Active *Mutator* Elements Suppress the Knotted Phenotype and Increase Recombination at the *Kn1-O* Tandem Duplication

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ABSTRACT

The *KNOTTED-1* (*KN1*) locus is defined by a number of dominant mutations that affect leaf development. The *Kn1-O* mutation is characterized by outpocketings of tissue along lateral veins of the maize leaf and by displacement of ligule tissue from the junction of the blade and sheath into the blade. *Kn1-O* results from a tandem duplication of 17 kb; each repeat includes the entire 8-kb *KN1* transcription unit. *Mutator* (*Mu*) transposable elements inserted at the junction of the two repeats diminish the mutant phenotype. The *Mu* insertions affect the *Kn1-O* mutation in several distinctive ways. (1) Two of the three *Mu* elements, a *Mu1* and a *Mu8* element, diminish the mutant phenotype only when active as indicated by hypomethylation; when methylated or inactive, the phenotype is comparable to the *Kn1-O* progenitor. (2) Additional rearrangements have arisen in these derivatives that further reduce the mutant phenotype. (3) A 100–2000-fold increase in the loss of one repeat occurs in the presence of *Mu* elements as compared to *Kn1-O* without elements. The high frequency of loss only occurs when the *Mu* elements are hypomethylated. The frequency is also influenced by the specific allele carried at the same locus on the homologous chromosome. Reciprocal exchange of flanking markers does not accompany the loss events. Various recombination models that address the events occurring at *Kn1-O* are presented.

TRANSPOSABLE elements affect gene expression in several different ways. Transposon insertions into coding regions often result in loss or diminution of gene expression. Insertions into regulatory regions may alter gene expression either by interrupting the normal regulatory sequences [see COEN *et al.* (1989) for review] or by placing the gene under the regulation of that element or unlinked elements (MASSON *et al.* 1987; CORCES and GEYER 1991). Transposable elements also affect gene expression by serving as a substrate for recombination. Crossing over between members of a transposon gene family may lead to inversions or deletions which in turn may alter regulation of nearby genes (LIM 1988; DAVIS, SHEN and JUDD 1987; NEVERS, SHEPHERD and SAEDLER 1986; ROBERTSON and STINARD 1987; MONTGOMERY *et al.* 1991). Dispersed transposons may also undergo recombination-mediated changes that do not alter the flanking DNA sequences, but may affect expression of adjacent genes (ROEDER and FINK 1982).

Transposable elements of the ROBERTSON's (1987) *Mutator* (*Mu*) system in maize are characterized by terminal inverted repeats of approximately 200 bp that flank unrelated internal sequences [for reviews see WALBOT (1991) and CHANDLER and HARDEMAN (1992)]. Most *Mu* elements do not encode a transposase and are considered nonautonomous. Recently, an element has been characterized in several laboratories that has the genetic features of an autonomous element (CHOMET *et al.* 1991; QIN, ROBERTSON and ELLINGBOE 1991; HERSHBERGER, WARREN and WALBOT 1991). Presumably, it encodes the transposase required for transposition of all nonautonomous elements. The *Mutator* system is characterized by a high forward-mutation rate presumably due to the numerous *Mu* elements present, and by occasional activity loss. In an inactive line, new mutant phenotypes are no longer generated (ROBERTSON *et al.* 1985), the *Mu* elements are methylated (CHANDLER and WALBOT 1986; BENNETZEN 1987), and *Mu* elements do not excise somatically (WALBOT 1986; BROWN and SUNDARESAN 1992). Inactivity is correlated with, and presumably a direct consequence of, loss or inactivation of the autonomous element (CHOMET *et al.* 1991; QIN, ROBERTSON and ELLINGBOE 1991; HERSHBERGER, WARREN and WALBOT 1991).

Mu insertions have been useful in defining regulatory regions of the *KNOTTED* (*KN1*) locus in maize. The locus is defined by a number of semidominant alleles that affect leaf morphology. Outpocketings of tissue, or knots, occur along the lateral veins. The ligule, a fringe of tissue normally found at the junction between leaf sheath and blade, is displaced into the blade, specifically along the lateral veins (BRYAN and

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SASS 1941; GELINAS, POSTLETHWAIT and NELSON 1969; FREELING and HAKE 1985). *KN1* was cloned by transposon tagging using a *Ds2* element (HAKE, VOLLBRECHT and FREELING 1989) and subsequently shown to encode a homeodomain-containing protein (VOLLBRECHT *et al.* 1991). While most dominant *Kn1* mutations result from the insertion of transposons into introns (HAKE, VOLLBRECHT and FREELING 1989; our unpublished data), the *Kn1-O* mutation is caused by a 17-kb tandem duplication (VEIT *et al.* 1990). The duplication leaves the surrounding regulatory sequences of the distal transcription unit unaltered, while the proximal transcription unit is placed in a novel context 500 bp from the repeat junction (see Figure 1). The phenotype of *Kn1-O* is fully penetrant. In addition, both juvenile and adult leaves display some level of ligule displacement or knots. From a screen of 10,000 heterozygous *Kn1-O*/+ plants in a *Mu* background, nine normal or near-normal appearing plants were identified. Southern analysis of DNA isolated from each individual plant demonstrated that five plants had lost one copy of the 17-kb repeat and four had sustained an insertion at the junction of the two repeats. Three of the four insertions are *Mu* elements (VEIT *et al.* 1990). No derivatives have been recovered that disrupt the normal context transcription unit. These findings suggest that the *Kn1-O* mutation is caused not by the duplication *per se*, but rather by the juxtaposition of novel sequences 5′ to the proximal transcription unit (see Figure 1).

To gain a better understanding of the *Kn1-O* mutation, we analyzed several independent *Mu*-induced derivatives. We find that the phenotypic effect of two *Mu* element insertions depends on whether the *Mu* elements are methylated; however, the effect of a third *Mu* insertion is independent of *Mu* methylation. The insertions generate subsequent rearrangements and increase the frequency of repeat loss at *Kn1-O*. The frequency of repeat loss is affected by the specific allele present on the homologous chromosome and by methylation of the *Mu* elements. Models are discussed concerning the recombination mechanisms involved in *Mu*-associated repeat loss at *Kn1-O*.

