Mu killer Causes the Heritable Inactivation of the *Mutator* Family of Transposable Elements in *Zea mays*

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ABSTRACT

Mutations in a number of genes responsible for the maintenance of transposon silencing have been reported. However, the initiation of epigenetic silencing of transposable elements is poorly characterized. Here, we report the identification of a single dominant locus, *Mu killer* (*Muk*), that acts to silence *MuDR*, the autonomous regulatory transposon of the *Mutator* family of transposable elements in maize. *Muk* results in the methylation of *MuDR* TIRs and is competent to silence one or several active *MuDR* elements. Silencing by *Muk* is not dependent on the position of the *MuDR* element and occurs gradually during plant development. Transcript levels of the *MuDR* transposase, *mudrA*, decrease substantially when *Muk* is present. The other transcript encoded by *MuDR*, *mudrB*, also fails to accumulate in the poly(A) RNA fraction when *MuDR* and *Muk* are combined. Additionally, plants undergoing *MuDR* silencing produce small, *mudrA*-homologous ~26-nt RNAs, suggesting a role for RNA-directed DNA methylation in *MuDR* silencing. *MuDR* elements silenced by *Muk* remain silenced even in plants that do not inherit *Muk*, suggesting that *Muk* is required for the initiation of *MuDR* silencing but not for its maintenance.

MUCH of the maize genome consists of a vast number of quiescent transposable elements (Sanmiguel *et al.* 1996). This inactivity is due to a variety of processes that have evolved to keep these potentially potent mutagens transcriptionally and transpositionally silent (Kumar and Bennetzen 1999). A number of genes have been identified in a variety of organisms that, when mutant, result in the activation of otherwise silenced transposons (for review see Okamoto 2001). The nature of these genes suggests that the initiation and maintenance of transposon silencing is a complex process that involves both transcriptional and post-transcriptional gene-silencing pathways. For example, in *Caenorhabditis elegans*, mutations in the RNaseD *mut-7* (Ketting *et al.* 1999) and the *Argonaute* family *rde-1* gene (Tabara *et al.* 1999)—both of which are involved in small RNA-based post-transcriptional silencing—activate otherwise silent transposons. Likewise, in *Chlamydomonas reinhardtii* a mutation in the DEAH-box RNA helicase *Mut6* can result in transposon reactivation (Wu-Scharf *et al.* 2000). In these cases the mutations relieve RNA-based post-transcriptional silencing and reverse previously established transposon inactivation. Mutations in other genes, such as the *Arabidopsis thaliana* SWI2/SNF2 chromatin remodeling factor *decrease in* DNA *methylation 1* (DDM1), reactivate silenced transposable elements not because they are necessary for RNA-based silencing, but because they are involved in the chromatin-based maintenance of the silenced state (Hirochika *et al.* 2000; Miura *et al.* 2001; Singer *et al.* 2001). Similarly, a mutation in the WD-repeat-containing gene *Mut11* in *C. reinhardtii* also reactivates silent transposons (Jeong *et al.* 2002). Similarities between *Mut11* and fungal transcriptional corepressors suggest a direct role for *Mut11* in transcriptional regulation of transposons (Zhang *et al.* 2002).

Nearly all screens designed to detect genes involved in transposon silencing have sought mutations that affect previously established silenced states (Okamoto 2001). Thus, the genes identified to date are specifically those involved in the maintenance (rather than in the establishment) of silencing. Very little is known about the initiation of the silenced state. Further, the transposon reactivation observed in both Arabidopsis and *C. reinhardtii* refers to populations of transposons without knowledge of the direct effects of the mutations on specific autonomous elements.

Three genes are known to affect the maintenance of the silenced epigenetic state of *Mutator* (*Mu*) and *Mutator*-like element (MULE) transposons. Mutants of DDM1 in *A. thaliana* result in global and heritable cytosine hypomethylation (Kakutani *et al.* 1999). Previously silenced hypermethylated MULEs are transcriptionally reactivated in a mutant *ddm1* inbred line (Singer *et al.* 2001). However, the DDM1 gene does not specifically target MULE elements. DDM1 targets other genomic sequences such as centromeric tandem repeats, retrotransposons, and some single-copy genes (Kakutani *et al.* 1996; Vielle-Calzada *et al.* 1999; Hirochika *et al.* 2000). Also in Arabidopsis, mutations in the PIWI/PAZ domain protein *Argonaute4* (*Ago4*) abolish RNA-based post-transcriptional silencing and result in transcriptional activation of silenced MULE elements (Ziberman *et al.* 2003). In maize, *modifier of paramutation 1* (*mop1*) mutant plants exhibit hypomethylation of previously si-

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lenced *Mutator* elements (Lisch *et al.* 2002). After several generations of inbreeding in a *mop1* mutant background, *Mutator* elements become transpositionally active (Lisch *et al.* 2002). In addition to its effects on *Mutator* transposons, the *mop1* mutation can prevent paramutation from taking place at three different loci (Dorweiler *et al.* 2000).

The initiation of epigenetic silencing has long been a recognized feature of the highly mutagenic family of *Mutator* transposable elements in maize (reviewed in Lisch 2002). Robertson found that self- or sibling-crossed active *Mutator* lines could maintain high levels of activity for several generations, but activity was abruptly lost in the fifth generation of inbreeding (Robertson 1983). To explain the loss of activity, he postulated the activity of a dominant negative regulatory mechanism, perhaps triggered by an increased copy number of the transposon. Similarly, others have since postulated the presence of a negative regulatory factor to explain enigmatic *Mutator* silencing results (Bennetzen 1994). In one such report, multiple *MuDR* elements became inactivated simultaneously (Martienssen and Baron 1994). This inactivation was associated with methylation of cytosine residues within the termini of the *MuDR* regulatory transposons. The simultaneous inactivation of multiple functional *MuDR* elements was consistent with the activity of a dominant negative regulator, but it was unclear what factors were important for inducing the silencing process. Analysis was complicated by the multiple *MuDR* elements segregating at various positions, as well as multiple deletion derivatives of *MuDR*. Consequently, any number of factors, including "poisoning" by deletion derivatives, *MuDR* elements at certain positions, and overall copy number may have been contributing to the silencing.

Given the complexity of most *Mutator* lines, identification of factors involved in *Mutator* regulation can be problematic. To reduce this complexity we have developed a simplified *Mutator* system. This minimal *Mutator* line (Chomet *et al.* 1991) is a W22-derived inbred line completely permissive for *Mutator* activity. The minimal line has only one functional *MuDR* element and a lone nonautonomous *Mutator* element, a *Mu1* insertion in the recessive reporter allele *a1-mum2*. Spotted kernels are generated by the *MuDR*-catalyzed excision of the *Mu1* element at *a1-mum2* late in somatic development, restoring *A1* function and pigment production. The single active *MuDR* element in the minimal line is located on the long arm of chromosome 2L and is designated *MuDR(p1)*. In a cross between a plant with an active hemizygous *MuDR(p1)* element in the *a1-mum2* minimal line and an *a1-mum2* minimal line plant with no *MuDR*, the progeny segregate nonspotted [no *MuDR(p1)*] and heavily spotted [*MuDR(p1)*] kernels in a 1:1 ratio.

In previous studies using the minimal *Mutator* line, three mechanisms for loss of *Mutator* activity were outlined (Lisch and Freeling 1994; Lisch *et al.* 1995). The first occurs when *MuDR* is absent due to genetic segregation. The second is the result of internal deletions in functional *MuDR* elements, which can be produced both somatically and germinally. The third involves the loss or reduction of *MuDR* activity due to position effects. All losses of activity observed to date in the minimal line can be traced to one of these three mechanisms. Spontaneous epigenetic silencing of a *MuDR(p1)* element has never been observed in the minimal *Mutator* line over the course of many generations and several hundred test crosses. Only when the minimal line was crossed to a different genetic background has silencing of the *MuDR(p1)* element in the minimal line been observed (Lisch and Freeling 1994).

Here we describe the identification and characterization of a new locus, *Mu killer* (*Muk*), which acts in a dominant manner to silence full-length *MuDR* elements at stable active positions. This silencing is associated with the absence of steady-state poly(A) *MuDR* transcripts, methylation of *Mutator* element terminal inverted repeats (TIRs), and the loss of germinal and somatic *Mutator* activity. Small ~26-nt sense and antisense RNAs homologous to the 5′ region of the *mudrA* transcript have also been identified in plants carrying both *MuDR* and *Muk*. Genetic analysis reveals that *Muk* is necessary for the initiation of *Mutator* silencing, but not for the maintenance of the subsequent silenced state.

MATERIALS AND METHODS

Generation of lines: *The a1-mum2 minimal Mutator line and the a1-mum2 minimal line tester:* The W22-derived minimal *Mutator* line was previously generated and described (Lisch *et al.* 1995). It contains one full-length functional *MuDR* element, as well as none of the other nonautonomous *Mutator* elements with the exception of one *Mu1* element in the allele *a1-mum2* (O'Reilly *et al.* 1985; Chomet *et al.* 1991). When an active *MuDR* element is present, the *Mu1* element excises out of *A1* late in somatic development, creating characteristic *Mutator* spotting in the kernel. When no *MuDR* activity is present, the *Mu1* element interrupts the *A1* gene and the kernel has no spotting. A hemizygous *MuDR* element on the long arm of chromosome 2 named *MuDR(p1)* (Chomet *et al.* 1991) is the single active element in the minimal *Mutator* line. This *MuDR(p1)* element has never been observed to become spontaneously epigenetically silenced in the minimal *Mutator* line. When the *MuDR(p1)* element is not present in the minimal *Mutator* line, the minimal line is referred to as the minimal *Mutator* line tester.

Although other *MuDR*-homologous sequences (*hMuDR* elements) are present in this (and all) maize backgrounds (Chomet *et al.* 1991; Rudenko and Walbot 2001), these sequences do not contribute to *Mutator* activity in the minimal line (Chomet *et al.* 1991; Lisch *et al.* 1995; this report).

