A mutation that prevents paramutation in maize also reverses *Mutator* transposon methylation and silencing

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Both paramutation and *Mutator* (*Mu*) transposon inactivation involve heritable changes in gene expression without concomitant changes in DNA sequence. The mechanisms by which these shifts in gene activity are achieved are unknown. Here we present evidence that these two phenomena are linked mechanistically. We show that mutation of a gene, *modifier of paramutation 1* (*mop1*), which prevents paramutation at three different loci in maize, can reverse methylation of *Mutator* elements reliably. In *mop1* mutant backgrounds, methylation of nonautonomous *Mu* elements can be reversed even in the absence of the regulatory *MuDR* element. Previously silenced *MuDR* elements are reactivated sporadically after multiple generations of exposure to *mop1* mutations. *MuDR* methylation is separable from *MuDR* silencing, because removal of methylation does not cause immediate reactivation. The *mop1* mutation does not alter the methylation of certain other transposable elements including those just upstream of a paramutable *b1* gene. Our results suggest that the *mop1* gene acts on a subset of epigenetically regulated sequences in the maize genome and paramutation and *Mu* element methylation require a common factor, which we hypothesize influences chromatin structure.

Although a great deal has been learned about the biochemistry and molecular biology of maize transposable elements, the means by which they are silenced epigenetically is only beginning to be understood. The most consistent molecular correlate with silencing in maize is cytosine methylation (1–4). The causal relationship between methylation of maize transposons and transposon activity, however, is not known. Work in maize and other systems such as *Caenorhabditis elegans* (5, 6) and *Arabidopsis* (7, 8) suggests that transposon regulation likely involves both transposon- (9, 10) and nontransposon-encoded gene products.

Methylation is a characteristic feature of inactivation of *Mutator* transposons (1, 3, 11, 12). The *Mutator* system includes a number of classes of elements; all classes share similar terminal inverted repeats (TIRs), but each carries unique internal sequences. *MuDR* is the regulatory element for the entire system, and the presence of active *MuDR* elements is required for transposition of the nonautonomous elements (11, 13). *MuDR* carries two genes, *mudrA* and *mudrB*. The *mudrA* gene is the presumptive transposase (14) and is absolutely required for all aspects of *Mutator* activity. Methylation of nonautonomous *Mu* elements is strictly correlated with the absence of functional *MuDR* elements and specifically with the absence of the *mudrA* gene product (11, 13, 15). Functional *MuDR* elements can be lost in three ways: segregation in genetic crosses, internal deletions, and epigenetic silencing correlated with methylation (16, 17). In *Arabidopsis*, mutations in *DDM1* (homologous to SWI2/SNF2, a component of a chromatin-remodeling complex) are known to result in both reduced methylation and sporadic activation of silenced *Mu*-like (8) and *Spm*-like (18) elements.

Paramutation is another phenomenon that results in the heritable alteration of gene expression (19). In maize, paramutation has been observed at the *r1*, *b1*, *pl1*, and *p1* loci, all of which are involved in flavonoid pigment production (reviewed in ref. 20). Paramutation occurs when a paramutagenic allele is heterozygous with a paramutable allele, resulting in a directed reduction in the expression of the paramutable allele. That change is heritable, and the paramutable allele can become paramutagenic.

Recently a mutation was isolated that prevents paramutation at *b1*, *r1*, and *pl1* (21). When this mutation, *mop1-1*, is homozygous, the normally low-expressing paramutagenic allele of *b1*, *B′*, expresses at the level of the highly expressing paramutable allele, *B-I*, which results in the dark-purple plant color characteristic of the *B-I* allele as opposed to the lighter and sporadic purple color characteristic of the *B′* allele. Further, when *B′* is heterozygous with *B-I* in a *mop1-1* homozygous background, paramutation is prevented, with both the *B′* and *B-I* phenotypes transmitted after out-crossing. In the absence of the *mop1-1* mutation only the *B′* phenotype is transmitted from a *B′/B-I* heterozygote, because *B-I* is changed into *B′* at a 100% frequency. The *mop1-1* mutation also prevents the establishment of paramutation at the *p1l* and *r1* loci, indicating that the *mop1* gene product is generally required for paramutation (21).

Herein we show that mutations in *mop1* can reverse *Mu* element methylation. We also show that somatic activity of previously silenced *MuDR* elements can be reactivated in a *mop1* mutant background. Because mutations in *mop1* affect multiple loci and phenomena, each of which requires different promoter sequences, we hypothesize that this gene operates on chromatin configuration rather than specific sequences.

Materials and Methods

Genetic Crosses. Throughout the article, a single allele listing indicates homozygosity, whereas heterozygous individuals are indicated with alleles separated by a slash (/). Following maize genetic nomenclature, recessive alleles are indicated by lowercase and dominant alleles are indicated by capitalization of the first letter. The gene is indicated by lowercase, and the protein is indicated by uppercase and no italics. All stocks carry functional alleles of the *c1* regulatory gene and all the anthocyanin biosynthetic genes except for *a1*, as indicated below. The alleles of the regulatory genes *r1*, *b1*, and *pl1* in each stock are indicated for each cross.

