A Position Effect on the Heritability of Epigenetic Silencing

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Abstract

In animals and yeast, position effects have been well documented. In animals, the best example of this process is Position Effect Variegation (PEV). In PEV, when genes are moved into close proximity to constitutive heterochromatin, their expression can become unstable, resulting in variegated patches of gene expression. This process is regulated by a variety of proteins implicated in both chromatin remodeling and RNAi-based silencing. A similar phenomenon is observed when transgenes are inserted into heterochromatic regions in fission yeast. In contrast, there are few examples of position effects in plants, and there are no documented examples in either plants or animals for positions that are associated with the reversal of previously established silenced states. MuDR transposons in maize can be heritably silenced by a naturally occurring rearranged version of MuDR. This element, Muk, produces a long hairpin RNA molecule that can trigger DNA methylation and heritable silencing of one or many MuDR elements. In most cases, MuDR elements remain inactive even after Muk segregates away. Thus, Muk-induced silencing involves a directed and heritable change in gene activity in the absence of changes in DNA sequence. Using classical genetic analysis, we have identified an exceptional position at which MuDR element silencing is unstable. Muk effectively silences the MuDR element at this position. However, after Muk is segregated away, element activity is restored. This restoration is accompanied by a reversal of DNA methylation. To our knowledge, this is the first documented example of a position effect that is associated with the reversal of epigenetic silencing. This observation suggests that there are cis-acting sequences that alter the propensity of an epigenetically silenced gene to remain inactive. This raises the interesting possibility that an important feature of local chromatin environments may be the capacity to erase previously established epigenetic marks.

Introduction

Whether or not a gene is expressed can depend as much on its location within the genome as its primary DNA sequence. Although proximity to enhancers and suppressors outside the core promoter can affect gene expression, the most dramatic position effects often involve epigenetic silencing of genes placed in proximity to inactive or heterochromatic regions of the genome. In animals, the best example of this process is Position Effect Variegation (PEV) in Drosophila melanogaster [1,2]. In PEV, when genes are moved into close proximity to constitutive heterochromatin, their activity can become unstable, resulting in variegated patches of gene expression. This process is regulated by a variety of proteins implicated in both chromatin remodeling [3–5] and RNAi-based silencing [6]. PEV appears to be the result of the spreading of a compacted chromatin state from heterochromatin to adjacent genes. Given that heterochromatin is largely composed of transposable elements, PEV can be seen as a breakdown in the normal process by which transposable elements and host genes are effectively sequestered from each other. The spread of heterochromatin can be blocked by insulating sites, such as those bound by Suppressor of Hairy-wing [7,8] and GAGA factor [9,10]. These proteins are competent to alter the silenced state by actively remodeling chromatin. Interestingly, some of the same proteins, such as GAGA factor, are also involved in the epigenetic regulation of homeobox genes during Drosophila development. These observations suggest that the process by which transposable elements are sequestered from the rest of the genome may have been recruited to regulate host gene expression as well.

Phenomena similar to PEV have also been observed in Schizosaccharomyces pombe. In this case, transgenes integrated into centromeric heterochromatin or silent mating type loci become silenced [1]. Many of the proteins that have been identified that influence this process are conserved among eukaryotes [11], and can affect gene silencing in species as diverse as Arabidopsis thaliana [12] and humans [13]. A number of proteins that influence centromeric silencing in S. pombe have orthologs in Drosophila that regulate PEV [11]. Thus, there are clear and consistent relationships between position effects, chromatin structure and epigenetic silencing.

Although a great deal is known about position effects in Drosophila and fission yeast, very little is known about it in plants. Indeed, there is conflicting evidence as to whether or not they exist at all in plants [14–16]. Certainly, transgenes equipped with minimal promoters can respond to local tissue-specific enhancers [17], but position-specific effects on the epigenetic state of genes, such as has been observed in Drosophila and yeast, have not been well documented. In plants, variations in expression of transgenes...
At various locations have been interpreted as “position effects”. However, the stochastic nature of transgene silencing, variations in copy number and sequence of integrated transgenes and sporadic tissue-culture induced epigenetic variation make interpretation of these experiments difficult.

Ideally, to prove a position effect, the effect should be reversible due to subsequent changes in position. Since transposable elements are mobile, they represent an ideal model for understanding the role of position in gene activity. Among transposable elements, the Mutator (Mu) transposons in maize are particularly useful because they transpose at a high frequency and can be epigenetically silenced in a controlled fashion [18]. Mutator is the most active known plant transposon. In Mu-active lines, Mu elements can duplicate at a 100% frequency; every element makes an average of one duplication every generation [19]. Insertions are into unlinked sites, and the overall mutation frequency in an active line can exceed 50 times that of background [20]. The system is regulated by MuDR elements, which carry two genes: mudaA and mudaB [18]. These genes encode MURA, the putative transposase, and MURB, a helper protein of unknown function. We have derived a minimal version of this transposon system, that includes a single active MuDR element and a single non-autonomous reporter element inserted into a color gene [21]. In the presence of an active MuDR element, the non-autonomous element excises from the color gene during somatic development, resulting in small sectors of revertant tissue. Unlike higher copy number Mu lines, the minimal line does not undergo spontaneous silencing. However, a single derivative of MuDR arose in the minimal line that can heritably silence one or many MuDR elements [22]. This derivative, called Mu killer (Muk), contains a portion of MuDR that has been duplicated and inverted. The Muk transcript forms a perfect 2.4 kb hairpin RNA, which is processed into 26 nt siRNAs [23]. These siRNAs trigger rapid degradation of the mudaA transcript, as well as methylation of the terminal inverted repeats (TIRs) and transcriptional silencing of one or many MuDR elements. After exposure to Muk, MuDR elements generally remain heritably and stably silenced even in the absence of Muk. The availability of the Muk locus has made it possible to target MuDR elements for heritable epigenetic silencing in a controlled and reproducible fashion by making the appropriate genetic crosses.

The minimal Mutator line began with a single active MuDR element that can move from place to place in the genome. It was therefore possible to examine the effects of Muk on duplicate copies of the same MuDR element at various positions. Given that Muk-mediated silencing of MuDR involves trans-acting siRNAs, it seemed likely that, regardless of position, all MuDR elements would be silenced in the presence of Muk. In fact, we have found that silencing is particularly effective when multiple MuDR elements are present (Sloktin and Lisch, unpublished data). However, it was also possible that the degree to which individual elements would remain heritably silenced in the absence of Muk might vary depending on the local context. A screen was developed that made it possible to isolate individual duplications of a single active MuDR element, expose them to Muk, and observe the degree of heritable activity in progeny plants that carried the transposed copies of MuDR but that lacked Muk. This screen lead to the identification of a MuDR element at a specific chromosomal location that failed to maintain a heritably silenced state. We suggest that this phenomenon represents the converse of PEV, in that cis acting sequences in this case are responsible for reversing, as opposed to triggering, epigenetic silencing. The existence of such a locus suggests that an important feature of the epigenome may be the capacity to reverse epigenetic silencing.

**Materials and Methods**

**MuDR Terminology**

All MuDR elements described in this manuscript were derived from a single MuDR element that had been genetically isolated and cloned previously [21]. We have found that there are variations in duplication frequency and somatic activity depending on the position of transposed copies of this element [24]. Therefore, the elements at various positions are given distinct position numbers, indicated by parentheses. Thus, the original element is designated MuDR(p1) and duplicates are given new position numbers as they are characterized.

**Maize Stocks**

The derivation of all families described in this manuscript is shown in Figure 1. This diagram follows standard conventions. Females are on the left and males on the right of the “x”. Unlinked loci are separated by a semicolon. MuDR elements at each position (designated “p4” or “p5”) are hemizygous for the insertion. All MuDR elements described here are derived from a single MuDR element originally present in the minimal Mutator line. The derivation of the minimal line, containing a single MuDR element and a single Mu1 element inserted into the a1-mum2 allele of the A1 gene was described in Chomet et al. [21]. In the presence of active MuDR elements, Mu1 excises from the a1-mum2 allele, resulting in characteristically small revertant sectors (spots). These sectors are most readily visualized in the outer layer of the kernel (the aleurone). In the absence of MuDR, the reporter element remains inserted in the A1 gene, and the kernels are uniformly colorless, or pale. The a1-mum2 allele has the additional advantage of being suppressible in the adult tissues (but not in the kernel). In the adult tissues, expression of a functional gene product from a1-mum2 is prevented by the presence of MuDR transposase (MURA), except when Mu1 excises from the allele. This results in characteristically small red (revertant) spots of color on a green (suppressed) background. When the transposase is lost, the adult tissue is
uniformly red because the a1-mum2 allele expresses in its absence [21]. This characteristic makes it possible to assay for transposase activity in mature plant tissue. In contrast, the aleurone layer of the kernels, a1-mum2 is not suppressible. Thus, in the absence of the transposase, the kernels are uniformly pale, as can be seen in Figure 2A. All individuals described in this work were homozygous for the a1-mum2 reporter allele. All crosses designated as “test crosses” represent crosses to the a1-mum2 tester, which lacks both functional MuDR elements and Mu killer. The genetic isolation, characterization and cloning of Muk was described in Slotkin et al. [22,23]. Genetic isolation and characterization of MuDR(p3) was described in Lisch et al. [24]. MuDR(p3) causes a distinctively low frequency of somatic excisions of Muf1 from a1-mum2 in the aleurone of the kernel. When MuDR(p3) transposes to a new position, somatic excision returns to a more typical frequency. Thus, germinally transmitted transpositions of MuDR from position 3 to a more typical position can be detected as heavily spotted kernels in a family segregating for weakly spotted kernels. With respect to the crosses of Muk to plants carrying MuDR(p3), previous work has demonstrated that, when Muk is used as a male parent there is little or no effect on excision of the reporter Muf1 element in the F1 aleurone, but a strong effect on MuDR elements in the F1 embryo and adult plant tissue [22]. Thus, transposed copies of MuDR(p3) can be easily detected as individual kernels with a high frequency of somatic excision of the reporter element in the aleurone, even when exposed to Muk derived from the male parent.

