Identification of a Regulatory Transposon That Controls the *Mutator* Transposable Element System in Maize

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ABSTRACT

The *Mutator* system of maize consists of more than eight different classes of transposable elements each of which can be found in multiple copies. All *Mu* elements share the ~220-bp terminal inverted repeats, whereas each distinct element class is defined by its unique internal sequences. The regulation of instability of this system has been difficult to elucidate due to its multigenic inheritance. Here we present genetic experiments which demonstrate that there is a single locus, *MuR1*, which can regulate the transposition of *Mu1* elements. We describe the cloning of members of a novel class of *Mu* elements, *MuR*, and demonstrate that a member of the class is the regulator of Mutator activity, *MuR1*. This conclusion is based on several criteria: *MuR1* activity and a *MuR*-homologous restriction fragment cosegregate; when *MuR1* undergoes a duplicative transposition, an additional *MuR* restriction fragment is observed, and *MuR1* activity and the cosegregating *MuR* fragment are simultaneously lost within clonal somatic sectors. In addition, the *MuR* element hybridizes to transcripts in plants with Mutator activity. Our genetic experiments demonstrate that the *MuR1* transposon is necessary to specify Mutator activity in our lines.

CERTAIN lines of maize, called "Mutator lines," generate a high frequency of mutations (ROBERTSON 1978). Mutations arising in Mutator lines often exhibit somatically unstable phenotypes characteristic of transposable element-induced alleles. The molecular characterization of *Mutator* induced mutations at *Adh1*, *Bz1*, *A1* and other loci, has established that transposable elements, called *Mu* transposons, are likely to cause the high rate of mutation (STROMMER *et al.* 1982; TAYLOR, CHANDLER and WALBOT 1986; O'REILLY *et al.* 1985).

Nine different classes of *Mu* transposons have been distinguished on the basis of the sequence heterogeneity within the element (BARKER *et al.* 1984; TAYLOR and WALBOT 1987; OISHI and FREELING 1987; TALBERT and CHANDLER 1988; TALBERT, PATTERSON and CHANDLER 1989; SCHNABLE, PETERSON and SAEDLER 1989; VARAGONA, FLEENOR and WESSLER 1987; QIN and ELLINGBOE 1990; V. CHANDLER, unpublished). All classes of *Mu* elements have in common the ~220-base pair (bp) terminal inverted repeats, but different classes have distinct unrelated internal sequences. Members within the same class share a strong sequence homology throughout the internal regions of the elements. Furthermore, the described classes can exist in multiple, heterogeneous copies and all elements shown to be capable of transposition generate a 9-bp genomic duplication upon insertion (BARKER *et al.* 1984; CHEN *et al.* 1987; SCHNABLE, PETERSON and SAEDLER 1989). At least six different elements representing four classes (*Mu1*, *Mu1.7*, *Mu1-del*, *Mu3*, *Mu7* and *Mu8*) are capable of transposition (STROMMER *et al.* 1982; TAYLOR and WALBOT 1987; OISHI and FREELING 1987; HARDEMAN and CHANDLER 1989). The other four classes (*Mu4*, *Mu5*, *Mu6* and *MuA*) have been identified by their DNA homology to the common ~220-bp inverted terminal repeats (TALBERT, PATTERSON and CHANDLER 1989; QIN and ELLINGBOE 1990; V. L. CHANDLER, unpublished). At least a few sequences characteristic of each class of element are found in most non-Mutator standard inbred lines of maize (CHANDLER, RIVIN and WALBOT 1986; TALBERT, PATTERSON and CHANDLER 1989; V. L. CHANDLER unpublished data). To date there is no evidence that any of these characterized *Mu* elements encode a transposase (LILLIS, SPIELMANN and SIMPSON 1985; ALLEMAN and FREELING 1986; see TALBERT, PATTERSON and CHANDLER 1989 for discussion), although, recently a *MuA* element has been shown to be homologous to a transcript associated with Mutator activity (QIN and ELLINGBOE 1990).

Unlike the well-defined controlling element systems *Ac-Ds* and *Spm*, the genetic basis of Mutator activity appears to segregate in a non-Mendelian or multigenic manner (ROBERTSON 1978; WALBOT 1986). When active Mutator stocks are outcrossed to standard non-Mutator lines, the typical result is that 90% of the

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progeny retain and 10% lose Mutator activity. However, these percentages are variable and simple mendelian ratios reflecting the segregation of only one or two regulatory genes are rarely observed. Mutator-inactive progeny are simultaneously altered relative to Mutator-active progeny with respect to a number of phenotypes associated with the *Mutator* system. First, the forward mutation rate is reduced, the elements are somatically stable and the high copy number of *Mu* elements is not maintained upon outcrossing (ROBERTSON *et al.* 1985; BENNETZEN 1987; WALBOT and WARREN 1988), indicating *Mu* elements are no longer transposing. Second, certain nucleotides in the ends of *Mu* transposons become methylated (CHANDLER and WALBOT 1986; BENNETZEN 1987; BENNETZEN, BROWN and SPRINGER 1988). Third, extrachromosomal *Mu* circles associated with Mutator activity are not detectable (SUNDARESAN and FREELING 1987). Finally, a reporter allele that is normally suppressed by Mutator activity and expresses a mutant phenotype, expresses the wild-type phenotype in *Mu*-inactive lines (MARTIENSSEN *et al.* 1990). In some, but not all cases, the loss of Mutator activity can be reversed by crossing to Mutator-active plants (ROBERTSON *et al.* 1985; BENNETZEN 1987; WALBOT 1986).

ROBERTSON and STINARD (1989) reported an unusual Mutator stock where the control of *Mu1* excision segregated 1:1 in a testcross, suggesting the presence of a single regulator locus. Using a similar line constructed independently in this laboratory we have identified a *Mu* transposon with regulatory activity and designated it *Mu-Regulator1* (*MuR1*). We have demonstrated that this gene is itself a transposon and is necessary to confer activity on different classes of *Mu* elements (this report; P. CHOMET, D. LISCH and M. FREELING, unpublished data).