Using *MuKiller* to Inactivate *MuDR* Elements. *MuKiller* (*MuK*), a dominant factor present in some lines but not in our minimal *Mutator* line, is competent to heritably silence *MuDR* elements (17). A plant carrying a single *MuDR* element at position 1 on chromosome 2L [*MuDR*(*p1*); ref. 11] with the genotype *B′ pl-sr*

Abbreviation: TIR, terminal inverted repeat.

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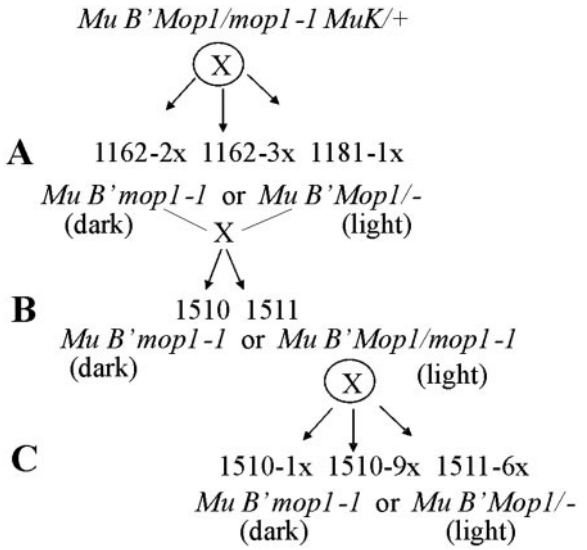

Fig. 1. Diagram of the crosses used to generate the families segregating *mop1-1* and silenced *Mu* elements that were analyzed in Table 1 and Fig. 2. Individual plants with the genotype indicated at the top of the diagram were self-fertilized. The resulting progeny from each family were segregating *mop1-1* (darkly pigmented plants) and wild type (lightly pigmented plants) as indicated in *A*. The symbol *Mop1*/− indicates that the other allele could have been *Mop1* or *mop1-1*. Dark and light individuals were crossed to generate the families shown in *B*. Several plants with the wild-type phenotype then were self-fertilized, and the resulting progeny were as indicated in *C*.

(allele of *pl1* that confers sun-red anthers and husks) *R-g* (*r1* allele that confers purple seed) *a1-mum2 MuDR*(*p1*) was crossed to a line carrying *MuK* and no active *MuDR* elements (*B′ pl-sr R-g a1-mum2 MuK*/+). The resulting plants were scored for the presence of newly silenced *MuDR* elements as indicated by no excision of the *Mu1* element from *a1-mum2* and methylation of *Mu1* elements in the presence of intact *MuDR* elements. The *a1-mum2* mutation contains a *Mu1* insertion in the 5′ promoter region of the *a1* anthocyanin biosynthesis gene (22). In the presence of active *MuDR* elements, excisions of *Mu1* can be scored as somatic purple sectors on the kernels. Of nine individuals examined in this family, three carried at least one full-length *MuDR* element and several methylated *Mu1* elements, which is consistent with the epigenetic silencing of *MuDR* elements by the segregating *MuK* activity.

The first ear of one such plant [*MuK*/+, silenced *MuDR*(*p1*), *B′ pl-sr R-g a1-mum2*] was crossed with a *mop1-1* homozygous *B′ Pl A1 r-g* (an *r1* allele that has no pigment and no paramutation activity) plant derived from an active *Mutator* stock and thus carried multiple *MuDR* and *Mu1* elements. The second ear was test-crossed by a plant lacking *MuDR* elements, wild-type *Mop1*, and homozygous for the *a1-mum2* reporter gene (11). None of the kernels from the second ear showed evidence of excisions of *Mu1* from *a1-mum2*, confirming the silencing of *MuDR* and the presence of *MuK* in the parent. Progeny from the first ear were self-fertilized, yielding *mop1-1*, *mop1-1*/*Mop1*, and *Mop1* (Fig. 1*A*). These progeny also were segregating *a1-mum2* versus *A1* and *R-g* versus *r-g*. Kernels with *A1* or *r-g* are fully purple or colorless, respectively, preventing the scoring of *Mu1* excision from *a1-mum2*. To generate a second generation of *mop1-1* homozygous plants and heterozygous siblings, *B′ mop1-1* plants resulting from the above cross were identified by their dark plant pigment and crossed to lightly pigmented *B′ mop1-1*/*Mop1* siblings, and the resulting progeny were scored for the *mop1-1* phenotype (Fig. 1*B*). To test for reversals of *Mu* element methylation, *B′ Mop1*/*mop1-1* progeny, which carried methylated *Mu* elements, were self-fertilized (Fig. 1*C*).

