Cloning of the *Mutator* Transposable Element *MuA2*, a Putative Regulator of Somatic Mutability of the *a1-Mum2* Allele in Maize

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ABSTRACT

The identification of the autonomous or transposase-encoding element of the *Mutator* (*Mu*) transposable element system of maize is necessary to the characterization of the system. We reported previously that a transcript homologous to the internal region of the *MuA* element is associated with activity of the *Mutator* system. We describe here the cloning of another *Mu* element, designated *MuA2*, that cosegregates with *Mutator* activity as assayed by somatic instability of the *a1-Mum2* allele. The *MuA2* element has features typical of the transposable elements of the *Mutator* family, including the 210-bp terminal inverted repeats. Several lines of evidence suggest that *MuA2* is an autonomous or transposase-encoding element of the *Mu* family: (1) *MuA2* cosegregates with a genetically defined element that regulates somatic mutability of the *a1-Mum2* allele; (2) *MuA2* is hypomethylated while most other *MuA2*-hybridizing sequences in the genome are extensively methylated; (3) the increase of the copy number of *MuA2* is concomitant with the increase of regulator elements; (4) *MuA2*-like elements are found in *Mutator* lines but not in non-*Mutator* inbreds. We propose that autonomous or transposase-encoding elements of the *Mu* family may be structurally conserved and *MuA2*-like.

TRANSPOSABLE element systems in maize are usually composed of two components: the autonomous element and nonautonomous elements. An autonomous element encodes the functions necessary for its own transposition and can also act in *trans* to promote the transposition of nonautonomous elements. In the *Ac-Ds* and *Spm* (*En*) systems, the autonomous element for each system is a single, structurally conserved element whereas nonautonmous elements are structurally heterogeneous. The *Ds* elements of the *Ac-Ds* system vary in structure, ranging from sequences derived from *Ac* by deletions to those that are virtually unrelated to the *Ac* element, except for the terminal inverted repeats (TIRs). Defective *Spm* (*dSpm*) elements of the *Spm* system are usually deletion-derivatives of the *Spm* element (reviewed by FEDOROFF 1989).

The *Mutator* (*Mu*) transposable element system of maize was discovered through its ability to induce mutations at an extremely high rate, usually 50–100-fold higher than spontaneous mutation rates (ROBERTSON 1978). Many unstable mutations isolated from maize plants exhibiting the *Mutator* trait have been shown to be due to the insertion of one of several structurally related transposable *Mu* elements. *Mu1* was the first member of the family cloned and was recovered from an unstable *Adh1* allele (BENNETZEN *et al.* 1984). It is approximately 1.4 kb and has TIRs of approximately 200 bp (BARKER *et al.* 1984). Subsequently, *Mu1.7* (*Mu2*), *Mu3*, *rcy:Mu7* and *Mu8* were also found inserted in *Mu*-induced mutations (TAYLOR and WALBOT 1987; OISHI and FREELING 1988; SCHNABLE, PETERSON and SAEDLER 1989; FLEENOR *et al.* 1990). Two other *Mu* elements, *Mu4* and *Mu5*, were cloned from non-*Mutator* stocks via their homology to the TIRs of the *Mu1* element (TALBERT, PATTERSON and CHANDLER 1989). These *Mu* elements share the TIRs with *Mu1* but the internal regions differ from *Mu1* and from each other, except for *Mu1.7* which is closely related to *Mu1*.

A fundamental difference between the *Mutator* system and the *Ac-Ds* and *Spm* systems has been the apparent non-Mendelian inheritance of *Mutator* activity. Crosses between a *Mutator* plant and a non-*Mutator* plant result in a stable loss of *Mutator* mutagenic activity in about 10% of the progeny, as assayed by the forward mutation rate in seedlings of the subsequent generation (ROBERTSON 1978). In a two-element system, an insertion of a defective element at a locus may suppress gene expression and the excision of the insertion is controlled by a regulatory element elsewhere in the genome. Inheritance of *Mutator* activity has also been studied by its ability to promote somatic reversion at alleles induced by *Mu* insertions (ROBERTSON *et al.* 1985; WALBOT 1986). These studies showed that stable alleles were derived from *Mu*-induced unstable aleurone alleles by outcrossing to

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non-*Mutator* plants and that stable derivatives could be reactivated by crossing to plants exhibiting *Mutator* activity. However, the crosses in which the inactivation or reactivation occurred did not produce ratios that would be expected if one or a few autonomous or transposase-encoding elements were segregating in a Mendelian fashion. It was not clear from these early studies if the *Mutator* system is a two-element system, like the *Ac-Ds* and *Spm* (*En*) systems. Nor was it known which previously cloned *Mu* elements, if any, encode transposase activity required for *Mu* transposition.

The first reported genetic evidence that the *Mutator* system may be a two-element system came from recent genetic analysis of maize lines carrying *Mu*-induced mutable *a1* alleles (ROBERTSON and STINARD 1989). These experiments demonstrated Mendelian segregation of elements or factors that regulate the excision of a nonautonomous *Mu1* element inserted at the *a1* locus. Some of these regulator elements have been mapped to specific chromosomal locations (D. S. ROBERTSON and P. STINARD, unpublished results).

The regulation of *Mutator* activity has been shown to be correlated with the copy number, the transposition, and/or the DNA modification of *Mu* elements. *Mu1*, the most active element in the *Mu* family, is found in multiple copies in *Mutator* lines but not in non-*Mutator* lines (BENNETZEN 1984; ALLEMAN and FREELING 1986). The active transposition of *Mu* elements is usually correlated with *Mutator* activity (ALLEMAN and FREELING 1986; BENNETZEN *et al.* 1987; WALBOT and WARREN 1988). The presence of the *Mu1*-like elements alone, however, is not sufficient for *Mutator* mutagenic activity since *Mu*-loss lines (loss of *Mutator* mutagenic activity) derived from crosses between *Mutator* and non-*Mutator* lines still retain *Mu1*-like elements (ALLEMAN and FREELING 1986; BENNETZEN *et al.* 1987). Loss of *Mutator* activity was also reported from intercrossing of diverse *Mutator* lines (ROBERTSON 1986). This loss is correlated with an increased copy number of *Mu1*-like elements and these *Mu* elements are usually hypermethylated (BENNETZEN 1987). Similar observations between DNA modification and loss of somatic *Mutator* activity have been reported (CHANDLER and WALBOT 1986). Another intriguing observation is that transposition and excision of *Mu* elements might be under separate genetic controls. A high mutation rate (presumably due to the transposition of *Mu* elements) has been observed in a few lines that have lost somatic instability of the *Mu*-induced allele, and some lines exhibit a high somatic instability but have a low germinal *Mutator* activity (ROBERTSON *et al.* 1988).