DNA Extraction and Southern Blot Analysis

DNA extraction and Southern blotting was as previously described [24]. Briefly, 10 micrograms of DNA was digested with a four-fold excess of restriction enzyme for a minimum of 2 hours, blotted and probed with a series of Mu-specific DNA fragments. Probes: The location of restriction enzyme sites and probes used are illustrated in Figure 3. The probes used to detect MuDR internal sequences (probes A and B) were as described in Slotkin et. al. [22]. The probe used for Muf1 (probe C) was as described in Chomet et al. [21]. The probe for the MuDR TIR was generated by amplifying genomic DNA with primers TIRAF (GAGAGAGGACGGTGACAGAAGTTCACTTATAGACGAAG) and TIRAR (AGGAGAGGACGGTGACAGAAGGAGTA), which generates a fragment of 219 bp that includes the entire TIR (TIRA) flanking the mudrA gene of MuDR.

Active MuDR elements, regardless of their position would be expected to yield a fragment of 445 bp when digested with HinfI. This size is consistent with a lack of methylation of both the HinfI site within the TIR adjoining mudrA (TIRA) of MuDR elements and of a second site within the first intron of mudrA. Methylation of
the TIR_Hinfl site of TIRA of MuDR elements will yield larger fragments whose size depends on the MuDR insertion sites. Based on the sequence of DNA flanking MuDR(p4) and MuDR(p5), if the TIR_Hinfl site (but not the internal Hinfl site) is methylated the expected fragment sizes are 640 bp and 1003 bp respectively. Similarly, the expected fragment size if the TIR_Hinfl site is methylated in Mu killer is 500 bp. In each case the expected fragment sizes were observed (Figure 4B).

Hypomethylation of Mu1_Hinfl sites has proved to be a highly reliable indicator of MURA activity in our lines; the loss of mudrA transcript is invariably associated with hypomethylation of this site [21, 24]. Methylation of Mu1 elements was examined using Hinfl digests probed with an internal fragment of Mu1, as described in Chomet et al. [21]. An unmethylated Mu1 element at a1-mum2 is expected to give a fragment size of 1.3 kb; a methylated Mu1 element at this locus gives a fragment size of 2.1 kb. In all cases, complete digestion of the DNA was confirmed by examination of the ethidium-stained gel.

To determine if full-length MuDR elements were present, Saci was used. MuDR elements have two SacI sites in the terminal inverted repeats (Figure 3). Digestion with this enzyme results in a diagnostic 4.7 kb fragment regardless of chromosomal position. The intensity of this fragment reflects the copy number of the element [24]. To detect transposed copies of MuDR, DNA samples were digested with EcoRI (Figure 2B) or XhoI (Figure 3B). These enzymes cut once within MuDR. Therefore, elements at various positions will give rise to unique fragment sizes.

Cloning MuDR(p4) and MuDR(p5)

Cloning of these elements was achieved using inverse PCR. Southern blot analysis had revealed that digestion of samples containing these elements with XhoI yielded MuDR terminal inverted repeat (TIR)-hybridizing fragments of 2.6 and 2.4 kb for MuDR(p4) and MuDR(p5) respectively (Figure 2). 10 micrograms of DNA containing one or the other element was digested with a four-fold excess of XhoI for 4 hours in a total volume of 20 microliters. The reaction was placed at 65 degrees C for 15 minutes, to heat inactivate the restriction enzyme. Two microliters of the reaction was then added to 1 microliter of DNA ligase, two microliters of ligase buffer and 15 microliters of water, and the resulting mixture was incubated for 2 hours at 25 degrees C. The reaction was then heat inactivated for 15 minutes at 65 degrees C. Two microliters of this reaction was then subjected to PCR amplification using primers specific to the MuDR TIR (TIRout: GCTGTCACCTTTCTGTTTGGCAGAT) and a MuDR internal sequence flanking the XhoI site (exon3R: CTAGCTGTGTTCTCAGTACTCC). These amplifications yielded products of 700 bp and 520 bp for samples containing MuDR(p4) and MuDR(p5) respectively, the expected sizes for these elements based on the XhoI restriction mapping data. Both strands of the PCR products were then sequenced using an ABI sequencer (Applied Biosystems). The sequences of MuDR obtained were identical to known MuDR sequences. The flanking sequences were used to design primers facing inwards towards the MuDR elements. These primers in combination with MuDR TIR primers were used to confirm that we had indeed cloned the elements. Flanking primers were used in combination with TIR-specific primers on DNA samples of plants segregating for each element. For MuDR(p5), primer p5hmK (CGATTAGCCGGGACCAAGAG) was used in combination with R1TIR2 (ATGTGACCCCTAGAGCA). In a family segregating for MuDR(p5) and MuDR(p4), these primers gave a product of 408 bp in three of three plants carrying only MuDR(p5) and zero of three plants carrying only MuDR(p4). To obtain sequences on

Figure 2. An ear derived from a plant carrying MuDR(p3) and Southern blot of DNA from plants grown from the test cross and the cross to a Muk homozygote. A) An ear derived from a plant carrying MuDR(p3) that was crossed as a female to a plant that was homozygous for Muk. Because Muk does not alter somatic excision frequency in F1 aleurone, changes in excision frequency from low to high could be used to screen for new insertions of MuDR(p3), as is indicated. Kerneks from this ear and the control test cross ear (MuDR(p3) x a1-mum2 tester, not shown) were separated by excision frequency and planted. B) Southern blot of DNA from plants grown from the test cross (lanes 1–9) and the cross to a Muk homozygote (lanes 11–14). In the top panel, the DNA was digested with EcoRI, used to distinguish MuDR elements at different positions based on size polymorphisms, and probed with an internal fragment of MuDR (probe B, Figure 3). The red arrows indicate new MuDR insertions. In the bottom panel, the DNA was digested with the methyl-sensitive enzyme HindIII and probed with an internal portion of Mu1 (probe C, Figure 3). The resulting fragments resulting from methylated and unmethylated HindIII sites in the end of the Mu1 element at the a1-mum2 reporter are as indicated. Following analysis of the DNA, each plant was then crossed to an a1-mum2 tester. The numbers below the blots indicate the percent frequency of the test crosses of plants in the lanes above them.

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the other side of the insertion, the available flanking sequences were used to search DNA databases for maize sequence matches. Perfect matches were used to extend the sequence, which were then used to design primers that would be expected to amplify when used in combination with a MuDR TIR primer. Primer p5flnkA (GGAGCGTGACAGGGGCGGCAGAT) was used with primer TIRAR (AGGAGAGACGGTGACAAGAGGAGTA). The same samples that yielded a product with the p5flnkB/RLTIR2 combination also yielded the expected 405 bp product. When the sequences of the DNA flanking the insertion were compared, they revealed the presence of a 9 bp target site duplication (GGCGTGCGC) diagnostic for Mu insertions. The strategy to confirm the MuDR(p4) was similar. The available sequence was used to design a flanking primer, p4flnkB (CGTGAAAGGTGGAGACTACTGGAA), which was used in combination with the MuDR TIRAR primer. A product of the expected size of 320 bp cosegregated with the presence of MuDR(p4), confirming that we had also cloned sequences flanking MuDR(p4).

