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The *mop1* (*mediator of paramutation1*) Mutant Progressively Reactivates One of the Two Genes Encoded by the *MuDR* Transposon in Maize

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ABSTRACT

Transposons make up a sizable portion of most genomes, and most organisms have evolved mechanisms to silence them. In maize, silencing of the *Mutator* family of transposons is associated with methylation of the terminal inverted repeats (TIRs) surrounding the autonomous element and loss of *mudrA* expression (the transposase) as well as *mudrB* (a gene involved in insertional activity). We have previously reported that a mutation that suppresses paramutation in maize, *mop1*, also hypomethylates *Mu1* elements and restores somatic activity to silenced *MuDR* elements. Here, we describe the progressive reactivation of silenced *mudrA* after several generations in a *mop1* background. In *mop1* mutants, the TIRA becomes hypomethylated immediately, but *mudrA* expression and significant somatic reactivation is not observed until silenced *MuDR* has been exposed to *mop1* for several generations. In subsequent generations, individuals that are heterozygous or wild type for the *Mop1* allele continue to exhibit hypomethylation at *Mu1* and *mudrA* TIRs as well as somatic activity and high levels of *mudrA* expression. Thus, *mudrA* silencing can be progressively and heritably reversed. Conversely, *mudrB* expression is never restored, its TIR remains methylated, and new insertions of *Mu* elements are not observed. These data suggest that *mudrA* and *mudrB* silencing may be maintained via distinct mechanisms.

TRANSPOSABLE elements make up at least half of the maize genome (SanMiguel *et al.* 1996). Given the mutagenic potential of transposons and their ubiquity in plant and animal genomes, it is not surprising that most transposable elements remain quiescent most of the time. Transposon activity is held in check by a well-conserved set of mechanisms that include both post-transcriptional and transcriptional components (Zilberman and Henikoff 2004). Mutants have been identified that reactivate quiescent transposons in a variety of species (Lippman *et al.* 2003) (reviewed in Okamoto and Hirochika 2001). In plants, loss of DNA methylation is often associated with transposon reactivation (Kato *et al.* 2003). Arabidopsis MULE (*Mutator*-like element) class II DNA transposons are reactivated in several recessive homozygous mutants, including *DDM1* (*DECREASE IN DNA METHYLATION1*) (Singer *et al.* 2001) and *MET1* (*METHYLTRANSFERASE1*) (Kato *et al.* 2003). Reactivation of Arabidopsis MULEs in a *ddm1* mutant background is associated with hypomethylation of terminal inverted repeats (TIRs), expression of the putative transposase, and new insertions (Singer *et al.* 2001). Hypomethylation is heritable in that it persists even in *ddm1*/+ progeny outcrossed from a *ddm1/ddm1* parent (Lippman *et al.* 2003). *ddm1* mutants also cause reactivation and mobilization of CACTA transposable elements, and, like MULE reactivation, this reactivation is heritable (Kato *et al.* 2004).

MET1 is a DNA methyltransferase that is also involved in maintaining global CG methylation (Bartee *et al.* 2001; Kankel *et al.* 2003). In *met1* mutants, MULEs become hypomethylated, and the transposase is expressed (Lippman *et al.* 2003). CACTA elements in Arabidopsis are strongly reactivated in lines double mutant for *MET1* and the DNA methyltransferase gene *CMT3* (*CHROMOMETHYLASE3*), which is involved in maintaining non-CG methylation.

In addition to chromatin-remodeling factors, genes involved in RNAi have been implicated in transposon silencing, both in animals (Aravin *et al.* 2001; Vastenhouw *et al.* 2003) and in plants (Lippman and Martienssen 2004). Indeed, the initiation and maintenance of transposon silencing almost certainly involves a complex interaction between RNAi and chromatin modification.

Arabidopsis is an excellent system in which to study the biochemistry and molecular biology of transposon reactivation in plants. The Arabidopsis genome is fully sequenced, and there are a number of mutants that affect transposon activity. However, the transposon systems in Arabidopsis are poorly characterized; the autonomous elements have not been generally identified, and the means by which the transposons were silenced in the first place is not known. In this respect, maize is unique

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in that it contains several DNA transposon systems that are highly active and that have been extensively characterized.

The *Mutator* family of transposons is the founding member of the MULE class of elements that are present in plants, fungi, and bacteria (reviewed by (Lisch 2002). All *Mutator* transposons are characterized by flanking 220-bp TIRs. The *Mutator* family consists of the autonomous element *MuDR* and at least nine classes of nonautonomous elements (Bennetzen 1996). *MuDR* carries two genes, *mudrA*, which encodes the transposase, and *mudrB*, a helper gene that is required for transposon integration (Lisch *et al.* 1995; Raizada and Walbot 2000). The *mudrA* gene is found in all autonomous MULEs in all species that carry MULEs; the *mudrB* gene is found only in the genus Zea (Lisch 2002). The *MuDR* TIRs contain the promoters for both *mudrA* and *mudrB* (Raizada *et al.* 2001). Methylation of sequences within the TIRs of all elements is correlated with *Mutator* silencing (Chomet *et al.* 1987, 1991; Martienssen and Baron 1994). A typical maize *Mutator* active line carries several active *MuDR* elements as well as up to several hundred nonautonomous elements, and duplication frequencies of these elements can average 100% (Alleman and Freeling 1986).

The large number of active autonomous and nonautonomous elements in the typical *Mutator* line makes it difficult to perform genetic analysis on the *Mutator* system. Therefore, we have developed a minimal *Mutator* line containing a single functional autonomous element, *MuDR(p1)*, at position one (p1) on chromosome 2L, and a single nonautonomous element, *Mu1*, in the *A1* color reporter gene (*a1-mum2*) (Chomet *et al.* 1991) (Lisch *et al.* 1995). This minimal line provides a simple system in which to easily track the excision and duplication events of a single *Mutator* element. Excision of *Mu1* from *a1-mum2* results in pigmented spots in the kernel aleurone, which is a direct reflection of transposase activity, as is hypomethylation of *Mu1* termini. Also, unlike typical *Mu* active lines, the minimal line does not exhibit spontaneous silencing, which would interfere with genetic analysis of factors influencing epigenetic regulation of the *Mutator* system.

We have identified and cloned a single dominant gene, *Mu killer* (*Muk*), which heritably and reliably silences the *Mutator* system via a double-stranded RNA mechanism that targets the 5′ region of *mudrA* (Slotkin *et al.* 2005). *Muk* activity results in silencing of *mudrA* in the first generation and silencing of *mudrB* by the next generation. *Muk* is a variant of *MuDR* that carries an inverted and duplicated portion of the transposon that includes TIRA and a portion of the *mudrA* gene, but that lacks *mudrB* or TIRB. In the presence of *Muk*, all *Mu* TIRs become methylated, and there is loss of somatic and germinal activity (Slotkin *et al.* 2003). Once silenced by *Muk*, *Mutator* elements do not become spontaneously reactivated. Thus, *Muk* can be used as a tool to reliably and heritably silence *MuDR* elements to study the dynamics of epigenetic *Mutator* regulation. This, along with the convenience of the minimal *Mutator* line, makes the maize *Mutator* system particularly useful for genetic analysis of transposon silencing and reactivation.

Previous work has demonstrated that a mutant that affects paramutation in maize, *mediator of paramutation1* (*mop1*) (Dorweiler *et al.* 2000), also reverses *Mutator* methylation and silencing (Lisch *et al.* 2002). Paramutation is the phenomenon whereby a silenced allele of a particular gene silences an otherwise active allele in *trans* (Brink *et al.* 1968). The *mop1* mutation prevents establishment of paramutation of the paramutable alleles of *b1*, *r1*, and *pl1* and increases RNA expression of the paramutagenic *b1* and *pl1* alleles (Chandler and Stam 2004). The *mop1* mutation also reverses *Mu1* TIR methylation in *Mutator* lines silenced by *Muk* as well as restoring low levels of somatic activity that was lost due to *Muk* silencing of *MuDR* (Lisch *et al.* 2002). While *mop1* affects both paramutation and *Mutator* TIR methylation, unlike *DDM1* or *MET1* in Arabidopsis, it neither affects global methylation in maize (Dorweiler *et al.* 2000) nor affects methylation of some other transposable elements, such as those just upstream of the paramutagenic allele of the *b1* locus (Lisch *et al.* 2002).