We recently described the cloning of the *MuA* element from a maize line with a *Mutator* background via its homology to the TIRs of *Mu1* (QIN and ELLINGBOE 1990). *MuA* hybridized to a transcript that is associated with *Mutator* activities. The molecular evidence presented was consistent with what would be expected for an autonomous or transposase-encoding element of the *Mutator* system. We propose that one or more elements of the *MuA* family encode transposase activity. Other *Mu* elements, including previously cloned elements and defective elements of the *MuA* family, would then be receptor elements. Since *MuA* was cloned from a maize line carrying no receptor alleles, we were unable to determine genetically if *MuA* regulates *Mu* transposition, or cross-hybridizes with *Mu* elements that confer *Mutator* activity. We identified a DNA fragment in Southern blots that hybridized to an internal fragment of *MuA*. This fragment cosegregated with an element or gene that has been genetically identified to regulate the somatic excision of the *Mu1* insertion of the *a1-Mum2* allele (ROBERTSON *et al.* 1985; O'REILLY 1985; ROBERTSON and STINARD 1989). In this study, we report the cloning of the *MuA2* element and present evidence that *MuA2* may be an autonomous or transposase-encoding element of the *Mu* family.

MATERIALS AND METHODS

Maize strains: Maize lines DR86-87-9849-7^{n} × 8849-1 (DR1) and DR87-4192-4^{n} × 5192-6 (DR6) (see ROBERTSON and STINARD (1989, Table 3) for the origin of these two lines) contain the *a1-Mum2* allele and segregate 1:1 for somatic *Mutator* activity. Somatic *Mutator* activity refers to the ability of an element or gene to support the mutable pattern of the *a1-Mum2* allele. Somatic mutability is classified into four categories (#1, #2, #3 and #4) ranging from fully stable (#1, colorless) to fully mutable (#4, spotted) according to ROBERTSON and STINARD (1989). In this study, kernels with extremely high frequency of spots (approximately over 90% of the aleurone covered with spots) are classified as #4^{+}. *Mutator* lines are defined as lines from which new mutants have been isolated. Non-*Mutator* lines are standard inbreds. Sources of various *Mutator* stocks and non-*Mutator* inbreds were described (QIN and ELLINGBOE 1990). Two *a1 sh2* tester lines, A632 and Oh43, were kindly provided by W. TRACY (University of Wisconsin-Madison).

DNA isolation and Southern blot analysis: Procedures for plant DNA isolation and Southern hybridization analysis were described previously (QIN and ELLINGBOE 1990). Probes used to identify *MuA2* were the *Eco*RI-*Eco*RV fragment and/or the *Hin*dIII fragment of *MuA* (Figure 1).

λ cloning: Plant DNA was digested with *Sac*I enzyme (Promega) and size-fractionated by gel electrophoresis (0.8% SeaKem GTG agrose, FMC). DNA from the region containing the 4.8-kb *MuA*-hybridizing *Sac*I band was excised from the gel and purified with Geneclean (Bio 101). The purified DNA was ligated to *Sac*I-digested λZap arms (Stratagene) and then packaged into phage particles. The library was screened with the *Eco*RI-*Eco*RV fragment of *MuA*.

Inverse polymerase chain reaction (PCR) amplifications: DNA was prepared from pooled leaf tissues from plants derived from spotted kernels and digested to completion with *Xho*I (Promega). The digested DNA was extracted with phenol:chloroform, ethanol-precipitated, and redissolved in 10 mM Tris (pH 8), 0.1 mM EDTA. The ligations were carried out with either the *Xho*I-digested total genomic

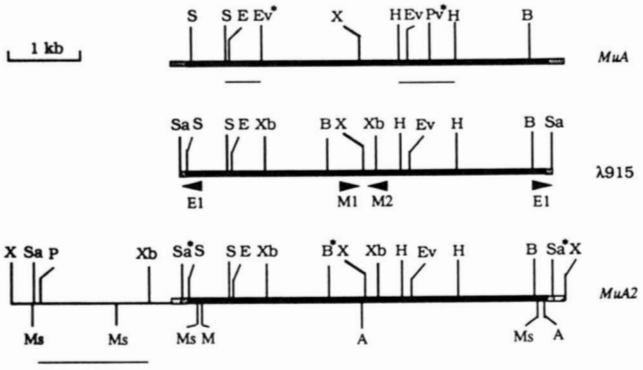

FIGURE 1.—The restriction maps of *MuA*, λ915, and *MuA2*. *MuA* or *MuA2* sequences are shown by the heavy lines. The hatched boxes show the TIRs. The flanking sequences are shown by thin lines. X, *Xho*I; Sa, *Sac*I; P, *Pst*I; Xb, *Xba*I; S, *Sal* I; Ms, *Msp*I; M, *Mlu*I; E, *Eco*RI; B, *Bam*HI; A, *Ava*I; H, *Hin*dIII; Ev, *Eco*RV. * denotes the sites that are different between *MuA* and *MuA2*. The bars under each map show the probes used in Southern hybridization analysis. Arrowheads (not drawn proportionally) under λ915 indicate the locations of the primers used in PCR amplifications.

