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Insertion of *Mu1* Elements in the First Intron of the *Adh1-S* Gene of Maize Results in Novel RNA Processing Events

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Maize transposable elements, when inserted in or near genes, alter expression by several transcriptional and post-transcriptional mechanisms. Three independent, unstable insertions of the transposable element *Mutator* (*Mu*) into the first intron of the *Alcohol dehydrogenase-1* (*Adh1*) gene have been shown to decrease expression [Strommer et al. (1982). Nature 300, 542–544]. We have developed an approach to elucidate the underlying molecular mechanisms responsible for the mutant phenotypes. *Mu1* elements were inserted into *Adh1-S* intron 1 in vitro to create plasmid facsimiles of the mutant alleles. The *Mu1* element was also inserted at novel positions within intron 1 to create new mutations. The *Mu1*/intron constructions were placed between the *Adh1-S* promoter/exon 1 segment and a reporter gene (firefly luciferase or β-glucuronidase), and these chimeric gene constructs were tested in transient assays in maize protoplasts. When compared with the appropriate control, the *Mu1* insertions decreased reporter gene expression to levels approximating the alcohol dehydrogenase enzyme activities observed for the *Adh1-S* mutants in vivo. The *Mu1* insertions also showed a polarity effect with luciferase expression increasing as the insertions were placed nearer the 3′ splice junction. In addition, *Mu1* insertions within a different intron, actin intron 3, also significantly reduced luciferase expression, indicating that *Mu1* insertions within introns are likely to diminish expression in many genes. The presence of the *Mu1* sequences was correlated with decreased levels of steady-state luciferase transcript. Deletion analysis of the *Mu1* element and RNase mapping indicate that the transposable element contains RNA processing signals in its central region that are largely responsible for the decrease in expression.

INTRODUCTION

Maize contains several families of active transposable elements that move within its genome. The best described are the *Ac/Ds* and *Spm* systems first discovered by McClintock (reviewed in McClintock, 1984). Robertson discovered another transposable element family, *Mutator*, which is distinguished by having a high rate of forward transposition (20 to 40 times that of *Ac*) (Robertson, 1978). There are at least nine characterized *Mu* elements having the same terminal inverted repeat (TIR) sequence but unique middle sequences (Walbot, 1991); however, the transposase-encoding *Mu* element has not yet been identified. The most common *Mu* element is 1.4 kb; these *Mu1* elements are generally present in 10 to 70 copies in active *Mutator* maize lines (Walbot and Warren, 1988).

Members of the *Ac/Ds* and *Spm/En* transposable element families have been shown to affect expression at both the transcriptional and post-transcriptional levels (reviewed in Fedoroff, 1989; Weil and Wessler, 1990). *Mu* insertions into genes can also result in altered expression. The most common effect is a null mutation when a *Mu* element inserts into a protein coding region (e.g., *Bronze-1*, Taylor et al., 1986; *Bronze-2*, McLaughlin and Walbot, 1987; and *Viviparous-1*, McCarty et al., 1989); excision of the element often restores phenotypically wild-type expression patterns (Levy et al., 1989). Insertions can also modulate gene expression. For example, a *Mu3* insertion into the promoter of the *Adh1* gene of maize affects both quantitative and tissue-specific expression patterns (Chen et al., 1987). The presence of a *Mu1* element in the 5′-untranslated leader region of *hcf106* suppresses gene expression when the *Mutator* system is active and the TIRs are unmethylated; expression is partially restored in *Mutator* inactive tissues in which the TIRs are methylated (Martienssen et al., 1989). A *Mu1* insertion in the promoter of the *Shrunken-1* gene results in a different transcription start site and reduced levels of *Sh1* mRNA (Ortiz et al., 1988). *Mu* elements may also contribute new RNA processing information: a cryptic 5′ splice junction has been proposed to be present in the *Mu* 1.7-kb element (Taylor et al., 1986).

From a *Mutator*-active line of maize, alleles of *Adh1-S* with *Mu1* insertions in the first intron were recovered as

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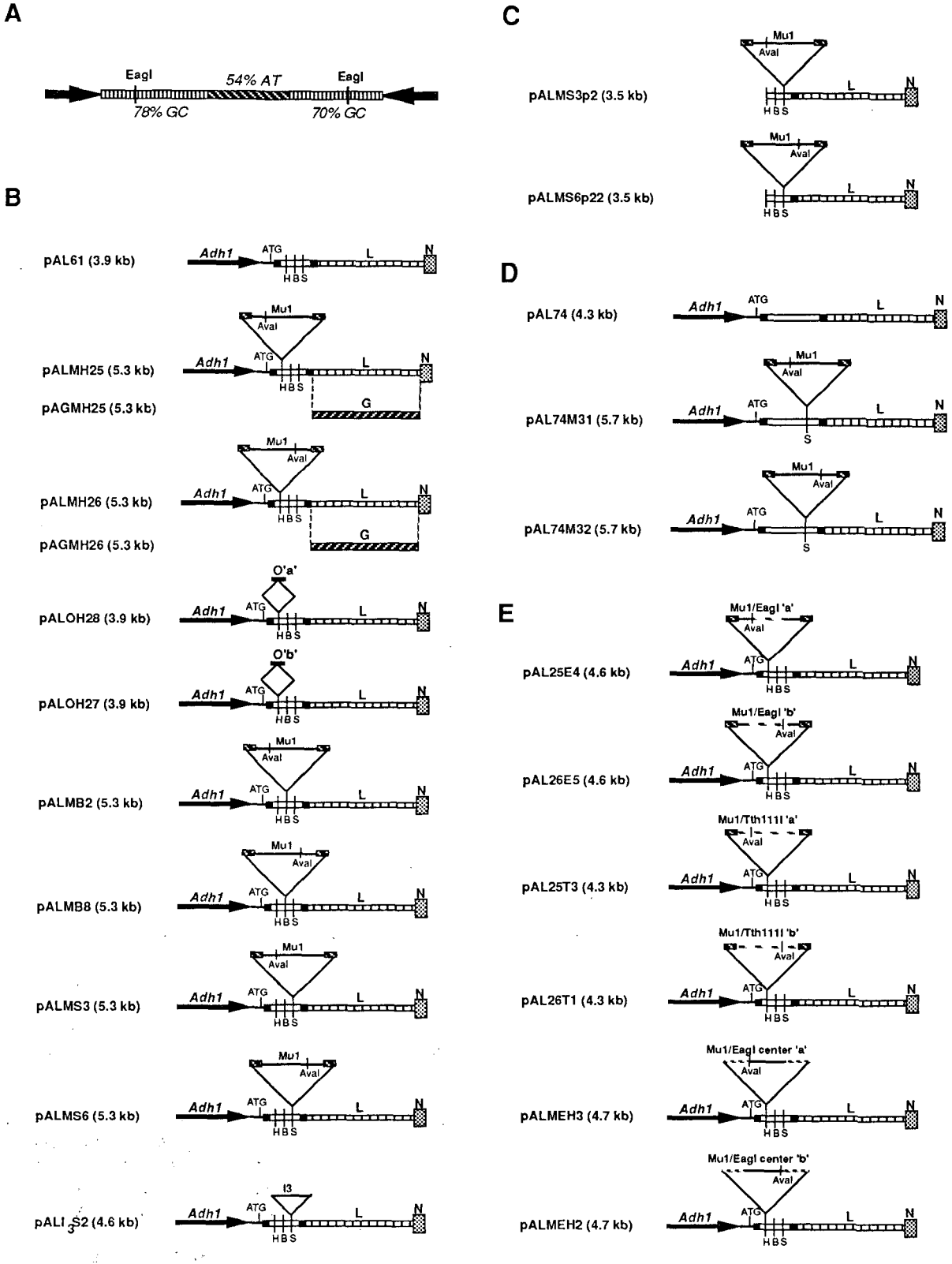

Figure 1. Expression Plasmids Used in This Study.

low-activity mutants (Freeling et al., 1982; Strommer et al., 1982; Bennetzen et al., 1984). Three of these mutants, *Adh1-S3034*, *Adh1-S4477*, and *Adh1-S4478*, were mutable and exhibited decreased levels of alcohol dehydrogenase (ADH) enzyme activity ranging from about 10% to 70% of wild-type activity (Strommer et al., 1982; Rowland and Strommer, 1985; Strommer and Ortiz, 1989). The amount of *Adh1*-hybridizing mRNA for the alleles was similarly decreased to about 10% to 70% of wild-type levels. Each of these alleles had a single *Mu* 1.4-kb insertion, in the same orientation, at different locations in the first intron of the *Adh1-S* gene. The *Mu* insertion from one of these alleles, *Adh1-S3034*, was sequenced and designated *Mu1* (Barker et al., 1984).

