TnpA *trans*-Activates Methylated Maize *Suppressor-mutator* Transposable Elements in Transgenic Tobacco

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ABSTRACT

The maize *Suppressor-mutator* (*Spm*) transposable element is subject to epigenetic inactivation in transgenic tobacco, as it is in maize. *Spm* inactivation in tobacco is correlated with increased methylation of sequences near the element's transcription start site. To determine whether element-encoded gene products can promote the reactivation of an inactive element, we investigated the effects of introducing individual CaMV 35S promoter-driven cDNAs for *tnpA*, *tnpB*, *tnpC* and *tnpD*, the element's four known protein-coding sequences. Introduction of the *tnpA* cDNA promoted the reactivation of the inactive resident *Spm* element, as judged by the appearance of regenerants with very early excision events and transposed elements. By contrast, the *tnpB*, *tnpC* and *tnpD* cDNAs had no affect on the activity of the resident *Spm* element. Similar results were obtained when the element-encoded cDNAs were introduced either by Agrobacterium-mediated retransformation or by a genetic cross. Reactivation of an inactive *Spm* by the *tnpA* cDNA is accompanied by reduced methylation of several methylation-sensitive restriction sites near the element's transcription start site. Maintenance of the reactivated *Spm* element in an active state requires the continued presence of the *tnpA* cDNA. Elimination of the *tnpA* cDNA locus by genetic segregation generally results in decreased element activity, as judged by a low frequency of excision events, and is accompanied by increased methylation of the element's 5′-end. Exceptions resembling the phenomenon of "presetting" are also observed in which progeny plants that did not receive the *tnpA* cDNA locus after meiotic segregation maintain high excision activity and exhibit low methylation levels.

THE *Spm* element was originally identified and studied genetically by McCLINTOCK (1954) [reviewed in Fedoroff (1989a)]. In recent years, the *Spm* element and the closely related *Enhancer* element identified by PETERSON (1953) have been cloned, sequenced and their activity reconstituted in transgenic plant systems (PEREIRA *et al.* 1986; MASSON *et al.* 1987; FREY *et al.* 1990; MASSON, STREM and FEDOROFF 1991). The *Spm* element is 8.3 kb long, has 13-bp terminal inverted repeats (TIR), and creates a 3-bp duplication upon transposition. The element encodes four alternatively spliced transcripts which code for a total of four proteins, designated TnpA, TnpB, TnpC and TnpD (MASSON *et al.* 1989). A combination of TnpA and TnpD gene products is both necessary and sufficient to promote transposition of the element, as well as of transposition-defective derivatives, termed *dSpm* elements.

McCLINTOCK (1957–1959, 1971) reported that *Spm* elements can undergo a type of inactivation that is both heritable and reversible. Active *Spm* elements in maize are methylated at sites throughout the element, but not in the immediate vicinity of the transcription start site (BANKS, MASSON and FEDOROFF 1988). Inactivation is correlated with increased methylation of sites both upstream of the element's transcription start site, as well as immediately downstream from it, within the extremely GC-rich first untranslated exon (BANKS and FEDOROFF 1989). *Spm* elements can exist either in an unstably inactive form that shows low heritability or in a very stably inactive form, termed *cryptic*, that shows high heritability. These differ primarily by the extent of methylation of the GC-rich first exon sequences: the greater the heritability of the inactive state, the greater the extent of first exon methylation (BANKS, MASSON and FEDOROFF 1988).

McCLINTOCK (1957–1959, 1971) observed that an inactive *Spm* could be reactivated transiently when a second, active element was introduced by a genetic cross. Furthermore, the *trans*-activating *Spm* element can have both a transient and a heritable effect (FEDOROFF and BANKS 1988; BANKS, MASSON and FEDOROFF 1988). The inactive *Spm* element is transcriptionally active in the presence of the *trans*-activating *Spm* and shows reduced methylation in the vicinity of the transcription start site (BANKS, MASSON and FEDOROFF 1988; KOLOSHA and FEDOROFF 1992). In addition, the presence of the *trans*-activating element promotes the heritable reactivation of both inactive and *cryptic Spm* elements (FEDOROFF 1989b). Because the *trans*-activating elements used in these experiments contained deletions affecting the coding se-

quences of all of the element encoded proteins except that of TnpA, it was postulated that TnpA is responsible for promoting the reactivation of inactive elements and that it functions as a positive autoregulatory protein in maintaining element activity (MASSON *et al.* 1987; FEDOROFF 1989b; KOLOSHA and FEDOROFF 1992).

To test the hypothesis that TnpA is required for maintenance of *Spm*'s genetic and transcriptional activity, as well as the reactivation of an inactive, methylated element, it was necessary to reconstitute the phenomenon of epigenetic inactivation and reactivation of the element in a transgenic system. Using transgenic tobacco lines containing both an intact *Spm* element and an excision assay construct in which a *dSpm* element resides in the leader sequence of a β-glucuronidase (GUS) gene, disrupting its expression (MASSON and FEDOROFF 1989), we show here that the *Spm* element becomes inactivated in tobacco, as it does in maize (FEDOROFF and BANKS 1988). The ability of the resident *Spm* element to promote excision of the *dSpm* from the GUS gene, giving somatic GUS-positive sectors, is a function of its copy number, and TnpA is the rate-limiting gene product. Plants with one or two elements often exhibit element inactivation and the resident inactive *Spm* is reactivated when a CaMV 35S-*tnpA* cDNA is introduced either by Agrobacterium-mediated retransformation or by a genetic cross. Neither the CaMV 35S-*tnpB*, the CaMV 35S-*tnpC*, nor the CaMV 35S-*tnpD* cDNAs affect the activity of the resident inactive *Spm*.

We show that *Spm* inactivation in transgenic tobacco is correlated with *de novo* methylation of the introduced element and that TnpA-mediated reactivation is accompanied by reduced methylation of the element's 5′ end. We present supporting molecular evidence that *Spm* reactivation reflects *dSpm* excision and transposition. We show that the continued presence of TnpA is generally necessary for the maintenance of the reactivated, 5′-terminal demethylated state of the *Spm* element. However, we also note that there are exceptions, resembling MCCLINTOCK's "presetting" effect (MCCLINTOCK 1963–1965), among the progeny of plants containing the 35S-*tnpA* cDNA. Such progeny lack the 35S-*tnpA* cDNA locus as a consequence of meiotic segregation, but maintain a high level of *Spm* activity and a low level of methylation, suggesting that TnpA is also responsible for promoting the heritable reactivation of an inactive *Spm*.