Generation of Seeds with a Silenced *MuDR* Element and Homozygous for *mop1-2*. Plants homozygous for a silenced *MuDR* element [*B′ a1-mum2 MuDR*(*p1*) *R-g*] were crossed to plants that were *B′ A1 r-g mop1-2*, an ethyl methanesulfonate-induced allele of *mop1* (21, 23), from a non-*Mutator* line (lacking full-length *MuDR* elements). The progeny plants heterozygous for *MuDR*(*p1*) were self-fertilized, and the resulting families were screened for spotted kernels. If *mop1-2* fully reactivated silenced *MuDR*(*p1*), then we would expect kernels homozygous for *a1-mum2* (1/4), *R-g*/*R-g* or *R-g*/*r-g* (3/4), homozygous for *mop1-2* (1/4) and carrying *MuDR*(*p1*) (3/4) to be spotted. Thus 100% reactivation would show 3.5% spotted kernels ($1/4 \times 3/4 \times 1/4 \times 3/4$). In addition to self-fertilization, some plants were crossed to plants that were homozygous for *mop1-2*, *a1-mum2*, and *R-g*, with the expected frequency for 100% reactivation to be 12.5% spotted kernels ($1/2$ *a1-mum2* $\times$ $1/2$ *MuDR*(*p1*) $\times$ $1/2$ *mop1-2*) for 100% reactivation.

DNA Preparation and Genomic Blotting. DNA preparation and genomic blotting were performed as described (21). A plasmid containing *Mu1* was as described (24). To generate an internal probe for *Mu1*, the plasmid was digested with *Ava*I and *Bst*EII, and the internal fragment was gel-isolated. An internal fragment of *MuDR* bounded by *Eco*RI and *Bam*HI was as described (11). The *b1* upstream SB (*Sal*I-*Bgl*II fragment) probe used to generate data shown in Figs. 2*F* and 5 was described previously (25, 26). Its location in *b1* is shown in Fig. 5*C*. As a control for partial digestion of DNA, blots with *Hin*fI-digested DNA samples were reprobed with a *Kpn*I fragment of the *a1* gene spanning a region of the coding sequence adjacent to (but not including) the *Mu1* insertion in this gene (22). As a control for blots with *Sac*I digests, the blots were probed with a single copy of *Pst*I fragment flanking but not including the *MuDR* insertion on chromosome 2L (13).

Results

Reduced Methylation of *Mu* Elements Correlates with the *mop1-1* Mutation. The *mop1-1* mutation was isolated originally in a *Mutator* active line (21) with multiple copies of both *MuDR* and *Mu1*, all of which were unmethylated (data not shown). To examine the effect of the *mop1-1* mutation on the process of *Mutator* silencing, a *mop1-1* homozygote that carried multiple active *MuDR* elements was crossed to a plant carrying *MuK*, a factor that dominantly inactivates the *Mutator* system (17). Fig. 1 shows a schematic of the crosses performed with this stock, and details are in *Materials and Methods*. In all cases, progeny were scored visually for *mop1-1* (dark-purple plants) versus wild-type (light-purple plants) phenotypes. In addition to their dark pigment, the *mop1-1* plants often were significantly shorter than their wild-type siblings, and some did not produce ears, consistent with previous observations of pleiotropic effects of this mutation (21). The methylation status of both the autonomous *MuDR* elements and the nonautonomous *Mu1* and related *Mu1.7* elements (hereafter referred to collectively as *Mu1*) was determined by using DNA blots. Table 1 summarizes the genotype and methylation status of *Mu1* elements for all the progeny.

Several plants resulting from the cross between the *mop1-1* mutant and the plant carrying *MuK* were self-fertilized (1162-2x, 1162-3x, and 1181-1x). An example of the methylation data for family 1162-3x is shown in Fig. 2 *A–C*. To determine the effect of *mop1-1* on *Mu1* methylation, DNA from the 21 plants in this family was digested with *Hin*fI and probed with an internal *Mu1* fragment (Fig. 2*A*). The *Mu1* and *Mu1.7* elements contain unique *Hin*fI sites near the ends of their TIRs (1). Complete digestion (no methylation) results in 1.4- and 1.7-kb fragments

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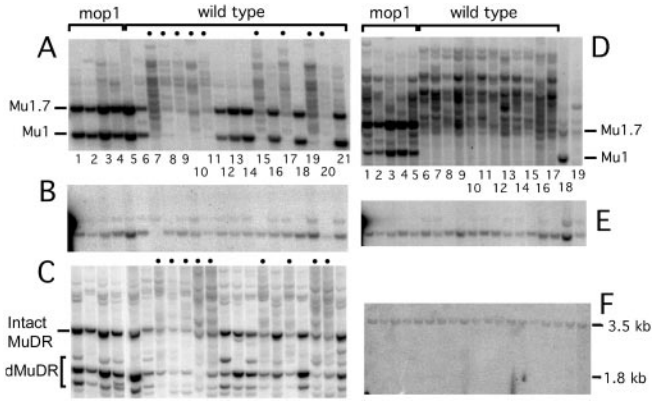

