Mutator Insertions in an Intron of the Maize *knotted1* Gene Result in Dominant Suppressible Mutations

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ABSTRACT

The *knotted1* (*kn1*) locus of maize is defined by a series of dominant mutations affecting leaf development. We recovered 10 additional mutant alleles in lines containing active *Mutator* transposable elements. Nine of these alleles contain *Mu1* or *Mu8* elements inserted within a 310-bp region of the *kn1* third intron. All five *Mu8* insertions are in the same orientation whereas both orientations of *Mu1* were recovered. Northern analysis showed that ectopic expression of *kn1* within developing leaves is correlated with the mutant phenotype for the four alleles analyzed. Transcript size was not altered. The effect of *Mu* activity, as measured by the extent of *Mu* element methylation or by the presence of the autonomous *MuDR* element, was investigated for two alleles. *Kn1-mum2*, containing a *Mu8* element, and *Kn1-mum7*, containing a *Mu1* element, required *Mu* activity for the knotted phenotype. We examined the effect of *Mu* activity on ectopic *kn1* expression in *Kn1-mum2* and found that the transcript was present in leaves of *Mu* active individuals only. We discuss possible mechanisms by which *Mu* activity could condition *kn1* gene expression.

TRANSPOSABLE elements are powerful mutagens for generating novel alleles of a locus (COEN *et al.* 1986; SCHWARZ-SOMMER and SAEDLER 1987; CORCES and GEYER 1991). In addition to insertions that simply block gene function, transposons may alter the timing or tissue specificity of gene action when they insert into regulatory regions (COEN *et al.* 1989; CHEN *et al.* 1987; BRADLEY *et al.* 1993). Transposons may provide alternate transcription start sequences (BARKAN and MARTIENSEN 1991) or alternative splicing sites (WEIL and WESSLER 1990; ORTIZ and STROMMER 1990), thereby modifying the mRNA sequence. Excision of a transposon may generate additional stable alleles due to imprecise excision (SOMMER *et al.* 1988; COEN *et al.* 1986). Transposable elements may also alter the regulation of the gene due to sequences contained within the transposon (MASSON *et al.* 1987; MARTIENSEN *et al.* 1990; TANDA and CORCES 1991).

Mutator (*Mu*) transposable elements were first recognized by DON ROBERTSON (1978) in a line of corn that exhibited an unusually high mutation rate. Genetic and molecular experiments have shown that the high rate of mutation results from the transposition and insertion of numerous *Mu* elements [for review, see CHANDLER and HARDEMAN (1992) and BENNETZEN *et al.* (1993)]. Most lines of corn contain several families of distinct *Mu* elements that are related by their 220-bp terminal inverted repeats; these families are distinguished by the sequences between the repeats. The majority of *Mu* elements are non-autonomous; their transposition requires

the presence of an autonomous element referred to as *MuDR* [previously called *MuR*, *MuA* and *Mu9* (CHOMET *et al.* 1991; QIN *et al.* 1991; HERSHBERGER *et al.* 1991)]. While *MuDR* has inverted repeats that are similar to non-autonomous elements, it is distinguished by internal unique sequences that encode *trans*-acting factors needed for transposition. In addition to its presumed role in regulating *Mu* transposition, *MuDR* can also influence the expression of genes that contain non-autonomous *Mu* element insertions.

Mu activity (*i.e.*, the presence of *MuDR*) can be determined by a number of methods. A somatic reversion assay takes advantage of a non-autonomous *Mu* element inserted in a color gene. The element excises in the presence of *Mu* activity to restore color gene function, thereby producing revertant sectors (WALBOT 1986; CHOMET *et al.* 1991; BROWN and SUNDARESAN 1992). A molecular assay distinguishes active from inactive elements based on the fact that the inactive elements are methylated (CHANDLER and WALBOT 1986; BENNETZEN 1987). Southern analysis using a *MuDR* probe can distinguish the autonomous element from related defective elements based on size of the hybridizing bands (CHOMET *et al.* 1991; D. LISCH, P. CHOMET and M. FREELING, manuscript submitted for publication). Finally, the phenotypes of certain *Mu*-induced mutations that are dependent upon the presence of *Mu* activity can also provide a reliable, independent marker for the presence of *Mu* activity (MARTIENSEN *et al.* 1990; CHOMET *et al.* 1991; LOWE *et al.* 1992; MARTIENSEN and BARON 1994).

The *knotted1* (*kn1*) gene was cloned by transposon tagging (HAKE *et al.* 1989a). The KN1 protein contains

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a homeodomain, suggesting that KN1 functions as a transcriptional regulator (VOLLBRECHT *et al.* 1991). KN1 is localized to nuclei of the apical meristem and a subset of cells in the stem; it is not found in wild-type leaf primordia (SMITH *et al.* 1992; JACKSON *et al.* 1994). The dominant *Kn1* mutations affect cell differentiation specifically along the lateral veins of the leaf blade (GELINAS *et al.* 1969; FREELING and HAKE 1985). These cells adopt fates of cells normally found in more basal positions on the leaf (HAKE 1992). Ectopic KN1 expression within developing leaves is correlated with the dominant phenotype (SMITH *et al.* 1992). The original allele, *Kn1-O*, is a tandem duplication of 17 kb (VEIT *et al.* 1990). Another allele, *Kn1-2F11*, results from the insertion of a *Ds2* element (HAKE *et al.* 1989a), a non-autonomous element of the (*Activator*) *Ac* family [see FEDOROFF (1989) for review]. A third allele, *Kn1-N* (gift of G. NEUFFER, University of Missouri) contains an *rDt* element in the fourth intron (HAKE 1992).

In this report, we describe the isolation of nine *Mu*-containing *Kn1* mutations. Significantly, the *Mu* elements have all inserted in a discrete region of one of the introns. The insertions do not alter the size of the *kn1* transcript, but result in ectopic expression of the transcript in leaves of mutant plants. At least two of these dominant alleles require *Mu* activity for expression of the knotted phenotype. We discuss mechanisms whereby suppressible insertions can produce dominant phenotypes.