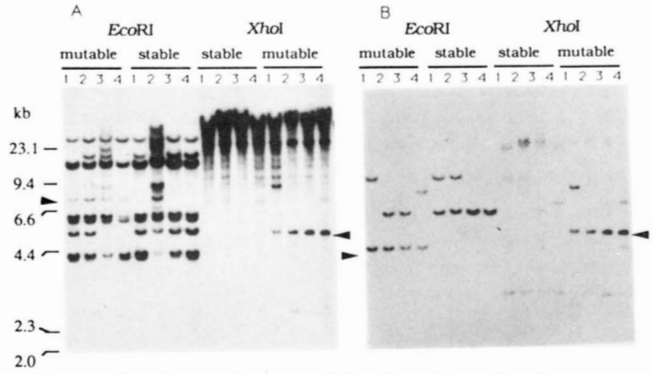

FIGURE 2.—Autoradiogram of Southern blots showing cosegregation of *MuA2* with somatic *Mutator* activity. Plants 1–3 are derived from DR6 and plant 4 from DR1. (A) The blot was probed with *Eco*RI-*Eco*RV fragment of *MuA*. (B) The same blot was reprobed with the *Pst*I-*Xba*I fragment flanking *MuA2*. Arrowheads indicate the bands cosegregating with *Mutator* activity.

DNA or size-selected *Xho*I fragments at a final concentration of 2 μg/ml DNA and 0.02 unit/ml T4 DNA ligase (BRL) at 16° for over 16 hr. The following oligonucleotides were used for PCR amplification: (E1) 5′ AACGGTAAACGCCGACAGGA 3′; (M1) 5′ GTAGCTAGATTGGCGGTGTC 3′; (M2) 5′ CCAGTGGAGTGTTTGCACCA 3′. PCR reactions were performed according to SAIKI (1990) except that BSA was used instead of gelatin. The cycling reactions were: 30 sec at 94°; 2 min at 55°; 5 min at 72° for 35 cycles, using an automated PCR machine (Perkin Elmer Cetus).

Plasmid cloning: PCR-amplified DNA products were gel-purified and digested with *Xho*I and *Sac*I restriction enzymes. After digestion, the DNA was repurified by phenol:chloroform extractions and ethanol precipitation. DNA was then ligated into *Xho*I- and *Sac*I-digested pBluescript DNA and transformed into *Escherichia coli* strain DH5α.

DNA sequencing: DNA sequencing reactions were performed by the dideoxy method using the Sequenase enzyme (U. S. Biochemical). The two ends of the *Sac*I fragment of the phage clone and plasmid clones flanking the *Sac*I fragment were sequenced on one strand using the M13 universal sequencing primer (U. S. Biochemical). Two plasmid clones flanking the left end and three flanking the right end were sequenced. The clones for each flanking region were from a single PCR. Sequence comparisons were made using the University of Wisconsin Genetics Computer Group software and documentation package.

RESULTS

Isolation of the *MuA2* element: Repeated outcrossing of maize lines carrying the *a1-Mum2* allele with non-*Mutator a1 sh2* tester lines resulted in lines that segregated for a single putative regulator element or gene (ROBERTSON and STINARD 1989). DR1 and DR6 (see MATERIALS AND METHODS) are two such lines. Somatic mutability in the two lines was shown to cosegregate with a *MuA*-hybridizing sequence (QIN and ELLINGBOE 1990). DNA from 20 plants derived from ten mutable kernels and ten stable kernels from DR1 or DR6 was digested with *Sac*I (which does not cut within *MuA*), Southern-blotted, and hybridized with the *Eco*RI-*Eco*RV fragment of *MuA*. A fragment of approximately 4.8 kb was found to be present in the DNA from mutable kernels but not from stable ones. This fragment was cloned into the *Sac*I site of λZap vector to yield λ915. The restriction map and the DNA sequence of the two ends of the *Sac*I insert of λ915 revealed that the fragment appears to consist of the internal region and part of the TIRs of a *MuA*-like element (Figure 1). To obtain DNA extending beyound this fragment, inverse PCR was used to clone the flanking sequences (OCHMAN, GERBER and HARTL 1988).

Genomic DNA from the same plants used in the cloning of the *Sac*I fragment was digested with *Xho*I, which cuts once within the *Sac*I insertion of λ915. The positions of the left and right flanking *Xho*I sites of the genomic DNA were determined by Southern blot analysis as approximately 4.8 kb and 2.9 kb from the internal *Xho*I site, respectively. This interpretation was based on the size of hybridizing bands that cosegregated with mutability when probed with the *Eco*RI-*Eco*RV fragment (Figure 2A) or the *Hin*dIII fragment (data not shown) of *MuA*. Two pairs of primers, E1/M1 and M2/E1 (Figure 1), were used to amplify the left and right flanking sequences, respectively. The predicted PCR products would be 2.5 kb for the left flanking region and 0.3 kb for the right flanking region. The amplified products were gel-purified, digested with both *Xho*I and *Sac*I, and cloned into the pBluscript plasmid vector.

Although the internal region and the missing portions of the TIRs of this *Mu* element were cloned separately, several lines of evidence were obtained to indicate that they all were from the same element. Southern blot analysis revealed that the cloned left end, designated MuLF, was contiguous to the element since both the internal *Eco*RI-*Eco*RV probe and the

TABLE 1

Sequence similarities between the TIRs of *MuA2* and those of several other *Mu* elements

	MuA		*Mu1*		*Mu2*		*Mu4*		*Mu5*		*Mu7*		*Mu8*	
MuA2	L	R	L	R	L	R	L	R	L	R	L	R	L	R
L	89	84	82	78	82	82	79	79	80	83	84	83	88	85
R	88	87	81	77	81	81	81	80	81	84	85	84	90	84

L, left TIR; R, right TIR. Sequence comparisons were made for the first 200 bp.

flanking *Pst*I-*Xba*I probe hybridized to the same *Xho*I fragment (Figure 2). A probe for the region flanking the other end of the element, designated MuRF, was not available because the flanking DNA had only three nucleotides. However, there were findings to suggest that the cloned right end was from the same *Mu* element. First, the size of the *Sac*I-*Xho*I fragment of the MuRF clones is 130 bp, close to the predicted 200 bp from Southern analysis (substraction of the 2.7-kb *Xho*I-*Sac*I fragment of λ915 from the 2.9-kb *Xho*I fragment identified on Southern blots with the *Hin*dIII probe). Second, sequence comparison of the PCR-amplified regions of the TIRs (Figure 3, from the *Sac*I site to the end of *MuA2*) showed only one base pair difference. A sequence similarity of nearly 100% was less likely to happen if the two ends were from different elements. Finally, the right end was amplified from DNA that had been *Xho*I-digested and size-selected to enrich for the DNA of the expected *Mu* end. The *Mu* element, composed of three cloned fragments, was therefore designated *MuA2*.