MATERIALS AND METHODS

Excision assay constructs and tobacco lines. The *Spm* and *dSpm* excision assay constructs were those previously described (MASSON and FEDOROFF 1989). The *Spm* clone used here lacked the third *Eco*RI site in the fourth intron of *tnpA*, as described for *SpmE3* in MASSON, STREM and FEDOROFF (1991). *Spm* was cloned into the single *Xho*I site of the binary vector pMON754 (kindly provided by S. ROGERS, Monsanto Co.), carrying the hygromycin phosphotransferase gene under the control of the cauliflower mosaic virus (CaMV) 19S promoter (ROGERS and KLEE 1987) as a selectable marker. The binary vector pMON530 (kindly provided by S. ROGERS), carrying the Tn*5* neomycin phosphotransferase II gene as a selectable marker under the control of the nopaline synthase promoter (SHAH *et al.* 1986), was used for the *dSpm* excision assay construct. A *dSpm* element was created by deletion of the two internal *Eco*RV fragments from *Spm* and inserted between the CaMV 35S promoter and the β-glucuronidase (GUS) reporter gene for the excision assay. Both binary vectors were transferred to *Agrobacterium tumefaciens* strain TiT37SE. *Nicotiana tabacum* SR1 leaf explants were cotransformed with the two different Agrobacterium populations, each harboring one of the two plasmids (ROGERS, HORSCH and FRALEY 1988). A number of cotransformed calli were selected, giving rise to callus lines *5*, *6*, *7*, *8*, *10* and *35*, respectively.

Phenotypic assay for *dSpm* excision: The visual assay for *dSpm* excision has been described previously (MASSON *et al.* 1989). Briefly, pieces of calli or regenerated shoots were incubated overnight in 0.5 mg/ml 5-bromo-4-choloro-3-indolyl β-D-glucuronic acid (X-glu; Clontech) at 37° (JEFFERSON, KAVANAGH and BEVAN 1987). The X-glu is converted to an insoluble blue dye in the presence of GUS in sectors of plant tissue where *dSpm* has excised.

Binary vectors with cDNA constructs for tobacco retransformation: The cDNAs derived from *Spm*-specific transcripts were described before (MASSON, STREM and FEDOROFF 1991). The CaMV 35S-*tnpA*, the CaMV 35S-*tnpB*, the CaMV 35S-*tnpC* and the CaMV 35S-*tnpD* cDNAs were inserted into a modified pCGN1548 (kindly provided by K. MCBRIDE, Calgene Inc.) binary vector carrying the npt II gene under the control of the mannopine synthase promoter as a selectable marker (MCBRIDE and SUMMERFELT 1990). A mutated acetolactate synthase gene (ALS) from *Arabidopsis thaliana*, which confers resistance to the sulfonylurea herbicide chlorsulfuron (HAUGHN *et al.* 1988), was inserted into pGCN1548 as follows. An ALS construct under the control of a double CaMV 35S promoter fused to the $C_{a/b}$ binding protein untranslated leader sequence (kindly provided by J. MAUVAIS, Du Pont Co.) was removed from pUC18 with *Sal*I and blunt-end ligated into the single *Asp*718 site of pCGN1548, designated pCGN1548\ALS, by using conventional cloning procedures (SAMBROOK, FRITSCH and MANIATIS 1989). A single *Asp*718 site flanking ALS was preserved by this procedure. The CaMV 35S-*tnpC* and CaMV 35S-*tnpD* cDNA expression cassettes (MASSON, STREM and FEDOROFF 1991) were removed from pUC19 with *Ava*I and blunt-end ligated into the single *Asp*718 site of the pCGN1548\ALS binary vector. The CaMV 35S-*tnpA* cDNA expression cassette was removed from the yeast vector pRS316 (SIKORSKI and HIETER 1989) with *Sal*I and *Bam*HI, and blunt-end ligated into the *Asp*718 site of pCGN1548\ALS. The CaMV 35S-*tnpB* expression cassette was removed from bluescript with *Xho*I and *Sma*I, and blunt-end ligated into the *Asp*718 site of pCGN1548\ALS. The binary vectors were transferred to Agrobacterium strain LBA4404 and the structure of each vector was verified by Southern blot hybridization analysis.

Retransformation of *Spm*- and *dSpm*-containing tobacco lines: Regenerating plantlets from callus lines *7*, *8* and *35* were grown on medium containing half strength Murashige and Skoog (MS) salts (Sigma), 0.8% agar (Difco), 1.5% sucrose (Sigma), 50 mg/liter i-inositol (Sigma), and 0.2 mg/liter thiamine-HCl (Sigma) in Magenta boxes. The

smallest leaves from each plant were used for Agrobacterium leaf disc transformation using Agrobacterium strain LBA4404 harboring either the *tnpA*, the *tnpB*, the *tnpC* or the *tnpD* cDNA expression cassette, respectively, as described by ROGERS, HORSCH and FRALEY (1988). The infected leaf discs were grown on medium containing MS salts, 3% sucrose, 100 mg/liter i-inositol, 0.4 mg/liter thiamine-HCl, 1.0 mg/liter 6-benzylaminopurine (GIBCO), 0.1 mg/liter naphthaleneacetic acid (GIBCO), 500 mg/liter carbenicillin (Sigma) and were subjected to triple selection with 20 mg/liter hygromycin B (Calbiochem), 50 mg/liter kanamycin monosulfate (Sigma), and 50 μg/liter chlorsulfuron (kindly provided by J. MAUVAIS, Du Pont Co.). Resistant callus lines were propagated on the same medium containing only 20 μg/liter chlorsulfuron.

Analysis of genomic DNA: DNA was isolated from tobacco plants grown in Magenta boxes or growth chambers as described by DELLAPORTA, WOOD and HICKS (1985). Genomic DNA (2 μg) was digested overnight with 20–50 units of restriction endonucleases as recommended by the supplier (Boehringer Mannheim, New England Biolabs). An internal control for completeness of digestion was employed as described by BANKS, MASSON and FEDOROFF (1988) when methylation sensitive endonucleases were used. Briefly, 10% of the restriction enzyme reaction mix containing the genomic DNA, the enzyme buffer, and the restriction enzyme, respectively, was removed and incubated with ^{32}P-labeled linear plasmid DNA containing at least one restriction site of interest at some distance from the cut ends. After an overnight incubation, the plasmid DNA was fractionated on an 0.5% agarose gel, dried down and exposed to x-ray film. The completeness of digestion of the labeled plasmid in the presence of genomic DNA was compared to a control digest and to undigested labeled plasmid, respectively. The genomic DNA digests were further processed only after monitoring that the individual DNA preparations did not inhibit complete plasmid digestion. The digested genomic DNA was fractionated on 0.7% agarose gels and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were hybridized with Geneclean (Bio 101)-purified DNA fragments labeled to a specific activity of 1×10^9 cpm/μg DNA with [^{32}P]dCTP by the random primer labeling method as recommended by the supplier (Amersham).