Several studies have addressed the mechanism(s) by which the *Mu1* elements in *Adh1-S* intron 1 act to decrease expression. Rowland and Strommer (1985), using nuclear run-off assays, suggested that the *Mu1* element affected transcription initiation but not RNA processing. In contrast, Vayda and Freeling (1986) concluded from their nuclear run-off assays that transcription initiation was unaffected but that transcription elongation was impeded in some way by the *Mu1* sequences. In a recent study, Ortiz and Strommer (1990) concluded that RNA processing of the *Mu1*-containing chimeric transcripts was altered by aberrant splicing and by cryptic polyadenylation during hypoxic stress; these conclusions were based on RNA gel blots and the sequences of several cDNAs of *Adh1-S3034* transcripts from hypoxic roots.

We have developed a new experimental approach to clarify further the molecular mechanisms responsible for the disruption of *Adh1* expression by *Mu1* insertions. Instead of studying the alleles in whole plants, plasmid facsimiles have been constructed using easily assayed reporter genes, i.e., firefly luciferase and β-glucuronidase (GUS). The plasmid constructs were assayed for reporter gene expression and transcript RNA structure in maize protoplasts. We report that the phenotypes of the facsimile alleles correlate well with those of the naturally occurring alleles in vivo. We describe both position and orientation effects of the *Mu1* element on expression. Deletion and insertion analysis of the internal region of *Mu1* suggested that the mutations are caused primarily by the presence of the central region and not by the TIRs. Our analysis of the transcript RNA suggested that in one orientation of the element transcription termination occurs, whereas the opposite orientation likely has an internal intron.

RESULTS

Facsimile Plasmid Reconstructions of the Mutable, *Mu1*-Induced *Adh1-S* Alleles

Three independent maize ADH-deficient mutants, *Adh1-S3034*, *Adh1-S4477*, and *Adh1-S4478*, have a *Mu1* element inserted into the first intron of the gene (Freeling et al., 1982; Strommer et al., 1982; Bennetzen et al., 1984). To assess the mechanism(s) by which the *Mu1* insertions acted to decrease expression, we recreated the relevant portions of the alleles by joining the *Adh1-S* promoter/exon 1/intron 1 region to the firefly luciferase or GUS reporter genes and then inserting *Mu1* (or segments of *Mu1*) at different restriction sites within intron 1. Because the ATG start codon is in *Adh1-S* exon 1, intron 1 must be correctly spliced to result in reporter gene expression. The structures of all expression plasmids are shown in Figure 1.

The *Adh1-S3034* allele contains a 1.4-kb *Mu1* (Figure 1A) insertion 2 bp upstream of the unique HindIII site within intron 1 (Bennetzen et al., 1984); we arbitrarily refer to the orientation of this insertion as "a." This cloned *Mu1* element was trimmed to remove most *Adh1* flanking sequence, was adapted with HindIII linkers, and was reinserted at the intron 1 HindIII sites of the firefly luciferase-expressing plasmid pAL21. The resulting plasmid, pALMH25, differed from the *Adh1-S3034* allele (in the intron 1 region) only by having an additional 7 bp upstream of the element (see Methods and Figure 1B). The plasmid pALMH26 has the *Mu1* element at the same HindIII site inserted in the opposite, or "b," orientation and, thus, represents a new mutation created in vitro.

Figure 1. (continued).

The plasmid name and size in kilobases (for the insert only) are given for each plasmid. All expression cassettes were inserted into pUC8. The ATG shown is the first present in the mRNA. *Adh1*, alcohol dehydrogenase promoter and exon 1 combination; L, firefly luciferase gene; G, β-glucuronidase (GUS) gene; N, nopaline synthase 3′ polyadenylation region; O, the excision oligomer (see Methods); H, HindIII; B, BglII; S, StuI.

(A) A schematic of the *Mu1* element. The TIRs are shown as arrows. Two GC-rich regions (positions 229 to 571 and 877 to 1176; Barker et al., 1984) surround a central AT-rich core (positions 572 to 876). The positions of the EagI restriction sites are shown.

(B) The pALMX series. See Methods for a detailed description of the plasmids.

(C) The promoterless pALMS3p2 and pALMS6p22 plasmids were derived from pALMS3 and pALMS6, respectively, by excising a HindIII fragment containing the *Adh1-S* promoter and 72 bp of intron 1.

(D) *Adh1-S* intron 1 of pAL61 was replaced with the third intron of the maize actin gene to yield plasmid pAL74; a *Mu1* insertion was made in both orientations at the StuI site.

(E) Deletions and insertions of internal *Mu1* regions were made using the pALMX plasmid series.

The *Mu1* element was also inserted at other locations within intron 1. The plasmid pALMS3 has the *Mu1* element inserted in the "a" orientation at the unique StuI site of intron 1; this is just 6 bp downstream of the *Mu* 1.4-kb insertion in the *Adh1-S4477* allele (Ortiz and Strommer, 1990) and, thus, pALMS3 closely approximates the structure of the *Adh1-S4477* allele. pALMS6 has a *Mu1* insertion at the same restriction site as pALMS3 but in the opposite, or "b," orientation. In two other plasmids, pALMB2 and pALMB8, the *Mu1* element was placed in the "a" and "b" orientations, respectively, at the unique, central BglII site of intron 1.

The *Adh1-S3034* allele is mutable (Freeling et al., 1982; Taylor and Walbot, 1985): when the element excises, ADH expression is often restored to phenotypically wild-type levels. Transposable element excision events can be imprecise (Döring and Starlinger, 1986); however, a precise excision of *Mu* can also occur for the *bz1::mu2* allele in vivo (A.B. Britt and V. Walbot, unpublished observation). To determine whether precise *Mu1* excision products were expressed at control levels, an oligomer of the *Mu1* footprint (the tandem 9-bp repeat created on *Mu1* insertion plus 7 bp of upstream flanking sequence) was inserted in the "a" and "b" orientations into the HindIII sites of pAL21; the resulting constructs are pALOH28 and pALOH27, respectively. As will be shown in the next section, the expression level of each facsimile plasmid was similar to the ADH expression level of the corresponding *Adh1-S* mutant in vivo.

Plasmid Reconstructions Mirror the in Vivo Phenotypes of the *Mu1*-Induced *Adh1-S* Mutations when Tested in a Transient Assay

To test the effects of *Mu1* element insertions on gene expression in the absence of *Mutator* transposition and excision activities, we chose Black Mexican Sweet (BMS), a non-*Mutator* maize line, as a source of protoplasts. The *Mu1* element does not encode the transposase and will not excise in the absence of transposase. The plasmids outlined in the previous section were tested in a transient expression assay by electroporation into BMS protoplasts. As shown in Table 1, pALMH25 and pALMS3 expressed luciferase activity at 3.5% and 22.4% of the pAL61 control value, respectively. To test whether the diminution in expression was an artifact of the reporter gene used, we replaced the luciferase coding region of pALMH25 with the GUS gene and found that expression was reduced to approximately the same degree (3.2% of the pAG8 control value for pAGMH25), indicating that quantitative expression is independent of the reporter gene used. We also tested the expected precise excision product of pALMH25 by replacing the *Mu1* sequence with an oligomer of the *Mu1* footprint; as shown in Table 1, pALOH28 expressed luciferase at 114.2% of the control value. Thus, as expected for a mutable allele, the excision of *Mu1* restored expression.

