The Plant Cell, Vol. 3, 73–85, January 1991 © 1991 American Society of Plant Physiologists

The *tnpA* and *tnpD* Gene Products of the *Spm* Element Are Required for Transposition in Tobacco

Patrick Masson,[a,b] Mary Strem,[a] and Nina Fedoroff[a,1]

[a] Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210

[b] Faculté des Sciences Agronomiques, Unité de Biologie Moléculaire et Physiologie Animale and Station d'Amélioration des Plantes, B-5800 Gembloux, Belgium

The maize *Suppressor-mutator* (*Spm*) element encodes four alternatively spliced transcripts designated *tnpA*, *tnpB*, *tnpC*, and *tnpD*. *tnpA* and *tnpB* are monocistronic, whereas *tnpC* and *tnpD* are dicistronic, and the protein-coding sequences of each transcript overlap extensively with those of one or more of the other transcripts. We have analyzed the role of the *Spm*-encoded gene products in element transposition by using cDNAs with a single open reading frame to (1) complement *Spm* elements with frameshift mutations and (2) complement each other in a tobacco transposition assay. We report that whereas the *tnpA* and *tnpD* gene products are essential for transposition, the *tnpB* and *tnpC* gene products are not. We have analyzed the structure of empty donor sites, new insertion sites, and potential transposition intermediates. We discuss the implications of our findings for the mechanism of *Spm* transposition.

INTRODUCTION

Suppressor-mutator (*Spm*) is a small maize transposable element capable of promoting both its own transposition and that of structurally related transposition-defective *dSpm* elements (reviewed in Fedoroff, 1989a). We recently reported that the *Spm* element encodes an unexpected variety of polyadenylated mRNAs, all of which are derived by alternative splicing from a primary transcript that is almost the element's full length (Masson et al., 1989). The purpose of our present investigation is to identify which of the several element-encoded gene products are necessary and sufficient for *Spm* transposase activity.

The *Spm* element is 8.3 kb long and its structure is depicted diagrammatically in Figure 1. The element is bounded by perfect 13-bp terminal inverted repetitions (IR; Pereira et al., 1986; Masson et al., 1987). The element's transcription unit commences 0.2 kb from its left, 5′ end and ends 0.4 kb from its right, 3′ end. Internally repetitive sequences, termed the *subterminal repetitive regions*, lie between the transcription unit and the IR at each element end (Pereira et al., 1986; Masson et al., 1987).

Sixteen exons have been identified among the several alternatively spliced products of the element's primary transcript (Masson et al., 1989). The element's most abundant processed polyadenylated transcript, identified and named *tnpA* by Pereira et al. (1986), contains 11 exons and is 2.5 kb long (Figure 1A). Of its 10 introns, nine are less than 0.2 kb in length, whereas the first is more than 4 kb long and contains two long open reading frames (ORFs), designated ORF1 and ORF2. The more recently identified *tnpB*, *tnpC*, *and tnpD* transcripts are 5 kb to 6 kb in length and are much less abundant than the *tnpA* transcript (Masson et al., 1989). They comprise all 11 of the *tnpA* transcript's exons and one, two, or three additional exons derived from the first intron sequence of the *tnpA* transcript (Figure 1A). The additional exons are derived almost entirely from the intronic ORF1 and ORF2 sequences. All four of the processed transcripts identified to date have one of two alternative first exons, which differ in length by 126 bp.

The *tnpA* and *tnpB* transcripts are monocistronic, whereas the *tnpC* and *tnpD* transcripts are dicistronic (Figure 1B; Masson et al., 1989). The *tnpA* transcript contains a single ORF, designated ORFA, which codes for a 68-kD DNA-binding protein that interacts with the element's *subterminal repetitive regions* (Gierl et al., 1988b). The *tnpB* transcript contains all of the *tnpA* exons and one additional long exon derived from the ORF1 sequence. It codes for a 171-kD ORF1-ORFA fusion protein (Masson et al., 1989). Hence, the predicted *tnpB* protein contains a domain that corresponds to the complete ORFA-encoded *tnpA* protein. The *tnpC* and *tnpD* transcripts contain two ORFs. The first codes for a 128-kD or 131-kD ORF1-ORF2 fusion protein and the sequence of the two tran-

[1] To whom correspondence should be addressed.

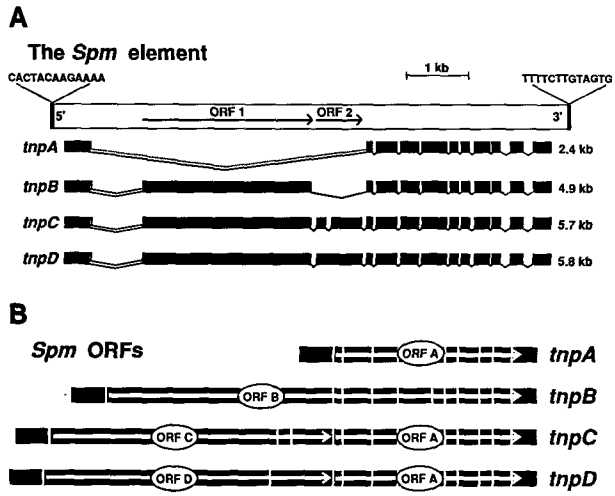

Figure 1. Structure of the *Spm* Element and *Spm*-Encoded Transcripts.

(A) The *Spm* element is represented by the open box. Arrows within the box show the location and length of the two large ORFs within the first intron of the element's *tnpA* transcript. The element's transcriptional polarity is indicated, and the sequences of its 13-bp IRs are shown above the ends of the diagram. The filled boxes below the diagram represent the exons of the various known alternatively spliced element transcripts, which are designated *tnpA*, *tnpB*, *tnpC*, and *tnpD*. The length of each transcript, without its poly A tail, is given to the right of each transcript. There are two first exons differing in length by 126 bp. The alternative first exon is represented by the open box behind exon 1.

(B) The diagrams show the major ORFs of each of the element's transcripts. The breaks in each diagram correspond to the locations of splice sites. ORFA encodes a 68-kD polypeptide and is present as a separate coding sequence in the *tnpA*, *tnpC*, and *tnpD* transcripts. ORFB encodes a 171-kD ORF1-ORFA fusion polypeptide, and ORFC and ORFD encode 128-kD and 131-kD ORF1-ORF2 fusion proteins, respectively.

scripts differs by the removal from the *tnpC* transcript of a 90-bp intron within ORF2 retained in the *tnpD* transcript (Figure 1B). The large ORFs of *tnpC* and *tnpD* have been designated ORFC and ORFD, respectively. In the *tnpC* and *tnpD* transcripts, ORFC and ORFD are followed by the entire ORFA sequence, with intervening translational stop codons in all three reading frames. Both transcripts, therefore, also separately encode the *tnpA* gene product. The *tnpB*, *tnpC*, and *tnpD* transcripts code for proteins that have a common domain encoded by most of ORF1, whereas the ORFC-encoded and ORFD-encoded proteins also have most of the ORF2-encoded domain in common.

The fact that the *Spm* element's multiple alternatively spliced transcripts code for proteins with extensive common domains complicates analysis of their functions by both traditional and contemporary genetic approaches. Almost all of the spontaneous *Spm* mutations that have been analyzed have proved to be intraelement deletions with pleiotropic or ambiguous phenotypic effects (reviewed in Fedoroff, 1983, 1989a). Using an assay for *Spm* transposition in transgenic tobacco, we determined that frameshift mutations introduced in vitro in both ORF1 and ORF2 reduce the ability of the *Spm* element to promote excision of a *dSpm* element inserted in the leader region of a β-glucuronidase (GUS) gene expressed from a cauliflower mosaic virus (CaMV) 35S promoter (Masson and Fedoroff, 1989; Masson et al., 1989). These observations suggested that the ORFs in the first intron of the sequence coding for the previously identified *tnpA* transcript encode polypeptides that participate in transposition and led to the identification of the *tnpB*, *tnpC*, and *tnpD* transcripts (Masson et al., 1989).

To make further progress in determining which of the four putative proteins encoded by the element are required for transposition, we have used cDNAs with a single protein coding sequence to complement *Spm* elements with frameshift mutations, as well as each other, in pro-

Table 1. The Ability of the *tnpA* cDNA To Complement Mutant *Spm* Elements Lacking Transposase Activity[a]

		No *tnpA* cDNA		With *tnpA* cDNA[b]	
Location of Frameshift Mutation in *Spm*	Mutated Site in *Spm*	Percent Positive Calli	Number of Calli Tested	Percent Positive Calli	Number of Calli Tested
tnpA exon 4	SpII	0	33	67	15
ORF1	Asp718	0	21	0	38
ORF2	EcoRI-2	0	34	0	50
tnpA intron 4	EcoRI-3	57	42	75	4
Control	No *Spm*	0	39	0	20[c]

[a] Transposase activity was detected by the appearance in transformed calli of many sectors of cells exhibiting GUS activity following *dSpm* excision from the p*dSpm*\GUS excision test plasmid (Masson and Fedoroff, 1989).