Fig. 2. (*A–C*) Methylation status of *Mu1* and *MuDR* elements in family 1162-3x, segregating for *MuK* and *mop1-1*. (*A*) A *Hin*fI digest of DNA from this family probed with a *Mu1* internal fragment. The lanes marked ''mop1'' are samples from plants that were homozygous for *mop1-1*. The lanes marked ''wild type'' are samples from plants that were either *mop1-1*/*Mop1* or *Mop1*/*Mop1*. The fragments expected for unmethylated *Mu1* and slightly larger *Mu1.7* elements are indicated. Individuals were scored as methylated (indicated by ●) if most of the *Mu1* and *Mu1.7* elements generated *Hin*fI fragments larger than the 1.4- and 1.7-kb fragment expected from complete digestion. The individual in lane 20 was retested, and the presence of methylated *Mu1* elements was confirmed (data not shown). (*B*) To control for complete digestion by the restriction enzyme, the blot shown in *A* was stripped and rehybridized with a fragment of the *a1* gene. The two fragments represent complete digestion by *Hin*fI. (*C*) A *Sac*I digest of the same DNA samples shown in *A* probed with an internal *MuDR* fragment. The 4.8-kb *MuDR* fragment diagnostic for the presence of unmethylated, intact *MuDR* elements is indicated. Smaller deleted versions of *MuDR* are indicated as *dMuDR*. The larger fragments observed in lanes 1–4 are sequences related to *MuDR* that are found in all maize lines (13), which serve as controls for loading differences and estimating the number of unmethylated, intact *MuDR* elements. (*D–F*) Reversal of *Mu1* element methylation. (*D*) A blot of *Hin*fI-digested DNA samples from family 1511, segregating *mop1-1* homozygotes (mop1) and *mop1-1*/*Mop1-1* heterozygotes (wild type) was probed with a *Mu1* fragment. As controls, the last two lanes contain DNA of plants with *Mu1* and carrying (lane 18) or lacking (lane 19) *MuDR*. (*E*) To control for complete digestion by the restriction enzyme, the blot shown in *D* was stripped and rehybridized with a fragment of the *a1* gene. (*F*) The same blot was stripped and rehybridized with the SB probe from a region upstream of the *b1* gene (see Fig. 5*C* for location). The 1.8-kb fragment is the size expected for no methylation, and the larger fragment represents methylation of a particular site upstream of the *b1* gene.

for *Mu1* and *Mu1.7*, respectively, whereas larger fragments are indicative of methylation in the TIRs. *Mul* methylation as assayed by *Hin*fI digestion has been a reliable indicator of transposon activity in previous studies (1, 13, 16). To control for complete digestion, the blot was probed with a fragment of the *a1* gene flanked by *Hin*fI sites that normally are not methylated (Fig. 2*B*). To assay for changes in methylation of *MuDR* termini, the same samples were digested with *Sac*I and probed with an internal *MuDR* fragment (Fig. 2*C*). *Sac*I cuts once near the ends of each of the *MuDR* TIRs, generating a 4.8-kb fragment if both sites are not methylated. As documented (13, 16), a plant is scored as having methylated *MuDR* elements if the expected 4.8-kb band is missing or reduced in intensity relative to the fragments seen in all maize lines and concomitantly larger fragments appear (Fig. 2*C*). Control hybridization with a single-copy clone from chromosome 2L revealed complete digestion by *Sac*I (data not shown).

In the four *mop1-1* homozygotes in family 1162-3x, the majority of the *Mu1* and *MuDR* elements were unmethylated (Fig. 2 *A* and *C*, lanes 1–4). In contrast, of the wild-type (*mop1-1*/*Mop1* or *Mop1*/*Mop1*) siblings, half (9/17) had mostly methylated *Mu1* elements, and half (8/17) had mostly unmethylated

Table 1. *Mu1* methylation in families (see Fig. 1) segregating for the *mop1-1* mutation

		Progeny phenotypes*	
	Families	*mop1-1*	Wild type
A	1162-2x†	3 (0)	7 (7)
	1162-3x‡	4 (0)	17 (9)
	1181-1x‡	3 (0)	7 (4)
B	1510	14 (0)	15 (15)
	1511	5 (0)	12 (12)
C	1510-1x†	1 (0)	13 (13)
	1510-9x†	3 (0)	7 (7)
	1511-6x†	3 (0)	11 (11)
Total plants		36 (0)	89 (78)

*Progeny were phenotyped based on pigment (dark for *mop1-1* and light for wild type). DNA from leaves was examined for the methylation status of *Mu1* as described in the Fig. 2 legend. The number methylated is indicated in parentheses.

†These families were derived from a parent that had all methylated *Mu* elements.

‡These families were derived from a parent that had predominantly unmethylated *Mu1* elements.

Mu1 elements (Fig. 2*A*, lanes 5–21). The *MuDR* elements were not methylated in *mop1-1* plants, nor were they methylated in wild-type individuals with mostly unmethylated *Mu1* elements (Fig. 2*C*). The wild-type plants with methylated *Mu1* elements did have the fragment diagnostic for the unmethylated full-length *MuDR* element, but it was reduced in intensity, and that reduction was accompanied by the appearance of additional, larger fragments (Fig. 2*C*). These results are consistent with the methylation of sites within the *MuDR* and *Mu1* TIRs seen previously in plants undergoing epigenetic silencing (1, 16, 17) and presumably are caused by the activity of *MuK* in this family.

