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The Maize Transposable *Ds1* Element Is Alternatively Spliced from Exon Sequences

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The null *wx-m1* allele contains a 409-bp Dissociation 1 (*Ds1*) element in exon 9 of the maize waxy (*Wx*) gene. In the absence of the autonomous Activator (*Ac*) element, the *Ds1* element cannot transpose, and this allele encodes several *Wx* transcripts that arise following alternative splicing of *Ds1* sequences from *Wx* pre-mRNA. Splicing involves the utilization of three 5′ splice sites and three 3′ splice sites. All but one of these splice sites are in *Ds1* sequences near the ends of the element. The presence of 5′ and 3′ splice sites near the *Ds1* termini and the element's small size and AT richness are features that distinguish *Ds1* elements from all other known *Ds* elements. It is suggested that these features may enhance the ability of *Ds1* to function as a mobile intron.

The insertion of maize transposable elements into genes does not always abolish gene expression. Certain Dissociation (*Ds*)- and defective-suppressor-mutator (*dSpm*)-containing alleles of the maize alcohol dehydrogenase 1 (*Adh1-Fm335*), waxy (*wx-m9*), and bronze (*bz-m13*, *bz-m13CS9*) genes display a stable intermediate or nonmutant phenotype in strains in which these elements cannot transpose, that is, in the absence of the autonomous Activator (*Ac*) or *Spm* element (16, 19, 21, 24). In addition to these examples, the null *Ds* alleles *wx-B4* and *Adh1-2F11* encode non-mutant-size *Wx* or *Adh1* transcripts (5, 32). Surprisingly, molecular cloning and DNA sequence analysis revealed that these leaky and null alleles contain exon insertions and that all, except *Adh1-Fm335*, contain insertions in translated exons (5, 6, 24, 27, 32).

To understand how genes containing large transposable elements in translated exons can encode functional products, wild-type-size products or both, we have focused on several *Ds* alleles of the waxy (*Wx*) gene. *Wx* encodes a starch granule-bound ADP-glucose glucosyl transferase that is responsible for amylose biosynthesis in the endosperm and pollen (20). The leaky *wx-m9* allele and the null *wx-B4* allele encode non-mutant-size *Wx* mRNAs, despite the presence of 4.3- and 1.4-kb *Ds* elements, respectively, in translated exons (32). For both alleles, the *Ds* elements can be alternatively spliced from pre-mRNAs if one of three 5′ splice sites clustered within 20 bp near the *Ds* terminus is joined to a 3′ splice site within the *Wx* gene adjacent to the *Ds* element (32). None of the resultant *Wx* mRNAs are wild type, because part of the *Ds* terminus remains in the mature message.

The splicing of the *Ds* insertion of the *wx-m1* allele is the focus of this report. Like *wx-B4*, this allele is null in the absence of *Ac* (14). The *Ds* insertion in *wx-m1* is in exon 9, is 409 bp in length, and is a *Ds1*-type element (33). Unlike the 4.3-kb *Ds* of *wx-m9* and the 1.4-kb *Ds* of *wx-B4*, which are deletion derivatives of the *Ac* element (6, 29), *Ds1* elements share only about 40 bp of homology with *Ac* sequences (22, 27). This 40 bp includes the 11-bp inverted repeats and most of the 20 bp adjacent to one terminus that contains the cluster of 5′ splice sites involved in *Ds* processing. Dennis et al. (4) demonstrated that *Ds1* elements can function as introns when they showed that the *Ds1* element of *Adh1-Fm335* is spliced from the untranslated leader of *Adh1*. However, they could not precisely determine the 5′ and 3′ splice sites or whether the *Ds1* element is alternatively spliced from pre-mRNA in a manner similar to that of the *Ac*-like *Ds* elements.

