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Functional Analysis of Deletion Derivatives of the Maize Transposon *MuDR* Delineates Roles for the MURA and MURB Proteins

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ABSTRACT

The regulatory transposon of the *Mutator* system of transposable elements in maize is *MuDR. MuDR* elements produce two transcripts, from genes *mudrA* and *mudrB*, encoding proteins MURA and MURB, respectively. Like many other transposons, *MuDR* elements often undergo deletions, usually of internal sequences. Analysis of a deletion that is restricted to the region encoding MURB demonstrates that this gene is not required to cause excisions of a reporter element, although it may be required for transposition or suppression of suppressible alleles. Conversely, a derivative that lacks the region encoding MURA but that produces MURB is nonfunctional for all aspects of Mutator activity. Northern analysis of these derivatives reveals that each of the two transcripts can be independently transcribed, and analysis using an antibody specific for MURB reveals that *mudrB* transcript can also be successfully translated and its product appropriately localized in the absence of *mudrA.* A third deletion derivative provides evidence for a source of previously reported antisense transcript.

TRANSPOSABLE elements were first identified because of their propensity to introduce variation into the genomes of their hosts. It has become clear from a large number of studies (Berg and Howe 1989) that the activity of transposons can also lead to a great deal of variation in their own genomes as well. In the *Ac/Ds* system of maize, for instance, an autonomous *Ac* element at the *Wx* locus was observed to change into a nonautonomous *Ds* element (McClintock 1963). Subsequent molecular analysis of *Ds* elements demonstrated that this kind of change had resulted from the loss of sequences between the terminal inverted repeats of the originally active *Ac* element (Fedoroff *et al.* 1983; Doring *et al.* 1984; Pohlman *et al.* 1984). Similarly, nonautonomous *dSpm* elements of the *Suppressor-mutator* system in maize are the result of deletions within autonomous *Spm* elements. Over the years several of these deletion derivatives have been characterized (reviewed by Fedoroff 1989). This phenomenon was also observed in Drosophila where, for instance, a large number of deletion derivatives of *P* elements have been identified (O'Hare and Rubin 1983; O'Hare *et al.* 1992; Gloor *et al.* 1993; Rasmusson *et al.* 1993).

In some cases, the altered structure of transposable elements in turn can lead to new kinds of regulation. In the best-studied example of this kind of regulatory evolution, some of the derivatives of *P* elements exhibit a negative effect on the activity of full-length *P* elements (Black *et al.* 1987; Robertson and Engels 1989; Gloor *et al.* 1993; Rasmusson *et al.* 1993). Often those negative effects vary in their severity depending on the chromosomal position of the deletion derivative being examined (Gloor *et al.* 1993). Among plant transposable elements, at least one deletion derivative of the maize *Spm* element encodes a product that can reduce the activity of a full-length version of the element (Cuypers *et al.* 1988).

With respect to the generation of diversity, the *Mutator* system of transposable elements in maize is of particular interest. The *Mutator* system is a diverse family of transposable elements in maize that was originally identified in a line of maize with an exceptionally high mutation rate (Robertson 1978). It is composed of at least five different classes of elements, all of which share homologous terminal inverted repeat sequences (TIRs), but each of which contains completely heterologous internal sequences (see Walbot 1991; Chandler and Hardeman 1992; and Bennetzen 1996 for reviews).

The regulatory transposon for the entire system is a member of the *MuDR* class of elements (Chomet *et al.* 1991; Hershberger *et al.* 1991; Qin *et al.* 1991; James *et al.* 1993). This 4.9-kbp transposon encodes two convergently transcribed transcripts of 2.8 and 1.0 kb, encoded by the genes *mudrA* and *mudrB*, respectively (Hershberger *et al.* 1995). On the basis of limited sequence similarity to bacterial transposases, *mudrA* has been hypothesized to be the transposase (Eisen *et al.* 1994). The presence of *MuDR* is required for all aspects of the

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activity of the entire system of transposable elements, and in all Mu-active lines examined to date both transcripts are present, suggesting that both open reading frames (ORFs) encode proteins necessary for high levels of Mutator activity. Mutator activity is defined as a constellation of behaviors, including high levels of transposition and excision, demethylation of TIRs, and the presence of supercoiled forms of *Mu* (reviewed by Chandler and Hardeman 1992). Active *MuDR* elements may also alter the mutagenic effects of *Mu* transposon insertions near the promoters of genes such as *hcf106* (Martienssen *et al.* 1990), *a1-mum2* (Chomet *et al.* 1991), *knotted-1* (Greene *et al.* 1994), and *Lg3-Or 211* (Fowler *et al.* 1996). In each of these cases expression of a gene is suppressed in the presence of the putative transposase.

The *Mutator* system is the most mutagenic of the known plant transposable element systems; the high frequency of duplicative transposition of multiple elements in an active Mutator maize stock can result in mutation frequencies 50 times that of background (Robertson 1978). Duplication frequencies can approach or even exceed 100%, so that even on outcrossing to non-Mutator stocks the number of elements, including *MuDR* elements, can rapidly increase (Alleman and Freeling 1986; Lisch and Freeling 1994). Perhaps as a consequence of this high transposition rate, *MuDR* elements also undergo frequent internal deletions (Chomet *et al.* 1991; Lisch and Freeling 1994; Hershberger *et al.* 1995; Lisch *et al.* 1995; Hsia and Schnable 1996). These deletions have been observed to occur at various times during development, resulting in either somatic or germinally transmitted events (Chomet *et al.* 1991; Lisch *et al.* 1995; Hsia and Schnable 1996).

Analysis of the structure of a number of deletion derivatives of *MuDR* elements has revealed that the junctions of these derivatives are consistent with a double-stranded gap repair mechanism (Hershberger *et al.* 1995; Hsia and Schnable 1996). Lesions that largely delete *mudrA* eliminate both excision and suppression activity (Chomet *et al.* 1991; Lisch and Freeling 1994; Lisch *et al.* 1995; Hsia and Schnable 1996). In the absence of functional *mudrA*, residues in the termini of *Mu1* elements were methylated, consistent with a lack of activity (Chomet *et al.* 1991). However, these analyses did not reveal how deletions within *MuDR* elements affect expression of each of the two genes encoded by this element, and thus the degree to which these genes are independent of each other in expression or function. Further, most maize lines examined to date carry heterogeneous complements of nonfunctional *MuDR* elements. Although these elements are not expressed in inactive lines, it was not known whether or not they are transcriptionally active in active lines.