MATERIALS AND METHODS

Plasmids, and statistical analysis: The genomic fragments used in Southern hybridization were derived from the lambda clones of *Kn1-2F11* (HAKE, VOLLBRECHT and FREELING 1989) and *Kn1-O* (VEIT *et al.* 1990). The *Mu* clones were either a 500 bp *Pst*I to *Pvu*II internal fragment of *Mu8* (FLEENOR *et al.* 1990) or the internal subclone of *Mu1*, pA/B5 (CHANDLER, RIVIN and WALBOT 1986). The frequency of loss of a repeat from the tandem duplication was statistically analyzed using Fisher's exact test (STEEL and TORRIE 1980) for 2 × 2 tables (computer program furnished by G. DALLAL, Malden, Massachusetts) (Table 1).

Alleles and genetic strategies: The *Adh1-F6* allele (D. SCHWARTZ, University of Indiana) is linked to the *Kn1-O* derivative alleles, *Adh1-F* and *Adh1-S* are linked to the wild-type *kn1* alleles. All three *Adh1* alleles can be distinguished on a starch gel (FREELING and BIRCHLER 1981). *Adh1* is approximately one map unit distal to *KN1* (J. MATHERN and S. HAKE, *Maize Genetics Coop. Newsl.* **63:** 2). The wild-type plants carry 0–2 inactive *Mu* elements and are referred to as non-*Mu* lines in the text. The crossing strategy to select for the *Mu*-induced derivatives of *Kn1-O* has been described (VEIT *et al.* 1990). Three derivatives were recovered that contain *Mu* elements, *Kn1-O174* (*174*), *Kn1-O169* (*169*), and *Kn1-O167* (*167*). A fourth derivative, *Kn1-O204*, was found in that experiment but does not contain a *Mu* element. A deletion of *Kn1* that was recovered in an X-ray mutagenesis experiment (HAKE, VOLLBRECHT and FREELING 1989; J. MATHERN and S. HAKE, *Maize Genetics Coop. Newsl.* **63:** 2) will be referred to as *Def(Kn1)O*; it was previously referred to as *Kn1-Od* (VEIT *et al.* 1990). The *169* revertants were isolated from a cross of *169 Adh1-F6/169 Adh1-F6* by wild-type (*kn1 Adh1-F/kn1 Adh1-F*) pollen. Two knotted individuals were noted in two separate plantings of 100 seedlings. *169b* was found by a similar strategy of crossing *169a Adh1-F6/169a Adh1-F6* plants by wild-type pollen. In general, the crosses were performed with the *Mu* derivative as female to avoid the possibility of pollen contamination.

Southern analysis, polymerase chain reaction (PCR) and sequencing: Genomic DNA was isolated primarily from seedling leaves (K. CONE, personal communication, *Maize Genetics Coop. Newsl.* **63:** 68), restricted, electrophoresed, blotted to nytran, and hybridized with appropriate probes labelled by the random priming method as described (SAMBROOK, FRITSCH and MANIATIS 1989). Primers flanking the ends of the duplicated regions of *Kn1-O* were used to amplify genomic DNA from plants that had lost one repeat. Primers flanking the central junction of the tandem repeat were used to amplify genomic DNA from plants that had lost the *Mu8* insertion. PCR conditions were as follows: 1 min 94°, 2 min 55° and 2 min 72° for 30 cycles using Promega reaction buffer and Taq polymerase. BV8 and E17 amplify the region that covers the distal end, BV1 and BV3 amplify the proximal end, BV1 and SH4 amplify the repeat junction as indicated in Figure 1: BV3 5′-GAAGTAACACAGGGTAATAGTATT-3′; BV1 5′-TCTAGTAACCAGCAC-3′; E17 5′-CAACTCTACAGGTCCTACAC-3′; BV8 5′-GTCGACGAGAATTAACCATAT-3′; SH4 5′-CTGGAGGCCAATGCAGCTGCCT-3′. PCR products were filled in with Klenow (SAMBROOK, FRITSCH and MANIATIS 1989), blunt-ligated into the *Eco*RV site of pSK$^+$ and sequenced in one direction using synthetic oligonucleotides to prime sequencing reactions using T7 DNA polymerase (Promega).

TABLE 1

Significance values of repeat in allelic comparisons

Alleles	*P* Values[a]	Allele	*P* Values[a]
169/+ vs. *169**/+	0.015	*169*/+ vs. *169/169*	0.045
174/+ vs. *174**/+	0.34	*174*/+ vs. *174/174*	0.76
169a/+ vs. *169a**/+	0.0073	*167*/+ vs. *167/167*	0.15
167/+ vs. *167**/+	0.069	*167*/+ vs. *167*/−	0.007
		167/174 vs. *167/167*	0.23
		167/169a vs. *167/167*	0.60

[a] The *P* values were obtained using Fisher's "exact test" for 2 × 2 tables. *P* values of 0.05 or smaller are considered significant, *P* values of 0.1 are only marginally significant. * denotes methylated; "+" is the wild-type allele; "−" represents the deletion.