The structure of *MuA2*: Comparison of *MuA2* with *MuA* by restriction analysis revealed that the linear structures of the two elements are similar, but differences in several restriction sites were found (see Figure 1). Sequence similarities of the TIRs of *MuA2* with those of *MuA* and several other *Mu* elements are given in Table 1.

Sequences of the TIRs of *MuA2* are shown in Figure 3. Approximately 97% similarity was observed for 210 bp. Only 1 bp was different in the first 189 bp. The sequences flanking *MuA2* are shown below:

CAGAGGTCGGgagataattg

. . . . . caattatctcGAG

Dots denote *MuA2* and small letters denote the sequences of the outmost 10-bp TIRs. The underlined letters show the first three nucleotides of the putative target site duplication. There were only 3 nucleotides flanking *MuA2* in the MuRF clones. If the target sequence were to begin with "GAG," as indicated at the right end, then the sequence of the left end would suggest that the target site sequence would have been of 8 bp. Due to a lack of the complete sequence data for the right target site duplication, this conclusion remains tentative.

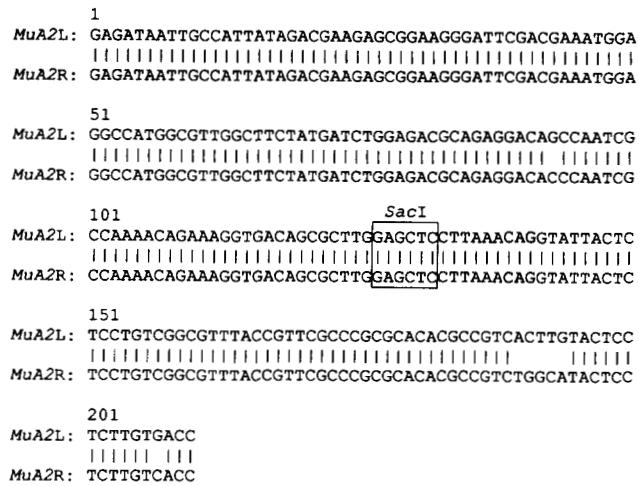

FIGURE 3.—Nucleotide sequences of the TIRs of *MuA2*. L, left end; R, right end. Position 1 is either the left or right end of the TIR.

Cosegregation of *MuA2* with somatic Mutator activity: The internal and flanking regions of *MuA2* were identified and cloned based on their cosegregation with somatic *Mutator* activity. We confirmed this cosegregation with Southern blot analysis using several enzymes that do not cut, or cut only once, within the *MuA2* element. This would reduce the possibility that the cosegregation was due to the comigration of two or more cross-hybridizing DNA fragments. Double digestions with enzymes that cut twice within the element were performed to determine if the band cosegregating with mutability contains an element that has an internal structure similar to *MuA2*, and to detect any possible DNA rearrangements in the cloning process.

DNA was prepared from 28 plants derived from either mutable or stable kernels from DR1 or DR6, both of which segregated for a single regulator element. DNA was digested with *Eco*RI which cuts once within *MuA2*. A representative of this Southern analysis is shown in Figure 2. A single *Eco*RI DNA fragment of approximately 7.5 kb (indicated by an arrowhead) cosegregated with somatic *Mutator* activity when probed with the *Eco*RI-*Eco*RV fragment of *MuA* (Figure 2A). A separate *Eco*RI DNA fragment of approximately 3.5 kb was found to cosegregate with somatic *Mutator* activity when the same blot was reprobed with the *Pst*I-*Xba*I fragment adjacent to *MuA2* (Figure 2B). The appearance of a similar size band as the one cosegregating with mutability in the second plant from a stable kernel (Figure 2A, *Eco*RI digestion) is likely due to the comigration of a different fragment of similar size since no 3.5-kb *Eco*RI band was detected in that plant when probed with the flanking *Pst*I-*Xba*I fragment (Figure 2B). When DNA from plants derived from either mutable or stable kernels from DR1 was digested with *Eco*RI and *Bam*HI, a 1.3-kb fragment expected for *MuA2* was found to cosegregate

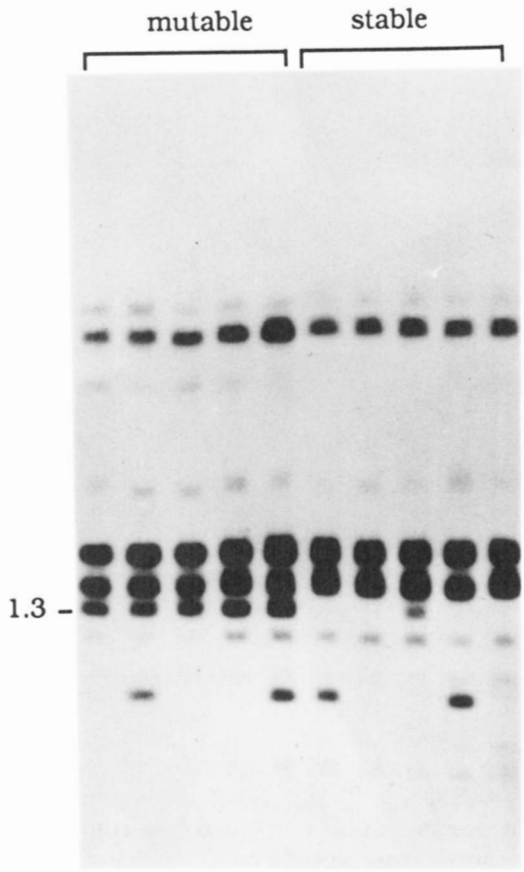

FIGURE 4.—Autoradiogram of a Southern blot of DNA from plants derived from either mutable or stable kernels of DR1 after *Eco*RI and *Bam*HI digestion. The blot was probed with the *Eco*RI-*Eco*RV fragment of *MuA*.

with *Mutator* activity (Figure 4). A band of similar size present in plant number 3 from a stable kernel is probably due to cross-hybridization with a different *Mu* element since the intensity of this band is lower than the one cosegregating with *Mutator* activity.