A total of 41 progeny from self-fertilizations of *mop1-1*/*Mop1* heterozygotes from this first generation were examined including 10 *mop1-1* homozygotes and 31 of their wild-type siblings (Table 1). The *Mu1* elements in all 10 of the *mop1-1* homozygotes from the three families were mostly unmethylated (1181-1x, 1162-3x, and 1162-2x). Among wild-type siblings the frequency of *Mu1* methylation in *Mop1*/*Mop1* and *Mop1*/*mop1-1* individuals depended on the methylation status of the parent, which varies because it sometimes takes more than one generation for *MuK* to silence the *Mutator* system fully (D.L., unpublished data). In the two families generated from the self-fertilization of plants that had unmethylated *Mu1* elements (1162-3x and 1181-1x), 13 of the 24 plants with the wild-type phenotype (*Mop1*/*Mop1* or *mop1-1*/*Mop1*) had methylated *Mu1* elements. In the family generated from a parent with methylated *Mu1* elements (1162-2x), all seven wild-type progeny had methylated *Mu1* elements. If *mop1-1* were not affecting methylation, we would have expected six of the *mop1-1* mutants to have had methylated *Mu1* elements based on the frequency of methylation in the wild-type sibs (20/31). The absence of any such plants is significant ($P = 0.05$ from χ^2 test).

The *mop1-1* Mutation Reverses the Effects of Previous Methylation of the *Mu* Elements. To test whether *mop1-1* reverses *Mu1* methylation, *mop1-1* homozygotes from the 1162-3x family were crossed to three different wild-type siblings with inactive methylated *Mu* elements. One ear produced no *mop1-1* plants, suggesting that the inactive parent was *Mop1*-homozygous. Progeny from the two other ears (1510 and 1511) segregated plants with the *mop1-1* phenotype. A methylation analysis of family 1511 is shown in Fig. 2 *D–F*. This family was derived from a cross between the two siblings, the DNA of which was analyzed in Fig.

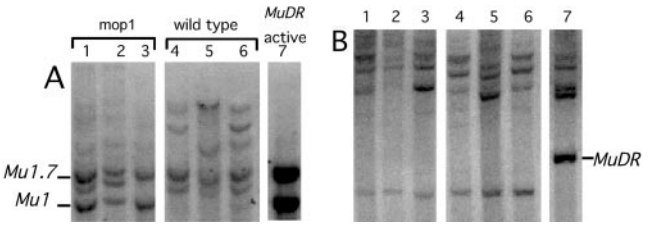

Fig. 3. Reversal of *Mu1* element methylation in the absence of *MuDR*. DNA was isolated from a family segregating for *mop1-1*/*mop1-1* (mop1, lanes 1–3) and *mop1-1*/*Mop1* or *Mop1*/*Mop1* (wild type, lanes 4–6). In *A* and *B*, lane 7 contains DNA from a *MuDR*-active individual. The diagnostic fragments for a full-length unmethylated *MuDR* element and unmethylated *Mu1* and *Mu1.7* elements are indicated. (*A*) A *Hin*fI digest of DNA was blotted and hybridized with the *Mu1* internal probe. The 1.7-kb fragment visible in lanes 1–6 represents an endogenous *Mu1.7*-homologous *Hin*fI fragment present in many maize lines (32). (*B*) DNA from the same family digested with *Sac*I, blotted, and probed with an internal fragment of *MuDR*. Digestions with additional methylation-insensitive enzymes confirmed that no intact *MuDR* elements were present (data not shown).

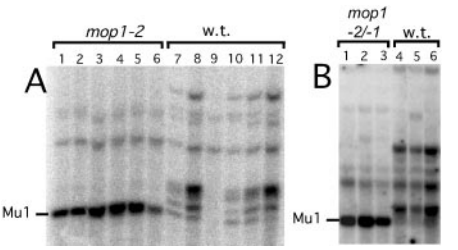

Fig. 4. Reversal of *Mu1* element methylation in *mop1-2* plants. (*A*) DNA was isolated from a family segregating *mop1-2* and wild type (*mop1-2*/*Mop1* and *Mop1*, w.t.). (*B*) DNA was isolated from a family that was segregating *mop1-2*/*mop1-1* and wild type (*mop1-2*/*Mop1*). A *Hin*fI digest of DNA was blotted and hybridized with the *Mu1* interval probe. The diagnostic fragment for an unmethylated *Mu1* element is indicated.

2*A*, lanes 4 and 15. In both families, all the resulting *mop1-1* plants carried predominantly unmethylated *Mu1* elements (Table 1 and Fig. 2*D*, lanes 1–5). In contrast, none of the wild-type *Mop1*/*mop1-1* plants carried any unmethylated *Mu1* elements (Fig. 2*D*, lanes 6–17). These results suggest that the *mop1-1* mutation reverses previously established *Mu1* element methylation. Additional plants heterozygous for *mop1-1* and carrying methylated *Mu1* elements were self-fertilized (Fig. 1*C*), and scored for both the *mop1-1* phenotype (dark-purple pigment) and methylation of *Mu1* elements (families 1510-1x, 1510-9x, and 1511-6x, Table 1). All seven of the *mop1-1* homozygotes had predominantly unmethylated *Mu1* elements relative to their wild-type siblings, all of which carried only methylated *Mu1* elements. Thus, the *mop1-1* mutation reliably reverses previously established methylation of *Mu1* elements.