We have identified and cloned two members of a new class of *Mu* elements that have not been described before. These elements have the *Mu* terminal inverted repeats, and hybridize with the *Mu5* element's extended terminal repeats. However, their internal sequences do not hybridize with those of *Mu1*, *Mu1.7*, *Mu3*, *Mu4*, *Mu5*, *Mu6*, *Mu7* or *Mu8*. In this paper we demonstrate that a member of this new class of *Mu* elements is the *MuR1* transposon, a regulator for the multiclass *Mutator* system. In addition, we show that the *MuR* elements are homologous to two transcripts present in plants carrying *MuR1*.

MATERIALS AND METHODS

Maize lines: The *a1-mum2* allele was originally isolated by D. S. ROBERTSON and the line containing *a1-mum2* in this study was originally obtained from S. DELLAPORTA. The *a1-dt sh2* tester line was obtained from B. MCCLINTOCK. The *a1-dt* allele has a stable, colorless phenotype in the absence of *Dt*. All lines used in this report lacked the *Dt* element and for this reason we simply refer to the *a-dt* allele as *a1*. Progeny segregating for the *MuR1* element(s) was obtained from the cross:

+MuR1; a1-mum2 Sh2/a1 sh2 × *a1 sh2/a1 sh2; −MuR1*

or

+MuR1; a1-mum2 Sh2/a1 sh2

× *a1-mum2 Sh2/a1 sh2; −MuR1.*

A Mutator stock, with a typical number of *Mu1* elements (20–60) was used to obtain new *sh1* mutants. Details of the stock construction were previously described (HARDEMAN and CHANDLER 1989). Eight *sh1* mutants isolated at a frequency of 1.1×10^{-4} were obtained (K. J. HARDEMAN and V. L. CHANDLER, unpublished). The *sh1* mutant relevant to this work was designated *sh1-A83* and contained a 4.0-kb insertion in the *sh1* gene.

Nucleic acid samples: Maize DNA from leaves was purified according to CONE (1989). RNA was isolated from leaves and immature ears by the guanidinium thiocyanate method and the sodium dodecyl sulfate (SDS) method (AUSUBEL *et al.* 1987). Plasmids containing *Mu1*, *Mu1.7*, *Mu4* and *Mu5* probes were previously described (TALBERT, PATTERSON and CHANDLER 1989). A plasmid containing the *Mu3* element was obtained from K. OISHI and a plasmid containing *Mu8* was obtained from S. WESSLER. Internal fragments of the *Mu6* and *Mu7* elements were from D. TURKS. The actin probe was obtained from R. MEAGHER (MEAGHER *et al.* 1983). A *Kpn*I fragment from the 5′ end of the *A1* gene was obtained from J. BROWN (O'REILLY *et al.* 1985).

Cloning: An ~8.5-kb sequence from the *sh1-A83* allele was cloned by ligating size-fractionated *Bcl*I-digested maize DNA into the *Bam*HI site of the λBv2 vector (obtained from N. MURRAY, University of Edinburgh). After *in vitro* packaging (HOHN 1979), the phage were plated and screened (BENTON and DAVIS 1977) using a *Sh1* clone, p17.6, obtained from C. HANNAH. An 8-kb *Pst*I fragment containing the entire *Mu* insertion, *sh1* sequences, and ~1.8 kb of the λ vector was subcloned into a pTZ18 vector (United States Biochemical). The site of insertion of the element in the *sh1* allele was determined by sequencing the two *Sst*I fragments (subcloned into a pTZ18 vector) which contained homology to both the *Mu*-termini and *Sh1*. Both strands were sequenced by the dideoxy chain termination method (SANGER, NICKLEN and COULSEN 1977) using the 17-mer universal primer (Pharmacia). An internal *Eco*RI-*Bam*HI fragment of the element at *sh1-A83* was designated Mu*.

A *Bcl*I clone containing most of the *MuR1* element was obtained by size-fractionating *Bcl*I digested DNA from a plant carrying *MuR1*. This DNA was ligated into the *Bam*HI site of λEMBL3 (purchased from Stratagene Inc.). The phage were in vitro packaged, plated and screened using the Mu* probe. A clone carrying the *MuR1*-diagnostic 1.4-kb internal *Xba*I fragment was restriction mapped further.

Southern and Northern blot hybridizations: Maize DNA was digested for 4 hr with a fourfold excess of restriction enzymes (conditions according to suppliers), electrophoretically separated through agarose gels, blotted to Duralon UV (Stratagene Inc.) and was UV cross-linked to the membrane using a Stratalinker apparatus (Stratagene Inc.) according to the manufacturer. Electrophoresis of RNA was through formaldehyde gels (AUSUBEL *et al.* 1987). Probes were prepared by the random priming method using a Prime-It kit (Stratagene Inc.) and ^{32}P-radiolabeled dCTP (Du Pont). Filters were prehybridized at 65° in 6 × SSC, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 × Denhardt's solution, 20 mM sodium phosphate, pH 7.2, 0.2 mg/ml dena-