Mu killer in the minimal Mutator line: We have previously described the epigenetic silencing of a single *MuDR* element from the minimal *Mutator* line when crossed to a full-color line (B-I, R-r, Pl-Rh) from a mixed genetic background (Lisch and Freeling 1994). The *MuDR* element in this particular minimal *Mutator* line individual was a transposed copy of the original *MuDR* element first cloned from the minimal line

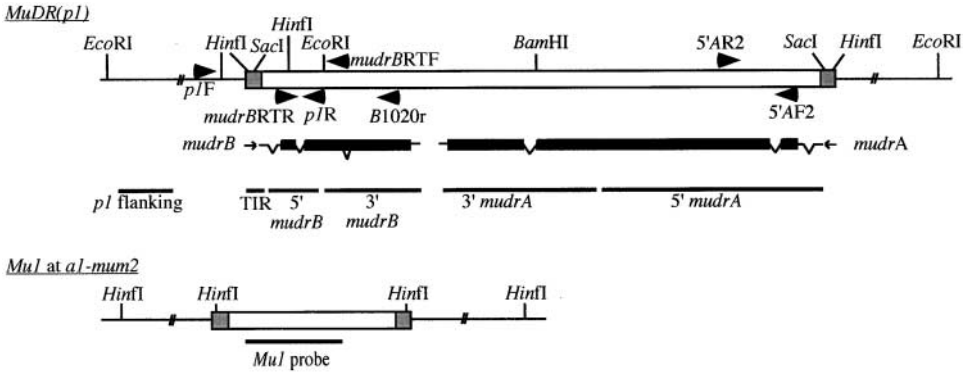

FIGURE 1.—Representation of *MuDR* at position 1 (*p1*) and *Mu1* at *a1-mum2*. Shaded boxes represent *Mutator* TIRs while open boxes are *Mutator* internal sequences that differ between *MuDR* and *Mu1*. Restriction sites used in this report are indicated. The *mudrA* and *mudrB* transcripts are shown just below the *MuDR* element as lines, with arrows indicating the direction of transcription. Exons are solid boxes while lines that angle down represent introns. Probes used in this report are indicated below the transcripts. Primers used in this report are shown as arrowheads.

[*MuDR(p1)*; LISCH and FREELING 1994]. Because it was not at the original position on the long arm of chromosome 2L, it was possible that the new chromosomal position was responsible for the silencing in this line. To test this, a hemizygous active *MuDR(p1)* element in the minimal *Mutator* line was crossed to an individual from the family that was exhibiting silencing. To exclude the possibility that previously silenced *MuDR* elements were necessary for inactivation, we crossed only silencing-line individuals that lacked full-length *MuDR* elements. Three of the nine progeny that carried *MuDR(p1)* had inactive hypermethylated *Mu1* TIRs as judged by *Hin*fI-digested Southern blots probed with *Mu1*. When test crossed, these plants gave rise to all nonspotted or very weakly spotted progeny kernels. In this and all subsequent families, *Mu killer* (*Muk*) activity is functionally defined as the presence of a previously active *MuDR* element that has become inactivated. Inactivation results in the hypermethylation of *Mutator* element TIR *Hin*fI sites while the *MuDR* element remains full length and at the same position.

The *Muk* locus has been introgressed into the minimal *Mutator* line for three generations. All *Muk*-carrying individuals in this report are in the minimal *Mutator* line background without any full-length *MuDR* elements and with only one *Mu1* element at *a1-mum2*.

Transposon-tagging line: The active *Mutator* transposon-tagging line used in this report was generated as in CHOMET (1994). The estimate of eight active *MuDR* elements in this line is based on analysis of the number of unique *Eco*RI fragments and the intensity of the *Sac*I internal fragments on Southern blots probed with a *mudrA* probe, as well as genetic segregation results.

DNA extraction and Southern blotting: DNA preparation and genomic Southern blotting were performed as previously described (DORWEILER *et al.* 2000). A total of 10 μg of maize genomic DNA was digested for >4 hr with an excess of 20 units of restriction enzyme. *Mutator* restriction sites used in this report are shown in Figure 1. Mature leaf tissue of leaf 8 was used for DNA extraction unless otherwise noted.

RNA extraction and Northern blotting: Total and Poly(A) RNA was isolated from the tips of immature ears using the Trizol reagent and manufacturer's directions (Invitrogen, San Diego). A total of 10 μg of RNA was run through a 1.5% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to uncharged Hybond nylon filters (Amersham, Arlington Heights, IL) in 20× SSC and fixed by UV crosslinking (Stratagene, La Jolla, CA) as specified by the manufacturer. Hybridization was performed in 0.5 M sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA, at 65° overnight. The most stringent wash was in 1× SSC, 0.1% SDS at 58° for 1 hr. Blots were stripped of radioactivity by washing with 1 liter of 10 mM Tris-Cl pH 7.4, 0.2% SDS at 75° for 1 hr.

Poly(A) RNA extraction: Poly(A) RNA was extracted from total RNA samples (see above), using the Oligotex mRNA mini kit (QIAGEN, Valencia, CA). A total of ~2 μg of poly(A) RNA was used in Northern analysis as described above.

Reverse transcription-PCR analysis of *mudrA*: The same immature ear total RNA samples used for Northern blotting were treated with DNase I (Invitrogen) and then reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and an oligo(dT) primer. Samples were amplified for 29 cycles using the primers 5′*A*F2, 5′ ATCCGGCATTGGGCGAAACA and 5′*A*R2, 5′ TTGTCCGTATCCAAACTTCCCT (see Figure 1 for primer locations) with an annealing temperature of 56°. PCR products were electrophoresed on a 1.5% agarose gel. Amplification of *mudrA* RNA generates a band of 241 bp, while amplification of the DNA gives a 386-bp band.

Reverse transcription-PCR analysis of *mudrB*: The same immature ear total RNA samples used above were treated with DNase I (Invitrogen) and then reverse transcribed using Superscript II reverse transcriptase (Invitrogen). Both the oligo(dT) primer and the *mudrB*-specific primer *B*1020r (5′ CCCATCACCAAGTTCATCATCA) were used to prime cDNA synthesis (see Figure 1). Samples were amplified for 20 cycles using the primers *mudrB*RTF, 5′ ATCTTGCCACCTTGTACC TCTGGA and *mudrB*RTR, 5′ AGATGCGCGGTATTTGTTGC TGAG (see Figure 1 for primer locations) with an annealing temperature of 59°. PCR products were electrophoresed on a 1.5% agarose gel and blotted to a nylon membrane as described above. The blot was hybridized with the 5′ *mudrB* probe as seen in Figure 1. Amplification of *mudrB* RNA generates a band of 241 bp, while amplification of the DNA produces a band of 314 bp.

Reverse transcription-PCR analysis of *ubiquitin* transcripts: The same oligo(dT)-primed cDNA used in the reverse transcription (RT)-PCR analysis of the *mudrA* and *mudrB* transcripts was amplified with primers specific for the *ubiquitin* transcript (MORENO *et al.* 1997) to ensure equal starting amounts of RNA. Amplification was done for either 29 cycles or 20 cycles followed by blotting to a nylon membrane (described above) and hybridizing (described above) with a *ubiquitin* probe (MORENO *et al.* 1997).

Assay to determine the presence of *MuDR(p1)*: The presence of a full-length *MuDR* element was assayed by Southern blots using DNA digested with *Sac*I and probing with any internal region of *MuDR* (see below for generation of probes). If a full-length *MuDR* element is present, a fragment of 4684

bp is visualized. To determine if the *MuDR* element was at the *p1* position, two different methods were used. First, PCR between the *MuDR(p1)* element and the *p1* flanking sequence was used. The *MuDR* primer *p1*F, 5′ ACCACATTCGATGA GGCCTT and the *p1* flanking primer *p1*R, 5′ GGATGTCGGG GGCGCAGAGA (see Figure 1 for primer locations) were used in the following PCR program: 94° for 3 min, 94° for 45 sec, 55° for 45 sec, 72° for 1 min, repeated for 30 cycles; and 72° for 5 min. An amplification product of 837 bp signifies that *MuDR(p1)* is present. Alternatively, an *Eco*RI-digested Southern blot was prepared and probed with a *p1* flanking probe (see below for generation of probes; Figure 1). If *MuDR(p1)* is present, a 3.9-kb fragment is produced, while if *MuDR(p1)* is not present, a 6.3-kb band is produced. To show that the *MuDR(p1)* was full length, the *Eco*RI-digested Southern blots were probed with a *mudrA*-specific probe (see below for generation of probes), which results in a 6.8-kb fragment for full-length *MuDR(p1)*. If both the flanking and the internal probes result in the expected fragment sizes, the *MuDR* element was presumed to be at the correct position and full length.

***Mutator* TIR methylation assay:** *Mutator* activity can be followed by the methylation status of the *Hin*fI restriction site present in all *Mu* element TIRs (Lisch *et al.* 1995). *Mutator*-active individuals have hypomethylated *Mu* TIRs and will produce a 1.3-kb band when digested with *Hin*fI and probed with the internal region of *Mu1*. Individuals without *MuDR* or with silenced *MuDR* elements have hypermethylated *Mu1* TIRs that are not digested by the methyl-sensitive *Hin*fI restriction enzyme, producing *Mu1* restriction fragments >1.3 kb (Lisch *et al.* 1995). The exact size of the inactive *Mu1* restriction fragment is dependent on the position of the hypermethylated *Mu1* element. In the allele *a1-mum2* the size of this fragment is 2.1 kb. Additional fragments that are the result of hybridization of the *Mu1* probe to MRS-A, a maize gene that is homologous to *Mu1*, can also be observed (Chandler *et al.* 1986). The *Hin*fI sites in this gene, which lacks *Mu* TIRs, are not affected by the presence or absence of *MuDR*.

The methylation and activity status of *MuDR(p1)* TIRs can also be assayed by restriction digestion using methyl-sensitive restriction enzymes *Hin*fI and *Sac*I. When using a *MuDR* TIR probe (see below for generation of probes), digestion of *MuDR(p1)* with *Hin*fI produces a 311-bp fragment when hypomethylated and a larger fragment of 497 bp when hypermethylated. Any *MuDR* internal probe can be used to assay the *Sac*I methylation status of *MuDR(p1)*. *Sac*I digestion of an active hypomethylated *MuDR(p1)* produces a fragment of 4684 bp and a larger fragment when hypermethylated.

Generation of probes: *Mu1 probe:* The plasmid that carries the probe for the internal region of *Mu1* has been previously described (Talbert and Chandler 1988). The *Mu1* internal probe is generated by gel isolating an internal *Ava*I/*Bst*EII fragment.

5′ mudrA probe: The 5′ *mudrA* probe was generated by PCR amplification from a *MuDR(p1)*-containing minimal *Mutator* line individual. The probe was sequenced to ensure that it was identical to *MuDR(p1)*. The 5′ *mudrA* probe was amplified using the primers 5′ ATCGCCAAAACAGAAAGGTGACAG and 5′ GCATGGACCAAAGGCACAAAAGAA. The touchdown PCR cycle used was 96° for 15 sec, 95° for 5 min, 95° for 45 sec, 64° − 0.5° per cycle for 30 sec, 72° for 2 min, back to 95° for 45 sec 19 times, 95° for 30 sec, 54° for 30 sec, 72° for 2 min + 1 sec/cycle, back to 95° for 30 sec 29 times, and 72° for 10 min. The PCR product was cloned using the TOPO TA cloning kit (Invitrogen).

3′ mudrA probe: The 3′ *mudrA* probe was also generated by PCR from a *MuDR(p1)*-containing *Mutator* minimal line individual. Again the probe was sequenced to ensure that it was identical to *MuDR(p1)*. The 3′ *mudrA* probe was amplified using the primers 5′ CATGCCCGATAGTGTGATTGAGAT and 5′ CTTTTCTTGGGGGTGATTTTCTTC. The same touchdown PCR program as above was used except the first-round annealing temperature was from 66° to 56° and the second-round annealing temperature was 55°. The PCR product was cloned using the TOPO TA cloning kit (Invitrogen).

