson (1983) demonstrated that inbreeding \textit{Mutator} lines (by sibling crosses of different \textit{Mutator} lineages) resulted in an initial increase in germinal \textit{Mutator} activity in the first generation, but, by the third inbred generation, activity was greatly reduced, and then lost completely after the fourth generation of inbreeding. This phenomenon was termed inbred-\textit{Mu}-loss. We initiated these studies with the expectation that somatic mutability and germinal activity would be greatly diminished by the third or fourth generation of self and/or sibling crosses. The inbreeding of the \textit{al-Mum2} lineage reported here has been even more intense than that of Robertson's (1983) previous experiment because we included three selfing generations in this lineage. The \textit{al-Mum2} inbred lineage was, therefore, unique.

We hypothesize that a change occurred in the regulation of \textit{Mutator} activity in this line, which enabled it to retain somatic mutability and germinal activity throughout extensive inbreeding. This change probably occurred in plant 83-8207-
2. This plant retained both somatic mutability and germinal activity, whereas its siblings, 83-8207-1, -3, and -4 had lost germinal activity as expected but retained somatic mutability. Subsequent generations of 83-8207-2 retained somatic mutability through the seventh generation of inbreeding and germinal activity through the sixth inbred generation.

This hypothesis was further reinforced when loss of somatic mutability in some plants was observed after the fifth generation of inbreeding. The plants (shown in Figure 1) in the left side of the pedigree (derived from 83-8207-1) demonstrated the expected and germinal inbred-Mu-loss, while 83-8207-2 and its subsequent progeny plants retained germinal Mutator activity. Loss of somatic mutability occurred only when plants from each side of the pedigree were crossed together (families 85-6151 X 85-5151). Thus, loss of somatic mutability in some plants may have been the result of crossing inbred-Mu-loss plants with plants having the putative, defective regulatory system for germinal activity. Lines that had reduced levels of somatic mutability were designated low lines. Further loss of somatic mutability in the next generation was detected in the progeny ears if two low lines were crossed together. Reciprocal crosses between a low line and a high line (such as 85-86-6558) produced progeny ears with mostly Class 4 seeds; in other words, the
high phenotype was dominant in this generation and not
dependent upon the direction of the cross. However, in the
next generation, reciprocal crosses between plants from Class
4 seeds from high/low crosses resulted in ears of with lower
Class averages. Plants from Class 4 seeds from low/high
crosses were also reciprocally crossed and yielded similar
results. Whenever plants from Class 2 seeds from low/low
crosses were crossed, the Class average of the ear was just
above 1.00 (stable, with less than 3% Class 2 seeds). No
reactivation of somatic mutability occurred in these crosses.

Once the loss of somatic mutability occurred in this
line, the maintenance of the inactive state was dependent on
the mutability state of the female parent. Reciprocal
crosses between plants from Class 2 and Class 4 seeds showed
a female effect on the mutability of the progeny kernels. In
these crosses, the level of mutability of the female parent
predicted the level of mutability observed in the progeny.
Similar results were obtained by Bennetzen (1985) and Walbot
(1986). Furthermore, Walbot reported that the events leading
to the initial loss of somatic mutability were also dependent
on the level of mutability seen in the female parent.

Additionally, we observed a putative "dosage" effect on
the maintenance of somatic mutability in Class 2 times Class
4 crosses. The capacity to maintain a low level of somatic
mutability in the progeny seeds of low lines crossed to
plants from Class 4 seeds was greater in Class 2 plants from a low/low background than in Class 2 plants from a low/high background. In other words, the background (i.e., low/low versus low/high) of a plant from a low mutability seed may determine its capacity to inactivate somatic mutability when crossed to plants from high mutability seeds or its capacity to maintain the low somatic mutability state of its progeny. Our data suggest that a first generation low line has less capacity to inactivate somatic mutability than a second generation low line. More crosses will need to be carried out to confirm this relationship.

Inbred-Mu-loss was shown to be accompanied by covalent DNA modification of certain restriction endonuclease target sites within Mu elements present in these stocks, presumably by methylation of cytosine residues (Bennetzen 1985, 1987). Modification of MUL elements has also been correlated with the loss of somatic mutability in bz2-mul, a Mutator-induced aleurone mutant (Chandler and Walbot, 1986). In a companion paper, we investigate the role of Mu element modification in the genetic phenomena described here.
REFERENCES


Walbot, V., and C. Warren. 1987. Regulation of Mu element copy number in maize lines with an active or inactive Mutator transposable element system. Submitted for publication.

SECTION II. MOLECULAR ANALYSIS OF \textbf{Mu} SEQUENCES IN A HIGHLY INBRED \textit{al-Mum2} MUTATOR LINE OF \textit{Zea mays} L.: RELATIONSHIPS BETWEEN SOMATIC MUTABILITY AND GERMINAL ACTIVITY AND \textbf{Mu} ELEMENT MODIFICATION.
Molecular analysis of Mu sequences in a highly inbred \textit{al-Mum2} Mutator line of \textit{Zea mays} L.: Relationships between somatic mutability and germinal activity and Mu element modification

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Robertson's Mutator maize lines contain a highly mutagenic transposable element system. Mutator induces new germinal mutation at a frequency 50-fold above the background level. Approximately 40% of the induced mutants are somatically unstable (mutable). It was previously shown that inbreeding Mutator lines resulted in the loss of germinal Mutator activity. Thus, we produced an inbred lineage (by selfing and sibling crosses) of the Mutator-induced mutable mutant, al-MumZ, to test an hypothesis that somatic mutability, like germinal activity, would be lost after three to four generations of inbreeding. On the contrary, a high level of somatic mutability was maintained through seven generation of inbreeding. Germinal activity was also maintained in some plants through the sixth inbred generation. We present results of Southern hybridization analyses with plants from the al-Mum2 lineage. We demonstrated that the Mu elements became modified upon the loss of somatic mutability in the aleurone tissue. However, no appreciable modification of Mu elements was observed in those plants which had lost germinal activity while retaining a high level of somatic mutability. A differential modification of restriction endonuclease target sequences present within the terminal
inverted repeats and the internal regions of Mu elements was observed. Additionally, Mu element copy number did not differ significantly in any of the plants in this lineage that were examined.
INTRODUCTION

The Mutator system in maize has the ability to induce new germinal mutations at frequencies much higher than the background mutation rate (Robertson, 1978). Approximately 40% of the mutants induced in Mutator lines were genetically unstable (mutable), and this suggested that transposable elements were involved in their induction. This was substantiated following the isolation of a DNA element from a mutable mutant allele of Adhl-S, adhl-S3034, induced in a Mutator background (Bennetzen et al., 1984). This element, called Mul, had many features characteristic of other transposable elements such as long terminal inverted repeats, and had caused the duplication of a short sequence of host DNA at the site of Mul insertion (Barker et al., 1984). Mutator lines contain approximately 10-70 copies of sequences similar in size and structure to Mul (Bennetzen et al., 1984; Barker et al., 1984). In addition to Mul elements, some Mutator lines contain one or more copies of a larger sized element structurally related to Mul. These elements, termed Mul.7, differ from Mul by the insertion of a 380 bp sequence, some restriction endonuclease polymorphisms, and a few small rearrangements (Taylor et al., 1986). Southern hybridization analyses demonstrated that both Mul and Mul.7 elements were
transposable (Alleman and Freeling, 1986; Taylor et al., 1986). To date, MuI elements have been cloned from three adhl alleles (including the original isolate) (Bennetzen et al., 1984; al-Mum2 (O'Reilly et al., 1985); bzl-mul (Taylor et al., 1986); and shh (Benjamin and Francis Burr, Brookhaven National Laboratory, Brookhaven, New York, personal communication); a Mul.7 element was associated with bzl-mul (Taylor et al., 1986). The implication that Mu elements are involved in Mutator activity has been further strengthened by the observation that non-Mutator lines of maize contain few Mul-homologous sequences (Bennetzen, 1984; Chandler et al., 1986; Roth et al., 1987).

It has been established that Mutator activity and Mu element transposition are co-regulated. Germinal Mutator activity can be lost either by outcrossing (outcross-Mu-loss) or inbreeding (inbred-Mu-loss) Mutator plants (Robertson, 1986). Outcross-Mu-loss occurs in about 10% of the outcross progeny (Robertson, 1978, 1985). Mu elements in these lines remained unmodified and upon subsequent outcrossing, dilution of Mu element copy number occurred by random segregation (Bennetzen et al., 1987). On the other hand, inbred-Mu-loss occurred during the inbreeding of Mutator stocks (by sibling crosses) after three to four successive generations (Robertson, 1983, 1986). Inbred-Mu-loss was accompanied by the modification of methylation-sensitive restriction
endonucleases target sites within \textit{Mu} elements which rendered them insensitive to digestion (Bennetzen \textit{et al.}, 1987; David Morris, Iowa State University, Ames, Iowa, personal communication). Bennttezen (1987) has shown that modification involved the methylation of cytosine residues at the 5' position in the sequence 5'-CG-3' and 5'-CNG-3'. Once \textit{Mu} elements were modified, modification was stably maintained upon outcrossing (Bennetzen, 1987). \textit{Mu} element modification, however, was first observed and correlated with the loss of somatic mutability in a \textit{Mutator}-induced mutant of \textit{Bz2}, \textit{bz2-mul} (Chandler and Walbot, 1986).