MATERIALS AND METHODS

Genetic stocks: The *Kn1* mutants arose in *Mutator* lines maintained collectively by S. HAKE and M. FREELING (HAKE *et al.* 1989b). Allelism was suggested by linkage of the knotted phenotype to *adh1*, which is 1 map unit distal to *kn1* on chromosome 1L. *ADH1* genotypes were determined by assaying scutellar slices (FREELING and SCHWARTZ 1973). Our use of nomenclature is according to the recommendations of the maize nomenclature committee (*Maize Genetics Cooperation Newsletter* 67: 171–73). The name and symbol of a gene locus is represented in lower case italics (*kn1*), the dominant alleles are designated with the first letter of the symbol capitalized (*Kn1*). The gene product, either RNA or protein, is KN1.

The dominant *Kn1* mutations were found in *Mu* screens, each screen containing approximately 1500 families of 20 kernels each, or in outcross populations to generate seed for screening (HAKE *et al.* 1989b). *Kn1-mum1* was found in the 1987 screen. *Kn1-mum2* was found in the outcross population of approximately 5000 *Mu* plants in 1988. *Kn1-mum3* was found in the 1989 screen; only tissue was taken, no seed was harvested. *Kn1-mum4* (90*620), *Kn1-mum5* (90*428) and *Kn1-mum6* (90*1234) were found in the 1990 screen (seed was obtained only from *Kn1-mum5*). *Kn1-mum7* (MF11333-1) and *Kn1-mum9* (MF11333-2) were isolated from open-pollinated *Mutator* stocks (5000 seeds planted). *Kn1-mum8* was a gift from N. SHEPARD (E.I. DuPont, Delaware). *Kn1-mum10* (91*1433) was found in the 1991 screen.

$\Delta sh\ bz$, a deletion of both *bronze1* (*bz1*) and *shrunk1* (*sh1*) (*sh bz X3*), was the primary non-*Mu* line used in our crosses (containing zero to a few inactive elements). It was a gift of J. MOTTINGER (University of Rhode Island). *bz-Mum9* is

a mutable allele of *bz1* that contains a *Mu1* element (BROWN *et al.* 1989) inserted at the single 3' intron/exon junction (DOSEFF *et al.* 1991). It was a gift from D. ROBERTSON (Iowa State University).

Probes: The *kn1* genomic fragment (H2) was derived from the *Kn1-2F11* genomic clone as previously described (HAKE *et al.* 1989a). The entire *kn1* cDNA, pOC5 (VOLLBRECHT *et al.* 1991) was used as a probe for RNA blot hybridization. The ubiquitin probe, a 700-bp *PstI/SacI* fragment from pskUBI, was a gift from P. QUAIL (University of California, Berkeley) (CHRISTENSEN *et al.* 1992). The *Mu1*-specific probe was pA/B5 (CHANDLER *et al.* 1986), the *Mu8*-specific probe was an internal 550-bp *PvuII/PstI* fragment subcloned from the *Mu8* element in *Kn1-mum1*. The *Mu** probe was a 1.3-kb *EcoRI/BamHI* fragment of *MuDR-1* (CHOMET *et al.* 1991).

Tissue dissections: Two tissue fractions were collected for RNA extraction, meristem enriched (ME) and leaf (L) fractions. To isolate ME and L fractions, 14-day seedlings (approximately three leaves visible) were harvested by cutting at the seed. The oldest six leaves were discarded, and the next three leaf primordia (plastochrons 4, 5 and 6, lengths of 0.5–2.5 cm) were collected, pooled and used for leaf RNA isolations. The meristem-enriched fraction included the leaf bases, stem, meristem and the most recently initiated leaf primordia. Tissue from six to eight individuals was pooled for each sample.

RNA isolation and northern blots: RNA extractions were performed as described previously (SMITH *et al.* 1992). Ten micrograms of total RNA in a volume of 5 μ l were glyoxylated with 10 μ l dimethyl sulfoxide, 2 μ l 10 $\times$ running buffer (0.1 M NaPO₄, pH 7.0), and 3 μ l deionized glyoxal (ethandial, Sigma) and incubated at 50° for 60 min. Samples were electrophoresed through a 1% agarose gel in 1 $\times$ running buffer and transferred to Nytran (Schleicher & Shuell) as described elsewhere (SAMBROOK *et al.* 1989). Deglyoxalization was performed by baking the filter at 80° for 2 hr followed by a brief wash in 200 mM Tris, pH 8.5. Hybridization was performed overnight as described (SMITH *et al.* 1992).

DNA analysis: DNA isolations were done by grinding 1–2 g of seedling leaf tissue under liquid nitrogen and transferring the powder into a microcentrifuge tube containing 500 μ l of urea extraction buffer (7 M urea, 30 mM NaCl, 5 mM Tris, 2 mM EDTA, 1% Sarcosyl, pH 8.0). Samples were vortexed gently, followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1). Nucleic acids were precipitated from the aqueous phase with isopropanol following the addition of 0.1 volume of 4.4 M ammonium acetate.

DNA samples were digested with the indicated enzymes according to the manufacturer's instructions, fractionated on 0.8% agarose gels, transferred to Nytran nylon membranes (Schleicher & Shuell) and UV cross-linked to immobilize the digested DNA. The filters were hybridized at 68° in a Robbins Scientific Hybridization Oven according to the manufacturer's suggestions.