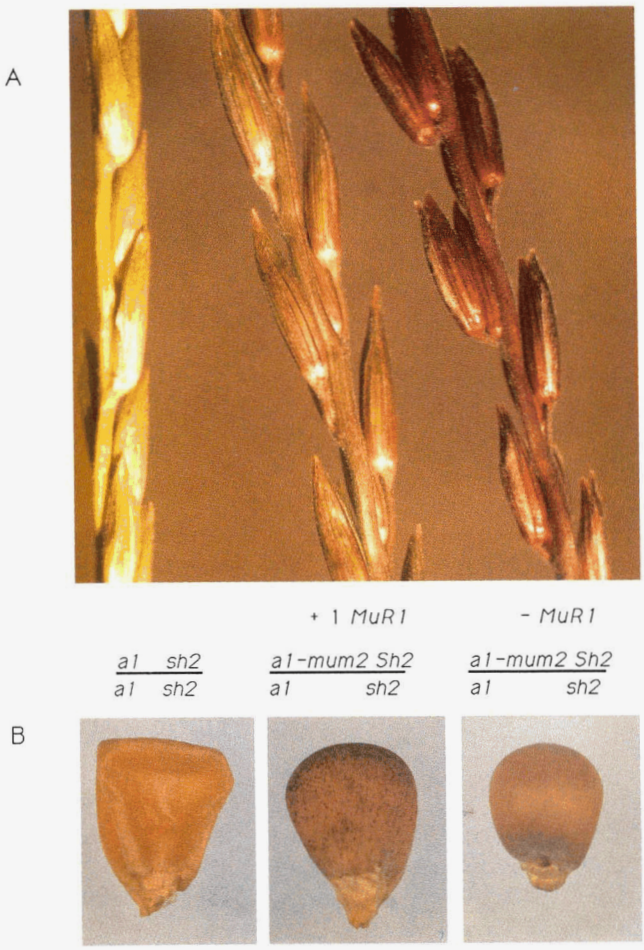

FIGURE 1.—Tassel branch (A) and seed phenotypes (B) of the *a1-mum2* allele in the presence and absence of *MuR1*. Plants carrying *MuR1* show small red clonal sectors of tissue on a green background. Plants lacking *MuR1* express red anthocyanin color (the intensity of red is dependent on genetic background). The suppression of the *a1-mum2* allele is not readily apparent in the aleurone layer of the kernels. The genotypes of the plants and seeds shown are listed between each structure, respectively.

tured salmon sperm DNA, and 10% sarcosine and hybridized in the same solution with the denatured probe for 6–12 hr. The filters were washed in 0.2 × SSPE, 0.1% SDS at 65° for 1.5 hr with three wash changes and exposed to Kodak X-Omat film with a Du Pont Cronex Lightning-Plus intensifying screen at −80° for varying lengths of time.

RESULTS

Mutator activity is controlled by the *MuR1* transposable element: To detect Mutator regulatory activity we used *a1-mum2*, an allele of the anthocyanin-conditioning gene *A1*. This mutant allele contains a *Mu1* insertion in the 5′ promoter region of the *A1* gene (O'REILLY *et al.* 1985). In the absence of Mutator activity this allele produces a pale colored (nonspotted) seed, whereas in the presence of Mutator activity, the allele produces a spotted phenotype (Figure 1B). To independently follow the segregation of the chromosome containing the reporter allele it was linked to *Shrunken2* (*Sh2*) which produces plump kernels. The recessive allele, *sh2*, was linked to a stable recessive *a1* allele in the non-Mutator tester stock (see MATERIALS AND METHODS for a further description of this allele). *A1* and *Sh2* are closely linked with only 0.2% recombination.

Testcrosses (as shown in MATERIALS AND METHODS) generated ears segregating 50% spotted, plump kernels and 50% nonspotted, plump kernels, thereby defining a locus (*MuR1*) which regulated somatic Mutator activity. Most ears from crosses of this type segregated for *MuR1* over a five generation period. Table 1 gives data for two consecutive generations of such crosses. These results suggested the presence of a single locus which was unlinked to *a1-mum2*, and was responsible for somatic excision of *Mu1*.

MuR1 activity was also necessary for the suppression

TABLE 1

Segregation data for families with one *MuR1* element

Plant No.	*MuR1* parent[a]	No. *Sh2* kernels		χ^2
		Mutable	Pale	
994-1	F	85	75	0.625
994-1	M	68	34	11.333[b]
994-2	F	60	72	1.091
994-3	F	64	60	0.129
994-4	F	68	53	1.860
994-5	F	55	42	1.742
994-6	F	50	51	0.010
994-6	M	47	50	0.093
994-7	F	89	55	8.028[b]
994-7	M	39	45	0.429
994-8	F	24	17	1.195
994-9	F	51	67	2.169
994-9	M	55	61	0.310
994-10	F	65	70	0.185
994-10	M	59	54	0.221
1277-2	F	94	61	7.026[b]
1277-2	M	61	50	1.090
1277-11	F	74	74	0.000
1277-14	F	87	65	3.184
1277-14	M	107	85	3.000
1277-17	F	110	113	0.040
1277-20	M	98	103	0.124

[a] *MuR1* carrying parent was used as either a male (M) or female (F) in the cross as outlined in MATERIALS AND METHODS.

[b] Ears with a significantly different χ^2 value at the 0.05 level for the segregation of one *MuR1* element.

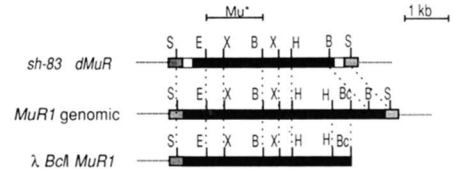

FIGURE 2.—Restriction map comparison of the *sh1-A83 dMuR* element, a genomic *MuR1* element, and the *Bcl*I clone containing most of *MuR1*. The location of the *Mu*-termini are indicated as a gray section and the approximate location of the *Mu5* homologous sequences are indicated as the cleared section of the *dMuR* in *sh1-A83*. The *dMuR* is the only element confirmed to contain the *Mu5* repeats; we have not tested the *Bcl*I clone or *MuR1* genomic sequences for hybridization to the *Mu5* repeat. The Mu* fragment is diagrammed above the map. Restriction fragment sizes of each element were directly compared on the same agarose gel. The genomic restriction map of *MuR1* was generated from southern blots of complete digests of genomic DNA. Dashed lines between the maps indicate the shared restriction sites and their positions. The nine bp target site duplication of *Sh1* sequence caused by the *dMuR* element is: AGAGAAACC, which includes the *Sh1* transcription start site (WERR *et al.* 1985). The restriction enzyme sites are abbreviated as follows: B, *Bam*HI; Bc, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; S, *Sst*I; X, *Xba*I.

of *A1* expression mediated by the *Mu1* element insertion in *a1-mum2*. In plants lacking *MuR1* activity (as assayed by nonspotted seed) we observed a strong red pigmentation in a variety of tissues whereas plants carrying *MuR1* expressed a mutable pattern of red somatic sectors over a green background (Figure 1A).

A new *Mu* transposon sequence identifies the *MuR1* locus: Most transposon systems are characterized by a regulatory element that encodes a transposase. In general, the regulatory elements are larger than their defective derivatives. At the time this study was initiated, the characterized *Mu* elements ranged in size from 1 to 2 kilobases (kb). Thus, we were interested in characterizing several larger *Mu* insertions (4–5 kb) at the *Shrunken1* (*Sh1*) locus that were potential candidates for regulatory elements based on their size. A description of the stocks and mutant isolation is outlined in MATERIALS AND METHODS.