The ADH enzyme levels reported for the *Adh1-S3034* and *Adh1-S4477* alleles in vivo are about 10% to 40% and 70% of wild-type activity, respectively, and, thus, the activities of the pALMH25 and pALMS3 plasmid reconstructions are quantitatively lower but qualitatively similar. Strommer and Ortiz (1990) have shown that the ADH expression levels of the *Adh1-S3034* and *Adh1-S4477* alleles can vary twofold to threefold depending on the nuclear background in which they are tested. In addition, previous determinations of ADH activities were probably done in active *Mutator* lines, and the excision of *Mu1* might restore full activity in some cells, resulting in increased ADH activity in a tissue homogenate. Thus, the differences between the ADH activities previously reported and the luciferase and GUS gene activities we found may result from a combination of the BMS nuclear background and the lack of *Mu1* excision.

We also tested mutations that were created by recombinant DNA methodology in vitro. The *Mu1* element was placed in intron 1 at different locations and in the opposite orientation from the *Mu1* element in the *Adh1-S3034* and *Adh1-S4477* alleles. As shown in Table 1, insertion of the *Mu1* element in the "a" orientation at the central BglII site within intron 1 also created a mutation; luciferase expression was reduced to 15.8% of the control value (pALMB2 compared with pAL61). This value is intermediate between the expression values when *Mu1* was placed at the upstream HindIII or the downstream StuI site (3.5% and 22.4% for the pALMH25 and pALMS3 plasmids, respectively). Thus, the *Mu1* insertion results in a less severe mutation as it is placed closer to the 3′ splice junction. This result is shown graphically in Figure 2.

The insertion of *Mu1* in the opposite, or "b," orientation in the HindIII, BglII, and StuI sites also significantly reduced expression, indicating that the *Mu1* element is mutagenic when present in the intron in either orientation. The luciferase and GUS expression for *Mu1* insertions at the HindIII (pALMH26, pAGMH26), BglII (pALMB8), and StuI (pALMS6) sites ranged from between 2% to 19.2% of the control value, as shown in Table 1. As with *Mu1* insertions in the "a" orientation, insertions in the "b" orientation were less severe as the element was placed closer to the 3′ splice junction (Figure 2).

A potential artifact in our assays could arise from promoter activity within *Mu1* leading to an additional luciferase-encoding transcript(s). We tested this possibility by deleting the *Adh1-S* promoter from the pALMS3 and pALMS6 constructs (Figure 1C) and transfecting these promoterless versions into BMS protoplasts. As shown in Table 1, neither the pALMS3p2 nor pALMS6p22 constructs displayed luciferase expression levels above background; this indicated that all of the luciferase and GUS activities from the expression plasmids depend on the *Adh1-S* promoter.

Table 1. *Mu1* Insertions in *Adh1-S* Intron 1 Affect Reporter Gene Expression

Plasmid	Insertion	GUS (pmol/min)	Corrected Luciferase	% Control
Mu1 insertions/luciferase				
A				
Control		0	(42) 0	0
pAL61	None	49.9	(118,512) 2414 ± 294	100
pALMH25	*Mu1* "a" at HindIII	34.4	(2454) 85 ± 8	3.5
pALMH26	*Mu1* "b" at HindIII	27.2	(1087) 48 ± 4	2
pALOH28	Oligo[a] "a" at HindIII	39.5	(108,652) 2757 ± 4	114.2
pALOH27	Oligo "b" at HindIII	52.2	(109,257) 2142 ± 358	88.7
pALI_3S2	729 bp I_3 at StuI	40.8	(116,193) 3173 ± 282	131.5
B				
Control		0	(44) 0	0
pAL61	None	11	(211,357) 19623 ± 3414	100
pALMB2	*Mu1* "a" at BglII	9.1	(23,022) 3096 ± 88	15.8
pALMB8	*Mu1* "b" at BglII	9.5	(15,777) 2018 ± 336	10.3
pALMS3	*Mu1* "a" at StuI	6.7	(23,466) 4400 ± 811	22.4
pALMS6	*Mu1* "b" at StuI	8.4	(25,742) 3772 ± 685	19.2
C				
Control		NA[b]	0	0
pAL61	None	NA	1140 ± 351	100
pALMS3p2 (no promoter)	*Mu1* "a" at StuI	NA	2 ± 0.15	0.2
pALMS6p22 (no promoter)	*Mu1* "b" at StuI	NA	2 ± 0.48	0.2
		Light Units/10 sec	Corrected GUS	
Mu1 insertions/GUS				
D				
Control		79	(0) 0	0
pAG8	None	528,395	(483) 92.6 ± 15.7	100
pAGMH25	*Mu1* "a" at HindIII	463,349	(12.8) 3.36 ± 0.04	3.6
pAGMH26	*Mu1* "b" at HindIII	435,822	(4.1) 1.13 ± 0.01	1.2

The Insertion column indicates the presence of an insertion at the listed restriction enzyme site within *Adh1-S* intron 1. The levels of reporter gene activity for each construct tested in the transient assay are shown. Twenty microliters to 50 μL of extract was assayed separately for each enzyme activity. The first column presents the raw data for the background or standardization marker (see Methods). For the pAL and pAG sets, the background expression plasmids were pCaG and pCaL88, respectively. The second column reports the enzyme activities for the test plasmids. The raw levels of luciferase or GUS activity are shown in parentheses and the ratios of test plasmid enzyme activity divided by background plasmid enzyme activity are the corrected values; the corrected values are expressed as light units per 10 sec per picomoles per minute (light units/GUS) for the pAL series and as picomoles per minute per light unit per 10 sec × 10^5 (GUS/light unit × 10^5) for the pAG series. No GUS-expressing standardization plasmid was used in part C; the luciferase expression values are expressed instead as a specific activity (light units per 10 sec per microgram of protein). Because the same mass of test plasmid was used in each trial, a final correction for the number of expressing plasmids was made by multiplying the enzyme activity by the ratio of the lengths (in base pairs) of the insertion plus and insertion minus plasmid pair. Each plasmid was tested in duplicate and the standard deviations are shown. The final column compares the expression of the control plasmid (pAL61 in parts A, B, and C; pAG8 in part D) with the plasmids containing an insertion element within intron 1. A, B, C, and D correspond to separate transfection experiments and, thus, the absolute expression levels between experiments are not directly comparable.

[a] Oligo, excision oligomer.

[b] NA, not applicable.

Mu1 Insertions within Actin Intron 3 also Reduce Expression

To ascertain whether *Mu1* insertions into introns were generally mutagenic or whether the effects were idiosyncratic to *Adh1-S* intron 1, we constructed a luciferase expression vector, pAL74, containing the 882-bp third intron of the maize actin gene (Shah et al., 1983). *Mu1* was inserted into the unique StuI site of actin intron 3 in both orientations (Figure 1D) and the expression plasmids were tested in the transient assay; see Table 2. pAL74M31 and pAL74M32, which contain the *Mu1* element in the "a" and "b" orientations, respectively, expressed at 11.1% and 17.7% of the control value. Thus, a *Mu1* element that transposed into other introns would be expected to reduce expression of the gene.

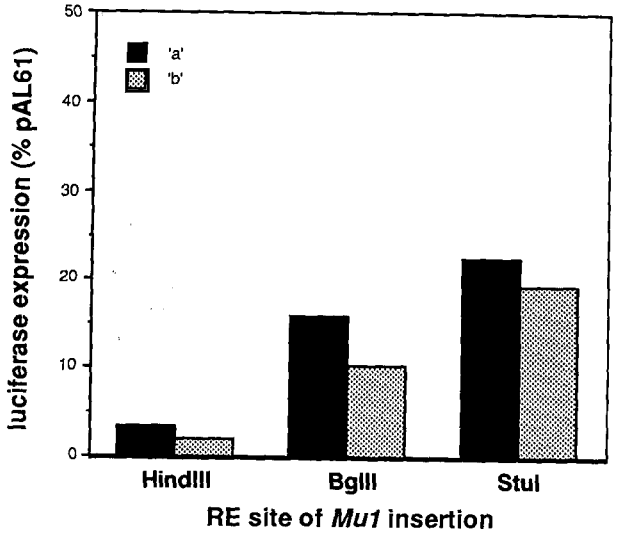

Figure 2. Luciferase Expression as a Function of *Mu1* Position and Orientation within *Adh1-S* Intron 1.