5′ and 3′ mudrB probes: The 5′ and 3′ *mudrB* probes were digested from a plasmid (pBMP1.3) carrying the entire *mudrB* gene from *MuDR(p1)*. pBMP1.3 is a *Bam*HI clone that includes the *mudrB* portion of *MuDR(p1)* as well as 4 kb of *p1* sequence flanking that element (Lisch *et al.* 1995). The 5′ *mudrB* probe was created by digesting pBMP1.3 with *Sal*I and *Eco*RI. The resulting 503-bp *mudrB*-specific fragment was then gel purified. The 3′ *mudrB* probe was generated by digesting pBMP1.3 with *Eco*RI and *Eco*RII followed by gel purification of the resulting 861-bp fragment.

MuDR TIR probe: The TIR probe was generated by amplification of the pBMP1.3 plasmid. The PCR primers used were 5′ GAGATAATTGCCATTATGGA and 5′ GATGTCGACCCCTA GAGC. The PCR product was cloned using the TOPO TA cloning kit (Invitrogen).

p1-flanking probe: The *p1*-flanking probe was generated by *Pst*I digestion of the pBMP1.3 plasmid. The 800-bp *p1*-specific fragment hybridizes to a single-copy sequence in the maize genome.

All DNA probes in this report were gel isolated and prepared by the random priming method using a Prime-It II kit (Stratagene) and ^{32}P-radiolabeled dCTP (Perkin-Elmer, Norwalk, CT). All blots were exposed to a Molecular Dynamics (Sunnyvale, CA) phosphor imaging screen, saved as TIFF files, and processed using Adobe Photoshop or Deneba Canvas programs.

Small RNA Northern analysis: Total RNA from seedling second leaves was extracted using RNAwiz (Ambion, Austin, TX). Total RNA was run on a 15% polyacrylamide gel containing 7 M urea. The gel was electroblotted at 100 V to charged Zeta-Probe blotting membrane (Bio-Rad, Richmond, CA). The hybridization conditions are the same as in Hamilton and Baulcombe (1999). Single-stranded sense or antisense RNA probes were generated by cloning the probes described above behind the T7, T3, or SP6 promoter. Run-off transcription was performed using a Maxiscript *in vitro* transcription kit (Ambion), and the hybridization was done overnight at 40°. The Northerns were washed twice for 15 min at 50° in 2× SSC, 0.2% SDS. Sizes of the hybridizing bands were estimated using single-stranded RNA oligos of known length homologous to *MuDR*. These control RNA oligos also served as a positive hybridization control.

RESULTS

***Mu killer* segregates as a single locus unlinked to *MuDR(p1)*:** In experiments described that employ an active *MuDR(p1)*-containing plant, the same *MuDR(p1)* male was also test crossed to the *a1-mum2* minimal *Mutator* line tester to ensure that the *MuDR(p1)* element did not epigenetically silence in the absence of *Muk*. In no case was such silencing observed.

To study the pattern of *Muk* inheritance, we crossed 16 plants that were heterozygous for *Muk* but lacked full-length *MuDR* (*Muk*/−; *a1-mum2*) to male minimal *Mutator* line plants hemizygous for *MuDR(p1)* (*MuDR (p1)*/−; *a1-mum2*). The progeny segregated two non-spotted kernels to one weakly spotted kernel to one heavily spotted kernel (see kernel phenotypes, Figure

TABLE 1

A single locus produces weakly spotted kernels

Cross[a]	Heavily spotted	Weakly spotted	Total kernels	Spotted kernels (%)	χ^2 [b]	% weakly spotted[c]	χ^2 [d]
Control	170	0	326	52.1	0.60	0.0	170.0*
1[e]	83	75	346	45.7	2.60	47.5	0.41
2	11	10	39	53.8	0.23	47.6	0.05
3	24	21	93	48.4	0.10	46.7	0.20
4	23	30	110	48.2	0.15	56.6	0.92
5	17	19	76	47.4	0.21	52.8	0.11
6	39	32	143	49.7	0.01	45.1	0.69
7	55	55	237	46.4	1.22	50.0	0.00
8	32	22	103	52.4	0.24	40.7	1.85
9	52	45	207	46.9	0.82	46.4	0.51
10	18	13	65	47.7	0.14	41.9	0.81
11	66	61	277	45.8	1.91	48.0	0.20
12	30	26	95	58.9	3.04	46.4	0.29
13	21	18	86	45.3	0.74	46.2	0.23
14	27	26	115	46.1	0.70	49.1	0.02
15	17	12	68	42.6	1.47	41.4	0.86
16	58	47	201	52.2	0.40	44.8	1.15
Total	573	512	2261	48.0	3.66	47.2	3.43

[a] All experimental families were generated by a cross of a female *Muk* heterozygote (*Muk/−* ; *a1-mum2*) by a plant hemizygous for *MuDR(p1)* (*MuDR(p1)/−* ; *a1-mum2*). The control cross was of an *a1-mum2* minimal *Mutator* line tester without *Muk* to a plant hemizygous for *MuDR(p1)*. All plants were homozygous for *a1-mum2*.

[b] χ^2 value for the expected one-to-one segregation of total spotted to nonspotted kernels if a single *MuDR* element were segregating.

[c] Percentage of total spotted kernels that were weakly spotted.

[d] χ^2 value for the expected one-to-one ratio if a single locus were responsible for weak spotting. * denotes ears with a significantly different χ^2 value at the 0.01 level for the expected segregation of a single locus associated with weak spotting.

[e] A subset of progeny from this cross was further analyzed in Table 2, generation 1.

3A). This ratio is consistent with the 1:1 segregation of a single dominant locus associated with weak spotting (Table 1). For one particular cross (cross 1 in Table 1), a subset of individuals from all kernel phenotypic classes was assayed for the presence of a full-length *MuDR(p1)* element and for methylation of the *Hin*fI site in *Mu1* TIRs (generation 1 in Table 2). Of the progeny analyzed, 16 of 17 plants grown from nonspotted kernels lacked full-length *MuDR(p1)*, 63 of 63 individuals grown from heavily spotted kernels had a full-length *MuDR(p1)*, and 61 of 61 individuals grown from weakly spotted kernels also had a full-length *MuDR(p1)*. Next we compared the *Mu1* TIR methylation status in plants grown from the heavily spotted kernels to that of plants grown from weakly spotted kernels (a subset of this data is presented in Figure 3C). A total of 58 out of 63 heavily spotted individuals had hypomethylated *Mu1* TIRs, while 55 of 61 weakly spotted individuals had hypermethylated *Mu1* TIRs (Table 2). Overall, 60 of 124 *MuDR(p1)* elements (48.4%) examined in this family were inactive. The 1:1 segregation of silenced to active *MuDR(p1)* elements demonstrates that *Mu killer* segregates as a single dominant Mendelian locus.

A total of 28 active *MuDR(p1)* plants from heavily spotted kernels and 15 silenced *MuDR(p1)* plants from weakly spotted kernels that were analyzed by Southern blot were then test crossed as female to the *a1-mum2* minimal *Mutator* line tester without *MuDR(p1)* (see Figure 2). In each instance, the active *MuDR(p1)* plants generated ears segregating 1:1 heavily spotted and nonspotted kernels (control generation in Table 7), while the inactive *MuDR(p1)* plants generated ~90% nonspotted kernels and ~10% weakly spotted kernels (generation 1 in Table 7).

If *Muk* segregates as a single locus unlinked to *MuDR(p1)*, then half of the progeny that lacked *MuDR(p1)* from the cross of a female *Muk* heterozygote with homozygous *a1-mum2* (*Muk/−* ; *a1-mum2*) to a plant hemizygous for *MuDR(p1)* with homozygous *a1-mum2* (*MuDR(p1)/−* ; *a1-mum2*) would be expected to carry *Muk*. To test this, 25 nonspotted kernels from the above cross were planted. Twenty-three of the resulting plants [none of which carried *MuDR(p1)*] were crossed as females to active *MuDR(p1)/−* ; *a1-mum2* individuals (Table 3). Thirteen of these crosses resulted in progeny exhibiting a 1:1 ratio of heavily spotted to pale kernels, consistent with the lack of *Muk* (crosses 1–13 in Table 3). The other 10 families gave a ratio of 2 nonspotted kernels to 1 weakly spotted kernel to 1 heavily spotted kernel, consistent with the presence of *Muk* in the female par-

TABLE 2

Weakly spotted kernels have hypermethylated TIRs even in the presence of *MuDR(p1)*

	Nonspotted			Heavily spotted			Weakly spotted		
Generation	*T*[a]	*p1*[b]	Hyper[c]	*T*[a]	*p1*[b]	Hypo[c]	*T*[a]	*p1*[b]	Hyper[c]
1[d]	17	1	17	63	63	63	61	61	55
2A[e]	12	0	12	30	30	30	0	0	0
2B[f]	20	0	20	15	15	15	13	12	13

[a] Total number examined by Southern blot.
[b] Presence of full-length *MuDR(p1)* determined by PCR and Southern blot.
[c] Methylation status as judged by *Hin*fI-digested Southern blots probed with *Mu1*. Hyper, hypermethylated TIRs; Hypo, hypomethylated TIRs.
[d] Plants used for this analysis were a subset from Table 1, cross 1. The plants were generated from the cross *Muk/−* ; *a1-mum2* × *MuDR(p1)/−* ; *a1-mum2*.
[e] Progeny of an *a1-mum2* plant [without both *Muk* and *MuDR(p1)*] crossed as female to *MuDR(p1)/−*; *a1-mum2*. Plants used for this analysis were a subset from Table 3, cross 3.
[f] Progeny of a *Muk/−* ; *a1-mum2* plant [without *MuDR(p1)*] crossed as female to *MuDR(p1)/−* ; *a1-mum2*. Plants used for this analysis were a subset from Table 3, cross 14.

ents (crosses 14–23 in Table 3). The segregation of *Muk* to 43.5% of nonspotted kernels further suggests that *Muk* is unlinked to *MuDR(p1)*. A subset of the progeny from these crosses was then analyzed for *Mu1* TIR methylation (generations 2A and 2B in Table 2). In a family that lacked *Muk* (segregating 1:1 for heavily to nonspotted kernels), 12 of 12 nonspotted individuals tested lacked *MuDR(p1)* and had hypermethylated *Mu1* TIRs, while 30 of 30 heavily spotted individuals had hypomethylated *Mu1* TIRs (generation 2A in Table 2). When the same *MuDR(p1)*-donating male parent was crossed to the nonspotted kernels that carried *Muk/−* (generation 2B in Table 2), 20 of 20 nonspotted kernels lacked *MuDR(p1)*, 15/15 heavily spotted kernels had a full-length *MuDR(p1)* and hypomethylated active *Mu1* TIRs, while 12 of 13 weakly spotted individuals tested had a full-length *MuDR(p1)* and hypermethylated *Mu1* TIRs.