Here we describe the structure, expression characteristics, and behavior of three deletion derivatives of *MuDR-1*, the single *MuDR* element resident in our low copy minimal Mutator line. This line is exceptional in that it carries a single functional *MuDR* element (*MuDR-1*) or derivatives of that element, making it possible to examine the expression characteristics and functions of individual deletion derivatives in relative isolation.

MATERIALS AND METHODS

Maize lines: The *a1-mum2* allele was originally isolated by D. S. Robertson and the line containing *a1-mum2* in this study was obtained from S. Dellaporta. The *a1-mum2* allele, which has a *Mu1* insertion in the promoter of the *a1* gene, is Mu-suppressible (Chomet *et al.* 1991). Plants lacking *MuDR-1* express red anthocyanin color, whereas plants carrying *MuDR-1* show small red clonal sectors of tissue on a green background; kernels show small red clonal sectors on a pale yellow background. The suppression of the *a1-mum2* allele is not readily apparent in the aleurone layer of the kernel, so that in the absence of *MuDR-1* the kernel is pale yellow. The *a1-dt sh2* tester line was obtained from B. McClintock. The *a1-dt* allele has a stable, colorless phenotype in the absence of *Dt*. All lines used in this report lacked the *Dt* element and for this reason we simply refer to the *a1-dt* allele as *a1*. Because the *Sh2* gene is tightly linked (0.2 map units) to *a1-dt*, the *sh2* allele serves as a marker for the presence of *a1-dt*. The tester, which lacks Mutator activity, will be referred to as the *a1 sh2* tester. Progeny segregating for the *MuDR-1* or *dMuDR* element(s) were obtained from the following crosses: *+MuDR-1 (or dMuDR); a1-mum2 Sh2/a1 sh2 X a1 sh2/a1 sh2; −MuDR-1, -dMuDR* or *+MuDR-1 (or dMuDR); a1-mum2 Sh2/a1 sh2 X a1-mum2 Sh2/a1 sh2; −MuDR-1, −dMuDR.*

Nucleic acid samples: Maize DNA from leaves of 2-wk-old seedlings was purified according to Cocciolone and Cone (1993). RNA was extracted from immature ears according to Kloeckener-Gruissem *et al.* (1992), with the following modification: after the RNA was precipitated using lithium chloride, the supernatant from the subsequent centrifugation was saved. DNA from that supernatant was precipitated using 1/10 volume sodium acetate and 1 volume 2-propanol. This DNA was used to confirm the condition of the *MuDR* elements in the same samples as were analyzed by RNA gel blots.

DNA and RNA gel blot hybridizations: DNA hybridizations were performed as described in Chomet *et al.* (1991). The DNA blots were washed in 0.2× SSPE, 0.1% SDS at 65°. RNA gel blot hybridizations were performed under the same conditions as the DNA blot hybridizations. However, the RNA blots were washed in 2× SSPE, 0.1% SDS at 65° for 1.5 hr.

Reverse transcriptase-based PCR amplification: For the RT-PCR experiment, ∼10 μg of RNA from various samples was incubated with 1 unit of DNaseI and 5.6 units of RNase inhibitor for 30 min at 37°. Following DNaseI digestion the samples were extracted once with phenol/chloroform, precipitated in 2-propanol, and then resuspended in water to a concentration of ∼1 mg/ml. The resulting RNA solution (2 μg) was added to a reaction containing reverse transcriptase buffer, 10 mm DTT, primer 8f at a concentration of 0.5 mg/ml, dTTP, dATP, dCTP, and dGTP each at a concentration of 10 mm, and 2.8 units of RNase inhibitor to a total volume of 20 μl. This mix was incubated for 10 min at 70°, then put on ice for 5 min. Reverse transcriptase (2 units) was then added, and the reaction was incubated for 2 hr at 37°. The volume was then adjusted to 100 μl using water, and 5 μl of this mixture was used in a PCR reaction using primers 7f and 1r.

PCR primers and amplification conditions: The PCR primers for cDNA synthesis, PCR amplification, and sequencing included the following sequences: 1r = CTCTGCTCCTG TGCGGATGGATTGTCC; 4r = CTGTTTTCTGTGTTGTTG AG; 7f = CACCACAAAAAATTGGATCCC; 8f = CACCAAT AGCTAGAAGAGGTC.

PCR reactions were performed using ~50 ng of genomic DNA or cDNA. The final concentrations of reagents in the 50-μl reactions were as follows: 200 mm dNTP, 1.5 mm $MgCl_2$, 50 mm KCl, 10 mm Tris-HCl (pH 8.3), and 0.4 mm of each primer. Amplification of genomic and cDNA sequences used the following conditions: melting, 94° for 1 min; annealing, 50° for 1 min; extension, 72° for 2 min. Samples were subjected to 35 cycles of amplification.

DNA sequencing and analysis: Gel-isolated RT-PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Chatsworth, CA) after gel electrophoresis, and the resulting DNA was used for direct sequencing. DNA samples were sequenced at the DNA sequencing facility at the University of California at Berkeley using the double-stranded dye termination technique on an ABI sequencer (Applied Biosystems, Foster City, CA). Sequencing of the derivative *d202* was performed using PCR primers 7f, 1r, and 4r as primers in the sequencing reactions.

Immunoblot and immunolocalizations: Immunoblot and immunocalizations were performed as described in Donlin *et al.* (1995). In each case in which a deletion derivative was subjected to these analyses, DNA from tissue samples from the same individual was subjected to DNA gel blot analysis to confirm the presence of the derivative. As a negative control, in each case either a sibling lacking the derivative or a closely related individual that lacked *MuDR* or *dMuDR* elements was also examined using each of the two methodologies.

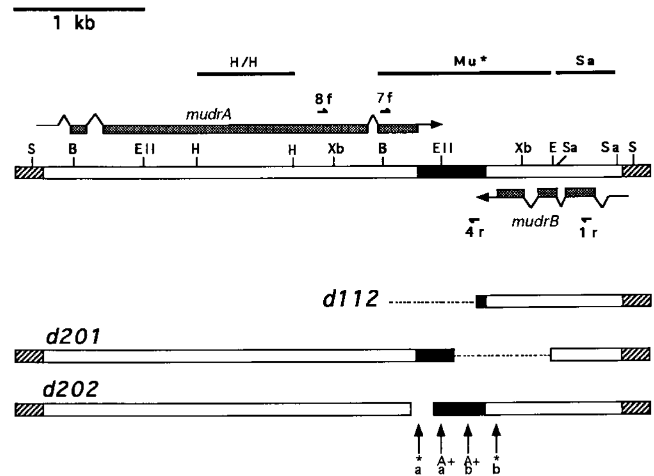

Figure 1.—Restriction mapping or sequencing data of three *dMuDR* elements. In the representation of *d112*, the dashed line represents the region in which the 3′ breakpoint of the deletion is found. In the representation of *d201*, the dashed line represents that region in which a deletion of 500 bp is found. The precise location of the deletion junction of derivative *d202* is as indicated. Transcribed regions *mudrA* (left) and *mudrB* (right) are as indicated (from Hershberger *et al.* 1995). ORFs encoding MURA and MURB are also as indicated. The shaded boxes on the ends represent the terminal inverted repeats. S, *Sac*I; Sa, *Sal*I; E, *Eco*RI; Xb, *Xba*I; EII, *Eco*RII; B, *Bam*HI; H, *Hin*dIII. Solid bars above the restriction map indicate probes referred to in the text. PCR primers, the sequences of which are given in materials and methods, are indicated by small arrows below and above the map. Polyadenylation sites are designated A+; translational stop codons are designated with asterisks. a, *mudrA*; b, *mudrB.*