Genomic DNA from the *Mu8*-containing *Kn1* alleles was polymerase chain reaction (PCR)-amplified using Promega *Taq* DNA polymerase after digestion with *PvuII*. The primers were: **PJ2** 5' GATCGATTCCATTGGAATG 3', contained within the *kn1* third intron, and **Mu15A-5** 5' GTCATCGTC-CAGAACTCGGA 3', designed from internal *Mu8* sequences (FLEENOR *et al.* 1990). Genomic DNA from the *Mu1*-containing alleles was first digested with *BstEII* prior to amplification. The *Mu* end primer, **MuE2** 5' GCGAATTCCATA-ATGGCAATTATCTC 3', a gift from B. K. GRUISSEM and M. FREELING, was used in combination with **PJ2**. Conditions were 25 cycles of 1 min, 94°; 1 min, 50°; and 1.5 min, 72°, in the buffer as suggested by the manufacturer, except 2 mM dGTP and 2 mM 7-deaza-dGTP (Sigma) were included in reactions

with the Mu15A-5 primer. PCR products corresponding to one end of the *Mu* insertion site were either directly sequenced or were filled in with Klenow and ligated into the *EcoRV* site of pBluescript SK⁺ (Stratagene). Sequencing was according to CHI *et al.* (1988).

RESULTS

Previously characterized *Kn1* mutants are dominant and have arisen spontaneously, often in transposon backgrounds (FREELING and HAKE 1985); they have not been recovered following non-targeted EMS mutagenesis (M. G. NEUFFER, personal communication). We screened for new *Kn1* mutants in lines carrying *Mu* transposable elements. Nine *Kn1* mutations arose from approximately 130,000 plants which were planted over the course of 5 years (see MATERIALS AND METHODS). One other mutation was obtained as a gift from N. SHEPHERD, DuPont, Delaware. We have designated the mutations *Kn1-mum1* to *Kn1-mum10*.

Identification and location of *Mutator* insertions:

The *kn1* locus codes for an mRNA of 1.6 kb and contains four introns 219, 98, 5056 and 690 bp in length (VOLLBRECHT *et al.* 1991). Southern blot analysis of DNA from the *Kn1* mutants that arose in *Mu* lines demonstrated that nine of them contained insertions of approximately 1.4 kb in the third intron (data not shown). Insertion polymorphisms at *kn1* were not detected in normal siblings or progenitor lines. One of the *Kn1* mutants, *Kn1-mum1*, was cloned as a *BclII* fragment using *kn1* genomic fragments as hybridization probes. Southern analysis and sequencing demonstrated the presence of an insertion 700 bp downstream of the site at which *Ds2* is inserted in *Kn1-2F11* (HAKE *et al.* 1989a). Partial sequence analysis of the insertion indicated identity to a *Mu8* element (data not shown) (FLEENOR *et al.* 1990). We determined by Southern analysis that four other alleles (*Kn1-mum2*, *Kn1-mum3*, *Kn1-mum5*, *Kn1-mum6*) contained *PvuII* and *PstI* sites (data not shown), which are diagnostic for the *Mu8* element. All five *Mu8* elements are in the same orientation. DNA samples were also digested with *NotI* and *BstEII*, restriction enzymes diagnostic for *Mu1* (HARDEMAN and CHANDLER 1993). Southern analyses demonstrated that the insertions in *Kn1-mum4*, *Kn1-mum7*, *Kn1-mum8* and *Kn1-mum10* contain *NotI* and *BstEII* restriction sites approximately 400 bp from each other (Figure 1), as found in the original *Mu1* element (BARKER *et al.* 1984). This analysis also revealed that the elements in *Kn1-mum4* and *Kn1-mum7* were in one orientation, and the elements in *Kn1-mum8* and *Kn1-mum10* were in the opposite orientation (Figure 1). Southern analysis did not reveal any polymorphism within a 10-kb region of the *kn1* gene for *Kn1-mum9* (GREENE 1993).

PCR was employed to amplify the insertion sites of the *Mu* elements at *kn1*. An oligonucleotide primer was designed from internal sequences of *Mu8* and used in combination with a *kn1*-specific primer to amplify se-

FIGURE 1.—Southern analysis of *Mu* induced *Kn1* alleles. DNA samples from: 1) *Kn1-mum4/+*, 2) *Kn1-mum7/+*, 3) *Kn1-mum8/+*, 4) *Kn1-mum10/+*, 5) *Kn1-mum2/+*, 6) *Kn1-mum9/+* were digested with *SacI* and *BstEII* or with *SacI* and *NotI*. *SacI* sites are indicated in Figure 2; *NotI* and *BstEII* sites are not present within the *kn1* gene. The resulting Southern was hybridized to the H2 probe which is indicated in Figure 2. The size of the *SacI*, *NotI* or *SacI*, *BstEII* fragment in the *kn1* + allele is 6.1 kb in length. Additional fragments are visible in the DNA of all individuals except *Kn1-mum9*. The fragment in the *Kn1-mum2* allele is increased to 7.5 kb. The 7.5-kb *SacI* fragment of *Kn1-mum4*, *Kn1-mum7*, *Kn1-mum8* and *Kn1-mum10* is digested to smaller size fragments with *NotI* and *BstEII*, indicating the presence of these restriction sites which are diagnostic for *Mu1*. The H2 probe does not extend far enough to detect fragments 5' of the *Mu* insertions.

quences from the *Mu8*-containing alleles (MATERIALS AND METHODS). A *Mu1* end primer, Mu-E2, was used in combination with the same *kn1* primer to amplify sequences from the *Mu1*-containing alleles. The Mu-E2 primer was also successfully used on *Mu8* alleles in further experiments. Subcloning and sequencing of the amplified fragments indicated the exact location of these insertions (Figure 2).

Nine independent *Kn1* mutations result from the insertion of *Mu* elements within an interval of 310 bp. Based on diagnostic restriction enzyme sites, both *Mu1* and *Mu8* elements are present. Four insertions fall in a 48-bp interval with two of these, a *Mu1* and a *Mu8* element, inserted at precisely the same location. The second cluster of four insertions, within a 54-bp interval, is located 208 bp farther downstream (Figure 2). One insertion lies between these clusters. The cluster of *Mu* insertions is approximately 700 bp downstream of the *Ds2* insertion site in *Kn1-2F11*.