Comparison of DNA modification of *MuA2* with *MuA2*-hybridizing sequences: Active *Ac/Ds* and *Spm* (*En*) elements of maize are hypomethylated whereas other cross-hybridizing sequences in the same genome remain extensively methylated (SCHWARTZ and DENNIES 1986; CHOMET, WESSLER and DELLAPORTA 1987; CONE, BURR and BURR 1986; FEDOROFF *et al.* 1988; SCHIEFELBEIN *et al.* 1988). We also found that *MuA2* was completely cleaved by *Xho*I, a methylation-sensitive enzyme that cuts once within *MuA2*, while most other *MuA2*-hybridizing sequences were poorly cleaved (Figure 2). Similar results were obtained with the methylation-sensitive enzyme *Hpa*II, which cuts twice within *MuA2* (Figure 7). The presence of other faint bands of low molecular weight in either *Xho*I or *Hpa*II digestions may be due to incomplete methylation of other *MuA2*-hybridizing elements or elements with less homology with the *MuA2* probe used. None of these bands was found to cosegregate with *Mutator* activity.

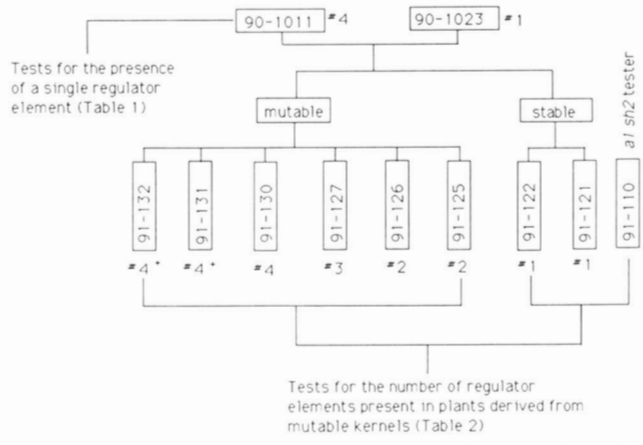

FIGURE 5.—Lineage relationships among plants used in the analysis of the amplification of the *MuA2* element. A number next to each plant indicates the mutability score.

Concomitant increase of *MuA2* copy number and regulator elements: Genetic studies on *Mu*-induced *a1-Mum* alleles showed the presence of a single putative regulator in some lines, as evidenced from the 1:1 segregation for somatic mutability. When plants from mutable kernels were testcrossed with *a1 sh2* tester lines, the segregation of mutable vs. stable kernels in the next generation occasionally became 3:1, 7:1 or 15:1 (ROBERTSON and STINARD 1989; M. QIN and A. H. ELLINGBOE, unpublished results). These ratios suggest the presence of two, three or four regulator elements, respectively, in these kernels. An explanation for this phenomenon was duplicative transposition of regulator elements. Experiments were performed to test whether *MuA2* could amplify from one generation to the next and if such amplification would result in an increased number of regulator elements.

The lineage relationship among plants used in this analysis is given in Figure 5. Plants 90-1011 and 90-1023 were derived from two kernels, one mutable and one stable, respectively, from an ear of DR1 (see MATERIALS AND METHODS) which segregated 1:1 for somatic mutability. The data suggest the segregation of a single regulator element which was shown to cosegregate with the cloned *MuA2* element (Figure 2). Plant 90-1011 was testcrossed with plant 90-123, or an *a1 sh2* tester, A632, or self-pollinated. The results of testcrosses are given in Table 2. The progeny ears segregated 1:1 (or nearly 1:1) and 3:1 for mutability in testcrosses and a self-pollination, respectively. The data suggest the presence of a single regulator in plant 90-1011.

Evidence for the MuA2 transposition: Amplification of the *MuA2* element was examined by Southern blot analysis for the appearance of new *Mu* bands and an increased intensity of bands characteristic of the *MuA2* element in the progeny plants. DNA from several progeny plants from the cross 90-1011 × 90-1023 was

TABLE 2

Tests for the presence of a single regulator element in plant 90-1011

Cross	90-1011	Mutable[a] #4+	#4	#3	#2	Total	Stable #1	Expected ratio	χ^2
A	× 90-1023	9	60	22	0	91	120	1:1	3.99*[b]
B	90-1023 ×	10	14	124	22	170	190	1:1	1.11
C	90-1357[c] ×	0	0	41	0	41	38	1:1	0.11
D	⊗[d]	14	95	25	0	134	40	3:1	0.40

Plants 90-1011 and 90-1023 were derived from either a mutable or a stable kernel, respectively, from an ear that segregated 1:1 for somatic *Mutator* activity.

[a] Mutable kernels were further classified into four categories according to the frequency of purple spots. #4+, highly mutable; #4, mutable; #3, intermediate; #2, low mutable with a few spots.

[b] * $\chi^2 > \chi^2(P = 0.05) = 3.84$.

[c] An *a1 sh2* tester, A632.

[d] Self-pollination.