[b] The *tnpA* cDNA was introduced using the p*tnpA::dSpm*\GUS plasmid (see Methods).

[c] A few of these calli had one or two GUS-positive sectors (see text).

moting transposition of a *dSpm* element in tobacco. To do this, the *tnpC* and *tnpD* transcripts were truncated, eliminating the *tnpA*-coding domain. Thus, the *tnpC* and *tnpD* cDNAs tested here contain only ORFC and ORFD, which encode the respective 128-kD and 131-kD ORF1-ORF2 fusion proteins (Figure 1B). We have designated these putative proteins the *tnpC* and *tnpD* gene products because ORFC and ORFD are the only coding sequences unique to the original *tnpC* and *tnpD* transcripts (Masson et al., 1989). A separate cDNA containing only ORFA was used to investigate the role of the *tnpA* gene product.

We present evidence that the *tnpA* and *tnpD* gene products, but not the *tnpB* or *tnpC* gene products, are directly required for transposition of a *dSpm* element in tobacco. We have characterized both the sites from which a *dSpm* element has excised and newly reinserted elements. We have observed *tnpA*-promoted and *tnpD*-promoted excision events resembling those supported by a complete *Spm* element, as well as several excision events accompanied by deletions. We have noted sequence similarities among *Spm* insertion sites and between insertion sites and the element's subterminal repeated sequences, suggesting a target sequence preference for insertion of the *Spm* element. In characterizing new *Spm* insertion sites using the inverse polymerase chain reaction technique (IPCR; Ochman et al., 1988), we have recovered end-joined *Spm* termini whose structure implies blunt-end cuts at *Spm* element ends during transposition.

RESULTS

Excision of a *dSpm* Element Requires the *tnpA*, but not the *tnpB*, Gene Product

In a previous report, we showed that frameshift mutations in ORF1 and ORF2 disrupt the ability of an *Spm* element to promote transposition of a *dSpm* element in tobacco (Masson et al., 1989). We concluded that *Spm* transposase function requires at least one of the polypeptides with domains encoded by these ORFs. However, we had no direct evidence that the *tnpA* gene product was also required. Because ORFA codes both for *tnpA* and a domain of *tnpB*, we first asked whether a frameshift mutation in ORFA disrupts the element's ability to *trans*-activate *dSpm* excision in tobacco. The results are reported in Table 1. None of 33 calli containing an *Spm* element with a frameshift mutation at the SpII site in exon 4 of the *tnpA* sequence exhibited the sectors of GUS-positive cells observed upon *Spm*-promoted excision of the *dSpm* element from the leader region of the GUS gene in the p*dSpm*\GUS excision assay plasmid (Masson and Fedoroff, 1989).

The foregoing result implies that either *tnpA* or *tnpB* (or both) is required for transposition. To distinguish among these possibilities, a *tnpA* cDNA cloned between a CaMV 35S promoter and nopaline synthase (NOS) 3′ terminator was introduced together with an SpII-mutated *Spm* element into tobacco cells with the p*dSpm*\GUS excision assay plasmid. As shown in Table 1, 10 of 15 transformed calli exhibited many GUS-positive sectors indicative of *dSpm* excision from the GUS gene. This result implies that the *tnpA* gene product, but not an intact *tnpB* gene product, is required for *dSpm* excision. By contrast, the *tnpA* cDNA did not complement mutant *Spm* elements with frameshift mutations in either ORF1 or ORF2, indicating that either *tnpC* or *tnpD* (or both) is also required for *dSpm* excision (Table 1).

The *tnpA* and *tnpD*, but not the *tnpC*, Gene Products Are Required for *dSpm* Excision

Because the *Spm* element's several different proteins are encoded by a single transcription unit and because there is evidence that the element encodes at least one autoregulatory gene product (Masson et al., 1987; Banks et al., 1988), it is possible that an element-encoded protein is required to activate transcription of the element and is, therefore, only indirectly involved in element mobility. To identify the *Spm* proteins that are both necessary and sufficient for transposase activity, we used cDNAs encoding only a single protein. These were the *tnpA* cDNA and truncated versions of the *tnpC* and *tnpD* cDNAs lacking ORFA (see Methods). The truncated *tnpC* and *tnpD* cDNAs retain only ORFC and ORFD, which code for the 128-kD and 131-kD ORF1-ORF2 fusion proteins unique to the original *tnpC* and *tnpD* transcripts, respectively (Masson et al., 1989). All of the cDNAs were expressed from a CaMV 35S promoter and followed by a NOS 3′ terminator sequence. The *tnpA*, *tnpC*, and *tnpD* cDNAs were tested singly and pairwise for their ability to promote excision of the *dSpm* from the GUS gene.

As shown in Table 2, none of the tested cDNAs was individually capable of promoting *dSpm* excision at a high frequency. By contrast, GUS-positive sectors indicative of *dSpm* excision were observed in calli containing both *tnpA* and *tnpD* cDNAs, but not in calli containing *tnpA* and *tnpC* cDNAs (Table 2). This observation implies that the *tnpA* and *tnpD* gene products are both directly involved in promoting *dSpm* excision. Moreover, a full-length *tnpD* cDNA with a frameshift mutation near the end of ORFD (*tnpD**) was unable to replace the nonmutant truncated *tnpD* cDNA, supporting the conclusion that an intact *tnpD* gene product is required (Table 2).

We have noted both in these and previous experiments that calli transformed with the p*dSpm*\GUS excision test plasmid alone occasionally exhibit a small number (one to three) of GUS-positive sectors (Tables 1 and 2; Masson and Fedoroff, 1989; Masson et al., 1989). The molecular events giving rise to these sectors will be discussed below. In addition, a small fraction of calli transformed with only

Table 2. Transposase Activity of *Spm* cDNAs[a]

	No *tnpA* cDNA		With *tnpA* cDNA[b]	
Spm cDNA[c]	Percent Positive Calli	Number of Calli Tested	Percent Positive Calli	Number of Calli Tested
tnpA	0	58[d]	0	9
tnpC	0	55	0	74
tnpD	1.9	104[d]	35.2	125
*tnpD**	5.3	19	3.6	28[d]

[a] Transposase activity was detected by the appearance in transformed calli of many sectors of cells exhibiting GUS activity following *dSpm* excision from the p*dSpm*\GUS excision test plasmid (Masson and Fedoroff, 1989).
[b] The *tnpA* cDNA was introduced using the p*tnpA::dSpm*\GUS plasmid (see Methods).
[c] Construction of the cDNA expression cassettes is described in Methods. The *tnpC* and *tnpD* cDNAs lacked the tnpA coding region, whereas the *tnpD** cDNA was mutated, full-length cDNA.
[d] A few of these calli had one or two GUS-positive sectors (see text).

the *tnpD* (1.9%) or *tnpD** (5.3%) cDNA or with both the *tnpD** and *tnpA* (3.6%) cDNAs exhibited GUS-positive sectors at a frequency higher than this background. These have been listed as positive in Table 2 because of the variability in sector number among independent transformants, although they generally exhibited fewer sectors than calli transformed either with an intact *Spm* element or with the *tnpA* and *tnpD* cDNAs. These results suggest that the *tnpD* protein alone may promote excision at a frequency lower than that observed in the presence of the *tnpA* gene product, but discernibly above background. The observation that a similar low frequency of *dSpm* excision was observed with the mutant *tnpD** cDNA both in the absence and presence of the *tnpA* cDNA suggests that the mutation disrupted the ability of the *tnpD* protein to interact with the *tnpA* protein.

tnpA and *tnpD* Promote *dSpm* Transposition

To verify that *dSpm* excision had occurred in calli with GUS-positive sectors of cells, DNA fragments containing the *dSpm* insertion site of the original GUS assay plasmid were amplified by the polymerase chain reaction (PCR), cloned, and sequenced. A total of 11 different empty donor site sequences were present among the 13 fragments recovered using DNA extracted from two different calli. Their sequences appear in Figure 2A. A majority of the empty donor site sequences are typical of those recovered after excision of a *dSpm* element in maize (Schwarz-Sommer et al., 1985). That is, the element had either excised perfectly, leaving the flanking sequences intact, or there were 1-bp to 4-bp deletions and inverted duplications at the former site of insertion. Four of the empty donor sites had slightly larger deletions of 15 nucleotides to 21 nucleotides, extending either in one or in both directions from the former insertion site. In three of the four empty donor sites, one of the deletion end points was immediately adjacent to a trinucleotide sequence in the original flanking DNA identical to the CAC trinucleotide terminus of the *dSpm* element (Masson et al., 1987). These observations confirm the expectation that calli with many sectors of GUS-positive cells contain different cell lineages in which the *dSpm* element has excised from its original insertion site within the leader of the GUS gene.