Processed x-ray films of Southern blot hybridizations involving methylation- sensitive restriction enzymes were scanned by a computing densitometer (Molecular Dynamics) and the intensity of each band of interest above background was determined separately. The percent cleavage of a particular methylation-sensitive restriction site was calculated by comparing the relative intensities of cleaved and uncleaved restriction fragments.

Segregation analysis of retransformed plants: Plants from different retransformed callus lines were grown to maturity in a growth chamber with 16 hr light/8 hr dark period at 25°. All developing flowers on the plants regenerated from callus lines *8* and from the retransformed plants *8*-45, *8*-47 and *8*-49 were male sterile. This might be due to the age of callus line *8* (2 years) or attributable to the initial T-DNA transformation events. By selecting anthers with slightly more pollen, manually selfed flowers gave pods with an average of only 20 seeds and a significant number of seeds could be obtained only by outcrossing with wild type SR1 pollen. The outcrossed progeny remained male sterile. The seeds were surface sterilized in 1% sodium hypochlorite for 15 min, washed three times with sterile water, and spread onto 0.8% agar plates containing half strength MS salts, 50 mg/liter i-inositol, and 0.2 mg/liter thiamine-HCl. Different antibiotics were added to the medium in order to analyze each resistence marker separately and in various combinations. Hygromycin B (20 mg/liter) was used to select for plants containing *Spm* T-DNA inserts, and 50 mg/liter kanamycin to select for plants containing *dSpm* excision assay T-DNA inserts. Plates containing the seeds were stored for 2 days at 4° in order to synchronize germination and scored 2 weeks after germination at room temperature. For retransformed lines, the number of insertion sites was determined genetically on selective media and the total number of copies was confirmed by Southern blot analysis. For example, the number of *Spm* (linked to hygromycin) and *dSpm* (linked to kanamycin) insertion sites in plants from callus line *8* was determined by germinating seeds from one selfed plant separately on hygromycin, kanamycin and hygromycin plus kanamycin. Hygromycin resistant progeny segregated 15:1, indicating two unlinked *Spm* sites, and kanamycin resistant plantlets segregated 3:1, indicating one *dSpm* site. The total copy number at each site was further determined by Southern blot hybridization. This analysis confirmed that line *8* contained two *Spm* copies and one *dSpm* copy. Of the progeny, 73% were resistant to hygromycin and kanamycin, and 10% were kanamycin resistant but hygromycin sensitive. Thus, kanamycin resistance segregated independently from hygromycin resistance, indicating that the *dSpm* insertion site was either unlinked or loosely linked to one of the *Spm* insertion sites. Plants from callus line *8* were retransformed with the CaMV 35S-*tnpA* construct linked to a mutant acetolactate synthase gene (ALS) as the third resistance marker. Copy number analysis by Southern blot hybridization indicated that all the retransformed lines tested contained one copy of the ALS marker. However, the ALS T-DNA also contained a kanamycin-resistance marker (also on the *dSpm* plasmid), which complicated the segregation analysis. Therefore, not all the progeny selected for both hygromycin and kanamycin resistance received the *dSpm* excision assay construct necessary for monitoring GUS positive sectors. Retransformed plants were outcrossed by wild-type SR1. Since the linkage relationship between the ALS T-DNA and the *Spm* and *dSpm* T-DNA were not known, one of three different linkage relationships is possible in the progeny with respect to kanamycin and hygromycin resistance. First, if the four insertion sites (two *Spm* sites, one *dSpm* site, one 35S-*tnpA* site) are unlinked, 56% (9/16) of the germinating plantlets should be resistant to both antibiotics and 67% (6/9) of the resistant plants should have GUS-positive sectors due to the presence of both an *Spm* and the *dSpm* excision assay T-DNAs, but only 50% (3/6) of these plants should contain the CaMV 35S-*tnpA*. Second, if there are two unlinked *Spm* loci and the CaMV 35S-*tnpA* construct is linked to the *dSpm* insertion site, 38% (3/8) of the germinating plants should be resistant to both kanamycin and hygromycin. All of these (3/3) should have GUS positive sectors and contain the CaMV 35S-*tnpA* construct. Third, if the CaMV 35S-*tnpA* construct is linked to one of the *Spm* loci, 63% (5/8) of the germinating plants should be resistant to both kanamycin and hygromycin. Of these, 60% (3/5) should have GUS positive sectors and 67% (2/3) of these plants, in turn, should contain the CaMV 35S-*tnpA*.

Terminology for transgenic plants: Throughout this paper, plants and callus lines will be referred to as follows. Independently transformed callus lines containing *Spm* and *dSpm* are numbered consecutively (*5*, *6*, *7*, *8*, etc.) and the numbers of consecutive plants regenerated from each is added after a hyphen (*8*-45, *8*-47, etc.). Callus lines obtained from plants retransformed with cDNAs contain the number of the parent plant and the cDNA or control vector used in parentheses, followed by a number that refers to the indi-

TABLE 1

***dSpm* excision frequency and *Spm* copy number**

Callus line	Excision frequency	*Spm* copy number
5	0	1
6	0	1
7	Very low	1
8	Medium	1–2
10	High	>5
35	Very high	>5

vidual callus line. For instance, *8*-45(*tnpA*-2) refers to callus line number 2 obtained from retransformation of plant *8*-45 with the *tnpA* cDNA. Callus lines from control vector retransformations are designated ALS in parentheses, followed by a number that refers to the individual callus line. The # sign denotes a particular progeny plant selected from kanamycin- and hygromycin-containing medium. For instance, F1#17 [*8*-47(ALS-2) × SR1] denotes F_1 progeny plant number 17 of a regenerated plant from callus line *8*-47(ALS-2) and outcrossed by an SR1 wild-type plant.

RESULTS

***Spm* activity in transgenic tobacco depends on element copy number:** The activity of *Spm* was assessed by its ability to promote excision of a *dSpm* element from the leader sequence of a bacterial β-glucuronidase (GUS) reporter gene expressed from a CaMV 35S promoter (MASSON and FEDOROFF 1989). Excision events give rise to somatic sectors of tissue expressing the GUS gene, which are detectable as sectors of blue-staining cells in the presence of X-glu. The number of blue sectors is a measure of the frequency of somatic excision, while the sector size reflects the time of excision during development of the organ or tissue tested. Plantlets regenerated from six different callus lines transformed with both the excision assay plasmid and an *Spm* element were tested for *Spm* activity. All of the callus lines initially showed *Spm* activity, as judged by the appearance of GUS-positive sectors throughout the callus tissue. As shown in Table 1, plantlets from two of the lines showed no excision events (lines *5* and *6*), those from two others exhibited low to moderate excision frequencies (lines *7* and *8*), and those from the last two showed early excision events as manifested by large blue sectors (lines *10* and *35*). The *Spm* copy number was determined by blot hybridization analysis (see MATERIALS AND METHODS). Lines *5*, *6* and *7* contain one copy of *Spm*, line *8* contains two copies and lines *10* and *35* contain more than five copies. Plants regenerated from callus lines with one to two copies that showed differences in excision frequency were selected for further studies.