Results

Transposed MuDR Elements Are Silenced by Muk

In order to screen for new insertions of single MuDR elements, we made use of a MuDR element that exhibits a position effect that results in reduced somatic excision of non-autonomous reporter element from a color gene in the aleurone (Figure 2A)(for alleles and stock construction see Materials and Methods). This effect on somatic excisions of the reporter is fully reversible; when MuDR(p3) transposes to a new position, the high frequency of excision and transposition more typical for MuDR are restored [24]. The advantage of using MuDR(p3) is that, in a family of kernels segregating for this element, new insertions of MuDR(p3) can be readily visualized as individual kernels exhibiting a high

Figure 3. Southern blot analysis of a family segregating for MuDR(p5), MuDR(p4) and Muk. Kernels were separated by somatic excision frequency and DNA was extracted from plants grown from those kernels. A) A SacI digest probed with a fragment of MuDR (probe B). The diagnostic 4.9 kb MuDR fragment is as indicated. The smaller Muk-specific fragment is as indicated, as is as the larger fragment that results from methylation of the SacI site in the Muk TIR (red arrow). B) An XhoI digest of the same samples probed with a second fragment of MuDR (probe A). Polymorphisms specific to MuDR at each of two positions are as indicated. C) A HindII digest of the same samples probed with an internal fragment of Mu1 (probe C). Fragments corresponding to unmethylated and methylated Mu1 elements in this background are as indicated. D) A restriction map of MuDR with probe regions as indicated. The red arrows indicate TIRs. E) A restriction map of Mu1 at a1-mum2.

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frequency of excisions. It should be emphasized that when \textit{Muk} is introduced through the male lineage, it has no immediate effect on the F1 aleurone, but it has a strong effect on the F1 embryo and the resulting plant [22]. Thus, individual kernels that inherit a transposed copy of \textit{MuDR(p3)} would be expected to exhibit a high frequency of excision, even in the presence of \textit{Muk}, but plants grown from those kernels would be expected to show reduced or absent \textit{MuDR} activity.

To perform the screen, one ear of a plant carrying \textit{MuDR(p3)} was crossed to the \textit{a1-mum2} tester (the control cross), and a second ear from the same plant was crossed to a plant that was homozygous for \textit{Muk}. These and subsequent crosses are portrayed in Figure 1. DNA from plants grown from weakly spotted and pale (non-spotted) sibling kernels derived from the control cross were examined by Southern blot for the presence of \textit{MuDR(p3)}. As expected, all progeny plants grown from weakly spotted kernels carried the diagnostic 6.8 kb \textit{MuDR(p3)} EcoRI fragment (Figure 2B, lanes 1–4). The other fragments hybridizing to this probe are inactive \textit{MuDR} homologs (\textit{hMuDR}s) that do not positively or negatively affect \textit{Mu} activity in this line [24,25]. Methylation of \textit{Mu1} at \textit{al-mum2} was also assayed because \textit{Mu1} methylation has proved to be a highly reliable indicator of \textit{MuDR} activity. The \textit{Mu1} elements in the individuals carrying \textit{MuDR(p3)} were unmethylated due to the presence of the \textit{MuDR(p3)}-derived transposase (Figure 2C, lanes 1–4). Sibling plants grown from non-spotted kernels that did not inherit \textit{MuDR(p3)} (lanes 5–9) carried methylated \textit{Mu1} elements, a consequence of the absence of a functional \textit{MuDR} element.

In the experimental cross (\textit{MuDR(p3)/-} x \textit{Muk/Muk}), only plants grown from heavily spotted kernels (which were expected to contain duplicate copies of \textit{MuDR(p3)}), were examined (Figure 2A, lanes 10–14). In each case, an EcoRI digest revealed that plants grown from these kernels contained at least one new \textit{MuDR} insertion (red arrows in Figure 2B). \textit{Mu} elements transpose duplicatively in the germinal lineage [24]. Therefore, the absence of \textit{MuDR(p3)} in plants grown from some of the heavily spotted kernels was not due to germally transmitted excisions of \textit{MuDR(p3)}. \textit{Mu} elements do, however, often transpose just prior to meiosis. Thus some of these plants carried \textit{MuDR(p3)}, while others carried only transposed copies of that element due to independent assortment of the donor and transposed elements. Previous work in our laboratory has demonstrated that although \textit{Muk} has no effect on \textit{MuDR} activity in the aleurone if \textit{Muk} is introduced via the male parent, it has a strong effect on \textit{MuDR} activity in the F1 embryo and plant [22]. This was observed in the plants grown from the heavily spotted kernels that carried transposed copies of \textit{MuDR(p3)}. Each plant contained \textit{Mu1} elements that were methylated, consistent with the loss of transposase in these plants due to the activity of \textit{Muk} [22] (Figure 2C, lanes 10–14). As described in Materials and Methods, the \textit{a1-mum2} allele is suppressible in the adult plant tissue, resulting in red plants in the absence of \textit{MuDR} activity and green plants with small revertant sectors in its presence [21]. This made it possible to monitor activity by observing plant color. All of the plants carrying \textit{Muk} in this experiment were red, consistent with the loss of \textit{MuDR} activity. We conclude from this experiment that each of these plants contained at least one newly transposed copy of \textit{MuDR(p3)}, and that \textit{Muk} was efficiently silencing all of these elements.

A Transposed Element Becomes Reactivated after the Loss of \textit{Muk}

To test for heritability of silencing, each plant carrying a transposed copy of \textit{MuDR(p3)} was crossed as a female to the \textit{a1-mum2} tester. Typically, the ears resulting from a cross of a plant carrying both \textit{Muk} and one or many \textit{MuDR} will exhibit a low frequency of spotted progeny kernels, most of which are only weakly spotted [22]. This was true for three of the five individuals examined, and these results are consistent with heritable silencing of transposed \textit{MuDR} elements in these plants. A fourth plant gave rise to a higher overall percent of spotted progeny (37\%), but these kernels were uniformly weakly spotted, and this family was not examined further. In contrast, one plant gave rise to an ear with an
unusually high proportion of heavily spotted kernels (Figure 2B, lane 13). Overall, the family derived from the test cross of this plant had 57% (83/147) spotted progeny kernels, roughly half of which (46/83) were more heavily spotted. This plant lacked \textit{MuDR(p3)} and contained two new \textit{MuDR}-hybridizing fragments, which we designated \textit{MuDR(p4)} and \textit{MuDR(p5)}. Progeny kernels were separated into classes based on excision frequency, with the expectation that excision frequency would reflect the degree of heritable activity. The more heavily spotted kernels are designated “heavy” and “medium” in Figure 3. Plants grown from representatives of each excision frequency class were then subjected to Southern blot analysis (Figure 3).

In order to detect the presence of full-length transposed \textit{MuDR} elements, a \textit{Sac}I digest of DNA from this family probed with a fragment of \textit{MuDR} was compared to an \textit{Xho}I digest, also probed with \textit{MuDR}. \textit{Sac}I cuts in the ends of \textit{MuDR} and gives rise to a diagnostic 4.7 kb fragment regardless of the element’s position; in this genetic background only full-length functional \textit{MuDR} elements yield a fragment of this size [24]. Because \textit{Mu} has sequence identity to \textit{MuDR} in the probe region [23], this derivative of \textit{MuDR} can also be observed as a 2.5 kb fragment (i.e. lanes 15,16 and 17 in Figure 3A). \textit{Sac}I sites in \textit{Mu} are subject to partial methylation (Slokin and Lisch, unpublished data), resulting in the larger, 4.2 kb fragment in plants with \textit{Mu} as well (red arrow, Figure 3A).

\textit{Xho}I cuts only once in \textit{MuDR}. Therefore, elements at various chromosomal positions give rise to unique fragment sizes (Figures 3B and 3D). Our analysis revealed that each of two \textit{Xho}I segregating fragments contributed to the intensity of the \textit{Sac}I internal fragment. When both \textit{Xho}I fragments were missing, so was the diagnostic \textit{Sac}I fragment. All spotted kernels gave rise to plants with one or the other \textit{Xho}I fragment; kernels that lacked both \textit{Xho}I fragments (Figure 3, lanes 30, 31, 33, 35 and 36) were uniformly pale and did not transmit spotted progeny kernels when plants grown from those kernels were test crossed. We conclude from this analysis that each \textit{Xho}I fragment represents a full-length \textit{MuDR} element that can condition somatic activity of the reporter. The element that gave the smaller \textit{Xho}I polymorphism was arbitrarily designated \textit{MuDR(p5)} and that which gave the larger polymorphism was designated \textit{MuDR(p4)}.

The DNA samples from this family were also digested with \textit{Hin}I, the methyl-sensitive enzyme that cuts in the ends of the reporter \textit{Mu1} element, and probed with \textit{Mu1}. Strikingly, most (8/10) of the individuals grown from the most heavily spotted kernels contained unmethylated \textit{Mu1} elements (Figure 3C). This reversal of \textit{Mu} element methylation has not been observed before, and suggests that some feature of the \textit{MuDR} elements in these plants had been altered. All plants that carried unmethylated \textit{Mu1} elements carried \textit{MuDR(p5)} and none of them carried \textit{MuDR(p4)} by itself. None of these plants carried \textit{Mu}, but 11/18 (61%) of plants grown from the more weakly spotted kernels did. None of the plants that were grown from weakly spotted kernels had hypomethylated \textit{Mu1}.

