Alternative Transcription Initiation Sites and Polyadenylation Sites Are Recruited During *Mu* Suppression at the *rf2a* Locus of Maize

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ABSTRACT

Even in the absence of excisional loss of the associated *Mu* transposons, some *Mu*-induced mutant alleles of maize can lose their capacity to condition a mutant phenotype. Three of five *Mu*-derived *rf2a* alleles are susceptible to such *Mu* suppression. The suppressible *rf2a-m9437* allele has a novel *Mu* transposon insertion (*Mu10*) in its 5′ untranslated region (UTR). The suppressible *rf2a-m9390* allele has a *Mu1* insertion in its 5′ UTR. During suppression, alternative transcription initiation sites flanking the *Mu1* transposon yield functional transcripts. The suppressible *rf2a-m8110* allele has an *rcy/Mu7* insertion in its 3′ UTR. Suppression of this allele occurs via a previously unreported mechanism; sequences in the terminal inverted repeats of *rcy/Mu7* function as alternative polyadenylation sites such that the suppressed *rf2a-m8110* allele yields functional *rf2a* transcripts. No significant differences were observed in the nucleotide compositions of these alternative polyadenylation sites as compared with 94 other polyadenylation sites from maize genes.

THERE are two broad categories of DNA transposons, autonomous and nonautonomous. Autonomous transposons encode all nonhost factors required for their own transposition. In contrast, the transposition of nonautonomous transposons is dependent upon factors encoded by autonomous transposons of the same family. Hence, only in the presence of factors encoded by the autonomous *Spm/En*, *Ac*, and *MuDR* maize transposons can the nonautonomous *dSpm/I*, *Ds*, and *Mu* transposons undergo excision and transposition (reviewed by Masson *et al.* 1991; Bennetzen *et al.* 1993; Bennetzen 1996; Kunze 1996; Fedoroff 1999). However, both autonomous and nonautonomous transposons can cause mutations when they insert into genes. The *Mutator* (*MuDR/Mu*) transposon family (Robertson 1978) has been widely used for gene mutagenesis and cloning (Bennetzen *et al.* 1993; Bennetzen 1996). Its 4.9-kb autonomous member, *MuDR*, mediates the transposition of the nonautonomous transposons, *Mu1–Mu8* (Schnable and Peterson 1986; Robertson and Stinard 1989; Chomet *et al.* 1991; Hershberger *et al.* 1991; Qin *et al.* 1991; Hsia and Schnable 1996). *MuDR* contains two open reading frames (ORFs), *mudrA* and *mudrB* (Hershberger *et al.* 1991; James *et al.* 1993). The *mudrA* gene encodes a DNA-binding protein with a region of sequence similarity to bacterial transposases (Eisen *et al.* 1994; Benito and Walbot 1997). The *mudrA* gene is necessary and sufficient for somatic excision of *Mu* transposons (Lisch *et al.* 1999; Raizada and Walbot 2000). The *mudrB* gene may be involved in suppression (Donlin *et al.* 1995; Lisch *et al.* 1999).

Many transposon-induced mutants exhibit unstable phenotypes as a result of DNA rearrangements such as excision. In addition, some transposon-induced alleles also exhibit instabilities that occur in the absence of DNA rearrangements (McClintock 1964, 1967; Martienssen *et al.* 1989). The mechanisms underlining these phenomena are not well understood.

The loss of a *Mu*-induced allele's capacity to condition a mutant phenotype is termed *Mu* suppression. *Mu* suppression was first reported at the *hcf106::Mu1* mutant allele, which has a *Mu1* insertion in its 5′ untranslated region (UTR; Martienssen *et al.* 1989). In the presence of active *MuDR* transposons, this allele conditions a pale-green seedling. In the absence of *MuDR*, the *hcf106::Mu1* allele can condition a nonmutant phenotype that consists of dark-green "normal" leaf tissue (Martienssen *et al.* 1989, 1990). In this suppressed state, alternative transcription initiation sites generate functional *hcf106* transcripts that are not present in plants that carry active *MuDR*. These alternative transcription initiation sites are in the 5′ terminal inverted repeat (TIR) of *Mu1* and in regions of the 5′ UTR of the *hcf106* genes that are 3′ of the *Mu1* insertion site (Barkan and Martienssen 1991).

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Mu suppression has been observed in several other *Mu*-induced mutants. Two suppressible *Knotted1* (*Kn1*) alleles arose via *Mu1* and *Mu8* insertions in the junction region of the *Kn1-0* repeats. This junction region contains the promoter region of the downstream copy of *Kn1-0* (Lowe *et al.* 1992). Two other suppressible *Kn1* alleles, *Kn1-mum2* and *Kn1-mum7*, have *Mu8* and *Mu1* insertions in the third intron of the *Kn1* locus (Greene *et al.* 1994). *Mu* suppression has also been observed with the *Les22-7* mutant allele, which has a *Mu1* insertion in its 5′ UTR (Hu *et al.* 1998), and with the *a1-mum2* allele, which carries a *Mu1* insertion 81 bp upstream of the transcription initiation site (Chomet *et al.* 1991). Some *Lg3* and *Rs1* alleles, which have *Mu* insertions in 5′ UTRs (*Lg3-Or422*, *Lg3-Or102*, and *Lg3-Or331*) or introns (*Lg3-Or211* and *Rs1-Or11*) also exhibit suppression (Girard and Freeling 2000). Hence, *Mu* insertions in promoter regions, 5′ UTRs, and introns can all generate suppressible alleles.

The *hcf106::Mu1* mutation and the genetically unlinked *Mu*-induced suppressible mutation *Les28* exhibit coordinated suppression and reactivation. The suppression of both mutant phenotypes is well correlated with hypermethylation of *Mu* transposons throughout the entire genome and also with hypermethylation of the region of the *hcf106* locus flanking the *Mu1* insertion (Martienssen and Baron 1994). As is true for *hcf106::Mu1* and *Les28*, *Mu* suppression of the four *Kn1* alleles is correlated with genomewide hypermethylation of *Mu* transposons (Lowe *et al.* 1992; Greene *et al.* 1994). At least two of these *Kn1* alleles (*Kn1-mum2* and *Kn1-mum7*) can be reactivated by crossing to *Mu*-active lines (Greene *et al.* 1994). Because loss of *Mu* activity is correlated with hypermethylation of *Mu* transposons (reviewed by Chandler and Hardeman 1992; Bennetzen *et al.* 1993) and the presence of MuDR (Chomet *et al.* 1991), and because expression of the 823 amino acids of MURA in transgenic plants is sufficient to result in demethylation (Raizada and Walbot 2000), it is likely that *Mu* suppression is caused by an absence of MURA.

Mu suppression has the potential to complicate efforts to clone genes via transposon tagging, because a critical step in such a project is to identify the particular *Mu*-containing restriction fragment length polymorphism or PCR fragment responsible for a *Mu*-induced mutation. This step is generally accomplished by identifying *Mu*-containing DNA fragments that cosegregate with the mutant phenotype through meioses. Because suppressed plants exhibit a wild-type phenotype even though they carry a *Mu* transposon in the target gene, suppression can mask the cosegregation between the mutant phenotype and the *Mu*-containing DNA fragment. *Mu* suppression can also lead to the loss of mutant phenotypes during backcrossing programs, which are becoming increasingly important with the adoption of new technologies such as RNA profiling and proteomics. These problems can be avoided if these analyses are conducted in an active *Mu* stock but this restriction limits the available genetic backgrounds.

Mu suppression also impacts technologies, such as Trait Utility System for Corn (Bensen *et al.* 1995) and *Mu* rescue (Walbot 1999), which are important components of maize functional genomics projects. Each of these projects utilizes *Mu* transposon sequence as bait to obtain *Mu*-insertion mutants through reverse genetics for functional analysis. This selection strategy does not exclude suppressible mutants, which can condition either mutant or normal phenotypes and thereby complicate the functional analysis of target genes. This is a particular concern because *Mu* transposons appear to exhibit a strong preference for insertion within the 5′ UTRs of at least one gene (Dietrich *et al.* 2002). On the other hand, *Mu* suppression during somatic development can produce clonal sectors on an unsuppressed background. Such chimeric plants provide a unique environment for analyzing mutants (Fowler *et al.* 1996).

Therefore, understanding the frequency and mechanisms of *Mu* suppression is critical to using *Mu* transposons to understand maize biology. Here we report the identification and characterization of three *Mu*-suppressible *rf2a* alleles. We found that *Mu* suppression can occur not only at alleles caused by *Mu* insertions in a 5′ UTR via the recruitment of alternative transcription initiation sites as reported previously, but also at an allele caused by a *Mu* insertion in the 3′ UTR. Polyadenylation sites within the TIRs of the inserted *Mu* transposon are recruited during *Mu* suppression of this new class of *Mu*-suppressible alleles. In addition, these studies establish that insertions of two additional classes of *Mu* transposons can generate suppressible alleles.