The *mop1-1* Mutation Reverses *Mu1* Methylation in the Absence of Full-Length *MuDR* Elements. Although *mop1-1* was derived from a *Mutator* line, in the process of propagating the stocks we identified several lineages that lacked intact, functional *MuDR* elements by using *Sac*I or *Xba*I or with an *Eco*RI/*Hin*dIII double-digest (data not shown). These stocks allowed us to test whether an intact *MuDR* element is required for *mop1-1*-mediated decreases in *Mu1* element methylation. We examined progeny from two families derived from the self-fertilization of plants heterozygous for *mop1-1* and carrying no intact *MuDR* elements. Eight *mop1-1* mutant and eight wild-type siblings were examined. The *Mu1* elements of all the *mop1-1* plants were unmethylated relative to the *Mu1* elements of their wild-type siblings (one family in Fig. 3*A*). Thus, reversal of *Mu1* element methylation occurs efficiently in *mop1-1* plants independent of intact *MuDR* elements (Fig. 3*B*). Normally, there is a very tight correlation between reduced *Mu1* element methylation and the presence of intact, active *MuDR* elements, suggesting that the *MuDR* transposase is required to prevent a default methylation pathway that targets *Mu* elements for inactivation (27). The *mop1-1* homozygous plants lacking functional *MuDR* elements provide the first exception to this rule.

A Second Allele of *mop1* Also Reduces *Mu1* Methylation. To eliminate the possibility that there was a factor modifying *Mu1* methylation that was only coincidentally linked to *mop1-1*, we examined a family segregating for a different recessive allele of *mop1*, *mop1-2*. This family was derived from a non-*Mutator* stock that had been ethyl methanesulfonate-mutagenized (21, 23). Non-*Mutator* stocks typically contain one to a few methylated *Mu1* elements but no *MuDR* elements (28). DNA from six *mop1-2* mutants and six wild-type siblings, *Mop1*/*mop1-2* and *Mop1*/*Mop1*, were digested with *Hin*fI and probed with *Mu1* (Fig. 4*A*). We also examined plants heterozygous for *mop1-1* and *mop1-2* (Fig. 4*B*). As with *mop1-1*, the *Mu1* elements in the *mop1-2* and *mop1-2*/*mop1-1* plants were mostly unmethylated relative to their wild-type siblings (Fig. 4 *A* and *B*).

The *mop1-1* Mutation Does Not Reverse Methylation of Sites Immediately Upstream of the *b1* Locus. Given that the *mop1-1* mutation correlates with reduced *Mu1* methylation, we wanted to determine whether methylation of sequences elsewhere in the genome were affected. Characterization of rDNA and centromere repeats had shown no methylation differences in these repeats between *mop1-1* and wild-type siblings (21). Previous studies identified a number of sites upstream of the *B′* transcription unit (GenBank accession nos. X70790 and S48060) that were methylated (25, 26). We tested whether these sites were undermethylated in *mop1-1*. In our experiments both *B′ mop1-1* and *B′ Mop1*/*mop1-1* plants were methylated equivalently at several sites as documented previously for *B′ Mop1* (25, 26). These sites include the *Hin*fI site 1.8 kb upstream of the transcriptional start site (Fig. 2*F*) and the *Apa*I, *Sal*I, and *Pvu*II sites (Fig. 5). Interestingly, these sites are all within sequences related to transposable elements, suggesting that methylation of transposon-related sequences in the promoter proximal region of *B′* is not reversed by *mop1-1*. These elements include a MITE element immediately upstream of the start of transcription and the *Muse* element (a distant relative of *MuDR*; V.L.C., D. Selinger, and M. Stam, personal observation). This region is not involved in *B′* paramutation (20, 25). Once key paramutation sequences are identified, it will be important to determine whether *mop1* mutations influence their methylation.

Progeny of Plants Homozygous for the *mop1-1* or *mop1-2* Mutations Do Not Show Restoration of *Mu* Element Transposition or Somatic Excision. To test for the effects of the *mop1-1* mutation on reactivation of *Mu* element transposition, the progeny of two *mop1-1* mutant plants and two of their wild-type siblings were assayed for the appearance of new *Mu1* fragments, which is consistent with new transposition events. A total of 30 progeny of *mop1-1* mutant plants were examined. These plants carried previously silenced *MuDR* elements, as well as multiple segregating *Mu1* elements, most of which were unmethylated in *mop1-1* homozygotes. Given the presence of an average of 12 *Mu1* elements in the parents of these families and the average transposition frequency of *Mu1* elements observed in the presence of *MuDR*(*p1*) (10% per element per generation; ref. 13), if *MuDR* elements were reactivated we would have expected at least 36 new restriction fragments representing transposition