Overall, 26/28 (93%) of plants grown from kernels exhibiting any spotting at all carried \textit{MuDR(p5)}. In contrast, only 17/28 (61%) of these plants carried \textit{MuDR(p4)}, as did 3/8 (38%) of the plants grown from pale kernels. These results are consistent with segregation of a single active \textit{MuDR} element (\textit{MuDR(p5)}) and a second, much more weakly active element (\textit{MuDR(p4)}). The presence of \textit{Mu} in roughly half of plants grown from the weakly spotted kernels demonstrated that this locus had been in the parent and was still competent to silence \textit{MuDR} elements.

Each plant from the above family was test crossed to determine the heritability of activity. The genetic ratios of spotted to pale kernels in the next generation were used to determine the copy number and degree of heritable activity of \textit{MuDR} elements in each plant. The resulting families demonstrated an unambiguous relationship between \textit{MuDR(p5)} and heritable activity as assayed by the number of spotted progeny kernels from these test crosses. Plants carrying only \textit{MuDR(p5)} and unmethylated \textit{Mu1} elements gave rise to an average of 50% spotted kernels, consistent with segregation of a single, fully active \textit{MuDR} element (Figure 4A and Table 1). Many of the plants examined that lacked \textit{Mu} and that carried \textit{MuDR(p5)} carried methylated \textit{Mu1} elements in the first generation following the loss of \textit{Mu}. This suggested that in the leaf tissue of these plants, \textit{MuDR} remained inactive. However, these plants exhibited a sectored phenotype with respect to expression of the suppressible \textit{a1-num2} allele in the first generation following the loss of \textit{Mu} (Figure 4D). This phenotype suggests that a reversal of \textit{MuDR(p5)} was occurring in these plants, but that it was incomplete. Supporting this hypothesis, after a second round of test crossing, these plants gave rise to an average of 49% heavily spotted progeny kernels (Figure 4A and Table 1). Together, these data suggest that \textit{MuDR(p5)} eventually reactivated in all cases, but in some plants reactivation was delayed. In contrast, plants carrying only \textit{MuDR(p4)} gave rise to a uniformly low frequency (5%) of very weakly spotted kernels, consistent with a more typically heritable silenced state. Thus, although both \textit{MuDR(p4)} and \textit{MuDR(p5)} had been exposed to \textit{Mu} in a previous generation, \textit{MuDR(p4)} remained silenced, whereas \textit{MuDR(p5)} eventually reverted to an active state in all cases once \textit{Mu} was lost.

Plants carrying both \textit{MuDR(p5)} and \textit{Mu} also gave rise to a high frequency (37%) of spotted kernels, an average of 17% of which were heavily spotted (Figure 4A and Table 1). This ratio is consistent with a second generation of escape from \textit{Mu}, where the progeny of these plants that carried \textit{MuDR(p5)} but that lacked \textit{Mu} had restored somatic activity. Thus, even after two successive generations of exposure to \textit{Mu}, plants carrying \textit{MuDR(p5)} retained the propensity to reactivate after \textit{Mu} was lost. In contrast, lineages carrying only \textit{MuDR(p4)} clearly lacked the propensity to reactivate even after only having been exposed to \textit{Mu} for a single generation.

We also examined methylation at \textit{MuDR} TIRs to see if the reversal of \textit{Mu1} TIR methylation was associated with a reversal of methylation at \textit{MuDR(p5)}. To do this, DNA that had been assayed for \textit{Mu1} methylation (Figure 3C) was again digested with \textit{Hin}I, blotted and probed with a \textit{MuDR} TIR fragment (Figure 4B). This analysis revealed that methylation at \textit{MuDR(p5)} and \textit{Mu1} correlated well.

It was important to show that reactivation of \textit{MuDR(p5)} is a reproducible phenomenon. To do this, a single plant carrying reactivated \textit{MuDR(p5)} was crossed to the \textit{a1-num2} tester and to a \textit{Mu} homozygote. Kernels from the resulting families were grown, assayed for \textit{Mu} element methylation, and test crossed. As expected, all of the plants from the \textit{a1-num2} test cross that inherited \textit{MuDR(p5)} were unmethylated at \textit{Mu1} (Figure 5A) and at \textit{MuDR(p5)} (Figure 5B). All of these plants gave rise to approximately 50% spotted progeny kernels (Table 2). These data confirmed that \textit{MuDR(p5)} remained active in a subsequent generation. In the family derived from the cross between the same \textit{MuDR(p5)}-containing plant and a \textit{Mu} homozygote, progeny plants contained methylated \textit{Mu1} and \textit{MuDR(p5)} (Figure 5A and B). Nevertheless, when these plants were test crossed, an average of 42% of the progeny kernels were spotted, indicating that \textit{MuDR(p5)} had again escaped heritable silencing (Table 2). Plants that did not inherit \textit{MuDR(p5)} did not give rise to any spotted progeny kernels, confirming that activity in this family was specific to \textit{MuDR(p5)}.

**\textit{MuDR(p4)} Reactivation Requires the Presence of an Active \textit{MuDR(p5)} Element**

After the loss of \textit{Mu}, \textit{MuDR(p4)} never reactivated when it was by itself (Table 1). However, \textit{MuDR(p4)} did become heritably
reactivated in the presence of a reactivated MuDR\(p5\) element, but only when MuDR\(p5\) was fully active in the generation immediately following the loss of Muk. Plants that carried an active MuDR\(p5\) element (as judged by hypomethylation of Hinfl sites in both MuDR\(p5\) and Mu1), also carried unmethylated MuDR\(p4\) elements (Figure 4B, lanes 7 and 8). When these plants were test crossed, they gave rise to an average of 77% spotted progeny, consistent with the independent segregation of two active MuDR elements (Table 1). To test this hypothesis, kernels from one of these families were planted and the resulting plants were subjected to Southern blot analysis (Figure 6A) and were test crossed (Table 3). In this family, both MuDR\(p5\) and MuDR\(p4\) cosegregated with Muk activity. All spotted kernels in this family carried either MuDR\(p5\), MuDR\(p4\) or both, while none of the pale kernels had either. Plants carrying either MuDR\(p5\) or MuDR\(p4\) gave rise to an average of 50% and 48% spotted kernels.

### Table 1. Activity of MuDR\(p5\) and MuDR\(p4\) in a family segregating for these elements and Muk.

| Genotype a | Sample b | meth c | hm | weak | pale | T spot | total | % spot | % hm |
|------------|----------|--------|-----|------|------|--------|-------|--------|------|
| PS no Muk unmethylated d | 1 | no | 97 | 0 | 93 | 97 | 190 | 51% | 51% |
| 3 | no | 60 | 0 | 67 | 60 | 127 | 47% | 47% |
| total | 157 | 0 | 160 | 157 | 317 | 50% | 50% |
| PS no Muk methylated e | 10 | yes | 123 | 0 | 127 | 123 | 250 | 49% | 49% |
| 18 | yes | 76 | 0 | 81 | 76 | 157 | 48% | 48% |
| total | 199 | 0 | 208 | 199 | 407 | 49% | 49% |
| PS Muk | 19 | yes | 17 | 14 | 25 | 31 | 56 | 55% | 30% |
| 21 | yes | 36 | 38 | 92 | 74 | 166 | 45% | 22% |
| 25 | yes | 36 | 50 | 181 | 86 | 267 | 32% | 13% |
| 28 | yes | 12 | 14 | 66 | 26 | 92 | 28% | 13% |
| total | 101 | 116 | 364 | 217 | 581 | 37% | 17% |
| Both, no Muk unmethylated d | 4 | no | 97 | 0 | 25 | 97 | 122 | 80% | 80% |
| 5 | no | 131 | 0 | 33 | 131 | 164 | 80% | 80% |
| 6 | no | 228 | 0 | 73 | 228 | 301 | 76% | 76% |
| 7 | no | 144 | 0 | 43 | 144 | 187 | 77% | 77% |
| 8 | no | 79 | 4 | 27 | 83 | 110 | 75% | 72% |
| 9 | no | 107 | 0 | 34 | 107 | 141 | 76% | 76% |
| total | 786 | 4 | 235 | 790 | 1025 | 77% | 77% |
| Both, no Muk methylated e | 2 | yes | 124 | 0 | 116 | 124 | 240 | 52% | 52% |
| 22 | yes | 63 | 9 | 64 | 72 | 136 | 53% | 46% |
| 26 | yes | 35 | 61 | 114 | 96 | 210 | 46% | 17% |
| total | 222 | 70 | 294 | 292 | 586 | 50% | 38% |
| Both Muk | 14 | yes | 47 | 42 | 106 | 89 | 195 | 46% | 24% |
| 15 | yes | 40 | 45 | 88 | 85 | 173 | 49% | 23% |
| total | 87 | 87 | 194 | 174 | 368 | 47% | 24% |
| P4 no Muk | 29 | yes | 5 | 9 | 224 | 14 | 238 | 6% | 2% |
| 34 | yes | 0 | 7 | 150 | 7 | 157 | 4% | 0% |
| total | 5 | 16 | 374 | 21 | 395 | 5% | 1% |
| P4 Muk | 32 | yes | 0 | 0 | 192 | 0 | 192 | 0% | 0% |
| 23 | yes | 3 | 6 | 252 | 9 | 261 | 3% | 1% |
| 24 | yes | 3 | 2 | 38 | 5 | 43 | 12% | 7% |
| total | 6 | 8 | 482 | 14 | 496 | 3% | 1% |
| Neither | 30 | 0 | 0 | 252 | 0 | 252 | 0% | 0% |
| 31 | 0 | 0 | 182 | 0 | 182 | 0% | 0% |
| 35 | 0 | 0 | 238 | 0 | 238 | 0% | 0% |
| 36 | 0 | 0 | 273 | 0 | 273 | 0% | 0% |