MATERIALS AND METHODS

Alleles of the *rf2a* gene: The *rf2a* gene (previously designated *rf2*; Skibbe *et al.* 2002) is one of the two complementary restorers of T-cytoplasm male sterility (reviewed by Schnable and Wise 1998; Wise *et al.* 1999), which encodes a mitochondrial aldehyde dehydrogenase (Cui *et al.* 1996; Liu *et al.* 2001). Plants that carry T cytoplasm and that are homozygous for mutant alleles of either *rf1* or *rf2a* are male sterile. All lines used in this study carry *Rf1* and *Rf2a* alleles unless otherwise indicated. The reference allele, *rf2a-R213*, is a spontaneous mutant carried by the inbred lines Wf9 and R213. All other described *rf2a* mutant alleles were isolated via transposon tagging experiments (Schnable and Wise 1994). Mutant alleles *rf2a-m8110*, *rf2a-m8122*, *rf2a-m9323*, *rf2a-m9390*, *rf2a-m9385*, and *rf2a-m9437* were all obtained from a *Mu*-containing population. The wild-type progenitor of *rf2a-m8110* and *rf2a-m9390* is *Rf2a-Q67*. The progenitor of *rf2a-m8122* and *rf2a-m9323* is *Rf2a-Q66*. The progenitor of *rf2a-m9437* is *Rf2a-B79* (Cui *et al.* 1996). Each of the *rf2a-m* alleles used in these experiments was backcrossed to the inbred line Ky21 for at least three generations. The *rf2a-m9385* allele was not included in this study because an adequate number of backcrosses had not been completed. The *rf2a-m8904* allele was isolated from

an *Spm/En* population. The inbred line B73 has the genotype *rf1Rf2a*.

Backcrosses of the *rf2a-m* alleles: Plants heterozygous for each *rf2a-m* allele (*rf2a-m/Rf2a*) carrying T cytoplasm were crossed as females by the inbred line Ky21. Progeny that carried the *rf2a-m* alleles were identified by DNA gel blot or PCR analyses. The 1.2-kb partial or 2.2-kb full-length *rf2a* cDNA was used as a hybridization probe against *Eco*RV- (for *rf2a-m9437*) or *Hin*dIII- (for the rest of the *rf2a-m* alleles) digested genomic DNA in DNA gel blot analyses. Genotypes of plants in families segregating for *rf2a-m* alleles were often confirmed via testcrosses: (T) *rf2a-R213/rf2a-R213* × *rf2a-m/Rf2a-Ky21* (or *Rf2a-Ky21/Rf2a-Ky21*). Segregation of male-sterile and male-fertile plants (1:1) in the resulting progeny confirmed the presence of a nonsuppressed *rf2a-m* allele in a particular male parent.

Male-fertility ratings: Phenotypes were scored in the morning during the period of pollen shedding for several days according to the rating system of Schnable and Wise (1994). Plants were scored as male fertile (F), full fertile, with >90% of the anthers exerted; semifertile ("F"), with <90% but more than half of the anthers exerted; semisterile ("S"), with <50% of the anthers exerted (usually only a few percent of anthers exerted); or male sterile (S), completely male sterile with no anthers exerted.

***Mu*-active lines:** *Mu*-active lines were derived either from the stocks used to generate the *rf2a-m* alleles (Schnable and Wise 1994) or from very similar crosses that also involved *Mu* stocks and the inbred R213.

***Rf2a* genomic clones:** Two overlapping *rf2a*-hybridizing genomic clones were obtained by screening B73 libraries. Both libraries were constructed using the λDASHII (Stratagene, La Jolla, CA) vector and were prepared by Pam Close and John Tossberg, respectively. Library screening conditions were as described by Xu *et al.* (1997). Phage inserts were subcloned into pBluescript SK or KS (Stratagene) vectors for further analysis or sequencing. Some of these fragments were sequenced by utilizing the TN1000 transposon system (Gold Biotechnology, St. Louis, adapted from Strathmann *et al.* 1991). Both DNA strands were completely sequenced unless otherwise indicated.

Clone rf2-DNA1 was obtained using a 900-bp probe (DD1, see Figure 1C of Cui *et al.* 1996) that includes the last two introns (9 and 10) and exons (10 and 11) of the *rf2a* gene. An alignment of the sequence of the entire 20,072-bp insert of rf2-DNA1 with the full-length *rf2a* cDNA (GenBank accession no. U43082) revealed that 353 bp from the 5′ end of the cDNA clone were not included in clone rf2-DNA1. Hence, another *rf2a* genomic clone (rf2-DNA2-65) was isolated from the second B73 genomic library using the full-length *rf2a* cDNA as probe. Sequencing and restriction mapping experiments revealed that the two *rf2a* genomic clones differ in the region 5′ of exon 2 but not in the region defined by exons 2 and 11. Furthermore, data from PCR amplification of B73 genomic DNA using various primer pair combinations and genomic mapping via DNA gel blot analyses indicate that the structure of rf2-DNA2-65, but not of rf2-DNA1, reflects the structure of the *Rf2a-B73* allele. On the basis of these results we propose that clone rf2-DNA1 is chimeric and contains the region 3′ of intron 1 derived from the *rf2a* gene, while the region 5′ of intron 1 is of unknown origin. Hence, to generate the complete genomic sequence of the *Rf2a-B73* allele, 5.2 kb of sequence from the 5′ end of clone rf2-DNA2-65 was combined with 12.6 kb of sequence from the 3′ end of clone rf2-DNA1 and deposited in GenBank (accession no. AF215823).

Mapping transposon insertion sites and identifying *Mu* transposons: The transposon insertion site in the *rf2a-m8122* allele was established during the cloning of the *rf2a* gene (Cui *et al.* 1996). Genomic restriction mapping was conducted on *rf2a-m8110*, *rf2a-m9323*, *rf2a-m9390*, and *rf2a-m9437* alleles using a variety of restriction enzymes. These DNA gel blots were hybridized with the *rf2a*-specific probes rf2-5m, C4-C6, B461-xq, and C1-C2 (see below). Using this method, it was possible to map the *Mu* insertions in three of the four alleles analyzed (*rf2a-m8110*, *rf2a-m9390*, and *rf2a-m9437*) to specific regions of the *rf2a* gene.

PCR reactions were also conducted on DNA from plants homozygous for each of the *rf2a-m* alleles to map and to identify *Mu* transposons. Each PCR reaction included a *Mu*-TIR primer and one of many primers specific to the exons of *rf2a*. Due to the large introns of this gene, most of these PCR reactions did not yield *rf2a*-specific products. After some of the transposon insertion sites were mapped via genomic restriction mapping, appropriate *rf2a*-specific primers were used (RF2C6 for *rf2a-m9390* and *rf2a-m9437* and RF2C1 for *rf2a-m8110*) to amplify these *rf2a-m* alleles (Figure 1). In these instances the transposon insertion sites were physically mapped via sequence comparisons between the resulting PCR products and the sequence of the *rf2a* gene (GenBank accession no. AF215823). The junction between the *rf2a* and *Mu*-TIR sequences in these PCR products defines the *Mu* insertion site. Because the TIRs of each class of *Mu* transposon contain diagnostic polymorphisms, it was possible to determine the identities of the *Mu* transposons inserted in *rf2a-m* alleles by comparing the *Mu*-TIR sequence contained in the allele-specific PCR products to the TIRs of all known *Mu* transposons. In addition, because the sequences of the two TIRs of most *Mu* transposons have one or more polymorphisms relative to each other, the orientations of the *Mu* insertions in *rf2a-m* alleles could also be determined.

To confirm the identity of the transposon inserted into the *rf2a-m9390* allele, genomic DNA samples from plants homozygous for *rf2a-m9390* and its progenitor Q67 were digested with *Eco*RV, *Hin*dIII, *Xba*I, *Eco*RV + *Hin*dIII, *Eco*RV + *Xba*I, and *Hin*dIII + *Xba*I and then hybridized with the *rf2a*-specific probe, rf2-5m. The same filter was stripped (Ausubel *et al.* 1999) and hybridized with a *Mu1*-specific probe. In each restriction digest the *rf2a*-hybridizing fragments were the same size as the *Mu1*-hybridizing fragments, which provided further support for the conclusion that the transposon inserted into *rf2a-m9390* is *Mu1*.

The identity of the *rcy/Mu7* transposon in *rf2a-m8110* was confirmed similarly with the *rf2a*-specific probe C1-C2 and a *rcy/Mu7*-specific probe. To further confirm the identity and orientation of the *rcy/Mu7* transposon insertion in *rf2a-m8110*, an *rf2a*-specific primer, RF2C2, which is ~0.1 kb 3′ of the *rcy/Mu7* insertion site in this allele, and an *rcy/Mu7* internal primer, Mu7-R, were used to amplify a 1.7-kb fragment from the 5′ end (*i.e.*, the rightmost end in Figure 1) of the *rcy/Mu7* transposon in *rf2a-m8110*. This PCR product was subcloned into a pGEM-T vector (Promega, Madison, WI) and >80% was sequenced. Sequence comparisons between this PCR product and *rcy/Mu7* (GenBank accession no. X15872) identified only a few nucleotide polymorphisms.