Southern blot analyses were used to map the insertions relative to *Sh1* restrictions sites. We cloned one insertion (*sh1-A83*) and a restriction map of this element is shown in Figure 2. This insertion hybridized to the *Mu1* terminal repeat probe indicating that this new insertion is a member of the Mutator family. Furthermore, two fragments near the ends of the insertion had homology to the 138-bp extended inverted repeats of *Mu5* (TALBERT, PATTERSON and CHANDLER 1989). The insertion failed to hybridize with the complete internal probes for the *Mu1*, *Mu1.7*, *Mu3*, *Mu4* and *Mu8* classes of elements. It also failed to hybridize to small internal probes of *Mu6* and *Mu7*. However, as our *Mu6* and *Mu7* specific probes do not represent the entire internal sequences of these elements we can not completely eliminate the possibility of limited sequence homology between the new element and *Mu6* or *Mu7*.

Cosegregation of the *MuR1* locus with a DNA fragment homologous to the new Mu transposon: To determine whether the new *Mu* transposon might be related to the *MuR1* locus, siblings segregating for a single *MuR1* element were examined. An internal *Eco*RI-*Bam*HI fragment of the new element, designated as the Mu* probe (Figure 2), was hybridized to Southern blots containing *Hin*dIII-digested DNA from 19 siblings, 10 with *MuR1* activity and 9 without *MuR1* activity (see Figure 1 for reporter gene phenotypes). As shown in Figure 3, the Mu* probe detected multiple restriction fragments in all samples. This result was expected since the other classes of *Mu* transposons are present in multiple, dispersed copies in all Mutator-active maize genomes. Importantly, a strongly hybridizing 11-kb fragment was found in the ten plants with *MuR1* activity and was absent in the plants lacking *MuR1* activity. The same cosegregation result was obtained in the analysis of other related families. We have examined a total of 94 progeny segregating for *MuR1* and observed zero recombinants of the *MuR1* element and this *Hin*dIII fragment (or a comparable 1.4-kb cosegregating *Xba*I fragment). Thus, the Mu* probe recognized a restriction fragment that is tightly linked to the *MuR1* locus. Although a few of the other Mu* cross-hybridizing fragments segregated in these populations, no other

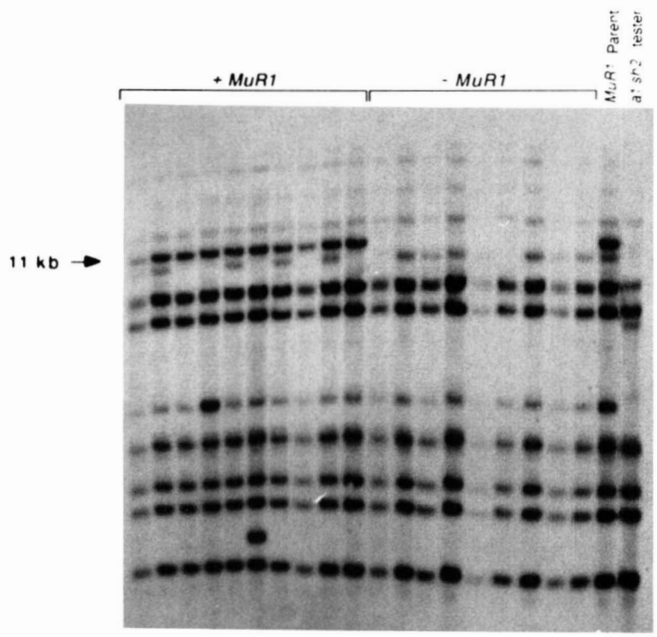

FIGURE 3.—Southern blot analysis of DNA from plants segregating for *MuR1* activity. DNA from plants containing (+*MuR1*) and lacking (−*MuR1*) was digested with *Hin*dIII, electrophoretically separated on a 0.8% agarose gel, blotted and hybridized to the Mu* probe. An 11-kb fragment (marked by an arrow) is present only in samples from *MuR1*-containing plants.

homologous fragment cosegregated with *MuR1*.

Duplicative transposition of *MuR1* was associated with duplication of a Mu* hybridizing fragment: Typically, most crosses of plants segregating for a single *MuR1* locus gave rise to mutable kernels which showed similar frequencies of spotting. However, in some testcrosses, including those in which plant 994-1 was the Mutator male parent, an excess of spotted, plump kernels was observed (Tables 1 and 2) and approximately half of the spotted kernels were more frequently sectored. Previous observations indicated that increased copies of *MuR1* in the endosperm produced an increase in the density of the *a1-mum2* mediated spotting pattern in these lines (P. CHOMET, D. LISCH and M. FREELING, unpublished). We reasoned that the increased spotting in the progeny of plant 994-1 was due to an increase in *MuR1* copy number. Fertilization by the gametes containing two *MuR1* copies would be initially detected by a frequent spotting pattern in the endosperm. The unusually high proportion of spotted, plump kernels might have resulted from a premeiotic duplicative transposition of *MuR1* during tassel development of plant 994-1, as this was observed in only the male transmitted gametes of the plant (Table 1).

To test this hypothesis, plants were grown from these heavily spotted kernels and testcrossed with *a1 sh2* plants. Ears resulting from these crosses (1176A family) often segregated 3 spotted:1 nonspotted, plump kernels consistent with the presence of a second nonlinked regulator gene. Sibling plants grown from

TABLE 2

***MuR1* segregation showing copy number changes**

Plant No.	*MuR1* parent[a]	No. *Sh2* kernels		No. *MuR1*[b]
		Mutable	Pale	
994-1	F	85	75	1
994-1[c]	M	68	34	2
1176A-1	F	5	98	—[d]
1176A-2	F	93	36	2
1176A-2[e]	M	89	13	3
1176A-3	F	97	20	3
1176A-4	F	69	20	2
1176B-1	F	64	63	1
1176B-2	F	56	56	1
1176B-3	F	70	62	1
1176B-4	F	30	40	1
8A-1	F	37	23	1
8A-1	M	35	65	—[d]
8A-2	F	71	8	3
8A-3	F	94	24	2
8A-3	M	73	29	2
8A-6	F	43	20	2
8A-6	M	66	19	2
8A-7	M	75	30	2
8B-1	F	55	42	1
8B-1	M	57	55	1
8B-4	M	40	76	—[d]
8B-6	F	38	41	1
8B-6	M	48	43	1
8B-7	F	49	44	1
8B-7	M	56	43	1

[a] *MuR1* carrying parent was used as either a male (M) or female (F) in the cross as outlined in MATERIALS AND METHODS.