We have subsequently followed *Muk* inheritance for three additional generations from the direct progeny of the initial *Muk* segregation crosses described here. *Muk* has continuously segregated as a single Mendelian locus unlinked to *MuDR(p1)*, which can silence *MuDR (p1)* in a reproducible manner. These data demonstrate that *Mu killer* is a single locus unlinked to *MuDR(p1)*, which can silence *Mutator* activity in a dominant fashion.

***Mu killer* silences *Mutator* activity when inherited from either the male or the female parent:** When inherited from the female parent, the dominant *Muk* silences *MuDR* and causes the weakly spotted *a1-mum2* phenotype characteristic of a silencing *Mutator* system (Figure 3A). However, when *Muk* is inherited from the male parent in the cross *MuDR(p1)/−*; *a1-mum2* × *Muk/−*; *a1-mum2*, heavily spotted and nonspotted kernels segregate in a 1:1 ratio, and no weakly spotted kernels are observed (Table 4). To determine if *Muk* acts only if inherited from the female parent, we reciprocally crossed *Muk/−*; *a1-mum2* plants with *MuDR(p1)/−* ; *a1-mum2* plants. Progeny of the reciprocal crosses were analyzed by Southern blot and test crossed as females to the *a1-mum2* minimal *Mutator* line tester (Table 5).

Progeny of the cross in which *Muk* was inherited from the female parent were separated into nonspotted, weakly spotted, and heavily spotted kernel phenotypic classes. From this cross, six of six plants grown from nonspotted and six of six plants grown from weakly spotted kernels had hypermethylated *Mu1* TIRs (Table 5). When crossed to the minimal *Mutator* line tester, all six individuals carrying inactivated *MuDR(p1)* transmitted only weakly and nonspotted kernels. In contrast, five of five plants grown from heavily spotted kernels showed hypomethylated *Mu1* TIRs and yielded at least 50% heavily spotted progeny kernels upon crossing to the *a1-mum2* minimal *Mutator* line tester (Table 5).

Progeny from the reciprocal cross, in which the

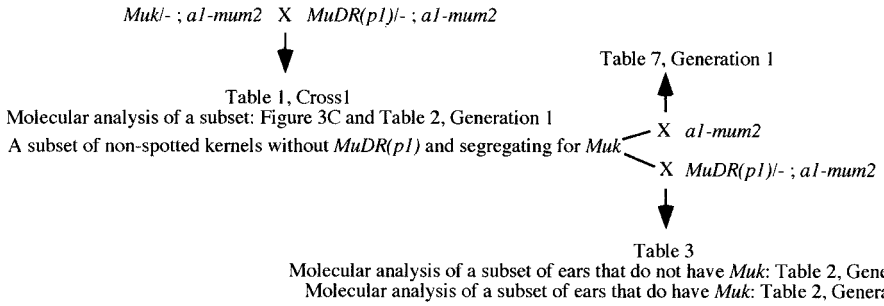

FIGURE 2.—Crossing scheme as described in RESULTS.

TABLE 3

Muk* is heritable as a single locus unlinked to *MuDR(p1)

	Progeny of a cross to *MuDR(p1)/−; a1-mum2*						
Cross[a]	Heavily spotted	Weakly spotted	Total kernels	Spotted kernels (%)	χ^2 [b]	% weakly spotted[c]	χ^2 [d]
1	21	2	45	51.1	0.02	8.70	15.7*
2	54	4	112	51.8	0.14	6.90	43.1*
3	46	0	86	53.5	0.42	0.0	46.0*
4	83	3	170	50.6	0.02	3.49	74.4*
5	38	0	67	56.7	1.21	0.0	38.0*
6	43	3	95	48.4	0.09	6.52	34.8*
7	45	5	94	53.2	0.38	10.0	32.0*
8	57	0	99	57.6	2.27	0.0	57.0*
9	99	0	189	52.4	0.42	0.0	99.0*
10	57	0	125	45.6	0.97	0.0	57.0*
11	28	0	61	45.9	0.41	0.0	28.0*
12	39	2	91	45.1	0.89	4.88	33.4*
13	48	1	93	52.7	0.27	2.04	45.1*
14	25	21	104	44.2	1.38	45.7	0.35
15	24	19	96	44.8	1.04	44.2	0.58
16	23	27	92	54.3	0.70	54.0	0.32
17	41	37	168	46.4	0.86	47.4	0.21
18	14	11	57	43.9	0.86	44.0	0.36
19	25	25	112	44.6	1.29	50.0	0.00
20	16	15	67	46.3	0.37	48.4	0.03
21	24	31	119	46.2	0.68	56.4	0.89
22	15	11	49	53.1	0.18	42.3	0.62
23	28	19	85	55.3	0.95	40.4	1.72

[a] Generated by crossing the nonspotted kernels without *MuDR(p1)* from the cross *Muk/−; a1-mum2* × *MuDR(p1)/−; a1-mum2* as female to an active *MuDR(p1)/−; a1-mum2* individual.

[b] χ^2 value for the expected one-to-one segregation of total spotted to nonspotted kernels if a single *MuDR* element were segregating.

[c] Percentage of total spotted kernels that were weakly spotted.

[d] χ^2 value for the expected one-to-one ratio if a single locus were responsible for weak spotting. * denotes ears with a significantly different χ^2 value at the 0.01 level for the expected segregation of a single locus associated with weak spotting.

Muk/− parent was male, were divided into heavily spotted and nonspotted kernel phenotypic classes; there were very few weakly spotted kernels. Of the 19 heavily spotted kernels tested, 11 had hypermethylated *Mu1* TIRs. When test crossed, all 11 yielded very few to no heavily spotted kernels and <10% weakly spotted kernels (Table 5). The eight progeny that had hypomethylated *Mu1* TIRs produced ears with near 50% heavily spotted kernels when crossed to the *a1-mum2* minimal *Mutator* line tester.

The observation that roughly half (57.9%) of the *MuDR(p1)* elements in a family in which *Muk* is inherited from the male are silenced suggests that *Muk* can silence *MuDR* when inherited from the male as well as the female parent. The poor correlation of *a1-mum2* spotting to *Mutator* activity when *Muk* is inherited from the male parent may be due to the dosage of *Muk* and *MuDR(p1)* in the triploid aleurone layer of the endosperm. When *Muk* is inherited from the female, the aleurone layer has the genotype *Muk/Muk/−; −/−/MuDR(p1)*, while when *Muk* is inherited from the male parent the aleurone genotype is *−/−/Muk; MuDR(p1)/MuDR(p1)/−*. Thus, it is likely that this imbalance in dosage of *Muk* and *MuDR* is responsible for the nonreciprocal *a1-mum2* kernel-spotting phenotypes.

***Muk* silencing of *Mutator* transposons is not dependent on number or position of *MuDR* elements:** To test if *Muk* silencing of *MuDR* is specific to *MuDR(p1)*, we crossed a *Mu*-tagging line containing an estimated eight active *MuDR* elements as a male to both an *a1-mum2* minimal *Mutator* line tester and a related plant heterozygous for *Muk* (Figure 4). Analysis of the transposon-tagging line showed that the *MuDR(p1)* element was not present in this line. For each cross 22 individual progeny were assayed by Southern blot and test crossed as female to the *a1-mum2* minimal *Mutator* line. From the control cross between the multiple-*MuDR* line and the *a1-mum2* tester, 19 of 22 (86.4%) individuals had active hypomethylated *Mu1* TIRs and produced heavily spotted kernels upon crossing to the *a1-mum2* minimal *Mutator* line tester (Table 6). The three individuals that were inactive all had at least one full-length *MuDR* element, based on Southern blots of DNA digested with *Sac*I and probed with an internal portion of *MuDR* (data not shown).

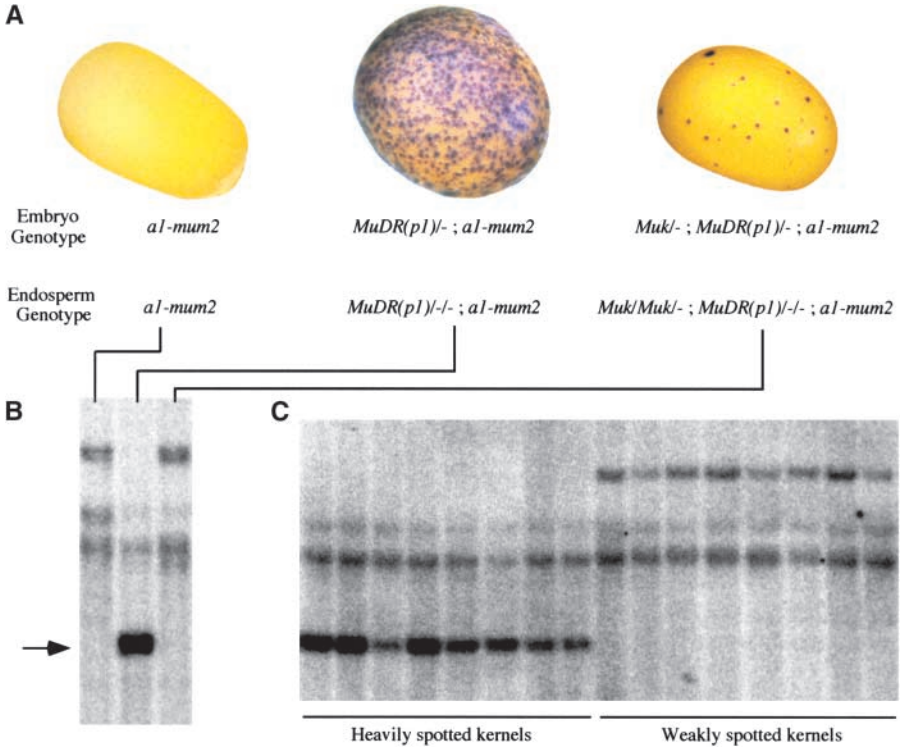

FIGURE 3.—*Mu killer* results in weakly spotted kernels and hypermethylated *Mu1* TIRs. (A) Nonspotted (left), heavily spotted (center), and weakly spotted (right) kernel phenotypes associated with the segregation of *MuDR(p1)* and *Muk* from the cross *Muk*/−; *a1-mum2* × *MuDR(p1)*/−; *a1-mum2*. All kernels are homozygous for *a1-mum2*. (B) *Hin*fI-digested Southern blots probed with *Mu1*. In the presence of an active *MuDR* element (heavily spotted kernel), *Mu1* is hypomethylated and produces a characteristic 1.3-kb band (arrow). In the absence of *MuDR* (nonspotted kernel) or with a *Muk*-silenced *MuDR* element, *Mu1* is hypermethylated and produces a *Hin*fI restriction fragment >1.3 kb. (C) Data from a typical *Muk*-segregating family generated from the cross *Muk*/−; *a1-mum2* × *MuDR(p1)*/−; *a1-mum2*. The heavily spotted kernels have an active *MuDR(p1)* element and no *Muk*, while the weakly spotted kernels have *MuDR(p1)* and *Muk*.