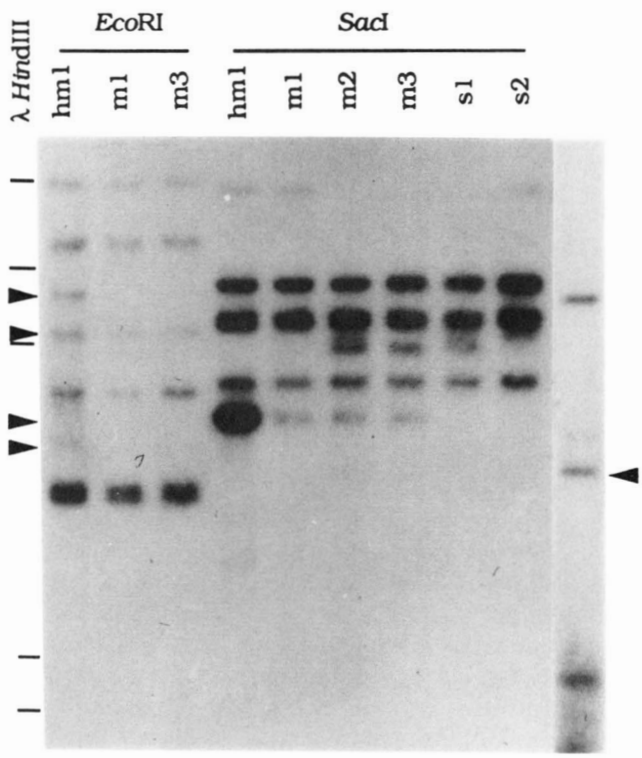

FIGURE 6.—Autoradiogram of Southern blot analysis showing the increase in copy numbers of *MuA2*. hm, highly mutable; m, mutable; s, stable. The number following each phenotype is the plant number. hm1 = 91-132; m1 = 91-130; m2 = 91-127; m3 = 91-125; s1 = 91-122; s2 = 91-121. Arrowheads on the left side indicate either the novel *Eco*RI bands (first and fourth arrowheads from the top), or the original *Eco*RI band (second), or the 4.8-kb *Sac*I band (third). The right most lane shows the single copy reconstruction (indicated by an arrowhead). The blot was probed with the *Eco*RI-*Eco*RV fragment of *MuA*.

digested with *Eco*RI and/or *Sac*I, Southern-blotted, and probed with the *Eco*RI-*Eco*RV fragment of *MuA*. The result is shown in Figure 6. Comparison of the *Eco*RI restriction patterns of several progeny plants with those of their parental generation (the restriction patterns of the parental generation were obtained

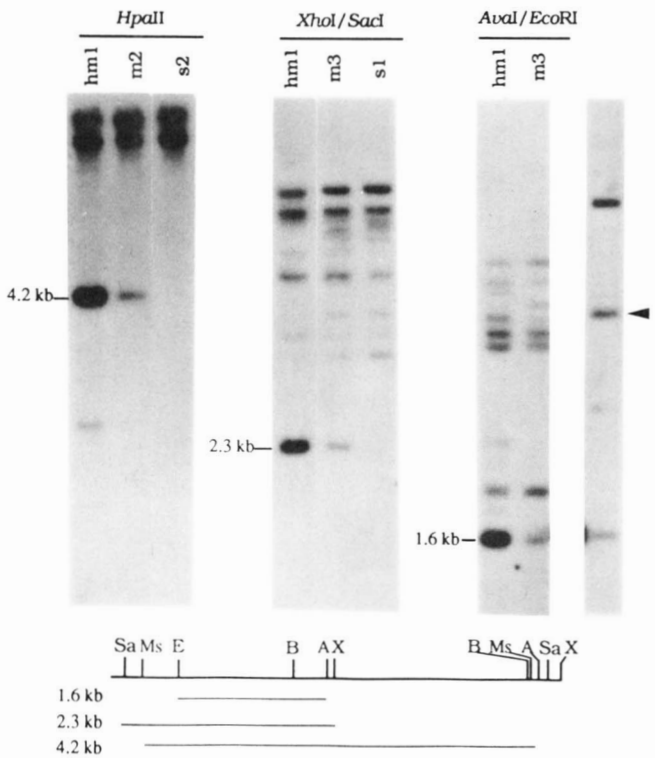

FIGURE 7.—Autoradiogram of a Southern blot, probed with the same probe as in Figure 6, showing that the amplified *Mu* copies have an internal structure characteristic of *MuA2*. The phenotypes and plant number are given in Figure 6. DNA fragments expected for *MuA2* with the enzymes used are indicated. The right most lane shows the single copy reconstruction (indicated by the arrowhead). The structure of *MuA2* and expected sizes of DNA fragments are shown in lower portion of the figure.

from a separate blot and not included) revealed two new bands (indicated by the first and fourth arrowheads on the left) in one of the progeny plants (Figure 6, lane 1, hm1 or plant 91-132). The *Sac*I digestion of the same DNA resulted in a greater intensity of the 4.8-kb *Sac*I fragment expected for *MuA2* (Figure 6, lane 4), suggesting that the new *Mu* bands were probably from the *MuA2* transposition. To obtain additional evidence that the new *Eco*RI bands were due to the newly transposed *MuA2* elements, the same DNA was digested with various restriction enzymes, Southern-blotted, and probed with the *Eco*RI-*Eco*RV fragment of *MuA*. The use of one methylation-sensitive enzyme in each digestion (*Hpa*II, *Xho*I and *Ava*I) reduced the possibility of the digestion of other cryptic *Mu* elements that may produce similar DNA fragments. All the plants from mutable kernels had bands of the sizes expected for *MuA2*, which were not present in plants from stable kernels (Figure 7). Furthermore, the intensities of these bands (Figure 7) were similar to the corresponding 4.8-kb *Sac*I fragment (Figure 6). Four more plants derived from mutable kernels from the same progeny population were analyzed with *Eco*RI and *Sac*I digestions. One of these four plants (plant 91-131) was also identified as having

TABLE 3

Segregation ratios and the putative numbers of regulator elements in progeny plants from cross B of Table 1

Cross[a]	Observed[b] M	S	T	Expected ratios	No. of elements[c]	χ^2
91-125/91-122	71	58	129	1:1	1	1.31
91-126/91-122	91	73	164	1:1	1	1.98
91-127/91-122	55	49	104	1:1	1	0.35
91-130/91-122	80	92	172	1:1	1	0.84
91-131/91-121	182	30	212	3:1	2	13.30**[d]
				7:1	3	0.51
91-122/91-131	206	83	289	3:1	2	2.15
91-110[e]/91-132	70	2	72	7:1	3	6.22*
				31:1	5	0.03
91-132/91-122	141	3	212	7:1	3	14.29**
				31:1	5	0.52

[a] Plant numbers can be found on Figure 5.
[b] M, mutable; S, stable; T, total.
[c] Expected number of regulatory elements based on segregation ratio.
[d] *, ** Observed segregation ratio, respectively, significantly (5%) or highly significantly (1%) different from the expected ratio.
[e] An *a1 sh2* tester, Oh43.

an increased copy number of *MuA2* (data not shown).