In a previous study (Lisch *et al.* 2002) we reported that *mop1* hypomethylates *Mu1* TIRs and facilitates somatic activity of silenced *MuDR* elements. Here, we describe in detail the reactivation of silenced *MuDR* elements after they have been exposed to the *mop1* mutation for multiple generations. We find that the TIR adjacent to *mudrA*, like *Mu1* TIRs, is subject to demethylation in a *mop1* background. We find that there is a parent-of-origin effect on the maintenance of the reactivated state and that levels of somatic activity become progressively higher in each generation in a *mop1* background, with excision frequencies approaching that observed in lines carrying active *MuDR* elements. We also find that after several generations in a *mop1* background, *MuDR* elements become heritably active even in the absence of the *mop1* mutation. Surprisingly, despite high levels of somatic activity, we do not find evidence for new insertions, and we also find that, while *mudrA* expression is fully restored, *mudrB* remains silenced, consistent with a previously defined role for *mudrB* in insertional activity (Lisch *et al.* 1999). Finally, while TIRA becomes hypomethylated in a *mop1* background, TIRB remains methylated. These data suggest that although both *mudrA* and *mudrB* are silenced by *Muk*, there are differences in the means by which that silencing is maintained. The progressive reactivation of silenced *mudrA* (but not *mudrB)* in a *mop1* background affirms that changes in the epigenetic state can be gradual and cumulative and supports the idea that, over time, the memory of the heterochromatic state can be lost in this mutant background.

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 and one nonautonomous *Mutator* element, the *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-mum2* late in somatic development, creating characteristic *Mutator* spotting in the kernel. When *MuDR* activity is absent the kernel is uniformly pale. 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 element does not become spontaneously epigenetically silenced in the minimal *Mutator* line. When *MuDR(p1)* is absent, the minimal line is referred to as the *a1-mum2* tester.

Although other *MuDR*-homologous sequences (*hMuDR* elements) are present in this (and all) maize background (Chomet *et al.* 1991; Rudenko and Walbot 2001), these sequences do not appear to contribute to *Mutator* activity in the minimal line either positively (Chomet *et al.* 1991; Lisch *et al.* 1995) or negatively (Slotkin *et al.* 2003, 2005).

mop1 in the minimal Mutator line: Silenced *MuDR(p1)* elements were generated by crossing *MuDR(p1)* to *Muk*. The resulting progeny were crossed to an *a1-mum2* tester and then self-fertilized. Resulting plants were self-fertilized and testcrossed to *MuDR(p1)* to screen for plants that lacked *Muk*. Plants that were homozygous for silenced *MuDR(p1)* [referred to as *MuDR(p1)**] and that lacked *Muk* were then crossed to *mop1* homozygotes. The resulting *mop1*-heterozygous progeny were crossed to a *mop1*-homozygous tester that carried the *a1-mum2* reporter. None of the resulting F_1 mutant kernels exhibited somatic mutability. A *MuDR(p1)** *mop1* mutant progeny of that cross was then self-fertilized. Again, none of the F_2 progeny kernels were spotted. An F_2 *mop1*-homozygous progeny plant carrying *MuDR(p1)** was then testcrossed to a *mop1*-heterozygous tester and the resulting ear had 3% spotted kernels. In the next, F_3, generation, one exceptional mutant individual carrying *MuDR(p1)** gave rise to 23% spotted kernels when testcrossed to a *mop1*-heterozygous tester. Spotted kernels from this cross were again testcrossed to *mop1*-heterozygous testers and again, one exceptional F_4 mutant individual gave rise to 21% spotted kernels. In the next generation (F_5), all *mop1*-homozygous individuals that carried *MuDR(p1)** gave rise to significant numbers of spotted kernels. In this and all subsequent generations the *mop1* mutant phenotype correlated well with the presence of large numbers of spotted progeny kernels. A schematic of all generations and crosses beginning with F_5 can be found in Figure 1.

The mop1/mop1;MuDR(p6) line*: The *mop1/mop1;MuDR(p6)** line was generated in a manner similar to that used to generate the *mop1/mop1;MuDR(p1)** line, using in this case a *MuDR* element at position 6 (*p6*) that had been previously silenced by *Muk* and then introgressed into the *mop1* mutant background for three generations. *MuDR(p6)* resulted from the transposition of *MuDR(p1)* in the minimal line and is therefore a duplicate of that original element in an identical genetic background (the minimal line).

DNA extraction and Southern blotting: DNA preparation and genomic Southern blotting were performed as previously described (Dorweiler *et al.* 2000). Maize genomic DNA (10 μg) was digested for >4 hr with an excess of 20 units of restriction enzyme. *Mutator* restriction sites and probes referred to in this report are shown in Figure 2.

RNA extraction and RT–PCR analysis of *mudrA* and *mudrB*: RT–PCR of *mudrA* and *mudrB* was performed as in (Slotkin *et al.* 2003). The same oligo(dT)-primed cDNA used in the RT–PCR analysis of the *mudrA* and *mudrB* transcripts was also amplified with primers specific for the alanine aminotransferase (*aat*) transcript to ensure equal starting amounts of RNA. Amplification was performed for 29 cycles using the primers *aat*F, 5′ ATGGGGTATGGCGAGGAT and *aat*R, 5′ TTGCAC GACGAGCTAAAGACT. Amplification of *aat* cDNA generates a band of 281 bp, while amplification of the DNA produces a band of 454 bp.

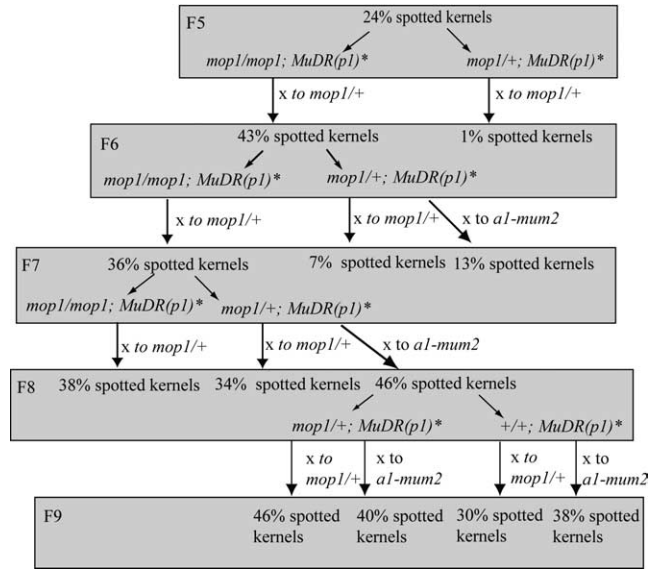

Figure 1.—Diagram of the crosses and generations discussed in this study. F_5 is the first generation in which a significant percentage of kernel spotting was seen. Each generation indicates the percentage of spotted-kernel progeny produced from the previous generation as well as the genotypes selected for the crosses produced in the next generation. Arrows indicate the testcross.

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 observed. To determine if the *MuDR* element was at the *p1* position, probing with the methyl TIRA probe that flanks *p1* and the TIRA with a *Hin*fI-digested blot (see below) assured the presence of *MuDR* at *p1*.

***Mutator* TIR methylation assay:** *Mutator* activity is associated with the methylation status of the *Hin*fI restriction site present in all *Mu* element TIRs (Lisch *et al.* 1995). *Mutator*-active individuals have hypomethylated *Mu1* 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 can also be observed that are the result of hybridization of the *Mu1* probe to MRS-A, a maize gene that is homologous to *Mu1* (Chandler *et al.* 1986). The *Hin*fI sites in this gene, which lack *Mu* TIRs, are not affected by the presence or absence of *MuDR*.