The 13.6% frequency of spontaneous silencing in this line is typical of a standard multiple-*MuDR Mutator* line (BENNETZEN 1996).

In the cross between the female *Muk* heterozygous plant and the same *Mutator* active individual, only 8 of 22 individuals had hypomethylated *Mu1* TIRs and produced heavily spotted kernels when crossed to the *a1-mum2* minimal *Mutator* line tester (Table 6). Fourteen of the 22 individuals had hypermethylated *Mu1* TIRs and produced only nonspotted kernels when crossed to the *a1-mum2* minimal *Mutator* line tester (Table 6). This 63.6% frequency of *Mutator* silencing is above the 50% expected for the segregation of *Muk*. However, if the 13.6% of spontaneous inactivation found in the cross of the transposon-tagging line to the *a1-mum2* minimal *Mutator* line tester is taken into account, the number of individuals silenced by *Muk* is approximately half.

The 1:1 segregation of silencing in the progeny of *Muk*/− crossed to an active transposon-tagging line without *MuDR(p1)* demonstrates that *Muk* can silence *MuDR* elements independent of their copy number or position in the genome.

***Muk*-induced hypermethylation of *Mutator* elements occurs gradually:** The *Muk*-induced weakly spotted kernel phenotype provides an excellent marker for de-

TABLE 4

Weakly spotted kernels are produced in genetic ratios only when *Muk* is inherited from the female parent

	Muk used as the female parent						*Muk* used as the male parent				
Cross[a]	Plant[b]	Heavily spotted kernels	Weakly spotted kernels	T[c]	Spotted kernels (%)	Cross[a]	Plant[b]	Heavily spotted kernels	Weakly spotted kernels	T[c]	Spotted kernels (%)
Muk/− × *MuDR(p1)*/−	1	33	30	122	51.6	*MuDR(p1)*/− × *Muk*/−	1	61	1	124	50.0
	2	45	39	164	51.2		2	20	0	37	54.0
	3	48	38	181	47.5		3	38	2	74	54.0
	4	36	34	136	51.8		4	18	0	37	48.6
	5	17	15	60	53.3		5	73	0	135	54.1

[a] All plants were homozygous for *a1-mum2*.
[b] Each number corresponds to a single *Muk*/− individual that was reciprocally crossed as a female (left) and a male (right).
[c] Total number of kernels examined.

TABLE 5

***Muk* silences *MuDR(p1)* when inherited from either the male or the female parent**

			Progeny when test crossed to *a1-mum2*[a]			
Muk parent[b]	Kernel phenotype	*Mu1* methylation status[c]	Heavily spotted kernels	Weakly spotted kernels	Total kernels	Spotted[d] kernels (%)
Female	Nonspotted	Hyper	0	0	132	0
Female	Nonspotted	Hyper	0	0	457	0
Female	Nonspotted	Hyper	0	0	168	0
Female	Nonspotted	Hyper	0	0	254	0
Female	Nonspotted	Hyper	0	0	78	0
Female	Nonspotted	Hyper	0	0	127	0
Female	Weakly spotted	Hyper	0	2	202	1.00*
Female	Weakly spotted	Hyper	0	22	263	8.37*
Female	Weakly spotted	Hyper	0	14	121	11.8*
Female	Weakly spotted	Hyper	0	2	60	3.33*
Female	Weakly spotted	Hyper	0	9	59	15.3*
Female	Weakly spotted	Hyper	0	8	61	13.1*
Female	Heavily spotted	Hypo	31	0	61	50.8
Female	Heavily spotted	Hypo	84	1	163	52.1
Female	Heavily spotted	Hypo	86	2	156	56.4
Female	Heavily spotted	Hypo	92	0	188	48.9
Female	Heavily spotted	Hypo	40	0	74	54.1
Male	Heavily spotted	Hyper	2	12	216	6.48*
Male	Heavily spotted	Hyper	0	7	68	10.3*
Male	Heavily spotted	Hyper	0	15	175	8.57*
Male	Heavily spotted	Hyper	0	7	44	15.9*
Male	Heavily spotted	Hyper	1	2	145	2.07*
Male	Heavily spotted	Hyper	1	1	60	3.33*
Male	Heavily spotted	Hyper	0	14	124	11.3*
Male	Heavily spotted	Hyper	0	5	150	3.33*
Male	Heavily spotted	Hyper	0	2	97	2.06*
Male	Heavily spotted	Hyper	0	8	51	15.7*
Male	Heavily spotted	Hyper	1	5	56	10.7*
Male	Heavily spotted	Hypo	174	0	328	53.0
Male	Heavily spotted	Hypo	50	0	90	55.6
Male	Heavily spotted	Hypo	132	1	275	45.4
Male	Heavily spotted	Hypo	37	0	67	55.2
Male	Heavily spotted	Hypo	107	0	202	53.0
Male	Heavily spotted	Hypo	55	5	112	53.6
Male	Heavily spotted	Hypo	71	0	130	54.6
Male	Heavily spotted	Hypo	10	2	18	66.7

[a] The *a1-mum2* minimal *Mutator* line tester was the male parent.

[b] From Table 4, plant 1.

[c] As judged by *Hin*fI-digested Southern blots probed with *Mu1*. Hyper, hypermethylated *Mu1* TIRs; Hypo, hypomethylated *Mu1* TIRs.

[d] * denotes ears with a significantly different χ^2 value at the 0.01 level for the expected segregation of a single *MuDR(p1)*.

termining which individuals have a silenced *MuDR* element. However, if the *MuDR* element was completely silenced by *Muk* in F_1 kernels, we would expect them to lack excisions altogether. To test how complete *MuDR* silencing is in individuals grown from weakly spotted kernels, we grew weakly spotted *Muk*/−; *MuDR(p1)*/−; *a1-mum2* F_1 kernels from the cross *Muk*/−; *a1-mum2* × *MuDR(p1)*/−; *a1-mum2*. DNA from 11 seedling L2 leaves, which are initiated early in embryogenesis, were digested with *Hin*fI and probed with the internal region of *Mu1*. In all cases the *Mu1* at *a1-mum2* in plants grown from weakly spotted kernels was only partly methylated—the digests produced both a hypomethylated 1.3-kb *Mu1* band and a larger hypermethylated *Mu1* band (Figure 5). Later in vegetative development, at leaf L6, we again isolated DNA from the same individuals, digested with *Hin*fI, and probed with *Mu1*. At this later developmental stage, the *Mu1* TIRs are completely methylated (Figure 5). The progressive inactivation of *Mutator* elements observed here is similar to that ob-

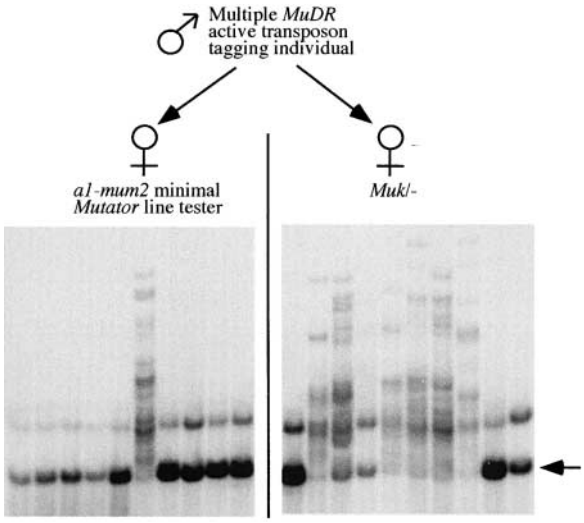

FIGURE 4.—*Muk* silences *MuDR* elements independent of their position or copy number. An active multiple *MuDR* transposon-tagging line individual was crossed as a male to both an *a1-mum2* minimal *Mutator* line tester and a *Muk* heterozygote. Progeny were scored by digesting with *Hin*fI and probing with the internal region of *Mu1*. The 1.3-kb fragment (arrow) is characteristic of hypomethylated *Mu1* TIRs and an active *Mutator* system. When crossed to the *a1-mum2* minimal *Mutator* line tester, 13.6% of progeny with at least one *MuDR* element were silenced, while when crossed to *Muk*/−, 63.6% were silenced.

served previously in more complex *Mutator* lines in which increasing portions of the plant tissue carried silenced *MuDR* elements as the plants developed (MARTIENSSEN and BARON 1994).

We also investigated the methylation status of *MuDR(p1)* TIRs in L6 leaves. Using the *Hin*fI restriction sites present in *MuDR* TIRs and in the flanking *p1* sequence, we were able to predict the sizes of hypomethylated as well as hypermethylated restriction fragments. The 497-bp TIR-hybridizing fragment in minimal *Mutator* line individuals with both *MuDR(p1)* and *Muk* demonstrates that the *Hin*fI restriction site in the *MuDR(p1)* TIR becomes methylated in *Muk* plants (Figure 6A). *Sac*I restriction sites in the *MuDR* TIR also become methylated in *Muk* plants, as seen in Figure 6B. In a line with multiple active *MuDR* elements present, the *Sac*I sites are not methylated and a 4684-bp band of all of the active *MuDR* elements is produced. When *Muk* is present in the same multiple *MuDR* line, the *Sac*I sites in the *MuDR* TIR are methylated and do not digest, producing various larger bands with size dependent on *MuDR* position. Due to the number of *MuDR*-hybridizing inactive background sequences present in all maize lines, the gradual methylation of the *MuDR* TIRs could not be assayed.

***MuDR* remains inactive multiple generations after silencing by *Mu killer*:** To determine the stability of the *Muk*-induced silenced state of *MuDR* in the absence of *Muk*, we crossed several *Muk*/− ; *MuDR(p1)*/− individuals grown from weakly spotted kernels as female to the *a1-mum2* minimal *Mutator* line tester over several generations (Table 7). The F_1 progeny (generation 1 in Table 7) yielded 9.0% weakly spotted kernels and 91.0% nonspotted kernels on 15 ears. The lack of heavily spotted kernels suggests that *MuDR* remains relatively inactive even when *Muk* is segregated away. Molecular analysis showed 46 out of the 46 individuals tested with *MuDR(p1)* had hypermethylated *Mu1* TIRs. Analysis of these 46 silenced individuals also showed no new *Mu1* insertions. Further analysis of *Muk*-silenced *MuDR* elements over three additional generations of crossing as female to the *a1-mum2* minimal *Mutator* line tester has shown that *MuDR(p1)* elements do not reactivate when segregated away from *Muk* (Table 7). Additionally, the decreasing trend in percentage of spotted kernels over the four generations (Table 7) suggests that *MuDR* may become more deeply silenced through time.