The entire *Mu1* element was inserted in both the "a" and "b" orientations at the *Adh1-S* intron 1 HindIII, BglII, and StuI sites of pAL61, and the resulting plasmids were tested in the transient assay. Luciferase expression levels are relative to the control plasmid pAL61 and are derived from Table 1. RE, restriction enzyme.

Mu1 Insertions Do not Cause a Mutation by Disrupting Critical Intron Sequences

We considered the possibility that *Mu1* insertions might interrupt an element important for the splicing of the intron 1-containing pre-mRNA, such as a potential lariat branch sequence. This seemed unlikely because insertion of the 729-bp Fnu4HI/SspI fragment of the central region of maize actin intron 3 into the StuI site had no adverse affect on expression (pALI$_3$S2 and Table 1; compare to pALMS3 with *Mu1* in the StuI site). This indicated that sequences at the StuI site could be interrupted without creating a mutation. Also, the insertion of the excision oligomer in both orientations at the HindIII site of intron 1 did not result in decreased luciferase activity (pALOH28 and pALOH27; Table 1). In addition, the *Adh1-S* intron 1 and actin intron 3 restriction sites used for the *Mu1* insertions can be removed without affecting reporter gene expression; this indicates that these sequences are not essential for intron processing (K.R. Luehrsen, unpublished observations).

Deletions and Insertions of Internal *Mu1* Sequence Affect Expression

An advantage of the plasmid-based expression assay is that relevant regions of the transposable element or the surrounding gene sequences can easily be modified by genetic engineering in vitro. The *Mu1* element, shown diagrammatically in Figure 1A, is 1376 bp in length and contains 215-bp and 213-bp TIRs. The approximately 1-kb region internal to the TIRs comprises two GC-rich regions (78% and 70%) surrounding a central AT-rich (54%) core. To determine which of these regions contributes to the mutant phenotype, we placed different fragments of the *Mu1* element at the intron 1 HindIII site (Figure 1E) and tested the resulting constructs in the transient assay; the luciferase expression data are shown in Table 3.

The deletion of central region fragments of *Mu1* resulted in significantly higher levels of luciferase expression. By deleting the central EagI restriction fragment (742 bp) of *Mu1*, the remaining insertion sequence, including both TIRs and some GC-rich sequence, mediated only a twofold reduction of luciferase expression (53.1% and 41.7% of the control value for pAL25E4 and pAL26E5, respectively); the expression was similar for both orientations of the *Mu1* partial fragment. RNase mapping indicated that the pre-mRNAs transcribed from the pAL25E4 and pAL26E5 constructs were spliced as efficiently as the pAL61 pre-mRNA (data not shown). A similar level of expression (63.3% and 38.9% of the control value for pAL25T3 and pAL26T1, respectively) was obtained when the entire central 1008-bp Tth111I fragment was deleted, leaving only the TIRs. Thus, insertions of the long TIRs appear, by themselves, to have only a modest impact on expression. We do not know whether the TIR sequences form a stem-loop structure in the nascent pre-mRNA in vivo, but their extended length suggests that an intramolecular reaction might be favored.

A much different result was obtained when the EagI central fragment was placed in either orientation at the intron 1 HindIII site. Plasmids pALMEH3 and pALMEH2, which contain the *Mu1* EagI central restriction fragment inserted at the HindIII site in the "a" and "b" orientations, respectively, reduced luciferase expression to lower levels (1.5% and 0.1%, respectively, of the control value) than seen for insertions of the entire *Mu1* element (6.7% and 2.2% of the control value for pALMH25 and pALMH26, respectively). Thus, the region(s) of *Mu1* most likely to cause reduced gene expression reside in the *Mu1* central region.

Mu1 Insertions Reduce Steady-State RNA Levels

Previous studies have determined that the steady-state transcript levels for the *Adh1-S3034* and *Adh1-S4477* alleles are reduced to between about 10% and 70% compared with the wild-type values (Strommer et al., 1982; Rowland and Strommer, 1985; Ortiz and Strommer, 1990). As shown in Figure 3, there was also a reduction in the levels of reporter gene transcript RNA in the transient

Table 2. *Mu1* Insertions in Actin Intron 3 Affect Reporter Gene Expression

Plasmid	Insertion	GUS (pmol/min)	Corrected Luciferase	% Control
Control		0	(43) 0	0
pAL74	None	686	(58,061) 85 ± 0	100
pAL74M31	*Mu1* "a" at StuI	685	(5366) 9 ± 1	11.1
pAL74M32	*Mu1* "b" at StuI	638	(7704) 15 ± 3	17.7

All calculations were performed as described in the legend to Table 1. The structures of the *Mu1* insertion plasmids are shown in Figure 1 and are described in Methods. The background (standardization) expression plasmid used was pCal$_1$Gc.

assay when *Mu* sequences were present in intron 1. When compared with the pAL61 control plasmid, luciferase-hybridizing transcript levels for the *Mu1* insertion plasmids were reduced to 10% to 68% when the entire *Mu1* element was placed within intron 1 (pALMH25, pALMH26, pALMS3, and pALMS6). Following the increased luciferase expression levels, the amount of luciferase-hybridizing RNA increased as the *Mu1* element was placed closer to the 3′ splice junction (pALMH25 and pALMH26 compared with pALMS3 and pALMS6, respectively).

Deletion of the central EagI fragment of *Mu1* led to an approximately sixfold to 10-fold increase in the luciferase-hybridizing RNA, when compared with plasmids containing the entire *Mu1* element (pAL25E4 and pAL26E5 versus pALMH25 and pALMH26, respectively). Thus, as the luciferase expression studies suggested, the TIRs with some internal flanking sequences have only a modest impact on expression. In contrast, the insertion of only the central EagI restriction fragment into intron 1 resulted in no detectable luciferase-hybridizing RNA (pALMEH3 and pALMEH2), consistent with the extremely low levels of luciferase activity expressed in the transient assay.

The Central AT-Rich Region of *Mu1* Contains RNA Processing Signals

From results in the previous sections, we deduced that the *Mu1* central region might be responsible for the mutations created by the transposable element. Plant intron sequences and 3′-untranslated leader region sequences are AT rich. The central 305-bp region of *Mu1* is 54% AT, and we hypothesized that cryptic splice sites or polyadenylation signals might be present in this region. We used RNase mapping to probe the structures of the transcripts generated from several of the expression constructs. Labeled antisense RNAs were made from the central *Mu1* EagI restriction fragment (Figure 1A) and were used to probe the *Adh1/Mu1* hybrid transcripts. As shown in Figure 4A, RNAs transcribed from plasmids pALMH25 and pALMS3 (*Mu1* in the "a" orientation at the HindIII and StuI sites) protected the full length of the probe (corresponding to read-through transcripts) and a fragment of 290 nucleotides (nt). Several minor protected fragments were also present. A second, shorter probe (BssHII; see Methods) derived from the EagI central *Mu1* fragment was also used.

Table 3. Insertions of Partial *Mu* Sequences in *Adh1-S* Intron 1 Affect Reporter Gene Expression

Plasmid	Insertion	GUS (pmol/min)	Corrected Luciferase	% Control
Control		0	(39) 0	0
pAL61	None	81.9	(124,870) 1467 ± 424	100
pALMH25	*Mu1* "a" at HindIII	52.4	(4577) 98 ± 47	6.7
pALMH26	*Mu1* "b" at HindIII	41.8	(1249) 32 ± 13	2.2
pAL25E4	EagI Δ "a" at HindIII	70.7	(53,940) 780 ± 234	53.1
pAL26E5	EagI Δ "b" at HindIII	33.2	(18,533) 611 ± 9	41.7
pAL25T3	Tth111I Δ "a" at HindIII	40.7	(36,909) 928 ± 353	63.3
pAL26T1	Tth111I Δ "a" at HindIII	89.6	(50,043) 571 ± 115	38.9
pALMEH3	EagI central "a" at HindIII	65	(1351) 22 ± 5	1.5
pALMEH2	EagI central "b" at HindIII	69.5	(165) 2 ± 1	0.1

All calculations were performed as described in the legend to Table 1. The structures of the *Mu1* insertion and deletion plasmids are shown in Figure 1 and are described in Methods. The background (standardization) expression plasmid used was pCaG.
[a] Δ = deletion.