Differences in *Spm* activity are correlated with methylation differences: Plantlets from line *7* had GUS-positive sectors at a very low frequency, while plantlets from line *8* had different sectoring frequencies. Figure 1 shows three examples. Plant *8*-47, *8*-45 and *8*-49 show low (Lfe), medium (Mfe) and high (Hfe) frequencies of excision, respectively. Since all three plants arose from the same callus line and should therefore have the same genotype, the observed variation may be epigenetic, as it is in maize (FEDOROFF and BANKS 1988). Inactivation of *Spm* elements has been observed in maize and correlated with methylation of C residues upstream and downstream of the element's transcriptional start site (BANKS, MASSON and FEDOROFF 1988; BANKS and FEDOROFF 1989). Therefore, an Hfe and an Lfe plant from callus line *8* were analyzed for methylation at the diagnostic *Sal*I site at nucleotide 266, just downstream of the transcription start site at nucleotide 209 (PEREIRA *et al.* 1986). The *Sal*I site was partially methylated in the Lfe plant *8*-47, whereas the site was almost fully cleavable in the Hfe plant *8*-49 (not shown). A negative correlation between element activity and methylation of the *Sal*I site at nucleotide 266 has been reported in maize (BANKS and FEDOROFF 1989).

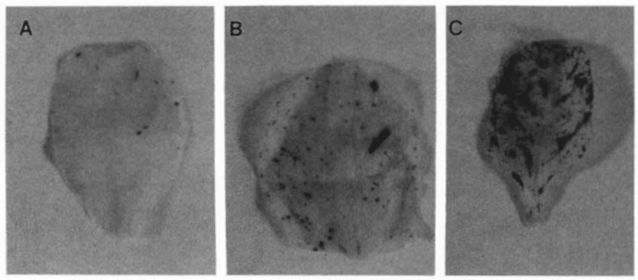

FIGURE 1.—*dSpm* excision phenotype of three plantlets regenerated from the same tobacco callus line. Excision is *trans*-activated by an *Spm* element and is detected in tissues when the incubation substrate (X-glu) is converted to an insoluble blue dye. Tissues were cleared with 100% ethanol for photography. (A) Plant *8*-47, low frequency of excision (Lfe) phenotype. (B) Plant *8*-45, medium frequency of excision (Mfe) phenotype. (C) Plant *8*-49, high frequency of excision (Hfe) phenotype.

Early *dSpm* excision is activated in the presence of the *tnpA* cDNA: It has been reported that a genetically inactive, partially methylated *Spm* element can be *trans*-activated by a second, fully active element in maize (BANKS, MASSON and FEDOROFF 1988). To determine whether element-encoded proteins can *trans*-activate *Spm* in tobacco, plants *8*-45, *8*-47 and *8*-49 were each retransformed individually with *tnpA*, *tnpB*, *tnpC* and *tnpD* cDNAs expressed from the CaMV *tnpB*, 35S promoter and carried on a *A. tumefaciens* transformation vector (see MATERIALS AND METHODS). These cDNAs carry the coding sequences for three of the four proteins encoded by alternatively spliced transcripts of the *Spm* element (MASSON *et al.* 1989). Two of the element-encoded proteins, TnpA and TnpD, have been shown to be necessary and sufficient for element transposition, while no function has been assigned to either TnpB or to TnpC (FREY *et al.* 1990; MASSON, STREM and FEDOROFF 1991). As a control,