This study presents evidence that the *wx-m1* allele encodes multiple transcripts that arise following alternative splicing of the *Ds1* insertion from pre-mRNA. The results indicate that despite the fact that *Ds1* and *Ac*-like *Ds* elements have only 40 bp of sequence homology, both classes of elements can be spliced in a similar manner. In addition, the *wx-m1 Ds* element can be alternatively spliced in a novel manner involving both 5′ and 3′ splice sites within element sequences.

MATERIALS AND METHODS

Maize strains. The *wx-m1* mutation was originally isolated by McClintock (14) and obtained for this study from Drew Schwartz. Nonmutant and *wx-m1* RNAs were isolated from strains homozygous for these alleles and also lacking active *Ac* elements in their genomes.

RNA preparation and blot analysis. Total and poly(A)$^+$ RNA was purified from the dissected endosperms of kernels harvested 18 to 21 days after pollination (26). For Northern (RNA) blot analysis, poly(A)$^+$ RNA samples were denatured at 65°C for 30 min in 2.2 M formaldehyde–50% (vol/vol) deionized formamide–5 mM $NaPO_4$ (pH 7.0), electrophoresed through 1% agarose containing 2.2 M formaldehyde, 0.2 M 3-(*N*-morpholino)propanesulfonic acid (MOPS), and 50 mM sodium acetate, and transferred to nitrocellulose as described by Thomas (28). Autoradiography was for 24 h with an intensifying screen.

PCR amplification and DNA sequencing. Three micrograms of poly(A)$^+$ RNA was used as substrate for single-stranded cDNA synthesis primed by oligo(dT) (8). Approximately 1 ng of cDNA and 350 ng of each primer were incubated in polymerase chain reaction (PCR) buffer (16.6 mM NH_4SO_4, 67 mM Tris [pH 8.8], 6.7 mM $MgCl_2 \cdot 6H_2O$, 6.7 mM EDTA) with 200 μM each deoxynucleoside triphosphate at 95°C for 5 min, and then 2.5 U of *Taq* polymerase (Perkin-Elmer-Cetus) was added and the solution was incubated at 60°C for 2 min and at 70°C for 40 min. The reaction mixture was then cycled 40 times for 1 min at 95°C and 4 min at 70°C and then finally incubated for 15 min at 70°C. Primers for PCR amplification of the *Ds1* insertion site were in exon 4 (5′-CGCGTGTTCGTTGACCACCC-3′) and exon 10 (5′-

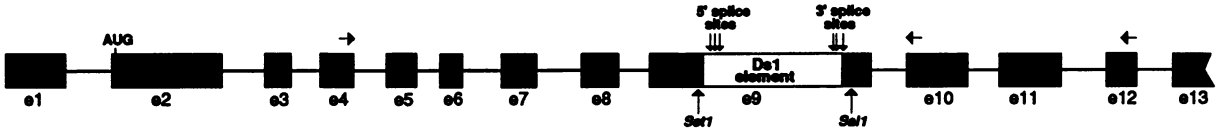

FIG. 1. Position of the *Ds1* element in the *wx-m1* allele. The positions of exons (e) 1 to 13 (of the 14 *Wx* exons) and the translation start are noted (12). The approximate positions of the alternative 5′ and 3′ splice sites used to splice *Ds1* sequences from pre-mRNA are shown. Only those restriction sites mentioned in the text are depicted. Horizontal arrows, positions of PCR primers in exons 4, 10, and 12.

CGCCTCCTTGTTCAGCGCCT-3′) or exon 12 (5′-TTTC CACATGGGCCGCCTCAGCGTC-3′) (see Fig. 1) and were based on the *Wx* sequence (12). The products of two independent amplifications from the same cDNA preparation were analyzed. Prior to DNA sequence analysis, regions containing the sites of *Ds1* insertion were subcloned into pUC118 (30) by using the *Sst*I and *Sal*I sites that flank the element (see Fig. 1). A total of 30 clones, representing five transcripts, were sequenced by the dideoxy chain termination procedure (23). In addition, at least three subclones representing each splicing event were sequenced. Comparison of these sequences with the *wx-m1* sequence (33) permitted the precise determination of 5′ and 3′ splice sites. Southern blot analysis of PCR products was as described previously (33).