The transposon insertion in the *rf2a-m8904* allele was identified by PCR amplification using primers rf2a-3320 and RF2C5UTRR, which flank the transposon insertion. Based on its sequence, this transposon is *Ds1* (GenBank accession no. AF010445).

Probes: DNA fragments used as probes in this study were obtained as follows. The 2.2-kb full-length *rf2a* cDNA fragment was obtained from plasmid prf273-11 with the restriction enzymes *Xho*I and *Eco*RI. The 1.2-kb partial *rf2a* cDNA was obtained from plasmid prf2a-1.2 with the restriction enzyme *Eco*RI. Plasmids prf273-11 and prf2a-1.2 contain the 1.2-kb and 2.2-kb *rf2a* cDNAs as described in Cui *et al.* (1996). The *rf2a*

5′ 0.15-kb probe, rf2-5m, was PCR amplified from plasmid prf273-11 using primers RF2C6 and RF2C8 (Figure 1). Another *rf2a* 5′ probe, C4-C6, was PCR amplified from plasmid prf273-11 using primers RF2C4 and RF2C6. Probes B461-xq (from 461 to 927 bp in GenBank accession no. U43082) and C1-C2 (from 1374 to 2029 bp in GenBank accession no. U43082) were PCR amplified from plasmid prf273-11 using primers rf2-B461 and rf2-xq or primers RF2C1 and RF2C2 (Figure 1). A 0.96-kb *Mu1*-specific fragment was isolated from plasmid pRB1 (Robertson *et al.* 1988) using the restriction enzyme *Mlu*I. The 0.16-kb *rcy/Mu7* probe was isolated from plasmid pSB9-in (Schnable *et al.* 1989) using restriction enzymes *Bam*HI and *Eco*RI. Maize GAPDH cDNA (GenBank accession no. X07156) was isolated from pGAPDH with the restriction enzymes *Eco*RI and *Hin*dIII. All probes were labeled with dCTP^{32} using a random primer labeling protocol (Ausubel *et al.* 1999).

Genotyping *rf2a-m* alleles via PCR: To determine which plants in a segregating family carry an *rf2a-m* allele, PCR amplifications were conducted using a *Mu*-TIR primer in combination with an appropriate *rf2a*-specific primer. Various primers that anneal to *Mu*-TIRs, such as XX153, were used in these PCR reactions. The *rf2a*-specific primers used for genotyping *rf2a-m* alleles include RF2C1 (for *rf2a-m8110* and *rf2a-m8122*) and RF2C6 (for *rf2a-m9390* and *rf2a-m9437*; Figure 1). PCR reactions were performed for 34 cycles as follows: denature at 94° for 40 sec; anneal primers for 40 sec at 55°–58° (depends upon the *Mu*-TIR primer used); extend at 72° for 2 min in the presence of 2.5 units of Taq polymerase (Promega) per reaction.

Primer sequences:

Mu7-R: 5′ TTCTCCGCCGTTGCCATCTC 3′
RF2C1: 5′ GCGTCGTTGGTGATCCGTTC 3′
RF2C2: 5′ CCAGGCTAGGGCAAATCTTAT 3′
RF2C4: 5′ AGCGGGAGACGAGCGAGGAC 3′
RF2C5: 5′ ATGCTGCGATTCCGTTTGGTG 3′
RF2C6: 5′ TCCTCACTCCCACACCAACC 3′
RF2C8: 5′ GCAGCAGGAGAAGCGGCAGGCAG 3′
RF2C9: 5′ GTGATGGGCTCCTCTACT 3′
XX153: 5′ CGCCTCCATTTCGTCGAATCC 3′
rf2-B461: 5′ ACAGATCTAAAGCTCCTCATTAAT 3′
rf2-xq: 5′ CCAACTTTCCAGGCATACATCA 3′
rf2a-3320: 5′ GAGGAACCAGTAGCGGAGGC 3′
RF2C5UTRR: 5′ GCTCCCGTTCGCAGTCG 3′

DNA and RNA gel blot analyses: Maize genomic DNA was isolated using a 1× CTAB procedure (Saghai-Maroof *et al.* 1984). About 10 μg of DNA was digested with the indicated restricted enzyme in a 30-μl reaction volume for >3 hr and then separated via electrophoresis through a 0.8% agarose gel. DNA was transferred to nylon filters (MSI, Westboro, MA) and hybridized with probes labeled with dCTP^{32} (Ausubel *et al.* 1999).

Total RNA from immature tassels (still in the whorl) was isolated according to Dean *et al.* (1985). Approximately 10 μg of total RNA was subjected to electrophoresis using a MOPS buffer system (Ausubel *et al.* 1999) and transferred onto Gene-Screen filters (NEN Research Products, Boston). The filters were hybridized with probes labeled with dCTP^{32} (Ausubel *et al.* 1999). The hybridization density of each band was quantified using a GS-710 densitometer (Bio-Rad Laboratories, Hercules, CA).

3′ and 5′ rapid amplification of cDNA ends: RNA was isolated from immature tassels (still in the whorl) using the Trizol reagent (GIBCO BRL, Rockville, MD) and treated with PCR-grade DNaseI (GIBCO BRL) according to the manufacturer's instructions. A total of 5 μg and 100 ng of RNA were used for 3′ and 5′ rapid amplification of cDNA ends (RACE) experiments, respectively, using kits obtained from GIBCO BRL. The *rf2a*-specific primers RF2C5 and RF2C9 (Figure 1) were used for 3′ and 5′ RACE, respectively. The position of RF2C9 in exon 2 allows the inadvertent amplification of genomic DNA to be detected. Primer RF2C 4 was used as a nested primer for the 5′ RACE experiments. RACE products were subcloned into the pGEM-T vector (Promega) and sequenced.

Analysis of polyadenylation sites: All GenBank records for which the organism was *Zea mays* (excluding chloroplast and mitochondrial genes) that had the feature of "polyA_site" were downloaded on March 22, 2000. This data set was then parsed for the feature of polyadenylation site. The few records that lacked sequence data downstream of the reported poly(A) site or that had only "A" bases downstream of this site were excluded. Only the most recent GenBank submission was used in those instances of multiple submissions of the same gene. Chi-square homogeneity tests were performed according to Steel and Torrie (1980).

RESULTS

Structure of the *rf2a* gene: The *rf2a* cDNA encodes a mitochondrially localized aldehyde dehydrogenase (Cui *et al.* 1996; Liu *et al.* 2001). Two overlapping genomic clones (rf2-DNA1 and rf2-DNA2-65) were obtained by screening B73 genomic libraries (materials and methods). Sequence comparisons of 17.8 kb derived from these genomic clones (GenBank accession no. AF215823) and the full-length *rf2a* cDNA clone (GenBank accession no. U43082) defined 11 exons and 10 introns (Figure 1). The extreme 5′ end of the genomic sequence (base positions 102–740) is 81.5 and 80.7% identical to the 3′ long terminal repeat (LTR; GenBank accession no. AF050449) and the 5′ LTR (GenBank accession no. U68407) of the maize Milt retrotransposon. In addition, a member of a newly defined class of retrotransposons, *DON QUIXOTE*, which is present in intron 5 (base positions 6763–13,927 of GenBank accession no. AF215823), contains a characteristic nucleic acid binding site that serves as the primer site for reverse transcription and an uninterrupted ORF of ~4 kb (positions 8335–12,129) that encodes predicted proteins with high degrees of sequence similarity to the reverse transcriptase, protease, integrase, and endonuclease typical of copia-like retrotransposons (Konieczny *et al.* 1991). A 0.6-kb region that is ~140 bp 5′ of exon 2 in rf2-DNA2-65 exhibits 91% sequence identity to the LTR of Grande1-4 retrotransposon (GenBank accession no. X97604). Two other regions of the *rf2a* gene (base positions 2065–3096, 5′ of the transcription initiation site and positions 4875–5270 in intron 1) exhibit ~88 and 85% sequence similarity, respectively, to the noncoding regions of various maize genes (GenBank accession nos. X06333, L40803, AF030385, AF031569, AF041044 and AJ005343, Z22760, AF043346, Z26824, AJ223471). These regions of sequence similarity have not been assigned any putative functions and do not exhibit characteristics of DNA transposons or retrotransposons.