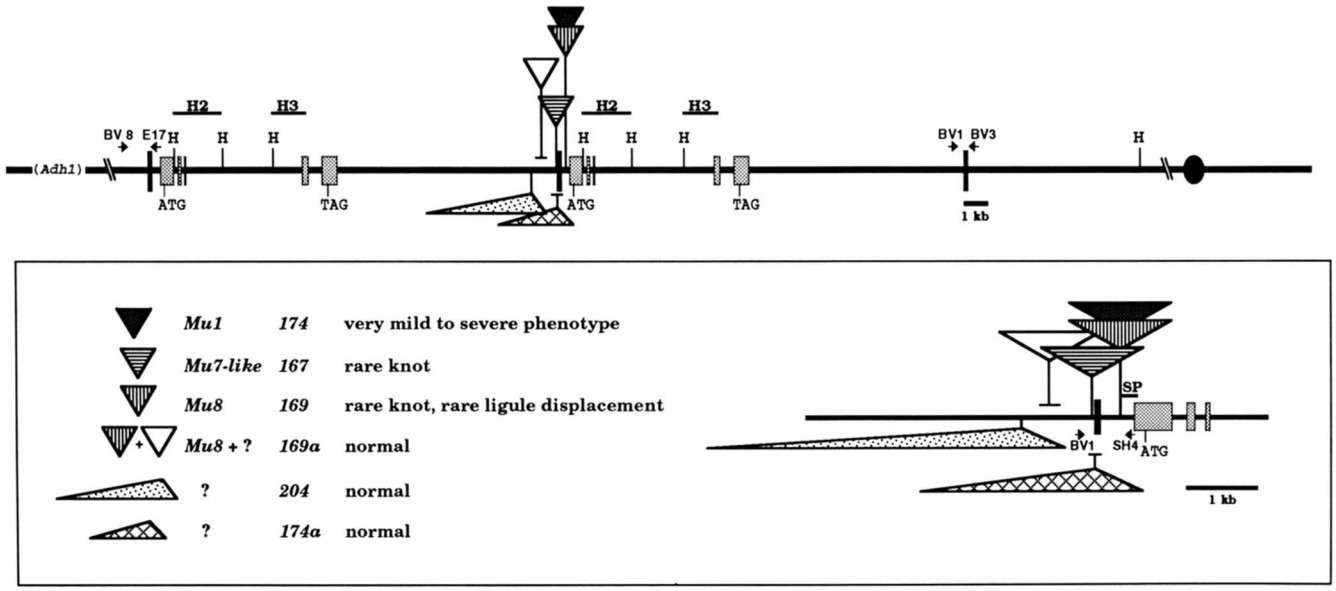

FIGURE 1.—Organization of the *Kn1-O* tandem duplication. The hatched boxes are exons, the solid line is non coding. The ATG and TAG show the start of translation and termination codons, respectively (VOLLBRECHT *et al.* 1991). The solid, vertical bars indicate the extent of the 17-kb region that is duplicated. The centromere is shown to the right (not to scale), *Adh1* is 1 map unit distal, and shown on the left. The lower box is a detail from the upper map. The non-*Mu* insertion of *Kn1-0204* is located 1105 bp distal to the repeat junction. The *Mu7*-like element of *Kn1-O167* (*167*) is located 6 bp distal to the repeat junction and 446 bp 5′ from the start of transcription. The *Mu1* element of *Kn1-O174* (*174*) and the *Mu8* element of *Kn1-O169* (*169*) are inserted in the same position, 310 bp proximal to the repeat junction and 120 bp 5′ from the start of transcription (VOLLBRECHT *et al.* 1991; VEIT *et al.* 1990). All three *Mu* insertions resulted in a 9-bp target site duplication. The position of the additional insertion in *169a* is approximate within 1 kb. The rearrangement of *174a* is near the repeat junction. H = *Hin*dIII. Fragments that were used as hybridization probes in Southern blots are H2, H3 or SP (in box). Short arrows at the ends of the duplication and across the repeat junction illustrate the position of primers for PCR and sequencing.

RESULTS

Phenotypic changes are correlated with further structural alterations: In the initial characterization of *Kn1-O Mu* derivatives (VEIT *et al.* 1990), it was noted that certain aspects of the mutant phenotype persisted (Figure 1). Plants carrying the *Kn1-O167* (*167*) allele display an occasional knot on an adult leaf. Plants with either the *Kn1-O169* (*169*) or *Kn1-O174* (*174*) allele often display mild ligule displacement on early leaves. Occasionally, *169* or *174* plants give rise to individuals displaying a severe knotted phenotype or a completely normal phenotype. The variants transmit the altered phenotype to their progeny.

To better understand the basis for these derivative phenotypes, progeny of *169* and *174* that appeared normal were analyzed by Southern analysis. We found that most of these individuals (93%) lost one repeat of the tandem duplication (for example, see lane 8 in Figure 2), but a few sustained further insertions or rearrangements at the locus while retaining the duplication. One such individual, *169a*, was identified in a sample of 10 sibling *169* plants. Southern blot analysis of DNA isolated from a *169* homozygote reveals two bands, 19 and 16.4 kb in length, when hybridized to the H3 probe (Figure 2). Similar analysis of a *169a* homozygote indicates that the 19 kb band is still

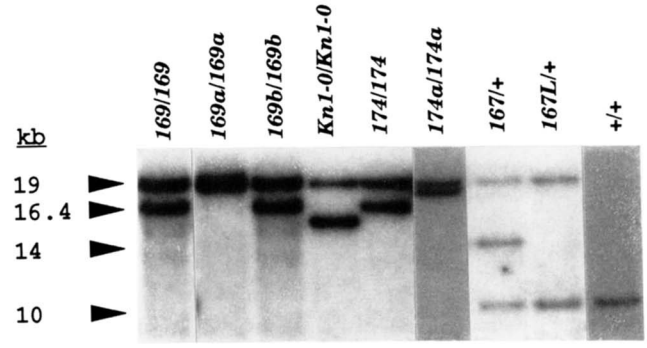

FIGURE 2.—Southern blot of *Mu* derivatives. DNA was digested with *Hin*dIII, electrophoresed and transferred to nytran. The blot was hybridized to H3 (Figure 1). The + refers to a wild-type allele. *167L* is used to designate a *167* individual that lost a repeat of the tandem duplication. The 19- and 21-kb bands of *169a* appear as a single broad band in this Southern blot.

present, but the 16.4-kb band has been replaced by a novel 21 kb band, appearing as a doublet in Figure 2. Further analysis shows that the *Mu8* insertion of *169* is retained in its normal location, and that in *169a* an additional 4.3-kb insertion is located approximately 500 bp distal to the junction of the two repeats (data not shown). *169b* was detected in a population of 54 *169a*/+ plants as the only individual with any knots (see MATERIALS AND METHODS for crosses). *169b* appears to have lost the 4.3 kb insertion of *169a* and may be a revertant to *169* (Figure 2).