RESULTS

The *wx-m1* allele. Molecular cloning and characterization of the *wx-m1* mutation were reported previously (33). Relevant to this study is the fact that the allele contains a 409-bp *Ds* element in exon 9 of the *Wx* gene (Fig. 1). The insertion site is not near either end of the exon (160 and 81 nucleotides [nt], respectively, from the 5′ and 3′ ends of exon 9). Furthermore, the sequence of the *Ds* element revealed that the insertion was a *Ds1* element; it is more than 95% similar to the *Ds1* element responsible for the *Adh1-Fm335* mutation (27).

Endosperms harboring the *wx-m1* allele are phenotypically null in the absence of the autonomous *Ac* element; they contain no amylose and have no *Wx* enzymatic activity (15, 33). Presumably, the presence of the *Ds1* element in exon 9 is incompatible with the production of a functional gene product. In a previous study (32), we demonstrated that another null *Ds* allele, *wx-B4*, encoded a wild-type-size *Wx* mRNA, despite the presence of a 1.4-kb *Ds* element in exon 13. The finding prompted the examination of *wx-m1* transcripts.

Northern blot analysis. The wild-type *Wx* gene encodes a 2.4-kb transcript (Fig. 2) (26). Analysis of poly(A)$^+$ RNA isolated from *wx-m1* endosperms (without *Ac* present) reveals multiple transcripts, one of about 2.8 kb and a broader band migrating at about 2.5 to 2.6 kb. Only the 2.8-kb transcript hybridizes with a *Ds1* specific probe (data not shown). From these data, we conclude that the 2.8-kb transcript is probably a pre-mRNA that includes *Wx* mRNA (2.4 kb) and the entire *Ds1* element (0.4 kb), whereas the 2.5- to 2.6-kb transcript(s) arises following the processing of most of the *Ds1* element from pre-mRNA. In addition, since the waxy gene contains 13 introns, the detection of a single *Ds1*-containing transcript implies that all normal introns are spliced prior to *Ds1* and that splicing of *Ds1* may be relatively inefficient.

Sequence of 5′ and 3′ splice sites. To determine precisely how *Ds1* sequences were removed from pre-mRNAs, *wx-m1* RNA was used as a template for the synthesis of single-stranded cDNA, which, in turn, served as template for PCR amplification. Primers derived from sequences in exons 4 and 10 were incubated with cDNA and amplified, as described in Materials and Methods. The multiple transcripts detected by Northern blots suggested alternative splicing of *Ds* sequences, and therefore PCR products were subcloned prior to sequencing rather than sequenced directly. Comparison of these sequences with the *wx-m1* genomic sequence (33) identified many alternatively spliced transcripts that utilize three 5′ splice sites and three 3′ splice sites, five of which were within the *Ds1* element (Fig. 3). In no case is *Ds1* removed precisely; part of one or both termini persists in the mature mRNA. In fact, none of the five identified transcripts maintains the correct reading frame; splicing of introns a through e results in the addition of 46, 31, 56, 67, and 62 nt, respectively, to the mature mRNA. If any 5′ splice site can be paired with any 3′ splice site, three of the nine possible combinations would restore the correct reading frame. Although it is possible that not all *wx-m1* transcripts were identified by the procedure used, the inability to detect an in-frame transcript is consistent with the null *wx-m1* phenotype.