The methylation and activity status of *MuDR(p1)* TIR at *mudrA* (TIRA) can also be assayed by restriction digestion

using methyl-sensitive restriction enzyme *Hin*fI. Digestion of TIRA with *Hin*fI produces a 607-bp fragment when the *Hin*fI site in TIRA is hypomethylated, and a larger fragment of 1052 bp when it is methylated. Additional fragments visible on Southern blots represent sequences homologous to *MuDR* (*hMuDRs*) that do not contribute either positively or negatively to *Mu* activity.

The methylation status of TIRA and TIRB can also be assayed using the restriction enzyme *Afe*I, which is blocked by CG methylation. A double digest of *Afe*I with *Bam*HI produces a 2552- bp *MuDR(p1)* fragment when the *Afe*I site is hypomethylated at TIRA and a 2875-bp fragment when *Afe*I is methylated using the methyl TIRA probe. An *Afe*I/*Bam*HI double digest produces a 4-kb fragment when the TIRB is hypomethylated at the *Afe*I site when using the *p1* flanking probe; when the *Afe*I site is methylated the fragment is ~7 kb and is difficult to resolve on a blot.

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.

Internal MuDR probe: The internal *MuDR* probe is generated by gel isolating an internal *Eco*RI/*Bam*HI fragment from the pBMP1.3 plasmid (Chomet *et al.* 1991).

Methyl TIRA probe: The methyl TIRA probe was generated by amplification using PCR primers TIRA methyl F, 5′ CGCGCA CGAGGAAGGCGTTCT, and TIRA methyl R, 5′ AGCACCCGT CGCTCCACTTCC. The PCR program was as follows: 94° for 2 min, 94° for 30 sec, 63° for 45 sec, and 72° for 40 min, and then repeated for 35 cycles at 72°, with a last step of 10 min. The PCR product was a 52-bp DNA band.

p1 flanking probe: The *p1* flanking probe was generated by the *Pst*I digestion of the pBMP1.3 plasmid. The 800-bp *p1*-specific fragment hybridizes to a single-copy sequence in the maize genome (Slotkin *et al.* 2003).

All DNA probes in this report were gel isolated and prepared by the random-priming method using a Prime-It II kit (Stratagene, La Jolla, CA) and ^{32}P-radiolabeled dCTP (Perkin-Elmer, Norwalk, CT). All blots were exposed to a Molecular Dynamics phosphorimaging screen and processed using Adobe Photoshop.

Genotyping for *mop1*: *mop1* is on chromosome 2 at locus 2.04. The primers umc1259 forward and reverse were used to amplify the SSR linked to *mop1* (umc1259 forward: 5′ CTCTTT GGTGGCAGAAGCAGAAT; umc1259 reverse: 5′ TAGCTAAA CTTGAGTGCTCTGCCC). umc1259 is tightly linked to the *mop1* locus in our minimal lines. When comparing the *mop1* mutant phenotype and a linked umc1259-size polymorphism, we have seen crossovers in <1% of individuals analyzed. Additional SSR markers used for mapping included umc1541 forward and reverse (for primer sequences and amplification conditions refer to http://www.maizegdb.org/).

RESULTS

Plants homozygous for *mop1* are hypomethylated at *mudrA* as well as *Mu1*: In the absence of transposase, nonautonomous elements such as *Mu1* become methylated at the TIRs (Chomet *et al.* 1991). This methylation is fully reversible when functional *MuDR* elements are introduced genetically, and it can be reintroduced *de novo* during development if the transposase is lost due to internal deletions within *MuDR* (Lisch *et al.* 1995). Thus, *Mu1* TIR methylation appears to be the default state that occurs in the absence of transposase. When otherwise functional *MuDR* elements are silenced by *Muk*, their TIRs become methylated as well (Slotkin *et al.* 2003). Previous work has shown that *Mu1* TIRs are hypomethylated in a *mop1*-homozygous background whether or not a functional *MuDR* is present, consistent with a role for MOP1 in the default methylation of *Mu* TIRs that occurs in the absence of the transposase (Lisch *et al.* 2002).

To determine whether the TIRs at *MuDR(p1)* elements silenced by *Muk* (designated *MuDR(p1)**) also become demethylated in a *mop1* background, we examined several families that were segregating for *mop1* heterozygotes and homozygotes and for *MuDR(p1)**. *Muk* was no longer present in any of these families, which had carried *MuDR(p1)** in a *mop1* mutant background for five or six generations. Methylation was assayed by examining Southern blots of DNA digested with *Hin*fI, which is blocked at certain overlapping CG sites. The blots were sequentially probed with an internal fragment of *Mu1* and a fragment flanking *MuDR(p1)* that included sequences adjacent to the TIR flanking *mudrA* (TIRA) as well as a portion of the TIR (Figure 2). All 43 individuals that genotyped as *mop1* homozygous and that contained *MuDR(p1)** exhibited hypomethylation at both *Mu1* and TIRA. Conversely, all 53 siblings that were *mop1* heterozygous and that contained *MuDR(p1)** remained methylated at both *Mu1* and TIRA (Figure 3A). These data demonstrate that the *mop1* mutation reverses the methylation of TIRA as well as *Mu1* TIRs.

Progressive restoration of high levels of somatic activity of silenced *MuDR(p1) elements in a *mop1* mutant background:** Plants silenced by *Muk* lose somatic activity of *Mutator* elements, including *Mu1* excision from the *a1-mum2* reporter allele (Slotkin *et al.* 2003), resulting in the loss of kernel spotting in the aleurone. In previous work, we observed that *mop1*-homozygous mutants restored somatic activity to previously silenced *MuDR(p1)** elements in a small percentage of kernels (3%) (Lisch *et al.* 2002). We wanted to know if somatic activity would increase if *MuDR(p1)** were carried in a *mop1* mutant background for additional generations. After four generations of exposure to the *mop1* mutant, a line was derived in which *mop1* mutants carrying *MuDR(p1)** consistently produced a high frequency of spotted kernels (see materials and methods and Figure 1 for derivation of this line). In one family in the F_6 generation, 4 *mop1* mutants gave a total of 43% spotted kernels. In contrast, 12 siblings that carried *MuDR(p1)** but that were heterozygous for *mop1* gave only 1% spotted kernels. None of the progeny of 4 plants that were *mop1* mutant but that lacked *MuDR(p1)** gave rise to spotted kernels (0/291) (data not shown), confirming that the somatic activity we observed is due to *MuDR(p1)*. A subsequent round of crossing (the F_7 generation) gave rise to a similar frequency of spotted kernels (Table 1A);

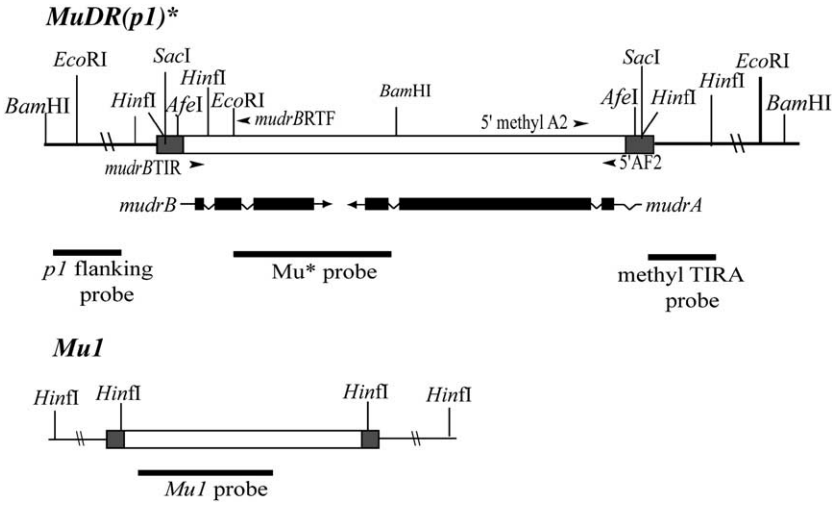

FIGURE 2.—Representation of *MuDR* at *p1* and *Mu1* at *a1-mum2*. Shaded boxes represent *Mutator* TIRs; open boxes are *Mutator* internal sequences that differ between *MuDR* and *Mu1*. The *mudrA* and *mudrB* exons are solid boxes just below the *MuDR* element; the lines that angle down represent introns. Arrows indicate the direction of transcription. Restriction sites used in this study are indicated. Probes used in this study are indicated below the transcripts. Primers used in this study are shown as arrowheads.