RESULTS

MURB alone is not sufficient to cause excisions or suppression of a suppressible allele: Restriction mapping revealed that derivative *MuDR-d112* (*d112*) carries only the sequences encoding *mudrB* (Figure 1). This derivative lacked an internal *Sac*I fragment, consistent with the lack of one terminal inverted repeat. An *Eco*RI digest probed with a 5′ flanking probe (not shown) demonstrated that the *Eco*RI site is present in this derivative. The derivative lacked the 5′ *Xba*I site as well as both of the *Bam*HI and *Hin*dIII sites. On the basis of the position of those sites in the flanking region outside of the element (not shown), we conclude that the deletion extends through one TIR to a point between the second *Bam*HI site (which is missing in the derivative) and the 3′ *Xba*I site (which is present). As expected on the basis of the restriction map, the derivative fails to hybridize to probe H/H (data not shown), consistent with the lack of this internal fragment. On the basis of these results, we conclude that derivative *d112* carries a deletion that includes nearly all of the region encoding *mudrA* as well as one of the two TIRs and ~1.5 kbp of flanking sequence (Figure 1). On the basis of restriction mapping data as well as Northern blot analysis (below) we conclude that this deletion does not extend into the region of *MuDR* encoding *mudrB.* Derivative *d112* is at the same chromosomal position as the full-length element first cloned on chromosome *2*L (Chomet *et al.* 1991).

Northern analysis reveals that only the *mudrB* transcript is expressed at detectable levels in individuals carrying this derivative (Figure 2). Immunolocalization also reveals that MURB is successfully translated and localized to the nucleus in the absence of MURA (Figure 3). Further, MURB is most prevalent in actively dividing cells, a pattern of localization that is very similar to that observed previously when this protein was produced from a full-length element (Donlin *et al.* 1995).

Individuals that carry derivative *d112* show no signs of Mutator activity. Kernels carrying *a1-mum2* and this derivative do not exhibit excisions, and plants grown from these kernels are red, consistent with the lack of suppression of *a1-mum2.* On the basis of these observations we conclude that although it can be transcribed, translated, and translocated in the absence of MURA, MURB by itself is not associated with these aspects of Mutator activity.

MURA alone is sufficient to cause excision of a reporter element: Derivative *d201* lacks a large portion of the gene encoding MURB. This derivative contains a deletion of 500 bp of the 861 bp between the *Eco*RI site and the 3′ *Eco*RII site in *MuDR* (Figure 1). Since the element lacks the 3′ *Xba*I site, we conclude that the deletion must remove nucleotides encoding at least 54 amino acid residues from the carboxy-terminal portion of MURB as well as nearly all of the 3′ untranslated portion of the *mudrB* transcript and, at least potentially, a portion of the intergenic region. Derivative *d201* is directly derived from the fully functional element first cloned on chromosome *2*L. However, because *d201* was

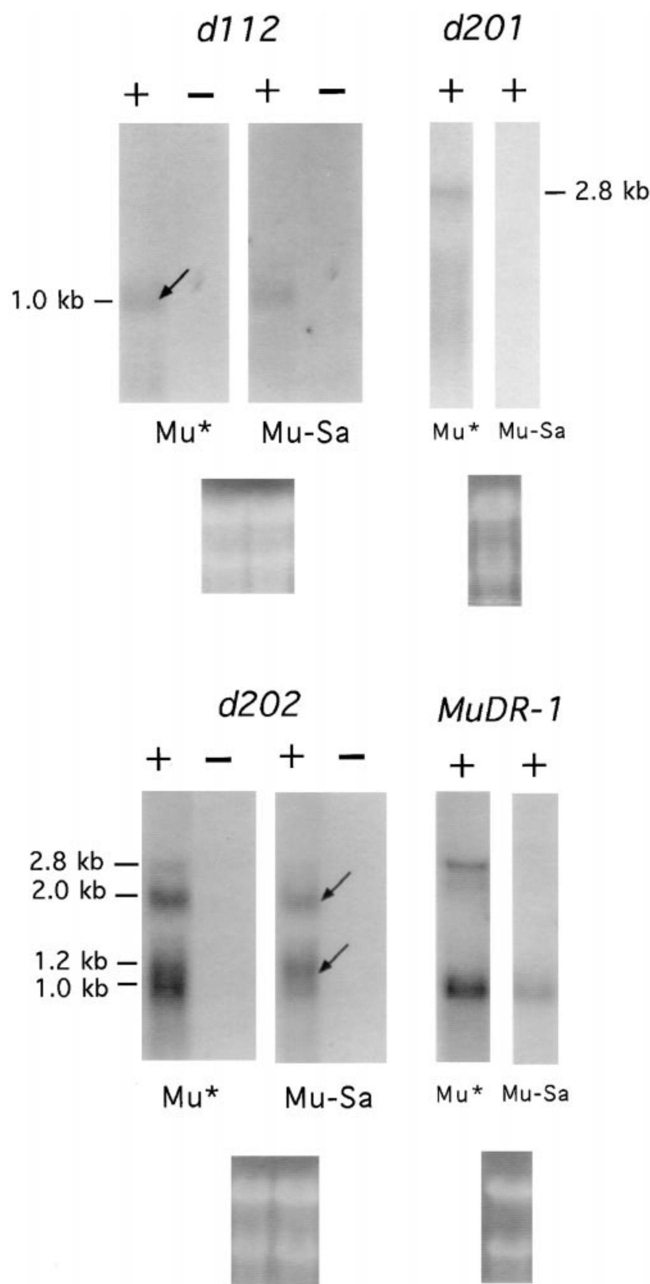

Figure 2.—RNA gel blot analysis of deletion derivatives. When two probes were used, as in the left-hand section of each part, RNA from a plant carrying the indicated element was probed with Mu*. In the right-hand section of these parts the same sample was probed with Mu-Sal. As a loading control, the bottom of each part is a photograph of the ethidium-stained samples before transfer. +, an individual carrying the indicated element; −, a sibling lacking the element.

derived from a transposed copy of that *MuDR* element, it is not located at that chromosomal position. Although some kernels display a *Mu1* excision frequency comparable to that observed in kernels carrying full-length elements, in most cases, kernels carrying *d201* exhibit few or no excisions of a reporter *Mu1* element from *a1-mum2* (Figure 4). This is also the case for the excision of a *Mu7* element from *bz-rcy* (data not shown).