Positions of transposons in *rf2a-m* alleles: The posi-

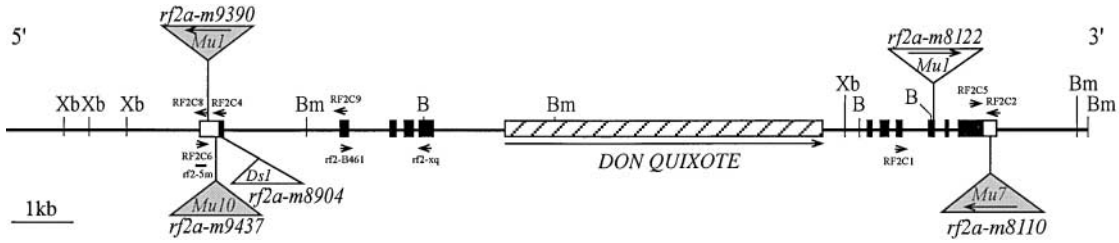

FIGURE 1.—The structure of the *rf2a* gene (GenBank accession no. AF215823). Solid and open boxes represent coding regions and 5′ or 3′ UTRs, respectively. Hatched box represents a *copia*-like retrotransposon, *DON QUIXOTE*. The positions of the transposon insertions responsible for the indicated alleles are indicated by triangles (not to scale). Solid triangles represent *Mu* insertions responsible for suppressible alleles. The arrows inside the triangles indicate the orientations of the transposon sequences in GenBank. The position of probe rf2-5m within exon 1 is shown as a bar underneath the 5′ UTR. The positions of PCR primers are indicated by arrows. Bm, *Bam*HI; Bg, *Bgl*II; Xb, *Xba*I.

tions of transposon insertions responsible for four of the five *Mu*-derived *rf2a-m* alleles (*rf2a-m8110*, *rf2a-m8122*, *rf2a-m9390*, and *rf2a-m9437*) were mapped via genomic restriction mapping and sequence comparisons between PCR products obtained using *rf2a-m* DNA templates in conjunction with *Mu*-TIR- and *rf2a*-specific primer pairs and the sequence of the *rf2a* gene (Figure 1). The specific class of *Mu* transposon responsible for each mutant and its orientation were determined via sequence analysis of the *Mu*-TIRs obtained from these PCR products. The *rf2a-m9390* allele has a *Mu1* transposon inserted into its 5′ UTR, 105 bp 5′ of the translation start codon. The identity of this *Mu1* transposon was confirmed by DNA gel blot analysis (MATERIALS AND METHODS). The *rf2a-m9437* allele contains a novel *Mu* transposon insertion in its 5′ UTR, 35 bp 5′ of the translation start codon. The TIR of this transposon differs from all other described *Mu* transposons. Therefore, this *Mu* transposon has been designated *Mu10* (GenBank accession no. AF231940). The *rf2a-m8110* allele arose via an *rcy/Mu7* insertion in the 3′ UTR, 30 bp 3′ of the translation stop codon. The identity of this *rcy/Mu7* transposon was confirmed by sequence analysis of a 1.7-kb fragment of it and DNA gel blot analyses. The *rf2a-m8122* allele has a *Mu1* insertion in exon 9 (CUI *et al.* 1996). It has not yet been possible to identify the molecular lesions associated with the *rf2a-m9323* and *rf2a-m9385* alleles. The *rf2a-m8904* allele contains a *Ds1* transposon a few base pairs downstream of the translation start codon. This 395-bp *Ds1* has three single nucleotide polymorphisms relative to the *Ds1* sequence in GenBank (accession no. AF010445).

Reanalysis of data from SCHNABLE and WISE (1994): Although one-half of the progeny from the testcross, (T) *rf2a-m8110/Rf2a* × *rf2a-R213/rf2a-R213*, would be expected to be male sterile, Schnable and Wise found only 4 of 26 progeny from this cross to be male sterile. A high rate of nonconcordance between the male sterile phenotype and a nearby marker (*wx1*) was also obtained from a similar testcross but involving *rf2a-m9390* allele (SCHNABLE and WISE 1994). After the *rf2a* gene was cloned and the positions of the *Mu* insertions responsible for these alleles were determined, genomic DNA samples from these testcross families were genotyped via PCR (MATERIALS AND METHODS). The results of these analyses are presented in Table 1. Families segregating for *rf2a-m8110* (92 2123) and *rf2a-m9390* (92 2148) contain three and six male-fertile plants, respectively, with the genotype of *rf2a-m/rf2a-R213*. Hence, in these families, these two alleles display only 70 and 40% penetrance, respectively. In contrast, a similar family but segregating for the *rf2a-m8122* allele, which has a *Mu1* insertion in the coding region, exhibited 100% penetrance (family 92 2126 in Table 1). The *rf2a-m9323* allele was not included in these genotyping experiments, but it has not exhibited low penetrance in the backcrossing program. Another allele, *rf2a-m9437* (family 92 2153), with a *Mu10* insertion in the 5′ UTR, also exhibited 100% penetrance in this generation. However, after three generations of backcrossing to the inbred line Ky21, the *rf2a-m9437* allele also began to show evidence of low penetrance (data not shown). These genotyping results establish that the low penetrance observed by SCHNABLE and WISE (1994) does not involve excisional loss of the *Mu* transposons. Because the *rf2a-m8122* allele (which has a *Mu1* insertion in the coding region) did not exhibit low penetrance in otherwise identical crosses (*e.g.*, family 92 2126, Table 1), it is not likely that the low penetrance of these alleles is conditioned by genetic suppressors (PRELICH 1999) of *rf2a* carried by the inbred line Ky21.

Reactivation of *rf2a-m* alleles: Because these three alleles (*rf2a-m8110*, *rf2a-m9390*, and *rf2a-m9437*) all have a transposon insertion in an UTR, this low penetrance could reflect *Mu* suppression, which occurs when a genome lacks *Mu* activity (MARTIENSSEN *et al.* 1989). One of the hallmarks of *Mu* suppression is the possibility of reactivation, whereby suppressed *Mu*-induced alleles are reactivated by the reintroduction of *Mu* activity via genetic crosses. To determine whether *rf2a-m8110* and/or *rf2a-m9390* can be reactivated (and thereby again condition mutant phenotypes), stocks carrying suppressed versions of these alleles were crossed to *Mu*-active lines. To minimize the confounding effects of ge-

TABLE 1

Comparison of the penetrance of suppressible and nonsuppressible *rf2a-m* alleles

Families	Alleles	No. of plants: F + "F" *Rf2a/rf2a*	No. of plants: F + "F" *rf2a-m/rf2a*	No. of plants: S + "S" *Rf2a/rf2a*	No. of plants: S + "S" *rf2a-m/rf2a*	Total no.	Penetrance[a] (%)
92 2123	*rf2a-m8110*	16	3	0	7	26	70
92 2148	*rf2a-m9390*	4	6	0	4	14	40
92 2126	*rf2a-m8122*	4	0	0	14	18	100
92 2153	*rf2a-m9437*	13	0	0	12	25	100

Random plants from the cross (T) *rf2a-m/Rf2a-Ky21* × *rf2a-R213/rf2a-R213* were genotyped via PCR using *rf2a*-specific and *Mu*-specific primers (MATERIALS AND METHODS). The number of suppressed mutant plants in each family is underlined. Plant phenotypes: F, fully male fertile; "F," more than one-half of the anthers exerted; "S," some anthers exerted; S, completely male sterile with no anthers exerted.

[a] Penetrance was calculated as the ratio of sterile plants (S + "S") with the genotype *rf2a-m/rf2a-R213* to the total number of plants with the genotype *rf2a-m/rf2a-R213*. On the basis of a chi-square homogeneity test the ratios of fertile *rf2a-m/rf2a* plants in families 92 2123 and 92 2148 are significantly different at 95% confidence level from those in families 92 2126 and 92 2153.

netic backgrounds, the *Mu*-active lines used in these crosses were closely related to the lines used for generating the *rf2a-m* alleles (SCHNABLE and WISE 1994). As a control, some male-fertile plants that carried suppressed versions of these two alleles were self-pollinated. No male-sterile plants appeared in the resulting progenies (96g 6104-05 and 98 6336 in Table 2). In contrast, various rates of reactivation (as determined by the frequency of male-sterile progeny) were obtained in the crosses with *Mu* stocks (Table 2). Hence these *rf2a-m* alleles can be reactivated via crosses to active *Mu* lines. This result is consistent with the hypothesis that the low penetrance of these alleles is due to *Mu* suppression.

The stability of the reactivation of *rf2a-m8110* was tested by crossing a reactivated (*i.e.*, male sterile) heterozygous plant, (T) *rf2a-m8110/rf2a-R213*, by (N) *rf2a-m8904/rf2a-m8904*. The *rf2a-m8904* allele was used for this cross instead of *rf2a-R213* because unlike *rf2a-R213* it does not accumulate a detectable amount of *rf2a* mRNA (CUI *et al.* 1996). About 100 of the resulting progeny were scored for male fertility (data not shown). Most were male sterile. Of 16 randomly selected male-sterile plants, 7 carried the *rf2a-m8110* allele. Hence, *rf2a-m8110* can be transmitted through meiosis in the reactivated state.