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*a* genotype of parents with respect to MuDR\(p5\) (P5), MuDR\(p4\) (P4) and Muk killer (Muk). Each parent plant was genotyped and then crossed to an a1-mum2 tester and the resulting frequencies of heavy/medium (hm), weakly spotted (weak) and pale kernels were tabulated.

*b* parent plant numbers correspond to lane numbers in Figure 3.

*c* methylation status of Mu1 in the parents of the families tabulated here, as determined by the blot in Figure 3C.

*d* parent plants had unmethylated Mu1 and MuDR elements.

*e* parent plants had methylated Mu1 and MuDR elements.

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respectively. Plants that carried both elements gave rise to an average of 78% spotted kernels, consistent with the independent assortment of two unlinked active MuDR elements (Table 3). Those that carried neither element did not give rise to any spotted kernels (data not shown). The elements also showed a positive dosage effect; the most heavily spotted kernels carried both elements (7/7) while the more moderate or weakly spotted kernels (19/19) carried a single MuDR element. These data demonstrate that both elements were active in this family, and that they were the only active elements present. Since MuDR(p4) alone never exhibited reactivation in this family (Table 1), we suggest that MuDR(p5) required the presence of active MuDR(p5) to become reactivated.

For comparison, we examined the heritable activity of MuDR(p4) in plants in which there had been a delay in MuDR(p5) reactivation. As described above, these plants carried methylated MuDR and Mu1 TIRs in the generation immediately after the loss of Muk (Figures 3 and 4). However, when these plants were test crossed, they gave rise to an average of 50% heavily spotted progeny kernels (Table 1). One plant and its progeny were examined in detail. In this plant, both MuDR(p5) and MuDR(p4) had remained at least partially inactive in the first generation after the loss of Muk (Figure 4B, lanes 9 and 10, and 4D). Despite having two potentially active elements, this and all similar families segregated only 50% spotted progeny kernels, as if only one of these two MuDR elements had become reactivated in this generation (Table 1). Southern blot analysis of progeny of this plant revealed that MuDR(p5), but not MuDR(p4), co-segregated with activity (Figure 7A). All the plants grown from spotted kernels in this family carried MuDR(p5), but the presence or absence of MuDR(p4) had no effect on activity; three of ten plants grown from spotted kernels carried MuDR(p4), as did seven of nine plants grown from pale kernels. This experiment demonstrated that MuDR(p4) was not active in this family. It also showed that in this generation, an active MuDR(p5) element had no influence on the

**Table 2. Recapitulation of Silencing and Reactivation of MuDR(p5) by Muk.**

| Genotype * | plant b | hm | weak | pale | T | spot total | %spot | %hm |
|------------|---------|----|------|------|---|-----------|-------|-----|
| P5 no Muk  | 1       | 64 | 0    | 62   | 64| 126       | 51%   | 51% |
|            | 2       | 55 | 0    | 71   | 55| 126       | 44%   | 44% |
|            | 3       | 69 | 0    | 57   | 69| 126       | 55%   | 55% |
|            | 4       | 18 | 0    | 35   | 18| 53        | 34%   | 34% |
|            | 5       | 72 | 52   | 83   | 124| 207       | 60%   | 35% |
|            | 6       | 39 | 0    | 49   | 39| 88        | 44%   | 44% |
|            | total   | 460| 53   | 357  | 513| 870       | 59%   | 53% |
| no P5      | 7       | 216| 0    | 0    |   |           | 0%    | 0%  |
|            | 8       | 111| 0    | 0    |   |           | 0%    | 0%  |
|            | 9       | 11 | 0    | 0    |   |           | 0%    | 0%  |
|            | 10      | 99 | 0    | 0    |   |           | 0%    | 0%  |
|            | 11      | 119| 0    | 0    |   |           | 0%    | 0%  |
|            | total   | 556| 0    | 0    |   |           | 0%    | 0%  |
| P5 with Muk| 13      | 59 | 49   | 88   | 108| 196       | 55%   | 30% |
|            | 14      | 34 | 22   | 80   | 56| 136       | 41%   | 25% |
|            | 15      | 34 | 41   | 102  | 75| 177       | 42%   | 19% |
|            | 16      | 38 | 35   | 110  | 73| 183       | 40%   | 21% |
|            | 17      | 26 | 31   | 94   | 57| 151       | 38%   | 17% |
|            | 18      | 23 | 32   | 83   | 55| 138       | 40%   | 17% |
|            | 19      | 36 | 31   | 59   | 67| 126       | 53%   | 29% |
|            | 20      | 7  | 17   | 153  | 24| 177       | 14%   | 4%  |
|            | 21      | 49 | 50   | 88   | 99| 187       | 53%   | 26% |
|            | total   | 306| 308  | 857  | 614| 1471      | 42%   | 21% |
| no P5      | 22      | 108| 0    | 0    |   |           | 0%    | 0%  |
|            | 23      | 54 | 0    | 0    |   |           | 0%    | 0%  |
|            | 24      | 29 | 0    | 0    |   |           | 0%    | 0%  |
|            | 25      | 64 | 0    | 0    |   |           | 0%    | 0%  |
|            | 26      | 216| 0    | 0    |   |           | 0%    | 0%  |
|            | 27      | 196| 0    | 0    |   |           | 0%    | 0%  |
|            | total   | 667| 0    | 0    |   |           | 0%    | 0%  |

*genotype of parent plants with respect to MuDR(p5) (P5) and Mu killer (Muk). Each plant was crossed to an a1-mum2 tester and the resulting frequencies of heavy/medium (hm), weakly spotted (weak) and pale kernels were tabulated. bplant numbers 1–15 correspond to lane numbers in Figure 5.

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**Figure 5. Genetic and Southern blot analysis of families segregating for MuDR(p5) and Muk.** A) A Hinfl digest of two families probed with an internal portion of Mu1. The first was derived from a cross between a plant carrying an active MuDR(p5) element and an a1-mum2 tester (lanes 1–12); the second was derived from a cross between the same plant carrying MuDR(p5) and a Muk homozygote. Methylated and unmethylated Mu1 elements at a1-mum2 are as indicated. Arrows indicate new insertions of Mu1 elements. B) DNA from representative individuals digested with Hinfl and probed with the mudrA TIR. Fragments resulting from methylated and unmethylated Hinfl sites within the TIR are as indicated, as is the fragment from Muk. Sample designations are the same as in panel A. C) Summarized frequencies of spotted kernels in progeny of test crosses of plants depicted in panel A.

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heritable activity of MuDR(p4). Plants that carried both MuDR(p5) and MuDR(p4), when test crossed, gave rise to only 50% spotted progeny (Table 4 and Figure 7B). Together, these results suggest that MuDR(p4) could be responsive to a reactivated MuDR(p5), but only in the generation immediately following the loss of Muk.

It is unclear as to precisely when MuDR(p5) must be active in order to alter the trajectory of MuDR(p4) silencing. Only those plants that showed hypomethylation at MuDR and Mu1 TIRs that were grown from more heavily spotted kernels gave rise to progeny with active MuDR(p4) elements. This suggests an active MuDR(p5) element was required quite early in development in order to reactivate MuDR(p4). The aleurone and the mature plant are the result of a double fertilization event. One sperm fuses with the egg cell of the female gametophyte to form the embryo. The second fuses to the diploid central cell to give rise to the triploid endosperm. The egg cell and the central cell are derived from a post-meiotic mitotic division in the female gametophyte. With that in mind, it is interesting to note that eight of ten heavily and medium spotted kernels gave rise to plants with hypomethylated Mu elements. In contrast, none of the plants grown from more weakly spotted kernels gave rise to plants with hypomethylated Mu elements. The fact that the methylation status of MuDR(p5) in the mature plant correlated so well with the phenotype of the kernels suggests that MuDR(p5) reactivation that was not delayed most often occurred prior to the post-meiotic mitotic cell division.