An increase in *KN1* transcript is found in *Kn1-mum*

leaves: We isolated RNA from *Kn1-mum1*, *Kn1-mum2*, *Kn1-mum5* and *Kn1-mum7* to determine the effect of *Mu* insertions on *KN1* transcript size and abundance. Total RNA from leaf and meristem-enriched tissue fractions (see MATERIALS AND METHODS) was used for northern analysis with the *kn1* cDNA as a probe (VOLLBRECHT *et al.* 1991). We included RNA from *Kn1-N* and normal (*kn1*+) sibling leaves as controls. The northern was also probed with the maize ubiquitin cDNA to assess RNA quality and quantity.

As previously reported for *Kn1-N* (SMITH *et al.* 1992), the *Kn1-mum* mutations result in ectopic expression of

FIGURE 2.—Location of the nine *MuI* and *Mu8* elements at the *kn1* locus. (A) A map of the *kn1* locus displays the position of the *Mu* insertions (open triangles) relative to the exons (boxes), the *Ds2* insert of *Kn1-2F11*, the unidentified insert in *Kn1-Z3* (unlabeled black triangle) and the *rDt* insert in *Kn1-N*. The H2 fragment used for Southern analysis is indicated. (B) The sequence displays the location of the *MuI* or *Mu8* insertions. The 9-bp duplication that resulted from each insertion is not indicated. The shaded triangles are *Mu8*, the empty triangles are *MuI*. All the *Mu8* elements are in the same orientation; the orientation of the *MuI* elements are indicated by the arrows. Diagnostic restriction enzyme sites are given: H = *HindIII*, S = *SacI*, B = *BclI*. PJ2 is a primer used in PCR experiments.

KN1 transcript in leaves (Figure 3, lanes 3–7). KN1 transcripts were not detected in the *kn1+* leaves (lanes 1–2 and our unpublished data). Approximately equal amounts of hybridizing transcript were detected in the meristem-enriched tissue of *kn1+* and *Kn1-mum* seedlings (lanes 8–12). The KN1 transcript is the same size, 1.6 kb, in all samples. The same size transcript has been detected using KN1 probes that extend over the first and second exon only (data not shown). No hybridization

signals have been detected using part of the third intron (H2, see Figure 2) as a probe (data not shown). Thus it appears that the *Mu* insertions have altered the tissue in which KN1 is expressed without altering the transcript itself.

***Mu* methylation is correlated with poor penetrance of the knotted phenotype in *Kn1-mum2* and *Kn1-mum7*:** *Kn1* mutants often vary in penetrance and expressivity (our unpublished data): the *Mu*-induced mutants are

FIGURE 3.—Northern blot analysis of *Kn1-mum* alleles. Lanes 1–7 are leaf tissue and lanes 8–12 are meristem-enriched tissue (see MATERIALS AND METHODS). The genotypes of the plants were as follows: lanes 1 and 12, *kn1+* (siblings of *Kn1-mum5* as determined by *adh1* genotype); lane 2, *kn1+ Δsh bz*; lanes 3 and 8, *Kn1-mum7/+*; lanes 4 and 9, *Kn1-mum5/+*; lanes 5 and 10, *Kn1-mum2/+*; lanes 6 and 11, *Kn1-mum1/+*; lane 7, *Kn1-N*. Panel A was probed with the *kn1* cDNA, panel B with the maize ubiquitin cDNA; 10 ug of total RNA were loaded per lane. The *Mu* insertions condition expression in leaves that is not found in *kn1+* leaves; however, the size of the *kn1* transcript is unaffected.

no exception. We sought to determine whether this variability was in response to changes in *Mu* activity. Our rationale was based on the fact that the phenotypes of a number of other *Mu*-induced mutations are dependent on *Mu* activity (MARTIENSSSEN *et al.* 1990; CHOMET *et al.* 1991; LOWE *et al.* 1992; MARTIENSSSEN and BARON 1994; L. SMITH and S. HAKE, unpublished data).

We examined *Mu* activity in a number of *Kn1-mum2* families with normal-appearing, *Mu8*-containing individuals (Table 1). DNA samples were cleaved with the restriction enzyme *HinfI*, and the Southern blots were hybridized to the *Mu8* probe (MATERIALS AND METHODS). Hypomethylated *Mu8* elements, indicative of *Mu* activity, produce a 1.34-kb hybridizing band that is internal to the *HinfI* sites of *Mu8*. Larger molecular weight bands are visible when the *Mu* elements are methylated or inactive, reflecting the digestion at adjacent genomic *HinfI* sites instead of those within *Mu* (CHANDLER and WALBOT 1986; BENNETZEN 1987). A Southern blot containing a few individuals from each family is shown in Figure 4. Of the 12 individuals that appeared normal, 10 had methylated *Mu8* elements. All eight knotted individuals carried hypomethylated elements (Figure 4A). Probing the blot with the *Mu1*-specific probe distinguished the same *Mu* active individuals from inactive ones (data not shown).

DNA samples from these same individuals were also digested with *SacI* and hybridized with *Mu**, a *MuDR*

TABLE 1

Correlation of *Mu* element methylation and knotted phenotype

Cross	Family no.	Knotted phenotype		Normal phenotype	
		Active	Inactive	Active	Inactive

<i>Kn1-mum2/+</i> × <i>Kn1-mum2/+</i> ^{a,b}					
244-12 × 243-4	1885	17	—	—	1
244-11 × 243-7	1894	9	—	2	1
244-9 × 243-7	1887	11	—	—	8
243-4 × 243-7	1886	11	—	—	1
244-2 × 243-4		15	—	—	—
244-11 × 243-4	1894	11	—	—	—
<i>Kn1-mum2/+</i> × <i>+/+</i> ^{a,c}					
2070-9 × 2036	2765	11	—	1 ^d	—
<i>Kn1-mum7/Kn1-mum7</i> × <i>Kn1-mum7/Kn1-mum7</i> ^b					
2235-1 × 2234-2		11	—	—	3
<i>+/+</i> × <i>Kn1-mum7/Kn1-mum7</i> ^c					
2508 × 2503-1	2602	12	—	—	27

^a Only individuals carrying the mutant allele, as determined by the linked *adh1* genotype, were analyzed.