174a, isolated from a sample of 20 *174* individuals, is also indistinguishable from wild type in appearance. The hybridization pattern of *174* is the same as *169* when Southern blots are hybridized to the H3 probe, due to the fact that similar sized elements inserted into the same location (VEIT *et al.* 1990). Southern analysis of *174a* demonstrates that the 16.4-kb band of *174* is absent and is replaced by a novel 18.5-kb band (Figure 2). The *Mu1* insertion of *174* is absent in *174a*, and instead, a 3.5-kb insertion is present in the region of the repeat junction (data not shown). Preliminary data suggest that a rearrangement of *Kn1-O* sequences has occurred. Consistent with the notion of a rearrangement, the alterations at *174a* are relatively stable; knotted progeny have not been found in a population of 500 heterozygous *174a*/+ individuals.

Mu insertions at *Kn1-O* were found at a frequency of 3 in 10,000, a frequency similar to the recovery of *yl* and *yg* mutants from *Mu* lines by ROBERTSON (1985). The subsequent derivatives, *174a* and *169a*, were recovered in relatively small populations, suggesting that the original insertions increased the rearrangement frequency at the locus. The propensity for transposable elements to cause additional rearrangements is also documented at other loci (TAYLOR and WALBOT 1985; B. KLOECKENER-GRUISSEM and M. FREELING, personal communication) [see COEN *et al.* (1989) for review].

Methylation of *Mu* elements in *174* and *169* correlates with increased severity of the knotted phenotype: We examined the DNA of individual derivatives that expressed a severely knotted phenotype to determine whether the *Mu* element had excised, resulting in reversion to the progenitor allele, *Kn1-O*. Of 28 *174* individuals examined, all retained the *Mu1* insertion, demonstrating that expression of the severe mutant phenotype was not related to an excision event. However, two knotted *169* plants that arose following pollination of a homozygous *169* female with wild-type pollen lost the insertion (data not shown). Within the limitations of Southern blot analysis, the *169* knotted revertants appear the same as *Kn1-O*. DNA was amplified using primers BV1 and SH4 (Figure 1). Sequencing of one PCR product showed that the revertant and *Kn1-O* progenitor sequence are identical (data not shown).

We examined the extent of *Mu* methylation among various derivatives since it is correlated with loss of *Mu* activity (CHANDLER and WALBOT 1986; BENNETZEN 1987) and in certain *Mu* induced mutations, the mutant phenotype is dependent on the element's activity state (MARTIENSSEN *et al.* 1990; BARKAN and MARTIENSSEN 1991). DNA samples from *174*/+ heterozygotes that varied in phenotype from severely knotted to almost normal were digested with *Hin*fI

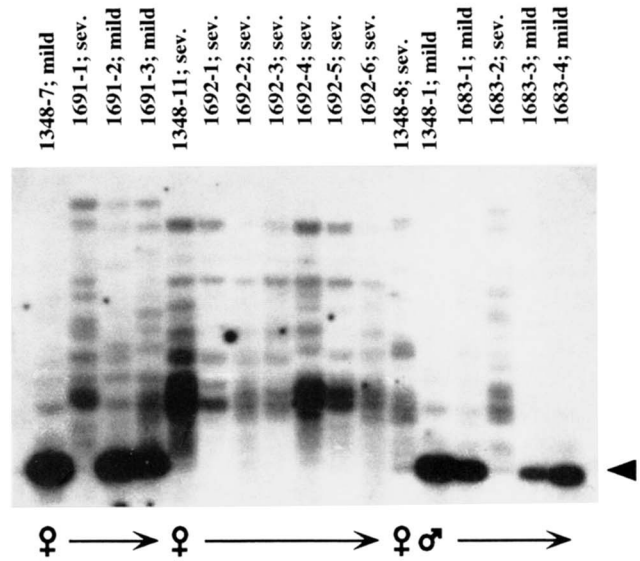

FIGURE 3.—Methylation of *Mutator* elements correlates with severity of the knotted phenotype in *174*. DNA was digested with *Hin*fI, electrophoresed, transferred to nytran and hybridized to the *Mu1* probe, pA/B5. The arrow indicates the 1.3-kb fragment present in unmethylated *Mu1* elements. Severely knotted and mildly affected plants are designated by "sev" and "mild," respectively. 1348-7, 1348-8, 1348-1 and 1348-11 were siblings. 1348-7 was crossed by a non-*Mu* male to generate the 1691 family. 1348-11 was crossed by a male of the same non-*Mu* line to generate the 1692 family. 1348-8 was crossed by 1348-1 to generate the 1683 family.

and the resulting Southern blot was hybridized with an internal *Mu1* fragment (Figure 3). Mildly knotted siblings, such as 1348-7 or 1348-1, carry primarily unmethylated *Mu1* elements, whereas severely knotted siblings, such as 1348-11 or 1348-8, carry primarily methylated elements. When 1348-7 was crossed to a non-*Mu* line, two of the three progeny examined remained unmethylated, one individual was methylated. When 1348-11 was outcrossed to the same non-*Mu* line, all six progeny examined were severely knotted and their DNA was methylated. The methylation of severely knotted 1348-8 was reversible in the cross to 1348-1, a mild individual. One of the four progeny examined was still severely knotted and carried methylated *Mu1* elements, while the other three were mildly affected and carried unmethylated elements (Figure 3). The ability to segregate severely knotted and mildly knotted (methylated and unmethylated) individuals in the same family suggests that the 1348 family is segregating for one autonomous *Mu* element (CHOMET *et al.* 1991; QIN, ROBERTSON and ELLINGBOE 1991; HERSHBERGER, WARREN and WALBOT 1991).