Because GUS-positive sectors of cells occasionally can be detected in calli containing the *dSpm*-disrupted GUS gene and no *Spm* element (Masson and Fedoroff, 1989; Masson et al., 1989), a *tnpD* cDNA, or both a *tnpA* and a *tnpC* cDNA (Table 1), an attempt was made to amplify and recover comparable fragments corresponding to the orig-

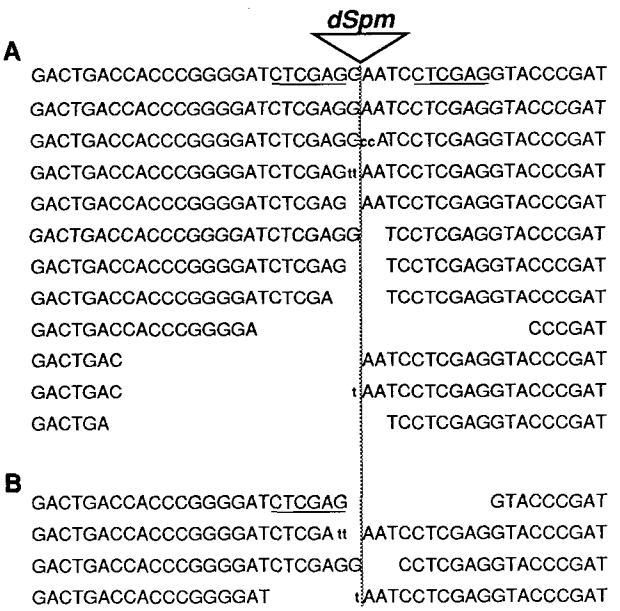

Figure 2. Nucleotide Sequences of Empty Donor Sites.

(A) DNA isolated from calli containing the p*dSpm*\GUS excision test plasmid, the *tnpA* and *tnpD* cDNAs.
(B) DNA isolated from calli containing only the p*dSpm*\GUS plasmid.
Empty donor site fragments were amplified by PCR, cloned, and sequenced (see Methods). The former *dSpm* insertion site is indicated by the vertical line. The first line shows the original sequence flanking the *dSpm* element, with the adjacent XhoI cloning sites underlined. All remaining lines show the sequences of empty donor site fragments. Gaps in the sequence represent deleted nucleotides, and lower-case letters represent inserted nucleotides.

inal *dSpm* insertion site from DNA of such calli. In most cases, no empty donor site fragment could be amplified, regardless of DNA concentration. However, it was possible to amplify fragments in the expected size range from one template DNA obtained from a callus containing only the p*dSpm*\GUS excision assay plasmid. Four different fragments lacking the *dSpm* element were cloned and their sequences appear in Figure 2B. The structure of the first fragment suggests that it arose by recombination between the XhoI sites flanking the *dSpm* element. However, the other three sequences shown in Figure 2B resemble those characteristic of empty *dSpm* donor sites, suggesting that the *dSpm* element may very occasionally excise in tobacco even in the absence of the *Spm*-encoded gene products necessary for high-frequency excision.

To determine whether excised *dSpm* elements reinsert into tobacco DNA in the presence of the *tnpA* and *tnpD* gene products, DNA sequences adjacent to *dSpm* termini were amplified by IPCR. DNA extracted from cells in which transposition was occurring, as judged by the presence of GUS-positive sectors, was partially digested with Sau3AI, religated under conditions favoring circle formation, and then used as a substrate for PCR amplification from primers homologous to and oriented away from element ends toward flanking sequences. This permits the identification of sequences initially adjacent to each end of the element (Ochman et al., 1988).

However, it is evident from the multiplicity of excision site sequences (Figure 2), the small size of GUS-positive sectors, the low abundance of empty donor sites, and the difficulty of detecting reinserted elements (Masson and Fedoroff, 1989) that any given reinserted element will be present in a very small fraction of the DNA molecules extracted from transformed tobacco tissue in which *dSpm* transposition is occurring. Indeed, the most abundant genomic *Spm*-homologous restriction fragment detectable in such tissue is that containing the *dSpm* element at the donor site on the integrated T-DNA (Masson and Fedoroff, 1989). Fragments containing *Spm* termini flanked by donor site sequences are readily amplified when no effort is made to prevent their amplification, providing evidence of the efficacy of the IPCR procedure (data not shown).

To favor amplification of sequences flanking transposed *dSpm* elements, recircularized DNA was cleaved with XhoI before amplification. Because there is an XhoI site immediately adjacent to each element terminus in the flanking sequence on the transforming T-DNA (Figure 2), cleavage with this enzyme should prevent amplification of the donor site fragments and favor amplification of sequences flanking newly transposed elements. And, indeed, after amplification of XhoI-treated template DNA, fragments could be detected that had homology to the *Spm* element, but not to the adjacent T-DNA sequences on the transforming plasmids (data not shown). Several such fragments were cloned from one amplification reaction containing template DNA isolated from a single transformed callus. Two of the fragments were sequenced and their termini and flanking sequences are shown in Figure 3B. Both elements are flanked by sequences that are different from the original plasmid flanking sequences, shown in Figure 3A. Both elements have an intact 5′ end and a short deletion at the 3′ end. Both deletion end points are within one of the 12-bp repeats characteristic of the *subterminal repetitive region* of the *Spm* element (Masson et al., 1987). Neither element is flanked by the 3-bp duplication characteristic of an *Spm* insertion in maize.

To show that the new sequences flanking the *dSpm* elements are present in the tobacco genome, the cloned DNA fragments were hybridized to DNA isolated from transformed and untransformed tobacco plants. As shown in Figure 4A, the *dSpm-15* fragment hybridized to two EcoRI fragments of approximately 7 kb and 8 kb (arrows). It also hybridized to a 3.7-kb EcoRI fragment in transformed tobacco DNA, as well as a 4.0-kb fragment in one of the DNA samples (lower bands). Because the *dSpm-15* fragment contains both *Spm* and flanking sequences, the blot was reprobed with an *Spm* probe to identify the fragments with homology only to the *Spm* element, which would include those derived from the initial insertion site, as well as elements at other locations. The *Spm* probe detected the 3.7-kb and 4-kb fragments, as well as the 8-kb fragment (upper arrow) in one of the DNA samples, but was not homologous to the other large fragment (lower arrow) in DNA from the first transformed plant (Figure 4B, lane 1) or either large fragment in DNA from the second transformed plant (Figure 4B, lane 2) or the untransformed plant (Figure 4B, lane 3). Because the *dSpm-15* fragment hybridizes to tobacco DNA fragments with no homology to *Spm*, we conclude that the *dSpm* termini are flanked by tobacco DNA in the *dSpm-15* fragment and were derived from a transposed copy of the element reinserted at a new site in the tobacco genome. The size of the *dSpm*-containing EcoRI fragment on the input plasmid was 3.7 kb, indicating that this fragment corresponds to the *dSpm* at the donor site on the input T-DNA. The other *Spm*-homologous fragments may be additional transposed elements or rearranged copies of the input plasmid.

End-Joined *Spm* Termini Can Be Amplified by IPCR from DNA of Tobacco Cells with Transposing Elements

When DNAs from tobacco calli with transposing *dSpm* elements were used as a template for IPCR, some of the fragments amplified were either of the length expected for adjacent termini with no intervening nucleotides or shorter. Upon cloning and sequencing of several such fragments, two types were identified, both of which consisted of the element's 3′ terminus directly ligated to its 5′ terminus. In one group of cloned fragments, both element termini were intact, whereas another cloned fragment had a 3-bp to 6-