Over the four generations, TIRs from all *Mu* elements tested remained hypermethylated and no new *Mu1* insertions were observed. This analysis included the occasional heavily spotted kernels from Table 7. The heavily spotted kernels in these families were not heritably reactivated. Eleven such kernels were subjected to *Hin*fI digestion, and none of them carried hypomethylated *Mu1* elements. Of these, six were also test crossed, and all of them gave rise to mostly nonspotted kernels (~8% weakly spotted kernels and no heavily spotted kernels). Thus, we suggest that the occasional heavily spotted kernel represents variation in the efficiency of the maintenance of the silenced state, rather than escape from it. In contrast to lines that carry silenced *MuDR(p1)* elements, nonsilenced *MuDR(p1)* elements remain active from generation to generation using both the *a1-mum2* reporter (control generation in Table 7) and TIR methylation status (data not shown). These data demonstrate that although the dominant *Muk* is required to silence *MuDR* elements, it is not required to maintain *MuDR* in an epigenetically silenced state. We have also observed that no new *Mu1* insertions were generated in progeny of plants carrying silenced *MuDR* elements, suggesting that *Muk* also silences germinal *Mutator* activity.

***Mu killer* results in decreased *mudrA* transcript levels:** To test whether the presence of *Muk* results in the loss of *MuDR* transcript, total RNA from immature second ears was isolated from F_1 plants derived from the cross *Muk*/− ; *a1-mum2* × *MuDR(p1)*/− ; *a1-mum2*. Each plant was genotyped for the presence of *MuDR(p1)* and *Muk*, crossed as female to the *a1-mum2* minimal *Mutator* line, and RNA from immature ears was subjected to Northern blot and RT-PCR analysis. *mudrA* transcript from total RNA was detected only in an active sibling that did not inherit *Muk* (Figure 7A). *Muk*/−; *MuDR(p1)*/−; *a1-mum2* individuals grown from weakly spotted kernels exhibited undetectable transcript levels compared to siblings that did not inherit *Muk* (Figure 7A). No *mudrA*

TABLE 6

***Muk* silences *MuDR* elements independent of their position or number**

a1-mum2 minimal *Mutator* line tester[a]				*Muk/−; a1-mum2*[b]			
Plant	*Mu1* methylation status[c]	*T*[d]	F_2 spotted kernels (%)	Plant	*Mu1* TIR methylation status[c]	*T*[d]	F_2 spotted kernels (%)
1	Hypo	106	100	1	Hypo	263	100
2	Hypo	224	100	2	Hypo	55	100
3	Hypo	57	100	3	Hypo	141	100
4	Hypo	182	100	4	Hypo	167	100
5	Hypo	175	100	5	Hypo	186	100
6	Hypo	197	100	6	Hypo	145	100
7	Hypo	220	100	7	Hypo	179	100
8	Hypo	281	100	8	Hypo	30	100
9	Hypo	174	100	9	Hyper	202	0
10	Hypo	203	100	10	Hyper	213	0
11	Hypo	182	100	11	Hyper	370	0
12	Hypo	272	100	12	Hyper	110	0
13	Hypo	250	100	13	Hyper	265	0
14	Hypo	139	100	14	Hyper	223	0
15	Hypo	255	100	15	Hyper	264	0
16	Hypo	259	100	16	Hyper	94	0
17	Hypo	172	100	17	Hyper	113	0
18	Hypo	208	100	18	Hyper	224	0
19	Hypo	65	100	19	Hyper	199	0
20	Hyper	175	1	20	Hyper	241	0
21	Hyper	70	0	21	Hyper	109	0
22	Hyper	213	0	22	Hyper	281	0

[a] Generated by crossing the *a1-mum2* minimal *Mutator* line tester by a multiple *MuDR*-tagging line individual and then crossing the F_1 progeny as female to the *a1-mum2* minimal *Mutator* line tester.

[b] Generated by crossing *Muk/−* ; *a1-mum2* by the same multiple-*MuDR*-tagging line individual and then crossing the F_1 progeny as female to the *a1-mum2* minimal *Mutator* line tester.

[c] *Mu1* TIR methylation status as judged by Southern blot using *Hin*fI digestion and *Mu1* probe. Hyper, hypermethylated *Mu1* TIRs; Hypo, hypomethylated *Mu1* TIRs.

[d] Total number of informative *a1-mum2/a1-mum2* kernels on the test-crossed ear.

transcript was detected in plants that lacked *MuDR(p1)* independent of the presence or absence of *Muk*. Probes detecting both the 5′ (data not shown) and 3′ (Figure 7A) ends of the *mudrA* transcript provided the same results.

Poly(A) transcript was extracted from the same total RNA samples. Northern analysis of the poly(A) RNA provided similar results as obtained using total RNA (data not shown). RT-PCR analysis of total RNA reverse transcribed using an oligo(dT) primer and amplified for 29 cycles provided identical results (Figure 7B). Only an active sibling from the family segregating *Muk* that did not inherit *Muk* provided detectable levels of polyadenylated *mudrA*. Similar results have been obtained with 12 different *MuDR(p1)/−* active sibling individuals, 22 *Muk/−* ; *MuDR(p1)/−* ; *a1-mum2* individuals grown from weakly spotted kernels, and 10 individuals without *MuDR(p1)*.

***Mu killer* results in decreased *mudrB* poly(A) RNA levels:** The same total RNA samples used in the *mudrA* expression analysis were used for expression analysis of the *mudrB* transcript. Total RNA and poly(A) RNA Northern blots were probed with the 5′ and 3′ regions of the *mudrB* gene. RT-PCR amplified for 20 cycles was also performed on cDNA primed with either a *mudrB*-specific primer (*B*1020r, see Figure 1) or an oligo(dT) primer. RT-PCR products were blotted to nylon and probed with the 5′ *mudrB* probe (see Figure 1).

Surprisingly, *mudrB* expression was still observed on Northerns using total RNA from *Muk/−* ; *MuDR(p1)/−* heterozygotes grown from weakly spotted kernels when hybridized with either the 5′ (data not shown) or the 3′ *mudrB* probe (Figure 8A). The *mudrA* transcript was absent in these same individuals. *mudrB* transcript of the correct size was present in all 21 *Muk/−*; *MuDR (p1)/−*; *a1-mum2* F_1 individuals tested. A total of 11 *MuDR(p1)/−* active siblings with no *Muk* also had *mudrB* transcript, while 10 siblings without *MuDR* were tested. In contrast, Northern analysis did not detect poly(A) *mudrB* transcript from the 21 *Muk/−*; *MuDR(p1)/−*; *a1-mum2* samples examined (Figure 8B). To verify these findings, RT-PCR was performed on the same RNA samples (Figure 8C). As with the Northern analysis, RT-PCR resulted in amplification of the *mudrB* transcript

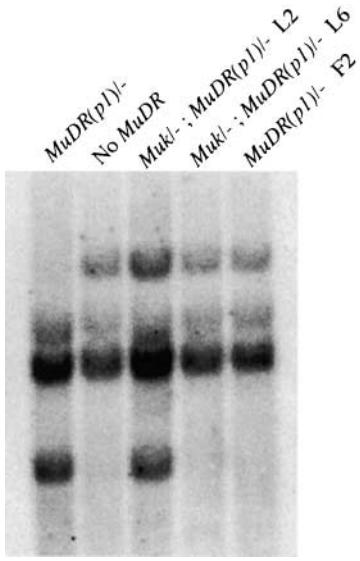

FIGURE 5.—*Mu* elements in *Muk/−*; *MuDR(p1)/−*; *a1-mum2* individuals grown from weakly spotted kernels become increasingly methylated through somatic development. Southern blots of DNA digested with *Hin*fI and probed with the internal region of *Mu1* show that L2 embryonic leaves are partially methylated, while the L6 leaf of the same individual has completely methylated *Mu1* TIRs. When crossed as female to the minimal *Mutator* line tester, progeny from the same *Muk/−*; *MuDR(p1)/−* individual with *MuDR(p1)* and without *Muk* are fully methylated (F_2). All plants are homozygous for the *a1-mum2* allele.

only in *Muk/−*; *MuDR(p1)/−*; *a1-mum2* samples when the cDNA was primed with a *mudrB*-specific primer and not when primed with an oligo(dT) primer. These data suggest that in *Muk/−*; *MuDR(p1)/−* heterozygotes, *mudrB* is still transcribed but not correctly processed into mature mRNA.

To determine if *mudrB* continues to be expressed in the total RNA fraction in the next generation, a *Muk/−*; *MuDR(p1)/−* heterozygote was crossed as female to the *a1-mum2* minimal *Mutator* line tester. *Muk* in the progeny was scored by crossing the plants as female to an active *MuDR(p1)/−* ; *a1-mum2* individual and assaying for the presence of *Muk*-induced silencing of the active *MuDR(p1)* element. Progeny with *MuDR(p1)* and no *Muk* one generation after initial silencing by *Muk* show no *mudrB* transcript in the total RNA fraction (Figure 8D). A total of 10 individuals with *MuDR(p1)* and without *Muk* were tested and *mudrB* transcript was undetectable in all of them.

Together, these data suggest that although *mudrA* total RNA transcript levels correlate with *Mu* TIR methylation and *Mutator* activity in weakly spotted *Muk/−*; *MuDR(p1)/−* F_1 heterozygotes, *mudrB* is silenced either by an alternative mechanism or at a different time than *mudrA*.