^b *Mu* activity was determined by *HinfI* digestion of genomic DNA and probing the Southern blots with *Mu8*.

^c *Mu* activity was determined by presence of spots at *bz-Mum9*.

^d The active, normal, individual did not contain the *Mu* insertion, either due to a reversion or recombination event between *adh1* and *kn1*.

probe. *Mu** hybridizes to a number of related elements in the genome, however, a 4.8-kb *SacI* fragment which is internal to *MuDR* (CHOMET *et al.* 1991), is the only one correlated with a single autonomous element segregating in the genome (D. LISCH, P. CHOMET and M. FREELING, manuscript submitted for publication). All the knotted individuals contain the 4.8-kb *SacI* fragment, indicative of the presence of *MuDR*; the normal-appearing individuals that contained methylated *Mu8* elements lacked the 4.8-kb *SacI* fragment (Figure 4B). We confirmed that all normal-appearing individuals still carried the *Mu8* element at *Kn1-mum2* by probing the *SacI* blot with the H2 probe of *kn1* (Figure 4C). Thus, the knotted individuals of *Kn1-mum2* were always *Mu* active. The normal individuals were mostly inactive, but exceptional individuals were found that carry *Mu* activity but do not show knots (Table 1).

We were interested to determine whether the knotted phenotype in *Mu1*-containing alleles was dependent upon *Mu* activity as in the *Mu8*-containing allele, *Kn1-mum2*. A family of fourteen *Kn1-mum7* homozygotes contained eleven knotted and three normal-appearing individuals. Southern analysis of *Mu* activity showed that the three normal-appearing individuals carried methylated *Mu1* elements, whereas the knotted individuals had hypomethylated *Mu1* elements (Figure 5). The presence of the *Mu* element at *kn1* was confirmed in all individuals by digestion with *SacI* and hybridization to the *kn1* H2 probe (data not shown).

Further analysis on *Kn1-mum7* penetrance took advantage of a *Mu1*-induced allele at the *bronze1* locus,

FIGURE 4.—Southern blot analysis of *Mu* activity in *Kn1-mum2*. Non-knotted individuals are marked with stars, the other individuals were all knotted. (A) DNA from *Kn1-mum2/kn1+* individuals was digested with *HinfI*, and the blot was hybridized to a *Mu8* probe. Complete digestion of *Mu8* results in a 1.34-kb band. The 2.0-kb band is present in our non-*Mu* $\Delta sh bz$ line. (B) DNA from the same individuals was digested with *SacI*, and the blot was probed with *Mu**. The 4.8-kb fragment is correlated with the presence of *MuDR* (D. LISCH, P. CHOMET and M. FREELING, manuscript submitted for publication). (C) The *SacI* blot was probed with H2, a fragment of the *kn1* gene, to verify that each individual was heterozygous for *Kn1-mum2*. All knotted individuals have hypomethylated *Mu8* elements and the 4.8-kb *MuDR* fragment.

FIGURE 5.—Southern blot analysis of *Mu1* activity in *Kn1-mum7*. DNA from non-knotted (marked with stars) and knotted siblings was digested with *HinfI* and probed with the *Mu1* probe, pA/B5, showing that knotted individuals had hypomethylated elements and normal-appearing individuals had methylated *Mu1* elements.

bz-Mum9. This allele conditions spotted kernels in the presence of *Mu* activity and non-spotted, bronze kernels in the absence of *Mu* activity (BROWN and SUNDARESAN 1992). A *Kn1-mum7; bz-Mum9* homozygote was crossed to a non-*Mu kn1+* homozygote that was carrying a deletion of both *bronze1* and *shrunk1*, $\Delta sh bz$. The resulting kernels segregated approximately 1:1 for spots. Of 24 plants grown from non-spotted kernels, all appeared normal with the exception of one that had a knot on the first leaf. The rest of the leaves on this plant were normal. Of 15 plants grown from spotted kernels, all but three were knotted. The knots were visible on seedling and adult leaves. DNA was isolated from the three normal plants in the spotted category, and Southern analysis showed that the *Mu1* elements were methylated (data not shown). Thus, every knotted *Kn1-mum7* individual was *Mu*-active and every normal *Kn1-mum7* individual was *Mu*-inactive, consistent with the results of *Kn1-mum2* in requiring *Mu* activity for the knotted phenotype.

Ectopic RNA expression in knotted *Kn1-mum2* leaves requires *Mu* activity: To determine whether the ectopic RNA seen in *Kn1-mum2* leaves requires *Mu* activity, RNA was isolated from *Mu*-inactive and *Mu*-active individuals. The *Mu* inactive individuals were the progeny of an inactive *Kn1-mum2* homozygote crossed to $\Delta sh bz$. The two *Mu*-active families came from crosses of *Mu*-active *Kn1-mum2* heterozygotes to $\Delta sh bz$. Knotted individuals were picked from these crosses for the RNA isolations. We included tissue from $\Delta sh bz$ and *Kn1-N* as controls. KN1 RNA was detected in leaf RNA from *KN1-N* and in the two *Mu*-active, knotted *Kn1-mum2* families (Figure 6A, lanes 3–5); it was not detected in the *kn1+; \Delta sh bz* leaf RNA nor in the *Mu*-inactive leaf RNA (lanes 1–2). KN1 RNA was detected in meristem tissue of inactive and active *Mu* families at comparably equal levels (lanes 6–9). We confirmed the presence of *Mu*-activity by probing the Northern blots with the *MuDR* probe, *Mu**. Our Northern blot shows that only RNA from the *Mu*-active, knotted families contain the 2.9-