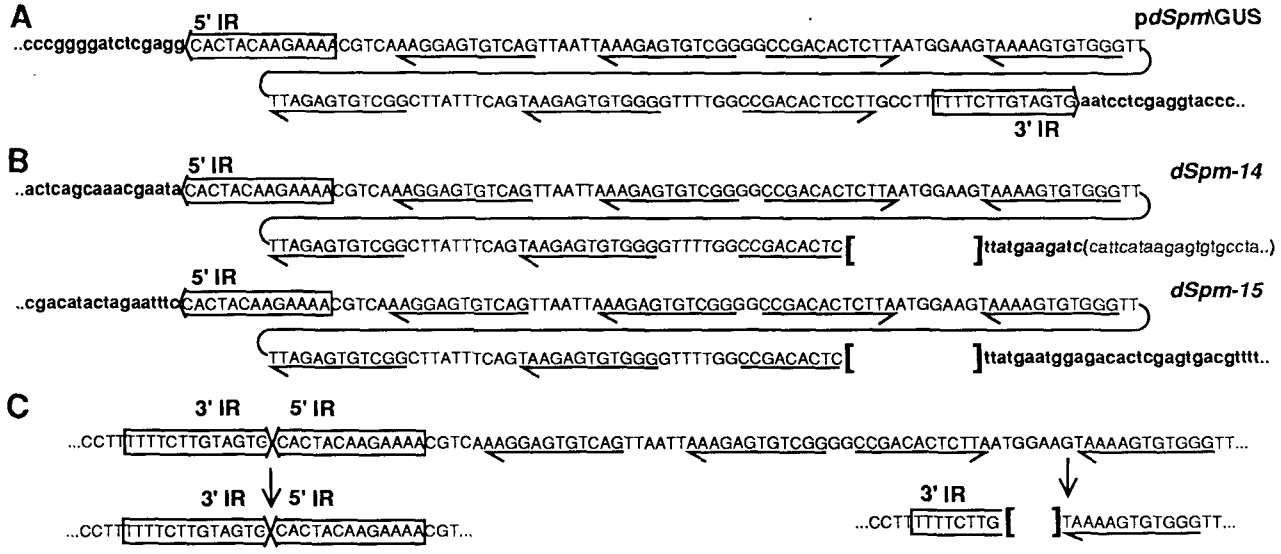

Figure 3. *Spm* Termini and Flanking Sequences.

(A) Donor site. The diagram shows the terminal and flanking sequences of the *dSpm* element in the p*dSpm*\GUS excision assay plasmid. The 5′-terminal and 3′-terminal sequences of the *dSpm* element are shown in capital letters, and the flanking plasmid sequences are shown in lower-case letters. The IRs are enclosed in boxes and the first several repeats of the *subterminal repetitive region* are underlined, with the arrowhead indicating the orientation of each repeat.

(B) New insertion sites. The diagram shows the terminal and flanking sequences of two reinserted *dSpm* elements. The sequences were obtained from fragments amplified by IPCR from DNA of calli containing the p*dSpm*\GUS excision assay plasmid and the *tnpA* and *tnpD* cDNAs. Element ends and flanking sequences are represented as in **(A)**. The bracketed gap in each sequence represents a deletion. The parentheses in the *dSpm-14* clone enclose the sequence that is beyond the first Sau3AI site and that may, therefore, be derived from either the 3′-flanking or the 5′-flanking sequence.

(C) End-joined *Spm* termini. The diagram shows sequences amplified by IPCR from DNA of calli containing the p*dSpm*\GUS excision assay plasmid and the *tnpA* and *tnpD* cDNAs. The two different structures recovered among such clones are shown below a representation of the expected sequence of the element with its IRs joined, along with part of the 5′ *subterminal repetitive region*. The bracketed gap in the sequence shown on the right represents the deleted *Spm* sequences, which include part of the 3′ IR, the entire 5′ IR, and part of the 5′ *subterminal repetitive region*.

bp deletion at the element's 3′ end and a 68-bp to 71-bp deletion terminating at the end of one of the repeats within the 5′ *subterminal repetitive region*. These sequences appear below a representation of the corresponding *Spm* sequences in Figure 3C.

To determine whether fragments with *Spm* termini joined end-to-end pre-exist in DNA from cells with transposing elements or are produced during the IPCR procedure, an effort was made to amplify end-joined *Spm* termini from uncut, unligated DNA. No such fragments could be amplified directly from DNA isolated from calli in which *dSpm* transposition was promoted by the *tnpA* and *tnpD* gene products or in which no *dSpm* excision was detectable by the histochemical assay for GUS activity (not shown). Fragments of the expected size were amplified from DNA isolated from calli in which *dSpm* transposition was promoted by an intact *Spm* element, but none had end-joined *Spm* termini without intervening nucleotides. One group of fragments contained only one element end and will be discussed below. The second group of the cloned fragments contained the two element ends separated by one copy of the two XhoI sites originally flanking the *dSpm* and the 4-bp sequence between the XhoI site and the end of the element in the original plasmid (Figure 2). Such a structure indicates that these adjacent termini were amplified from *Spm* circles that arose by intramolecular recombination between the XhoI sites in the p*dSpm*\GUS excision assay plasmid. The ability to recover such a structure provides evidence that the IPCR technique is capable of detecting end-joined *Spm* termini if they exist before ligation.

Thus, end-joined *Spm* termini have been recovered only from DNA of calli in which transposition is occurring and only after the cleavage and ligation steps of the IPCR procedure. Because cleavage sites for the restriction enzymes used in the IPCR were not present between element ends in the clones with end-joined termini, the IPCR step required for recovery of end-joined termini is likely to be

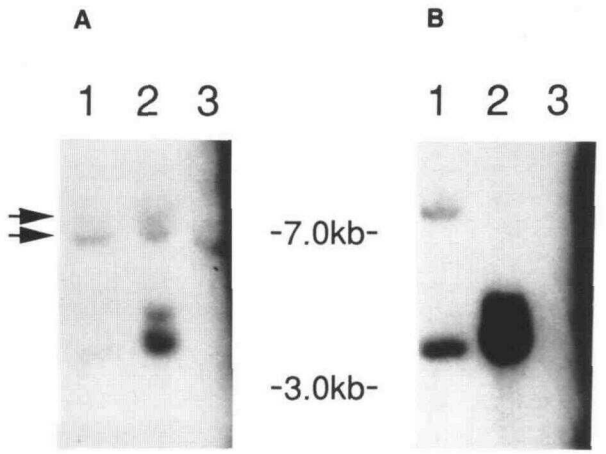

Figure 4. Blot Hybridization Analysis of Sequences Flanking a Transposed *dSpm* Element.

DNA was isolated from tobacco calli containing the p*dSpm*\GUS plasmid and the *tnpA* and *tnpD* cDNAs (lanes 1 and 2) or untransformed tobacco plants (lane 3).
(A) The EcoRI-digested DNA was first probed with a cloned DNA fragment (*dSpm-15*) containing 180 bp of flanking sequences and 270 bp of *dSpm* sequence.
(B) The EcoRI-digested DNA was then reprobed with a cloned 780-bp 3′-terminal fragment of the *Spm* element.

the circularization step. We conclude that fragments of the type shown in Figure 3C probably arose from excised linear forms of the *dSpm*. The observation that element ends are joined very precisely in at least one group of clones suggests that *Spm* excision occurs by double-strand cuts exactly at element ends.

Insertion Site Sequence Similarities

As mentioned above, the second group of fragments amplified directly from DNA of calli in which *dSpm* transposition was promoted by an intact *Spm* element contained only the element's 3′ end. Each fragment also contained the oligonucleotide primer sequence employed for amplification of the 5′ end separated from the element's 3′ end by a sequence not present in the original plasmid. Each fragment contained a different nonplasmid sequence flanking the element's 3′ end, whose lengths were 167 bp, 212 bp, and 222 bp (not shown). Therefore, it is likely that these fragments were derived from transposed copies of the element. The fact that such fragments were recovered implies that there were sequences of sufficient homology to the 28-bp 5′ *Spm* primer near the element's end to permit amplification of the intervening fragment. Because the 3′ end of the primer that was used overlaps one of the element's subterminal repeats, as shown in Table 3 (primer 103), it appears most probable that the sequence similarity permitting amplification is to the element's subterminal repeat. The implication of this finding is that sequences similar to the repeats of the *Spm* element's *subterminal repetitive region* are located near *Spm* insertion sites.

The foregoing observation prompted a comparison of *Spm* insertion sites with the consensus repeat sequence of the element's *subterminal repetitive region* (*SRR* consensus). In Figure 5, the *SRR* consensus sequence has been aligned vertically with sequences surrounding the transposed *dSpm* copies recovered from tobacco (*dSpm-14* and *dSpm-15*), as well as two *Spm* insertion sites in maize DNA. A comparison of the sequences reveals both striking identities between insertion site sequences, as well as similarities between the *SRR* consensus and the insertion sites (Figure 5). The sequence immediately adjacent to the element at the *dSpm-14* and the *dSpm-15* insertion site is identical to the *SRR* consensus sequence at 6 and 7 of 12 nucleotides, respectively (Masson et al., 1987). Similarly, there is a 6-bp of 12-bp identity between the *SRR* consensus repeat and the *Spm* insertion site in the maize *a-m2* allele and a 5-bp of 12-bp identity with a sequence immediately adjacent to the *bz-m13* insertion site. Similarities of the same magnitude have been noted previously between the end points of intraelement deletions and the subterminal consensus repeat sequence (Masson et al., 1987). As discussed in more detail below, these observations suggest that a common target site

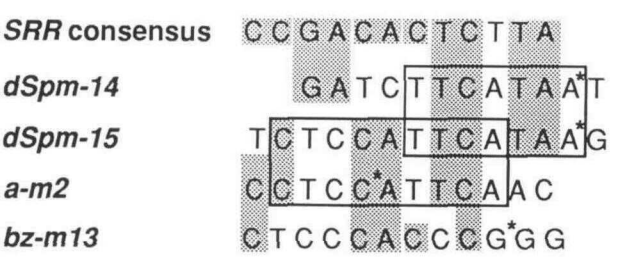

Figure 5. Similarities between *Spm* Insertion Sites and *subterminal repetitive regions*.