[b] Minimum number of independently segregating *MuR1* to obtain a nonsignificant χ^2 value at the 0.05 level.

[c] Plant 994-1M was the *MuR1* parent for family 1176. Family 1176 individuals marked A arose from heavily spotted kernels, and individuals marked with a B arose from medium spotted kernels.

[d] The observed ratio is significantly different at the 0.05 significance level from the expected ratios for all possible numbers of independently segregating *MuR1*.

[e] Plant 1176A-2M was the *MuR1* parent for family 8. Family 8 individuals marked A arose from heavily spotted kernels and individuals marked with a B arose from medium spotted kernels.

kernels displaying medium spot densities all continued to segregate 1 spotted:1 nonspotted plump kernels (Table 2, 1176B family). From the data presented in Table 1, we can also infer that a germinal duplicative transposition event to an unlinked site occurred in approximately 20% of the plants which originally carried one *MuR1* element.

This genetic material provided the means to test unequivocally whether the *MuR1* transposon hybridized to the Mu* probe. DNA samples from sibling plants deduced to carry either one or two *MuR1* loci by genetic analysis, the grandparent plant (994-1), and the tester line were digested with *Hin*dIII, Southern blotted and hybridized with the Mu* probe. The expected 11-kb fragment associated with *MuR1* was present (Figure 4; dark arrow) in the grandparent plant (994-1) but was absent in the plants lacking activity and was absent in the *a1 sh2* homozygote

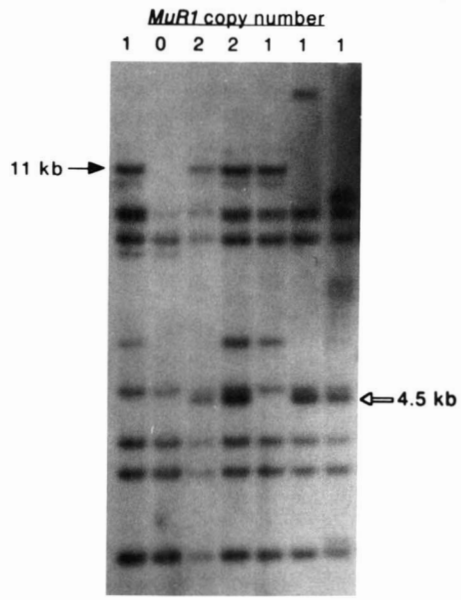

FIGURE 4.—Identification of a unique Mu* hybridizing fragment after duplicative transposition of *MuR1*. DNA from plants with either two *MuR1* copies (plants 8A-3 and 8A-6), one *MuR1* copy (plants 8B-1, 8B-6 and 8B-7), or zero *MuR1* copies (a1 sh2 tester line) was digested with *Hin*dIII, electrophoretically separated on a 0.8% agarose gel, blotted and hybridized to the Mu* probe. See Table 2 for genetic analyses of these plants. The open arrow points at the new 4.5-kb fragment found in both DNA samples from plants carrying two *MuR1* copies and in plants 8B-1 and 8B-6 which has one *MuR1* element. The closed arrow indicates the original 11-kb band.

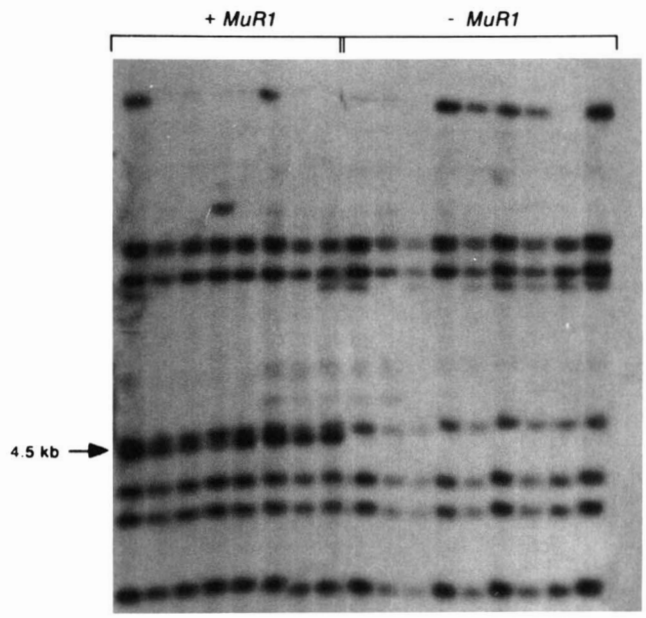

FIGURE 5.—The novel 4.5-kb Mu* hybridizing fragment cosegregates with *MuR1* activity. DNA from *MuR1*-segregating progeny of a plant carrying the new 4.5-kb fragment (8B-6) was digested with *Hin*dIII, electrophoretically separated on a 1.0% agarose gel, blotted and hybridized to the Mu* probe. The arrow points at the segregating 4.5-kb fragment.

tester (known to genetically lack Mutator activity). In all the testcross progeny deduced to carry two *MuR1* genes by genetic analysis, a unique 4.5-kb fragment was detected (Figure 4; hollow arrow) in addition to the original 11 kb fragment and the other Mu*-homologous fragments. This new 4.5-kb fragment was not present in the 994 family (data not shown). In progeny plants segregating for only one element, either the original 11-kb or the new 4.5-kb band was present, but not both (Figure 4).