FIGURE 6.—Dependence on *Mu* activity for *Kn1-mum2* ectopic expression. RNA was isolated from leaves (lanes 1–5) and meristem-enriched tissues (lanes 6–9) (see materials and methods). The genotypes of the plants are as follows: lanes 1 and 6, *kn1+ Δsh bz*; lanes 2 and 7, *Kn1-mum2/+ (Mu-inactive)*; lanes 3 and 8, *Kn1-mum2/+ (Mu-active)*; lanes 4 and 9, *Kn1-mum2/+ (Mu-active)*; lane 5, *Kn1-N*. (A) The blot was probed with the *kn1* cDNA. (B) The blot was probed with the ubiquitin cDNA probe. (C) The blot was probed with the *Mu** probe. Residual ubiquitin staining is seen in all lanes (*). (D) DNA isolated from the same individuals was digested with *HinfI* and probed with *Mu8*. Lane 1, *kn1+ Δsh bz*; lane 2,

FIGURE 7.—Southern blot of reactivated *Kn1-mum7* individuals. Normal-appearing *Kn1-mum7* individuals were crossed to *Mu* active, *kn1+* lines. The resulting progeny were moderately to severely knotted. DNA samples from these individuals were digested with *HinfI* and the blot probed with *Mu1*. While all the individuals have some unmethylated elements, the variation in the degree of hypomethylation appears to correlate with the phenotypic severity.

and 1.0-kb transcripts (Figure 7C), which are found only in *Mu*-active lines (CHOMET *et al.* 1991; HERSHBERGER *et al.* 1991). DNA isolated from the same individuals and analyzed by *HinfI* digestion confirmed the Northern analysis (Figure 6D). We conclude from these experiments that the ectopic expression found in mutant leaves requires *Mu* activity and is not conditioned solely in response to the insertion's presence.

Reactivation of the knotted phenotype by reintroduction of *Mu* activity: *Mu* elements are inactive due to the absence of the autonomous *MuDR* element (CHOMET *et al.* 1991; HERSHBERGER *et al.* 1991; D. LISCH, P. CHOMET and M. FREELING, manuscript submitted for publication). When *MuDR* is reintroduced by a genetic cross, inactive non-autonomous elements become active once again (MARTIENSSSEN *et al.* 1990; BROWN and SUNDARESAN 1992). We predicted that *Mu* inactive *Kn1-mum2* or *Kn1-mum7* individuals would again express the knotted phenotype when crossed to active lines. The closely linked *adh1* locus provided a means of determining the *kn1* genotype of individuals involved in the crosses (see MATERIALS AND METHODS).

From a segregating family (Table 1; 1887), we selected individuals that were homozygous for *Kn1-mum2* and *Mu*-inactive, or homozygous for *kn1+* and *Mu*-active, as determined by *adh1* genotype and *HinfI* digestions using the *Mu8* probe. Plants of either genotype are normal in appearance. Two *Kn1-mum2* plants were crossed to *Mu* active individuals as well as to the non-*Mu*, *Δsh bz* line. Seedlings from each cross were examined for the appearance of knots (Table 2). Twenty-six out of 39

Kn1-mum2/+ (Mu-inactive); lanes 3 and 4, *Kn1-mum2/+ (Mu-active)*. *KN1* transcripts accumulate in leaves only when the plant carries *Mu* activity.

TABLE 2
Reactivation of knotted phenotype by crossing in *Mu* activity

Cross	Progeny	
	<i>Kn</i>	<i>N</i>
<i>kn1</i> + <i>adh1-F/kn1</i> + <i>adh1-F</i> ; <i>MuDR</i> × <i>Kn1-mum2 adh1-S/Kn1-mum2 adh1-S</i> 1887-18A × 1887-17D	26	13
<i>Kn1-mum2 adh1-S/Kn1-mum2 adh1-S</i> × <i>kn1</i> + <i>adh1-F/kn1</i> + <i>adh1-F</i> 1887-17D × 1895	0	26
<i>kn1</i> + <i>adh1-F/kn1</i> + <i>adh1-F</i> ; <i>MuDR</i> × <i>Kn1-mum2 adh1-S/Kn1-mum2 adh1-S</i> 1887-18B × 1887-17E	6	6
<i>kn1</i> + <i>adh1-F/kn1</i> + <i>adh1-F</i> × <i>Kn1-mum2 adh1-S/Kn1-mum2 adh1-S</i> 1895 × 1887-17E	0	10
<i>Kn1-mum7 adh1-F/Kn1-mum7 adh1-F</i> × <i>kn1</i> + <i>adh1-S/kn1</i> + <i>adh1-S</i> ; <i>MuDR</i> 2431-4 × 2431-5	22	14
<i>Kn1-mum7 adh1-F/kn1</i> + <i>adh1-S</i> × <i>kn1</i> + <i>adh1-S/kn1</i> + <i>adh1-S</i> ; <i>MuDR</i> 2602A-3 × 2587	4	4
<i>kn1</i> + <i>adh1-S/kn1</i> + <i>adh1-S</i> × <i>Kn1-mum7 adh1-F/kn1</i> + <i>adh1-S</i> 2576 × 2602A-3	0	32

The linked *adh1* allele was used to determine the genotype at *kn1*. All progenitors were normal in appearance.

progeny from one cross to the *Mu* active line showed a knotted phenotype, whereas none of the 26 progeny from the same parent crossed to Δsh *bz* showed any signs of knots. Progeny from the second *Mu*-inactive *Kn1-mum2* parent gave 6 out of 12 knotted individuals when crossed to the *Mu* line, but 0 out of 10 when crossed to Δsh *bz*.