The sequence shown on the top line (*SRR* consensus) is the 12-bp consensus repeat sequence of the *Spm* element's *subterminal repetitive regions* (Masson et al., 1987). There are nine repeats within the first 180 bp of the element's 5′ end and 14 repeats in the 3′-terminal 300 bp. Below the *SRR* consensus are the sequences flanking the reinserted *dSpm* element in tobacco clones *dSpm-14* and *dSpm-15*, as well as the *Spm* insertion site in the maize *a-m2* allele (Masson et al., 1987; Schwarz-Sommer et al., 1987) and the *dSpm* insertion site in the *bz-m13* allele (Ralston et al., 1988; Schiefelbein et al., 1988). The sequences at or immediately adjacent to the insertion site, indicated by a star, have been aligned with the *SRR* consensus repeat and identities are highlighted by the cross-hatching. Boxes enclose sequence identities between insertion sites.

Table 3. Sequences of the Oligonucleotides Used as Primers in PCR Amplification

Primer No.	5′bp[a]	Sequence	3′bp[a]
Empty donor site fragments			
101		GGAGAGGATCCGCTGAAATCACC	
102		TATCTGCAGCGGCGAACTGATCGTT	
Transposed *dSpm* elements			
103	199	GAATTTAGGGATCCATTCATAAGAGTGT	172
104	7913	TTGTGTCGACATGGAGGCTTCCCATCCGGGGA	7944
105	145	ATTAAAAGCCTCGAGTTCATCGGGA	121
106	8020	AGGTAGTCGACTGATGTGCGCGC	8042
107	28	GACACTCGAGTGACGTTTTCTTGTAGTG	1
108	8020	AGGTAGCTTACTGATGTG	8037

[a] The location within the *Spm* sequence of the 5′-terminal and 3′-terminal nucleotides of each primer used two amplified sequences flanking transposed *dSpm* elements.

recognition mechanism functions in *Spm* transposition and in the origin of element-promoted deletions.

DISCUSSION

The *tnpA* and *tnpD* Gene Products Are Both Necessary and Sufficient for *dSpm* Transposition in Tobacco

We have provided evidence that both the *tnpA* and *tnpD* gene products are directly involved in *Spm* transposition. The ability of a *tnpA* cDNA to complement a full-length *Spm* element with an inactivating frameshift mutation in ORFA implies that an intact *tnpB* gene product is not required for transposition, but that the *tnpA* gene product is required. Because we have not yet tested the ability of *tnpB* to carry out the same complementation, we do not know whether the *tnpA* domain of the *tnpB* protein can replace the *tnpA* protein in promoting transposition.

Because the *tnpA* cDNA was unable to complement an element with a frameshift mutation in either ORF1 or ORF2, it appeared likely that at least two gene products were required for transposition, *tnpA* and either *tnpC* or *tnpD* (or both). It was also formally possible that one gene product is necessary only because it regulates production of a second gene product directly involved in transposition. This possibility was eliminated by the inability of the *tnpA*, *tnpC*, and *tnpD* cDNAs to support high frequency *dSpm* transposition individually. The observation that *dSpm* transposition occurs in cells containing the *tnpA* and *tnpD* cDNAs, but not in cells containing the *tnpA* and *tnpC* cDNAs, implies that the *tnpA* and *tnpD* gene products are both directly involved in transposition. However, these results do not rule out the possibility that one or both also have a second enzymatic or regulatory function in element expression or transposition.

The results of molecular analyses of empty donor sites and sequences flanking *dSpm* elements provide clear evidence that the *tnpA* and *tnpD* gene products promote transposition of the *dSpm* element from its original location to new sites in the tobacco genome. Although a majority of the empty donor sites resemble those observed in maize and tobacco with a complete *Spm* element, we recovered several fragments with unusually long deletions surrounding the former insertion site (Schwarz-Sommer et al., 1985; Masson and Fedoroff, 1989). Moreover, there were 3′-terminal deletions in the *dSpm* termini within both of the cloned fragments in which novel sequences flanked the element. Several observations support the inference that the deletions are associated with the transposition process. First, the end points of several of the donor site deletions coincide with a trinucleotide in a flanking sequence that is identical with the trinucleotide sequence at the end of the element. Second, all of the intraelement deletions terminate at the end of an internal repeat within the element's *subterminal repetitive region.* A similar coincidence of deletion end points with the subterminal repeats is characteristic of spontaneous element-promoted intraelement deletions arising in maize (Tacke et al., 1986; Masson et al., 1987). Third, one of the PCR-amplified, cloned fragments with end-joined *Spm* termini has a deletion similar to those found in transposed *dSpm* elements (Figure 3C). That is, the deletion also ends next to a subterminal repeat. Although the sample size is not large enough to support a firm conclusion, these observations suggest that the transposition process in tobacco cells containing the *tnpA* and *tnpD* cDNAs may be somewhat more error prone than it is in cells containing an intact *Spm* element (Masson and Fedoroff, 1989; Pereira and Saedler, 1989). If this is indeed the case, then perhaps either the *tnpA* and *tnpD* gene products are present at the wrong relative or absolute concentrations or an additional element-encoded gene product is required for high-fidelity transposition.

Spontaneous *dSpm* Excision in Tobacco

In these and previous experiments, we have noted that tobacco tissues containing a *dSpm*-disrupted GUS gene show occasional sectors of GUS-positive cells (Masson and Fedoroff, 1989; Masson et al., 1989). Such sectors occur at a sufficiently low frequency so that in most cases we were unable to amplify the fragments expected if the *dSpm* is eliminated without a major rearrangement of flanking sequences. A successful amplification of fragments in the expected size range from the DNA of one callus containing just a *dSpm*-disrupted GUS gene permitted the cloning and characterization of several different

sequences lacking the original *dSpm* element. One of these could have arisen by a rearrangement unconnected with *dSpm* excision, whereas the rest had a structure typical for an empty donor site (Figure 2B), suggesting that a *dSpm* element occasionally excises in tobacco by a mechanism typical of the transposition process.

Transposable elements that have IRs similar to or identical with those of the *Spm* element, as well as internal homologies to the *Spm* ORF1 sequence, have been isolated from both *Antirrhinum majus* (*tam1*, Gierl et al., 1988a) and *Glycine max* (*tgm1*, Rhodes and Vodkin, 1988). Because of these similarities, it has been suggested that *Spm* is a member of a transposable element "superfamily" (Gierl et al., 1988a). The occasional excision of the *dSpm* element in tobacco suggests that *Nicotiana* might also contain a member of the *Spm* superfamily, whose transposase has some affinity for *dSpm* ends and occasionally promotes its excision.

Functions of *Spm*-Encoded Gene Products and the Mechanism of Transposition

Although we have established that both the *tnpA* and *tnpD* gene products are required for transposase function, the precise role of each in transposition is not yet known. There is homology between ORF1 and sequences within other elements belonging to the *Spm* superfamily (Gierl et al., 1988a; Rhodes and Vodkin, 1988), and ORF1 contains a putative zinc finger domain common to DNA-binding proteins (Vodkin and Vodkin, 1989). Moreover, low but significant levels of *dSpm* transposition are observed with only the *tnpD* and only the *tnpD** cDNAs. Therefore, it presently appears most likely that it is the *tnpD* gene product that binds to and cleaves element ends.

The results of the present experiments provide evidence that the *tnpA* protein also functions as part of the transposase. The *tnpA* gene product markedly increases the low transposition frequency observed with a nonmutant *tnpD*, but has no effect on that observed with the *tnpD** cDNA. The *tnpD** cDNA has a frameshift mutation near the 3′ end of the coding sequence that should truncate the protein prior to a region of homology between the *tnpD* protein and the *rev* protein of HIV 2 (Guyader et al., 1987; Masson et al., 1989). These observations establish the importance of this portion of the *tnpD* sequence in transposase function and suggest that the carboxyl-terminal end of the molecule is not required for catalytic function, but for binding the *tnpA* protein to form the active transposase complex. These functions of the postulated *tnpA-tnpD* complex in the initial excision of the *Spm* element from the donor site are represented diagrammatically in Figure 6A.