10 *mop1*-homozygous mutants gave a total of 36% spotted kernels (360/989). Again, only individuals that were *mop1* homozygous and carried *MuDR(p1)** gave rise to significant numbers of spotted kernels (Figure 3B); the 17 *mop1*-heterozygous plants gave rise to only 7% spotted kernels (192/2700) (Table 1B and Figure 3B).

Although the frequency of spotted kernels was consistently much higher in the F_6 and F_7 generations than in earlier generations, there was significant variation between individual ears, suggesting a stochastic component to the degree of activity achieved by *MuDR* in this mutant background. Similarly, there was a range of spotting intensity among individual kernels on a given ear. Despite this variation, the proportion of heavily and medium spotted kernels was clearly much higher in progeny of *mop1* mutant plants than in their heterozygous siblings (21% *vs.* 3%). The frequency of excision in these kernels is indistinguishable from that

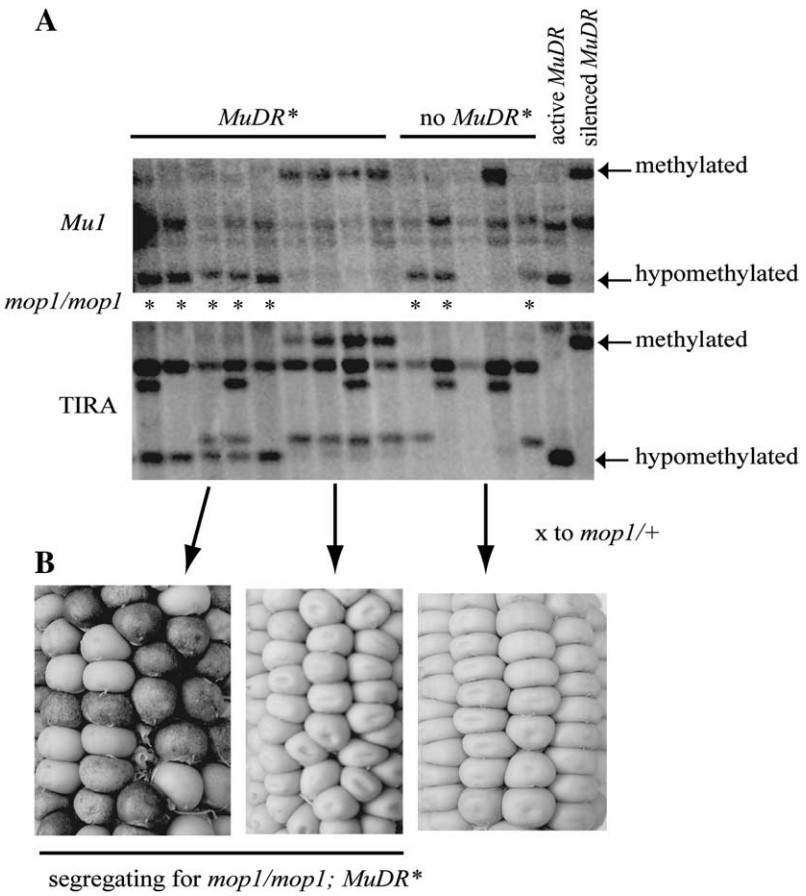

FIGURE 3.—(A) Southern blots of a family from the F_7 generation segregating for the *mop1* mutation as well as the presence of *MuDR(p1)**. The same blot was probed for *Mu1* and then stripped and reprobed with TIRA. The genotype of each individual is indicated. Individuals that genotype as *mop1* homozygous ("*") are hypomethylated at both *Mu1* and TIRA. Individuals that are *mop1* heterozygous are methylated at both *Mu1* and TIRA. Individuals that lack *MuDR(p1)** are missing fragments corresponding to both methylated and hypomethylated TIRA. Active *MuDR(p1)* and *MuDR(p1)** silenced by *Muk* are presented as controls. (B) Ear progeny of a cross between indicated individuals represented by the Southern blot by a *mop1/+* tester. Individuals that are *mop1/mop1;MuDR(p1)** gave rise to ~36% spotted kernels when crossed to *mop1/+*. Conversely, individuals that are *mop1/+;MuDR(p1)** gave rise to very few spotted kernels when crossed to *mop1/+*. Individuals lacking *MuDR(p1)**, regardless of *mop1* phenotype, gave rise to no spotted kernels.

TABLE 1

Somatic excision activity is restored in an *mop1* background

Cross[a]	Heavy/medium spotted	Weakly spotted	Pale	Total	Spotted (%)	Heavy/medium spotted (%)
		A. *mop1/mop1*; *MuDR(p1)** siblings				
1	44	33	90	167	46	26
2	8	0	33	41	20	20
3	15	0	33	48	31	31
4	17	3	47	67	30	25
5	27	10	28	68	57	42
6	12	21	105	138	24	9
7	38	30	83	151	45	25
8	12	20	106	139	24	9
9	38	29	84	150	45	25
10	1	0	20	21	5	5
Total	212	148	629	989	36	21
		B. *mop1/+*; *MuDR(p1)** siblings				
1	14	9	164	187	12	7
2	10	8	104	122	15	8
3	0	2	104	106	2	0
4	2	10	178	190	6	1
5	2	1	146	149	2	1
6	13	4	35	52	33	25
7	4	15	140	159	12	3
8	10	22	170	202	16	5
9	0	5	251	256	2	0
10	8	16	77	101	24	8
11	0	1	16	17	6	0
12	1	4	216	221	2	0
13	3	4	204	211	3	1
14	3	13	173	189	8	2
15	1	0	130	131	1	1
16	2	5	200	207	3	1
17	0	0	200	200	0	0
Total	73	119	2508	2700	7	3

[a] In the F_7 generation *mop1/mop1*;*MuDR(p1)** (A) and *mop1/+*;*MuDR(p1)* siblings (B) were crossed to *mop1/+* testers.

observed in kernels carrying a fully active *MuDR(p1)* element.

These results indicate that extended exposure of *MuDR(p1)** to the *mop1/mop1* background can restore high levels of *MuDR(p1)** activity with respect to somatic excisions. However, in these generations *mop1* heterozygotes grown from heavily spotted kernels derived from *mop1* mutant parents had methylated *Mu1* and *MuDR* TIRs (Figure 3A) and did not transmit a significant number of spotted kernels (Table 1 and Figure 3B), demonstrating that in these generations *MuDR(p1)** was not heritably reactivated by *mop1*.

Somatic excision activity is largely dependent upon the female parental genotype: We observed that in a *mop1/mop1*;*MuDR(p1)** × *mop1/+* cross, each ear had more than the expected 25% spotted kernels, presuming kernel spotting is dependent upon the kernel genotype being *mop1* homozygous and carrying *MuDR(p1)**. This suggested that at least a subset of the spotted kernels were not *mop1* homozygous. This turned out to be the case. Of 135 individuals genotyped, only 41% of the spotted kernels derived from the above cross were *mop1* homozygous (data not shown). This suggested that kernel spotting is dependent upon the female parent being *mop1* homozygous, and that the genotype of the kernel itself is irrelevant to kernel- spotting intensity.