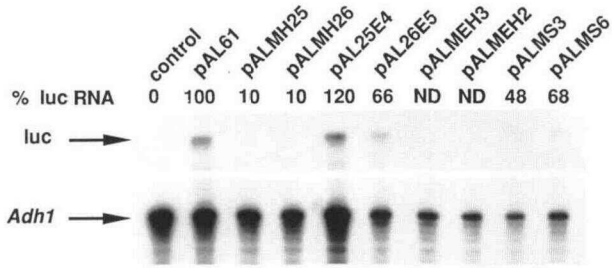

Figure 3. Insertion of *Mu1* Sequences in *Adh1-S* Intron 1 Results in Reduced Levels of Steady-State Transcript RNA.

Total RNA from BMS protoplasts transfected with the expression plasmids shown was isolated and hybridized with ^{32}P-labeled antisense RNA made using the pJD350 probe (luciferase) and the pBS*Adh1* probes (*Adh1*). RNase mapping was completed as described in Methods and the protected fragments were separated by PAGE. The amount of each protected fragment was determined by laser densitometry and is expressed as a percentage of the pAL61 control level. The quantity of luciferase-hybridizing RNA was standardized with the amount of *Adh1*-hybridizing fragment detected. ND, not detectable.

The same family of fragments was observed, but the major protected band was reduced to 165 nt. The same sets of bands were seen with both insertion sites of the *Mu1* element (HindIII or StuI sites), indicating that the processing signals were within *Mu1* and functioned independently of the gene context. The same pattern of protected fragments was also observed for transcripts derived from a *Mu1* insertion in actin intron 3 (data not shown), confirming that the processing signals were not affected by the surrounding intron sequences. No protected RNAs were seen when transcript RNA from the promoterless pALMS3p2 plasmid was probed: this indicated that the partial protected fragments seen did not originate within *Mu1* but were derived from transcripts initiated from the *Adh1-S* promoter (data not shown).

The RNase mapping shown in Figure 4A did not distinguish whether the partially protected transcripts were products of alternative splicing or polyadenylation. Thus, a second probe that spanned the StuI site and contained intron 1 and luciferase, but no *Mu1*, sequences was used to protect RNA transcribed from the plasmid pALMS3. Because the probe is not homologous along its entire length with the *Mu1*-containing transcript RNA, it was expected to be split into fragments corresponding to transcript regions upstream and downstream of the point of *Mu1* insertion. As shown in Figure 5, the molar amount of protected RNA is about threefold greater for the fragment corresponding to the region upstream of the *Mu1* element when compared with the fragments representing the regions downstream of it; thus, only 28% of the transcripts traversed the entire element. For comparison, RNA transcribed from pALI$_3$S2, a plasmid containing an insertion of actin intron 3 sequence at the StuI site of intron 1, was also probed; the insertion of actin intron 3 fragment did not reduce expression (Table 1). For the pALI$_3$S2 plasmid, 93% of the transcripts traversed the insertion sequence.

We conclude that the partially protected fragments observed with the EagI central region probe represented transcript termination at one major and possibly several minor sites in *Mu1*. The transcript termination that we observed was likely the result of RNA processing by polyadenylation (see Discussion and Ortiz and Strommer, 1990). The region where the major protected fragments map is shown in Figure 6. The RNA processing events within *Mu1* are not efficient for pALMS3 because about 28% of the transcript represented read-through products. Correctly spliced RNA, lacking the intron and the inserted *Mu1* sequences, also represented read-through transcript; presumably, ADH activity in vivo and the luciferase and GUS activities reported here are a consequence of these correctly spliced, read-through transcripts.

We also probed transcript RNAs derived from the pALMH26 and pALMS6 plasmids in which the *Mu1* element is inserted in the "b" orientation. As shown in Figure 4B, when using the EagI central region probe only a faint trace (<5% of all *Mu1*-containing transcripts) of full-length transcript was observed. Most of the transcript RNA was present as two partially protected fragments, 320 nt and 280 nt, that map to the central AT-rich region of *Mu1*. Again, the protected fragments observed were independent of the position of *Mu1* insertion, indicating that the RNA processing signals were completely contained within *Mu1*. The same-sized fragments were observed for RNase mapping of transcript RNA derived from a *Mu1* insertion into actin intron 3, indicating that the surrounding intron sequences did not alter the RNA processing pathway (data not shown). No protected fragments were seen for transcript RNA derived from the promoterless pALMS6 plasmid, establishing that the protected fragments observed were initiated at the *Adh1-S* promoter (data not shown). Protection of the same pool of RNA with a shorter version of the *Mu1* internal probe (BstNI probe; see Methods) showed that the 320-nt band remained, whereas the 280-nt band was shortened to 230 nt, the length difference between the first and second probes. RNase mapping using the probe that spans the StuI insertion site demonstrated that about 71% of transcripts (Figure 5) proceeded through the *Mu1* element; this indicated that transcription termination was possibly occurring at a low level in the "b" orientation of *Mu1*. If transcription termination was solely responsible for the protected bands, however, each would have been reduced by the size difference between the full-length and BstNI probes. We hypothesize that the *Mu1* element contains an intron in the central AT-rich region with the 320-nt fragment mapping to the downstream exon and the variable-length fragment mapping to the upstream

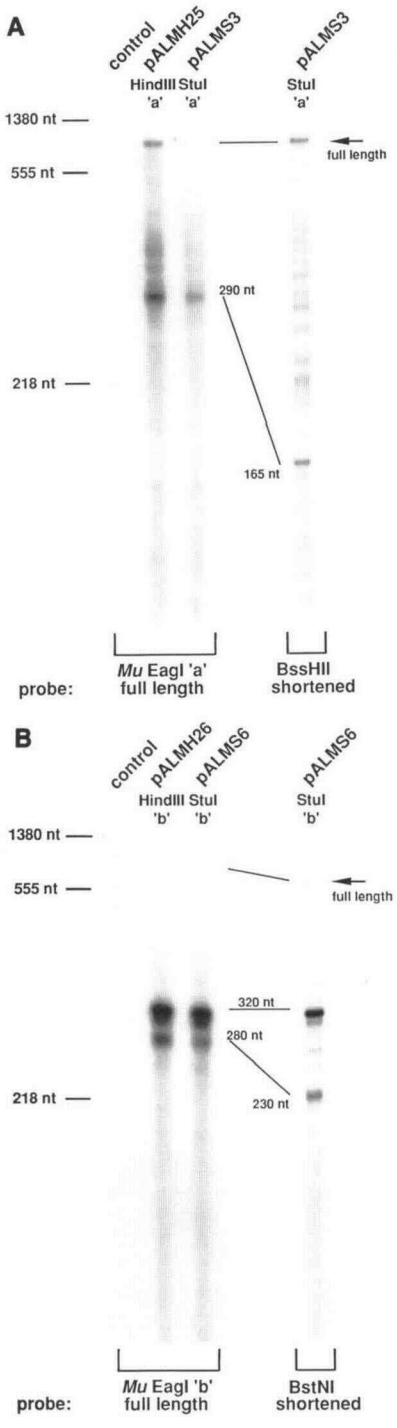

Figure 4. *Mu1*-Containing Transcripts Show Unexpected RNA Processing Events.

Total transcript RNA from the expression plasmids shown was probed by RNase mapping using ^{32}P-labeled antisense RNA derived from the central region of *Mu1*.

exon. Mapping of the protected fragments to the *Mu1* sequence suggests that the internal intron is about 150 bp in length and is derived from the central AT-rich region [positions 795 to 646 of *Mu1* (Barker et al., 1984), although other splice acceptor sequences are possible].