We found a similar correlation between increased knotted phenotype and methylation of *Mu* elements in *169* progeny. However, the correlation of methylated *Mu* elements and a knotted phenotype does not extend to *167* or *169a* (data not shown). Although most *167* plants analyzed carry unmethylated *Mu* elements, one individual and its progeny were found that

carry methylated *Mu* elements. These individuals appeared normal in phenotype. The *Mu8* element in *169a* is also methylated (data not shown) and these individuals are normal in appearance. Since *169a* has a *Mu8* element in the same position as *169*, the additional insertion is the likely cause of the persistent normal phenotype despite methylation of the resident *Mu8* element. Thus, *Kn1-O* alleles containing either a *Mu1* or a *Mu8* element 5′ to the transcription start site are regulated by methylation of the *Mu* elements, suggesting that the position of the element is critical to the regulation and not the type of element itself.

Tandem duplication instability is enhanced by *Mu* insertions: *Kn1-O* spontaneously reverts to wild type at a frequency of approximately 10^{-4} in the absence of active transposable elements. Southern analysis revealed that the normal appearing plants lost one repeat of the tandem duplication (VEIT *et al.* 1990). In progeny of *167*, *169* and *174*, the repeat containing the *Mu* element is lost at a higher frequency (10^{-1}-10^{-2}). To further assess the effect *Mu* insertions have on the frequency of repeat loss, the following crosses and progeny analyses were performed. Individuals that were homozygous or heterozygous for the *Mu* insertions at *Kn1-O* were analyzed by Southern blots to ensure that the duplication and *Mu* insertion were present and to determine whether the *Mu* elements were methylated. The plants were outcrossed as male or female to lines that are wild-type for *KN1*. The wild-type lines carry 1–2 inactive *Mu* elements and differ in restriction fragment lengths when hybridized to *KN1* probes. The wild-type *kn1* alleles are linked to either *Adh1-F* or *Adh1-S*, while the *Mu Kn1-O* derivatives are linked to *Adh1-F6*. Progeny from the heterozygotes backcrossed to wild type yield two segregant classes; those homozygous for the wild-type allele, and those heterozygous for the derivative allele. The heterozygotes were selected by screening for *Adh1-F6*. Seedlings were scored phenotypically for the knotted phenotype and apparently normal individuals were examined by Southern analysis. Individuals that lost one repeat of the tandem duplication always carried the 19-kb band from the *Mu Kn1-O* parent and a 10 kb band from the wild-type parent (see Figure 2 for example). All progeny from *167* and *169a* were examined by Southern analysis for the presence of the tandem duplication.

The repeat loss occurs approximately 100 times more frequently in homozygous *Mu* derivatives, *174*, *167* and *169* than in *Kn1-O* (Figure 4, B, C and D; 5/80, 2/35 and 2/59 losses per analyzed progeny). The repeat loss occurred at a slightly higher frequency when the *167* or *169* parent was heterozygous with wild-type (Figure 4, G and J, 15/89 and 3/14), but was not statistically different when heterozygous with a different *Mu* derivative (see Table 1 for significance values). The mode of transmission, whether from the male or female, did not alter the frequency. However, the loss of one repeat was significantly reduced in progeny of *167* when the *167* parent was heterozygous for a deletion (Figure 4I, 1/94). The deletion, *Def(Kn1)O*, removes the entire tandem duplication but not more than two or three map units within the long arm of chromosome *1* (HAKE, VOLLBRECHT and FREELING 1989; J. MATHERN and S. HAKE, *Maize Genetics Coop. Newsl.* **63:** 2). Thus, the repeat is lost at a higher frequency when it contains a *Mu* insertion. The loss appears sensitive to the influence of the specific allele on the homologous chromosome, suggesting that the loss is mediated by a recombination event.

We examined genomic DNA of an individual that lost a repeat of the tandem duplication to determine if basepair changes occured during the loss event. The individual was self-pollinated and DNA was prepared from homozygous individuals identified by the linked *Adh1* allele. Primers BV8 and E17 were used to amplify sequences at the distal end of the remaining repeat, and primers BV1 and BV3 were used to amplify sequences at the proximal end (Figure 1 and MATERIALS AND METHODS). The sequence matched the ends of the entire tandem duplication of *Kn1-O*, suggesting that the repeat containing the *Mu* element was lost precisely with no base pair alterations (data not shown).

Lack of flanking marker exchange during repeat loss: To investigate the mechanism involved in the repeat loss, flanking markers served to monitor the occurrence of coincident or correlated reciprocal exchange. The *Kn1-O* derivative alleles are linked to *Adh1-F6*, whereas wild-type *kn1* alleles are linked to *Adh1-F* or *Adh1-S*. *ADH1* is approximately 1 map unit distal to *KN1*. The *KN1* alleles are also distinguished by different *Hin*dIII fragments proximal to the central junction of the tandem duplication when hybridized to H3. The *Hin*dIII fragments result from a polymorphic restriction site outside the tandem duplication and a conserved *Hin*dIII restriction site in the tandem duplication. A 19-kb and a 10-kb fragment are present in *Kn1-O* and wild-type lines, respectively (Figures 1 and 2). All 27 individuals that arose from heterozygous parents and lost a repeat retained both parental markers, suggesting that conventional crossing over, as monitored by flanking marker exchange, did not occur between the derivative and the wild-type homolog. However, we selected individuals that carried the linked *Adh1-F6* allele, therefore, we could have only detected one of the two possible exchange events. Thus, it appears that intrachromosomal recombination or gene conversion are the most likely mechanisms for the loss of a repeat of the duplication (see FOGEL, WELCH and MALONEY 1989).