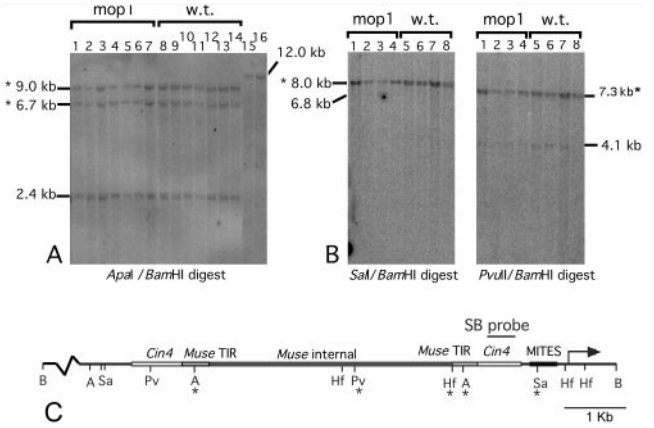

Fig. 5. DNA blots and summary map of digests of DNA from mutant (mop1) and wild-type (w.t.) individuals using several methyl-sensitive enzymes. Hybridization was with the SB probe upstream of the *b1* gene (shown in *C*). (*A*) *Apa*I/*Bam*HI digestions. Lanes 15 and 16 are *mop1-1* homozygous and wild-type individuals, respectively, digested with only *Bam*HI. (*B*) *Sal*I/*Bam*HI and *Pvu*II/*Bam*HI digestions. The fragments marked with asterisks represent cutting at the *Bam*HI site in the *b1* coding region and not cutting at one or more of the indicated upstream sites. (*C*) Restriction map of the region upstream of the start of transcription of *B′*. Sites marked with asterisks are methylated partially or completely in all genotypes tested (*B′ Mop1* or *Mop1*/*mop1-1* or *B′ mop1-1*). The other sites are unmethylated in all genotypes tested. A, *Apa*I; B, *Bam*HI; Pv, *Pvu*II; Hf, *Hin*fI; Sa, *Sal*I.

events (12 progenitor *Mu1* elements × 10% transposition frequency × 30 *mop1-1* individuals). No new fragments were observed in any of the progeny.

To explore this issue further, we used a more sensitive assay, reactivation of somatic excision of a reporter *Mu1* element. Non-*Mutator* plants that lacked full-length *MuDR* elements and were homozygous for *mop1-2* were crossed to plants homozygous for a single *MuDR* element [*MuDR*(*p1*)] that had been silenced by using *MuKiller*. The resulting plants were either self-fertilized or crossed to plants homozygous for *a1-mum2* and *mop1-2*. The resulting ears were screened for the appearance of spotted kernels. If *mop1-2* fully activated the silenced *MuDR* element, 3.5% of the progeny kernels from the self-fertilizations or 12.5% of the test-cross progeny kernels should have excisions (*Materials and Methods*). Twenty-two families consisting of 6,324 progeny kernels resulting from self-fertilization and three families with a total of 710 kernels generated from out-crosses to *a1-mum2* testers were examined. The expected number of spotted kernels from these crosses was 221 and 89, respectively. No spotted kernels were observed in any of these families, indicating no reactivation of the silenced *MuDR* element.

***MuDR* Can Be Reactivated in Progeny of Plants Homozygous for the *mop1-2* Mutation for Multiple Generations.** One concern with the above experiment is that *MuK* was potentially segregating, possibly preventing reactivation by *mop1-2*. It also was possible that the presence of *MuK* in previous generations may have set up a chromatin state that was more difficult to activate, and multiple generations in the presence of *mop1-2* might be required for activation. To determine whether *MuDR* could be reactivated after multiple generations of exposure to *mop1* mutants in a non-*MuK* background, additional crosses with *mop1-2* were done. In all cases, plants homozygous for the *mop1-2* mutation and heterozygous for the *MuDR*(*p1*) element were either self-fertilized or out-crossed with *mop1-2*/*Mop1*, *a1-mum2* (no *MuDR*) plants. Progeny were subjected to DNA gel-blot analysis to determine which carried *MuDR*(*p1*) and unmethylated *Mu1* elements, and their pigment phenotype was scored for *mop1-2* versus wild type. Kernels from all ears were scored for spots indicative of excision of *Mu1* from *a1-mum2*.

Progeny from one plant in which silenced *MuDR*(*p1*) had been in a *mop1-2* homozygous background for two consecutive generations were examined. No spotted kernels were observed in the 288 progeny kernels from the self-fertilization or the 241 kernels from the out-cross. To examine a third consecutive generation, seeds from the out-cross were planted, and all plants were crossed with *mop1-2 a1-mum2* testers, resulting in 33 ears. Of the six ears from plants homozygous for *mop1-2* and carrying *MuDR*(*p1*), four had spotted kernels (29 of the 965 kernels were spotted). The excision frequency in these kernels was variable, ranging from only a few excisions per kernel to a frequency typical for a single active *MuDR* element. In contrast, in 13 ears generated from plants heterozygous for *mop1-2* and that carried *MuDR*(*p1*), 0 of 3,309 kernels were spotted. Similarly, in 9 ears generated from plants homozygous for *mop1-2* but that lacked *MuDR*(*p1*), 0 of 1,900 kernels were spotted, and in 5 ears generated from plants heterozygous for *mop1-2* but that lacked *MuDR*(*p1*), 0 of 989 kernels were spotted. The presence of spotted kernels only in the plants homozygous for *mop1-2* and carrying *MuDR*(*p1*) strongly suggests that the mutation is responsible for the sporadic reactivation of *MuDR* in these plants.