Together, these data suggest that the window of opportunity for activation of MuDR(p4) by MuDR(p5) may be a very narrow one. Indeed, it may be restricted to the gametophyte, or even meiosis II.

All Aspects of MuDR Activity Are Restored Following Reactivation

Although somatic excision of a reporter element is a reliable indicator of Mu activity, it only represents one aspect of that activity, which only requires MURA transposase function [26,27].
Insertional activity, either of the reporter element or of MuDR itself requires both mudcA and mudcB expression. The analysis portrayed in Figure 5 demonstrated that a reactivated MuDR(p5) element could cause new insertions of Mu1. When Mu1 is methylated, the size of the fragments following digestion varies depending on the position of the element. The element at a-mum2 is 2.1 kb. Other sizes present in single individuals represent independent new insertions of Mu1. The presence of new Mu1 fragments in progeny of plants that carried reactivated MuDR(p5) (Figure 5A, lanes 8 and 13) indicates that this element can cause new insertions of Mu1, consistent with reactivation of both mudcA and mudcB functions.

We also examined the propensity of reactivated MuDR(p5) and MuDR(p4) to duplicate themselves by test crossing a series of individuals that carried active versions of either MuDR(p5) or MuDR(p4). In each case, the plants were derived from a family that had segregated genetically for a single active MuDR element. In the absence of new duplications of these MuDR elements, the expectation would be that each resulting family would also segregate 50% spotted progeny kernels. Ratios significantly higher than 50% are the result of MuDR duplication events [21]. The frequency of ears showing ratios of spotted kernels significantly greater than 50% provides an estimate of the duplication frequency, which we have shown can vary from position to position [24]. Of 100 ears derived from plants carrying silenced MuDR(p5) in the presence of Muk, none had ratios significantly greater than 50% spotted kernels (data not shown), indicating that MuDR(p5) does not transpose in the presence of Muk. In contrast, following reactivation we found that both MuDR(p4) and MuDR(p5) were competent to transpose at a frequency of 10% for MuDR(p5) and 18% for MuDR(p4) (data not shown). These data demonstrate that although both somatic and transpositional activity of MuDR(p5) is repressed in the presence of Muk, both manifestations of activity are restored once Muk is lost via genetic segregation.

We also wanted to confirm that the “rescue” of MuDR(p4) in the previous experiment was due to the presence of a reactivated MuDR(p5) element. To test the effects of Muk on MuDR(p4) in the absence of MuDR(p5), plants carrying active MuDR(p4) were crossed to Muk heterozygotes, and the resulting plants were then test crossed (Figure 1). As before, unlike MuDR(p5), which showed clear evidence of reactivation following the loss of Muk, MuDR(p4) remained heritably silenced (Table 5). Thus, MuDR(p4) in the absence of an active MuDR(p5) element showed a typical pattern of heritable silencing after being exposed to Muk.

In order to replicate the “rescue experiment”, a plant carrying active MuDR(p5) and MuDR(p4) elements was crossed to a Muk homozygote. Progeny plants were genotyped and test crossed (Figure 8). Plants that carried only MuDR(p4) and Muk gave an average ratio of spotted kernels of 6%, consistent with our previous result that MuDR(p4) without Muk was heritably silenced. Plants that carried Muk with MuDR(p5) alone or with MuDR(p4) gave an average frequency of spotted progeny of 48%, consistent with reactivation of MuDR(p5) following the loss of Muk. Progeny of this cross that carried both MuDR(p5) and MuDR(p4) but that lacked Muk were test crossed again. One individual gave rise to a ratio of spotted kernels of 68%, consistent with the independent segregation of two active elements. In the next generation, somatic activity segregated with both elements; plants carrying both MuDR(p4) and MuDR(p5) gave rise to a 75% ratio, and those with either MuDR(p4) or MuDR(p5) by itself gave rise to roughly 50% ratios (Table 6). These data strongly support the

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**Table 3. Activation of MuDR(p4) by MuDR(p5).**

| genotype | plant | spotted | pale | total | %spot |
|----------|-------|---------|------|-------|-------|
| P4+P5    | 2     | 137     | 40   | 177   | 77%   |
|          | 4     | 145     | 37   | 182   | 80%   |
|          | 6     | 56      | 13   | 69    | 81%   |
|          | 7     | 97      | 30   | 127   | 76%   |
|          | total | 435     | 120  | 555   | 78%   |
| P5 only  | 9     | 82      | 88   | 170   | 48%   |
|          | 10    | 45      | 61   | 106   | 42%   |
|          | 12    | 48      | 47   | 95    | 51%   |
|          | 16    | 84      | 72   | 156   | 54%   |
|          | 20    | 89      | 81   | 170   | 52%   |
|          | total | 348     | 349  | 697   | 50%   |
| P4 only  | 21    | 31      | 31   | 62    | 50%   |
|          | 22    | 13      | 26   | 39    | 33%   |
|          | 23    | 70      | 59   | 129   | 54%   |
|          | 24    | 108     | 113  | 221   | 49%   |
|          | 25    | 79      | 95   | 174   | 45%   |
|          | total | 301     | 324  | 625   | 48%   |

*plant numbers correspond to lane numbers in Figure 6. Note that only a subset of the plants were test crossed.

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**Figure 7. Genetic and Southern blot analysis of a family derived from a plant that carried MuDR(p5) and MuDR(p4) in which reactivation was delayed and both elements were still methylated in the first generation following the loss of Muk. A) XhoI digests of a family segregating for MuDR(p5) and MuDR(p4), in which the female parent carried methylated MuDR(p5) and MuDR(p4). Kernels were separated into classes based on somatic excision frequency, planted, and the resulting progeny plants were subjected to Southern blot analysis. B) Summarized frequency of spotted kernels in progeny of test crosses of plants depicted in panel A.

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hypothesis that, although MuDR(p4) is invariably silenced in the absence of MuDR(p5), a reactivated MuDR(p5) element can cause MuDR(p4) to reactivate as well.

Table 4. Lack of heritable reactivation of MuDR(4).

| genotype | plant * | spotted | pale | total | %spot |
|----------|---------|---------|------|-------|-------|
| P5 only  |         |         |      |       |       |
| 2        | 89      | 90      | 179  | 50%   |
| 5        | 65      | 67      | 132  | 49%   |
| 6        | 82      | 83      | 165  | 50%   |
| 8        | 62      | 57      | 119  | 52%   |
| 9        | 101     | 101     | 202  | 50%   |
| 10       | 47      | 47      | 94   | 50%   |
| total    | 446     | 445     | 891  | 50%   |
| P5+P4    |         |         |      |       |       |
| 1        | 91      | 101     | 192  | 47%   |
| 3        | 57      | 59      | 116  | 49%   |
| 4        | 49      | 39      | 88   | 56%   |
| total    | 197     | 199     | 396  | 50%   |
| P4 only  |         |         |      |       |       |
| 11       | 0       | 145     | 145  | 0%    |
| 14       | 0       | 120     | 120  | 0%    |
| 15       | 5       | 104     | 109  | 5%    |
| 16       | 0       | 57      | 57   | 0%    |
| 17       | 0       | 145     | 145  | 0%    |
| 18       | 6       | 82      | 88   | 7%    |
| 19       | 0       | 103     | 103  | 0%    |
| total    | 11      | 756     | 767  | 1%    |
| neither  |         |         |      |       |       |
| 12       | 0       | 95      | 95   | 0%    |
| 13       | 0       | 98      | 98   | 0%    |
| total    | 0       | 193     | 193  | 0%    |

*plant numbers correspond to lane numbers in Figure 7.
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Table 5. Heritable silencing of MuDR(p4) by Muk.

| Genotype    | plant | spotted | pale | total | %spot |
|-------------|-------|---------|------|-------|-------|
| P4 no Muk   | 1     | 87      | 116  | 203   | 42.9% |
|             | 2     | 186     | 174  | 360   | 51.7% |
|             | 3     | 89      | 87   | 176   | 50.6% |
|             | 4     | 69      | 51   | 120   | 57.5% |
|             | 5     | 65      | 33   | 98    | 66.3% |
|             | 6     | 44      | 44   | 88    | 50.0% |
|             | 7     | 112     | 151  | 263   | 42.6% |
|             | 8     | 79      | 76   | 155   | 51.0% |
| total       | 731   | 732     | 1463 | 50.0% |
| P4 with Muk | 1     | 5       | 338  | 343   | 1.5%  |
|             | 2     | 1       | 308  | 309   | 0.3%  |
|             | 3     | 20      | 252  | 272   | 7.4%  |
|             | 4     | 15      | 252  | 267   | 5.6%  |
|             | 5     | 5       | 112  | 117   | 4.3%  |
| total       | 46    | 1262    | 1308 | 3.5%  |
doi:10.1371/journal.pgen.1000216.t005