Northern analysis of *d201* demonstrates that although the *mudrA* transcript is present, the *mudrB* transcript is not detectable, indicating that the deleted region in *d201* is required for the production of detectable levels of this transcript (Figure 2). Further, an antibody specific to MURB failed to detect the protein encoded by the small transcript (Figure 5). These data confirm that MURA by itself can cause excisions of a reporter element in the absence of detectable levels of *mudrB* transcript or MURB protein, demonstrating that MURB is not required for this function. They also demonstrate that production of MURA is not dependent on the presence of MURB.

A deletion derivative lacking *mudrB* is unable to prevent methylation and appears to be unable to transpose: *Hin*fI sites within the termini of *Mu* elements are characteristically methylated in the absence of Mutator activity (Bennetzen 1987). To determine the effect of the presence of *d201* on the status of those sites within the *Mu1* element at *a1-mum2*, DNA from plants carrying *d201* and siblings lacking the element were digested with *Hin*fI, blotted, and probed with a fragment of the *A1* gene (Figure 6). Successful digestion of the sites results in a fragment of 0.6 kb. A lack of digestion of both sites results in a 2.1-kbp fragment. Additional fragments present in only a subset of the samples (indicated by "a1-dt") are those associated specifically with the *a1-dt* allele of *A1*, which was segregating in the families examined. In each case examined, the *Mu1* at *a1-mum2* was modified to the same degree in plants carrying *d201* and siblings lacking the element, indicating that the presence of *d201* does not alter the methylation status of this element. This was the case even though the plant containing *d201* depicted in Figure 6 was grown from a kernel exhibiting a high frequency of *Mu1* excisions. This suggests that MURA is unable by itself to prevent modification of nonautonomous elements.

Despite the sometimes-frequent excisions conditioned by *d201*, in no case has this element been observed to transpose in the absence of a full-length *MuDR-1* element. This was the case even when kernels carrying *d201* exhibited a high frequency of excisions of *Mu1* from *a1-mum2*. A total of 45 plants grown from such high excision kernels were test crossed as females to *a1-mum2/a1 sh2* testers. In the resulting ears the ratios of highly spotted to weakly spotted kernels were variable and quite similar to like ratios on the ears from which their parents were picked. Further, none of the resulting ears segregated for significantly more than 50% spotted kernels, suggesting that the element had not transposed (data not shown).

Molecular analysis of plants carrying *d201* gave a similar result; no transposition of the element was observed. In order to assay for changes in copy number of *d201*, families segregating for the element were digested with *Sac*I and *Eco*RI. Duplications of the element were detected as changes in the intensity of the internal 4.2-kbp *Sac*I fragment or in the appearance of unique new

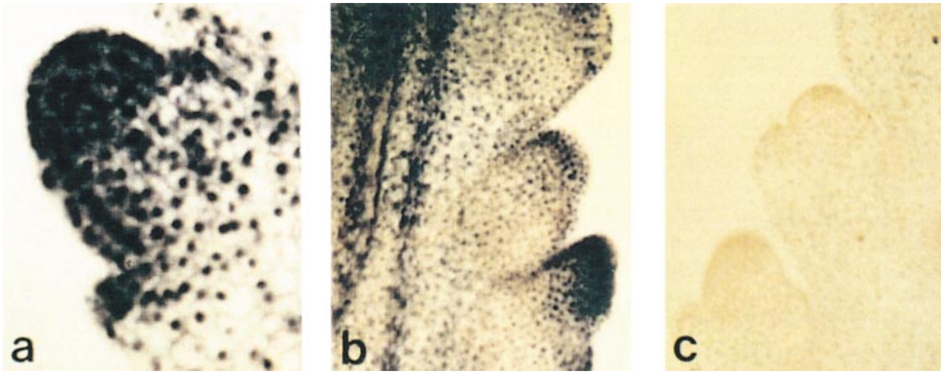

Figure 3.—Immunolocalization of MURB in a developing tassel. (a) Localization in the tassel primordium of a plant that is hemizygous for *d112* in the minimal line. (b) Localization of *d112* in a tassel primordium at lower magnification. (c) Localization in an *a1-mum2/a1 sh2* tester that lacks *MuDR-1* or its derivatives.

*Eco*RI fragments. In the family portrayed in Figure 7, a family generated from a cross between a plant carrying *d201* and an *a1-mum2/a1 sh2* tester was subjected to Southern blot analysis. DNA from plants grown from spotted and pale kernels was extracted, digested with *Sac*I, blotted, and probed with Mu*. As expected, all of the plants grown from spotted kernels carried *d201.* Further, a number of plants grown from nonspotted kernels also carried the element, indicating that *d201* is not always able to cause somatic excision of *Mu1.* DNA from a total of 64 plants carrying *d201* was digested with *Sac*I and probed with Mu*. In all cases the 4.2-kbp internal fragment remained at a relative intensity, consistent with the presence of a single copy of the deletion derivative. Further, *Eco*RI digests of the DNA from 37 of these plants did not reveal the appearance of unique new fragments, consistent with a lack of duplicative transposition of *d201.* Finally, 84 progeny of individuals carrying *d201* and at least one *Mu1* element (the one at *a1-mum2*) that had been crossed to *a1-mum2/a1 sh2* testers were examined by Southern blot for the presence of new *Mu1* fragments. In no case was *Mu1* observed to transpose. In contrast, the full-length *MuDR-1* element at position 1 has been observed to duplicate itself and a *Mu1* element at a frequency of between 10 and 20%, which was itself an exceptionally low transposition frequency for a *MuDR* element (Chomet *et al.* 1991; Lisch *et al.* 1995). These data suggest that *d201* transposes at a frequency that is significantly lower than that reported for *MuDR-1* at position 1 ($P < 0.01$). Indeed, it may be altogether incapable of causing transposition.