Amplification of regulator elements: If *MuA2* is a regulator element, plants with an increased copy number of *MuA2* would be expected to have an increased number of mutable kernels in the next generation. Some of these plants were grown in the greenhouse and testcrossed with sibling plants from stable kernels or with an *a1 sh2* tester, Oh43. The data are summarized in Table 3. The two plants, 91-131 and 91-132, with an increased copy number of *MuA2*, as determined by Southern blot analysis, also showed an increased number of regulator elements in the progeny tests whereas plants maintaining an apparent single copy of *MuA2* still showed 1:1 segregation for somatic mutability in their progeny plants (plants 91-125, 91-126, 91-127 and 91-130). The difference between the three copies of *MuA2* in plant 91–132 based on the *Eco*RI digestion (Figure 6, the two new *Eco*RI fragments plus the original copy indicated by the second arrowhead on the left) and the five copies of regulator elements based on the segregation ratios in the progeny tests (Table 3, crosses 91-110/91-132 and 91-132/91-122) can be explained by an underestimation of new *Eco*RI fragments because of comigration with the parental *MuA2*-hybridizing bands. Southern blot analyses and progeny tests involving plant 91-131 indicated three copies of *MuA2* and three regulator elements (Table 3, cross 91-131 × 91-121), respectively. The deficiency of mutable kernels in the "reciprocal" cross (Table 3, 91-122 × 91-131) is likely due to the reduced somatic mutability observed when lines with somatic instability are crossed as males to tester lines, as has been described before [D. S. ROBERTSON (unpublished data, *Maize Genetics News Letter* **59:** 10–11, 1985) and WALBOT (1986)].

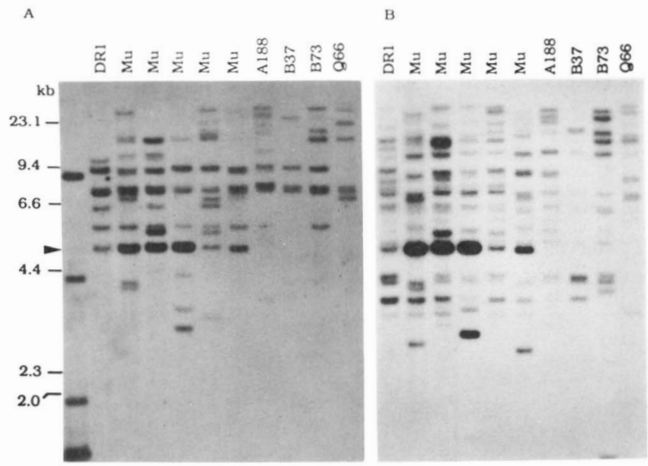

FIGURE 8.—Autoradiogram of Southern blot analysis of DNA from *Mutator* and non-*Mutator* plants after *Sac*I digestion. The band of approximately 4.1 kb (the *Eco*RI-*Bam*HI fragment of *MuA*) of the first lane is a two-copy reconstruction, assuming 5 μg DNA were loaded in each lane. DR1, a plant from a mutable kernel from DR1 (also as a single copy control). The blot was probed with the *Eco*RI-*Eco*RV fragment of *MuA* (A) and reprobed with the *Hin*dIII fragment of *MuA* (B). The arrowhead indicates the 4.8-kb *Sac*I band expected for *MuA2*.

Southern blot analysis of *Mutator* lines and non-*Mutator* inbreds: We have found that sequences homologous to the internal regions of *MuA* are heterogeneous and present in multiple copies in both *Mutator* and non-*Mutator* maize lines. We reasoned that *MuA*-homologous elements in non-*Mutator* inbreds and most of elements in *Mutator* lines may be structurally different from transposase-encoding *Mu* elements. By comparing the internal structures of these *MuA2*-hybridizing sequences with enzymes that cut twice within the elements, we should be able to distinguish elements structurally similar to *MuA2* from elements that have gross changes in the internal structures. DNA from plants derived from five *Mutator* lines and four non-*Mutator* inbreds was digested with *Sac*I and probed with the *Eco*RI-*Eco*RV fragment (Figure 8A) or the *Hin*dIII fragment of *MuA* (Figure 8B). The 4.8-kb *Sac*I fragment (indicated by an arrowhead) was detected in all the plants from the *Mutator* lines with both probes but absent from the four inbreds. Other bands were either present in both *Mutator* and non-*Mutator* plants or absent from some of the *Mutator* plants screened. Furthermore, different plants from different *Mu* lines had different copy numbers of the 4.8-kb *Sac*I fragment, varying from one (Figure 8A, lane 6 from the left) to approximately ten (Figure 8A, lanes 3, 4 and 5), based on densitometer scanning. Most of the variations may represent the copy number differences in different *Mu* lines.

DISCUSSION

We have described the cloning of a novel *Mu* element, designated *MuA2*, and presented evidence that

MuA2 has properties expected for a regulator or transposase-encoding element of the *Mutator* system.