The *tnpA* protein has been shown to bind both in vitro and in vivo to certain of the repeats within both the 3′ and 5′ *subterminal repetitive regions* (Gierl et al., 1988b; Grant et al., 1990). And although there is both genetic and molecular evidence that *tnpA* has regulatory functions (Masson et al., 1987; Banks et al., 1988; Fedoroff, 1989b), the results of the present experiments demonstrate that the *tnpA* gene product participates directly in transposition. In view of its ability to bind to the subterminal repeats (Gierl et al., 1988b), we postulate that *tnpA* binding to both *subterminal repetitive regions* brings element ends together for transposition. Our finding that there are similarities between the subterminal consensus repeat sequence and a sequence at or near an *Spm* insertion site (Figure 5) suggests that *tnpA* protein also binds the excised element to the recipient site (Figure 6B). Because the similarity between the insertion site and the subterminal consensus repeat sequence can be centered on the insertion site, immediately adjacent to it or at some distance from the insertion site, we postulate that *tnpA*-insertion site binding can occur throughout the length of the *subterminal repetitive region*. Thus, we propose that the function of *tnpA* protein is to assemble a complex containing both element ends and the recipient site, making use of the multiple *tnpA* binding sites at each element end (Figure 6B).

Our ability to recover end-joined *Spm* termini by IPCR of DNA from cells with transposing elements, but not without IPCR manipulations, implies that there is a linear form of the *Spm* element with blunt, ligatable ends, but little or none of the free circular form. This, in turn, implies that the element is excised by double-stranded cuts precisely at element ends as the first step in transposition (Figure 6A). Although negative results in efforts to amplify very rare molecules by PCR techniques cannot be considered conclusive, our failure to recover end-joined *Spm* termini from unligated DNA bears the implication that circular transposition intermediates are not abundant, as they are in cells with transposing *Mu* elements (Sundaresan and Freeling, 1987).

If double-strand breaks at the ends of *Spm* elements initiate transposition, the free ends of the adjacent DNA must initially have a similar structure. Yet empty donor sites are characterized by small deletions and duplications at the former insertion site. Two different mechanisms have been proposed to explain the structure of empty donor sites. Saedler and Nevers (1985) proposed a staggered-cleavage-repair mechanism, whereas Coen et al. (1986) postulated the formation of a hairpin at each free cleaved end of the donor site, followed by recleavage, repair, and rejoining at the donor site. In the light of our recovery of precisely end-joined *Spm* termini, it appears more likely that the initial fate of the free blunt donor site ends is to be ligated in a hairpin structure, as postulated by Coen et al. (1986); therefore, we have depicted the cut donor site ends as hairpins (Figure 6A). Subsequent single-strand cleavage and repair of such hairpins readily give rise to the types of inverted duplications and deletions observed at empty donor sites (Figure 2).

The structure of the *Spm* insertion site in the maize *a-m2-7991A1* allele originally isolated by McClintock pro-

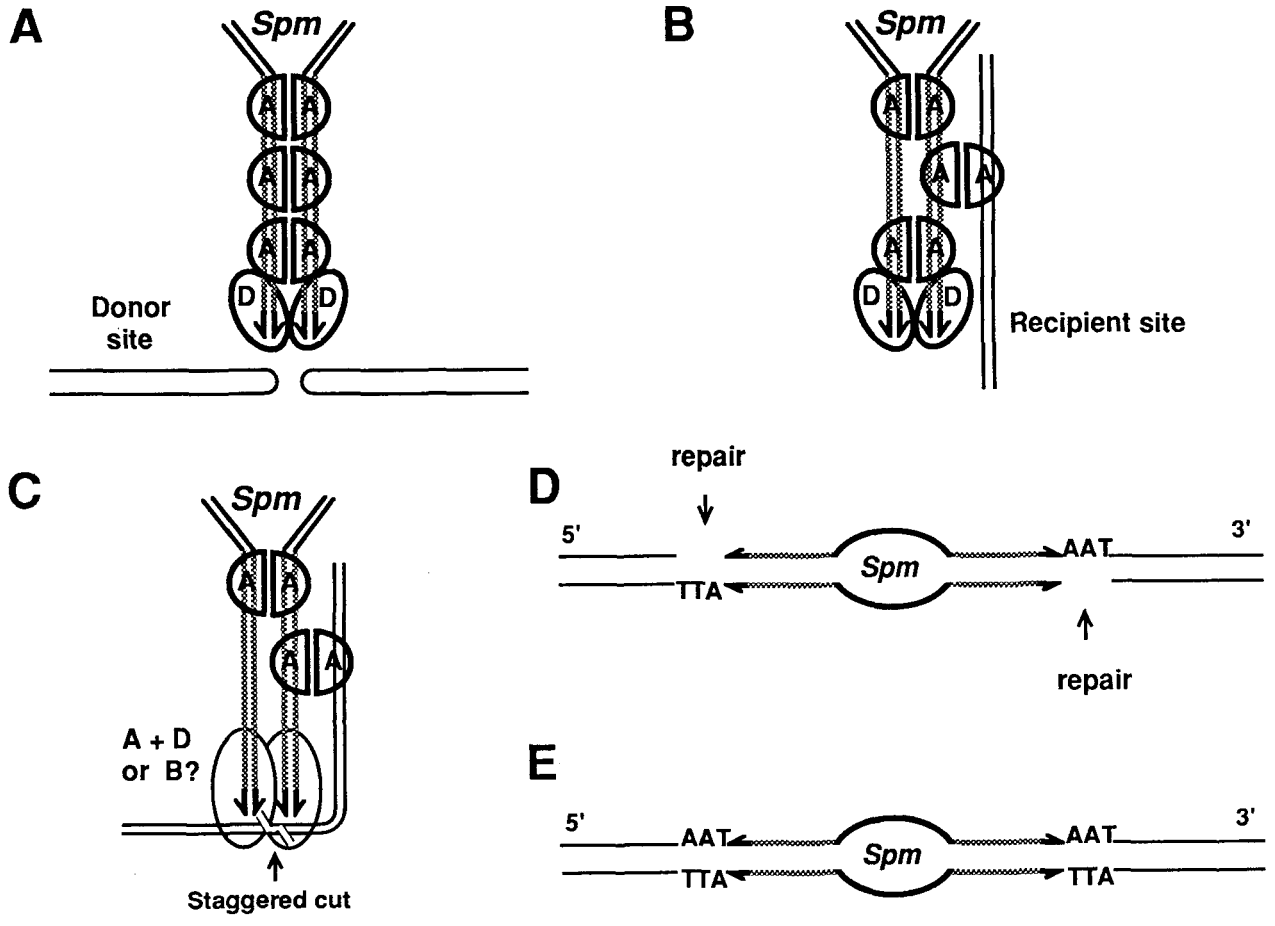

Figure 6. A Model of *Spm* Transposition.

(A) Excision of the *Spm* element is proposed to occur after *tnpA*-mediated association of the element ends, followed by binding of *tnpD* to both *tnpA* and the element's IRs. Free donor site ends are represented as hairpin structures arising by ligation of 5′ to 3′ ends (see text).

(B) Transposition complexes are proposed to form by *tnpA*-mediated association of the *Spm-tnpA-tnpD* complex with a new DNA sequence having some homology to the *tnpA* binding site within the element.

(C) Staggered cleavage at the recipient site is postulated to occur either within or at some distance from the site of homology with the *tnpA* binding sequence and require the participation of either the *tnpA-tnpD* complex or the *tnpB* protein.

(D) and **(E)** Insertion of the *Spm* element is postulated to occur by ligation of one strand of the element to the recipient site at each end **[(D)]** followed by repair synthesis and religation to generate the 3-bp flanking duplication **[(E)]**. Each line corresponds to one strand of a DNA duplex. The *Spm* element's IRs are represented by split arrows and the *subterminal repetitive regions* are represented by stippled lines.

vides support for the foregoing postulates about the initial steps in the transposition process (Masson et al., 1987). In this allele, there is an inverted duplication (AAT) of the 3-bp sequence (ATT) immediately adjacent to the 3′ end of the *Spm* element. The duplication is likely to have been generated as a result of an abortive transposition event in which only one element end was cleaved and the donor site underwent hairpin formation, cleavage, and repair to generate the inverted duplication and was then religated to the unaltered blunt-ended terminus of the element.