Autonomous transposition of a deletion derivative: In contrast to all other derivatives examined to date, one deletion derivative, *d202*, is unique in that it has been observed to transpose autonomously. Like *d201*, derivative *d202*, which was initially identified because of its association with very low frequency excisions, can also condition a more typical *Mu1* excision frequency

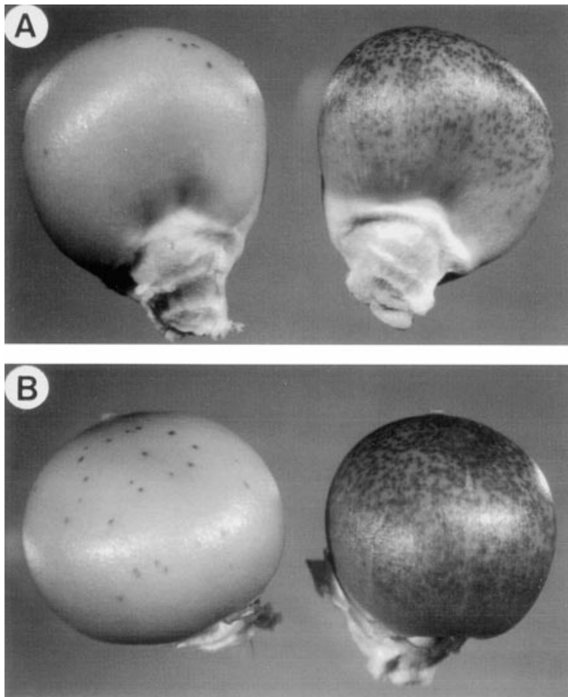

Figure 4.—Examples of the range of kernel excision frequencies of *Mu1* from *a1-mum2* associated with deletion derivatives (A) *d201* and (B) *d202.*

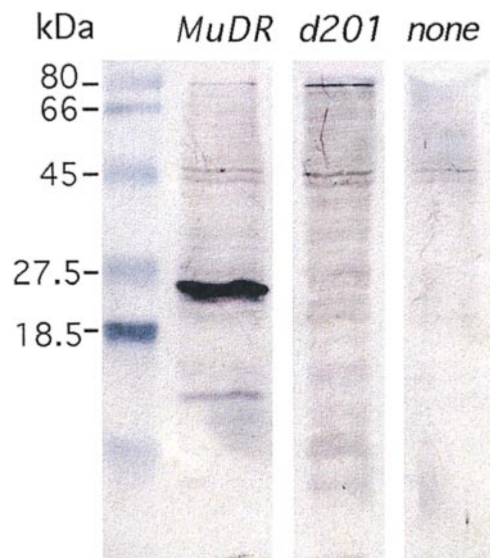

Figure 5.—Immunoblot analysis of derivative *dMuDR-201* in the minimal line. Lane 1, a plant carrying *MuDR-1.* Lane 2, a plant carrying *dMuDR-201.* Lane 3, a sibling of the plant analyzed in lane 2 that carries no *MuDR* or *dMuDR* derivatives.

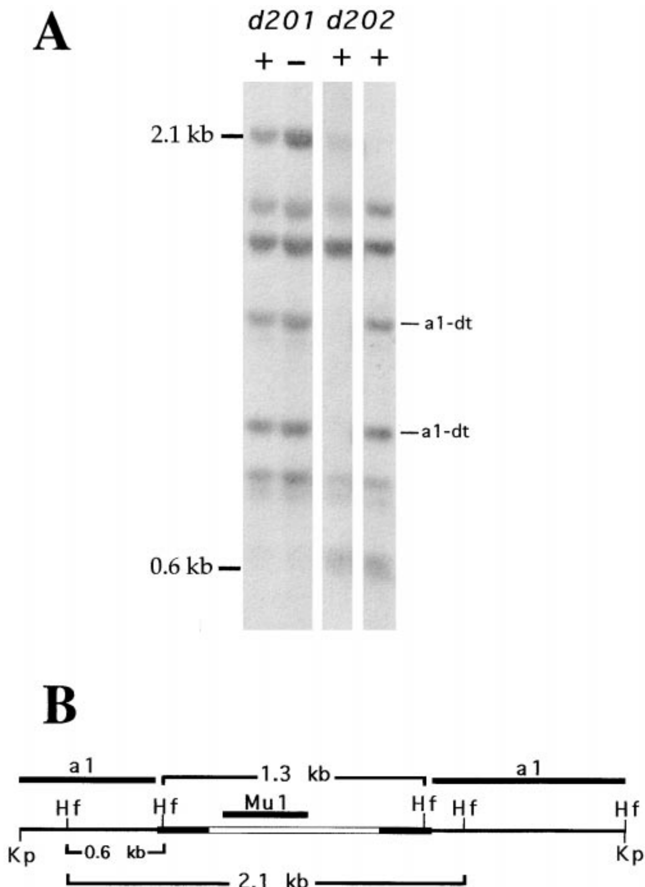

Figure 6.—(A) DNA gel blot of *Hin*fI digests of DNA from plants carrying deletion derivatives *d201* and *d202.* The blot was probed with a *Kpn*I fragment from the *A1* gene. Lane 1, *d201* in a plant grown from a heavily spotted kernel. Lane 2, a sibling lacking *d201.* Lanes 3 and 4, two plants grown from heavily spotted kernels carrying *d202.* The fragments labeled a1-dt are those associated specifically with the *a1-dt* allele of *A1*, which was segregating in these families. (B) A restriction map of *a1-mum2.* The bars above the map indicate probes used in the analysis. K, *Kpn*I; Hf, *Hin*fI.

(Figure 4). The same was true when this element was combined with *bz-mum9*, which carries a *Mu1* element in the *bronze* gene (data not shown). Sequence analysis (see below) revealed that this derivative carries a deletion of 174 bp between and including residues 3094 and 3267 (Figure 1).

To determine if *d202* could transpose, plants carrying the element were digested with *Hin*dIII and *Sac*I. A transposition event would be expected to result in the presence of a new, unique *Hin*dIII fragment and an increased intensity of the diagnostic 4.52-kbp *Sac*I fragment. Figure 8 shows a pair of DNA gel blots from individuals carrying *d202.* The DNA was digested with *Hin*dIII (Figure 8A) and with *Sac*I (Figure 8B) and the resulting blot was hybridized to Mu*. The *Hin*dIII digests (Figure 8A) revealed the presence of a new *MuDR-1*-homologous fragment in DNA from one of these plants (indicated). Densitometric analysis of the *Sac*I

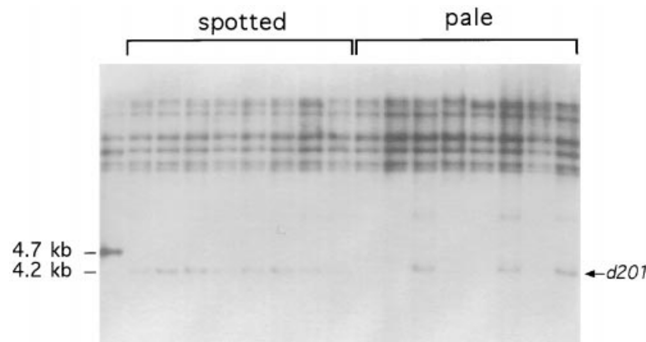

Figure 7.—DNA gel blot of a family segregating for derivative *d201.* The first lane represents a related individual carrying two copies of *MuDR-1.*

digests of DNA from that plant revealed that the diagnostic 4.52-kbp fragment exhibited a twofold increase in intensity, consistent with the duplication of *d202* in this individual. When this plant was test crossed to an *a1 sh2/a1-mum2* tester, ~3/4 (99/120) of the progeny kernels carrying *a1-mum2* were spotted, consistent with the independent segregation of two *d202* elements.