In this paper, we present the results of molecular hybridization experiments with an \textit{al-Mum2} inbred line. This lineage had maintained germinal activity through six, and somatic mutability through seven, generations of inbreeding (Roth \textit{et al.}, 1987). We show that the \textit{Mu} elements present in this lineage became modified upon the loss of somatic mutability. However, no appreciable modification was observed in those plants which had lost germinal activity while retaining a high level of somatic mutability. No difference in \textit{Mu} element copy number was observed in any of the plants examined.
MATERIALS AND METHODS

Maize stocks

The Mutator-induced al mutable mutant, al-Mum2 (Robertson et al., 1985; O'Reilly et al., 1985), was produced by applying Purple Mutator (fully colored kernels) pollen onto the silks of an al sh2 tester during the summer of 1980. The plants were either intercrossed or selfed each generation after an outcross to purple standard in 1982 as shown in the pedigree in Figure 1. Significant loss of somatic mutability first occurred in the 1985-86 winter nursery crosses (Roth et al., 1987). Genetic crosses with this material have been described elsewhere (Roth et al.; 1987).

Classification of al-Mum2 somatic mutability

Seeds from the al-Mum2 ears were scored according to the scale shown in Figure 2: Class 1, stable; Class 2, low mutability; Class 3, medium mutability; Class 4, high mutability; Class 5, purple (Roth et al., 1987).

Isolation of maize DNA

Maize nuclear DNA was isolated exactly according to the protocol of Rivin and coworkers (1982) from individual plants at the 2 to 4 leaf stage, or from several one week old seedlings. The plants were grown in an environmental growth chamber or greenhouse sandbench.
The original isolate, 80-1223-2, was selfed and in the following generation outcrossed to a purple standard line. Subsequent selfing or sibling crosses are indicated. $\times$ = al-Mum2 sibling crosses (or outcrosses to purple standard as indicated), $\otimes$ = self pollination.
Fl Mu x al sh2

80-1223-2
(original isolate)

Purple stand. x 81-82-5504

82-2153

Purple stand. x 81-82-5503 x Purple stand.

82-2154

82-83-2581

83-8207-1

83-8207-2

84-1119

84-1120

85-6151

85-5151

85-86

Different ears
than the '85 -'86 ears

85-86 (-5558 x -5559) → 86 (-607, -609, -821) → 86 (-8109, -8110, -9109, -9110) → 86 (-809)

85-86 (-5558 x -6558) → 86 (-819) → 86 (-811)

85-86 (-5559 x -5558) → 86 (-621) → 86 (-817)

85-86 (-5559 x -6558) → 86 (-617) → 86 (-817)

85-86 (-5559 x -6559) → 86 (-611, -807) → 86 (-807)

85-86 (-6558 x -6559) → 86 (-613, -813) → 86 (-823)

85-86 (-6559 x -6558) → 86 (-615, -619, -815) → 86 (-823)

(Duplicate planting of
Class 4 highly
mutable line,
85-86-6558)
Figure 2. Classification scale for al-Mum2 and stable alleles

From crosses involving al-Mum2, the range of somatic mutability between al stable (Class 1, top kernel) and A1 stable (purple, Class 5, bottom kernel) includes: Class 2 (first row of three kernels), Class 3 (second row), and Class 4 (third row) kernels.
Restriction endonuclease digestion and gel electrophoresis of maize DNA

Restriction endonucleases obtained from New England Biolabs (Beverly, MA) and Bethesda Research Labs (Gaithersburg, MD) were used according to the manufacturers' instructions. High molecular weight DNA (5-7 μg) was digested with restriction endonucleases at a concentration of 6-8 units/μg of DNA for 3 to 4 hours, and fractionated by electrophoresis on 0.8% agarose gels made with TBE (0.09 M Tris, 0.09 M boric acid, 0.0025 M Na₂EDTA) for 16-18 hours at 45V. Gels were stained with ethidium bromide (1.5 μg/ml) in distilled water. The stained gels were photographed under short wave ultraviolet light with an MP4 camera and Type 55 film (Polaroid).

Southern transfer and DNA hybridization

DNA fragments in gels were nicked with ultraviolet light under a germicidal hood, denatured, and transferred to Genatran (Plasco) using Southern's (1975) procedure. Filters were baked for 2 hours at 80°C and prehybridized in 50% (v/v) formamide; 3 x SSC; 5 x Denhardt's solution; 100 μg/ml sonicated and denatured salmon sperm DNA; and 1% SDS, for 6 hours to overnight. Hybridizations were carried out at 42°C for 36 hours in prehybridization solution with the addition of 5% dextran sulfate and 25 ng of probe DNA. Filters were
then rinsed two times with 250 ml of wash buffer (0.1% SDS, 0.1X SSC) at room temperature followed by three, 30 to 45 minute washes at 65° C with pre-heated wash buffer. The filters were blotted dry on paper towels and exposed to Kodak XAR-5 film with one Lightning-Plus (Dupont) intensifying screen for 1-3 days at -70° C. Filters were stripped for rehybridization by two, 20 minute washes with 2000 ml of wash buffer heated to 95-100° C. Rehybridization was carried out as described before.

DNA probes

A DNA probe specific for the internal region of Mul was prepared from plasmid pRB1. This plasmid was constructed by inserting a 2.9 kb HindIII-PstI fragment of pMJ9 containing the whole of Mul (Bennetzen et al., 1984) into the polylinker sequence of plasmid pSP64 (Promega Biotec). The internal fragment of Mul was released by digestion with Aval and TaqI, and purified by electrophoresis on an agarose gel (Maniatis et al., 1982). A 9 kb maize ribosomal tandem repeat probe was isolated from pZmR1 (obtained from Dr. David Grant, Pioneer Hi-bred International, Johnston, Iowa) by digestion with EcoRI and purified in a similar manner. This probe was used to test for the complete digestion of DNA samples, and to standardize the amount of DNA in each electrophoresis lane. Purified fragments were labeled to a specific activity
of at least $4 \times 10^8$ cpm/µg by random hexamer priming (Feinberg and Vogelstein, 1983) using a kit (Pharmacia).

**Mu element copy number estimation**

The number of copies of Mu elements per diploid genome in individual plants was estimated in two ways. In the first, genomic DNAs were cleaved with EcoRI, which has no target sites within Mu elements. Thus, counting individual bands in autoradiographs was used to determine the copy number of Mu elements. Because the plants used in this analysis were highly inbred and assumed to have a high degree of homozygosity of Mu element insertions, the actual copy number per diploid genome was twice the number of bands (each band consisting of two similarly sized fragments). The number of Mu bands was probably underestimated in this type of analysis because accurate counting of more than 20 individual bands was impossible due to the large number of bands not resolved from each other on the autoradiograph. In the second method, the number of Mu copies was estimated by densitometric scanning of autoradiographs of genomic DNAs digested with Tth1111I and comparison to copy number reconstructions. The latter were made by digesting amounts of pRBl, representing 10, 25, and 50 copies of Mu per diploid genome, with Tth1111I or HinfI. Following hybridization to MuAT, the filters were routinely stripped and rehybridized to
the ribosomal DNA probe to check that each DNA sample had been completely digested, and to calibrate the amount of DNA in each electrophoresis lane.
RESULTS

The \textit{al-Mum2} inbred lineage was produced initially to investigate whether inbreeding would inactivate somatic mutability. We reported earlier (Roth \textit{et al.}, 1987) that somatic mutability was maintained through seven generations of inbreeding, although loss of mutability occurred in some of the progeny after the fifth inbred generation. Germinal activity was also maintained through six generations of inbreeding, although loss of activity occurred in some of the progeny after the third generation of inbreeding.