Small ~26-nt RNAs are found in plants with *MuDR* and *Muk*: To test if RNA-based post-transcriptional gene silencing of *MuDR* was occurring in *Muk* plants, small RNA Northern blots were used. RNA from the second leaf of seedling plants in a family segregating for *Muk* and *MuDR* were examined for the presence of small RNA molecules. These plants were generated from the cross *Muk/−*; *a1-mum2* × *MuDR(p1)/−*; *a1-mum2*. A species of small RNA of ~26 nt is present in only F_1 individuals with both *MuDR(p1)* and *Muk* (Figure 9). This small RNA species hybridizes with both sense- and antisense-transcribed RNA probes complementary to the 5′ region of the *mudrA* transcript. This result has been tested on a total of 14 *MuDR(p1)/−*; *Muk/−*; *a1-mum2* individuals; 10 *MuDR(p1)/−*; *a1-mum2* siblings without *Muk*; and 10 each of control *MuDR(p1)/−*;

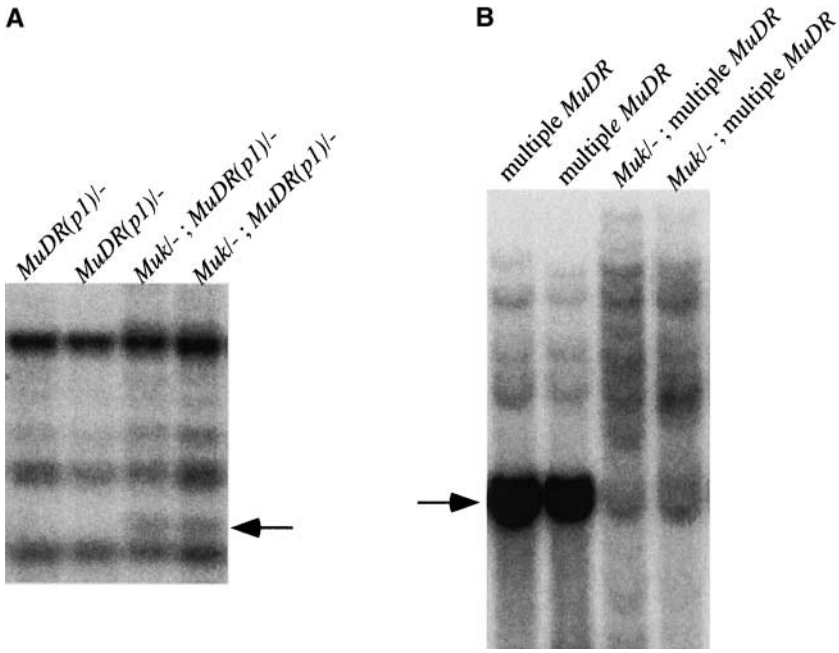

FIGURE 6.—*MuDR* element TIRs become methylated when *Muk* is present. (A) *MuDR(p1)* in the minimal *Mutator* line has the methylated *Hin*fI restriction product (arrow) only when *Muk* is present. *Hin*fI sites are present in the the *p1* flanking DNA, *MuDR* TIR, and *mudrB* portion of *MuDR* (see Figure 1). The center *Hin*fI restriction site in the TIR is the site that becomes methylated. The methylated band that hybridizes to the TIR probe is 497 bp. (B) *MuDR* elements from a multiple-*MuDR* line have methylated TIRs only when *Muk* is present. *Sac*I sites are found in each of the *MuDR* TIRs (see Figure 1). When not methylated and probed with an internal region of *MuDR*, a 4684-bp band (arrow) is produced. When the *Sac*I TIR sites are methylated, larger bands are generated.

TABLE 7

***MuDR(p1)* elements inactivated by *Muk* remain silenced for multiple generations**

Generation	Heavily spotted kernels	Weakly spotted kernels	Nonspotted kernels	No. of crosses	Spotted kernels (%)
Control[a]	5202	96	4508	28	54.1
1[b]	0	148	1490	15	9.0
2[c]	3	35	353	5	9.7
3[d]	6	37	1475	5	2.8
4[e]	2	46	3695	35	1.3

[a] Spotted F_1 seed from the cross *a1-mum2* × *MuDR(p1)/−* ; *a1-mum2* crossed as female to the *a1-mum2* minimal *Mutator* line tester.

[b] Weakly spotted F_1 seed from the cross *Muk/−* ; *a1-mum2* × *MuDR(p1)/−* ; *a1-mum2* crossed as female to the *a1-mum2* minimal *Mutator* line tester.

[c] Weakly spotted seed from generation 1 crossed as female to the *a1-mum2* minimal *Mutator* line tester.

[d] Weakly spotted seed from generation 2 crossed as female to the *a1-mum2* minimal *Mutator* line tester.

[e] Weakly spotted seed from generation 3 crossed as female to the *a1-mum2* minimal *Mutator* line tester.

a1-mum2 individuals, control *Muk/−* ; *a1-mum2* individuals, and control *a1-mum2* individuals without *MuDR(p1)* or *Muk*. The only individuals that show the small RNA band of ~26 nt are the plants with both *MuDR(p1)* and *Muk*. Small RNAs homologous to the rest of *MuDR* were not found (data not shown).

Directed attempts to identify *Mu killer*: Deletion derivatives of transposons in Drosophila and maize have been implicated in repressing the activity of their cognate full-length elements (Cuypers *et al.* 1988; Lee *et al.* 1998). Antisense *MuDR* RNA has been detected in both minimal and complex *Mutator* lines, which in at least one case is due to read-through of a *MuDR* deletion derivative (Lisch *et al.* 1999). This antisense RNA could conceivably trigger RNA-mediated *Mutator* inactivation. In addition to deletion derivatives present in most *Mutator* lines, all maize lines contain multiple inactive *MuDR*-homologous sequences (*hMuDR*s; reviewed in Walbot and Rudenko 2002). It is possible that one of these inactive background elements expresses an aberrant transcript that can cause *Mutator* silencing.

To explore these possibilities we attempted to locate a *MuDR*-homologous sequence cosegregating with *Mutator* silencing by Southern blot. Ten methylation-insensitive restriction enzymes and five probes that cover the entire *MuDR* element (including the TIRs) have been used without detecting cosegregation between *Muk* and any *MuDR*-related sequence (data not shown). Special care was taken on these Southern blots to ensure that no small hybridizing fragment that cosegregates with *Muk* was missed. Further, total RNA and poly(A) Northern analysis of >35 individuals also suggests that no detectable aberrant *MuDR* homologous transcript is associated with *Muk*. Although it remains a formal possibility that *Mu killer* is a *MuDR*-homologous element, to have escaped detection, this element would have to be significantly diverged from functional *MuDR* elements.

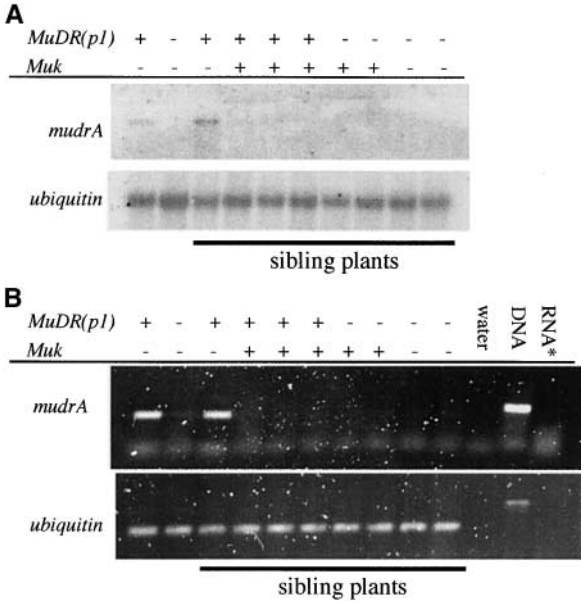

Figure 7.—Expression analysis of the *mudrA* transcript in a family segregating *MuDR(p1)* and *Muk* from the cross *Muk/−* × *MuDR(p1)/−*. Both total RNA Northerns (A) and poly(A) RNA Northerns (not shown) provide similar results. In both, the *mudrA* transcript does not accumulate in individuals without *MuDR* or with *Muk*. The *mudrA* probe used hybridizes to the 3′ end of *mudrA*, as shown in Figure 1. (B) DNase I-treated RNA reverse transcribed with an oligo(dT) primer and amplified using *mudrA* exon primers for 29 cycles provides similar results as with Northern analysis. The polyadenylated *mudrA* transcript is not present in individuals with both *MuDR(p1)* and *Muk*. RT-PCR controls include a water sample without nucleic acid, a DNA sample that is larger due to the presence of an intron, and a sample of DNase I-treated RNA that was not reverse transcribed (RNA*). *mudrA*-specific primers used in RT-PCR are shown in Figure 1. In both A and B, + denotes the presence of hemizygous *MuDR(p1)* or heterozygous *Muk*, while − denotes the absence of *MuDR(p1)* or *Muk*.

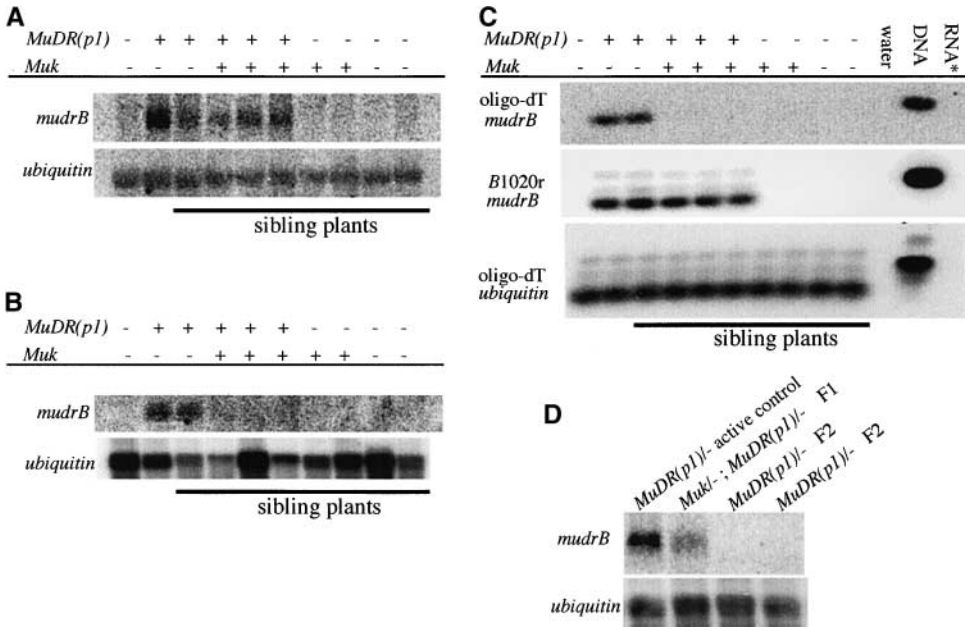

FIGURE 8.—Expression analysis of the *mudrB* transcript in a family segregating *MuDR(p1)* and *Muk* from the cross *Muk*/− × *MuDR(p1)*/− (A–C), as well as one generation after initial silencing by *Muk* (D). Unlike *mudrA* expression, total RNA Northerns (A) show *mudrB* still accumulates in *Muk*/−; *MuDR(p1)*/− F_1 individuals. Poly(A) RNA Northerns (B) do not show *mudrB* accumulation in the same *Muk*/−; *MuDR(p1)*/− samples. The 3′ *mudrB* probe (see Figure 1) was used in A and B, while the 5′ *mudrB* probe produced similar results (data not shown). (C) DNase I-treated RNA was reverse transcribed with either a *mudrB*-specific primer (*B*1020r) or an oligo(dT) primer and amplified using *mudrB* exon primers for 20 cycles, then blotted, and probed with the 5′ *mudrB* probe (see Figure 1). RT-PCR analysis provides similar results as with Northern analysis. The polyadenylated *mudrB* transcript is not present in individuals with both *Muk* and *MuDR(p1)*; however, nonpolyadenylated transcript does accumulate. Although *mudrB* is still transcribed in F_1-silencing plants, we assume no functional protein is produced because of the lack of poly(A) *mudrB* transcript. RT-PCR controls include a water sample without nucleic acid, a DNA sample that is larger due to the presence of an intron, and a sample of DNase I-treated RNA that was not reverse transcribed (RNA*). *mudrB*-specific primers used in RT-PCR are shown in Figure 1. To determine if *mudrB* continues to accumulate in the total RNA fraction one generation after initial silencing by *Muk*, we crossed *Muk*/−; *MuDR(p1)*/− individuals from A–C to the *a1-mum2* minimal *Mutator* line and examined progeny by total RNA Northern blot (D). One generation after initial silencing (F_2), nonpolyadenylated *mudrB* transcript does not accumulate. In A–C, + denotes the presence of hemizygous *MuDR(p1)* or heterozygous *Muk*, while − denotes the absence of *MuDR(p1)* or *Muk*.