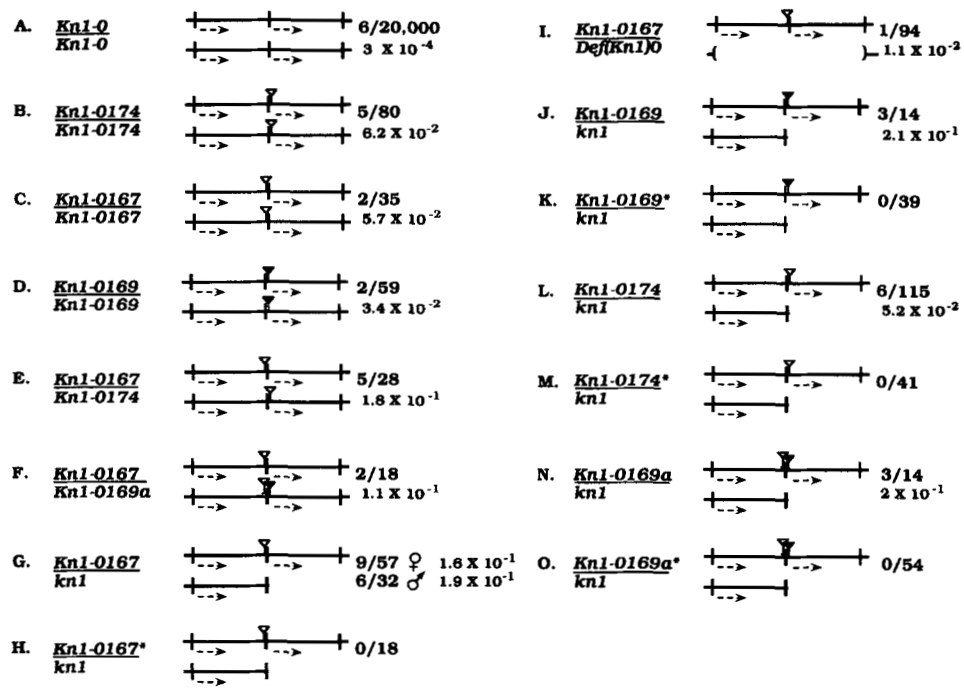

FIGURE 4.—Repeat loss is enhanced by *Mu* insertions. The parental alleles are diagrammed for each genotype analyzed. The distance between the vertical bars represents the 17-kb repeat. The arrows refer to the *Kn1-O* transcriptional unit. The triangles depict the *Mu* insertion of the *Kn1-O* derivatives. *kn1* is the wild-type allele. The derivative parent was crossed by *kn1* plants. ♀ or ♂ denote which parent was used as the *Mu* derivative parent. In general, the derivative was crossed as the female. Families with methylated *Mu* elements at *Kn1-O* are indicated by an asterisk (*). The fraction is the number of individual repeat losses observed over the number of progeny analyzed.

Methylation of *Mu* decreases the frequency of repeat loss: We exploited derivatives that varied in the state of *Mu* methylation to determine whether *Mu* activity modulates the frequency of repeat loss. We detected no repeat loss in 41 plants that arose from a *174*/+ parent with methylated *Mu* elements or in 39 plants from a *169*/+ parent with methylated *Mu* elements. We also did not detect any losses in 18 progeny from a methylated *167*/+ parent. The frequency of loss for *167*, *169* and *174* was higher in the unmethylated state (15/89, 3/14 and 6/115, respectively; Figure 4, G, J and L).

We also examined the progeny of the *167* hemizygotes to ensure that the low frequency of repeat loss was due to the hemizygous condition and not because the elements were methylated. We examined 20 of the 94 progeny (Figure 4I) by *Hinf*1 digestion, and found the *Mu* elements were all hypomethylated (data not shown).

Although the *169a* derivative sustained a further insertion, the *Mu8* element is retained in the original position as in the *169* progenitor (data not shown). The *Mu8* element is methylated in *169a*. We detected no instance of repeat loss in 54 progeny from a *169a*/+ heterozygote. The lowered frequency could reflect the presence of the second insertion or the methylated element. To distinguish between these alternatives, *169a* was crossed to *167* plants that carried active *Mu* elements in order to reverse the methylation (BENNETZEN 1987; MARTIENSSEN *et al.* 1990; BROWN and SUNDARESAN 1992). The *169a*/*167* heterozygote was then crossed to a wild-type line carrying a different *Adh1* allele, *Adh1-F*. DNA was prepared from a few individuals to determine which carried the *169a* allele and to ensure that the *Mu* elements were no longer methylated. The *169a*/+ individual was crossed again to a similar wild-type line and the individuals carrying the *169a* allele were identified by the linked *Adh1-F6* polymorphism. The progeny were analyzed for the presence of the tandem duplication by Southern analysis. Among 14 progeny derived from a *169a*/+ parent with active *Mu* elements, three individuals lost a repeat compared to zero in 54 when *169a* carried methylated *Mu* elements. This finding indicates that the reduced frequency of repeat loss in *169a* is associated with *Mu* methylation and not with the additional rearrangement. The destabilization of the tandem duplication in the presence of active *Mu* elements suggests that the putative *Mu* transposase may play a role in the high frequency of loss events.

DISCUSSION

Mu elements inserted at the *Kn1-O* tandem duplication profoundly affect the locus in a number of ways. Depending on their position and whether they are methylated, the phenotype varies from severely knotted to indistinguishable from wild-type. Further rearrangements have occurred in the presence of the

Mu elements, most notably are the losses of one copy of the tandem repeat. The frequency of repeat loss is affected by the allele carried on the homologous chromosome and by whether the *Mu* element is methylated.

Two insertions, a *Mu1* and a *Mu8* element located upstream from the start of transcription, diminish the mutant phenotype only when hypomethylated. When the elements are methylated, the phenotype is identical to the *Kn1-O* progenitor. *Mu* methylation affects the expressivity of another maize mutant, *hcf106* (*high chlorophyll flourescence*) (MARTIENSSEN *et al.* 1990). This recessive mutation is caused by a *Mu1* insertion in the untranslated leader of the transcription unit. When *Mu1* is active, *i.e.*, not methylated, a pale green and lethal phenotype is manifested and mRNA accumulation is prevented. However, when the element is methylated, transcription is initiated within the *Mu* element and immediately downstream, permitting an apparent wild-type phenotype (BARKAN and MARTIENSSEN 1991). The interaction we describe is analogous in that methylation returns the phenotype to that of the progenitor, only in our case the progenitor is knotted in phenotype. Unlike the findings at *hcf106*, we have no evidence for transcription initiating in the *Mu* element. The *Kn1-O* transcripts are the same size on Northern blots in the presence or absence of *Mu* insertions (unpublished data). This difference is possibly due to the fact the *Mu* insertions at *Kn1-O* are further 5′ from the transcription start site.