\textbf{Loss of somatic mutability is accompanied by modification of Mu elements}

In the previous paper describing the \textit{al-Mum2} inbred lineage, we reported that loss of somatic mutability occurred in some of the progeny of the fifth inbred generation propagated during 1985 (Roth \textit{et al.}, 1987). The progeny ears of the previous generation contained mostly Class 4 seeds (greater than 99%). Plants from these highly mutable seeds were expected, from previous reports (Chandler and Walbot, 1986), to contain numerous, unmodified \textit{Mu} elements. To test this, siblings of the 1982 and 1984 plants in this lineage were analyzed by Southern blot hybridization. Individual
plant DNAs were digested with HinfI or \textit{Tth}III, which have single target sites within each of the \textit{Mu} and \textit{Mu}1.7 terminal inverted repeats. Digestion with \textit{Tth}III was used to determine the types of \textit{Mu} elements (\textit{Mu}1 and \textit{Mu}1.7) present in each plant. \textit{Tth}III is insensitive to methylation of its target sequences, and Chandler and Walbot (1986) have shown that both unmodified and modified \textit{Mu} elements are cleaved by this restriction endonuclease. The hybridization profiles we obtained are shown in Figure 3. Two bands, 1.35 and 1.0 kb in size were detected and represented \textit{Mu}1 and \textit{Mu}1.7-like elements, respectively, present in the genomes of all these plants. The hybridization profiles obtained after HinfI digestion confirmed this result. The expected 1.3 and 1.6 kb bands, corresponding to \textit{Mu}1-like and \textit{Mu}1.7-like elements, were also observed. No bands larger than 1.6 kb, expected if \textit{Mu} elements were modified, were detected. Thus, all the \textit{Mu} elements in these plants were unmodified. The copy number of the \textit{Mu} elements in each plant was estimated from EcoRI hybridization profiles. The number of bands generated in each sample was counted to give an estimation of the \textit{Mu} copy number in each 1982 and 1984 plant (Figure 4, left panel). We detected approximately 18-23 \textit{Mu}-specific bands, equivalent to about 35-40 copies of \textit{Mu} elements per diploid genome, assuming that most of the bands contained two \textit{Mu}-specific fragments. These results were confirmed by comparing the

DNA isolated from individual plants, was digested to completion with HinfI or Tth1111, fractionated on 0.8\% agarose gels, transferred to Genatran, and hybridized to a MuAT probe. Lanes 82-2154a and 82-2154b, and 84-1120a and 84-1120b contained DNA from sibling plants grown from Class 4 seeds from \textit{al-Mum2} ears produced in 1982 and 1984, respectively. Q60 lane: DNA from hybrid Q60 digested with Tth1111 was included as a negative control. Mu1 HinfI lane: HinfI-digested Mu1 positive control. Molecular weights were estimated from lambda DNA fragments generated by \textit{PstI} digestion.
Maize genomic DNA was digested to completion with EcoRI, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized to a MuAT probe. Lanes 82-2154a and 82-2154b, and 84-1120a and 84-1120b contained DNA from sibling plants grown from Class 4 seeds from al-Mum2 ears produced in 1982 and 1984, respectively. Lanes Class 2, 3, and 4 contained DNA isolated from sibling plants grown from 1985 progeny seeds (85-6151/5151) of the designated mutability levels. Mu1 HinfI lane: HinfI-digested Mu1 positive control. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
Tthl1II hybridization profiles to copy number reconstructions (data not shown).

Onset of the loss of somatic mutability occurred only in some plants after the fifth generation of inbreeding. We used Southern blot hybridization to determine if this was correlated with a decrease in Mu element copy number (Robertson et al., 1985), modification of the Mu elements (Chandler and Walbot, 1986; Bennetzen, 1987), or both. Figure 4 (right panel) shows the EcoRI hybridization profiles of individual plants grown from Class 2, 3, and 4 seeds of ear 85-6151-8/5151-3T. This was one of the fifth inbred generation progeny ears which exhibited the first significant loss of somatic mutability in the al-Mum2 lineage. The Mu element copy numbers in these plants were similar to the 1982 and 1984 progenitors. Again, approximately 20 bands (equivalent to about 40 copies per diploid genome) were detected for each sample. No detectable difference in copy number was observed between plants from different Classes of kernels. These results concur with Bennetzen (1985), who reported that in the mutable line, bz-Mum4, loss of somatic mutability was not accompanied by a significant decrease in Mu element copy number.

To determine if the modification of Mu elements had occurred in the low mutable seeds, genomic DNAs were examined by digestion with Tthl1II and HinfI. The hybridization
profiles are shown in Figure 5. Bands corresponding to Mu-like and Mu1.7-like elements were seen in the TthllI profiles. Digestion with HinfI, however, produced different patterns in each plant. If all the Mu elements were unmodified, two bands, 1.3 kb and 1.6 kb in size, would be expected. These were, in fact, the predominant bands seen in the highly mutable Class 4 sample. Class 3 and Class 2 samples had increasing numbers of modified, compared to unmodified, elements, which was evident from the greater number of high molecular weight bands seen in these samples. In other words, the results indicated that a decrease in somatic mutability was accompanied by an increase in Mu element modification. This result was not an artifact of incomplete digestion by HinfI. Rehybridization of this blot with a maize ribosomal DNA probe showed that the DNA samples were equivalently digested and no significant loading differences existed between the lanes (data not shown).

Loss of germinal activity is not accompanied by modification of Mu elements in the al-Mum2 inbred lines which retain somatic mutability.

Plant 84-1119-9 was derived from the selfed progeny of plant 83-8207-1 which had no Mutator activity as measured by Robertson's standard test (Roth et al., 1987). Therefore, it was assumed that plant 84-1119-9 also had no Mutator.
Figure 5. Southern hybridization analysis of Mu elements in individual 1985 al-Mum2 plants grown from Class 2, 3, and 4 seeds.

Maize genomic DNA was digested to completion with Hinfl and Tth111I, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized to a MuAT probe. Lanes designated Class 2, 3, and 4 contained DNA isolated from plants grown from seeds of that mutability level. Q60 lane: DNA from hybrid Q60 digested with Tth111I was included as a negative control. Mu1 Hinfl lane: Hinfl-digested Mu1 positive control. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
activity, even though the ear produced by this plant showed no loss of somatic mutability. Robertson (1986) showed that plants which lose germinal activity by inbreeding did not regain activity in subsequent crosses (Donald S. Robertson, Iowa State University, Ames, IA, personal communication). It has been shown that in these inbred Mu-loss lines, Mu elements were irreversibly modified about the HindIII, and other restriction endonuclease, target sites (Bennetzen, 1987; David W. Morris, Iowa State University, Ames, Iowa, personal communication). In order to quantitate the Mu elements and determine if they were modified in the progeny of 84-1119-9, DNA was isolated from individual plants from Class 4 seeds of the selfed progeny, digested with EcoRI, TaqI, and HindIII, and hybridized to MuAT. The results are shown in Figure 6. The three sibling plants tested contained both Mu1 and Mu1.7 elements, deduced from TaqI profiles. The Mu element copy number was about 35 to 45 per diploid genome in each plant, and similar to other plants in this lineage. Thus, no change in copy number occurred upon the loss of germinal Mutator activity. Equally important, no modification of the Mu elements was observed, as can be seen from the HindIII profiles.

To demonstrate that populations of sibling plants of the 1982 and 1984 progenitors to 85-6151/5151 and the 83-8207-1 progenitor of 84-1119-9 had no significant modification,
Figure 6. Southern hybridization analysis of Mu elements in plants from Class 4 seeds and no germinal activity.

Maize genomic DNA was digested to completion with EcoRI, HinfI, or Tth111I, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized to a MuAT probe. Lanes a, b, and c contained DNA from individual sibling plants from ear 84-1119-9. Q60 lane: DNA from hybrid Q60 digested with Tth111I was included as a negative control. Mu1 HinfI lane: HinfI-digested Mu1 positive control. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
hybridization analyses were performed on DNA pooled from fifty seedlings derived from each of the following plants used to produce the αl-Mum2 inbred lineage (see Figure 1): 81-82-5503-6; 82-2154-5; 82-83-3582-6; 83-8207-1; 83-8207-2; and 84-1120-4. The HinfI hybridization profiles of each of these samples are shown in Figure 7. No significant modification of Mu elements was seen in any of these lines.

The weakly hybridizing high molecular weight bands seen in some lines most likely represented Mul-homologous sequences observed in many other Mutator and nonMutator lines (Chandler et al., 1986; Roth et al., 1987). From our results, we concluded that Mu element modification is correlated with the loss of somatic mutability, but is not always associated with the loss of germinal activity.

The nature of Mu element modification varies between plants from seeds of different mutability classes