A similar test for reactivation of the knotted phenotype in *Kn1-mum7* was performed. A *Mu*-inactive, *Kn1-mum7* homozygote with no knots was crossed to a *Mu* active *kn1*+ sibling. Twenty-two of 36 progeny were knotted. Eleven knotted progeny that varied in the intensity of knots were examined by *HinfI* digestions. The diagnostic *HinfI* blot for *Mu1* methylation showed that the most knotted individuals contain primarily the 1.3- and 1.7-kb hypomethylated fragments, whereas the moderately knotted individuals also contain larger fragments indicating that some of the *Mu* elements are methylated (Figure 7). This result suggests that the *Kn1-mum7* individuals may have been chimeric for *Mu* activity (MARTIENSSSEN *et al.* 1990). An inactive *Kn1-mum7* heterozygote from family 2602 (Table 1) was also crossed to a *Mu*-active line. Four of the eight progeny were knotted, while a cross to Δsh *bz* yielded 0 out of 32 knotted individuals (Table 2). Thus, both *Kn1-mum2* and *Kn1-mum7* are capable of reactivation by the reintroduction of *MuDR*.

DISCUSSION

We isolated nine *Kn1* mutants in lines containing active *Mu* elements. Interestingly, we determined that all of these alleles contain either a *Mu8* or a *Mu1* element in the third intron of the *kn1* locus. These *Mu* insertions condition ectopic KN1 expression in young leaves, presumably leading to the dominant knotted phenotype. We established that the knotted phenotype of *Kn1-mum7* and *Kn1-mum2* requires *Mu* activity by crossing

inactive, normal *Kn1-mum* individuals by *Mu*-active, *kn1*+ individuals and regaining the knotted phenotype.

The nine *Mu* insertions lie within a 310-bp region of the 5-kb third intron, suggesting that this region is a "hotspot" for *Mu* element insertion. Clustering of *Mu* insertions occurs to some degree in mutant alleles of *adh1* and *bz1* [reviewed by BENNETZEN *et al.* (1993)]. A knotted phenotype is also produced by insertion of a *Ds2* element in the same intron approximately 700 bp 5' to the *Mu* insertion sites (HAKE *et al.* 1989a). Two additional alleles are associated with insertions; an uncharacterized insertion lies 1 kb 3' to the *Mu* insertion sites in the *Kn1-Z3* allele, and an *rDt* element lies in the fourth intron of the *Kn1-N* allele (our unpublished data). *Kn1-O*, caused by a tandem duplication of 17 kb, is the only mutation that is not correlated with a transposon insertion (VEIT *et al.* 1990). One other potential exception is *Kn1-mum9*. Although this mutation arose in a *Mu* line, we have not found an insertion at *kn1*. It is either a mutation resulting from a small change that is not detectable by Southern analysis, or a mutation at a closely linked gene which caused a phenotype very similar to knotted. A mutation of *knox3*, a *kn1*-like homeobox gene also tightly linked to *adh1* (our unpublished data), might provide a possible explanation for *Kn1-mum9*.

kn1-hybridizing transcripts were detected in young leaf tissue of four of the *Kn1* mutants. KN1 RNA was not detected in total RNA of normal leaves. The hybridizing RNA species isolated from mutant meristems or leaves appears to be the same size as that from normal siblings. We can not, however, rule out the possibility that low levels of aberrant mRNAs are transcribed from *Kn1-mum* alleles. Analysis of three other insertion alleles, *Kn1-N*, *Kn1-2F11* and *Kn1-Z3*, also demonstrates an increase in KN1 transcript in mutant leaves but no change in size (SMITH *et al.* 1992; GREENE 1993). Based

on the fact that the same size transcript is produced by a variety of insertions, we would argue that altered splicing or termination is not the major mechanism responsible for knot formation.

The knotted phenotype of *Kn1-mum2* and *Kn1-mum7* requires *Mu* activity. An absolute correlation exists between the presence of the knotted phenotype and hypomethylated elements for both alleles, in addition, all normal-appearing *Kn1-mum7* plants had methylated *Mu* elements. We also showed that *kn1* transcripts do not accumulate in leaves of inactive *Kn1-mum2* individuals and have similar unpublished data for *Kn1-mum7*. We performed a genetic test to demonstrate the dependence of the knotted phenotype on *Mu* activity. Non-knotted, methylated *Kn1-mum2* and *Kn1-mum7* individuals became knotted when *MuDR* was re-introduced through a genetic cross. The reactivation was usually less than 100%. The lowered penetrance is probably due to the fact that the *Mu* parents were from families that segregated active and inactive individuals and thus they could have been heterozygous for *MuDR*.

A similar requirement for the autonomous element occurs with the *Ds2* insertion allele, *Kn1-2F11* (HAKE *et al.* 1989a). The phenotype is mild to normal-appearing in the absence of *Ac*; it is much stronger in the presence of *Ac* with certain *Ac* elements having a greater effect (E. VOLLBRECHT and S. HAKE, unpublished data). Transposon dependent mutations have also been documented with other *Mu* insertions (MARTIENSSEN *et al.* 1990; LOWE *et al.* 1992; MARTIENSSEN and BARON 1994; L. SMITH and S. HAKE, unpublished data). The recessive *hcf106* mutation contains a *Mu1* element near the transcription initiation site. *hcf106* plants that lack *Mu* activity appear normal due to transcription that initiates from an outward promoter in the terminal inverted repeat of the *Mu1* element producing a normal *hcf106* translational product. Transcription fails to occur in *Mu* active lines thus leading to a null phenotype (BARKAN and MARTIENSSEN 1991). A *MuDR* gene product is thought to bind to the active element and block transcription. A dominant *Mu*-dependent mutation, *Les28*, has been recently described that conditions localized necrotic regions on the leaf blade only when *Mu* elements are hypomethylated (MARTIENSSEN and BARON 1994). Using genetic lines that contain both the *hcf106* and *Les28* mutations, MARTIENSSEN and BARON identified plants that coordinately lost both phenotypes in sectors containing methylated *Mu* elements.