DISCUSSION

Previously, Freeling and coworkers isolated and described three *Adh1-S* mutants derived from a *Mutator* line of maize (Freeling et al., 1982; Strommer et al., 1982; Bennetzen et al., 1984). Each of these mutants had a *Mu1* insertion in the same orientation but at three different sites in the first intron of the gene. By using a novel experimental approach, we have addressed the question of how the *Mu1* elements that had transposed into *Adh1-S* intron 1 caused a decrease in expression. Facsimile reconstructions of the mutable alleles were joined to reporter genes and tested in a maize protoplast transient expression assay. The protoplasts used were derived from a non-*Mutator* line of maize, BMS, and, thus, expression results were obtained in a nuclear background devoid of *Mutator* transposition and excision activities. The luciferase and GUS expression from the plasmid reconstructions was found to mimic the ADH expression levels of the naturally occurring *Adh1-S3034* and *Adh1-S4477* alleles tested in vivo. The *Adh1-S3034* allele is mutable and excision of the *Mu1* element is correlated with reversion to wild-type expression levels; our plasmid reconstruction of a precise excision of *Mu1*

(A) The "a" orientation of *Mu1* is represented in the transcript RNA. Using the full *Mu1* EagI central fragment as a probe, the full length of the EagI region as well as a major partial protected fragment of 290 nt was observed. A shorter central probe was made by restricting the EagI insert with BssHII and transcribing an RNA 86 nt less than the full length of the insert. When the shorter BssHII probe was used, the same array of fragments was observed but the major protected band was reduced to 165 nt. The major protected fragments detected with the full-length and BssHII probes likely map to the same end point; however, the size difference between the protected fragments is about 125 nt and, thus, is greater than the size difference (86 nt) between the full-length and the shorter BssHII probe. This discrepancy might result from the inability to precisely size large protected fragments on a sequencing gel.

(B) The "b" orientation of *Mu1* is represented in the transcript RNA. Protection assays done using the full-length EagI antisense probe protected two discrete bands of 320 nt and 280 nt; only a small amount of full-length protected probe was observed. A shorter central probe was made by restricting the EagI insert with BstNI and transcribing an RNA 51 nt less than the full length of the insert. When the shorter probe was used, the 320-nt band remained, whereas the 280-nt band was reduced to 230 nt, the length difference between the full-length and shortened probes.

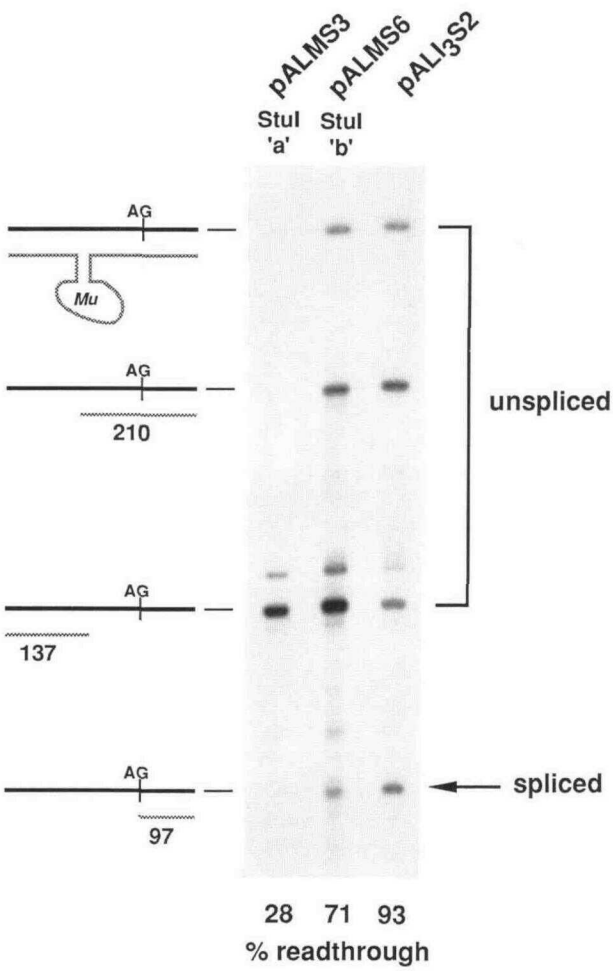

Figure 5. Transcript Termination Occurs within the "a" Orientation of *Mu1*.

Transcript RNA derived from the pALMS3 and pALMS6 expression plasmids was probed with *Adh1-S* intron 1/luciferase sequences spanning the StuI insertion site. By comparing the molar ratios of the protected fragments corresponding to the regions upstream or downstream of the *Mu1* insertion, an estimation of the read-through transcript was made; this value is shown for each expression construct. For comparison to the *Mu1*-containing transcripts, transcript RNA from an expression plasmid with actin intron 3 sequence inserted at the intron 1 StuI site was probed; this insertion sequence did not reduce luciferase expression or splicing efficiency (Table 1 and data not shown). The origin and length of each protected fragment are shown in a schematic to the left of the gel; the full-length protected probe is 347 bp.

also restored full expression. The *Mu1* insertions of *Adh1-S3034* and *Adh1-S4477* caused a decreased level of steady-state *Adh1-S* mRNA; the steady-state luciferase-hybridizing RNA levels were also reduced for the cognate reconstructions. Thus, we were able to reproduce the whole plant phenotypes of the alleles in the transient expression assay. We also made several new mutations in which the *Mu1* element was placed in the opposite orientation and at different locations within intron 1; these also resulted in a diminution of expression. In addition, chimeric expression constructs having *Mu1* insertions within the third intron of the maize actin gene were tested in the transient assay; expression was again decreased, indicating that *Mu1* transpositions to other introns are likely to cause a mutation in the affected gene. These new mutations added to our understanding of the impacts of *Mu* insertions within an intron.

The *Mu1* element has several exceptional structural features including unusually long terminal inverted repeats (about 215 bp) and internal GC-rich regions surrounding a central AT-rich core. Because the facsimile *Mu1*/intron reconstructions can be readily altered in vitro and then tested in the transient assay, we could dissect the *Mu1* element and quantitate the contribution of its different sequence features to expression. When the central EagI or Tth111I central restriction fragments of *Mu1* were ex-

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                                                        CGGCCGCGCACATCC
                                                        EagI
351
TCCTCGCCGCGAGCGCGTCCGCGGACGCGCGCGCCTTCTCGGGCCTCCACGCCGGGGACCTCCTCCTCCC

421
GCGCGCCAACGAGGCCGCCGCCGCCGCGGACCGGGCGCTCCGCGTGCTCCTCAAGTTCCCCGCGGTGTCA
BssHII
491
CTGTCGTCCTCGCCCAAGAAGTCCGCCTCGCCGCCGCCGGCCCCGGAGGCGAGGAAGGAGTACCCGCCCG

561
ACCTGACGCTGCCGGACCTCAAGAGCGGGCTGTTCAGCACCGACGAGCAACGGACGCACCTTCCACCTGT

631
CCTAGCACTAATTACTCCTAAATTCATCATACACCAAAGTTTCAATTAGTAAAAGGTTTTGGTATTATTT

701
TCTTTACAAGACTAAAAGCATCCACTCGTATTTGCCATGGAAATATTGCCAAAATGGTTACCGAAGGAAC

771
CAATGAAAAATGGCGGTATCCGTCCACCTGTAGCTTGTCCAAGGCTCTTCCAGCAGAGTAGTGCCGCCAT

841
GACGATTGACAGAGACACGAGACGAAACAAGCTGAAGGTCCCCGCGGCGTCACTGTCGTCCTCGCCGAAG

911
AAGTCCGCCTCGCCGCCGCCGGCCCCGGAGGCGAGGAAGGAGTACCCGCCCGACCTGACGCTGCCCACCG

981
TGTCGCCGCCGCCGCCCAACGGCCTCGGCGACATGCTCAGCCCAGCGGCCTGGCCCTCCTCCCCCGCGAG

1051
CAGGCTCAACAAGGCCGCGCTCGGCGGCGGCCG
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Figure 6. The Central Region of *Mu1* Is Responsible for the RNA Processing Signals.

