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

Ben Greene, Richard Walko¹ and Sarah Hake

Plant Gene Expression Center, ARS-USDA and University of California at Berkeley, Albany, California 94710

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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; Coe and Geer 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 Martienssen 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; Martienssen et al. 1990; Tanda and Coe 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 Hershberger (1991)]. 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 (Martienssen et al. 1990; Chomet et al. 1991; Lowe et al. 1992; Martienssen and Baron 1994).

The knotted1 (kn1) gene was cloned by transposon tagging (Hake et al. 1989a). The KN1 protein contains...
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. 1999; 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, Kn1-2P11, 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 rDi 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 II. ADH1 genotypes were determined by assaying scutellar slices (Freeling and Schwartz 1975). Our use of nomenclature is according to the recommendations of the maize nomenclature committee (Maize Genetics Cooperation Newsletter 67: 171–75). 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*260), Kn1-mum5 (90*282) and Kn1-mum6 (90*1254) 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.L. DuPont, Delaware). Kn1-mum10 (91*1433) was found in the 1991 screen.

Δsh 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. Möttger (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 (Doege et al. 1991). It was a gift from D. Robertson (Iowa State University).

**Probes:** The kn1 genomic fragment (H2) was derived from the Kn1-2P11 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 Mu 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 MuDk-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 µl were glyoxylated with 10 µl dimethyl sulfoxide, 2 µl 10× running buffer (0.1 M NaPO4, pH 7.0), and 3 µl deionized glyoxal (ethandial, Sigma) and incubated at 50° for 60 min. Samples were electrophoresed through a 1% agarose gel in 1× 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 µl 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' GATCGATTCCA'ITTGGAATG 3', contained within the kn1 third intron, and Mu1SA 5' GTCATCTGC- CAGAACCTGGA 3', derived from internal Mu8 sequences (Fleenor et al. 1990). Genomic DNA from the Mu-containing alleles was first digested with BstEI prior to amplification. The Mu end primer, MuE2 5' CCCAATCCATCATGGCAATATCCT 3', a gift from B. K. Grussheim 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 Cui et al. (1988).

RESULTS

Previously characterized Knl 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 Knl mutants in lines carrying Mu transposable elements. Nine Knl 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 Knl-mum1 to Knl-muml0.

Identification and location of Mutator insertions:
The knl 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 Knl 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 knl were not detected in normal siblings or progenitor lines. One of the Knl mutants, Knl-mum1, was cloned as a BciI fragment using knl 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 Knl-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 (Knl-mum2, Knl-mum3, Knl-mum5, Knl-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 Knl-mum4, Knl-mum7, Knl-mum8 and Knl-mum10 contain NotI and BstEII restriction sites (data not shown). Additional fragments are visible in the DNA of all individuals except Knl-mum9. The fragment in the Knl-mum2 allele is increased to 7.5 kb. The 7.5-kb SacI fragment of Knl-mum4, Knl-mum7, Knl-mum8 and Knl-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.

An increase in Knl transcript size is found in Knl-mum leaves: We isolated RNA from Knl-mum1, Knl-mum2, Knl-mum5 and Knl-mum7 to determine the effect of Mu insertions on Knl transcript size and abundance. Total RNA from leaf and meristem-enriched tissue fractions (see MATERIALS AND METHODS) was used for northern analysis with the knl cDNA as a probe (Vollbrecht et al. 1991). We included RNA from Knl-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 Knl-N (Smith et al. 1992), the Knl-mum mutations result in ectopic expression of
KN1 transcript in leaves (Figure 3, lanes 3–7). KN1 transcripts were not detected in the knl+ leaves (lanes 1–2 and our unpublished data). Approximately equal amounts of hybridizing transcript were detected in the meristem-enriched tissue of knl+ and Knl-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 Knl-mum2 and Knl-mum7: Knl mutants often vary in penetrance and expressivity (our unpublished data): the Mu-induced mutants are**
Correlation of Mu element methylation and knotted phenotype