If the new 4.5-kb fragment was derived from a duplication of the original *MuR1* locus, it too should confer *MuR1* genetic activity. Progeny analysis of 40 individuals from a plant carrying only the 4.5-kb Mu*-homologous fragment (plant 8B-6) showed that this fragment cosegregated with *MuR1* activity as was previously shown for the 11-kb fragment. A subset of this segregating population is shown in Figure 5. Therefore, either the original (11-kb *Hin*dIII) or the newly transposed (4.5-kb *Hin*dIII) *MuR1* gene was sufficient to confer somatic excision activity to *Mu1*. Taken together these results demonstrate that the Mu* probe is homologous to a regulatory element, *MuR1*, which is itself a transposable element. Furthermore, one *MuR1* transposon, independent of its chromosomal location, is necessary to encode Mutator activity. We will refer to the transposable element we cloned from *sh1-A83* as a *derivative of MuR* (*dMuR*).

We cloned a *Bcl*I fragment containing most of a *MuR1* element from a line which segregated for a single element. Restriction analysis comparing *MuR1* in the *Bcl*I clone, the *dMuR* element at *Sh1* and a proposed genomic restriction map of *MuR1* is shown in Figure 2. Genomic mapping data indicated that in our segregating lines the *MuR1* element had unique internal restriction fragments that were not shared by other *MuR* homologous sequences, such as a 1.4-kb *Xba*I and a 1.9-kb *Eco*RI-*Hin*dIII fragment (see Figure 2). As shown by the restriction map, the *MuR1* clone contains all such restriction sites so far examined. Interestingly, restriction analysis of the *dMuR* element, cloned from an unrelated Mutator line, demonstrated that it is very similar to *MuR1* except for a putative deletion of approximately 0.9 kb near one end of the element. This region encompasses the *Bcl*I and one of the *Hin*dIII sites found in *MuR1*. The 1.4-kb *Xba*I and a 1.9-kb *Eco*RI-*Hin*dIII internal fragments are unique only in our *MuR1* segregating lines and are not likely unique in other unrelated Mutator lines since the *sh-83dMuR* element contains these restriction fragments.

Somatic loss of Mutator activity reflects the loss of the 11-kb *MuR1* fragment: Somatic sectors of pale red tissue on a background of green tissue appeared in plants heterozygous for *a1-mum2* and carrying a single *MuR1* locus. Those sectors represented losses of *MuR1* activity by two criteria: (1) the pale red phenotype within the sectors is the same as that of

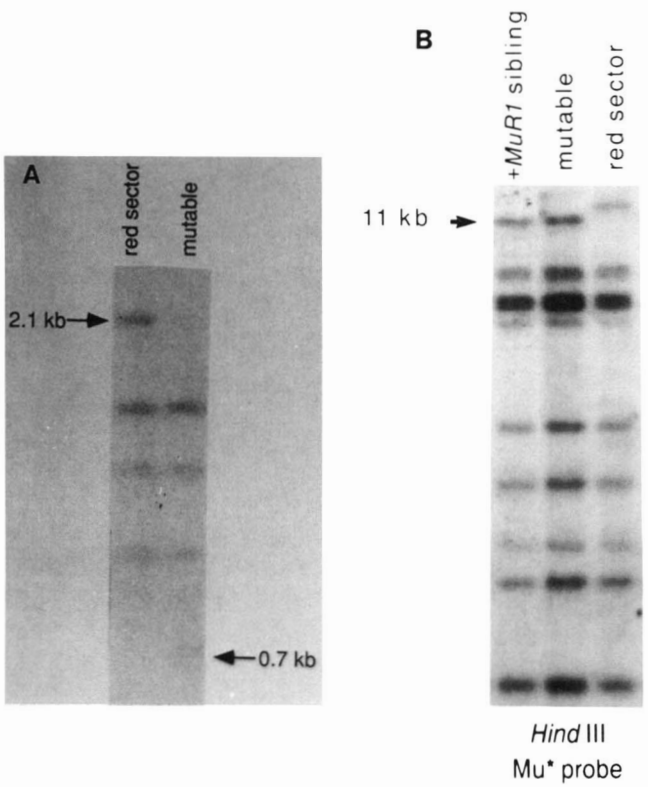

FIGURE 6.—Loss of *MuR1* activity in a somatic sector correlates with the loss of the 11-kb *MuR1* fragment. DNA was prepared from a red sector of sheath tissue (red sector) and an adjoining region of tissue which still showed *MuR1* activity (mutable). With the red sector, efforts were made to remove the nonpigmented tissue before isolating DNA. Panel A shows a Southern blot hybridized to the *A1* probe of the DNA samples digested with *Hin*fI, a methylation sensitive enzyme. The 2.1-kb fragment represents the entire *Mu1* element methylated at the *Hin*fI sites and flanked by *A1* sequence. The 0.7-kb fragment represents complete digestion at the *Hin*fI site in the *Mu1* terminal inverted repeat. The other *A1* hybridizing fragments are from regions outside of the *Mu1* insertion in *a1-mum2* and from the other allele and are found in both samples. Panel B is a Southern analysis of a different but comparable sector. The DNA was digested with *Hin*dIII, electrophoretically separated on a 0.8% gel, and hybridized to the Mu* probe. The 11-kb *MuR1* fragment (marked by the arrow) is present in the sample from the mutable tissue but is absent in the DNA from the red sector. An additional, larger *MuR*-homologous fragment is detected in the red sector.

plants carrying the *a1-mum2* allele but lacking *MuR1* (Figure 1) and (2) *Hin*fI digestion of the DNA from tissue of many independent sectors revealed the *Mu1* element inserted at *a1-mum2* was methylated in its inverted terminal repeats (Figure 6A). CHANDLER and WALBOT (1986) and BENNETZEN (1987) have shown that modification of methylation sensitive sites in *Mu1* ends correlates with the lack of Mutator activity. Given that *MuR1* activity was lacking in these sectors, we investigated what happened to the *MuR1* fragment in these sectors.