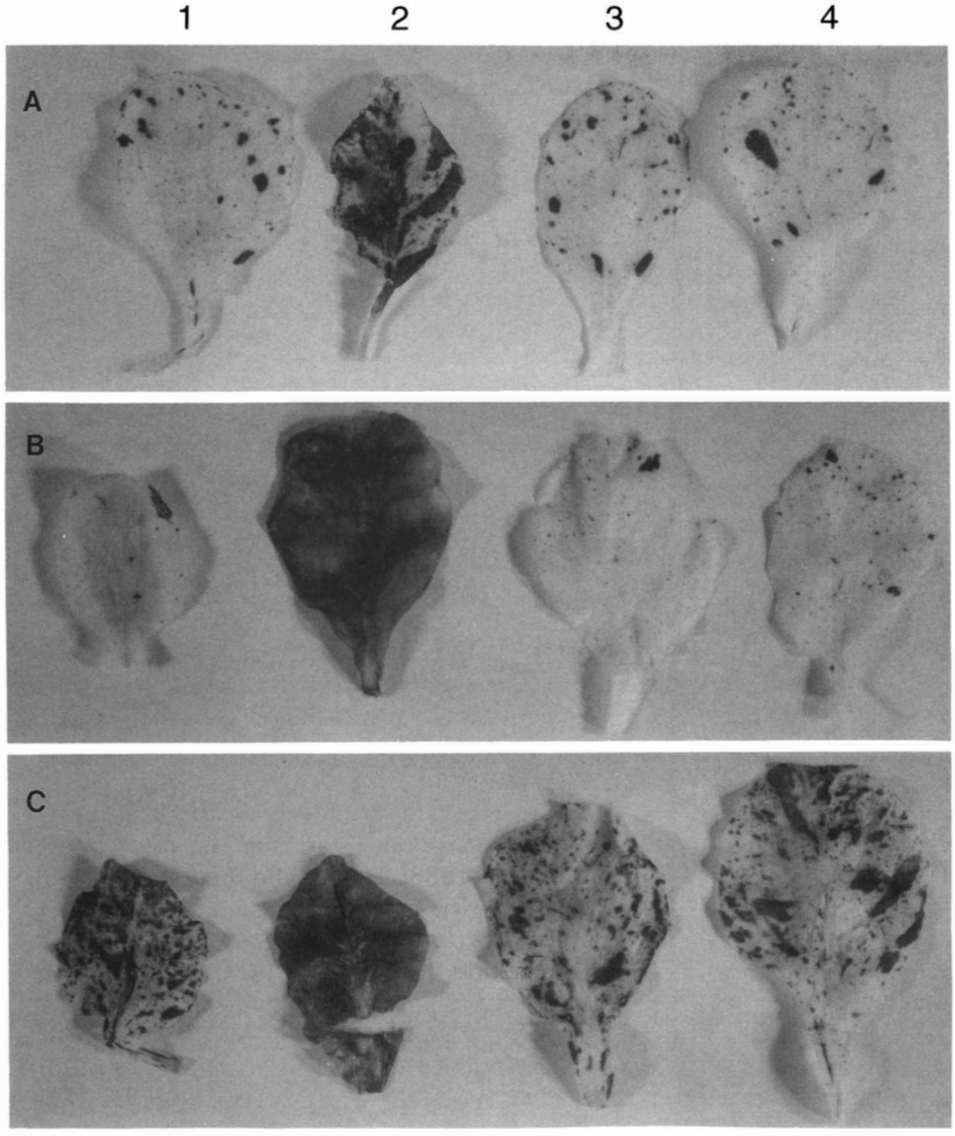

FIGURE 2.—Excision phenotype of plants regenerated from callus line *8* and retransformed with different *Spm* cDNA expression cassettes. Leaves were incubated with X-glu and cleared with 100% ethanol for photography. (Panel A) Plant *8*-45; (panel B) plant *8*-47; (panel C) plant *8*-49. (Lane 1) Retransformation with pCGN1548\ALS control vector. (Lane 2) Retransformation with pCGN1548\ALS containing CaMV 35S-*tnpA*. (Lane 3) Retransformation with pCGN1548\ALS containing CaMV 35S-*tnpC*. (Lane 4) Retransformation with pCGN1548\ALS containing CaMV 35S-*tnpD*.

the same plants were retransformed with the *A. tumefaciens* vector used for gene transfer. The results are shown in Figure 2. Neither the vector alone, nor the *tnpB* (not shown), the *tnpC* or the *tnpD* cDNAs had any effect on the excision frequency in any of the lines. By contrast, retransformation with the *tnpA* cDNA led to very early excison events, as manifested by large sectors or completely GUS-positive leaves. Similar results were obtained with the Lfe, Mfe and Hfe lines and were highly reproducible. All plants regenerated from a given retransformed callus line showed similar somatic sectoring patterns. The results are summarized in Table 2. For transformants obtained from plant *8*-49, *8*-47 and *8*-45 retransformed with the *tnpA* cDNA, 88%, 94% and 100% of the obtained callus lines showed early excision events, respectively. By contrast, when plants of the callus line *35*, which had a high *Spm* copy number and showed very early excision events, were retransformed with the same constructs, no differences were seen against the high background of excision for any of the element-encoded cDNAs. Thus the presence of the *tnpA* cDNA stimulates early excision events in plants with few *Spm* elements, but has no effect in plants with many copies of the element.

Similar results were obtained when the *tnpA*, *tnpC* and *tnpD* cDNAs were introduced by genetic crosses. Plants regenerated from callus line *8* were crossed by plants harboring either the *tnpA*, the *tnpC*, or the *tnpD* cDNA construct. Plant *8*-63 had a low excision frequency and selfed progeny of this plant containing both an *Spm* and the excision assay plasmid exhibited almost no GUS-positive sectors, suggesting that the resident *Spm* had been inactivated. Plant *8*-69, on the other hand, had a moderate to high initial excision frequency, as did a majority of its selfed progeny. When either plant was crossed by a plant carrying a *tnpA* cDNA, the resulting progeny exhibited large

TABLE 2

***dSpm* excision frequencies of plants retransformed with *Spm* specific cDNAs**

Plant	Excision frequency, GUS sectoring phenotype				
	Original	Control vector	*tnpA*	*tnpC*	*tnpD*
7-45	O-vLfe[a]	O-vLfe (7/7)[b]	L.s.[c] (5/5)[b]	(ND)[d]	O-vLfe (4/4)[b]
8-45	Mfe[e]	Lfe[f]-Mfe (8/8)	L.s. (8/8)	Lfe-Mfe (8/8)	Lfe-Mfe (8/8)
8-47	Lfe	Lfe (8/8)	L.s. (16/17) Lfe (1/17)	Lfe (8/8)	Lfe (8/8)
8-49	Hfe[g]	Mfe-Hfe (8/8)	L.s. (7/8) Mfe-Hfe (1/8)	Mfe-Hfe (8/8)	Mfe-Hfe (8/8)
35-41	vHfe[h]	vHfe (4/4)	vHfe (4/4)	vHfe (4/4)	vHfe (4/4)
35-45	L.s.	L.s. (4/4)	L.s. (4/4)	L.s. (4/4)	L.s. (4/4)
Total	6	39	46	32	36

[a] No *dSpm* excision to very low frequency of excision.
[b] Number of retransformed callus lines exhibiting the indicated GUS sectoring phenotype per total lines analyzed.
[c] Large sectors, very early *dSpm* excision events.
[d] Not determined.
[e] Medium frequency of *dSpm* excision.
[f] Low frequency of *dSpm* excision.
[g] High frequency of *dSpm* excision.
[h] Very high frequency of *dSpm* excision, many small sectors.

GUS-positive sectors indicative of early excision events (not shown). Introduction of either *tnpD* or *tnpC* cDNAs into the same background by a genetic cross had no effect. Thus the presence of the *tnpA* cDNA promotes activation of *dSpm* excision whether the element-encoded cDNA is introduced by retransformation or by a genetic cross, while *tnpC* and *tnpD* cDNAs have no effect.

Molecular evidence that the *tnpA* cDNA stimulates both transposition and excision: To determine whether the *tnpA* cDNA stimulates transposition, DNA was extracted from plants *8*-47 and *8*-49 and analyzed by Southern blot hybridization. An internal *Eco*RV restriction fragment missing from the *dSpm* was used to detect *Spm* transposition events. As shown in Figure 3A, *Spm*-homologous DNA fragments that differ in size from those of the original construct are present only in the plants containing the *tnpA* cDNA. It is likely that the novel, element-homologous restriction endonuclease fragments (Figure 3A) arise by transposition of the element, as previously shown for *Spm* and *dSpm* in tobacco (MASSON and FEDOROFF 1989; FREY *et al.* 1990; MASSON, STREM and FEDOROFF 1991). Moreover, parental and retransformed plants contain the same number of *Spm* copies, indicating that there was neither a gain nor a loss of *Spm* sequences upon retransformation with the *tnpA* cDNA or consequent on transposition (not shown).

GUS DNA was used as an empty donor site probe to detect *dSpm* excision. As shown in Figure 3B, empty donor sites were detected in plants retransformed with *tnpA*, but not in plants retransformed with the control vector or in plants that did not show early excision events. Hence large GUS-positive sectors are the consequence of early somatic *dSpm* excision events. Thus we conclude that both excision and transposition of *Spm* elements are stimulated in the presence of the *tnpA* cDNA.