Deletions can result in the production of antisense message: Northern analysis demonstrated that several transcripts were associated with *d202* (Figure 2). Two aberrant transcripts were visible, one 1.2 kbp in length and the other 2.0 kbp. Both aberrant transcripts hybridized to probes from regions encoding each of the two ORFs (Figure 1, probes Mu-Sa and Mu*). Because the two transcripts are produced in opposite directions and from opposite strands, the presence of a 2.0-kbp tran-

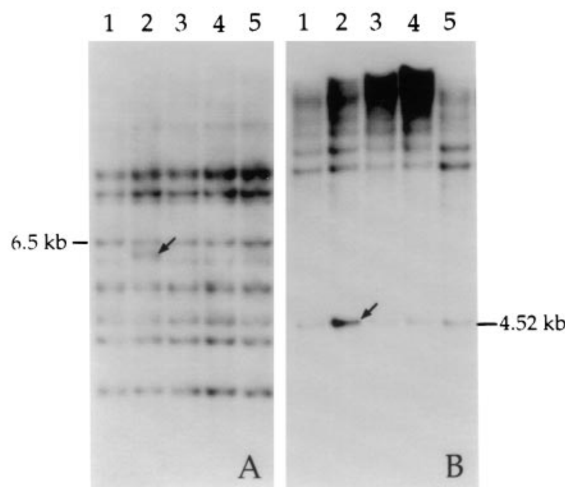

Figure 8.—DNA gel blot of (A) *Hin*dIII and (B) *Sac*I (B) digests of DNA from plants carrying *d202.* In both cases the blots were hybridized to Mu*. In A, the 6.5-kbp fragment is that previously identified as *d202* at the position at which it was first identified. The arrow indicates the presence of a new *MuDR-1*-homologous fragment in lane 2. In B, the 4.52-kbp *Sac*I fragment is the size expected for *d202.* The arrow indicates the fragment in lane 2 that is twice as intense as the same fragment in the other lanes.

script homologous to regions encoding both genes was consistent with the production of a hybrid transcript containing the sense version of one transcript and the antisense version of the other transcript. To test this hypothesis, reverse transcriptase-based PCR (RT-PCR) was used to specifically amplify the transcript expected to be produced if transcription were initiated from the *mudrB* promoter and continued through the intergenic region and into sequences normally expected to encode *mudrA*. This aberrant transcript would be expected to contain sequences identical to the normal *mudrB* transcript and sequences that are complementary to the 3′ end of the normal *mudrA* transcript.

To specifically amplify antisense product, both RNA and DNA from individuals carrying *d202*, and from siblings that lacked the derivative, were isolated. RNA and DNA from an individual carrying a full-length copy of *MuDR-1* were also isolated. The RNA from each of these samples was digested with DNaseI, and DNA was synthesized from the RNA samples using primer 8f (Figure 1) to direct an initial round of first-strand cDNA synthesis using reverse transcriptase. Because this initial round of DNA synthesis used a primer that was identical to a sequence in the normal *mudrA* transcript, it would only be expected to result in a product if a transcript complementary to the normal transcript were present. The resulting product was subjected to PCR amplification using a nested primer (primer 7f, Figure 1) identical to a sequence in normal *mudrA* transcript and a second primer that is complementary to a sequence in the normal *mudrB* transcript (primer 1r, Figure 1). Controls included individual siblings that lacked the derivative as well as closely related individuals that carried only a full-length *MuDR* element. Each sample was also amplified either with or without DNaseI digestion.

Given that there are a number of *MuDR*-homologous sequences in this genetic background, we expected to see some amplification from all DNA samples not subjected to DNaseI digestion. As expected, in those samples a number of fragments resulted from our PCR amplification (Figure 9, lanes 3, 6, 9, and 11). Among these fragments, one was unique to the DNA sample from the individual carrying *d202* (lane 3, arrow). This fragment was missing in a sibling lacking the derivative (lane 6) and in a related individual carrying a full-length element (lane 9); its size was that expected for a fragment amplified from the deletion derivative (∼1500 bp). The fragment unique to the individual carrying the full-length *MuDR-1* element (lane 9, arrow) was of the size (∼1700 bp) expected for the amplification of this region from a full-length element.

To demonstrate that DNaseI digestion was complete and that our PCR reactions were not simply amplifying residual DNA, we amplified DNA samples that had been subjected to DNaseI digestion but had not undergone a round of cDNA synthesis. No product was amplified from these samples (lanes 2, 5, and 8). Similarly, when