Discussion

Our results demonstrate that in *mop1* mutants, in which paramutation is inhibited at three genes, cytosine methylation of *Mu* elements is reduced. One explanation, based on the activation of transposons by "genomic shock" (29), is that the demethylation of *Mu* elements is an indirect effect of genomic stress caused by the defect in *mop1*. We think this explanation is unlikely for several reasons. First, although many *mop1* mutants show pleiotropic developmental effects and look stressed, some *mop1* mutants look quite normal (21), yet without exception all *mop1* mutants show reduced methylation of *Mu* elements. For example, one *mop1-2* homozygote was runty and five were healthy, yet all six had reduced *Mu1* methylation (Fig. 4*A*). Second, we have isolated two paramutation mutants, *rmr1-1* and *rmr2-1*, that have no obvious pleiotropic developmental phenotypes (23), but *Mu1* elements in these mutants show reduced methylation (D.L., unpublished data). Third, we have examined *Mu* element methylation in plants that clearly were under stress, yet we have never seen reduced methylation of *Mu* elements in lines that were silenced previously. Thus, we favor an alternative explanation, which is that the product of the *mop1* gene is involved in maintaining silent states, potentially by mediating chromatin changes that influence DNA methylation of certain sequences.

Our observation that *mop1* mutants affect methylation of both *Sac*I (lacks CG or CXG sequences) and *Hin*fI (has CG sequence) sites suggests that the wild-type *mop1* product may interact with both *de novo* and maintenance methylation pathways. The change in methylation did not lead to an immediate reactivation of transposition. However, after several generations of exposure of an epigenetically silenced *MuDR* element to a *mop1* mutant background, *MuDR* activity was restored.

Because both *Mu* elements and paramutagenic alleles are affected by mutations in *mop1*, it is probable that these two genetic systems share targets for epigenetic modification. Clearly, the targets are not something as broad as all methylated sequences, because not everything that is methylated is altered by mutations in *mop1*. The targets for silencing are unlikely to be specific sequences or specific chromosomal locations, because there is little sequence similarity between the genetic elements affected by *mop1* mutations, and the affected sequences are distributed throughout the genome. It is also unlikely that *mop1* mutations are simply reversing silenced sequences within condensed heterochromatin, because *Mu* elements typically insert within or nearby single-copy sequences (30). However, not all

Mu1 elements are unmethylated in *mop1-1* homozygotes (e.g., see Figs. 2*D* and 3*A*). Thus, there may be variation in the capacity of the *mop1-1* mutation to reverse *Mu1* methylation as a function of the element's chromosomal position or chromatin context.

Although there is an excellent and immediate correlation between reduced *Mu* element methylation in plants homozygous for *mop1* mutations, reactivation of *Mu* element somatic excision was delayed and sporadic. The lack of immediate reactivation of silenced *MuDR* elements in *mop1* mutant backgrounds suggests that methylation is only part of the process of *Mu* element silencing. Previous studies with *Mutator* lines demonstrated that methylation follows rather than causes inactivation (13). In these experiments, methylation of the nonautonomous elements is restored rapidly after the loss of a single *MuDR* element because of deletions within the *MuDR* element during somatic development or loss of the element after genetic segregation, suggesting *MuDR* activity prevents *Mu1* methylation. Our data suggest that *MuDR* methylation is separable from *MuDR* silencing, because removal of methylation does not cause immediate reactivation. Experiments with *MOM* mutants in *Arabidopsis* (31), which showed reactivation of silenced transgenes in the presence of continued methylation, also indicate that methylation and silencing are separable.

Our experiments demonstrate that MOP1 is required for *Mu* element methylation, but the absence of MOP1 only gradually results in the reversal of *MuDR* silencing, which is similar to the activation of transposons seen with *ddm1* mutations in *Arabidopsis* (8, 18). Multiple mutations have been isolated that affect paramutation (23), and mutations in three genes, *rmr1*, *rmr2* and *mop3*, also reduce *Mu* element methylation (D.L. and V.L.C., unpublished data). It will be interesting to examine whether silenced *MuDR* elements would be reactivated more rapidly in double- or triple-mutant backgrounds. The gradual, sporadic increase in *MuDR* activation by *mop1* mutants contrasts with the immediate increase in the transcription of the paramutant *B′* and *Pl′* alleles in all homozygous *mop1* mutant plants (21). The two phenomena are distinct at the transcriptional level as the transposons are silenced completely in wild-type maize and *Arabidopsis* plants, whereas transcription is reduced but detectable from the paramutant *B′* and *Pl′* alleles. The slower response to *mop1* mutations by transposons versus paramutant alleles may reflect distinct chromatin states.

Until we know the identity of *mop1*, we can only speculate on its specific mode of action. One possibility is that there is a general chromatin code for directing both methylation and transcriptional repression. We hypothesize that the two genes, *mop1* in maize and *DDM1* in *Arabidopsis*, function early to interpret the code, because mutations in these genes affect both methylation and transcriptional repression. In contrast, the *MOM* gene may act downstream, translating the chromatin code into transcriptional repression but not methylation. Although the phenotypes of *mop1* and *DDM1* are very similar, there is an interesting difference in that *DDM1* mutants have a global effect on DNA methylation, whereas the targets of *mop1* appear more limited. Further studies should reveal whether this difference is because these two genes encode distinct functions or because of differences between chromatin regulation in maize and *Arabidopsis*.

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