The *tnpD* cDNA is functional: Since retransformation with *tnpD* had no effect on the *Spm* element, the CaMV 35S-*tnpD* construct used in these experiments was independently tested for function. Tobacco was cotransformed with a GUS excision assay plasmid carrying the CaMV 35S-*tnpA* cDNA (MASSON and FEDOROFF 1989) and a vector carrying the CaMV 35S-*tnpD* cDNA. Since TnpA and TnpD are both required for *dSpm* transposition, this assay tested the integrity of the *tnpD* cDNA. All of the callus lines tested (5/5) exhibited large GUS-positive sectors, showing that the *tnpD* cDNA encoded functional TnpD.

Strong expression of the *tnpA* gene is necessary for the maintenance of the *Spm* activity: To determine whether the high level of *tnpA* expression supported by the 35S-*tnpA* construct is necessary for the maintenance of *Spm* activity in the next generation, retransformed plants containing either the *tnpA* cDNA or only the vector were crossed by wild type SR1 plants and selected progeny were tested for GUS-positive sectors. Seeds were germinated on semisolid medium containing hygromycin and kanamycin to select for both the *Spm* and either the *dSpm* excision assay T-DNA or the *tnpA* cDNA containing T-DNA (or both), since both the *dSpm* and the cDNAs were linked to kanamycin (see MATERIALS AND METHODS). Because callus *8* already contained two independently segregating hygromycin loci and one kanamycin locus segregating independently of hygromycin (see MATERIALS AND METHODS), the segregation analysis was complicated by the introduction of another kanamy-

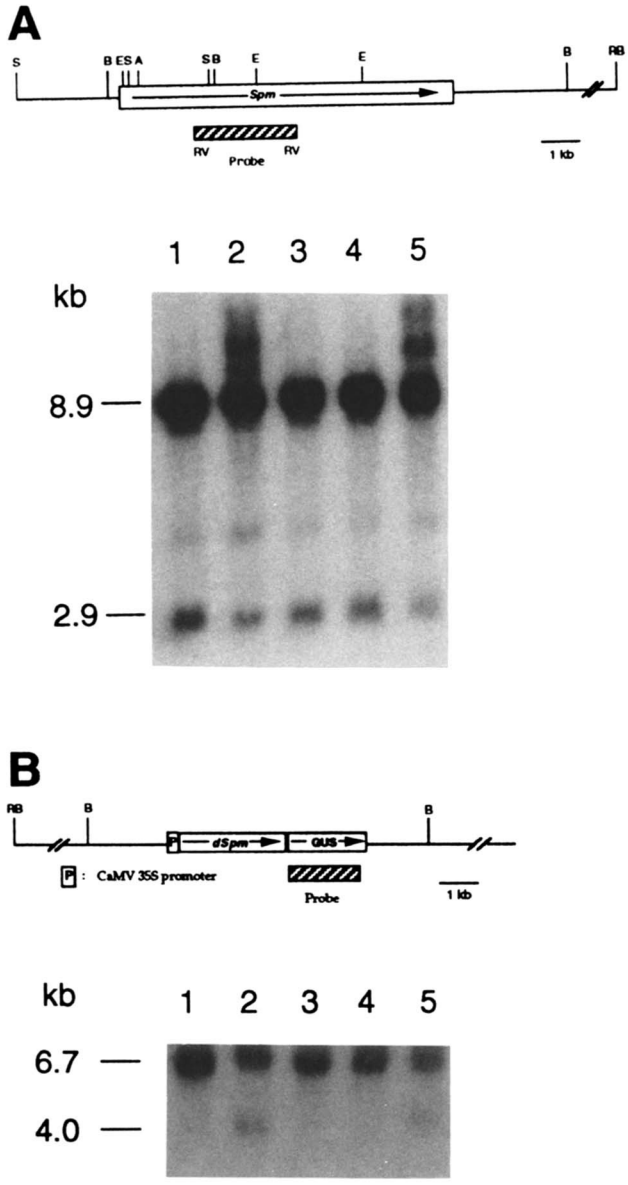

FIGURE 3.—Southern blot hybridization analysis of genomic DNA from plants *8*-47 and *8*-49 retransformed with CaMV 35S-*tnpA* or the acetolactate synthase (ALS) containing control vector. Genomic DNA was digested with *Bam*HI, separated on agarose gels, transferred to nitrocellulose, and hybridized with the indicated probe. (A) Analysis of *Spm* transposition. Shown in the upper part is a partial restriction map of the T-DNA region of pMON754 containing *Spm*. The internal *Eco*RV fragment specific to *Spm* but not to *dSpm* used as probe is shown. Bands of sizes different than 2.9-kb and 8.9-kb are due to *Spm* transposition. Lanes 1, *8*-47(ALS-1); 2, *8*-47(*tnpA*-7), callus line with early excision events; 3, *8*-47(*tnpA*-4), callus line without early *dSpm* excision events; 4, *8*-49(ALS-3); 5; *8*-49(*tnpA*-7). (B) Analysis of *dSpm* excision. A partial restriction map of pMON530 containing *dSpm* separating GUS from the CaMV 35S promoter is shown in the upper part. The GUS coding region used as a probe is shown below. A 4-kb fragment is expected if *dSpm* excises, the original construct gives a 6.7-kb fragment. Lanes 1–5 are the same as in A. A refers to *Ava*I, B to *Bam*HI, E to *Eco*O109, S to *Sal*I, RB to the right border sequence of the T-DNA, GUS to the β-glucuronidase open reading frame, and P to the CaMV 35S promoter. For terminology concerning the plant material refer to MATERIALS AND METHODS.

TABLE 3

F_1 segregation analysis of retransformed plants

Genotype[a]	H^+K[b] Percent resistant	H^+K[b] Total analyzed	GUS^+[c]	GUS^+ phenotype Spots[d]	GUS^+ phenotype Large sectors[e]
8-45(*tnpA*-2)[f]	66	76	76	11	89
8-47(*tnpA*-0)	45	77	77	30	70
8-47(*tnpA*-7)	49	81	68	22	78
8-49(*tnpA*-7)	41	137	84	4	96
8-45(ALS-2)	61	88	13	100	0
8-47(ALS-2)	54	105	9	100	0
8-49(ALS-1)	54	113	20	100	0

[a] Plants regenerated from retransformed callus lines, outcrossed by SR1.
[b] Percent hygromycin and kanamycin resistant F_1 progeny.
[c] Percent F_1 progeny with GUS-positive spots or sectors.
[d] Percent GUS-positive F_1 progeny with small spots or sectors.
[e] Percent GUS-positive F_1 progeny with large sectors, early *dSpm* excision events.
[f] Number in parentheses indicates different callus lines obtained from retransformation with 35S-*tnpA* (linked to modified acetolactate synthase, ALS) or with the ALS control vector.