Hybridization analyses were carried out on DNA pooled from fifty seedlings derived from the 85-86-5558 and -6558 families. The plants tested exhibited progeny which differed in the extent of kernel mutability and retention of germinal activity: (1) 85-86-5558-1: low somatic mutability and no germinal activity; (2) 85-86-5558-3: low somatic mutability and germinal activity; (3) 85-86-6558-7: high somatic mutability and no germinal activity; and (4) 85-86-6558-4:
Maize genomic DNA was isolated from seedlings grown from fifty kernels of each ear, digested to completion HinfI, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized to a MuAT probe. Sample lanes correspond to families in Figure 1: 81-82-5503-6, 82-2154-5, 82-83-3582-6, 83-8207-1, 83-8207-2, and 84-1120-4. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
high somatic mutability and germinal activity. In addition to \textit{HinfI}, modification of \textit{Mu} elements was assayed using \textit{HpaII} to test if modification occurred in the internal regions of \textit{Mu} elements as well as the extreme ends of the terminal inverted repeats. The internal regions of \textit{Mu} elements contain nine target sites for the methylation-sensitive restriction endonuclease, \textit{HpaII}. If unmodified \textit{Mu} elements were present in these lines, then, upon digestion with \textit{Tth}III and \textit{HpaII} in combination, the majority of the hybridizing bands would be 350 bp and less in size. If the internal regions of \textit{Mu} elements were modified, some or all of the \textit{HpaII} sites would be blocked and the expected molecular weights of the hybridizing bands would be greater than 350 bp, up to the sizes of the bands generated by \textit{Tth}III alone. DNA was isolated from fifty seedlings, digested with \textit{HinfI}, \textit{Tth}III, or \textit{Tth}III/\textit{HpaII}, blotted, and hybridized to \textit{MuAT}. The results are shown in Figure 8. Each set of sample lanes, 1 through 4, corresponds to the four sources of plant material mentioned before (e.g., lanes labeled "1", denote DNA of plants from 85-86-5558-1, and so on). Hybridization profiles generated from \textit{Tth}III digestion showed the two bands expected from \textit{Mul}-like and \textit{Mul.7}-like elements (1.0 and 1.35 kb, respectively). Digestion with \textit{HinfI} generated two bands, representing unmodified \textit{Mul}-like and \textit{Mul.7}-like elements, from all samples. The hybridization profiles
Maize genomic DNA was digested to completion with
HinfI, TthIII, or TthIII and HpaII in combination,
fractionated by electrophoresis on 0.8% agarose
gels, transferred to Genatran, and hybridized to a
MuAT probe. Lanes: (1) 85-86-5558-1, low somatic
mutability and no germinal activity, (2) 85-86-5558-
3, low somatic mutability and germinal activity, (3)
85-86-6558-7, high somatic mutability and no
germin al activity, (4) 85-86-6558-4, high somatic
mutability and germinal activity, and (5) 82-2154-5,
a positive control for high somatic mutability,
germin al activity, and no Mu element modification.
Mu HinfI lane: HinfI-digested Mu positive
control. Molecular weights were estimated from
lambda DNA fragments generated by PstI digestion.
obtained from digestion with both Tth111I and HpaII differed in some samples, however. Plants from low mutability seeds (lanes 1 and 2) generated two predominant bands equal in size to those seen in the Tth111I digests only, indicating that most of the HpaII sites within the Mu elements in these lines were modified. Lanes 3 and 4 contained DNA samples from plants grown from high mutability seeds: only one predominant band, 350 bp, was present, the smaller bands having run off the end of the gel, showing that most Mu elements were unmodified at these sites. The blot shown in Figure 8 was stripped and rehybridized to a maize ribosomal DNA probe to test for complete sample digestion. The results, shown in Figure 9, demonstrated similar rDNA hybridization profiles for each sample and confirmed that the digestions were equivalent.

In summary, these results showed once again that modification of Mu elements was closely associated with loss of somatic mutability, and did not correlate with loss of germinal activity. In the low mutability lines, however, HpaII sites, rather than HinfI sites, were modified. We hypothesize that two types of Mu element modification occurred in this lineage, both leading to a decrease in somatic mutability. In the earlier results, loss of somatic mutability was associated with the modification of HinfI
Figure 9. Rehybridization with a rDNA probe to test for complete digestion

The filter probed with MuAT in Figure 8 was stripped and rehybridized with to a maize ribosomal tandem repeat probe to test for complete sample digestion. Lanes: (1) 85-86-5558-1, (2) 85-86-5558-3, (3) 85-86-6558-7, (4) 85-86-6558-4, and (5) 82-2154-5. Mu HinfI lane: HinfI-digested Mu1 plasmid. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
sites in Mu elements, however, not all Mu elements were modified. Presumably, in some cells, the Mu element at the al-Mum2 locus was unmodified and contributed to the low level of somatic mutability seen in these lines. In the latter results, modification of HpaII sites had occurred in most, if not all, of the Mu elements present in the low mutability lines. The HinfI sites of these elements, however, remained unmodified, presumably allowing the extreme ends of the terminal inverted repeats to interact with a transposase, resulting in element excision, albeit at a very low frequency. Thus, the modification of sequences in the internal portion of Mu elements also correlated with the loss of somatic mutability even though little or no modification of the terminal sequences was observed.
In this paper, we have reported the results of Southern hybridization analyses of Mu elements in a highly inbred al-Mum2 lineage. It was previously demonstrated that this line maintained somatic mutability through seven generations of inbreeding (Roth et al., 1987). Germinal activity was lost in several plants after the third inbred generation (83-8207-1, -3, -4), but one sibling plant, 83-8207-2, retained this activity through to the sixth generation. Some loss of somatic mutability was observed in the progeny ears of some plants after the fifth generation of inbreeding. We hypothesized that a defect occurred in Mutator regulation in plant 83-8207-2, allowing somatic mutability and germinal activity to function under a circumstance where normal activity would cease.

Chandler and Walbot (1986) first reported that modification of Mu elements occurred in plants from seeds that had lost somatic mutability. Bennetzen (1987) extended these studies and deduced that Mu element modification was most likely due to the methylation of cytosine bases within the elements. Modification was also correlated with the loss of germinal activity which occurred following several generations of inbreeding (Bennetzen, 1987; David W. Morris, Iowa State University, Ames, Iowa, personal communication).
Modification of transposable elements, presumably by methylation, has been associated with the inactivation of Ac (Chomet et al., 1987) and the bacterial transposon, IS10 (Roberts et al., 1985).

In this study, we found that the onset of modification, assayed using HindIII digestion, correlated well with the loss of somatic mutability in al-Mum2 when testing sibling plants from different Classes of seeds from a single ear (which exhibited a broad range of mutability Classes, indicating the onset of the loss of somatic mutability). This result was in agreement with the results of Chandler and Walbot (1986). In addition, the degree of Mu element modification was proportional to the level of somatic mutability. Thus, when many reversion events were observed (Class 4), relatively few Mu elements were modified. Conversely, when few somatic reversion events occurred, a larger number of Mu elements were modified. It was assumed that when many Mu elements in a plant are modified, the chance of modification of the Mu element at al-Mum2 in any cell increases, and vice versa. Also, no change in element copy number was observed upon loss of somatic mutability in these plants.

In plants from seeds that retained somatic mutability, but whose progenitors had lost germinal activity, no modification of Mu elements was observed. These results were not due to exceptional plants being inadvertently selected...
for analysis because in tests with DNA pooled from fifty seedlings, no appreciable modification of Mu elements was observed. We concluded from these results that DNA modification was not necessarily correlated with the loss of germinal activity. This may mean that somatic mutability (which involves the excision of a Mu element from a mutant locus) and germinal activity (which involves, for the most part, replication and transposition of a Mu element at a mutant locus) may be differentially regulated.

We observed that two types of Mu element modification occurred: one involving modification of sequences at the ends of the terminal inverted repeats (HinfI sites); the other, within the internal Mu sequences (HpaII sites). Different patterns of restriction endonuclease target site modification were previously reported by Bennetzen (1987). He demonstrated the partial modification of target sites for the methylation-sensitive restriction endonucleases AvaII, BglII and SstII. In some lines, even EcoRII, HpaII, and MspI showed incomplete modification. Therefore, the lack of modification about the HinfI target sites in the samples in Figure 8 was not exceptional.

It is possible that the different patterns of Mu element modification seen in these studies may be the result of the putative defect in Mutator regulation present in this lineage. However, we must point out that in these, and
similar studies, it was assumed that the \textit{Mu} element modification patterns observed in DNA isolated from whole plants, reflected the modification state of \textit{Mu} elements in the aleurone tissue. In addition, the mutability level of the mature plant was presumed to be the same as that in the aleurone even though these tissues were derived from two independent fertilization events.
REFERENCES


SECTION III. THE PREVALENCE OF MUTATOR ACTIVITY AND THE CHARACTERIZATION OF Mu1-HOMOLOGOUS DNA SEQUENCES IN WIDELY DIVERSE LINES OF MAIZE
The prevalence of Mutator activity
and the characterization of MuI-homologous
DNA sequences in widely diverse lines of maize

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Robertson's Mutator lines of maize are defined by their ability to induce new germinal mutations at a frequency up to 50-fold above the background mutation level. Transposable elements, called Mu1 elements, have been implicated as the causative agents of Mutator activity. They are found in high copy number in Mutator lines and many Mutator-induced mutant alleles are associated with Mu1 insertions, or structural derivatives of this element. We report here the presence of a mutator activity (as defined by Robertson's standard test) in two lines of maize that were not derived from Robertson's original Mutator stock. Each of these lines contained low numbers of sequences homologous to Mu1. However, correlation between mutator activity and the presence of these sequences could not be demonstrated. In further studies, fourteen widely diverse lines of maize obtained from different geographic locations, and several cornbelt inbred lines and varieties were also shown to contain a low copy number of Mu1-homologous elements by Southern blot hybridizations. The origin of Robertson's Mutator system is considered in the light of these results.
INTRODUCTION

Active transposable element systems furnish organisms with a source of possible genetic change by their intrinsic mobility. Moving from one chromosomal location to another, they produce mutations by inserting into or near genes, or by causing chromosomal rearrangements such as deletions, duplications, and inversions (see reviews, Shapiro, 1983; Nevers et al., 1985). Repetitive DNA sequences with structures similar to transposable elements have been found in most organisms. McClintock (1984) hypothesized that these sequences have the potential to become mobilized provided a stress which challenges an organism be applied. However, studies of transposable element systems have been limited to those organisms that are amenable to thorough genetic analysis, such as maize. Many transposable element systems have been described in this plant. Several of them, such as Ac-Ds and Spm (En-I), have been well characterized on the genetic level, and, lately, the elements responsible for their activity have been isolated by molecular cloning and their structures analyzed extensively (Fedoroff et al., 1983; Döring et al., 1984; Pereira et al., 1985).