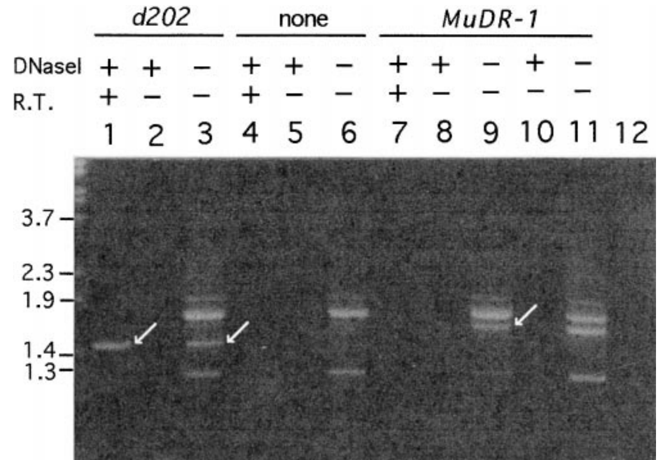

Figure 9.—RT-PCR analysis of transcript associated with *d202*. Lane 1, RNA from a plant carrying *d202*, digested with DNaseI, reverse transcribed, and then PCR amplified. Lane 2, the same RNA sample digested with DNaseI, *not* reverse transcribed, and then PCR amplified. Lane 3, DNA from the same plant amplified using the same PCR primers. Lane 4, RNA from a sibling that lacked the deletion derivative digested with DNaseI, reverse transcribed, and then PCR amplified. Lane 5, RNA from the same plant as is in lane 4 digested with DNaseI, *not* reverse transcribed, and then PCR amplified. Lane 6, DNA from the same plant PCR amplified. Lane 7, RNA from a plant carrying *MuDR-1(p1)* digested with DNaseI, reverse transcribed, and PCR amplified. Lane 8, the same sample digested with DNaseI, *not* reverse transcribed, and then PCR amplified. Lane 9, DNA from the same plant PCR amplified. Lane 10, DNA from a different plant also carrying *MuDR-1(p1)* digested with DNaseI and PCR amplified. Lane 11, DNA from the same plant as in lane 10 *not* digested with DNaseI and then PCR amplified. Lane 12, a water-only PCR amplification negative control for lanes 1–11.

the same PCR reactions were performed on independently derived samples of *MuDR-1*-containing genomic DNA that had also been subjected to DNaseI treatment, no product was amplified (lane 10).

Most importantly, of the RNA samples that had undergone DNaseI digestion, cDNA synthesis, and PCR amplification, only the one from the individual carrying *d202* yielded a product (Figure 9, lane 1, arrow). That product was the size predicted if amplification was of the aberrant transcript associated with the deletion derivative.

To more precisely determine the nature of this aberrant transcript, the PCR product produced from the aberrant transcript associated with *d202* was partially sequenced. The resulting sequence was identical to that reported for *MuDR* (Hershberger *et al.* 1991, 1995), with the exception of a deletion of 174 bp between and including residues 3094 and 3267. Because amplification was successful using primer 1r, we conclude that the transcript must extend at least as far as residue 4494 in the 3′ direction. As has been observed in a number of other deletion derivatives, the junction of the deletion includes one copy of a short, direct repeat (AAGAA in this case) found in the original sequence. Surprisingly,

the deletion also removed the last 17 predicted amino acids of MURA as well as the normal translational stop codon. The derivative retained the normal *mudrA* polyadenylation sites, most of the 3′ untranslated region, and the entire intergenic region (Hershberger *et al.* 1995).

This 2.0-kbp aberrant transcript included both the second and the third intron from *mudrB*, suggesting that neither of these introns is efficiently spliced from the aberrant transcript. In contrast, although the third intron of the normal *mudrB* transcript is only rarely spliced (<10%) from full-length elements, the second intron is normally spliced with high efficiency (Hershberger *et al.* 1995).

On the basis of this analysis, it appears that the larger of the two aberrant transcripts associated with *d202* contains nucleotide sequences that are identical to the 3′ end of *mudrA* and sequences that are complementary to at least part of *mudrB*, presumably due to transcription initiating from the promoter associated with *mudrB* and proceeding past its normal transcriptional stop signals into the region encoding the large transcript. As a consequence of this read-through, *d202* produces an antisense *mudrA* mRNA. This derivative is active by both the excision (Figure 4) and the transposition assays (Figure 8). However, as is apparent in Figure 4, excisions driven by *d202* were often less frequent and consistently more variable than those of *d202*'s progenitor, *MuDR-1.* Finally, in contrast to the results obtained for *d201*, *Mu1* at *a1-mum2* is incompletely hypomethylated in the presence of *d202*, even in DNA from plants grown from heavily spotted kernels (Figure 6). This is most apparent when the plant carrying *d202* was homozygous for *a1-mum2.*

DISCUSSION

Specific deletion derivatives are associated with specific transcripts: Although a large number of derivatives of *MuDR* have now been reported (Chomet *et al.* 1991; Hershberger *et al.* 1995; Lisch *et al.* 1995; Hsia and Schnable 1996), the minimal line in which the above elements have been described provides some unique advantages. First and most importantly, the only transcripts observed in this line are those associated with a single previously cloned *MuDR* element or its derivatives (Figure 2). Thus, for the first time it is possible to associate specific transcripts with specific deletion derivatives and to ascribe specific roles to those transcripts. In addition, the use of the minimal line makes it possible to determine the source of aberrant transcripts, such as truncated or read-through messages. In more complex Mutator backgrounds, expression analysis of deletion derivatives has not been possible because of the presence of large numbers of additional, transcriptionally active elements. Finally, the minimal line used in these experiments is also well inbred (>10 generations) and so variations in various aspects of Mutator activity, such as excision frequency or suppression of reporter element, are most likely due to changes in the *Mutator* system itself rather than background effects.

MURB is not by itself associated with Mutator activity: Previous work (Lisch and Freeling 1994; Hsia and Schnable 1996) had demonstrated that deletions within the region encoding *mudrA* eliminated excision and suppression function, but it was not known how these deletions effected transcription from *MuDR.* The deletion in *d112* extends through all of *mudrA*, but *mudrB* remains intact. Transcript analysis revealed the presence of a 1.0-kbp transcript that hybridizes with a *mudrB*-specific probe and that cosegregates with this derivative (Figure 2). Immunoblot analysis revealed that this transcript is translated and the protein is translocated to the nucleus primarily in mitotically active cells (Figure 3). These data demonstrate that expression of MURB is largely independent of MURA.

Because *d112* is not associated with either excision of *Mu1* from *a1-mum2* or suppression of this suppressible allele, it does not appear that MURB is sufficient by itself to condition any aspect of Mutator activity. Of course, as we have not sequenced the entire derivative, we cannot say for certain that the small protein associated with *d112* is not altered in some subtle way that has destroyed its normal function. However, we note that additional derivatives that carry large deletions in the regions encoding *mudrA*, but that can produce large quantities of *mudrB* transcript, are also incapable of causing excisions of reporter elements (D. Lisch, unpublished results; Lisch and Freeling 1994; Hsia and Schnable 1996), lending weight to the argument that MURB is not by itself capable of conditioning these events.

MURA is sufficient to cause excisions of a reporter element: Deletion derivative *d201* is missing a large portion of *mudrB.* Although it contains a portion of the region encoding *mudrB*, including the promoter and all of the first exon, at a minimum, it lacks sequences including the third exon, the termination codon, and it may lack sites that are normally polyadenylated (Hershberger *et al.* 1995) as well as a portion of the intergenic region. Derivative *d201* appears to produce only *mudrA* transcript and is not associated with detectable amounts of *mudrB* transcript (Figure 2) or MURB (Figure 3). Our Northern blot analysis used total RNA, so there is the possibility that very small quantities of RNA are still produced. However, the lack of detectable truncated *mudrB* transcript suggests that in the absence of the deleted sequences *mudrB* transcript is produced in very small (or nonexistent) quantities compared to the normal level of expression. Alternatively, the expected truncated transcript may be unstable. Given the continued presence of the appropriate promoter elements in the Mu termini, the latter explanation is perhaps more likely. In either event, the net result is the lack of detectable MURB protein.