The observation that transposed *Spm* elements are flanked by 3-bp target site duplications (Schwarz-Sommer et al., 1985) implies a 3-bp staggered cleavage of the target site (Figure 6C). This must be followed by ligation of one strand of the element to target site, repair, and religation of the second strand to generate the flanking duplication (Figure 6D). In the present model, we postulate that target site selection involves recognition of *tnpA* binding site similarity within a rather large region, but that the precise insertion sequence may or may not resemble the

tnpA binding site. Rather, once the element ends and the target DNAs are associated by a *tnpA* bridge, we postulate that cleavage can occur at some distance from the *tnpA* binding site (Figures 6B and 6C).

We do not yet know whether an additional element-encoded gene product is required for correct insertion of the transposed element. Both of the transposed elements cloned out of DNA from a callus in which transposition was promoted by *tnpA* and *tnpD* had terminal deletions and neither was flanked by the 3-bp direct duplication typical for transposed *Spm* elements. The deletion could have occurred either during excision, as implied by the structure of the deleted end-joined *Spm* termini (Figure 3C), so that the two different insertions arose from the same deleted element. Alternatively, the deletions may have occurred after insertion of an intact element, although the identity of the intraelement deletion end points within the two reinserted copies makes this alternative less likely. Such insertion and deletion anomalies may be consequent on expression of both *tnpA* and *tnpD* cDNAs from strong promoters, with the result that the *tnpD* gene product is present at a much higher concentration relative to the *tnpA* gene product in cDNA-containing cells than it is in cells containing a complete *Spm* element. If this interpretation is correct, then the only element-encoded proteins in the transposase complex are *tnpA* and *tnpD*. Thus, staggered cleavage of the insertion site, ligation of element ends to the recipient site, and subsequent repair of single-stranded gaps (Figures 6C, 6D, and 6E) require no additional *Spm*-encoded proteins, although additional cellular enzymes are likely to be involved.

An alternative possibility, which we have also incorporated into the transposition model depicted in Figure 6, is that another *Spm*-encoded gene product participates in staggered cleavage at the recipient site and insertion of the element. In the model, we attribute this function to *tnpB* because (1) it has a complete *tnpA* domain, (2) it has the putative DNA binding domain common to other elements of the *Spm* superfamily (Gierl et al., 1988a; Rhodes and Vodkin, 1988), and (3) it lacks the ORF2 domain that appears to be important for *tnpD* function (Figure 6C).

Other Element-Encoded Gene Products

Despite its virtual structural identity with the *tnpD* cDNA, the *tnpC* cDNA cannot replace it in promoting *dSpm* transposition in tobacco. The protein-coding sequences of the two cDNAs differ by the removal of a 90-bp intron from the 5′ region of ORF2 in the *tnpC* but not the *tnpD* cDNA. Thus, the two putative proteins differ by only 30 amino acids. Cuypers et al. (1988) have analyzed an internally deleted *dSpm* element, designated *En-I102*, which gives rise to an abundant transcript that encodes an internally deleted ORF1-ORFA fusion protein. The genetic effect of the element is to reduce the transposition frequency of a standard element present in the same genome, suggesting that the protein encoded by this element may actually be a competitive inhibitor of the element's transposase. Similarly, transposition of the *Drosophila* P element is negatively regulated by a repressor protein that is encoded by an alternatively spliced version of the same element transcript that encodes the transposase and, therefore, shares much of its protein sequence (Rio et al., 1986; Robertson and Engels, 1989; Misra and Rio, 1990). Also, a truncated form of the vp16 protein inhibits activation of early transcription of HSV-1 by the vp16 protein in mammalian cells (Friedman et al., 1988). We hypothesize that *tnpC* (and perhaps *tnpB* as well) may be such a negative regulator of element activity by virtue of its extensive sequence overlap with other element-encoded gene products.

METHODS

Mutagenesis of *Spm* and cDNA Construction

A frameshift mutation was introduced in the *tnpA* coding sequence by cleaving a Bluescript KS$^-$ plasmid containing a cloned *Spm* element with SpII, filling in, and religating the cut ends (Sambrook et al., 1989). The mutant *Spm* element was cloned into the unique XhoI site of the pMON754-XhoI binary vector (Rogers and Klee, 1987). Other *Spm* frameshift mutations used here were described in Masson et al. (1989). A *tnpA* cDNA was constructed by completing a partial cDNA clone obtained by screening a maize cDNA library prepared in λgt11 (Maniatis et al., 1982; Pereira et al., 1986; Masson et al., 1987). The *tnpA* cDNA was completed by adding oligonucleotides synthesized in vitro, using conventional cloning techniques (Maniatis et al., 1982; R. Surosky, K.C. Chow, and N. Fedoroff, unpublished data). Full-length *tnpC* and *tnpD* cDNAs were constructed from the PCR-amplified, cloned cDNA fragments described in Masson et al. (1989) using conventional cloning procedures (Sambrook et al., 1989). The ORFA sequences were deleted from the full-length *tnpC* and *tnpD* cDNAs to produce the truncated cDNAs by mutagenizing the spacer region separating ORFC/D from ORFA (Masson et al., 1989; Sambrook et al., 1989). This was done using an oligonucleotide overlapping the spacer region and the 5′ end of the *tnpA* sequence (5′-CATAATTTCTAGACTCGAGCAGGATGG-3′) that introduced an XbaI and an XhoI site into the spacer. This yielded truncated *tnpC* and *tnpD* cDNAs flanked by XbaI sites. A mutated *tnpD* cDNA (*tnpD**) with two 1-bp deletions at the end of ORF2 [G residues deleted at positions 4718 and 4819 of the *Spm* sequence (Masson et al., 1987)] was constructed by replacing the wild-type ORF2 sequences of the full-length *tnpD* cDNA with a cDNA fragment carrying the mutations recovered during the PCR amplification of the *tnpD* cDNA (Masson et al., 1989).

The *tnpA*, the truncated *tnpC* and *tnpD*, and the full-length *tnpD** cDNAs were inserted between the CaMV 35S promoter and NOS terminator sequences of the pBI221 plasmid (Clontech Co., Palo Alto, CA), generating *tnpA*, *tnpC*, *tnpD*, and *tnpD** plant expression cassettes. All of the cDNA expression cassettes are flanked by AvaI sites and were moved into the StuI site of the pMON754 binary vector for *Agrobacterium*-mediated T-DNA transformation of plants (Rogers and Klee, 1987), generating the

p*tnpA*, p*tnpC*, p*tnpD*, and p*tnpD** plasmids, respectively. The structure of the plasmids was verified by resequencing the cDNAs before (*tnpA* and *tnpD*) or after (*tnpC* and *tnpD**) their introduction into the pMON754 plasmid. The p*dSpm*\GUS reporter plasmid was previously described (Masson and Fedoroff, 1989). The p*tnpA*::*dSpm*\GUS plasmid was constructed by inserting the *tnpA* expression cassette into the unique ClaI site of p*dSpm*\GUS.

All plasmids were transferred to *Agrobacterium tumefaciens* strains 3111SE or TiT37SE. These were used to infect tobacco SR1 leaf discs as described in Rogers et al. (1988). The structure of the *tnpC* cDNA present in the *Agrobacterium* culture used to transform tobacco was verified once again by recloning the plasmid into *Escherichia coli* and sequencing the *tnpC* cDNA, as described by Masson et al. (1989). Expression of *tnpA*, *tnpC*, *tnpD*, and *tnpD** was verified by RNA gel blot analysis of poly A^+ RNA from calli transformed with plasmids carrying the respective cDNAs.

Analysis of *dSpm* Transposition in Transgenic Tobacco Calli

Transgenic tobacco calli were selected and assayed for β-glucuronidase activity as described in Masson and Fedoroff (1989). DNA was extracted from transfected calli as described by Dellaporta et al. (1985). Empty donor site sequences were PCR amplified using oligonucleotides primers 101 and 102 (Table 3), as described in Masson et al. (1989). Amplified fragments were cloned in Bluescript KS^- (Stratagene Co.) and sequenced, using the Sequenase II sequencing kit (U.S. Biochemical), as recommended by the supplier.

Transposed copies of *dSpm* were cloned by IPCR using a modification of the procedure described by Ochman et al. (1988). One microgram of total DNA was partially cleaved with Sau3AI and religated at a concentration of 0.5 μg/mL (Ochman et al., 1988). The religated DNA was cleaved with XhoI, which cuts p*tnpA*::*dSpm*\GUS at two sites immediately adjacent to the *dSpm* element. This step was included in the IPCR protocol to avoid the preferential amplification of nontransposed *dSpm* sequences. Because *dSpm* excision is detectable in many small sectors of cells within a callus (see Masson and Fedoroff, 1989) and each sector may carry a reinserted element at a different location, only a small fraction of the DNA molecules in the DNA prepared from a callus is expected to contain any given newly reinserted element, whereas the majority will contain the element at its original insertion site within the transforming T-DNA.