cin locus (linked to the *tnpA* cDNA), whose linkage relationship to the other T-DNAs is unknown. Nonetheless, depending on whether the new T-DNA is unlinked to the others, or linked to one of them, it can be calculated that between 38% and 63% of the progeny from outcrossed plants should germinate on both kanamycin and hygromycin (see MATERIALS AND METHODS). As shown in Table 3, between 41% and 66% of the progeny from seven different plants were resistant to both drugs. This is close to the frequency range of plants expected to contain both the *Spm* element and either the *dSpm* excision assay construct or the *tnpA* cDNA or both. Furthermore, it was expected that between 60% and 100% of the resistant progeny would show GUS-positive sectors due to the presence of the *Spm* element and the *dSpm* excision assay construct (see MATERIALS AND METHODS). Among the progeny of plants retransformed with the *tnpA* cDNA, between 68% and 84% exhibited GUS-positive sectors (Table 3). This is close to the frequency range of plants expected to contain the *Spm* element, the excision assay construct, and the segregating *tnpA* cDNA. By contrast, among the progeny of plants retransformed with the control vector, only 9–20% showed GUS-positive sectors. This is well below the expected frequency, since 60–100% of the plants should receive both the *dSpm* excision assay construct and the *Spm* element. A somewhat larger fraction (20%) of the progeny from the Hfe *8*-49 parental plant showed GUS-positive sectors than of the progeny from the Mfe (13%) and Lfe (9%) plants *8*-45 and *8*-47, respectively. These contrasting observations suggest that the *Spm* element is being inactivated in plants that lack the CaMV 35S-*tnpA*.

Moreover, the number of progeny with GUS-posi-

tive sectors from retransformed plants containing the *tnpA* cDNA deviates from the expected value in the opposite direction. Depending on their genetic constitution, as few as 50% of the plants in some progenies should exhibit the large GUS-positive sectors characteristic of plants containing the CaMV 35S-*tnpA*. However, no less than 70% of progeny from retransformants containing the *tnpA* cDNA showed the large GUS-positive sectors (Table 3). For example, 50% of the progeny of outcrossed plants from callus lines *8*-47(*tnpA*-7) and *8*-47(*tnpA*-0) should have lost the CaMV 35S-*tnpA* by segregation, yet 78% and 70%, respectively, of the progeny exhibited the characteristic large GUS-positive sectors. Thus, while most of the progeny that received only the *Spm* and the excision assay T-DNAs showed just a few late *dSpm* excision events, the excess of progeny showing early excisions suggest that some of the progeny that did not receive the 35S-*tnpA* cDNA continued to exhibit early excison events. It was confirmed by Southern blot hybridization analysis that progeny plant number 46 of an outcrossed plant obtained from callus line *8*-47(*tnpA*-0) lacked the CaMV 35S-*tnpA* but showed large GUS-positive sectors. The sibling plant number 10, on the other hand, also lacked CaMV 35S-*tnpA*, but showed only a few GUS-positive sectors. These observations suggests that some of the progeny showed a memory effect following loss of the *tnpA* cDNA by genetic segregation.

Introduction of the CaMV 35S-*tnpA* cDNA results in decreased methylation of *Spm*: Reactivation of genetically inactive *Spm* elements in maize is associated with reduced methylation of sequences in the vicinity of the transcription start site (BANKS, MASSON and FEDOROFF 1988; BANKS and FEDOROFF 1989). To determine whether TnpA-promoted activation of *Spm* in tobacco is correlated with reduced methylation, we analyzed the *Spm* element in DNA from transgenic plants with methylation-sensitive restriction enzymes. The plants whose DNA was analyzed included the Lfe plant *8*-47, the Hfe plant *8*-49, a primary regenerant of each plant retransformed with CaMV 35S-*tnpA*, as well as regenerants retransformed with the control vector and several progeny plants from the primary regenerants outcrossed by wild-type SR1 tobacco and selected for *dSpm* excision. We examined the extent of methylation of the *Eco*O109 site at nucleotide 118 at the element's 5′ terminus upstream of the transcription site at nucleotide 209 (PEREIRA *et al.* 1986), as well as the *Sal*I site at nucleotide 266 and the *Ava*I site at nucleotide 488, both of which are in the GC-rich sequence downstream from the transcription start site. The same analysis also provided information about the methylation state of restriction sites elsewhere in the *Spm*, including the *Sal*I site at nucleotide 2372, and the *Eco*O109 sites at nucleotides 3569 and 6385 (Figure 4A). The results of the analysis of the *Eco*O109 site at nucleotide 118 (*Eco*O109-118) for the *8*-47 series are shown in Figure 4, B and C. The *Eco*O109-118 site is more extensively methylated in the primary regenerant retransformed with the control vector (Fig. 4B, lane 3), and in its outcrossed progeny (Figure 4B, lanes 4 and 5), than it is in the parental plant *8*-47 (Figure 4B, lane 2). This increased methylation is associated with a very low frequency of *dSpm* excision (Figure 4D). By contrast, the *Eco*O109-118 site is almost fully sensitive to cleavage in the primary regenerant retransformed with CaMV 35S-*tnpA* (Figure 4C, lane 1). The same is true for outcrossed progeny of primary regenerants containing the CaMV 35S-*tnpA* (Figure 4C, lanes 5–8). Two different results were obtained when the CaMV 35S-*tnpA* was segregated away in the outcrossed progeny. In some plants the *Eco*O109-118 site of the *Spm* showed increased methylation (Figure 4C, lane 2) and there was a decrease in the size and frequency of GUS-positive sectors (see Figure 4E, leaf 2). In other plants, the demethylated state persisted (Figure 4C, lane 4) and such plants exhibited the large GUS-positive sectors diagnostic for *dSpm* excision events and characteristic for the presence of 35S-*tnpA* (see Figure 4E, leaf 4).

A similar result was obtained for the *Sal*I site at nucleotide 266 (*Sal*I-266, Figure 5A). The results of the analysis for the *8*-49 series is shown in Figure 5, B and C. Whereas the *Sal*I-266 site in the parental plant *8*-49 was only slightly methylated (Figure 5B, lane 1), all the progeny of the outcrossed plants that had been retransformed with the control vector exhibited an increase in methylation at this site (Figure 5B, lanes 4–8). Increased methylation was correlated with a reduction in the *dSpm* excision frequency among the progeny plants. Again, the *Sal*I-266 site of the primary regenerant retransformed with CaMV 35S-*tnpA* was completely cleavable (Fig. 5C, lane 2), as was the *Sal*I-266 site in the progeny of these plants outcrossed by SR1 (Figure 5C, lanes 4–6). Thus the *Sal*I-266 site remained unmethylated in the presence of CaMV 35S-*tnpA*. The *Sal*I site in the coding region at position 2372 was partially methylated in the primary regenerant containing the CaMV 35S-*tnpA* cDNA (Figure 5C, lane 2). However, partial *Sal*I methylation in the coding region was not correlated with a decrease in the frequency and delay in the timing of *dSpm* excision events (see Figure 2C, lane 2). Futhermore, the *Sal*I-266 site of progeny from primary regenerants outcrossed to SR1 and selected for the presence of CaMV 35S-*tnpA* was fully cleavable (Figure 5C, lanes 4–6). Demethylation of the *Sal*I-266 site is correlated with early excision events in the progeny plants. The methylation analysis of the *Ava*I site at nucleotide 488 (*Ava*I-488) further downstream