Southern blot analysis of PCR products. There is an apparent discrepancy between the size of *wx-m1* transcripts detected on Northern blots (about 100 to 200 nt larger than wild-type *Wx* mRNA, following *Ds1* splicing) (Fig. 2) and the additional nucleotides that remain after *Ds1* splicing (31 to 67 nt when introns a to e are processed) (Fig. 3). To determine whether the presence of *Ds1* in exon 9 alters additional RNA processing events, other regions of *wx-m1* transcripts were

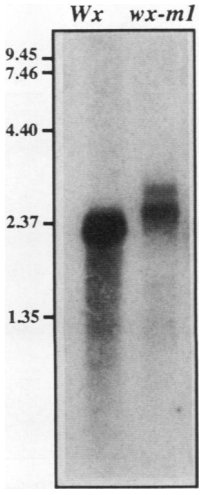

FIG. 2. Northern blot analysis of *Wx* RNAs encoded by the *wx-m1* allele and compared with nonmutant (*Wx*) transcripts. The amounts of poly(A)$^+$ RNA loaded were 0.5 μg (*Wx*) and 2.5 μg (*wx-m1*). The molecular weight markers are in kilobases.

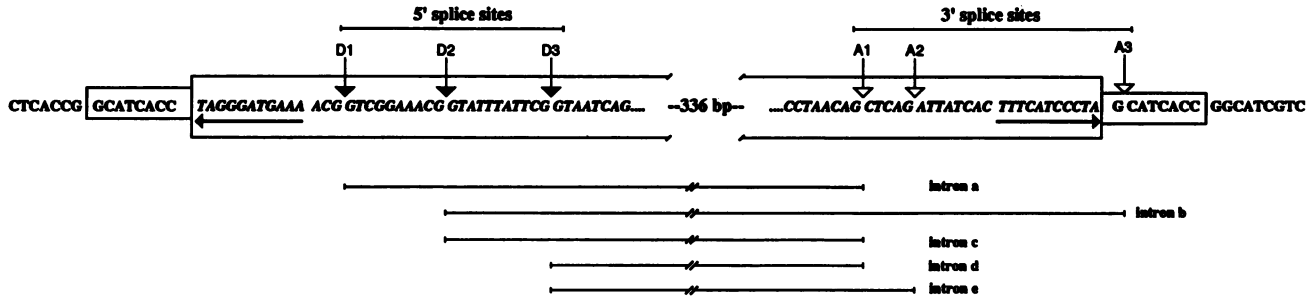

FIG. 3. Alternative splicing of the *Ds1* element from *Wx* pre-mRNA. The positions of alternative 5′ and 3′ splice sites with respect to the sequence of the *Ds1* element (italics) and flanking DNA are shown. Horizontal arrows underline the 11-bp inverted repeat at the *Ds1* termini that are adjacent to the 8-bp direct repeat of *Wx* sequence generated upon insertion (boxed). Alternative use of the 5′ and 3′ splice sites results in introns a through e.

compared with the *Wx* transcript. To this end, *Wx* and *wx-m1* cDNAs were amplified between exons 4 and 12 and were analyzed on a Southern blot (Fig. 4) either before (lanes 1 and 2) or after (lanes 3 and 4) digestion with *Sal*I (see Fig. 1 for the positions of primers and the *Sal*I site). The undigested *wx-m1* products migrate as two predominant bands that are approximately 100 and 400 nt larger than the single wild-type band (Fig. 4, lanes 1 and 2). Digestion with *Sal*I reveals that the altered migration is confined to the larger *Sal*I fragment that corresponds to exons 4 through part of exon 9 (Fig. 1). This larger fragment encompasses all of the alternative splice sites shown in Fig. 3. In contrast, the smaller *Sal*I fragment, corresponding to part of exon 9 through exon 12, is indistinguishable from the wild-type fragment, indicating that this region is correctly spliced. A similar comparison of amplified sequences between exons 2 and 7 also failed to detect additional RNA processing events among *wx-m1* transcripts (data not shown). Taken together, these data support the idea that the presence of *Ds1* sequences leads exclusively to the addition of an alternatively spliced intron in the waxy gene.