A Duplicate Copy of MuDR(p5) Remains Inactive Following Exposure to Muk

If the reactivation effect we observe for MuDR(p5) were a function of position, then we would expect that, if this element transposed to a new position, it would exhibit a more typical heritable response to Muk. To test this hypothesis, plants carrying MuDR(p5), a transposed copy of this element at a second unlinked position and Muk were test crossed (Figure 9). Resulting progeny plants grown from spotted kernels were genotyped for MuDR(p5) and Muk and test crossed a second time (Table 7). Plants carrying MuDR(p5) that lacked Muk gave rise to ears that segregated for one or more active MuDR elements and averaged 55% spotted progeny kernels. In contrast, siblings that inherited only the second MuDR element and not MuDR(p5) gave rise to a much lower frequency of spotted kernels (5%), consistent with the kind of heritable silencing that is typical for MuDR elements after having been exposed to Muk. These results suggest that, while MuDR(p5) reactivates once Muk is segregated away, the duplicate copy of this element remained heritably silenced. These data strongly suggest that the reduction of heritable silencing at MuDR(p5) is a function of chromosomal position and not sequence, since this effect can be reversed following transposition.

MuDR(p5) Is Inserted into the 5’ UTR of a Conserved Gene Near a GA-Rich Sequence

In order to determine the local chromosomal environment around MuDR(p5) and MuDR(p4), inverse PCR was used to clone sequences flanking the insertions. DNA from plants carrying either...
element was digested with XhoI, which gives rise to fragments of 2.6 and 2.4 kb corresponding to MuDR(p4) and MuDR(p5) respectively (Figure 3B). The DNA was then ligated and primers specific to MuDR were used on the circularized fragments to amplify fragments of the expected sizes (see Materials and Methods for details). The products were sequenced on both strands, and the resulting sequences were used to design flanking primers. These primers were then used with MuDR-specific primers on DNA from families segregating for MuDR(p4) or MuDR(p5). In each case, these primer pairs specifically amplified a product only in samples containing the MuDR elements (data not shown). Sequences were extended using publicly available maize genomic sequences, and these sequences were used to design primers matching DNA sequences present to the other side of each element. Nine base pair target site duplications, a characteristic feature of Mu insertion, were identified in each case. Further, XhoI and Hinfi sites in the flanking sequences obtained from the public databases also correlated well with data obtained from Southern blot restriction data.

Given its propensity to reactivate, we were particularly interested in sequences flanking MuDR(p5). This element was inserted into the 5’ UTR just 4 base pairs proximal to the start codon of a putative ORF of unknown function (Figure 10), which we designate here Hemera, after the Greek goddess of the day, who was believed to disperse the night’s mist each morning. Genes homologous to Hemera are also present in dicots, including papaya, grape, Arabidopsis and poplar. Although some of these sequences carry GA or TC-rich regions near the putative start of translation, their positions are not conserved between species (Figure S1).

Table 6. Recapitulation of MuDR(p4) reactivation with MuDR(p5).

| Genotype | plant | spotted | pale | total | % spot |
|----------|-------|---------|------|-------|--------|
| P5       | 1     | 102     | 111  | 213   | 47.9%  |
|          | 2     | 105     | 107  | 212   | 49.5%  |
|          | 3     | 137     | 188  | 325   | 41.1%  |
|          | 4     | 80      | 70   | 160   | 56.3%  |
|          | 5     | 59      | 60   | 119   | 49.6%  |
|          | 6     | 46      | 49   | 95    | 48.4%  |
|          | 7     | 79      | 100  | 179   | 44.1%  |
|          | 8     | 65      | 76   | 141   | 46.1%  |
|          | total | 673     | 761  | 1444  | 47.3%  |
| P4       | 9     | 91      | 107  | 210   | 49.0%  |
|          | 10    | 69      | 63   | 132   | 52.3%  |
|          | 11    | 74      | 106  | 189   | 43.9%  |
|          | total | 234     | 276  | 531   | 48.0%  |
| P5+P4    | 12    | 57      | 22   | 81    | 72.8%  |
|          | 13    | 150     | 44   | 201   | 78.1%  |
|          | 14    | 152     | 53   | 206   | 74.3%  |
|          | 15    | 79      | 34   | 115   | 70.4%  |
|          | total | 438     | 153  | 603   | 74.6%  |

Figure 9. A graphic representation of a lineage in which MuDR(p5) and a duplicate copy of that element were crossed to a Muk heterozygote. Percent figures refer to the summarized frequency of spotted progeny kernels derived from each cross.

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tester x p5

80%

p5; p(new) x Muk/Muk

69%

p5; p(new); Muk x tester

55%

p5 x tester

p(new) x tester

5 %

Figure 9. A graphic representation of a lineage in which MuDR(p5) and a duplicate copy of that element were crossed to a Muk heterozygote. Percent figures refer to the summarized frequency of spotted progeny kernels derived from each cross.

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A Position Effect on Silencing

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Table 7. A transposed copy of MuDR(p5) is heritably silenced by Muk.

| Genotype * | plant | spotted | Pale | Total | % Spot |
|------------|-------|---------|------|-------|--------|
| P5         | 1     | 31      | 30   | 61    | 50.8%  |
|            | 2     | 110     | 107  | 217   | 50.7%  |
|            | 3     | 60      | 63   | 123   | 48.8%  |
|            | 5     | 167     | 76   | 243   | 68.7%  |
|            | 6     | 54      | 61   | 115   | 47.0%  |
|            | 7     | 115     | 47   | 162   | 71.0%  |
|            | 8     | 99      | 133  | 232   | 42.7%  |
|            | 9     | 90      | 26   | 116   | 77.6%  |
|            | 10    | 89      | 53   | 142   | 62.7%  |
|            | 11    | 60      | 59   | 119   | 50.4%  |
|            | 12    | 62      | 233  | 295   | 21.0%  |
|            | 13    | 98      | 30   | 128   | 76.6%  |
|            | 14    | 115     | 79   | 194   | 59.3%  |
|            | 15    | 94      | 100  | 194   | 48.5%  |
|            | 16    | 151     | 52   | 203   | 74.4%  |
|            | 17    | 119     | 127  | 246   | 48.4%  |
|            | 18    | 78      | 34   | 112   | 69.6%  |
| total      | 1751  | 1453    | 3204 | 54.7% |
| P(new)     | 19    | 0       | 210  | 210   | 0.0%   |
|            | 20    | 53      | 112  | 165   | 32.1%  |
|            | 21    | 6       | 65   | 71    | 8.5%   |
|            | 22    | 7       | 195  | 202   | 3.5%   |
|            | 23    | 4       | 72   | 76    | 5.3%   |
|            | 24    | 7       | 203  | 210   | 3.3%   |
|            | 25    | 0       | 216  | 216   | 0.0%   |
|            | 26    | 0       | 42   | 42    | 0.0%   |
|            | 27    | 0       | 30   | 30    | 0.0%   |
|            | 28    | 0       | 273  | 273   | 0.0%   |
| total      | 77    | 1418    | 1495 | 5.2%  |

*all plants lacked Muk. Plants either carried MuDR(p5) or they lacked that element but carried a second element (P(new)).

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**Discussion**

The experiments described here detail a position effect that alters the heritability of the silenced state of a maize transposon. The experiments were possible because of the absence of spontaneous Mu transposon silencing in our lines and the availability of a single silencing locus (Muk) that can reliably and heritably silence MuDR elements. Because heritability is the rule for MuDR silencing by Muk, it was possible to screen for exceptions to this rule in order to uncover variation in the ability of chromosomal positions to maintain silencing over multiple generations. One such exception is MuDR(p5), which fails to maintain silencing. The fact that a transposed copy of MuDR(p5) showed a more typically heritable pattern of Muk-induced silencing demonstrates that MuDR(p5) is exceptional because of its position rather than its sequence.

To our knowledge, this is the only known example of a specific locus competent to reverse epigenetic silencing of flanking sequences. In plants, a related (albeit reversed) phenomenon can be found at the FLC locus in Arabidopsis thaliana. In that case, the FLC gene is apparently competent to alter the activity of neighboring genes via an epigenetically mediated pathway [28]. When a T-DNA encoded resistance gene is integrated near the FLC gene, its expression is down regulated in response to cold temperatures, and this down-regulation is dependent at least in part on components of the small RNA mediated silencing pathway. The difference is that FLC attracts factors that down-regulate gene expression, and Hemera apparently attracts factors that reverse silencing. Nevertheless, in each case it appears that there are cis-acting sequences that can alter the epigenetic state of inserted genes.