Because the *rf2a-m9390* allele became suppressed

TABLE 2

Reactivation of suppressed *rf2a-m* alleles

Families	Alleles	Crosses	No. of fertile progeny	No. of sterile progeny	Reactivation ratio[a] (%)
97 5432	*rf2a-m9390*	(T) *rf2a-m/rf2a-m* (F)[b] × *rf2a-R213/Rf2a* (*Mu* on)	36	20	71
97 5433-34	*rf2a-m9390*	Same as above	114	21	30
96g 6104-05	*rf2a-m9390*	(T) *rf2a-m/rf2a-m* (F) self	20	0	0
97 5435-36	*rf2a-m8110*	(T) *rf2a-R213/rf2a-R213* (*Mu* on) × *rf2a-m/rf2a-R213* (F)	18	95	68
97 5437	*rf2a-m8110*	Same as above	1	9	80
97 5446	*rf2a-m8110*	(T) *rf2a-m/rf2a-R213* (*Mu* on) × *rf2a-m/rf2a-m* (F)	55	50	48
98 6336	*rf2a-m8110*	(T) *rf2a-m/rf2a-m* (F) self	15	0	0

Mu-active lines (indicated as "*Mu* on") were crossed to plants carrying suppressed *rf2a-m* alleles. The resulting progenies were scored for male-fertile plants. Each row in the table is from a single reactivation cross. *Mu*-active lines are progenies of *Mu*-active parents confirmed by seedling tests.

[a] The reactivation ratio was calculated by dividing the number of male-sterile plants by the number of male-sterile plants expected if the *rf2a-m* alleles displayed 100% penetrance. This formula was adjusted to account for the segregation of homozygous *rf2a-R213* plants in families 97 5435–36 and 97 5437.

[b] (F) plants are homozygous for *rf2a* mutants but male fertile.

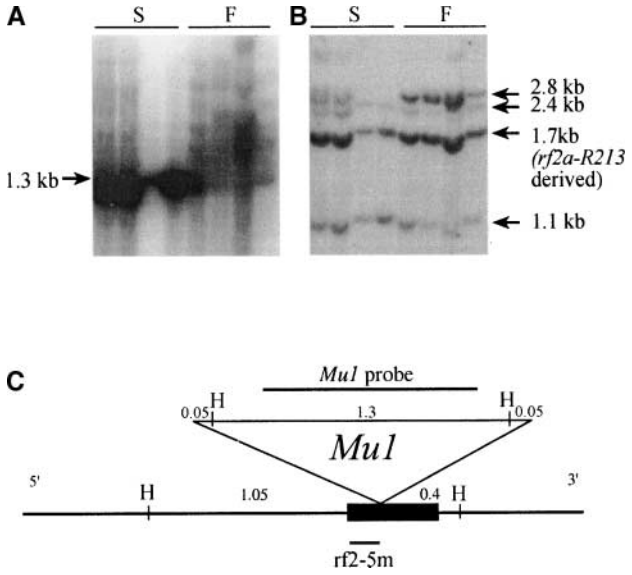

FIGURE 2.—Methylation status in suppressed and nonsuppressed plants with the genotype *rf2a-m9390/rf2a-R213*. DNA isolated from young leaves of plants from families 96 2209 and 96 2214 was digested with *Hin*fI. (A) DNA gel blot was hybridized with the *Mu1*-specific probe illustrated in C. (B) The same blot was stripped and hybridized with the *rf2a*-specific probe rf2-5m (C). (C) The *Mu1* insertion and *Hin*fI sites in the *rf2a-m9390* allele. All plants carry T cytoplasm and their phenotypes are indicated as F, fully male fertile, and S, completely male sterile with no anthers exerted. H, *Hin*fI; distances between restriction sites are indicated in kilobases.

early in the backcrossing program, male-sterile plants homozygous for *rf2a-m9390* were not initially available for mRNA accumulation analyses. To generate unsuppressed stocks homozygous for this allele, some unsuppressed heterozygous plants, (T) *rf2a-m9390/rf2a-R213* from an early generation, were crossed by suppressed (*i.e.*, male fertile) plants with the genotype (T) *rf2a-m9390/rf2a-m9390*. About 300 progeny were screened for male-sterile (*i.e.*, unsuppressed) plants. Only 19 were obtained. When these 19 plants were genotyped, it was found that they all had the genotype *rf2a-m9390/rf2a-R213*; *i.e.*, no male-sterile *rf2a-m9390* homozygous plants were obtained. The reason for this unbalanced result is not known.

Methylation and *Mu* suppression of *rf2a-m9390*: There is a good correlation between the methylation of *Mu*-TIRs and *Mu* activity (reviewed by BENNETZEN 1996). In previously reported studies of *Mu* suppression, hypermethylation of *Mu* transposons throughout the genome was generally correlated with *Mu* suppression, although exceptions were found (MARTIENSSEN *et al.* 1990; LOWE *et al.* 1992). To further test whether the low penetrance of *rf2a-m9390* is due to *Mu* suppression, the methylation status of *Mu1* elements in the genome was determined via DNA gel blot hybridization following *Hin*fI digestion (Figure 2C; CHANDLER and WALBOT 1986). *Mu1* elements with hypomethylated *Hin*fI sites (which are therefore susceptible to restriction digestion) in their TIRs will yield *Mu1*-hybridizing fragments of 1.34 kb. In contrast, *Mu1* elements with methylated *Hin*fI sites will yield larger hybridizing fragments (Figure 2C). Although the *Mu1* probe can also cross-hybridize with *Mu2* transposons, there were few *Mu2* transposons in the families analyzed in this study. The results obtained using genomic DNA isolated from young leaves of plants carrying *rf2a-m9390* are summarized in Table 3. All male-sterile (*i.e.*, not suppressed) and semisterile ("S," partially suppressed) plants in families segregating for recently reactivated *rf2a-m9390* (families 96 2209, 96 2214, and 96 2218) from the reactivation experiment contained mostly hypomethylated *Mu1* elements (Table 3). Of the 13 analyzed fertile (*i.e.*, suppressed) plants, 11 contained completely or mostly methylated *Mu1* elements; the two exceptions contained both methylated and hypomethylated elements. Examples of these analyses are shown in Figure 2A. Generally, fertile (*i.e.*, suppressed) plants exhibited a higher degree of methylation than did sterile (*i.e.*, not suppressed) plants. A chi-square homogeneity test showed that the ratios of 0 methylated:4 hypomethylated male-sterile plants *vs.* the 11 methylated:2 hypomethylated fertile plants are significantly different ($\chi^2 = 9.59$). This result is consistent with the hypothesis that the low penetrance of *rf2a-m9390* is due to *Mu* suppression. Interestingly, plants from a family (family 92 2148) that had just begun to show suppression after outcrossing to inbred lines exhibited a higher level of *Mu* methylation in general (Table 3) than did plants from the reactivated families.

MARTIENSSEN *et al.* (1990) reported that suppression is also correlated with hypermethylation of the *Mu1* transposon inserted in the *hcf106::Mu1* allele. To detect the methylation status of the *Mu1* insertion in *rf2a-m9390*, an *rf2a* probe (rf2-5m), was hybridized to *Hin*fI-digested genomic DNA from the segregating progenies (the results are shown in Table 3). This probe hybridizes to a 1.1-kb fragment bounded by the *Hin*fI site 5′ of the probe in the *rf2a* promoter region and by the *Hin*fI site in the 3′ TIR (*i.e.*, leftmost end in Figure 2C) of the *Mu1* transposon. The methylation status of the *Mu1* inserted in *rf2a-m9390* was scored by determining the signal strength of the 1.1-kb *rf2a*-hybridizing fragment relative to the 2.4- and 2.8-kb *rf2a*-hybridizing fragments. If the 3′ *Hin*fI site in the *Mu1* transposon is susceptible to *Hin*fI digestion (*i.e.*, if it is hypomethylated), a 1.1-kb *rf2a*-hybridizing fragment will be detected (*e.g.*, the lanes of "S" in Figure 2B). If this site is not susceptible to *Hin*fI digestion (*i.e.*, if it is methylated), either the 2.4-kb (the 5′ *Hin*fI site in the *Mu1* transposon is digested) or the 2.8-kb (neither of the two *Hin*fI sites in the *Mu1* transposon is digested) *rf2a*-hybridizing fragment will be detected (*e.g.*, the "F" category in Figure 2B). A strong correlation was observed between the methylation status of the *Mu1* transposon in *rf2a-m9390* and the methylation

TABLE 3

Correlation between male fertility of *rf2a-m9390/rf2a-R213* plants and the methylation status of *Mu1* transposons within their genomes and within *rf2a-m9390*

		No. of plants			
		All *Mu1*		*Mu1* from *rf2a-m9390*[a]	
Families	Phenotypes[b]	Methylated[c]	Hypomethylated[d]	Methylated[e]	Hypomethylated[f]
96 2209	S	0	4	0	3
96 2214	"S"	0	2	0	2
96 2218	"F"	0	0	0	0
	F	11	2	5	2
92 2148	S	1	1	1	1
	"S"	0	2	0	2
	"F"	2	0	2	0
	F	2	0	2	0

[a] Not all plants were subjected to this analysis.
[b] Plant phenotypes: F, fully male fertile; "F," more than one-half of the anthers exerted; "S," some anthers exerted; S, completely male sterile with no anthers exerted.
[c] Plants were scored as "methylated" if they contained *Mu1*-hybridizing fragments >1.34 kb and had no or few 1.34-kb *Mu1*-hybridizing DNA fragments (*e.g.*, see F in Figure 2A).
[d] Plants were scored as "hypomethylated" if prominent 1.34-kb *Mu1*-hybridizing DNA fragments were identified (*e.g.*, see S in Figure 2A).
[e] Plants were scored as "methylated" if no or few *rf2a*-hybridizing 1.1-kb DNA fragments and strong *rf2a*-hybridizing 2.4-kb and/or 2.8-kb DNA fragments were observed (*e.g.*, see F in Figure 2B).
[f] Plants were scored as "hypomethylated" if a strong *rf2a*-hybridizing 1.1-kb DNA fragment and weak 2.4-kb and/or 2.8-kb fragments were observed (*e.g.*, see S in Figure 2B).

status of other *Mu1* transposons in the genome (compare A and B in Figure 2).