DISCUSSION

In a previous report, Dennis et al. (4) described the splicing of a *Ds1* element inserted in the untranslated leader of *Adh1* in the *Adh1-Fm335* mutation. Because of low levels of *Adh1-Fm335* mRNAs, they could not directly determine the sequence of *Adh1-Fm335* cDNAs. Rather, they used S1 nuclease analysis to estimate the splice sites responsible for *Ds1* processing. In this way, they deduced that the boundaries of the *Ds1* intron were a 5′ splice site at D1 (Fig. 3) and a 3′ splice site at an AG dinucleotide that included the terminal A of the *Ds1* element and the first nucleotide of the 8-bp direct repeat generated upon insertion (equivalent to A3) (Fig. 3). Since the 3′ splice site contains both *Ds1* and host sequences, they concluded that for splicing to occur, the *Ds1* element has to insert adjacent to sequences that can provide a 3′ splice site.

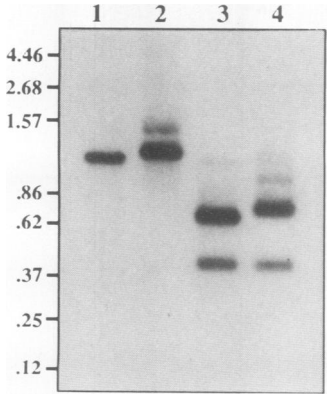

FIG. 4. Southern blot analysis of PCR products from *Wx* and *wx-m1* cDNAs between exons 4 and 12. Lane 1, *Wx*, undigested; lane 2, *wx-m1*, undigested; lane 3, *Wx* with *Sal*I; lane 4, *wx-m1* with *Sal*I. The probe was a full-length *Wx* cDNA. The molecular weight markers are in kilobase pairs.

In this report, the splicing of a second *Ds1* element is described; in addition, positions of the 5′ and 3′ splice sites responsible for *Ds1* processing have been determined directly. These results extend the previous findings in two important ways. First, the *wx-m1 Ds1* element can be alternatively spliced from pre-mRNA when one of three 5′ splice sites is joined to one of three 3′ splice sites. Although none of the five *wx-m1* transcripts described here restore the correct reading frame, other combinations of these 5′ and 3′ splice sites could maintain the reading frame. For example, an intron utilizing D2 and A2 (Fig. 3) would add 51 nt, or 17 codons, to the mature mRNA. Similarly, D1 with A3 would add 7 codons, while D3 with A3 would add 14 codons. Second, in addition to detecting alternative splice sites, this study identified introns that utilize *Ds1* sequences for both 5′ and 3′ splice sites. In fact, the processing of four of the five *Ds1*-derived introns detected in this study involves *Ds1*-encoded donor and acceptor sites (Fig. 3; introns a, c, d, and e). In contrast, splicing of the *wx-B4* and *wx-m9 Ds* elements was accomplished by the utilization of alternative 5′ splice sites in the *Ds* terminus and a 3′ splice site within the waxy gene adjacent to the *Ds* element (Fig. 5; 3′ splice sites are shown only for the *wx-m1 Ds1* element) (32). Thus, the *Ds1* element may be a better mobile intron than *Ac*-like *Ds* elements, because *Ds1* splicing does not require a 3′ splice site in host sequences near the insertion site. Experiments are currently under way to determine whether the *Ds1* sequences are sufficient for splicing or whether adjacent DNA influences splicing efficiency or splice site selection.

The use of 5′ and 3′ splice sites near the termini of transposable elements has been reported previously. A *dSpm* element inserted in the maize *A2* gene is spliced by 5′ and 3′ splice sites that are 16 and 2 nt, respectively, from the ends of the element (17). In *Drosophila melanogaster*, the retrotransposon 412 is alternatively spliced from the first

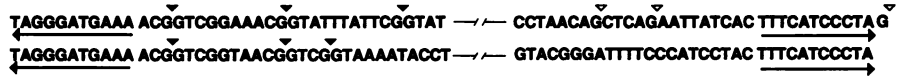

FIG. 5. A comparison of the sequence at the ends of *Ds1* and *Ac*-like *Ds* elements (*Ac/Ds*). Solid and open arrowheads, positions of 5′ and 3′ splice sites, respectively. The 5′ and 3′ splice sites for *Ds1* were determined in this study; the 5′ splice sites for *Ac*-like *Ds* elements were from the *wx-m9* and *wx-B4* elements (32).

untranslated exon of the vermilion gene by 5′ and 3′ splice sites located near the ends of the element within the long terminal repeats (7).