The sequence of the central EagI restriction fragment in the "a" orientation is shown. The AT-rich region is in italics. The major partially protected fragment (165 nt or 290 nt) noted in Figure 4A maps to the region denoted by the thick underline (—); this likely maps a point(s) of polyadenylation in the transcript RNA. An animal-like polyadenylation consensus sequence is shown by the wavy underline (〰); Ortiz and Strommer (1990) have described cDNAs ending immediately downstream of this sequence. The relevant BssHII site used in synthesizing the shortened probe of Figure 4A is shown.

cised, removing 742 bp or 1008 bp of sequence, respectively, luciferase expression was restored to levels only about twofold below the control value. The restoration of luciferase expression was observed for these deletions of *Mu1* sequence in both the "a" and "b" orientations. Because correct splicing is required to produce the approximately 10% to 70% of ADH (or luciferase and GUS) activity found in the mutants, we interpret these results as suggesting that the presence of TIR sequences alone does not significantly impair the ability of the intron to be recognized and spliced. In a complete *Mu1* element, the TIRs may form a stem-loop structure in vivo; if so, then the GC-rich internal *Mu1* sequences might be effectively "hidden" from the maize splicing machinery, allowing efficient intron splicing. It has been shown in mammalian transient assays that an exon sequestered in a stem-loop structure is not as efficiently recognized by the splicing machinery (Solnick and Lee, 1987).

Insertion of the central 746 bp (the EagI fragment) of the *Mu1* element, in the absence of the TIR sequences, reduced luciferase expression to levels that are about fivefold to 10-fold below those observed for the entire *Mu1* element. RNA processing signals (polyadenylation in the "a" orientation and splicing in the "b" orientation) found in this central region partly explain the decrease in expression. Intron AT-richness is necessary for efficient splicing of dicot introns (Goodall and Filipowicz, 1989). For 64 maize exons, GC content averages 64% (36% AT), whereas for 57 introns the average GC content is only 41% (59% AT; K.R. Luehrsen, unpublished observations). Thus, if AT-richness is a requirement for the splicing of monocot introns, the addition of *Mu1* sequences might interfere with the recognition of those AT-rich regions necessary for the efficient splicing of *Adh1-S* intron 1.

RNase mapping of several of the facsimile reconstructions showed that RNA processing signals were embedded in the central region of the *Mu1* element. This was surprising in that the *Mu1* element has not been found to be transcribed in vivo. In the "a" orientation of *Mu1*, we have presented evidence demonstrating that transcription termination signals exist within the central AT-rich region. These signals are not efficient because about 28% of the transcripts proceed through *Mu1* (in pALMS3); all of the reporter gene activity depends on these read-through transcripts. This conclusion is consistent with two previous reports. Nuclear run-off assays were used to analyze the *Adh1-S3034* allele (Vayda and Freeling, 1986), and it was found that the *Adh1-S* transcript RNA did not effectively traverse the *Mu1* element, suggesting that it was polyadenylated or terminated. Sequence analysis of seven cDNA clones (Ortiz and Strommer, 1990) derived from the *Adh1-S3034* and *Adh1-S4477* alleles under hypoxic stress demonstrated that polyadenylation was occurring within the AT-rich central region of *Mu1* as well as in the TIR sequence. Curiously, these polyadenylated RNAs were observed only in hypoxic tissues.

Transcripts containing the opposite, or "b," orientation of *Mu1* appear to contain an intron within the central AT-rich region of *Mu1*. The putative *Mu1* intron is in an AT-rich (54%) region and is surrounded by two GC-rich regions (78% and 70%); this is consistent with the local sequence requirements for the splicing of dicot introns (Wiebauer et al., 1988; Goodall and Filipowicz, 1989). What is perplexing is that the internal *Mu1* intron appears to be more efficiently spliced than the *Adh1-S* intron 1 in which it is contained, as evidenced by the ability to detect *Mu1*-hybridizing sequences in the RNase mapping procedure (Figure 4B). Either the *Mu1* intron is recognized more efficiently than the *Adh1-S* intron 1 or each is recognized by the spliceosome, but the *Mu1* intron is spliced preferentially as a result of steric hindrance of multiple splicing complexes in the same region of RNA. In some of the cDNAs examined by Ortiz and Strommer (1990), an internal intron within the "a" orientation of *Mu1* was observed. A cDNA representing alternative splicing of the *Mu1* 5′ donor to the 3′ acceptor of intron 1 was also recovered.

We found that the *Mu1* element intron insertions had position-specific effects on expression. When the entire *Mu1* element was inserted in the "a" or "b" orientations within intron 1, expression was decreased to about 2% to 20% in all cases. There is a polarity effect whereby upstream insertions result in a more severe reduction in reporter gene expression and transcript levels; this was observed for both the "a" and "b" orientations of *Mu1*. It has been shown that for some maize genes and chimeric gene constructs, an intron placed in the transcription unit is necessary for high levels of expression (Callis et al., 1987; McElroy et al., 1990); this enhancement effect appears to be a consequence of splicing per se rather than an increase in transcription initiation. The presence of *Mu1* sequences, especially insertions near the 5′ splice junction, might affect intron recognition and decrease splicing efficiency, negating the intron enhancement effect. Ortiz and Strommer (1990) have suggested that there is a competition between the splicing and polyadenylation complexes to recognize their respective signals in the same region of a nascent transcript. When the *Mu1* element is situated closer to the 3′ splice junction, more of the intron sequence is exposed before the polyadenylation signal is transcribed, thus increasing the likelihood of intron recognition and splicing. The splicing of the intron/*Mu1* sequences from the pre-mRNA effectively removes the polyadenylation signal, allowing normal elongation and greater ADH activity. Evidence from the transient assays corroborates this hypothesis. Less *Mu1*-hybridizing sequence was found for the construct in which *Mu1* was placed closer to the 3′ splice junction (pALMS3 versus pALMH25; Figure 4A), although about 5 times more luciferase-containing transcript was present (Figure 3). This difference is likely to be the consequence of more luciferase-hybridizing transcripts being correctly spliced, resulting in fewer transcripts truncated by polyadenylation.

Constructs with *Mu1* insertions in the "b" orientation also exhibited a gradient of increasing reporter gene expression when the element was placed nearer to the 3′ splice junction. Transcripts including *Mu1* in the "b" orientation likely splice an intron wholly contained within the element. Because this *Mu1* intron is contained within *Adh1-S* intron 1, resulting in an intron within an intron, there may be a competition between them for the splicing machinery. When the *Mu1* element is placed nearer the 3′ splice junction of intron 1, more of intron 1 is exposed before the transcription complex proceeds through *Mu1*, increasing the likelihood that intron 1 will be recognized and spliced preferentially. The preferential splicing of intron 1 is consistent with the higher levels of luciferase activity observed for the *Mu1* insertions situated closer to the intron 1 3′ splice junction.

In summary, we have described a plasmid-based approach to test the impact of transposable element insertions on gene expression. We have shown that the luciferase and GUS expression from plasmid reconstructions of two *Mutator*-derived *Adh1-S* mutable alleles mirrored the ADH expression phenotypes measured in vivo. In addition, the levels of transcript RNAs from the expression constructs were both qualitatively and quantitatively similar to those described for the mutant *Adh1-S* alleles. The ability to manipulate the *Mu1* element in vitro allowed the creation of new mutants by genetic engineering and permitted a flexibility in the study of the mutant *Adh1-S* alleles that was not possible using only plant materials.

METHODS

Reagents

Restriction and DNA modifying enzymes were obtained from New England Biolabs, Bethesda Research Laboratories, or Pharmacia LKB Biotechnology Inc. and used according to the manufacturers' specifications. ^{32}P-CTP was purchased from Amersham Corp. The pBSSK(−), pBSKS(−), and pBSKS(+) vectors are products of Stratagene. pUC plasmids have been described (Vieira and Messing, 1982).

Plasmid Constructions

Base Plasmids

pAL61, pAL74, pAG8, pCaL88, pCaG, and pCaI$_1$Gc have been described in detail (Luehrsen and Walbot, 1990). Briefly, pAL61 contains the *Adh1-S* promoter/exon 1/intron 1 combination in a translational fusion with the firefly luciferase gene. pAG8 is similar to pAL61 except that the luciferase gene is replaced with the GUS gene. pAL74 was derived from pAL61 by replacing *Adh1-S* intron 1 with the third intron of the maize actin gene (Shah et al., 1983). pCaL88 and pCaG have the luciferase and GUS genes, respectively, driven by the cauliflower mosaic virus 35S promoter. pCaI$_1$Gc is similar to pCaG except that it has *Adh1-S* intron 1 inserted between the cauliflower mosaic virus 35S promoter and the luciferase gene.

pALMX Series

The ClaI/BamHI *Adh1-S* intron 1-containing fragment from p911 (see Callis et al., 1987; obtained from M. Fromm), was inserted into the ClaI/BamHI sites of pBSKS(−). A TT > GC change was made at positions 197 to 198 (for the numbering scheme, see Dennis et al., 1984) by oligo-mediated site-directed mutagenesis to create an additional HindIII site. The BclI/BamHI mutagenized intron fragment was used to replace the intron 1 fragment of pAL61 to result in the plasmid pAL21.