To determine whether or not the genotype of the male parent had any effect on kernel spotting in the progeny, we performed a series of exact reciprocal crosses of F_7 plants, whereby *mop1/mop1*;*MuDR(p1)** individuals were crossed to and by *mop1/+* plants (Figure 4). When the female parent was *mop1/mop1*;*MuDR(p1)**, 36% of the kernels were spotted, 11% of the total being heavy and medium spotted (Table 2A). In contrast, when the male parent was *mop1/mop1*;*MuDR(p1)**, only 19% of the kernels were spotted, and only 1% of which

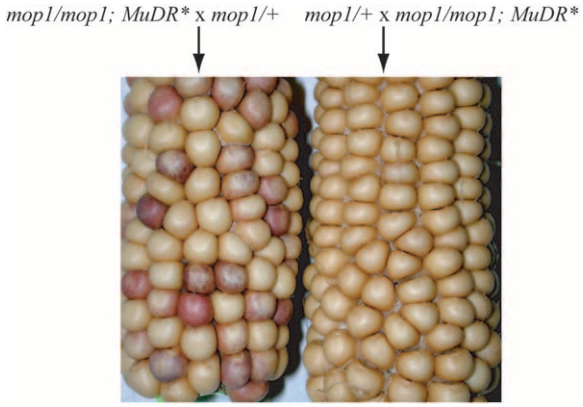

FIGURE 4.—Ears resulting from an exact reciprocal cross between *mop1/mop1*;*MuDR(p1)** and *mop1/+*.

were heavy or medium spotted (Figure 4 and Table 2A). A subset of the spotted progeny kernels from several reciprocally crossed individuals were genotyped for *mop1*. As seen previously, 51% of all spotted kernels genotyped from the female cross were *mop1/+*; the rest were *mop1/mop1* (Table 3). Interestingly, almost all (93%) of the spotted kernels from the male cross were *mop1* homozygous. We also performed reciprocal crosses of *mop1/mop1*;*MuDR(p1)** to *a1-mum2*, which is wild type for *Mop1*. In these crosses the percentage of spotted kernels (31%) (290/921) (Table 2B) produced when *mop1/mop1*;*MuDR(p1)** was female and crossed by an *a1-mum2* male was similar to that seen when the female *mop1/mop1*;*MuDR(p1)** was crossed to a *mop1/+* male. In the reciprocal cross, there were fewer spotted kernels (12%) (136/1174) (Table 2B), and there were no heavily or medium spotted kernels.

Together, these data suggest that when the female parent is *mop1/mop1*;*MuDR(p1)**, kernel- spotting intensity is independent of the kernel genotype; conversely, when the male parent is *mop1/mop1*;*MuDR(p1)**, kernel-spotting intensity is largely dependent upon the kernel genotype.

MuDR(p1)* **becomes heritably reactivated after multiple generations in a *mop1* background:** To comprehensively examine the cumulative effects of *mop1* on *MuDR(p1)** in the same environment, three generations of kernels (F_6, F_7, and F_8) were planted simultaneously in the summer of 2003. In all generations, *mop1* homozygotes and heterozygotes carrying reactivated *MuDR(p1)** were crossed as female to *a1-mum2* (wild type for *Mop1*) and/or *mop1/+*. We found that in the F_6 and F_7 generations, as noted above, all *mop1/+*;*MuDR(p1)** progeny plants were methylated at *Mu1* or TIRA (Table 4). When testcrossed, these plants gave rise to few or no spotted kernels (Table 5 and Figure 5). Surprisingly, in the next (F_8) generation, all *mop1/+*;*MuDR(p1)** plants grown from spotted kernels (50 of 50) were hypomethylated at TIRA (Table 4). Of these 50 *mop1/+*;*MuDR(p1)** plants that were hypomethylated at TIRA, roughly half (29) were also hypomethylated at *Mu1*; the remaining 21 were not (Table 4). When *Mu1*-hypomethylated *mop1/+*;*MuDR(p1)** (F_8) plants were crossed to either *a-1mum2* or *mop1/+* (resulting in the F_9 generation), they gave rise to an average of 47% spotted kernels (Table 6), matching the percentages found in cases where the parent had been *mop1/mop1*;*MuDR(p1)** . In contrast, sibling plants that were methylated at *Mu1* (but were hypomethylated at TIRA) gave rise to only 3% (80 of 2286) spotted kernels. These data suggest that in the F_8 generation, although there was a heritable effect on TIRA methylation in all *mop1*-heterozygous progeny that showed high levels of somatic activity in the aleurone, only a subset of those progeny were active enough to hypomethylate *Mu1* in the embryo, and it was those individuals that went on to transmit continued somatic activity in a subsequent generation.

One family in the F_9 generation that was segregating for both *mop1/+* and homozygous wild- type individuals, was testcrossed by *mop1/+* or to *a1-mum2* (Table 7). *MuDR* activity continued to transmit in the resulting ears. Notably, one ear, derived from a cross between a homozygous wild-type plant and an *a1-mum2* tester segregated 38% for spotted kernels, demonstrating that the somatic activity of *MuDR(p1)** persisted even in the absence of even one copy of the *mop1* mutant allele.

We wanted to know whether the hypomethylation of *Mu1* in *mop1/+*;*MuDR(p1)** individuals was the result of a generic effect on *Mu1* due to its long-term exposure to *mop1* (because *mop1* has an effect on *Mu1* independent of *MuDR* activity) or whether it was the result of reactivated *MuDR(p1)** transposase (which can hypomethylate *Mu1* elements independent of *mop1).* To test this, we examined siblings in the F_8 generation that were *mop1/+* but that lacked *MuDR(p1)**. *Mu1* was methylated in all (20 of 20) of these individuals (data not shown), suggesting that MOP1 was present and competent to mediate default methylation of TIRs but that this process is prevented in plants that carry reactivated *MuDR(p1)** due to activity of the transposase in sibling plants that carried heritably reactivated *MuDR* elements.

mudrA* but not *mudrB* expression is restored in *mop1*-reactivated *MuDR(p1)*:** When silenced by *Muk*, *MuDR(p1)** loses expression of polyadenylated and non-polyadenylated *mudrA* and polyadenylated *mudrB* transcript. By the next generation, both *mudrA* and *mudrB* become transcriptionally silenced, even in the absence of *Muk* (SLOTKIN *et al.* 2003). We wanted to see whether or not the *mop1* mutation was able to restore expression of these genes. In the F_6 and F_7 generations, plants that were *mop1/mop1*;*MuDR(p1)** expressed polyadenylated *mudrA*, but *mop1/+*;*MuDR(p1)** sibling plants did not, nor did plants that were *mop1* mutant but that lacked *MuDR(p1)** (Figure 6A). By the F_8 generation however, *mudrA* expression was maintained in *mop1/+*;*MuDR(p1)** individuals that are also hypomethylated at *Mu1* (Figure 6B) and that transmitted significant

TABLE 2

Somatic activity exhibits a maternal effect in a *mop1* background

A. *mop1/mop1*; *MuDR(p1)** × *mop1/+*						*mop1/+* × *mop1/mop1*; *MuDR(p1)**				
Cross[a]	h/m[b]	w[c]	T[d]	Spotted (%)	h/m (%)	h/m[b]	w[c]	T[d]	Spotted (%)	h/m (%)
1	47	52	203	49	23	1	27	187	15	1
2	26	49	141	53	18	14	93	355	30	4
3	7	14	50	42	14	3	74	347	22	1
4	8	45	231	23	3	0	6	206	3	0
5	18	24	103	41	17	6	2	204	2	3
6	1	10	21	52	5	0	26	226	12	0
7	65	32	196	49	33	2	64	236	28	1
8	3	38	137	30	2	6	68	260	28	2
9	11	11	60	37	18	6	58	183	35	3
10	12	51	155	41	8	3	61	225	28	1
11	0	5	8	63	0	0	4	204	2	0
12	2	28	139	22	1	0	1	231	0	0
13	12	6	133	14	9	2	28	136	22	1
14	0	42	126	33	0	0	42	199	21	0
15	2	47	172	28	1	1	6	22	32	5
Total	214	454	1875	36	11	44	556	3221	19	1

B. *mop1/mop1*; *MuDR(p1)** × *a1-mum2*						*a1-mum2* × *mop1/mop1*; *MuDR(p1)**				
Cross[a]	h/m[b]	w[c]	T[d]	Spotted (%)	h/m (%)	h/m[b]	w[c]	T[d]	Spotted (%)	h/m (%)
1	4	8	21	57	19	0	14	84	17	0
2	6	25	70	44	9	0	10	35	29	0
3	0	4	204	2	0	0	0	200	0	0
4	7	41	96	50	7	0	6	56	11	0
5	16	48	143	45	11	0	18	118	15	0
6	4	11	32	47	13	0	1	81	1	0
7	5	28	73	45	7	0	0	100	0	0
8	4	6	20	50	20	0	4	20	20	0
9	3	0	11	27	27	0	4	104	4	0
10	9	1	34	29	26	0	26	118	22	0
11	3	11	23	61	13	0	15	55	27	0
12	0	0	100	0	0	0	0	25	0	0
13	16	30	94	49	17	0	38	178	21	0
Total	77	213	921	31	8	0	136	1174	12	0

[a] In each pair of crosses (numbered), an individual plant was reciprocally crossed as a female (left) or as a male (right) to an *mop1/+* (A) or an *a1-mum2* (B) tester.
[b] Heavy/medium spotted kernels;
[c] Weakly spotted kernels; and
[d] Total number of kernels.

numbers of spotted progeny kernels when testcrossed (Table 6). However, polyadenylated *mudrB* expression was not restored in a *mop1* mutant background in any individuals, even in later generations in which *MuDR(p1)** activity had become independent of the *mop1* mutant.