Accumulation of *rf2a* mRNA in plants homozygous for suppressed *rf2a-m* alleles: Prior RNA gel blot analysis identified a plant homozygous for *rf2a-m9390* that accumulated *rf2a* mRNA at the same level as wild-type plants (Cui *et al.* 1996). Because the entire young tassel of this plant had been collected for RNA in that study, the male fertility status of this plant could not be determined. To overcome this problem in subsequent experiments, only a few branches of each young tassel were collected from plants homozygous for the *rf2a-m9390* or *rf2a-m8110* alleles. An RNA gel blot analysis of these individuals is shown in Figure 3. Hybridization with a full-length *rf2a* cDNA detected an mRNA that accumulated to the same or only slightly lower levels in fertile plants (*i.e.*, suppressed) that were homozygous for either *rf2a-m8110* or *rf2a-m9390* as accumulated in wild-type controls. In addition, at the level of resolution provided by RNA gel blot analyses, these mRNAs were indistinguishable in size from those that accumulated in the wild-type controls.

Native transcription initiation sites are not used in suppressed *rf2a-m9390* plants: Because the *rf2a-m9390* allele contains a *Mu1* insertion in its 5′ UTR, the *rf2a* transcripts that accumulate in the suppressed *rf2a-m9390* plants (Figure 3) could be derived from alternative transcription initiation sites downstream of the native sites (*i.e.*, in *Mu1* and/or in its flanking region) as has been reported for *hcf106::Mu1* (Barkan and Martienssen 1991). Alternatively, these transcripts could arise via readthrough transcription of the *Mu1* transposon followed by the splicing of the *Mu1* from the transcripts. To distinguish between these two possibilities, a probe (rf2-5m) upstream of the *Mu1* transposon (Figure 1) was hybridized to RNA from suppressed plants homozygous for *rf2a-m9390*. The results of this experiment are shown in Figure 4. Because *rf2a-m9390* transcripts could not be detected with the rf2-5m probe (Figure 4B), it can be concluded that transcription initiation in suppressed *rf2a-m9390* does not occur from the native transcription initiation sites of *rf2a*. Instead, transcription of this allele (Figure 4A) must initiate downstream of the position of probe rf2-5m (Figure 1), likely from the 5′ TIR (*i.e.*, the rightmost TIR in Figure 1) of *Mu1* and/or of an *rf2a* flanking region.

Transcription initiation sites in *rf2a-m9390*: Initially 5′ RACE experiments were conducted on RNA extracted from the inbred line Q67, which is homozygous for the wild-type progenitor of *rf2a-m9390* (Cui *et al.* 1996). The primer (RF2C9) used for the first-strand synthesis was located at position +182 relative to the start of translation and the nested primer (RF2C4) was located at position +37. DNA gel blot analysis revealed that the resulting RACE products were ~290 bp, a result that is consistent with the size of the longest *rf2a* cDNA clone isolated to date (prf273-11), which begins at position −253. Cloned 5′ RACE products obtained using RNA

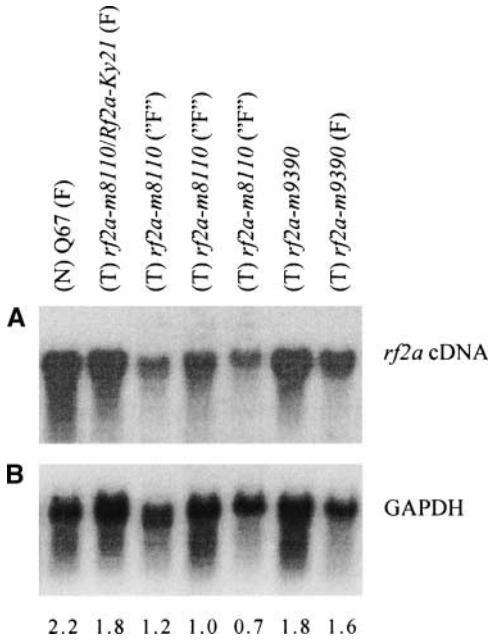

FIGURE 3.—The accumulation of *rf2a* mRNA in many suppressed plants is as high as or only slightly lower than that in wild type. An RNA gel blot containing RNA isolated from young tassels of the indicated genotypes was hybridized with a full-length *rf2a* cDNA probe (A) and the loading control maize GAPDH (B). The density of each band was quantified using a GS-710 densitometer (Bio-Rad Laboratories). The density ratio of each band in A to its corresponding band in B is listed below B. All plants were homozygous for the indicated *rf2a* alleles unless otherwise noted. The inbred line Q67 is homozygous for the wild-type progenitor (*Rf2a-Q67*) of *rf2a-m8110* and *rf2a-m9390*. (T), T cytoplasm; (N), N cytoplasm. The male fertility phenotypes were denoted unless unavailable. F, fertile; "F," semifertile.

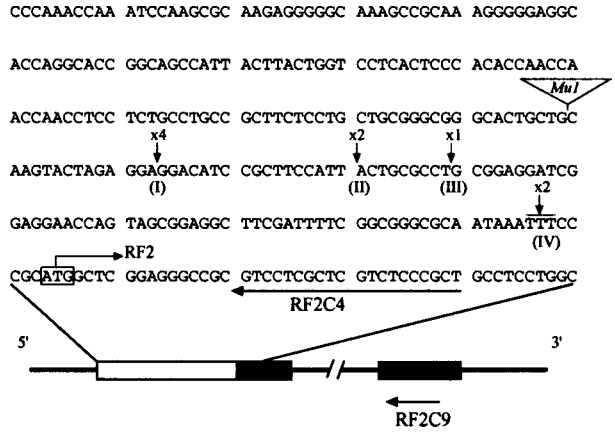

FIGURE 5.—Alternative transcription initiation sites in the suppressed *rf2a-m9390* allele. The open and solid boxes indicate the 5′ UTR and coding regions of exons 1 and 2, respectively. Horizontal arrows indicate the primers used for the 5′ RACE experiments. Sequence is provided for the *rf2a* 5′ UTR and the beginning of the coding region. The translation start codon is boxed. The triangles indicate the position of the *Mu1* insertion in *rf2a-m9390*. The vertical arrows indicate the transcription initiation sites revealed by 5′ RACE using tassel RNA from a male-fertile plant homozygous for the *rf2a-m9390* allele. The number of clones (if there are multiple clones) isolated with each initiation site is indicated above the arrows. Alternative transcription initiation sites are designated by roman numerals.

extracted from a plant homozygous for suppressed *rf2a-m9390* were considerably smaller than those obtained from the inbred line Q67. Sequence analysis of nine of the RACE products from *rf2a-m9390* revealed multiple transcription initiation sites, all of which were located between the position of the *Mu1* insertion (−105) and the translation start codon (Figure 5). This result is similar to what has been observed during suppression of *hcf106::Mu1* (BARKAN and MARTIENSSEN 1991).

Alternative polyadenylation sites in the TIR of *rcy/Mu7* are used during suppression of *rf2a-m8110*: The *rf2a-m8110* allele contains an *rcy/Mu7* insertion in its 3′ UTR. As is true for *rf2a-m9390*, suppressed plants homozygous for *rf2a-m8110* accumulate *rf2a* mRNA that is indistinguishable from wild type by RNA gel blot analyses (Figure 3). On the basis of the analysis of cDNA clones, two *Rf2a* alleles (*Rf2a-B73* and *Rf2a-W22*) use polyadenylation sites >140 bp 3′ of the site of the *rcy/Mu7* insertion in the *rf2a-m8110* allele (Figure 6). In addition, 3′ RACE experiments have established that this is also true for the *Rf2a-Q67* allele, which is the wild-type progenitor of *rf2a-m8110* (Figure 6). These results indicate that the *rf2a-m8110* allele either uses the native polyadenylation sites but with the *rcy/Mu7* transposon subsequently spliced out or uses alternative polyadenylation sites. 3′ RACE was used to identify the polyadenylation sites used by suppressed *rf2a-m8110* and to thereby distinguish between these two possibilities. As shown in Figure 6, five alternative polyadenylation sites were detected in two independent 3′ RACE experiments. Some sites were recovered in both experiments. All identified sites are within the 3′ TIR (*i.e.*, leftmost TIR in Figure 6) of the *rcy/Mu7* transposon insertion in *rf2a-m8110*.