<table>
<thead>
<tr>
<th>Cross</th>
<th>Family no.</th>
<th>Knotted phenotype</th>
<th>Normal phenotype</th>
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<td>1 1 1 1 1 1</td>
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<tr>
<td></td>
<td>244-11 X 243-7</td>
<td>1894 9 9 9 9 9</td>
<td>2 2 2 2 2 2</td>
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<tr>
<td></td>
<td>244-9 X 243-7</td>
<td>1887 11 11 11 11 11</td>
<td>8 8 8 8 8 8</td>
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<tr>
<td></td>
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<td>1886 11 11 11 11 11</td>
<td>1 1 1 1 1 1</td>
</tr>
<tr>
<td></td>
<td>244-12 X 243-4</td>
<td>1894 11 11 11 11 11</td>
<td>1 1 1 1 1 1</td>
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<td></td>
<td>2070-9 X 2056</td>
<td>2765 11 11 11 11 11</td>
<td>1 1 1 1 1 1</td>
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<tr>
<td>Knl-mum2/+ X +/- c</td>
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<td>11 11 11 11 11 11</td>
<td>3 3 3 3 3 3</td>
</tr>
<tr>
<td></td>
<td>2508 X 2503-1</td>
<td>2602 12 12 12 12 12</td>
<td>27 27 27 27 27 27</td>
</tr>
</tbody>
</table>

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

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

Mu activity was determined by presence of spots at bzmum9.1

The active, normal, individual did not contain the Mu insertion, either due to a reversion or recombination event between adhl and knl.

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 (Martienssen et al. 1990; Chomet et al. 1991; Lowe et al. 1992; Martienssen and Baron 1994; L. Smith and S. Hake, unpublished data).

We examined Mu activity in a number of Knl-mum2 families with normal-appearing, Mu8-containing individuals (Table 1). DNA samples were cleaved with the restriction enzyme Hinfl, 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 Hinfl sites of Mu8. Larger molecular weight bands are visible when the Mu elements are methylated or inactive, reflecting the digestion at adjacent genomic Hinfl 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 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 contained 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 Knl-mum2 by probing the SacI blot with the H2 probe of knl (Figure 4C). Thus, the knotted individuals of Knl-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, Knl-mum2. A family of fourteen Knl-mum7 homoyzogates 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 knl was confirmed in all individuals by digestion with SacI and hybridization to the knl H2 probe (data not shown).

Further analysis on Knl-mum7 penetrance took advantage of a Mu1-induced allele at the bronze1 locus,
Southern blot analysis of Mu activity in Knl-mum2. Non-knotted individuals are marked with stars, the other individuals were all knotted. (A) DNA from Knl-mum2/knl+ individuals was digested with Hinfl, 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 Ash 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 knl gene, to verify that each individual was heterozygous for Knl-mum2. All knotted individuals have hypomethylated Mu8 elements and the 4.8-kb MuDR fragment.

Southern blot analysis of Mu1 activity in Knl-mum7. DNA from non-knotted (marked with stars) and knotted siblings was digested with Hinfl 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 Knl-mum7; bz-Mum9 homozygote was crossed to a non-Mu knl+ homozygote that was carrying a deletion of both bronze1 and shrunken1, Δsh 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 Knl-mum7 individual was Mu-active and every normal Knl-mum7 individual was Mu-inactive, consistent with the results of Knl-mum2 in requiring Mu activity for the knotted phenotype.

Ectopic RNA expression in knotted Knl-mum2 leaves requires Mu activity: To determine whether the ectopic RNA seen in Knl-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 Knl-mum2 homozygote crossed to Δsh bz. The two Mu-active families came from crosses of Mu-active Knl-mum2 heterozygotes to Δsh bz. Knotted individuals were picked from these crosses for the RNA isolations. We included tissue from Δsh bz and Knl-N as controls. KN1 RNA was detected in leaf RNA from KN1-N and in the two Mu-active, knotted Knl-mum2 families (Figure 6A, lanes 3–5); it was not detected in the knl+;Δsh 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-
A

\[1.6 \text{ kb} \]

B

\[1.9 \text{ kb} \]

C

\[2.9 \text{ kb} \]

\[1.0 \text{ kb} \]

D

\[2.0 \text{ kb} \]

\[1.34 \text{ kb} \]