The observation that *d201* is capable of causing excisions of *Mu1*, sometimes at a frequency approaching

that associated with full-length elements (Figure 4), demonstrates that MURB is not required for excision of *Mu1* elements. However, although it is capable of conditioning a high frequency of *Mu1* excisions, *d201* is not associated with a high or even moderate frequency of new insertions. An attractive explanation for this observation is that MURB is required for reinsertion but not excision of *Mu* elements. Because these data represent a negative result that may reflect only a reduced frequency of these events, it is possible to surmise only that *d201* is significantly less efficient than a full-length *MuDR-1* element at conditioning new insertions. Further, because *d201* is at a new position, we cannot exclude the possibility that position effects are playing a role in the observed variations in the ability of this element to cause excisions of *Mu1* (Figure 4).

In connection with our observation that MURA has independent activity, it is interesting to note that although genome database searches using *mudrA* sequences detect similar sequences in Arabidopsis and rice, there are no known sequences that share significant similarities to *mudrB* (D. Lisch, unpublished results). As the functional analysis of these sequences has not been performed, it is not known if these represent active elements. However, sequences previously identified as being similar to bacterial transposases are also conserved in the elements from Arabidopsis and rice, as is a conserved zinc finger domain, indicating functional similarities to *mudrA* (D. Lisch, unpublished results). It may be that *mudrA* and *mudrB* evolved independently and that *mudrB* function is specific in some way to the requirements of the *Mutator* system in maize. Alternatively, *mudrB* may have been lost due to internal deletions in these other species, or *mudrB* sequences simply may have diverged more due to less stringent selection for their preservation. It will be interesting to determine whether or not the elements in these heterologous species have full transpositional activity in the absence of *mudrB.*

The large variations in *Mu1* excision frequency associated with *d201* are as yet unexplained. The variation is quite sporadic; it does not segregate in a manner consistent with the presence of a few modifying loci, nor are high-frequency excisions heritable (D. Lisch, unpublished results). Alternatively, it may be that *d201* is subject to some degree of epigenetic modification, and that variations in epigenetic modification of the element could explain the observed variations in excision frequency of *Mu1.* We also cannot exclude the possibility that there are additional factors in our minimal line that modify the activity of *d201.* We believe this is unlikely, however, given the extremely inbred nature of the line, as well as the absence of evidence for genetic segregation of variations in excision frequency. Regardless of the excision frequency associated with it, *d201*, like *d112*, is not associated with suppression of *a1-mum2*, suggesting that MURB may be necessary but not sufficient for suppression function. Nor is *d201* associated with hypomethylation of *Mu1* at *a1-mum2* (Figure 6), suggesting some role for MURB in that process. Previous work has demonstrated that deletions within *mudrA* are associated with hypermethylation of *Mu1* elements, suggesting that MURB is not sufficient to mediate hypomethylation of nonautonomous elements (Lisch *et al.* 1995).

A deletion can result in the production of antisense message: Our analysis of the transcripts associated with *d202* demonstrates one mechanism by which the previously reported *MuDR* antisense transcript can be produced (Hershberger *et al.* 1995; Joanin *et al.* 1997). Four distinct transcripts are produced by this derivative: two transcripts characteristic of the full-length element and two aberrant transcripts (Figure 2). The larger aberrant transcript is composed of both sense *mudrB* and antisense *mudrA* sequences. Surprisingly, read-through from the *mudrB* promoter occurs despite the fact that the deletion is in the region containing stop signals not for *mudrB*, but for *mudrA.* Despite this, the *mudrA* transcript is present and is of the correct size (Figure 2), and it is transcription from the *mudrB* promoter that appears to extend past its normal stop signals. This result suggests that there are sequences in the transcribed 3′ region of *mudrA* that are necessary for the normal transcription of *mudrB.*

The region between the translational stop signals in the two normal transcripts is highly repetitive, and has a high potential for secondary structure (Hershberger *et al.* 1995). It may be that in *d202* disruption of this secondary structure has effected the normal termination of transcription of *mudrB.* Whatever the cause, the result is a read-through into *mudrA* and thus the production of antisense message.

Antisense message has been detected before in Mutator lines (Hershberger *et al.* 1995). Here a particular antisense message is linked with a particular *Mu* element. All previously reported antisense messages were detected in lineages with multiple *MuDR* and *dMuDR* elements. In our minimal line this antisense message was not detected in individuals carrying only *MuDR-1*, suggesting that expression of antisense message (at least that complementary to primer 8f) is not necessarily associated with the normal function of full-length *MuDR* elements in the tissues analyzed.

Because the antisense message is at least potentially capable of interfering with the normal sense message, it has been suggested that the presence of antisense message may be indicative of a negative regulatory pathway (Hershberger *et al.* 1995; Joanin *et al.* 1997). Because the excision frequency associated with *d202* varies considerably (Figure 4B), and it appears that both normal and antisense transcripts are produced by this derivative (Figure 2), an intriguing possibility is that the observed variation may be due to changes in the relative quantities of normal *vs.* antisense transcript. If this were the case, then our expectation would be that the relative quantity of the antisense version of the transcript would

be higher in those aleurones that show lower frequencies of *Mu1* excisions. However, analysis would be complicated if the presence of antisense transcript resulted in post-transcriptional silencing of the element.

Alternatively, variations in excision frequency associated with *d202* could be a result of direct changes in MURA. The deletion includes the last 17 predicted amino acids of MURA as well as the normal translational stop codon. Some of the residues that are lost are similar to nuclear localization signals in other plant genes (Hicks *et al.* 1995). Thus, reductions in excision frequencies associated with *d202* may be the result of reductions in the effective quantity of MURA within the nucleus. Changes in copy number of *d202*, as can be observed in Figure 8, could result in increased overall levels of protein that could compensate for a reduced efficiency of translocation. Indeed, the progeny of the individual carrying the transposed copy of *d202* showed a marked increase in excision frequency (data not shown). This hypothesis would suggest that this derivative should show a marked decrease in the extent of nuclear localization of MURA compared with a full-length element and that changes in excision frequency should be accompanied by quantitative changes in the level of MURA protein. The recent isolation of MURA protein in yeast (Benito and Walbot 1997) should make it possible to test these hypotheses directly.

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