Robertson's Mutator system has attracted a great deal of attention recently as a unique transposable element system compared to the other systems characterized in maize (see
review, Lillis and Freeling, 1986). The Mutator maize line was first described by D.S. Robertson as a stock of maize which generated a high frequency of mutations at a variety of different loci at rates 50-fold above the spontaneous mutation frequency (Robertson, 1978). Approximately 40% of the new mutations induced in this line were unstable (mutable) which suggested that transposable elements were responsible for their induction. This was confirmed when a mutable mutant allele of *Adhl-S*, *adhl-S3034*, isolated from a Mutator background, was shown to contain a DNA insertion, approximately 1.4 kilobase (kb) pairs in size (Strommer et al., 1982). The insertion was subsequently cloned, named *Mul* (Bennetzen et al., 1984), and sequenced (Barker et al., 1984). The nucleotide sequence demonstrated that *Mul* had some features characteristic of previously cloned transposable elements, such as long terminal inverted repeats, and had caused the duplication of a short sequence of host DNA at the site of *Mul* insertion. Mutator maize lines contained approximately 10-70 copies of sequences similar in size and structure to *Mul* (Bennetzen et al., 1984; Barker et al., 1984). Southern hybridization analyses demonstrated that both *Mul* and *Mul.7* elements were transposable (Alleman and Freeling, 1986; Taylor et al., 1986). In addition to *Mul* elements, some Mutator lines contained one or more copies of a larger element structurally related to *Mul*. These elements,
termed Mul.7, differ from Mul by the insertion of a 380 bp sequence, some restriction endonuclease target site polymorphisms, and a few small rearrangements (Taylor et al., 1986). They were also shown to be transposable.

It was generally supposed, until quite recently, that maize lines other than Mutator (referred to as nonMutator lines), did not contain DNA sequences homologous to Mul. Bennetzen (1984), for example, reported that only Mutator lines contained multiple copies of sequences homologous to Mul. However, Chandler and coworkers (1986) later reported the presence of Mul-homologous DNA sequences in eight nonMutator stocks of maize which consisted of six cornbelt inbred lines and two other varieties. Each line tested by hybridization analyses with a probe representing the internal portion of Mul had less than one to three copies of Mul-like sequences per haploid genome. Additionally, sequences homologous to the terminal inverted repeat region of Mul were present in approximately 40 copies per genome.

We have extended the previously described studies by determining the prevalence of Mutator activity as defined by Robertson's standard test for the induction of new mutants, in several maize inbred lines and varieties. In addition, we surveyed these lines and a wide variety of lines of diverse geographic origin for the presence of Mul-homologous sequences using the Southern blot hybridization technique. We report
the presence of mutator activity in two lines of maize unrelated to Robertson's Mutator stocks, and the presence of Mu-homologous sequences, in low copy numbers, in all the maize lines we examined.
MATERIALS AND METHODS

Maize stocks

The nonMutator maize stocks used in this study were obtained from several sources. The origins of the inbred and hybrid lines are described in Table 1. The widely diverse maize lines described in Table 2 were obtained from the USDA Central Region Plant Introduction Station, Ames, Iowa). Stocks were propagated at Iowa State University, Curtiss Farm. Plants to be used for DNA isolations were grown in a growth chamber.

Measurement of mutator activity

NonMutator maize lines listed in Table 1 were tested for mutator activity as previously described (Robertson, 1978, 1983).

Isolation of maize DNA

Maize nuclear DNA was isolated according to the protocol of Rivin and coworkers (1982) from pooled seedlings at the 2 to 4 leaf stage of growth.
Table 1. Inbred and hybrid lines used in this study

<table>
<thead>
<tr>
<th>Line</th>
<th>Origin</th>
<th>Mutator Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mu-Pion (B73 (WSP)&lt;4)</td>
<td>Dr. Donald Duvick</td>
<td>yes (weak)</td>
</tr>
<tr>
<td></td>
<td>Pioneer Hi-Bred Intl.</td>
<td></td>
</tr>
<tr>
<td>B73 (progenitor of B73 (WSP)&lt;4)</td>
<td>Dr. Donald Duvick</td>
<td>yes (weak)</td>
</tr>
<tr>
<td></td>
<td>Pioneer Hi-Bred Intl.</td>
<td></td>
</tr>
<tr>
<td>Gaspé Flint</td>
<td>Dr. Walton Galinat</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>Univ. of Connecticut</td>
<td></td>
</tr>
<tr>
<td>Wilber's Knobless Flint</td>
<td>Maize Genetics Coop.</td>
<td>no</td>
</tr>
<tr>
<td>B37 (nonMutator)</td>
<td>Dr. Wilbert Russell</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>Iowa State University</td>
<td></td>
</tr>
<tr>
<td>B37 (&quot;Mutator&quot;)</td>
<td>Dr. Wilbert Russell</td>
<td>yes (weak)</td>
</tr>
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<td></td>
<td>Iowa State University</td>
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<tr>
<td>A632</td>
<td>Dr. H. Green</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>Molecular Genetics Inc.</td>
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<tr>
<td>Mo17</td>
<td>Dr. Wilbert Russell</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>Iowa State University</td>
<td></td>
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<tr>
<td>B73 (ISU)</td>
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<td>no</td>
</tr>
<tr>
<td></td>
<td>Iowa State University</td>
<td></td>
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<tr>
<td>Q60 (Q66/Q67)</td>
<td>Dr. Donald Robertson</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>Iowa State University</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Mutator activity measured by Robertson's (1978) standard test.
Table 2. Origin diverse lines from the Plant Introduction Station

<table>
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<th>Origin</th>
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<th>Variety</th>
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<td>228166</td>
<td>USSR</td>
<td>FT</td>
<td>N/A</td>
</tr>
<tr>
<td>433656</td>
<td>Italy</td>
<td>-</td>
<td>Bianco Perla</td>
</tr>
<tr>
<td>210402</td>
<td>So. Africa</td>
<td>DT</td>
<td>Robyn</td>
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<tr>
<td>244067</td>
<td>Yemen</td>
<td>FT ST DT</td>
<td>N/A</td>
</tr>
<tr>
<td>279022</td>
<td>Spain</td>
<td>FT DT</td>
<td>Enano</td>
</tr>
<tr>
<td>239109</td>
<td>Yugoslavia</td>
<td>FT</td>
<td>N/A</td>
</tr>
<tr>
<td>405705</td>
<td>People's Republic of China</td>
<td>FT</td>
<td>Chan II inbred</td>
</tr>
<tr>
<td>207528</td>
<td>Afghanistan</td>
<td>FT DT</td>
<td>N/A</td>
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<td>221703</td>
<td>Indonesia</td>
<td>DT</td>
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<td>W. Pakistan</td>
<td>DT</td>
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<td>Nepal</td>
<td>FT</td>
<td>N/A</td>
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<td>Thailand</td>
<td>FT</td>
<td>Baby Sugar Sweet</td>
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<td>346433</td>
<td>W. Indies</td>
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<td>N/A</td>
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<tr>
<td>291390</td>
<td>China</td>
<td>FT DT</td>
<td>Early Variety</td>
</tr>
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aPlant Introduction Station number.
bSeed types include: FT, flint; DT, dent; ST, starchy; and FL, floury.
cVariety name; N/A, not applicable.
Restriction endonuclease digestion and gel electrophoresis of maize DNA

Restriction endonucleases obtained from New England Biolabs (Beverly, MA) and Bethesda Research Labs (Gaithersburg, MD) were used according to the manufacturers' instructions. High molecular weight DNA (5-7 μg) was digested with restriction endonucleases at a concentration of 6-8 units/μg of DNA for 3 to 4 hours, and fractionated on 0.8% agarose gels made with TBE (0.09 M Tris, 0.09 M boric acid, 0.0025 M Na2EDTA) for 16-18 hours at 45V. Gels were stained with ethidium bromide (500 μg/ml) in distilled water. The stained gels were photographed under short wave ultraviolet light with an MP4 camera and Type 55 film (Polaroid).

Southern transfer and DNA hybridization

DNA fragments in gels were nicked by ultraviolet light under a germicidal hood, denatured, and transferred to Genatran (Flasco) using Southern's (1975) procedure. Filters were baked for 2 hours at 80°C and prehybridized in 50% (v/v) formamide; 3 x SSC; 5 x Denhardt's solution; 100 μg/ml sonicated and denatured salmon sperm DNA; and 1% SDS, for 6 hours to overnight. Hybridizations were carried out at 42°C for 36 hours in prehybridization solution with the addition of 5% dextran sulfate and 25 ng of probe DNA. Filters were then
rinsed two times with 250 ml of wash buffer (0.1% SDS, 0.1x SSC) at room temperature followed by three, 30 to 45 minute washes at 65° C with wash buffer pre-heated to 65° C. The filters were blotted dry on paper towels and exposed to Kodak XAR-5 film with one Lightning-Plus (Dupont) intensifying screen for 1-3 days at -70° C. Filters were stripped for rehybridization by two, 20 minute washes with 2000 ml of wash buffer heated to 95-100° C. Rehybridization was carried out as described before.