Polyadenylation sites are preferentially located at the 3′ end of a YA (*i.e.*, UA, CA, or GA) sequence in at least

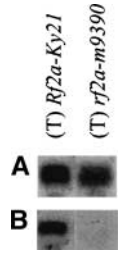

FIGURE 4.—RNA gel blot analysis reveals that the suppressed *rf2a-m9390* allele does not utilize native transcription initiation sites. About 10 μg of RNA from young tassels was loaded in each lane. Full-length *rf2a* cDNA (A) and rf2-5m, a 5′ fragment (see Figure 1) of the *rf2a* gene (B), were used as hybridization probes. All plants were homozygous for the indicated alleles.

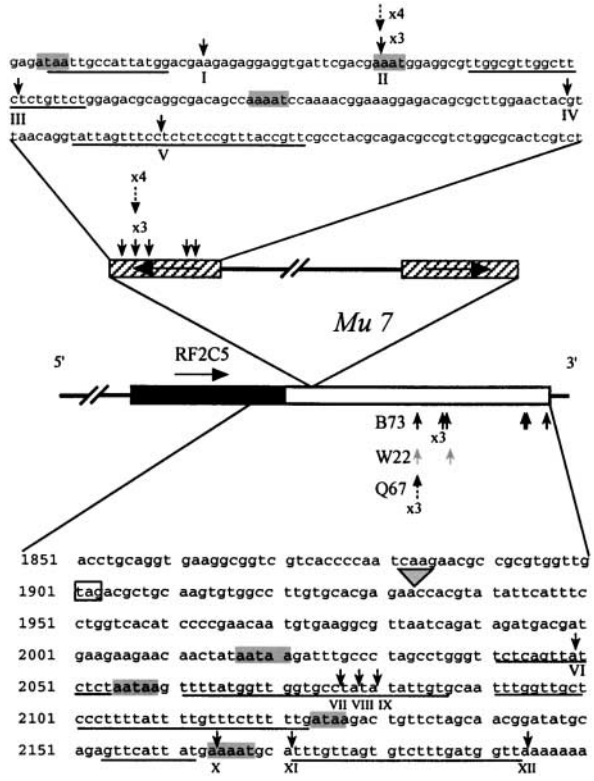

FIGURE 6.—Polyadenylation sites used in suppressed *rf2a-m8110* plants and in wild-type *Rf2a* plants. The solid and open boxes indicate the translated and 3′ UTR regions of the last exon of the *rf2a* gene, respectively. The triangle represents the *rcy/Mu7* transposon insertion responsible for the *rf2a-m8110* mutation. The two hatched boxes containing horizontal arrows represent the TIRs of the *rcy/Mu7* transposon. The vertical arrows above the TIRs indicate the polyadenylation sites used in the suppressed plants as revealed by 3′ RACE experiments. Each arrow represents one event unless otherwise indicated. The solid and dashed arrows indicate the events revealed in the first and the second 3′ RACE experiments, respectively. The primer used in the 3′ RACE experiment is indicated as a horizontal arrow labeled as RF2C5. The *rcy/Mu7* TIR sequence in which the polyadenylation sites were revealed is shown above the transposon. The 3′ UTR sequence of *Rf2a-B73* is shown below. The arrows below the gene structure represent the polyadenylation sites used by the *Rf2a-B73* (solid arrows), *Rf2a-W22* (shaded arrowhead), and *Rf2a-Q67* (dashed arrows) alleles as determined by sequence analysis of cDNA clones (B73 and W22) or 3′ RACE (Q67). Polyadenylation sites are designated by roman numerals. The translation stop codon, TAG, is boxed. The AAUAAA-like PE elements are shaded. U-rich regions flanking polyadenylation sites are underlined.

some eukaryotes (reviewed by ROTHNIE 1996; WAHLE and RUEGSEGGER 1999). To determine whether this pattern holds for maize, the polyadenylation sites associated with *Z. mays* records in GenBank were examined. Fifty-eight records with a total of 94 polyadenylation sites were obtained. Of these 94 sites, 73 fit the YA rule (Table 4). Similarly, 6 of the 7 polyadenylation sites within the 3′ UTR of the *rf2a* gene are YA. In contrast,

TABLE 4

Summary of 94 polyadenylation sites from 58 maize genes in GenBank

	Polyadenylation site[b]				
5′ base[a]	A	U	C	G	Total
A	0	3	0	0	3
U	33	3	4	2	42
C	25	2	2	1	30
G	15	1	2	1	19
Total no.	73	9	8	4	94

[a] Base immediately 5′ of the polyadenylation site.
[b] Polyadenylation sites were determined as (1) The "U," "C," and "G" sites annotated in the GenBank records unless they are followed by an "A," in which case this "A" was assumed to be the polyadenylation site and (2) the "A" sites annotated in GenBank unless they are preceded by an "A," in which case the 5′-most "A" was designated as the polyadenylation site.

only 2 of the 5 alternative polyadenylation sites within *rcy/Mu7* (GA, GA, UC, AC, and CC) fit this pattern. A χ^2 homogeneity test ($\chi^2 = 3.67$) indicated that the rate of YA polyadenylation sites in *rcy/Mu7* at the 95% confidence level is not significantly different from that in other maize genes.

DISCUSSION

***Mu* suppression occurs at a high frequency:** *Mu* transposons are widely used for the functional analysis of the maize genome, including gene tagging, gene discovery, and reverse genetics. It has been known for over a decade that in the absence of *Mu* activity, some *Mu*-insertion alleles lose the capacity to condition a mutant phenotype (MARTIENSSEN *et al.* 1989). This phenomenon, *Mu* suppression, has important implications for the use of *Mu* transposons in understanding maize biology.

Of the thousands of mutant alleles derived from *Mu* stocks, only 12 have been described as suppressible (MARTIENSSEN *et al.* 1990; CHOMET *et al.* 1991; GREENE *et al.* 1994; HU *et al.* 1998; GIRARD and FREELING 2000). These suppressible alleles arose via *Mu* insertion into promoters (3 alleles), 5′ UTRs (5), and introns (4). The low rate at which suppressible alleles have been described probably underestimates the rate at which they arise. This is because many suppressible alleles are likely to have been incorrectly characterized as not transmissible/heritable and were therefore not further analyzed. Consistent with this hypothesis, we found that 65 of 75 *Mu* insertions in the *glossy8* gene were in the promoter or in the 5′ UTR and are therefore potentially suppressible (DIETRICH *et al.* 2002).

In this study a collection of five *Mu*-derived *rf2a* mutant alleles originally obtained via a phenotypic screen

was backcrossed to a non-*Mu* inbred line (Ky21) for at least three generations. Two alleles (*rf2a-m9390* and *rf2a-m8110*) with *Mu* insertions in noncoding regions exhibited low penetrance early in this backcrossing program. A third allele, *rf2a-m9437*, exhibited low penetrance in later generations (data not shown). The low penetrance of these three alleles is not the result of DNA rearrangements and at least *rf2a-m8110* and *rf2a-m9390* can be reactivated by the introduction of *Mu* activity. The *Mu1* transposon in *rf2a-m9390* is methylated in fertile plants, further confirming the relationship between the low penetrance and the absence of *Mu* activity. Furthermore, on the basis of its low penetrance and *Mu* insertion site, *rf2a-m9437* is also likely to be a suppressible allele. Thus three of the five *Mu*-insertion alleles analyzed appear to be *Mu* suppressible. Hence, it is clear that *Mu*-suppressible alleles arise frequently enough to seriously impact the outcome of genetic experiments. In retrospect, suppression probably accounts for our lack of success in identifying a *Mu*-containing DNA fragment that cosegregated with any of the five *Mu*-induced *rf2a* alleles except *rf2a-m8122* (Cui *et al.* 1996). An even greater proportion of suppressible alleles are likely to be recovered by reverse genetic screens (*e.g.*, Dietrich *et al.* 2002), because such alleles would not have been prescreened by phenotypic selection as was true for this collection of *rf2a-m* alleles.