The cleaved, circularized DNA was PCR amplified according to Masson et al. (1989), using oligonucleotides 103 (*Spm* nucleotides 172 to 199) and 104 (*Spm* nucleotides 7913 to 7944) as primers (Table 3). The orientation of the primers is such that they prime DNA synthesis away from the element's center, toward and beyond its termini. The PCR-amplified products were purified on a Sephadex CL-6B column (Boehringer Mannheim) as recommended by the supplier and subjected to a second round of PCR amplification using oligonucleotides 105 (*Spm* nucleotides 121 to 145) and 106 (*Spm* nucleotides 8020 to 8042) or 107 (*Spm* nucleotides 1 to 28) and 108 (*Spm* nucleotides 8020 to 8037) as primers (Table 3). PCR products were further purified on Sephadex CL-6B or Sephacryl S-400 (Boehringer Mannheim), cloned into the Bluescript KS^- plasmid (Stratagene Co.), and sequenced using the Sequenase II sequencing kit (U.S. Biochemical). When XhoI was omitted from the IPCR protocol, fragments with homology to both *Spm* termini and the GUS gene in the p*tnpA*::*dSpm*\GUS plasmid were readily amplified. Several such fragments were cloned and sequenced to verify that the *Spm* termini were flanked by donor site T-DNA plasmid sequences. The ability to recover such fragments provided an internal control for the efficacy of the IPCR procedure.

DNA was extracted from untransformed and transformed tobacco plants and calli (Dellaporta et al., 1985), digested with EcoRI, electrophoretically fractionated on a 0.7% agarose gel, transferred to GeneScreen-*Plus* membrane (Du Pont), and hybridized to a gel-purified DNA fragment labeled with ^{32}P by the random-priming procedure (U.S. Biochemical).

ACKNOWLEDGMENTS

This work was supported by National Institute of Health Grant 5-R37-GM34296. Patrick Masson is a Chercheur Qualifié of the Fonds National de la Recherche Scientifique of Belgium. We thank George Rutherford and Kyr Gaganidze for excellent technical support and Jo Ann Banks, King-Cheun Chow, Vladimir Kolosha, and Tami Seddon for discussions.

Received September 6, 1990; accepted October 26, 1990.

REFERENCES

Banks, J.A., Masson, P., and Fedoroff, N. (1988). Molecular mechanisms in the developmental regulation of the maize *Suppressor-mutator* transposable element. Genes Dev. **2,** 1364–1380.

Coen, E.S., Carpenter, R., and Martin, C. (1986). Transposable elements generate novel spatial patterns of gene expression in *Antirrhinum majus*. Cell **47,** 285–296.

Cuypers, H., Dash, S., Peterson, P.A., Saedler, H., and Gierl, A. (1988). The defective *En-I102* element encodes a product reducing the mutability of the *En/Spm* transposable element system of *Zea mays*. EMBO J. **7,** 2953–2960.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1985). Maize DNA miniprep. In Molecular Biology of Plants: A Laboratory Course Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp. 36–37.

Fedoroff, N.V. (1983). Controlling elements in maize. In Mobile Genetic Elements, J. Shapiro, ed (New York: Academic Press), pp. 1–63.

Fedoroff, N.V. (1989a). Maize transposable elements. In Mobile DNA, D.D. Berg and M.M. Howe, eds (Washington, DC: ASM Press), pp. 375–411.

Fedoroff, N. (1989b). The heritable activation of cryptic *Suppressor-mutator* elements by an active element. Genetics **121,** 591–608.

Friedman, A., Triezenberg, S., and McKnight, S. (1988). Expression of a truncated viral *trans*-activator selectively impedes lytic infection by its cognate virus. Nature **335,** 452–454.

Gierl, A., Cuypers, H., Lütticke, S., Pereira, A., Schwarz-Sommer, Z., Dash, S., Peterson, P.A., and Saedler, H. (1988a).

Structure and function of the *En/Spm* transposable element system of *Zea mays*: Identification of the suppressor component of *En*. In Plant Transposable Elements, O. Nelson, ed (New York: Plenum Press), pp. 115–119.

Gierl, A., Lütticke, S., and Saedler, H. (1988b). *TnpA* product encoded by the transposable element *En-1* of *Zea mays* is a DNA binding protein. EMBO J. **7,** 4045–4053.

Grant, S.R., Gierl, A., and Saedler, H. (1990). *En/Spm*-encoded *tnpA* protein requires a specific target sequence for suppression. EMBO J. **9,** 2029–2035.

Guyader, M., Emerman, M., Sonigo, P., Clavel, F., Montagnier, L., and Alizon, M. (1987). Genome organization and *trans*-activation of the human immunodeficiency virus type 2. Nature **326,** 662–669.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Masson, P., and Fedoroff, N.V. (1989). Mobility of the maize *Suppressor-mutator* element in transgenic tobacco cells. Proc. Natl. Acad. Sci. USA **86,** 2219–2223.

Masson, P., Surosky, R., Kingsbury, J., and Fedoroff, N.V. (1987). Genetic and molecular analysis of the *Spm-dependent a-m2* alleles of the maize *a* locus. Genetics **177,** 117–137.

Masson, P., Rutherford, G., Banks, J., and Fedoroff, N. (1989). Essential large transcripts of the maize *Spm* transposable element are generated by alternative splicing. Cell **58,** 755–765.

Misra, S., and Rio, D.C. (1990). Cytotype control of *Drosophila* P element transposition: The 66 kd protein is a repressor of transposase activity. Cell **62,** 269–284.

Ochman, H., Gerber, A.S., and Hartl, D.L. (1988). Genetic applications of an inverse polymerase chain reaction. Genetics **120,** 621–623.

Pereira, A., and Saedler, H. (1989). Transpositional behavior of the maize *En/Spm* element in transgenic tobacco. EMBO J. **8,** 1315–1321.

Pereira, A., Cuypers, H., Gierl, A., Schwarz-Sommer, Z., and Saedler, H. (1986). Molecular analysis of the *En/Spm* transposable element system of *Zea mays*. EMBO J. **5,** 835–841.

Ralston, E.J., English, J.J., and Dooner, H.K. (1988). Sequence of three *bronze* alleles of maize and correlation with the genetic fine structure. Genetics **119,** 185–197.

Rhodes, P.R., and Vodkin, L.O. (1988). Organization of the *Tgm* family of transposable elements in soybean. Genetics **120,** 597–604.

Rio, D.C., Laski, F.A., and Rubin, G.M. (1986). Identification and immunochemical analysis of biologically active *Drosophila* P element transposase. Cell **44,** 21–32.

Robertson, H.M., and Engels, W.R. (1989). Modified P elements that mimic the P cytotype in *Drosophila melanogaster*. Genetics **123,** 815–824.

Rogers, S.G., and Klee, H. (1987). Pathways to plant genetic manipulation employing *Agrobacterium*. In Plant DNA Infection Agents, T. Hohn and J. Schell, eds (New York: Springer-Verlag), pp. 179–203.

Rogers, S.G., Horsch, R.B., and Fraley, R.T. (1988). Gene transfer in plants: Production of transformed plants using Ti plasmid vectors. In Methods for Plant Molecular Biology, A. Weissbach and H. Weissbach, eds (New York: Academic Press), pp. 425–436.

Saedler, H., and Nevers, P. (1985). Transposition in plants: A molecular model. EMBO J. **4,** 585–590.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Schiefelbein, J.W., Raboy, V., Kim, H.Y., and Nelson, O.E. (1988). Molecular characterization of *Suppressor-mutator* (*Spm*)-induced mutations at the *bronze-1* locus in maize: The *bz-m13* alleles. In Plant Transposable Elements, O.E. Nelson, ed (New York: Plenum Press), pp. 261–278.

Schwarz-Sommer, Z., Gierl, A., Cuypers, H., Peterson, P.A., and Saedler, H. (1985). Plant transposable elements generate the DNA sequence diversity needed in evolution. EMBO J. **4,** 591–597.

Schwarz-Sommer, Z., Shepherd, N., Tacke, E., Gierl, A., Rohde, W., Leclercq, L., Mattes, M., Berndtgen, R., Peterson, P.A., and Saedler, H. (1987). Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays*. EMBO J. **6,** 287–294.

Sundaresan, V., and Freeling, M. (1987). An extrachromosomal form of the *Mu* transposons of maize. Proc. Natl. Acad. Sci. USA **84,** 4924–4928.

Tacke, E., Schwarz-Sommer, Z., Peterson, P.A., and Saedler, H. (1986). Molecular analysis of states of the *A* locus of *Zea mays*. Maydica **31,** 83–91.

Vodkin, M.H., and Vodkin, L.O. (1989). A conserved zinc finger domain in higher plants. Plant Mol. Biol. **12,** 593–594.