The correlation of knotted phenotype with methylated *Mu* elements does not apply to all the *Mu* insertions or their derivatives. Plants with a *Mu7* insertion immediately distal to the repeat junction (*167*) are nearly normal whether the insertion is methylated or not. The methylation dependence in *169* does not occur when an additional insertion is present distal to the repeat junction (*169a*). Finally, the non-*Mu* insertion of *Kn1-O204* also distal to the junction (VEIT *et al.* 1990) completely abolishes the mutant phenotype (Figure 1). Thus, insertions proximal to the junction affect expression depending on their state of methylation, but distal insertions abolish expression regardless of the state, further implicating sequences distal to the junction as causing the *Kn1-O* mutant phenotype (VEIT *et al.* 1990).

Different recombination mechanisms exist by which repeated sequences increase or decrease their copy number (PETERSON and LAUGHNAN 1963; JACKSON and FINK 1985; MALONEY and FOGEL 1987; WELCH, MALONEY and FOGEL 1990; reviewed by PETES and HILL 1988). One such mechanism involves unequal crossing over between repeats on homologous chromosomes and is generally accompanied by an exchange of flanking markers (STURTEVANT and MORGAN 1923; STADLER and NEUFFER 1953; MANGELSDORF and GALINAT 1964; DOONER and KERMICLE 1971). The change in repeat copy number we documented in the *Mu* derivatives of *Kn1-O* is not accompanied by flanking marker exchange. Moreover, the frequency of repeat loss is increased 100–2000-fold compared to the frequency at *Kn1-O* without insertions. To rationalize the loss events observed, we present three different but plausible mechanisms (Figure 5).

In mechanism A, repeats on one chromatid undergo reciprocal exchange. Such crossovers have been inferred in yeast (JACKSON and FINK 1985; MALONEY and FOGEL 1987), but are less frequent than other recombination events. Because the repeats of *Kn1-O* are identical, we have no data to support or eliminate this mechanism.

In mechanism B, unequal crossing over between repeats on sister chromatids would also fail to generate flanking marker exchange. Sister chromatid exchange has been inferred for repeat number changes at the Bar duplication of Drosophila that occur without flanking marker exchange (PETERSON and LAUGHNAN 1963). It is the primary mechanism of copy number change in tandem repeats of rDNA in yeast (PETES 1980). However, it is not the principal mechanism implicated in copy number change at other loci (JACKSON and FINK 1985; FOGEL, WELCH and MALONEY 1988). The predicted reciprocal products at *Kn1-O* would be one chromatid with three 17-kb repeats, two of which contain *Mu* insertions, and one chromatid with a single 17-kb repeat. Plants with three repeats carrying *Mu* elements might not be distinguished by phenotype. However, they could be identified via Southern analysis, manifested as a shift in hybridization intensity between the 19- and 14-kb bands from an approximately equal ratio (Figure 2) to a 1:2 ratio. We detected a hybridization intensity shift in one individual among 177 tested (data not shown), suggesting that though sister strand exchange does occur, this exchange reaction does not represent the primary mechanism involved in loss of a repeat.

In mechanism C, gene conversion occurs between sister or non-sister chromatids, resulting in loss of one repeat. Gene conversion is defined as a nonreciprocal exchange of information from one DNA duplex to another. The information is transferred with high fidelity, and in most cases, the exchange shows parity, or equal directionality of transfer [see FOGEL, MORTIMER and LUSNAK (1981) for review on gene conversion]. Experiments in yeast demonstrate that double strand breaks are highly efficient at initiating gene conversion (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981; NICHOLAS *et al.* 1989). The broken chromosome is the recipient for the genetic information. In the model for gene conversion of SZOSTAK, ORR-WEAVER and ROTHSTEIN (1983), a double strand