It has previously been established that the insertion of *Mu* transposons into promoter regions, 5′ UTRs, and introns can generate suppressible alleles. By determining the structure of the *Rf2a-B73* allele and the insertion sites of the *Mu* transposons responsible for four of five *rf2a-m* alleles, this study not only identified two suppressible alleles with *Mu* insertions in the 5′ UTR of the *rf2a* gene (*rf2a-m9390* and *rf2a-m9437*), but also for the first time demonstrated that a *Mu* insertion into a 3′ UTR can result in a suppressible allele (*rf2a-m8110*). Only the apparently nonsuppressible *rf2a-m8122* allele arose via a *Mu* insertion in an exon.

The *Rf2a-B73* allele spans >17 kb (Figure 1) and contains many small exons and introns as well as two large introns (the largest of which is >7 kb). It is worth noting that if a reverse genetic screen that depends upon PCR-based detection of *Mu* insertions (Bensen *et al.* 1995) were to be conducted on the *rf2a* gene, it is likely that most of the *rf2a-m* alleles described in this report would have been missed. This is because as a result of the presence of two large introns, only *rf2a* primers from a small portion of the gene can amplify the *rf2a-m* alleles. For example, *Mu*- and *rf2a*-specific (such as RF2C6, Figure 1) primers from the 5′ end of the *rf2a* gene fail to amplify DNA templates from plants that carry *rf2a-m8122* or *rf2a-m8110* (data not shown). Similarly, under all PCR conditions tested, *Mu* and *rf2a* primers from the 3′ end of the gene fail to amplify DNA from plants that carry *rf2a-m9390* and *rf2a-m9437*. Instead, such a reverse genetic screen would most likely identify *Mu* insertions in introns that would be unlikely to confer a mutant phenotype. These results point to the importance of having a gene structure available before performing reverse genetics and/or using a large number of primer pairs when conducting reverse genetic screens in species such as maize, which may have large introns.

Suppressible alleles described previously arose via the insertion of a variety of *Mu* transposons: *Mu1*, *Mu3*, *Mu8*, and *MuDR* deletion derivatives (Barkan and Martienssen 1991; Lowe *et al.* 1992; Greene *et al.* 1994; Hu *et al.* 1998; Girard and Freeling 2000). Three *rf2a-m*-suppressible alleles, *rf2a-m9390*, *rf2a-m8110*, and *rf2a-m9437*, are *Mu1*-, *rcy/Mu7*-, and *Mu10*-induced, respectively. These results indicate that insertions of at least six different classes of *Mu* transposons can generate suppressible alleles.

***Mu* suppression occurs to alleles that have a *Mu* insertion in the 5′ UTR:** Three mutant alleles that arose via *Mu* transposon insertions in 5′ UTRs recruit alternative transcription initiation sites during suppression. The *hcf106::Mu1*, *Lg3-Or422*, and *rf2a-m9390* alleles have *Mu1*, *Mu3*, and *Mu1* insertions at positions −34 (Barkan and Martienssen 1991), −238 (Girard and Freeling 2000), and −105 relative to the presumed translation start codons, respectively. The alternative transcription sites recruited during suppression of *hcf106::Mu1* and *rf2a-m9390* exhibit a significant difference in relation to their *Mu1* insertions. In the suppressed *hcf106::Mu1* allele, alternative transcription sites are recruited both from the downstream TIR of the inserted *Mu1* transposon and from the region of the 5′ UTR that is 3′ of this *Mu1* insertion. In contrast, all of the transcription initiation sites recruited during suppression of *rf2a-m9390* are located 3′ of the *Mu1* insertion; *i.e.*, there is no evidence that this allele recruits transcription initiation sites from within *Mu1*. This difference is not due to the orientation of the transposons; the *Mu1* transposons responsible for the *hcf106::Mu1* allele and the *rf2a-m9390* allele are inserted in the same relative orientation. However, the positions of all these alternative transcription initiation sites are similar relative to the presumed translation start sites: *hcf106::Mu1* uses positions −7 to −80 and *rf2a-m9390* uses positions −6 to −91. This observation suggests that there may be a downstream *cis*-acting signal that interacts with the promoter to determine the locations of transcription initiation sites.

The *rf2a-m9437* allele also arose via a *Mu* insertion in the 5′ UTR. Unlike *rf2a-m9390*, which exhibited evidence of suppression very early in the backcrossing program, *rf2a-m9437* did not begin to exhibit evidence of suppression until after three generations of backcrossing to the inbred line Ky21 (Table 1 and data not shown). Interestingly, the position of the *Mu10* insertion responsible for *rf2a-m9437* (base pair −35 relative to the presumed translation start codon) precludes the

use of all but one of the alternative transcription initiation sites used by *rf2a-m9390*.

***Mu* suppression affects an allele that has a *Mu* insertion in its 3′ UTR:** Analysis of *rf2a-m8110* revealed a novel mechanism of *Mu* suppression. This allele has an *rcy/Mu7* transposon insertion 30 bp downstream of the stop codon in the 3′ UTR. Sequence analysis of cloned 3′ RACE products revealed that during suppression the *rf2a-m8110* allele recruits novel polyadenylation sites from within the TIR of the *rcy/Mu7* transposon. This is the first report that *rcy/Mu7* contains polyadenylation sites. Because the TIRs of the 10 classes of *Mu* transposons are well conserved, analysis of the *rf2a-m8110* allele suggests that the recruitment of alternative polyadenylation sites might be a general mechanism by which mutants containing other classes of *Mu* insertions in their 3′ UTRs could be suppressed. This is similar to the finding that the long terminal repeats of retroviruses can provide polyadenylation sites for human cellular transcripts (Mager *et al.* 1999; Baust *et al.* 2000). Thus, the TIRs of *Mu* transposon may serve as polyadenylation sites for maize genes and thereby contribute to the generation of genetic diversity.

Polyadenylation sites in *Mu1* were identified previously following the analysis of truncated transcripts produced by the *adh1-s3034* allele, which contains a *Mu1* insertion in its first intron (Ortiz and Strommer 1990). However, these alternative polyadenylation sites differ from those in the current report in that they are located in the central (*i.e.*, non-TIR) region and the 3′ TIR of the *Mu1* transposon.

In eukaryotes, polyadenylation occurs following the cleavage of the hnRNA, which produces a 3′ -OH. The positions at which cleavage, and hence polyadenylation, occur are controlled by *cis*-acting elements that are usually located between the stop codon and the cleavage site. In mammals, the polyadenylation signals consist of the AAUAAA positioning element (PE) and the U- or UG-rich downstream element (DE; Wahle and Ruegsegger 1999; Zhao *et al.* 1999). Plant and yeast genes contain AAUAAA-like PEs and U- or UG-rich upstream elements (UEs), which resemble the DEs of mammals (reviewed by Rothnie 1996; Hunt and Messing 1998; Wahle and Ruegsegger 1999). In addition, the polyadenylation sites of rice and Arabidopsis genes are often located in U-rich regions (Graber *et al.* 1999).

Seven polyadenylation sites were detected in *Rf2a* alleles (VI–XII in Figure 6). Each of these sites is 20–50 bases downstream of an AAUAAA-like PE and in the vicinity of a U-rich flanking region (each ~50% U). No obvious UE was detected in the *rf2a* gene. Similarly, all five polyadenylation sites in the TIR of *rcy/Mu7* (I–V in Figure 6) are located in the vicinity of U-rich regions and 20–50 bp downstream of one of the three AAUAAA-like PE elements that exist in this TIR.

The fact that only the TIR polyadenylation sites are used in suppressed *rf2a-m8110* plants indicates either that transcription does not proceed through the *rcy/Mu7* transposon to the native polyadenylation sites or that the TIR sites are preferred over the native sites by the polyadenylation machinery. One hypothesis to explain such a preference would be the presence of an as-yet-unidentified polyadenylation signal upstream of the position of the *rcy/Mu7* insertion. The spatial separation between this upstream signal and the native sites caused by the 2.2-kb *rcy/Mu7* insertion in the *rf2a-m8110* allele might cause such an upstream signal to interact more efficiently with the nearby TIR sites than with the native sites.

In vitro and *in vivo* polyadenylation experiments demonstrated that cleavage occurs preferentially at the 3′ end of a YA (*i.e.*, UA, CA, or GA) sequence (reviewed by Rothnie 1996; Wahle and Ruegsegger 1999). In mammals, this YA consensus was observed in ~70 of 100 polyadenylation sites (Sheets *et al.* 1990). YA was also found to be the most frequent sequence at the polyadenylation sites in the yeast *cyc1* gene (Guo and Sherman 1995) and several plant genes (Wu *et al.* 1995). Our analysis of 94 polyadenylation sites from 58 maize genes confirmed that this rule also holds for maize; 73 of 94 polyadenylation sites fit the YA pattern (Table 4). In contrast, only 2 of 5 polyadenylation sites in the TIR of the *rcy/Mu7* transposon inserted into *rf2a-m8110* follow this rule. In mammals, the preferred bases at the first position of the YA consensus are C ⩾ U > G, where C accounts for ~60% of sites (Sheets *et al.* 1990). In contrast, UA is most common (45%) among maize polyadenylation sites.

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