We suspect that *MuA2* may regulate somatic mutability of the *a1-Mum2* allele because it cosegregated with *Mutator* activity in more than 50 plants (approximately half from mutable kernels and half from stable kernels) from two lines that segregated 1:1 for a single putative regulator. Additional lines of evidence in support of, or consistent with, this conclusion came from the following observations. First, *MuA2* was hypomethylated relative to the bulk of other *MuA2*-hybridizing sequences in the same genome. Second, the increase of *MuA2* copy number was concomitant with an increased number of regulators. Third, although there were many copies of *MuA2*-hybridizing sequences in all lines tested, intact *MuA2* elements were found only in *Mutator* lines. Furthermore, because of the cross-hybridization between *MuA* and *MuA2*, the *MuA*-hybridizing transcript that has been shown to be associated with *Mutator* activities (QIN and ELLINGBOE 1990) should also hybridize to *MuA2*. The transcript, in fact, could be produced from *MuA2*. Finally, the TIRs of *MuA2* have high sequence similarity with those of other *Mu* elements, ranging from 77 to 90% (Table 1), a characteristic similar to the *Ac-Ds* and *Spm* systems in which the autonomous *Ac* and *Spm* elements also share the TIRs with their nonautonomous elements. Together, the data are consistent with a concept that *MuA2* may promote the transposition of the *Mu1* insertion at the *a1-Mum2* allele (or any nonautonomous *Mu* insertions in general) by providing the transposase activity.

The data presented here suggest the transposition of the *MuA2* element. Transposition events were detected by the appearance of new *MuA2*-hybridizing elements at different sites in two out of eight progeny plants tested (the molecular data for plant 91-132 is shown in Figure 6). An increased copy number of *MuA2* in the same plant when the DNA was digested with *Sac*I and several other enzymes (Figures 6 and 7) supports the idea that the transposed elements were from *MuA2*. Since a plant with newly transposed *MuA2* elements (Figure 6, indicated by the first and fourth arrowheads on the left) also retained the original copy of *MuA2* (Figure 6, indicated by the second arrowhead on the left), *MuA2* may transpose via a replicative mechanism, similar to the *Mu1* transposition (ALLEMAN and FREELING 1986). The amplification of the *MuA2* elements is also consistent with previous genetic studies on the behavior of regulator elements (ROBERTSON and STINARD 1989). It may also explain in part the appearant non-Mendilian inheritance of *Mutator* activity and *Mu*-induced mutations.

In outcrosses of plants with a single regulator, variation in mutability is frequently observed among mutable kernels from ears that segregate 1:1 for mutability. Eight plants that we analyzed were grown from selected kernels with different mutability scores, six of which are given in Figure 5. Two plants with increased copy number of *MuA2* (Figure 6 and Table 3, 91-132 and 91-131) also had higher mutability (#4^+). Whether this reflects a dosage effect needs further examination.

Autonomous *Ac* and *Spm* transposable elements are both structurally conserved elements for each system. We have found that when DNA from diverse *Mu* lines and non-*Mu* inbreds was restricted with *Sac*I, which cuts within the TIRs of the *MuA2* element, the 4.8-kb band characteristic of *MuA2* was found in *Mu* lines but not in non-*Mu* inbreds (Figure 8). Similar results were obtained with an *Eco*RI and *Bam*HI double digestion (data not shown). The copy numbers of the 4.8-kb *Sac*I fragment varied from one to approximately ten in plants from different *Mu* lines, this may reflect the copy number differences of *MuA2*-like elements. This is also in agreement with the non-Mendelian segregation of the *Mutator* trait. These data suggest that autonomous *Mu* elements may be structurally conserved and similar, if not identical, to *MuA2*. The final proof must await the determination of DNA sequences of *MuA2* and other autonomous *Mu* elements.

The association of *MuA2*-like elements with germinal *Mutator* activity would also suggest that *MuA2*-like elements encode the functions required for germinal *Mutator* activity as well as somatic *Mutator* activity. This is also in agreement with our previous Northern blot analysis of maize lines with various *Mutator* activities in which the *MuA*-hybridizing transcript of similar size is associated with both germinal mutagenic activity and somatic mutability (QIN and ELLINGBOE 1990). The difference between germinal and somatic *Mutator* activities may be due to the copy number differences of active *MuA2*-like elements as well as actively transposing nonautonomous *Mu* elements. While a single copy of *MuA2*-like element may be sufficient to promote the somatic excision of a nonautonomous *Mu* insertion at a given locus, only lines with certain numbers of active *Mu* elements (autonomous as well as nonautonomous) may exhibit detectable germinal *Mutator* activity. Genetic studies have shown that most *a1-Mum2* and *a1-Mum3* stocks segregating for a single regulator have no detectable germinal *Mutator* activity (ROBERTSON and STINARD 1989). The amount of the *MuA*-hybridizing transcript of two such lines was also found lower than that of germinally active lines (QIN and ELLINGBOE 1990).

The target site duplication is found in almost all transposable element insertions and the size of the duplication is usually conserved within each system. We compared the left and right flanking sequences of *MuA2* for the presence of the target site duplication.

From the limited data, an 8 bp target duplication was inferred. This is different from the 9-bp target duplications found in all the *Mu* insertions that have been analyzed thus far except *MuA* which was also found to be flanked by an 8-bp target duplication (QIN and ELLINGBOE 1990). Variations of target duplications have also been reported in bacterial insertion sequences (GALAS and CHANDLER 1989). It is possible that the cleavage of the target occurred with a 9-bp spacing, but a base was subsequently removed from one or the other of the cleaved target ends by nuclease action. An alternative possibility is duplication of 9 and 8 bp reflects a variation in the spacing of the initial cleavage, possibly due to sequence variations in the TIRs and/or structural variations in the internal regions of different *Mu* elements. More sequence data from the *MuA* family will be needed to determine if this one base pair difference is common to the *MuA* family or represents one of the few exceptions.

The cloning of the *MuA2* element is an important step toward the understanding of *Mu* regulation. For example, loss of *Mutator* activity by outcrossing and intercrossing have been proposed to be due to the segregation and the DNA modification of transposase-encoding elements, respectively. These hypotheses can now be tested with respect to the segregation and/or the DNA modification of *MuA2*-like elements. The *Mutator* system has become increasingly interesting because of its high mutagenic activity. It may now be possible to introduce the *Mutator* system into other heterologous systems, similar to the work done with the *Ac-Ds* and *Spm* systems (reviewed by HARING *et al.* 1991).

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