Hybridization probes

The DNA probes were prepared from plasmid pRB1 which consists of a 2.9 kb HindII-PstI fragment of the adhl-S3034 mutant allele including the entire Mul element (Bennetzen et al., 1984), subcloned into pSP64 (Promega Biotec). The probes (Figure 1) were: (1) a HinfI fragment of Mul (MuH) consisting of almost the entire Mul element; (2) an internal AvaI-TaqI fragment of Mul (MuAT); and (3) a HindIII-Mul fragment of Mul, specific for the terminal inverted repeat sequences. The fragments were generated from pRB1 using the appropriate restriction endonucleases, purified on agarose gels (Maniatis et al., 1982), and labeled to a specific activity of at least 4 x 10^8 cpm/μg by random hexamer priming (Feinberg and Vogelstein, 1983) using a kit (Pharmacia).
Figure 1. *Mul* hybridization probes

"Mutator" activity in a Mutator-Pioneer line of maize

In 1982, we found that a modified B73 maize line with the WSP type of cytoplasm had weak mutator activity as measured by Robertson's standard test (Robertson, 1978). This stock, which was subsequently termed Mutator-Pioneer (Mu-Pion), was developed by Dr. Don Duvick, Pioneer Hi-Bred International, Johnston, IA, who provided the seeds for testing. Seven plants of the original Mu-Pion line (B73(WSP)<4) were outcrossed to ISU standard lines, and plants from two of the outcross progeny ears were selfed to determine if any seedling mutants had been induced by the Mu-Pion parents. Four selfed ears of 112 outcross plants tested segregated for new mutants, which represented a mutation frequency eighteen times higher than the control rates (3.6% compared to 0.2%). The frequency observed was somewhat lower than that of most Mutator stocks, but still significant.

In 1983 and 1984, additional outcresses of the Mu-Pion stocks were made and subsequently tested for the induction of new mutants. Reciprocal crosses were also made with standard lines to determine if the WSP cytoplasm had an effect on the induction of mutants. The results of these tests are given in Table 3. The outstanding feature of these crosses was that the mutator phenotype (as measured by the induction of new
Table 3. Summary of mutation frequencies in outcrosses of the original Mutator-Pioneer (Mu-Pion) stock

<table>
<thead>
<tr>
<th>Plant Number</th>
<th>Total Number of the Mu-Pion</th>
<th>Outcross Parent Plants</th>
<th>Segregating Selfed Mutants</th>
<th>% Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>82-074-1</td>
<td>93</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>82-074-2</td>
<td>48</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>82-074-4</td>
<td>89</td>
<td>1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>82-074-7</td>
<td>42</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>82-074-8</td>
<td>46</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>82-074-9</td>
<td>98</td>
<td>2</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>82-074-10</td>
<td>94</td>
<td>2</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

B. Crosses of Mu-Pion as Females

<table>
<thead>
<tr>
<th>Plant Number</th>
<th>Total Number</th>
<th>Outcross Parent Plants</th>
<th>Segregating Selfed Mutants</th>
<th>% Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>83-8073-1</td>
<td>41</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>83-8073-2</td>
<td>53</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>83-8073-3</td>
<td>26</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>83-8073-9</td>
<td>53</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

C. Reciprocal Crosses of Mu-Pion

<table>
<thead>
<tr>
<th>Plant Number</th>
<th>Total Number</th>
<th>Outcross Parent Plants</th>
<th>Segregating Selfed Mutants</th>
<th>% Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>84-5225 ♂</td>
<td>42</td>
<td>1</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>84-5226 ♂</td>
<td>48</td>
<td>3</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>84-5227 ♂</td>
<td>47</td>
<td>1</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>84-5228 ♂</td>
<td>46</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>84-5229 ♂</td>
<td>44</td>
<td>1</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>84-4230 ♂</td>
<td>47</td>
<td>1</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>84-5231 ♂</td>
<td>44</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>84-5232 ♂</td>
<td>47</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Totals 1048 12 1.15

Total of Families Segregating for Mutants 509 12 2.4
mutants) was erratic in expression, in that only some of the crosses gave rise to new germinal mutations. Additionally, no significant differences were seen when Mu-Pion stocks were reciprocally crossed, thus providing no evidence for the involvement of the WSP cytoplasm in the induction of mutants. Further tests for mutator activity were carried out with the B73 and SK2(WSP) progenitors of Mu-Pion (the latter line being the source of WSP), as well as the original WSP cytoplasm line (isolated from WF9). A low level of mutator activity (approximately 2.0%) was observed in the B73 progenitor of Mu-Pion, as well as in other Pioneer B73 stocks (Table 4). However, no mutator activity was observed in either the SK2(WSP) progenitor, or in the original WSP cytoplasm line (data not shown). Taken together, these data suggest that the mutator activity found in the Mu-Pion stocks was not contributed by the WSP cytoplasm of this stock or the nuclear components of WF9 or SK2. The likely source of the genetic instability in this line was, therefore, the B73 recurrent parent (perhaps enhanced by combination with the WSP cytoplasm).

In further studies, Mu-Pion plants were crossed together to determine if an increase in mutator activity would occur in the progeny. Robertson had shown earlier that crossing Mutator plants resulted in a three-fold increase in Mutator activity in the progeny (Robertson, 1983). No increase in
Table 4. Summary of mutation frequencies in outcrosses of the B73 progenitor of Mu-Pion stock and other Pioneer B73 stocks

<table>
<thead>
<tr>
<th>Plant Number</th>
<th>Total Number</th>
<th>Number of ears</th>
<th>% Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Outcross</td>
<td>Segregating</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Selfed</td>
<td>Mutants</td>
<td></td>
</tr>
</tbody>
</table>

A. B73 progenitor of Mu-Pion

<table>
<thead>
<tr>
<th>Plant Number</th>
<th>Total Number</th>
<th>Number of ears</th>
<th>% Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>82-83-4528</td>
<td>49</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>82-83-4529</td>
<td>31</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>84-5305</td>
<td>45</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>84-5306</td>
<td>49</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

B. Other Pioneer B73 stocks

<table>
<thead>
<tr>
<th>Plant Number</th>
<th>Total Number</th>
<th>Number of ears</th>
<th>% Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>82-83-4532</td>
<td>55</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>82-83-4533</td>
<td>55</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>82-83-4536</td>
<td>45</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td>82-83-4537</td>
<td>50</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
mutator activity was observed, however, in the progeny of the intercrossed Mu-Pion plants, termed Mu-Pion<sup>2</sup>. These Mu-Pion<sup>2</sup> plants were intercrossed once more, and then outcrossed to standard lines to determine mutator activity. One of the outcrosses had three plants segregating for new mutants, two of which were mutable. The mutation frequency obtained was about 7.0% and was comparable to that of the original Mutator stocks isolated by Robertson (1978).

The presence of a mutator activity in the Mu-Pion stocks prompted us to examine their genomic DNAs for the presence of Mu-homologous DNA sequences. In the initial analyses, nuclear DNA was digested with restriction endonucleases EcoRI, PstI, BamHI, and HindIII, which do not cleave within Mul. The digested DNAs were fractionated on agarose gels, blots made, and hybridized to a probe specific for the entire Mul element (MuH). The hybridization profiles are shown in lanes 1 through 8 in Figure 2. Approximately three to four hybridizing bands were visible in each lane, indicating that three to four copies of Mu-homologous sequences per genome were present in this line. To determine if these sequences were similar in structure to Mul, Mu-Pion DNA was cleaved with HinfI or Tth1111 and hybridized to MuH. Both of these endonucleases have a single target site within each of the Mul terminal inverted repeats (Barker et al., 1984); thus, if Mul-like elements were present, digestion with these endonucleases
Figure 2. Southern hybridization analysis of Mu-Pion DNA

Mu-Pion DNA samples were digested to completion with various restriction endonucleases, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized with a MuH probe. EcoRI (lanes 1 and 2), PstI (lanes 3 and 4), BamHI (lanes 5 and 6), HindIII (lanes 7 and 8), HinfI (lane 9), Tth111I (lane 10), and HinfI-digested MuH plasmid control (lane 11). Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
would generate fragments similar in size to control digests of Mul. The results are shown in Figure 2, lanes 9-11. Three bands 1.7, 2.1 and 2.3 kb in size were detected following HinfI digestion (lane 9). Although a band at 1.3 kb, expected if Mul-like elements were present, was not detected, the 1.7 kb band may have been derived from the larger, structurally-related derivative of Mul, called Mul.7 (Taylor et al., 1986). However, the bands generated by TthIII digestion (lane 10) did not confirm that Mul.7-like elements were present in this line. Two bands, 2.5 and 2.6 kb in size, were detected, but a 1.3 kb band, expected if Mul.7-like elements were present, was not seen. We concluded, therefore, that the Mu-homologous sequences present in this line were probably quite distinct in structure from either Mul or Mul.7 elements.