DNA from pale red sectors and adjoining mutable tissue was analyzed by Southern blotting. One example is shown in Figure 6B. The sector was obtained from a plant that contained the 11-kb *Hin*dIII fragment. This red sector, which lacked *MuR1* activity, also lacked the 11-kb fragment. Similar results were obtained from plants which carried the 4.5-kb *MuR1* fragment. The adjoining leaf tissue which continued to show *MuR1* activity contained the appropriate *Hin*dIII fragment, serving as a control. All the other *MuR*-homologous fragments appeared in all samples independent of the activity of *MuR1* with the exception of a new *MuR* hybridizing fragment which appeared in the DNA from the red sector. The appearance of a new fragment has been observed in some, but not all, of the other red sectors examined (data not shown). It is unclear if this fragment represents an alteration of the original *MuR1* fragment or is an alteration or duplication of another *MuR* sequence. Similar results, showing loss of the *MuR1*-containing fragment, were obtained using several other restriction enzymes suggesting the 11-kb fragment was lost and not absent due to incomplete digestion of the DNA. Furthermore, Southern analysis of DNA from six independent sectors indicated that the unique, internal *Eco*RI-*Hin*dIII fragment of *MuR1* was lacking in all six red sector samples (data not shown). The consistent lack of the *MuR1* fragment in mutator-loss sectors provides independent conformation that *MuR1* is necessary for mutator activity.

Transcripts homologous to *Mu are associated with *MuR1*:** To determine whether there were transcripts homologous to *MuR* that correlated with both *MuR1* and Mutator activity in general, we performed Northern blot hybridizations. Figure 7 shows the results of hybridization of the Mu* probe to a blot carrying poly(A)$^+$ RNA from four siblings, two containing (+*MuR1*) and two lacking *MuR1* (−*MuR1*) from a population segregating 1:1 for *MuR1*. Two transcripts, migrating at approximately 2.5 and 0.9 kb were homologous to the Mu* probe, and were present in segregants carrying *MuR1* only. These transcripts may represent a *MuR1* encoded transcript involved in *MuR1* activity, or represent transcripts from defective *MuR* elements that are regulated by Mutator activity. We have examined other Mutator-active, Mutator-inactive and non-Mutator stocks from diverse backgrounds and found the 2.5-kb transcript and the smaller 0.9-kb transcript in all Mutator-active stocks examined to date (data not shown), supporting the hypothesis that both poly(A)$^+$ RNA species are involved in Mutator activity.

To further analyze the putative transcriptional units of *MuR1*, two additional probes flanking Mu*, Mu E-S and Mu B-H (see Figure 7), were also hybridized to similar Northern blots. The resulting data are summarized in Figure 7. Whereas Mu* hybridized to both the 2.5-kb and the 0.9-kb transcripts, the Mu B-H probe hybridized only to the larger transcript. No

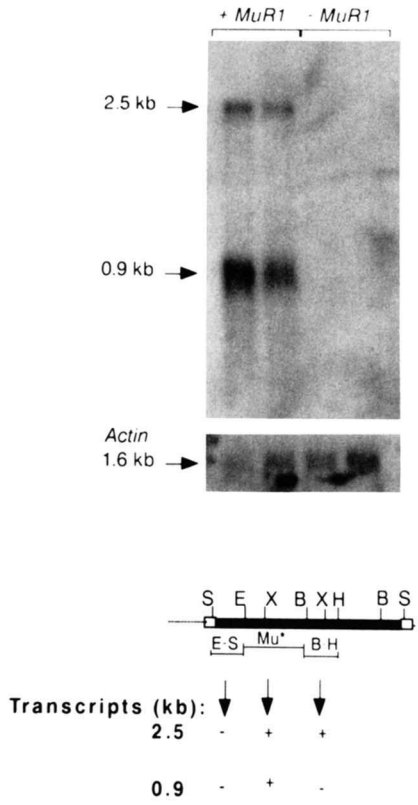

FIGURE 7.—Identification of Mu*-homologous transcripts associated with Mutator activity. A sample of 1 μg of poly(A)$^+$ RNA from immature ears of *MuR1* containing (+*MuR1* lanes) and *MuR1* lacking (−*MuR1* lanes) plants was separated on a 1% formaldehyde agarose gel and hybridized to the Mu* probe. The 2.5-kb and 0.9-kb RNA species are indicated by arrows. The Mu* probe was washed off the membrane (according to the manufacturer) and the blot was hybridized with the maize *Actin* probe (lower panel) to show each lane contained approximately equal amounts of intact RNA. Distinct restriction fragments of *dMuR* were used as probes to additional Northern blots and the results are summarized in the diagram below the blot. A "+" indicates that the particular restriction fragment probe detected the indicated transcript. A "−" indicates no signal was detected.

hybridization signal on Northern blots was detected with the Mu E-S probe. Therefore, it is likely that the 0.9-kb transcriptional unit is contained within the limits of the Mu* sequence and that the 2.5-kb transcriptional unit extends to the right side of that sequence.

DISCUSSION

A member of a previously unidentified class of *Mu* elements was cloned from a *Mu*-induced *shrunken1* allele. We have shown that this 4.0 kb element molecularly identifies the genetically characterized *MuR1* element, which regulates the activity of *Mu* elements. There are multiple fragments homologous to *MuR1* in all maize plants examined, even plants lacking *MuR1* or Mutator activity. The 4.0-kb element cloned from *sh1-A83* appears to be incomplete, as a restriction analysis comparison between this *dMuR* and *MuR1* indicates the *dMuR* element is missing 0.9-kb relative to *MuR1* (Figure 2). Many of the *dMuR* elements are likely to be rearrangement, insertion or deletion variants of each other much like other nonautonomous elements of controlling element systems such as *Dissociation* (*Ds*) or *dSpm* (FEDOROFF, WESSLER and SHURE 1983; PEREIRA *et al.* 1985). At least three different families of *Ds* elements have been described, each family containing multiple members that are heterogeneous in size (SACHS *et al.* 1983; BURR and BURR 1981; FEDOROFF, WESSLER and SHURE 1983; DORING *et al.* 1984). Molecular analyses of other *dMuR* elements we have cloned may show their familial relationships.