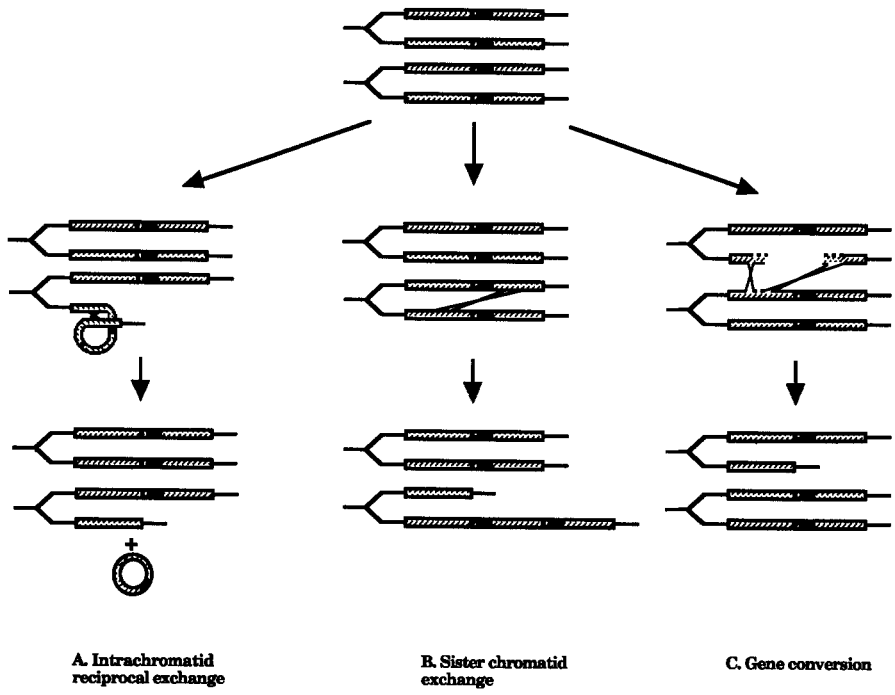

FIGURE 5.—Mechanisms for repeat loss. In these diagrams all four chromatids carry the tandem duplication, illustrated as a speckled box. The *Mu* element (solid box) is located just to the right of the central junction of the two repeats. The alleles are drawn as homozygous, but could be heterozygous. In mechanism A, pairing between two repeats on one chromatid occurs. The circle represents one of the recombination products obtained. In mechanism B, sister chromatid exchange gives rise to one chromatid with three repeats and one chromatid with one repeat. In mechanism C, a double strand break initiates gene conversion. The central region of the tandem repeat is lost by exonuclease activity. A free 3′ end invades the homologous region of the wild-type repeat on the non-sister chromatid. The gene conversion event proceeds as described (SZOSTAK, ORR-WEAVER and ROTHSTEIN 1983). The gene conversion could also occur between sister chromatids.

break is enlarged to a gap and a free 3′ end invades the homologous region on the donor duplex. The invaded duplex serves as a template to repair the double strand gap.

We propose that gene conversion may be the operative mechanism responsible for loss of one repeat in the *Kn1-O* derivatives. In support of this view is the dependence on active *Mu* elements and the effect of the allele on the homologous chromosome. Neither of these factors would be expected to affect recombination frequency in mechanisms A or B. Gene conversion could either replace both repeats with one repeat, or replace the *Mu*-containing repeat with the wild-type repeat while maintaining the tandem duplication. The latter event would appear as an excision and may account for the two *169* revertants we detected. Gene conversion was proposed by DOSEFF, MARTIENSSEN and SUNDARESAN (1991) to explain certain *Mu* excision events occurring at the maize *bz1-mum9* allele. Sequence analysis of somatic excision sites suggested that gene conversion occurred using neighboring *bronze* sequences as a template. Gene conversion was also documented in tobacco after selective pressure for recombination between closely linked inverted duplications (TOVAR and LICHTENSTEIN 1992). In Drosophila, precise excision of a *P* element at the *white* locus is clearly attributable to gene conversion (GLOOR *et al.* 1991). The frequency of *P* element excision was also affected by the allele carried on the homologous chromosome. Excision failed to occur when the allele was homozygous or heterozygous with a deletion (ENGELS *et al.* 1990).

The increase in repeat loss in unmethylated *Mu* lines suggests that a transposase is involved since the transposase is presumably not present in lines with methylated *Mu* elements (CHOMET *et al.* 1991; QIN, ROBERTSON and ELLINGBOE 1991; HERSHBERGER, WARREN and WALBOT 1991). Transposable elements at the *P* locus in maize also increase recombination. The *P* gene, which conditions red pericarp (STYLES and CESKA 1977, 1989), is comprised of two 5.2-kb direct repeats separated by an 8.2-kb sequence. The spontaneous loss of one repeat and intervening sequence, resulting in colorless pericarp, occurs at the frequency of 3.1×10^{-4} somatically. The frequency increases 300 fold in the *P-ovov* allele, in which the autonomous element, *Ac*, has inserted between the two repeats (ATHMA and PETERSON 1991). Transposases are thought to initiate excision by causing a staggered double strand break (SAEDLER and NEVERS 1985). The broken chromosome ends that result from a double strand break following *Mu* excision could initiate gene conversion.

We found no evidence for exchange of flanking

markers in the 27 individuals for which it would be possible to detect. Although 50% of meiotic gene conversions are accompanied by conventional genetic exchange of outside flanking marker exchange in yeast (FOGEL, MORTIMER and LUSNAK 1981), gene conversion-like events such as mating type switching in yeast and *P* element excision in Drosophila may not be accompanied by flanking marker exchange (MCGILL, SHAFER and STRATHERN 1989; GLOOR *et al.* 1991). These results suggest that recombination events initiated by certain double strand breaks, such as transposon excision, are resolved by other mechanisms than those operating during meiotic recombination.

We found a 14-fold decrease in frequency of repeat loss when the *Mu* derivative was heterozygous with a deletion compared to heterozygous with wild-type. Hemizygosity also decreased the frequency of *P* element excision (ENGELS *et al.* 1990). However, hemizygosity did not affect loss of one repeat at the *A^b-Peru* complex in maize, which consists of a serial duplication of the *A1* gene (LAUGHNAN 1961). We found 1/94 losses from *Kn1-O167/Def(Kn1)O*, while Laughnan reported a frequency of loss at approximately 10^{-3} for *A^b-Peru* when paired with a deficiency. We propose that *Mu* element-induced gene conversion occurs at a higher frequency than sister chromatid exchange or intrachromatid recombination events which may not be affected by the homolog.

We can not argue unequivocally that the recombination events we are documenting are mitotic or meiotic. The allele carrying the *Mu* insertion was generally the female parent, thus, loss of the duplication that occured premeiotically could be observed as an ear sector. Preliminary evidence from one ear has shown both a three kernel sector and scattered events (data not shown). Either there were numerous, small (1 kernel) mitotic sectors or a coincidental clustering of three meiotic events. We tend to favor the latter possibility, especially when considering the fact that homologous chromosomes are not known to pair during mitosis and yet, we see an effect of the homologous chromosome on repeat loss.

The duplication of *Kn1-O* is highly recombinagenic in the presence of *Mu* insertions. Not only is there a greater than 100-fold increase in recombination, but new rearrangements also occur at a high frequency. Transposons such as *Mu* are intrinsic to the polymorphism found in the maize genome and are part of the restructuring of genomes throughout evolution. In balance with the transposon-induced alterations are mechanisms such as gene conversion of active transposons that act to remove these highly recombinagenic sites and thereby temper the rapid rate of genome change (NICHOLAS *et al.* 1990).

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