DISCUSSION

Transposable elements are present in most eukaryotic genomes in multiple copies. Generally, only a subset of the elements present is competent to catalyze their own transposition, and it is likely that even these autonomous elements vary in competence depending on their regional chromatin context. In cases where transposons have been reactivated as a result of mutations in genes responsible for silencing, the transposons examined have been treated as a relatively uniform population (reviewed in OKAMOTO 2001). However, it is likely that only a subset of any given family of transposons is reactivated. In the case of *ddm-1* reactivation, for instance, it is clear that only some MULE elements (those located in heterochromatin) were reactivated, suggesting that the position of the elements plays a role in the nature of their silenced state (MIURA *et al.* 2001; SINGER *et al.* 2001). Further, because the autonomous element in these systems has not been identified, it has not been possible to determine which specific autonomous transposon in a given genome has actually been reactivated.

In addition to the heterogeneity of transposon populations, due to the nature of the screens used, only those genes necessary for continued maintenance of transposon silencing have been identified. Little is known about those factors that can initiate *de novo* silencing. Although many of the mechanisms involved in maintenance of silencing are almost certainly involved in its initiation, additional factors are involved. For instance, although it is clear from work in a variety of systems (particularly those involving transgenes) that double-stranded RNA can trigger silencing, it is likely that the double-stranded RNA trigger is not sufficient to initiate silencing in all cases (TIJSTERMAN *et al.* 2002). This is almost certainly true in *Mutator* silencing. Since *mudrA* and *mudrB* are transcribed convergently from opposite strands, some read-through transcription can and does occur in *Mutator*-active plants that do not exhibit transposon silencing (HERSHBERGER *et al.* 1995; RUDENKO and WALBOT 2001).

Ideally, analysis of initiation of transposon silencing should utilize a single transposon at a known chromosomal position that can be reproducibly and reversibly inactivated. In this respect, the minimal *Mutator* line presents a unique opportunity to examine the process by which transposons become inactivated. Because this line contains a single active autonomous transposon at a known position, it is possible to examine changes in chromatin configuration, transcription, and transpositional activity simultaneously during the process of silencing.

We have demonstrated that the dominant *Muk* locus

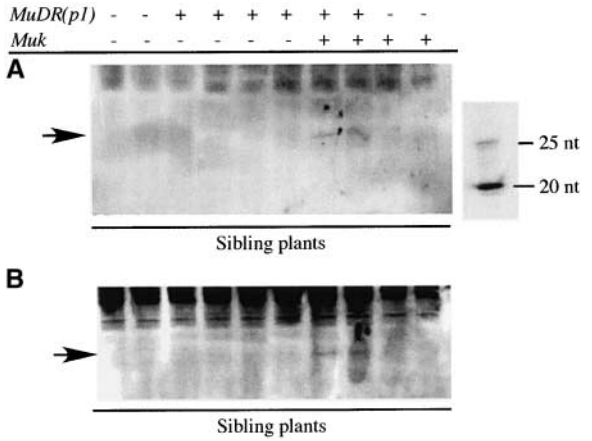

FIGURE 9.—Northern blot analysis of total leaf 2 RNA probed with the antisense strand of the 5′ region of *mudrA*. Small ~26-nt RNAs are present only in plants with both *MuDR(p1)* and *Muk*. The ~26-nt RNA band (arrow) hybridizes to both sense (data not shown) and antisense probes. Larger hybridizing bands are products of *hMuDR* elements and do not contribute to *Mutator* activity. Two different Northerns with different individuals are shown in A and B. The size range of products shown in A is ~18–45 nt. The size of the band specific to *Muk*/−; *MuDR(p1)*/− individuals was estimated using known single-stranded RNA molecules of 20 and 25 nt that hybridize to the 3′ end of *mudrA*. The size standard also served as a positive hybridization control. Northerns showing the ~26-nt RNAs were stripped of radioactivity and reprobed with the antisense transcript of the 3′ region of *mudrA* to hybridize the size standards. +, presence of hemizygous *MuDR(p1)* or heterozygous *Muk*; −, absence of *MuDR(p1)* or *Muk*.

is competent to silence multiple *MuDR* elements independent of their position. Silencing by *Muk* is initiated regardless of the gender of the parent from which *Muk* is inherited. However, the weakly spotted kernel phenotype associated with *Muk*-induced silencing of the *Mutator* system is apparent only when *Muk* is inherited from the maternal parent, presumably due to dosage effects in the triploid endosperm. Whether *Muk* acts differently in the embryo (and not the endosperm) when inherited from the male or female parent remains to be investigated.

The *Muk* silencing of active *MuDR(p1)* elements is not dependent on the presence of a previously silenced *MuDR* element. The silencing appears to be progressive during plant development and is complete by the production of the mature sixth leaf. As with *Mu1* TIRs, the TIRs of *MuDR* become methylated when silenced by *Muk*. Importantly, the stable inactive state of a *Muk*-silenced *MuDR(p1)* element can be propagated for multiple generations in the absence of *Muk*, suggesting that *Muk* is not required for the maintenance of the silenced state. Finally, the decreasing proportions of weakly spotted kernels in subsequent generations suggest that a *Muk*-silenced *MuDR* element that has segregated away from *Muk* may become gradually more inactive over several generations.

MuDR silencing by *Muk* is associated with the loss of polyadenylated *mudrA* and *mudrB* transcript as well as the transient presence of nonpolyadenylated *mudrB* transcript. The observed differences in the total RNA *mudrA* and *mudrB* transcript levels suggest differential regulation of these two genes. The *mudrA* gene is the putative transposase, and analysis of deletion derivatives has revealed that the loss of *mudrA* gene product is sufficient to result in *Mu* element methylation (LISCH *et al.* 1999). Thus, it is tempting to suggest that *Muk* acts directly on *mudrA* and that *mudrB* is then lost because it requires *mudrA* for continued expression. However, deletions that remove *mudrA* do not result in the loss of *mudrB* transcript or protein (LISCH *et al.* 1999), suggesting that the loss of *mudrB* transcript is not simply due to the loss of *mudrA*. The loss of polyadenylated *mudrB* transcript, followed in the next generation by loss of the remaining nonpolyadenylated *mudrB* transcript, suggests that *Muk* also affects *mudrB*, possibly later or by an alternate mechanism than it affects *mudrA*. Previous studies on the spontaneous inactivation of *MuDR* have found that the subcellular location of *mudrB* is altered in *Mutator*-active *vs.* -inactive plants (RUDENKO *et al.* 2003). Rudenko and co-workers found a higher proportion of nuclear-retained *mudrB* transcript in *Mutator*-silencing plants than in *Mutator*-active plants. In general, the majority of polyadenylated transcript is present in the cytoplasm, while nonpolyadenylated transcript is located in the nucleus (HUANG and CARMICHAEL 1996). Thus, our finding that nearly all of the *mudrB* transcript in *Muk*/−; *MuDR(p1)*/− F_1 plants is nonpolyadenylated is consistent with previous results in *Mutator*-silencing plants (RUDENKO *et al.* 2003).

By the second generation after silencing by *Muk*, nonpolyadenylated *mudrB* is no longer present. Thus, it appears that the continued expression of nonpolyadenylated *mudrB* is associated with the initiation, but not the maintenance of silencing. It is not clear whether the presence of nonpolyadenylated and potentially nuclear-localized *mudrB* is a cause or an effect of silencing. Previous workers have suggested a role for increased retention of nuclear-localized transcript in the process of silencing (RUDENKO *et al.* 2003). In those experiments, although the percentage of nuclear *mudrB* transcript increased, this was largely due to the loss of polyadenylated transcript; the total amount of *mudrB* transcript in the nucleus remained relatively constant. Similarly, we observe a dramatic change in the proportion of polyadenylated to nonpolyadenylated *mudrB* transcript. However, that change is due primarily to the loss of polyadenylated *mudrB*, not an increase in nonpolyadenylated *mudrB*. Thus, in each of these experiments, increased nuclear retention *per se* is unlikely to be the cause of silencing, since it was not a variable associated with the process of silencing. One scenario

to explain the available data is that silencing triggers the loss of cytoplasmic polyadenylated RNA (first from *mudrA* and then from *mudrB*) and those changes in turn lead to transcriptional inactivation of first *mudrA* and then *mudrB*. By the next generation, transcriptional repression of both *mudrA* and *mudrB* has been achieved. Nuclear run-on experiments will be used to address these issues directly.

Most intriguingly, at least the initial stages of *MuDR* silencing are associated with the production of small, ~26-nt RNA molecules that are homologous to both strands of the 5′ end of the *mudrA* gene. Since the ~26-nt RNA is found only when probed with the 5′ region of *mudrA*, this further suggests that *Muk* affects *mudrA* before or differently than *mudrB*. Approximately 26-nt RNAs are part of a larger family of newly identified small RNAs that may have properties different from those of the ~21-nt siRNAs known to be associated with RNA degradation (Hamilton *et al.* 2002). Hamilton *et al.* (2002) found that in plants the smaller ~21-nt siRNAs are involved in the degradation of the target mRNA, but the presence of longer ~26-nt RNA correlates with systemic silencing and methylation of homologous DNA. Since *Mu* TIR methylation appears to be initiated early in development in tissues where ~26-nt RNAs are observed, we suggest that these longer small RNAs may be the trigger for *MuDR* element methylation.

Despite efforts to locate a *MuDR*-homologous sequence associated with *Muk*, none has been found. Also, regardless of its effects on *Mu* methylation, *Muk* is not linked to *mop1* or either of the two maize DDM1 homologs, CHR101 or CHR106 (data not shown). Further, *Muk* does not affect the methylation status of the maize centromeric or ribosomal RNA repeats (data not shown), suggesting that *Muk* is not a global chromatin-remodeling gene such as DDM1.

Future experiments will focus on *Mu killer*'s effects on transcriptional activity of *mudrA* and *mudrB*, small RNA production, and DNA methylation at various stages of development. Additionally, we will investigate chromatin compaction, histone methylation, and acetylation of *MuDR(p1)* when it is being silenced by *Muk*. We are particularly interested in embryonic tissues in which the process of silencing may be initiating. Because we can separate initiation from maintenance of *MuDR* silencing, it will also be interesting to examine each of these variables in plants that carry a silenced *MuDR* element but that lack *Muk*. The availability of *Muk* and the single-copy minimal *Mutator* line should make it possible to carefully dissect a number of aspects of transposon silencing.

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