Furthermore, no genetic or molecular evidence has been obtained which would suggest that the Mu-homologous sequences in this line were transposable. Efforts were made to produce insertional mutants at the Waxy (Wx) and Shrunken (Sh) loci, for which DNA probes are available for molecular analysis, by crossing tester plants homozygous for wx and sh as males with Mu-Pion plants. However, no sh or wx mutants were isolated from approximately 8 x 10^5 kernels analyzed. Evidence for the transposition of Mu elements can also be obtained by
comparative Southern blot hybridization analyses of parent and progeny genomic DNAs (Alleman and Freeling, 1986; Bennetzen et al., 1987). We observed no change in the hybridization profiles of parent and progeny plants which strongly suggested that the Mu-homologous sequences did not undergo frequent, if any, transpositions in this line (data not shown).

The prevalence of mutator activity and Mu-homologous sequences in North American inbred lines and varieties of maize

The presence of mutator activity in the Mu-Pion line prompted us to test a variety of cornbelt inbred and other varieties for a similar activity using Robertson's standard test. A low level of mutator activity was detected in an accession of B37 obtained from Dr. Wilbert Russell of Iowa State University. This line was termed, B37 ("Mutator"). Individual plants from this accession were tested by Robertson's standard test, and induced new mutations at a rate of between 1.0% and 2.0%. Another accession of B37 tested, however, did not induce any new mutants, thus it was termed, B37 (nonMutator). The other lines listed in Table 1 were also tested for mutator activity, but none of these lines showed a mutation rate significantly higher than the control lines.

In another series of experiments, the lines shown in Table 1 were examined for the presence of sequences homologous to Mu1 by Southern blot hybridization analyses. In the first
analysis, DNA samples from cornbelt inbred lines B37 ("Mutator"), B37 (nonMutator), A632, Mo17 and B73 (ISU) were digested with EcoRI and hybridized to a probe consisting of the internal fragment of Mul (MuAT). Because the cloned Mul element does not contain any target sequences for EcoRI, Mu-homologous sequences at different genomic locations will be separated on differently sized DNA fragments. The first panel of Figure 3 shows that each line contained only 2 to 3 Mu-homologous bands. Rehybridization of the blot with MuT (Mul terminal repeat probe) also detected only 2 to 3 bands in all the lines tested (data not shown). However, only one, or at most two, of these bands were the same size as those detected by hybridization with MuAT. Taken together, these results suggested that at least three types of Mu-homologous sequences were present in these lines; (1) sequences consisting of both the internal region and one or both of the terminal inverted repeats of Mul; (2) sequences homologous only to the internal portion of Mul; and (3) sequences homologous only to the terminal inverted repeats of the Mul. These results differ in one respect from those of Chandler and coworkers (1986). They reported that in lines they tested, sequences homologous to the terminal inverted repeats predominated over sequences homologous to the internal portion of Mul. We, however, have been unable to substantiate this observation in our lines.
Figure 3. Southern hybridization analysis of EcoRI, Tth111I, and HinfI digests of DNA from several cornbelt inbred lines of maize.

DNA samples from five different cornbelt inbred accessions were digested to completion with EcoRI, Tth111I, and HinfI, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized with a MuAT probe. 1, B37 ("Mutator"); 2, B37 (nonMutator); 3, A632; 4, Mo17; 5, B73 (ISU). Mul lanes: HinfI-digested Mul positive control. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
To determine if the Mu-homologous DNA sequences present in these lines were similar in structure to Mu1, genomic DNAs were cleaved with Tth111I or HinfI, and hybridized to MuAT. Lanes 1 and 2 of the second panel of Figure 3 shows that Tth111I digestion of B37 ("Mutator") and B37 (nonMutator) generated, amongst others, a band 1.0 kb in size, expected if elements similar in structure to Mu1 were present. In the HinfI profiles of these lines, however, a band 1.3 kb in size, predicted if Mu1-like elements were present, was not seen. We hypothesize that Mu1-like elements were present in these lines, however, their digestion by HinfI was inhibited. The inhibition of HinfI digestion of Mu-homologous elements, and their complete digestion by Tth111I, is similar to the DNA modification seen in Mu1 elements from inactive Mutator lines (Chandler and Walbot, 1986; Roth et al., 1987). Modified Mu elements are unable to undergo transposition (Bennetzen et al., 1987); therefore, the Mu1-like elements present in B37 ("Mutator") were unlikely to have contributed to the low level of mutator activity seen in this line. It is noteworthy, in this respect, that the hybridization profiles of both B37 lines ("Mutator" and nonMutator) were identical, providing further evidence that all the Mu-homologous sequences in these lines were inactive. Furthermore, these results are in agreement with Chandler et al. (1986), who have also reported the presence of modified Mu1-like elements in another
nonMutator accession of B37. The hybridization profiles of
the other inbred lines tested here were different from the B37
profiles and from each other. Furthermore, they clearly did
not contain sequences structurally similar to Mul.

Two other varieties of maize were tested for the presence
of Mu-homologous sequences. These were Gaspé Flint, obtained
from Dr. Walton Galinant, and Wilber's Knobless Flint,
provided by the Maize Genetics Cooperation Stock Center.
Hybridization analyses had indicated that sequences homologous
to Mul were present in both lines (data not shown) and a
detailed analysis was carried out to determine if any of those
sequences bore a structural resemblance to Mul. The results
are shown in Figure 4. Many of the bands seen in the
hybridization profiles of Gaspé Flint were characteristic of
Mul. Indeed, the Mul-like elements present in this line
contained sites for restriction endonucleases that cut within
the central region of Mul (such as BstEII and SstII) as well
as the terminal inverted repeats (see also Figure 1).
Furthermore, the Mul-like elements were completely digested
with HinfI and were, therefore, unmodified. They were present
in low copy numbers, estimated to be less than 5 per diploid
genome from densitometric comparison to copy number
reconstructions made from pRBl (data not shown). Although the
Mul-like elements present in Gaspé Flint were unmodified, they
were most likely inactive, because no mutator activity was
Figure 4. Southern hybridization analysis of DNA from Gaspé Flint and Wilber's Knobless Flint

DNA samples from Gaspé Flint (G), Wilber's Knobless Flint (W) and a Mutator (M) control line of maize were digested to completion with restriction endonucleases, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized with a MuH probe. Each DNA sample was cleaved with the following restriction endonucleases: SstII, HinfI/SstII, HinfI, and HinfI/BstEII. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
detected in this line. Robertson et al. (1985) and Bennetzen et al. (1987) have reported that loss of Mutator activity in some outcross progeny of Mutator lines was associated with decreased copy numbers of Mul elements (less than 10 per diploid genome) which did not undergo further transposition. Alternatively, small deletions, or other types of molecular lesions, undetectable by the method of analysis used here, may have been responsible for their apparent inactivity.

Smaller Mu-homologous sequences were also present in this line and in Wilber's Knobless Flint. In each of the hybridization profiles where HinfI was used singly or in combination with a restriction endonuclease that has target sites within Mul, the same sized band, approximately 400 bp, was seen. We hypothesize that this sequence was a deletion derivative of a Mul-like element that had retained both of the terminal inverted repeat sequences.

Mu-homologous sequences are found in maize lines from many geographic locations.

Thus far, our results, together with those reported by Chandler et al. (1986), had shown that sequences homologous to Mul were present in all North American maize lines tested. We were interested, however, to determine if Mu-homologous sequences were present in maize lines derived from other parts of the world. Therefore, similar hybridization analyses were
carried out on the Plant Introduction Station maize accessions listed in Table 2. All lines tested contained sequences homologous to Mu1. Hybridization with MuAT detected 3 to 9 bands in each of the lines (Figure 5). Rehybridization to MuT detected 3 to 4 bands, some of which were similar in size to those obtained with the cornbelt inbred lines; that is, sequences with homology only to the internal region of Mu1, to the terminal inverted repeat only, or to both regions of the Mu1 element, were present in these lines. However, because more bands hybridized to the MuAT probe, the internal region was more predominant than the terminal inverted repeat sequences in most of these lines.