Phenotypes that are dependent on trans-acting factors have been well described in *Drosophila* [reviewed by KUBLI (1986), MODOLELL *et al.* (1983), RUTLEDGE *et al.* (1988), MOUNT *et al.* (1988), and BIRCHLER and HIEBERT (1989)]. For example, the mutagenic effects of the *gypsy* transposable elements are in great part due to the binding of modifier proteins, such as that encoded by *suppressor of hairy wing* (SUHW) (PARKHURST *et al.* 1988).

The binding of SUHW to *gypsy* elements inserted within coding regions can result in qualitative transcript changes (CAMPUZANO *et al.* 1986; DORSETT *et al.* 1989; HOOVER *et al.* 1993). Binding of SUHW to *gypsy* insertions in regulatory regions can also lead to quantitative transcription changes (PEIFER and BENDER 1986; CAMPUZANO *et al.* 1986; CORCES and GEYER 1991; JACK *et al.* 1991). One specific example is the *gypsy* element inserted in the 5' region of the homeodomain-encoding *cut* locus between a long distance enhancer and the promoter. The element blocks expression of *cut* when the SUHW protein is present (JACK *et al.* 1991). Mutations that affect the SUHW binding site in *gypsy* or that affect the SUHW protein itself restore the phenotype to wild-type (HOOVER *et al.*, 1992). The SUHW protein must be present at the time the enhancer is active (DORSETT 1993). By analogy, we would expect an effect of *MuDR* only when *MuDR* is coexpressed with *kn1* in the leaf.

Any model we propose to explain the *Kn1-mum* alleles must take into account the dominant, gain of function, nature of the transposon-induced mutants. Dominant *Hairy wing* mutations of the *achaete-scute* complex in *Drosophila melanogaster* results from overexpression of truncated *achaete-scute* transcripts due to *gypsy* and *copia* insertions (CAMPUZANO *et al.* 1986). The overexpression rather than the truncation is considered to be the cause of the mutant phenotype since rearrangements that do not result in truncated transcripts produce the same dominant phenotype and recessive phenotypes occur in the absence of a functional gene product (CAMPUZANO *et al.* 1986; BALCELLS *et al.* 1988). Another example is the insertion of a *tom* transposable element in the 3' region of the homeobox gene, *Om(1D)*, in *Drosophila ananassae* (TANDA and CORCES 1991). Expression of the *Om(1D)* gene is increased sevenfold in the eye imaginal disc in the mutant flies. Mutations resulting from insertion of *tom* transposons are almost always associated with dominant phenotypes that affect eye morphology (HINTON 1984), presumably due to the presence of an eye enhancer within the *tom* transposon (TANDA and CORCES 1991).

We propose that either *MuDR* turns *kn1* on, *i.e.*, specifically activates the gene during leaf development, or *MuDR* interferes with a negative element. KN1 is normally abundant in the meristem and certain cells of the unexpanded stem. It is not present in leaves nor in the cells that will initiate leaf primordia. In *Kn1* mutants, KN1 is present in a restricted group of provascular cells beginning at plastochron five (SMITH *et al.* 1992). *MuDR* may be capable of interacting with the transcriptional machinery in that selected group of provascular cells to allow expression of *kn1*. The specificity of expression, however, is not likely to reside with *Mu* since *Mu* insertions are not correlated with any tissue specific defects. The provascular localization more likely resides in the *kn1* gene itself since ectopic expression of *kn1* driven by

a CaMV 35S promoter produces a similar mutant phenotype as the transposon-induced mutants (R. WILLIAMS and P. LEMAUX, personal communication).

An alternative model proposes that there are silencer elements present in the large *kn1* intron that function to repress transcription in leaves. The binding of the *MuDR* product to the *Mu* elements blocks access of a repressor to these silencers. Since there are at least two different insertion sites that produce a conditional mutant phenotype, we might propose that multiple silencer elements exist. A model for an intron-located silencer has been proposed by BRADLEY and coworkers (1993) to explain the *ovulata* mutation of the *plena* locus in snapdragon. The *plena* gene is normally expressed only in the inner two flower whorls. A *Tam3* insertion in the intron leads to a dominant gain of function phenotype due to expression in all four whorls (BRADLEY *et al.* 1993).

The penetrance and expressivity of morphological mutations is often variable. Transposon dependence is one of many ways by which the phenotype of a mutation can be affected. All of our transposon-induced *Kn1* mutants vary in penetrance and the *Kn1-mum* mutants are no exceptions. We had one *Kn1-mum2* family that had active individuals without a knotted phenotype (Table 1) and have also seen active *Kn1-mum5* and *Kn1-mum1* individuals without knots (unpublished data). One possible explanation is that non-knotted, *Mu* active *Kn1-mum* individuals have subtle changes at *kn1* that render them inaccessible to the *MuDR*. Another possibility is that the *MuDR* element in these *Mu* active non-expressive individuals is in a different position in the chromosome. The chromosomal position of *P* elements in *Drosophila* affects their ability to excise (MISRA and RIO 1990, and references within). *MuDR* position effects have been shown to affect the transposition rate of *MuDR* itself as well as the excision of a *Mu1* element from the *a* locus (D. LISCH, P. CHOMET and M. FREELING, manuscript submitted for publication). If *MuDR* suffers position effects that prevent its transcription in the same provascular cells in which *kn1* is ectopically expressed, we might expect that it could no longer activate the mutant alleles. Alternatively, if the interaction between *MuDR* and *Kn1-mum* alleles requires high levels of *MuDR* expression, we might find that there are positions in the genome that reduce *MuDR* expression below a threshold level. Finally, there may be other modifiers in the genome that affect *kn1* expression and can account for the variable penetrance (GREENE and HAKE 1993).

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