The *Mu1* element from the *Adh1-S3034* allele was cloned into pBR322 to give plasmid pLT1 (obtained from L. Taylor). The *Mu1* element with bits of flanking *Adh1-S* sequence was excised as a Sau3AI/HindIII fragment and subcloned into the BamHI/HindIII sites of pBSSK(−). Unidirectional deletions toward the upstream Sau3AI site were made with exonuclease III, repaired with mung bean nuclease and adapted with HindIII linkers (New England Biolabs No. 1050). Deletions ending immediately upstream of the 9-bp host duplication flanking the *Mu1* element were selected and sequenced. One deletion variant, pKL23, had a deletion end point 1 bp upstream of the 9-bp host duplication sequence (TTTTGGGGA, positions 5 to 13; see Barker et al., 1984).

Plasmid facsimiles of mutable alleles previously characterized and new mutations were constructed by placing the *Mu1* element at different locations within intron 1 in both orientations. pALMH25 and pALMH26 were made by inserting the *Mu1* element from pKL23 in orientations "a" and "b," respectively, into the intron 1 HindIII site of pAL21. pALMH25 represents the "a" orientation and differs from the *Adh1-S3034* allele (in the insertion region) by having an additional 7 bp added upstream of the *Mu1* element. pALMH26 has the *Mu1* element inserted in the opposite, or "b," orientation and, thus, represents a novel mutation created in vitro. pALMB2 and pALMB8 have the HindIII *Mu1* fragment from pKL23 [excised with HindIII, filled in with Klenow, and adapted with BamHI linkers (New England Biolabs No. 1021)] inserted in orientations "a" and "b," respectively, in the intron 1 BglII site of pAL61; both constructions are new mutations created in vitro. pALMS3 and pALMS6 have the HindIII *Mu1* element from pKL23 (excised with HindIII and filled in with Klenow) inserted in the "a" and "b" orientations, respectively, into the StuI site of pAL61. The *Adh1-S4477* allele has a *Mu1* element inserted 6 bp upstream of the same StuI site (Ortiz and Strommer, 1990) and, thus, the plasmid pALMS3 has a structure similar to that of the *Adh1-S4477* allele. pALMS6 is a new mutation created in vitro.

pALMS3p2 and pALMS6p22 were derived from pALMS3 and pALMS6, respectively, by removing the HindIII fragment containing the promoter, exon 1, and 72 bp of intron 1.

pALI$_3$S2

The 729-bp Fnu4HI/SspI (positions 1552 to 2281; see Shah et al., 1983, as corrected in Luehrsen and Walbot, 1990) internal fragment of maize actin intron 3 was excised, the overhangs were filled in with Klenow, and the blunt-end fragment was ligated into

the StuI site of pAL61. The orientation of the insert results in the correct strand of the intron being represented in the pre-mRNA.

pALOX Series

pALOH28 and pALOH27 have an oligomer, representing the sequence remaining after a precise *Mu1* excision, inserted into the intron 1 HindIII sites of pAL21 in the "a" and "b" orientations, respectively. The sequence of the oligomer is

AGCTTGGATTTTGGGGA | TTTTGGGGAAGCT

where the | symbol represents the position of the excised *Mu1* insertion.

pAL74M Series

pAL74M31 and pAL74M32 have the *Mu1* element from pKL23 (excised with HindIII and filled in with Klenow) inserted into the actin intron 3 StuI site in the "a" and "b" orientations, respectively.

pALM Deletion and Insertion Series

pAL25E4 and pAL26E5 were made by restricting pALMH25 and pALMH26, respectively, with EagI and religating the cohesive ends. pAL25T3 and pAL26T1 were made by restricting pALMH25 and pALMH26, respectively, with Tth111I, filling in the overhangs with Klenow, and religating the blunt ends. pALMEH3 and pALMEH2 were constructed by inserting the EagI central fragment of *Mu1* [overhanging ends filled in with Klenow and adapted with HindIII linkers (New England Biolabs No. 1050)] in the "a" and "b" orientations, respectively, into the HindIII site of pAL61.

RNase Mapping Probe Plasmids

pBSI$_1$/luc3 has been described in detail (Luehrsen and Walbot, 1990); briefly, the BglII/XbaI fragment from pAL61 containing the intron/luciferase junction fragment was cloned into the BamHI/XbaI sites of pBSKS(−). pJD350 was obtained from J.R. de Wet and is the ClaI/SspI luciferase fragment (positions 1418 to 1749; for numbering, see de Wet et al., 1987) inserted into the AccI/EcoRV sites of pBSKS(+). The plasmid pBS*Adh1* was used to synthesize the antisense *Adh1* probe used to detect cellular *Adh1-F* mRNA. It was constructed by cloning the BalI (adapted with New England Biolabs No. 1022 HindIII linkers)/HindIII fragment of an *Adh1* cDNA (positions 934 to 2602 of the genomic fragment; Dennis et al., 1984) into pBSKS(−); the probe protects a 152-nt fragment of the *Adh1-F* mRNA. pBSMu31 was constructed by cloning the EagI central *Mu1* fragment (positions 336 to 1076; for numbering, see Barker et al., 1984) into the EagI site of pBSKS(−). The full-length (748-nt) "a" and "b" orientation probes were made by restricting pBSMu31 with EcoRI and XhoI, respectively, and using the appropriate RNA polymerase to synthesize the probe. Shortened, truncated antisense probes were made by restricting the *Mu1* EagI fragment with BssHII or BstNI for the "a" (662-nt) and "b" (697-nt) orientation probes, respectively.

Transient Assay Analysis

The tissue culture line used in all experiments was BMS; this material was maintained as described previously (Fromm et al., 1987). Electroporation was carried out essentially as reported (Fromm et al., 1987) except that the protoplasts were subjected to a 15-min heat shock at 45°C and placed on ice for ≥30 min before electroporation. Twenty five micrograms of the test plasmid was used in each transfection assay; each test plasmid was done in duplicate cuvettes and the results were averaged. In addition, 5 μg to 20 μg of a plasmid expressing a second reporter gene enzyme activity was added to each transfection assay. The values of the second expression plasmid were used to correct for differences in protoplast viability and recovery between the test plasmid transfections. The protoplasts were allowed to recover for 20 hr to 24 hr before harvesting. A sonic extract in 400 μL of luciferase extraction buffer (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol) was prepared, and 20-μL aliquots were assayed for either luciferase (de Wet et al., 1987) or GUS (Jefferson et al., 1986) activity as described. Activity data are expressed in terms of light units (photons) per 10 sec (luciferase) per picomoles methylumbelliferyl converted per minute (GUS) when the test plasmid expressed luciferase or as picomoles methylumbelliferyl per minute (GUS) per light unit per 10 sec (luciferase) when the test plasmid expressed GUS. The expression values reported for each set of expression plasmids represent data from duplicate cuvettes in a single experiment with one batch of protoplasts; all expression plasmids were tested several times.

RNA Purification and RNase Mapping

Total RNA was purified from electroporated protoplasts 20 hr to 24 hr after electroporation. Standard procedures were used (Callis et al., 1987) with modifications as described (Luehrsen and Walbot, 1990). RNase mapping was completed using published procedures (Krieg and Melton, 1987; Goodall and Filipowicz, 1989). In all cases, the lengths of the protected fragments were less than that of the probe used, indicating complete RNase digestion. RNase mapping autoradiograms were exposed using two intensifying screens; those scanned with an LKB laser densitometer were exposed without an intensifying screen. When comparing the relative intensities of protected fragments, the appropriate correction for labeled C-content was applied.

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