The hybridization analyses carried out to determine in any of the Mu-homologous sequences detected in this lines bore a structural resemblance to Mu1 are shown in Figures 6 and 7. In the Tth1111 profiles (Figure 6), only two lines, in lanes 9 and 10, generated bands characteristic of Mu1. Only the sample in lane 9, however, generated a 1.3 kb band upon HinfI digestion (Figure 7) similar in size to the Mu1 control digestion. Thus, we concluded that the line in lane 9 contained at least one copy of a Mu1-like element that was unmodified; the Mu1-like element presumed to be present in the sample in lane 10 was impervious to HinfI digestion and, therefore, modified. Of note is the observation that many of the lines shared Mu-homologous bands that were similar in
Figure 5. Southern hybridization analysis of EcoRI digests of DNA from widely diverse lines of maize

DNA samples from diverse lines of maize were digested to completion with EcoRI, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized with a MuAT probe. The country of origin of each line was: 1, USSR; 2, Italy; 3, South Africa; 4, Yemen; 5, Spain; 6, Yugoslavia; 7, People’s Republic of China; 8, Afghanistan; 9, Indonesia; 10, West Pakistan; 11, Nepal; 12, Thailand; 13, West Indies; and 14, China. Lane 15: hybrid Q60 (Q66/Q67). Mul: HinfI-digested Mul plasmid control. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
Figure 6. Southern hybridization analysis of Tth1111I digests of DNA from widely diverse lines of maize

DNA samples from diverse lines of maize were digested to completion with Tth1111I, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized with a MuAT probe. The country of origin of each line was: 1, USSR; 2, Italy; 3, South Africa; 4, Yemen; 5, Spain; 6, Yugoslavia; 7, People's Republic of China; 8, Afghanistan; 9, Indonesia; 10, West Pakistan; 11, Nepal; 12, Thailand; 13, West Indies; and 14, China. Lane 15: hybrid Q60 (Q66/Q67). Mul: HinfI-digested Mul plasmid control. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
Figure 7. Southern hybridization analysis of Hinfl digests of DNA from widely diverse lines of maize

DNA samples from diverse lines of maize were digested to completion with Hinfl, fractionated on 0.8% agarose gels, transferred to Genatran, and hybridized with a MuAT probe. The country of origin of each line was: 1, USSR; 2, Italy; 3, South Africa; 4, Yemen; 5, Spain; 6, Yugoslavia; 7, People's Republic of China; 8, Afghanistan; 9, Indonesia; 10, West Pakistan; 11, Nepal; 12, Thailand; 13, West Indies; and 14, China. Lane 15: hybrid Q60 (Q66/Q67). Mul: Hinfl-digested Mul plasmid control. Molecular weights were estimated from lambda DNA fragments generated by PstI digestion.
size. For example, a 15 kb band was present in most lines in the EcoRI hybridization profiles (Figure 5). In TthlII profiles, two bands, 5.75 and 2.9 kb, were prevalent (Figure 6), and 1.8 and 1.6 kb bands were common in the HinfI profiles (Figure 7). This suggested to us that some of the Mu-homologous sequences were conserved in structure in many of these diverse lines of maize.
DISCUSSION

Mutator in maize was characterized only ten years ago (Robertson, 1978). The purpose of this study, which is the most extensive to date, was to determine how prevalent mutator systems are in maize, and to survey different maize lines for the presence of DNA sequences homologous to Mul, the transposable element implicated in Mutator activity (Bennetzen et al., 1984). These studies are important in that they may provide insights into the origin of the Mutator system.

In some earlier studies, Bennetzen (1984) reported that no strong cross-hybridizing Mu-homologous sequences were present in several non-Mutator lines of maize that he tested. In some of our early work, we, too, were unable to detect Mul-homologous sequences in Q60, B70, B77, W23, some accessions of A188, B73, and twelve commercial hybrid lines. Present day studies, however, (Chandler et al., 1986; and this paper), have substantiated that Mu-homologous sequences are present in most, if not all, maize lines that have thus far been examined. The difference in the results obtained earlier from present day work may be explained as follows. Hybridization techniques tended to vary in different laboratories. For example, different washing conditions can profoundly affect the stringency of hybridization. High stringency washes tend to exclude partially homologous DNA hybrids. With the advent
of nylon membranes (e.g., Genatran, Plasco) for Southern blotting and DNA labeling by random hexamer priming, the sensitivity of hybridization analyses has greatly increased over previous methods, which employed nitrocellulose membranes and probes labelled by nick-translation. Also, standardized hybridization conditions are being adopted by many laboratories. In the experiments described here, we used the hybridization procedure described by Chandler et al. (1986).

We have demonstrated a mutator activity, measured by Robertson's standard test, in the modified B73 line with the WSP type of cytoplasm (Mu-Pion). The expression of activity has proven to be erratic from generation to generation. However, a Mu-Pion\(^2\) line was observed to have a level of mutator activity similar to the original Mutator line (Robertson, 1978). Further experiments need to be carried out to determine if activity is maintained in subsequent generations. Mu-homologous sequences, distinct in structure from either Mul or Mul.7, were present in low copy number in this line. We have not been able to demonstrate the transposition of these sequences, however. Therefore, the presence of Mu-homologous elements in this line cannot be correlated with the observed mutational frequency. The molecular characterization of mutable mutant alleles generated in this line is necessary to identify the transposable elements responsible for the mutator activity. However, thus
far, mutations at loci for which DNA probes are available have not been recovered.

We have also observed a low level of mutator activity in an accession of B37, termed B37 ("Mutator"). Another accession of B37, B37 (non-Mutator), however, had no detectable mutator activity. The mutator activity present in the B37 ("Mutator") line could have been due to the activation of a dormant system present in the non-Mutator acquisition of B37. Both of these lines contained Mul-like elements which we determined to be modified because the restriction sites in their termini were not accessible to cleavage by Hinfl. It seems unlikely, therefore, that they play a role in mutator activity in B37 ("Mutator").

We also found DNA sequences homologous to the Mul element in cornbelt inbred maize lines, other North American varieties, and lines of diverse geographical origin. All stocks tested contained sequences homologous to the internal and terminal portions of the element. The internal portion of the element is found in approximately two to three copies per genome in the cornbelt inbred lines and in two to nine copies per genome in the Plant Introduction Station samples. These results are similar to those of Chandler and coworkers (1986). They found one to three copies of the internal portion of Mul in six cornbelt inbred lines, Wilber's Knobless Flint, and Black Mexican Sweet. They also detected approximately forty
copies of the \textit{Mu} terminal repeated sequence in each line. We, however, could not substantiate this last observation in our lines. There appeared to be considerable conservation in the sizes of bands hybridizing to \textit{MuAT} in the Plant Introduction Station accessions. This suggested that the structures of some of the \textit{Mu}-homologous sequences were similar in most of these lines. Confirmation of this must await the molecular cloning of these sequences, which is also necessary to determine if they are modified in the genome.

In summary, these results suggest that the \textit{Mutator} system in maize is probably not the result of a recent uptake of foreign genetic material, such as a viral transformation, as previously suggested by Bennetzen (1984). More likely, the \textit{Mutator} system, is an example of what McClintock (1984) has called a "previously silent mobile genetic element system" which was activated by the "reaction" of the maize to a traumatic genomic shock. It is surprising, however, that few genetically active mutator systems have been found given the prevalence of the DNA sequences homologous to the \textit{Mu} element in all lines of maize.
REFERENCES


Robertson's Mutator system in maize has been studied extensively by Robertson and others since its discovery (Robertson, 1978a) nearly a decade ago. Fundamental questions concerning the regulation of the high mutagenic activity of the system and its origin remain to be answered. Only in recent years, with the advent of molecular genetic techniques, have we begun to relate genetic phenomena to events occurring at the molecular level.

The research reported in this dissertation adds to the body of evidence helping us to understand fully this complex system. The first part of this research described the genetic analyses pertaining to the maintenance of somatic mutability and germinal activity in a highly inbred line of the Mutator-induced A1 allele, A1-Mum2.

Somatic mutability was shown to be retained through to the seventh generation of inbreeding, while germinal activity was retained through to the sixth inbred generation. This observation was unexpected because previously reported results showed that Mutator activity was lost completely after only the third or fourth generation of inbreeding (Robertson, 1986b; Walbot, 1986; Bennetzen et al., 1987). We hypothesize that a functional change occurred in plant 83-8207-2 which resulted in a defective mechanism for Mutator regulation.
Further genetic analyses of plants in this lineage are necessary before this hypothesis can be substantiated fully. Some loss of somatic mutability was observed after the fifth generation of inbreeding. The events that led up to this loss were not dependent on the direction of the cross performed. However, further inactivation of somatic mutability in subsequent crosses was dependent on the mutability level of the female parent.

The characterization of the Mu elements present in the al-Mum2 lineage by molecular hybridization analyses are reported in Section II. We demonstrated that the Mu elements became modified upon the loss of somatic mutability in the aleurone tissue. However, no appreciable modification of Mu elements was observed in those plants which had lost germinal activity while retaining a high level of somatic mutability. A differential modification of restriction endonuclease target sequences present within the terminal inverted repeats and the internal regions of Mu elements was observed.

The work presented in Section III describes the prevalence of mutator activity in various lines of maize. We detected a germinal mutator activity in only two lines of maize (nonMutator lines) that were not derived from Robertson's original stock. However, we showed that these lines, as well as other cornbelt inbreds, varieties, and fourteen widely diverse lines of maize obtained from different
geographic locations, contained a low copy number of sequences homologous to \textit{Mu} by Southern blot hybridizations. Our results suggest that low numbers of \textit{Mu}-homologous sequences are endogenous to almost all maize lines. Therefore, the likely origin of Robertson's \textbf{Mutator} system could be explained by McClintock's postulation that transposable element systems can be "activated" in maize lines by a traumatic genomic shock.

Finally, working on a maize transposable element system for my doctoral dissertation research has been tedious and frustrating at times, however, the effort has been rewarding, and professionally and intellectually stimulating. It is obvious that many more questions concerning Robertson's \textbf{Mutator} system need to be asked, and answered. It is my guess that only then will the complexities of this system be fully realized.
ADDITIONAL LITERATURE


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