To determine whether the differential reactivation of *mudrA* and *mudrB* expression in a *mop1* background was due to a position effect of *MuDR** at *p1*, we examined *mudrA* and *mudrB* expression at another silenced *MuDR* locus, position 6 *(p6). MuDR(p6)** had been introgressed into a *mop1* background for at least three generations and exhibited a small percentage of kernel spotting, around 2% (data not shown). We found that *mop1/mop1*;*MuDR(p6)** individuals exhibited *mudrA* but not *mudrB* expression (Figure 7), just as was seen with *MuDR(p1)**. This suggests that the differential reactivation of *mudrA* and *mudrB* is a generic effect of the *mop1* mutation and not due to a position effect on *MuDR*.

We wished to discover the methylation status of TIRB *vs.* TIRA to see if there was a methylation correlate with the differential *mudrA* and *mudrB* expression in a *mop1* background. Unfortunately our primary methylation assay, the *Hin*fI digest, did not produce conclusive data as the fragment sizes around *mudrB* were too small to

TABLE 3

Frequency of *mop1/mop1* mutants among spotted progeny of *mop1/mop1*;*MuDR(p1) plants**

mop1/mop1; *MuDR(p1)** × *mop1/+*						*mop1/+* × *mop1/mop1*; *MuDR(p1)**					
Cross[a]	*m/m*[b]	*m/+*[c]	T[d]	*m/m* (%)	*m/+* (%)	Cross[a]	*m/m*	*m/+*	T	*m/m* (%)	*m/+* (%)
1	15	13	28	54	46	1	12	0	12	100	0
2	8	5	13	62	38	2	11	0	11	100	0
3	13	13	26	50	50	3	17	1	18	94	6
4	8	19	27	30	70	4	14	4	18	78	22
Total	44	50	94	49	51	Total	54	5	59	93	7

[a] Genotype of a subset of spotted kernels from reciprocal crosses.
[b] *mop1/mop1*.
[c] *mop1/+*;
[d] Total embryos from spotted kernels genotyped for *mop1*.

resolve on a Southern blot. Therefore, we looked to another restriction site, *Afe*I, which is found in both TIRA and TIRB and that is blocked by CG methylation. We digested DNA from a family segregating for *mop1* and *MuDR(p1)** with *Afe*I and *Bam*HI (which cuts once within *MuDR* and which is not methyl sensitive) and then probed the blot with the methyl TIRA probe (as mentioned previously) and subsequently with a *p1* flanking probe (Figure 8). We found that in individuals that were *mop1/mop1*;*MuDR(p1)** the TIRA was hypomethylated, but the TIRB remained methylated, indicating that at the *Afe*I site there is a difference in methylation between TIRA and TIRB.

New transpositions are not observed in a *mop1* background: One of the effects of *Mutator* silencing is the loss of new *Mu* element transpositions. In an active *Mutator* minimal line, new transpositions by *Mu1* and *MuDR(p1)* occur a rate of 10–20% transpositions per generation (Lisch *et al.* 1995). When new *MuDR(p1)* transpositions occur, the resulting ears exhibit a higher percentage of spotted kernels than that expected from an ear segregating for only *MuDR(p1)* (50% expected spotted kernels in a cross *MuDR(p1)/*− × −/−). We wanted to see if the *mop1* mutation restored transposition of either *MuDR(p1)** or *Mu1* in silenced *Mutator* lines. To test this, we examined Southern blots for the appearance of unique fragments consistent with new transposition events, and we looked for ears that segregated for significantly more than 50% spotted kernels. *Hin*fI digests of *mop1*-heterozygous progeny of *mop1* mutant plants were probed with *Mu1*, and *Bam*HI and *Eco*RI digests were probed with an internal fragment of *MuDR*. If full transpositional activity were restored, we would expect that between 10 and 20% of the individuals seen would exhibit new transpositions of either *Mu1* or *MuDR(p1)**. No new insertions of *Mu1* were observed in 437 progeny of *mop1/mop1* plants that carried reactivated *MuDR(p1)**. The same was true for the 119 progeny examined by Southern blot for evidence of new insertions of *MuDR*. In addition, of the 932 ears we examined that were derived from *mop1*-homozygous plants carrying reactivated *MuDR(p1)**, we have not detected any with a percentage of spotted kernels significantly greater than 50%. This suggests that while the *mop1* mutant can reactivate *Mutator* somatic activity, it fails to reactivate transpositional activity either in *cis* (*MuDR* insertions) or in *trans* (*Mu1* insertions). Because *mudrB* regulates transposition, and because in a *mop1* mutant *mudrB* remains silenced, the lack of transposition in a *mop1* mutant is further evidence for the relationship between transpositional activity and *mudrB* activity.

DISCUSSION

Unlike the immediate and heritable reactivation of silenced transposons in a *ddm1* mutant background

TABLE 4

Hypomethylation of *mop1/+* progeny of mutant individuals over several generations

Cross	Hypo TIRA[a]	Hypo *Mu1*[b]	Meth *Mu1*[c]	Total TIRA	Hypo TIRA (%)	Hypo *Mu1* (%)	Meth *Mu1* (%)
F_6	0	0	0	75	0	0	0
F_7	0	0	0	27	0	0	0
F_8	50	29	21	50	100	58	42

[a] Number of *mop1/+* progeny plants with hypomethylation at TIRA among heavily spotted mop1/+ progeny plants.
[b] Of plants that were hypomethylated at TIRA, number that were also hypomethylated at *Mu1*.
[c] Of plants that were hypomethylated at TIRA, number that were methylated at *Mu1*.

TABLE 5

***MuDR(p1)** activity becomes heritable after several generations in an *mop1* background**

Cross[a]	Heavy/medium spotted	Weakly spotted	Pale	Total	Spotted (%)	Heavy/medium spotted[b] (%)	No. of crosses
			A. Crosses by a *mop1/+* testers				
F_6 *mop1/mop1*	241	64	411	716	43	34	4
F_6 *mop1/+*	6	13	1276	1295	1	0	12
F_7 *mop1/mop1*	212	148	627	987	36	21	10
F_7 *mop1/+*	73	119	2508	2700	7	3	17
F_8 *mop1/mop1*	124	31	248	403	38	31	5
F_8 *mop1/+*	644	272	1813	2729	34	24	17
F_9 *mop1/+*	504	179	799	1482	46	34	5
			B. Crosses to *a1-mum2* testers				
F_7 *mop1/mop1*	734	177	1028	1939	47	38	15
F_7 *mop1/+*	108	105	1416	1629	13	7	11
F_8 *mop1/mop1*	126	71	191	388	51	32	3
F_8 *mop1/+*	260	98	411	770	46	34	6
F_9 *mop1/+*	342	164	789	1285	39	26	7

[a] Generation (F_X) and genotype of the female plants carrying *MuDR(p1)** crossed to the indicated male genotypes; all individuals from a particular generation are siblings.
[b] Percentage of heavy/medium spotted of total kernels.