FIGURE 6.—Dependence on Mu activity for Knl-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, \( knl^+ \Delta sh \); lanes 2 and 7, \( Knl-mum2/+ \) (Mu-inactive); lanes 3 and 8, \( Knl-mum2/+ \) (Mu-active); lanes 4 and 9, \( Knl-mum2/+ \) (Mu-active); lane 5, \( Knl-N \). (A) The blot was probed with the knl 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 HinII and probed with Mu8. Lane 1, \( knl^+ \Delta sh \); lane 2, and 1.0-kb transcripts (Figure 7C), which are found only in Mu-active lines (CHOMET et al. 1991; HERSBERGER et al. 1991). DNA isolated from the same individuals and analyzed by HinII 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; HERSBERGER 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 (MARTIENSSEN et al. 1990; BROWN and SUNDARESAN 1992). We predicted that Mu inactive Knl-mum2 or Knl-mum7 individuals would again express the knotted phenotype when crossed to active lines. The closely linked adh1 locus provided a means of determining the knl 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 \( Knl-mum2 \) and Mu-inactive, or homozygous for \( knl^+ \) and Mu-active, as determined by adh1 genotype and HinII digests using the Mu8 probe. Plants of either genotype are normal in appearance. Two \( Knl-mum2 \) plants were crossed to Mu active individuals as well as to the non-Mu, \( \Delta sh \) line. Seedlings from each cross were examined for the appearance of knots (Table 2). Twenty-six out of 39
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 Knl-mum 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 Knl-mum7 was performed. A Mu-inactive, Knl-mum7 homozygote with no knots was crossed to a Mu active knl+ 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 Mu methylation showed that the most knotted individuals contain primarily the 1.3-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 Knl-mum7 individuals may have been chimeric for Mu activity (MARTIENSENS et al. 1990). An inactive Knl-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 Knl-mum2 and Knl-mum7 are capable of reactivation by the reintroduction of MuDR.

**DISCUSSION**

We isolated nine Knl 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 knl 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 Knl-mum7 and Knl-mum2 requires Mu activity by crossing inactive, normal Knl-mum individuals by Mu-active, knl+ 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 Knl-Z3 allele, and a rDtl element lies in the fourth intron of the Knl-N allele (our unpublished data). Knl-0, caused by a tandem duplication of 17 kb, is the only mutation that is not correlated with a transposon insertion (VETT et al. 1990). One other potential exception is Knl-mum9. Although this mutation arose in a Mu line, we have not found an insertion at knl. 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 knl-like homeobox gene also tightly linked to adh1 (our unpublished data), might provide a possible explanation for Knl-mum9.

knl-hybridizing transcripts were detected in young leaf tissue of four of the Knl 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 Knl-mum alleles. Analysis of three other insertion alleles, Knl-N, Knl-2F11 and Knl-Z3, also demonstrates an increase in KN1 transcript in mutant leaves but no change in size (SMITH et al. 1992; GREENE 1993). Based

**TABLE 2**

Reactivation of knotted phenotype by crossing in Mu activity

<table>
<thead>
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<th>Cross</th>
<th>Progeny</th>
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<td>knl + adh1-F/knl + adh1-F, MuDR × Knl-mum2</td>
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<tr>
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</tr>
<tr>
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<tr>
<td>1887-18B × 1887-17E</td>
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</tr>
<tr>
<td>knl + adh1-F/knl + adh1-F × Knl-mum2 adh1-S</td>
<td>0</td>
</tr>
<tr>
<td>1895 × 1887-17E</td>
<td>10</td>
</tr>
<tr>
<td>Knl-mum7 adh1-F/Knl-mum7 adh1-F × knl +</td>
<td>22</td>
</tr>
<tr>
<td>adh1-S/knl + adh1-S; MuDR</td>
<td>14</td>
</tr>
<tr>
<td>2431-4 × 2431-5</td>
<td>4</td>
</tr>
<tr>
<td>Knl-mum7 adh1-F/knl + adh1-S × knl + adh1-S</td>
<td>4</td>
</tr>
<tr>
<td>2692A3 × 2587</td>
<td>4</td>
</tr>
<tr>
<td>knl + adh1-S/knl + adh1-S × Knl-mum7 adh1-F</td>
<td>0</td>
</tr>
<tr>
<td>1895 × 1895</td>
<td>32</td>
</tr>
</tbody>
</table>

The linked adh1 allele was used to determine the genotype at knl. All progenitors were normal in appearance.
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 reintroduced 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 Mu 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 that 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 mutations. 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 plastochnon 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 knl 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 exists. A model for an intron-located silencer has been proposed by Bradley and coworkers (1993) to explain the ovulate 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 whors (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 Knl mu mutants vary in penetrance and the Knl-mum mutants are no exceptions. We had one Knl-mum2 family that had active individuals without a knotted phenotype (Table 1) and have also seen active Knl-mum5 and Knl-mum1 individuals without knots (unpublished data). One possible explanation is that non-knotted, Mu active Knl-mum individuals have subtle changes at knl that render them inaccessible to the MuDR. Another possibility is that the MuDR element in these Mu active nonexpressive individuals is in a different position in the chromosome. The chromosomal position of P elements in Drosophila affects their ability to excise (Misra 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 knl is ectopically expressed, we might expect that the MuDR element in these Mu active nonexpressive individuals requires high levels of MuDR expression, which might explain why 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 knl expression and can account for the variable penetrance (Greene and Hake 1993).

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