Interestingly, the kind of epigenetic resetting we see with MuDR(p5) is typical in animals, although the role of position remains poorly understood. In certain cell types at certain times, massive changes in patterns of histone and DNA methylation are observed. This process, which is thought to be required for the elimination of some epigenetic marks and their replacement with others, is particularly pronounced in the pre-implantation embryo of mammals [29]. The same is true of primordial germ cells, where this process of epigenetic reprogramming is thought to be involved in the restoration of totipotency [30]. In mammals, exceptional instances in which DNA methylation is not lost, are associated with imprinted genes and deeply silenced transposons [31]. In some cases it has been shown that a close association between transposon and host gene can lead to heritable changes in phenotype. For instance, the Agouti viable yellow (A/vy) locus in mice is under the control of an IAP retrotransposon. Hypomethylation of this element results in expression of the gene and yellow coat color. Epigenetic variants of this allele can be transmitted from generation to generation, and it is hypothesized that the heritable epigenetic effects of on A(vy) are due to a failure to remove epigenetic marks due to the close association of the IAP element with the coding sequence [32].

In Drosophila, changes in the efficiency of epigenetic resetting can have important consequences. A hyperactive version of a JAK kinase, hop/Tum-1, causes tumor formation. It does so because counteracts heterochromatic gene silencing, which is an important regulatory pathway for tumor suppression [33,34]. Enhancers of the hop/Tum-1 allele included several components of the heterochromatin formation pathway, including HP1 and several Suppressors of variegation mutations, which were first identified due to their effects on position effect variegation. Remarkably, not only can hop/Tum-1 cause tumors in one generation, but it can increase the propensity for the wild-type offspring of mutant flies to have tumors as well [35]. It is hypothesized that the hop/Tum-1 mutation antagonizes the normal process by which epigenetic states are reset each generation by allowing genes that should be heritably silenced to take on a heritably active state.

Plants are distinct from animals in the sense that they lack a dedicated germ line. Instead, somatic meristem tissue differentiates into germinal cells each generation. A wealth of information suggests that the result of this difference is that epigenetic changes in plants are more readily transmitted from generation to generation [36]. Nevertheless, it is likely that in plants, as in animals, at least a subset of genes in are reset each generation in order to ensure that the epigenetic state of each embryo is roughly equivalent. DNA methylation, for instance, increases in the meristem as it ages, and these changes must presumably be reversed each generation. If Hemera may represent a gene whose epigenetic state must be reset each generation. If Hemera were epigenetically silenced in the floral meristem and upregulated in the embryo, for instance, then perhaps that epigenetic regulation must be relieved during or following meiosis. It will be interesting to see if differences in
expression levels of \textit{Hemera} correlate with changes in chromatin configuration or DNA methylation, and whether or not these changes correlate with changes at \textit{MuDR(p5)}.

It should be emphasized that the variation we observe is not in the propensity to become silenced; \textit{MuDR(p5)} is effectively silenced by \textit{Muk}. Given that \textit{MuDR(p5)} TIR sites are methylated at the \textit{Hin}I site, it is also unlikely that this element is exclusively inactivated at the post-transcriptional level. Rather, the effect we observed appears to be specifically associated with the efficiency with which transcriptional silencing of this element is heritably propagated in the absence of the trigger. The loss of methylation at \textit{MuDR(p5)} may not be a passive process; our assay for methylation, a \textit{Hin}I digest, depends on methylation of a CG site. Since CG methylation can be maintained passively through the activity of maintenance methyl-transferases such as MET1, the loss of methylation at this site may reflect an active de-methylation process. Active demethylation has been observed as a consequence of DNA glycosylase activity in plants, and is often associated with repetitive elements such as transposons [39,40]. It will be interesting to see whether or not the reversal of methylation we see at \textit{MuDR(p5)} is due to similar activity in maize. It will be particularly interesting if mutations of maize DNA glycosylase genes affect \textit{MuDR(p5)} reactivation.

We do not know the cause of the position effect on \textit{MuDR(p5)}. The fact that this element is inserted into an expressed portion of a gene may have been sufficient to reverse silencing, but \textit{Mu} elements often insert into or near genes and nearly all \textit{MuDR} elements are silenced when high copy number \textit{Mu} lines are crossed.
to *Muk* [22]. The presence of GA repeats near the insertion is intriguing, as GA repeats have been associated with programmatic changes in chromatin structure and in particular with the active replacement of histones [9]. Although we have not established that this is the case at *MuDR(p5)*, we do note that the rice and *B. distachyon* homology of *Heman* also have GA-rich sequences just upstream of the start of the ORFs. Although the sequence of the GA-rich regions in the maize, rice and *B. distachyon* genes are not similar in sequence, they do have similar sequence composition (100%, 89% and 96% GA respectively). These blocks of sequences are roughly the same distance from the first ATG of each gene, 82 bp, 83 bp and 89 bp for maize, rice and *B. distachyon* respectively. Given the phylogenetic distance between these species (roughly 50 million years [41]), the conserved positioning of these blocks at the same distance from the start of translation in each gene suggests that they may have a conserved function.

In addition to the position effects we observed, our data also suggests that epigenetically determined states of competency can change over time. Specifically, we provided evidence that a silenced *MuDR(p4)* element could respond to a reactivated *MuDR(p5)* element, but only for a brief period of time. This was revealed because of variations in the rate at which *MuDR(p5)* became reactivated. In some cases, it was immediately after the loss of *Muk*, as evidenced by the high level of somatic activity in the aleurone and the complete loss of methylation in the growing F2 plants (Figures 3 and 4). In these cases, when *MuDR(p4)* was also present, it too was reactivated. However, in those cases in which *MuDR(p5)* reactivation was delayed (weakly spotted kernels, variegated *al-num2* suppression and TIR methylation), *MuDR(p4)* was not reactivated. In the subsequent generation, even though *MuDR(p5)* had become fully reactivated, it had no effect on a previously silenced *MuDR(p4)*. We hypothesize that silencing of *MuDR* elements is a progressive process that involves successively deeper silenced states, from responsive to a second, active element, to refractive to that element. Thus, immediately after *Muk* was lost due to genetic segregation, *MuDR(p4)* silencing was not completely established, and so this element was responsive to active *MuDR(p5)*. After a round of meiosis, *MuDR(p4)* had become fully refractive to *MuDR(p5)*. Perhaps passage through meiosis of a previously silenced transposon acts as a check-point, during which provisionally established silenced states are made more permanent. If our interpretation of the data is correct, then the epigenetic state of *MuDR(p4)* can change over time, even once the silencing trigger (*Muk*) has been lost. This is consistent with what we know about silencing mechanisms in plants, in which chromatin remodeling factors, DNA methylation and siRNAs form a self-reinforcing loop [42]. *MuDR(p4)* silencing may represent an illustration of how this process can deepen a silent state over time, resulting in a shift from competency to respond to a second, active element to a refractive state in the course of a generation. Similarly but conversely, *MuDR(p5)* may represent a process by which silenced states can be reversed over time through the activity of cis-acting factors. The delay in *MuDR(p5)* reactivation in many of the plants examined suggests that reactivation, like silencing, can be a progressive process. Our data suggest that even after a trigger is lost, a series of additional and progressive changes can continue to occur. This is perhaps the most fascinating aspect of epigenetic modifications: time matters. Changes triggered in one generation can manifest themselves over multiple subsequent generations.

Historically, an emphasis has been on mechanisms by which epigenetic information is propagated from generation to generation, a classic example being paramutation [43]. Our data suggest that an equally important process may be the erasure of epigenetic modifications that have occurred in plants in the meristem prior to meiosis. The cis-acting factors that appear to be responsible for reversing *MuDR(p5)* silencing may provide an important clue concerning the mechanism of this erasure.

**Supporting Information**

**Figure S1** A representation of the region immediately upstream of the putative start of translation of homolog of *Heman* in poplar (two paralogs), papaya, *Arabidopsis*, and grape. Sequences in green are the GA-rich sequences identified near the insertion. Sequences in blue are TC-rich sequences. Sequences in red are presumed coding sequences.

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**Figure S2** A representation of the region immediately upstream of the putative start of translation of the gene into which *MuDR(p4)* is inserted, and the homolog of that gene in rice. Sequences in green are the GA-rich sequences identified near the insertion. Sequences in blue are TC-rich sequences. Sequences in red are presumed coding sequences.

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**Author Contributions**

Conceived and designed the experiments: DL. Performed the experiments: JS. Analyzed the data: JS MF DL. Contributed reagents/materials/analysis tools: MF DL. Wrote the paper: DL.

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