(Lippman *et al.* 2003; Kato *et al.* 2004), reactivation of *MuDR* elements in a *mop1* mutant background occurs only gradually and stochastically. Only after multiple generations of exposure to the *mop1* mutation do we observe evidence of heritable activity of silenced *MuDR* elements in the absence of the mutation. This process is reminiscent of the gradual appearance of epimutations, such as *fwa,* after several generations in a *ddm1* mutant background (Kakutani *et al.* 1996; Soppe *et al.* 2000). Interestingly, the promoter and upstream portion of the FWA transcript are composed of a transposable element (Lippman *et al.* 2004). These data suggest that the effects

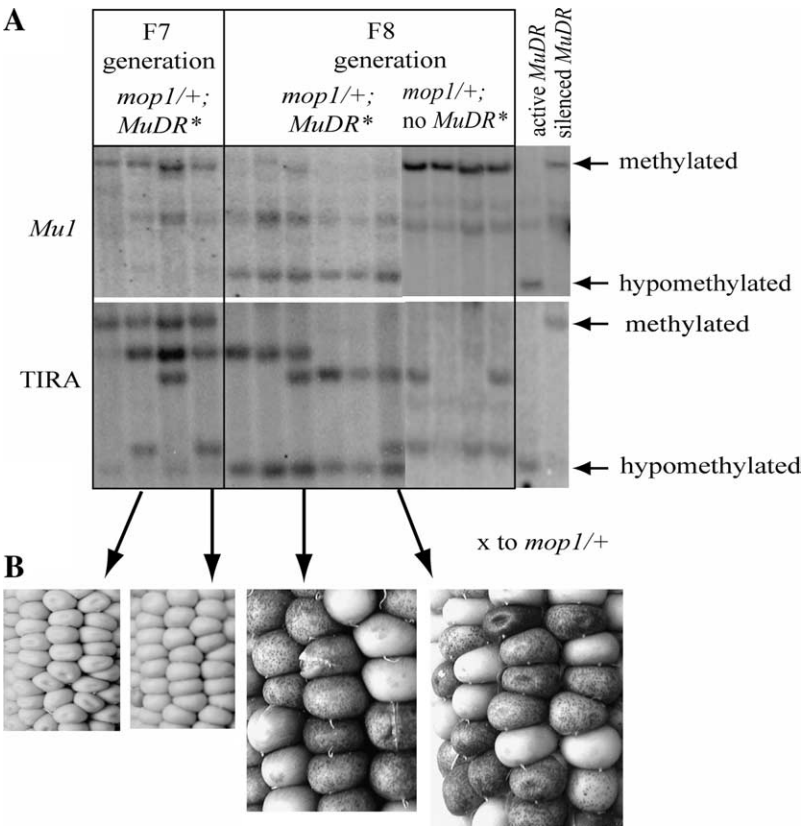

Figure 5.—(A) *Mu1* and TIRA Southern blots of individuals from both F_7 and F_8 generations. All individuals are *mop1/+*. Note that in the F_7 generation, all individuals are methylated at both *Mu1* and TIRA. In the F_8 generation in this figure, all individuals that are *mop1/+;MuDR(p1)** are hypomethylated at *Mu1* and TIRA, indicating that *MuDR(P1)** has been heritably reactivated in this generation. Individuals lacking *MuDR(p1)** are methylated at *Mu1* and lack the methylated or the hypomethylated TIRA fragments. (B) Ear progeny of a cross between indicated individuals represented by the Southern blot to a *mop1/+* tester. *mop1/+* individuals from the F_7 generation gave rise to few or no spotted-kernel progeny when outcrossed to a *mop1/+* tester. However, *Mu1*-hypomethylated *mop1/+;MuDR(p1)** individuals from the F_8 generation gave rise to ~46% spotted kernels when outcrossed to *mop1/+*.

TABLE 6

Persistence of somatic activity even in the absence of the *mop1* mutation: *Mu1* methylation of *mop1/*;+*MuDR(p1) individuals, F_8 generation, and progeny**

h[a]	Cross to	Spotted	Total	Spotted (%)	m[a]	Cross to	Spotted	Total	Spotted (%)
1	*mop1/+*	80	184	43	1	*mop1/+*	3	123	2
2	*mop1/+*	48	99	48	2	*mop1/+*	1	112	1
3	*mop1/+*	108	207	52	3	*a1-mum2*	4	204	2
4	*a1-mum2*	58	123	47	4	*mop1/+*	3	158	2
5	*mop1/+*	178	334	53	5	*a1-mum2*	6	192	3
6	*mop1/+*	66	222	30	6	*a1-mum2*	7	207	3
7	*mop1/+*	127	258	49	7	*mop1/+*	1	121	1
8	*mop1/+*	96	172	56	8	*mop1/+*	2	202	1
9	*a1-mum2*	120	256	47	9	*a1-mum2*	4	203	2
10	*a1-mum2*	46	92	50	10	*a1-mum2*	14	214	7
11	*mop1/+*	96	263	37	11	*mop1/+*	27	227	12
12	*a1-mum2*	168	327	51	12	*mop1/+*	5	186	3
13	*a1-mum2*	144	280	51	13	*mop1/+*	3	137	4
Total		1335	2817	47	Total		80	2286	3

As indicated in Table 4, all plants were hypomethylated at TIRA, but only about half were also hypomethylated at *Mu1*. h, plant had hypomethylated *Mu1* element; m, plant had methylated *Mu1* element. Plants were crossed by either *a1-mum2* or *mop1/+* testers as indicated.

[a] Plants grown from the F_8 generation kernels that were spotted.

of missing one component of the silencing machinery (MOP1 in this case, DDM1 in Arabidopsis) can be cumulative over time, perhaps because the loss of one component results in destabilization of a complex, which in turn leads to the loss of additional components and subsequent additional destabilization. Since a number of factors involved in the construction of stabilized chromatin appear to be mutually reinforcing (*i.e.*, DNA methylation and histone modification (Johnson *et al.* 2002; Soppe *et al.* 2002), it is reasonable to assume that, although the loss of one component may not have an immediate effect of gene activity, the absence of reinforcement of the silenced state could eventually result in destabilization of the silencing chromatin. Several other mutations that affect both paramutable alleles and *Mu* element methylation have been identified (Lisch and Hollick, unpublished data). It will be interesting to see how efficiently double mutants reactivate silenced *MuDR* elements.

The absence of both new insertions and *mudrB* expression in our reactivated lines is consistent with earlier data suggesting a role for *mudrB* in *Mu* element transposition (Lisch *et al.* 1999) (Raizada and Walbot 2000). Like our reactivated *MuDR* elements, deletion derivatives that lack *mudrB*, as well as transgenes that express only *mudrA*, can condition only excisions. Differential reactivation of two genes on the same transposon has not been observed previously. It suggests that the silenced state of these two genes differs in some way. Either *mudrB* is simply more deeply silenced than *mudrA* or the silenced state of the two genes is qualitatively different. There is some evidence that the latter may be the case, since TIRA becomes hypomethylated in a *mop1* background but TIRB does not. Further, although both

TABLE 7

Crosses from one family of the F_9 generation segregating for wild type

Cross[a]	h/m[b]	w[c]	Pale	T[d]	Spotted (%)	h/m (%)	Kernels (% wt)
mop/+; *MuDR(p1)** × *a1-mum2*	2	6	93	101	8	2	75
mop/+; *MuDR(p1)** × *a1-mum2*	62	48	145	255	43	24	75
mop/+; *MuDR(p1)** × *a1-mum2*	7	12	140	159	12	4	75
+/+; *MuDR(p1)** × *mop1/+*	1	10	102	113	10	1	75
+/+; *MuDR(p1)** × *mop1/+*	45	39	68	152	55	30	75
+/+; *MuDR(p1)** × *a1-mum2*	38	14	84	136	38	28	100

[a] Plants in this generation were either heterozygous for *mop1* or homozygous for *Mop1*.

[b] Heavy/medium spotted kernels.

[c] Weakly spotted kernels; and

[d] Total number of kernels.