Mutator-active stocks contain *MuR*-homologous transcripts that are absent in non-Mutator and Mutator-inactive stocks. Presence of these transcripts correlates with *MuR1* activity. These transcripts do not hybridize with the *Mu* termini, and are therefore unlikely to result from the simple readthrough from a gene into an endogenous *dMuR* element. Thus, our working hypothesis is that one or both of these transcripts encodes a product involved in Mutator activity. Isolation and characterization of these transcripts should reveal clues as to their role in Mutator activity.

Unlike other two-element systems in maize, each of which usually segregate for a single regulatory element, ROBERTSON's Mutator system does so only very rarely. Upon outcrossing, most progeny from a Mutator plant receive Mutator activity. To explain the unusual segregation, ROBERTSON (1978) and BENNETZEN (1984) suggested that multiple copies of a regulatory element would have to exist and such copies would have to undergo premeiotic amplification to be passed on to most progeny. Although *Mu1* transposons behave according to these expectations (ALLEMAN and FREELING 1986), *Mu1* does not regulate the Mu system (LILLIS, SPIELMANN and SIMPSON 1985; P. Chomet, unpublished). Our data show that *MuR1* does undergo premeiotic duplicative transposition events which result in the increase in *MuR1* copy number. Although the frequency of duplication we observed may not completely explain the unusual segregation patterns of Mutator activity, it is possible that multiple *MuR1* elements act synergistically to increase their rate of duplication. This possibility is currently being tested.

WALBOT (1986) has argued that the segregation of Mutator activity is such that simple, mendelian regulatory genes cannot be the proximate cause of the loss of Mutator activity in her lines. This conclusion stemmed from observations in which the active state of Mutator, as assayed by somatic instability of *Mu1*, can become weakly active with only a few kernels showing somatic excisions, or the active state can become inactive through repeated self pollinations. It

is likely that these data are not explained simply by meiotic segregation of regulatory elements. We do know that *MuR1* can functionally replace Mutator activity when Mutator activity was lost via outcrossing (P. CHOMET, D. LISCH and M. FREELING, unpublished data). Furthermore, we show here that inactivity in our lines is primarily due to the lack of the *MuR1* element, although we do note exceptional plants which give rise to non-mendelian loss of Mutator activity (see Table 2). Therefore, it is possible that genetic loci, such as *MuR1*, are responsible for the segregation of Mutator activity but the unusual behaviors may be caused by stable or metastable secondary modifications (such as DNA methylation) to these regulating loci rendering them functionless.

The simultaneous occurrence of the loss of Mutator activity, the methylation of *Mu1*, and the loss of the *MuR1* restriction fragment in somatic sectors, suggests that the loss of *MuR1* is associated with the gain of *Mu1* methylation. Furthermore, loss of *MuR1* through mendelian segregation is also associated with *Mu1* methylation (P. CHOMET, D. LISCH and M. FREELING, unpublished data). One possibility for such results is that *Mu1* methylation is the consequence of the loss of *MuR1* in these instances. It would follow then that *Mu1* methylation is the "ground state" in the absence of *MuR1* activity. By this reasoning, the presence of *MuR1* product(s) should block *Mu1* methylation. A DNA footprint in the *Mu1*-end sequence associated with Mutator activity has been detected (Z. ZHAO and V. SUNDARESAN, personal communication). We suggest that *MuR1* product(s) bind to these protected nucleotides.

The mechanism underlying *MuR1* loss in somatic sectors is unclear. It is possible that the element was altered or rearranged during the transposition process. The appearance of a new fragment in some of these sectors would be consistent with such an assertion. The new fragment may represent a rearrangement derivative at the *MuR1* location or at a new genomic position. In either case, it is likely these new fragments appearing in the red sectors are not complete *MuR1* elements since analysis of independent sectors indicated that a unique, internal restriction fragment, associated with *MuR1*, was lacking in the red sectors (data not shown). More detailed analyses to further elucidate these alterations are underway.

Genetic studies on the *Cy* controlling element system demonstrated that *Cy* is a transposable element capable of regulating *Mu1* and *Mu7* (SCHNABLE and PETERSON 1988; SCHNABLE, PETERSON and SAEDLER 1989). *Cy* can segregate as a single mendelian element and has been observed to increase its copy number (SCHNABLE and PETERSON 1988). Presumably, this means that *Cy* is a regulatory element of the Mutator system, functionally analogous to *MuR1*. Molecular analyses will demonstrate if the sequence of *MuR1* is homologous to that of *Cy*.

Recently, a ninth class of *Mutator* elements has been described. One member of the family, designated *MuA*, was cloned and shown to be homologous to a 3.5-kb transcript associated with Mutator activity (QIN and ELLINGBOE 1990). Although no evidence indicates that *MuA* is the autonomous element, it is possible that *MuA* and *MuR1* are members of the same class of *Mu* elements, as they have similar but not identical restriction maps. The reason for the discrepancy in size between the reported 3.5-kb transcript and the 2.5-kb transcript we observe is not clear. Different electrophoresis conditions and RNA markers might have led to different size estimations. Alternatively, the two elements may be hybridizing to different transcripts. A direct comparison between the two elements will clarify the differences.

The recent insertion of the *dMuR* element into *Sh1* caused a 9-bp host site direct duplication. All other recently transposed *Mutator* elements also produced a 9-bp duplication (BARKER *et al.* 1984; CHEN *et al.* 1987; SCHNABLE, PETERSON and SAEDLER 1989). Such host site duplications are a common feature of transposable elements and the extent of the duplication is specific for each family of interacting elements. Since all elements within a family are under the control of a common transposase, the host-site duplication length is likely a reflection of the shared transposase-mediated insertion mechanism. This suggests the transposition of *MuR* elements, along with the other *Mutator* elements, is controlled by a similar mechanism, likely regulated by *MuR1*.

Although *Mu* transposons are excellent insertional mutagens in maize, it has been difficult to clone some *Mu*-induced mutations due to the complexity of the system. The identification of a regulatory element will greatly facilitate the use of the Mutator system as a transposon tag in maize. Furthermore, a demonstration of *Mu* transposition in transgenic systems would prove to be a valuable tool for insertional mutagenesis and cloning in other plants as well. Sequences homologous to *MuR1* have been detected in other monocots (D. LISCH, unpublished data). An efficient transposon-tagging system for major crops other than maize would be particularly valuable.

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