The *Ds1* family represents an intriguing class of transposable elements. On the basis of genetic criteria, *Ds1* and the *Ac*-like *Ds* elements are virtually indistinguishable; both transpose in the presence of an active *Ac* element and both display a negative dosage effect in response to increased *Ac* dosage (for a review, see reference 31). In addition to these genetic similarities, both classes of elements can be spliced from exon sequences in a similar manner. Given these similar properties, it is remarkable that *Ds1* elements and *Ac*-like *Ds* elements have only about 35 to 41 bp of homology (Fig. 5). This includes the 11-bp inverted repeat termini and two of the three 5′ splice sites adjacent to one terminus (Fig. 5, solid arrowheads). The origin of the rest of the 409-bp *Ds1* element is unknown; however, over 75% is made up of A and T residues. Recently, Goodall and Filipowicz (9) demonstrated that plant introns have a positive requirement for AU-rich sequences. It has already been suggested that the presence of both 5′ and 3′ splice sites near the *Ds1* termini makes this class of elements more intronlike. Perhaps the additional features that distinguish *Ds1* from *Ac*-like elements, including its short length and its AT richness, also makes it more intronlike. The success of *Ds1* elements, which are present in the genomes of maize and its wild relatives (22), may reflect the evolution of *Ds* into a better intron.

The evolutionary origin of introns is a question that has fascinated molecular biologists. Models involving intron insertion during evolution have become more attractive since the discovery of mobile group I introns in fungal mitochondria (11). The spread of these self-splicing introns to identical positions in genes lacking the intron is facilitated by intron-encoded, sequence-specific endonucleases (3). The reversibility of the self-splicing reaction of group I and group II introns provides a second possible mechanism for intron insertion (1, 2, 18). Recently, Lambowitz (13) hypothesized that some group I and II self-splicing introns have evolved into mobile elements, thus becoming the ultimate genome parasite that can insert into genes with little effect on gene expression.

What is the role, if any, of maize elements in the origin of introns, and how do the maize *Ds* elements compare with group II mobile introns? Insertion of the maize elements provides a mechanism for the addition of introns into nuclear genes. Lack of such a mechanism has been used to support the theory that all eukaryotic introns are of ancient origin. In addition, a possible role for transposable elements in the origin of nuclear introns has been discounted, because known elements lack 5′ and 3′ splice sites at their termini (25). The *wx-m1 Ds1* element has 5′ and 3′ splice sites near but not within the terminal inverted repeats. In fact, the 5′ splice sites in *Ds1* represent the first GT residues encountered at one terminus, whereas the 3′ splice sites are the last AG residues encountered at the other terminus. Prior studies have suggested that *cis* requirements for transposition reside within the *Ds* inverted repeats (10). Thus, the positions of 5′ and 3′ splice sites within the *Ds1* element may reflect an evolutionary compromise between the ability to transpose and the ability to be spliced. Possibly as a result of this compromise, *Ds* elements are poor introns because they are imprecisely removed from pre-mRNA, but they are superb transposable elements, with few constraints as to where they can insert. In contrast, group II mobile introns are superb introns that are precisely removed from pre-mRNA, but they are poor transposable elements because they have very restricted target sites. These contrasting strengths and weaknesses may reflect the original function of each class of element: group II introns may be introns evolving into transposable elements (13), whereas *Ds1* elements may be transposable elements evolving into introns.

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