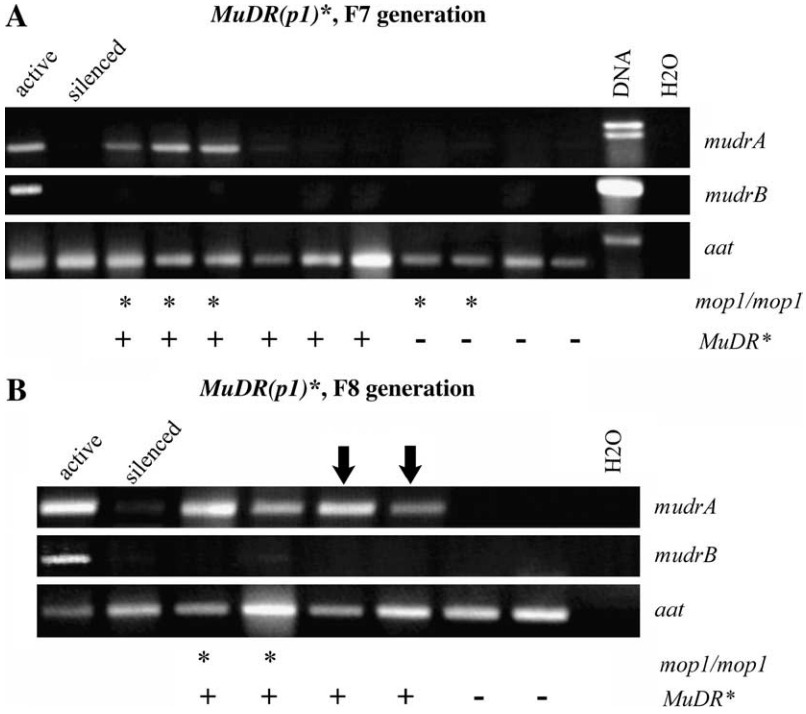

FIGURE 6.—*mudrA* and *mudrB* expression. (A) RT–PCR of both *mudrA* and *mudrB* in the F_7 generation. Individuals that are *mop1/mop1*; *MuDR(p1)** express *mudrA*, but *mop1/+*;*MuDR(p1)** individuals and those that lack *MuDR(p1)** do not express *mudrA*. However, *mop1/mop1*;*MuDR(p1)** individuals do not express *mudrB*. (B) RT–PCR of *mudrA* and *mudrB* in the F_8 generation. *mop1/+*; *MuDR(p1)** as well as *mop1/mop1*;*MuDR(p1)** individuals now express *mudrA*, in conjunction with the heritable reactivation of *MuDR(p1)** in this later generation. Once again, *mudrB* is still not expressed in any individual, not even *mop1* homozygotes. *aat* is the cDNA control.

genes are silenced after exposure to *Muk*, their mode of silencing appears to be different. *Muk* is an inverted duplicated version of *MuDR* that lacks the *mudrB* gene. It produces a long inverted repeat transcript that is homologous to the *mudrA* gene that produces small (23 and 26 nt) *mudrA*-homologous RNA molecules that are amplified when *Muk* silences *MuDR* elements. No such RNA molecules homologous to *mudrB* are observed (SLOTKIN *et al.* 2005). The *mudrA* gene is transcriptionally silenced by the immature ear of F_1 plants carrying both *MuDR* and *Muk*. In contrast, the *mudrB* gene remains transcriptionally active in these same F_1 immature ears, and it is only in the next generation that *mudrB* becomes transcriptionally silenced (SLOTKIN *et al.* 2003). This process requires that *mudrB* be in *cis* to *mudrA*; when a deletion derivative of *MuDR(p1)* that carries only *mudrB* is placed in *trans* to *MuDR(p1)*, it is not silenced in the presence of *Muk* (SLOTKIN *et al.* 2005). Thus, *mudrB* silencing is mediated via *mudrA* silencing, most likely via a distinct pathway that involves a signal that spreads in *cis* from *mudrA* to *mudrB*. If this is the case, then the chromatin at silenced *mudrB* may well be qualitatively different from that at *mudrA*.

The identity of *mop1* remains mysterious, so we can only speculate as to the mechanism of *MuDR* reactivation. The fact that this mutation affects both paramutation and *MuDR* activity but neither global methylation nor methylation of some transposon sequences upstream of the maize paramutagenic *b1* allele implies a relatively restricted (or partially redundant) role in maize gene silencing. This is reinforced by our observation that only one of the two genes encoded by *MuDR* is reactivated in a *mop1* background. The relative specificity of the *mop1* mutation may be because the *Mop1* gene is qualitatively different from mutations in Arabidopsis that affect transposon activity, or because the more global functions of those genes have been partitioned among multiple genes in maize. This possibility is supported by the existence of multiple mutations that affect both *Mutator* methylation and paramutation (HOLLICK and CHANDLER 2001; LISCH and HOLLICK, unpublished data).

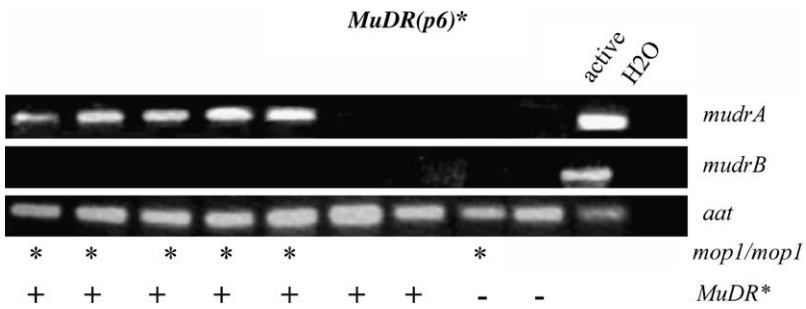

FIGURE 7.—*mudrA* and *mudrB* expression at another *MuDR* position (*MuDR(p6)**). Individuals that are *mop1/mop1*;*MuDR(p6)** express *mudrA* but not *mudrB*, similar to what is observed in reactivated *MuDR(p1)**.

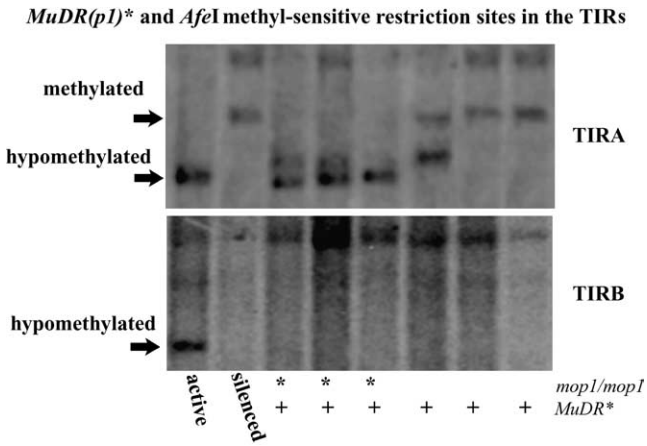

FIGURE 8.—Methyation analysis of TIRA *vs.* TIRB. Shown is a Southern blot depicting individuals carrying *MuDR(p1)** and that are *mop1/mop1* or *mop1/+*. Individuals were digested with *Afe*I (a methyl-sensitive enzyme that cuts once within each TIR) and *Bam*HI (not methyl sensitive and that cuts once within the *MuDR* element as well as outside each TIR). The Southern blot was probed first with the methyl TIRA probe and then stripped and reprobed with the *p1* flanking probe. *mop1/mop1*;*MuDR(p1)** individuals are hypomethylated at TIRA but are not hypomethylated at TIRB. Active *MuDR* is hypomethylated at both TIRA and TIRB. Silenced *MuDR* and *mop1/+*;*MuDR(p1)** individuals are hypomethylated at neither TIR.

An understanding of the differences between *mudrA* and *mudrB* silencing and resulting differences in chromatin at the *mudrA* and *mudrB* promoters at various stages of reactivation, should shed light on the relationship between the means by which genes are silenced and the nature of the silenced state once achieved. The evidence to date suggests that the initiation and maintenance of silencing is far more complex than simple "on" and "off" states. History, position, context, and timing may all play a role in these processes. The cumulative reactivation of *mudrA* in a *mop1* background suggests that changes in chromatin memory through several rounds of meiosis can be gradual, and that there are intermediate chromatin states between silenced and active chromatin that may in themselves have unique regulatory consequences and interesting implications for our understanding of heritable changes in the chromatin state.

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