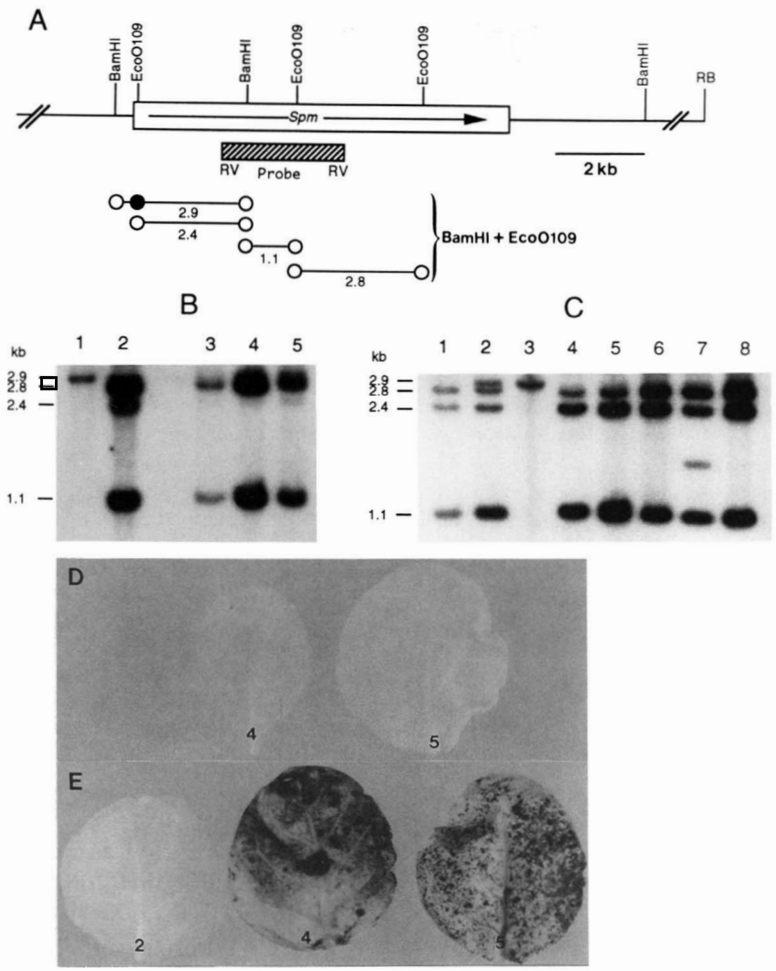

FIGURE 4.—Analysis of the methylation state of the *Eco*O109 site at nucleotide 118 in the 5′-end of *Spm* in genomic DNA of plants from *8*-47 and retransformed with CaMV 35S-*tnpA* or control vector by Southern hybridization, and GUS staining pattern in leaves from the same plants. DNA was treated as indicated in Figure 3 and leaves as in Figure 2. (A) Partial restriction map of *Spm* inserted in pMON754 depicting the restriction sites analyzed is shown. The internal *Eco*RV (RV) fragment specific to *Spm* but not to *dSpm* used as probe is shown. For analysis of the *Spm* 5′-end, a 2.4-kb restriction fragment is expected after double digest with *Bam*HI and *Eco*O109 (○) and a 2.9-kb fragment in the case of *Eco*O109 methylation (●). The 1.1-kb and 2.8-kb fragments come from internal *Eco*O109 fragments. (B) Methylation analysis of the *Eco*O109 site at the *Spm* 5′-end of DNA from plants without CaMV 35S-*tnpA*. Lanes 1 and 2, *8*-47; 3, *8*-47(ALS-1); 4, F1#17 [*8*-47(ALS-2) × SR1]; 5, F1#36 [*8*-47(ALS-1) × SR1]. DNA in lane 1 was digested with *Bam*HI and in lanes 2–5 with *Bam*HI and *Eco*O109, respectively. (C) Methylation analysis of the *Eco*O109 site at the *Spm* 5′-end of DNA from plants retransformed with CaMV 35S-*tnpA*. Lanes 1, *8*-47(*tnpA*-7); 2; F1#10 [*8*-47(*tnpA*-0) × SR1]; 3 and 4, F1#46 [*8*-47(*tnpA*-0) × SR1]; 5, F1#47 [*8*-47(*tnpA*-0) × SR1]; 6, F1#53 [*8*-47(*tnpA*-7) × SR1]; 7, F1#54 [*8*-47(*tnpA*-7) × SR1]; 8, F1#55 [*8*-47(*tnpA*-7) × SR1]. DNA in lane 3 was digested with *Bam*HI and in lanes 1, 2, 4–8 with *Bam*HI and *Eco*O109, respectively. (D) GUS sectoring phenotype of plants used for the methylation analysis shown in B. Numbers in leaves correspond to lane numbers in B. (E) GUS sectoring phenotype of plants used for the methylation analysis in C. Numbers in leaves correspond to the lane numbers in panel C. For terminology concerning transgenic plants refer to MATERIALS AND METHODS.

from the transcription start site gave similar results, except that some methylation was maintained in the plants retransformed with CaMV 35S-*tnpA* .

Overall, six methylatable sites in the *Spm* sequence were analyzed in 22 plants and the percent cleavage of each site was determined by densitometry (see MATERIALS AND METHODS). The results, shown in Table 4, clearly indicate that the presence of CaMV 35S-*tnpA* in retransformed plants is correlated with high *Spm* activity and with reduced methylation of sequences near the transcription start site, whereas no clear trend was detectable for methylatable restriction sites elsewhere in the *Spm* sequence.

Introduction of the *tnpA* cDNA by a genetic cross results in reactivation of methylated inactive *Spm* elements in the F_2 generation: The CaMV 35S-*tnpA* cDNA was introduced into plants with extensively methylated, inactive *Spm* elements by genetic crosses. Progeny plant number 36 of an outcrossed plant from callus line *8*-47(ALS-2) and progeny plant numbers 12 and 41 of an outrossed plant from callus line *8*-49(ALS-2), which showed little *Spm* activity and extensive methylation at the *Eco*O109-118, *Sal*I-266 and *Ava*I-488 sites, were crossed by plants that contained only the CaMV 35S-*tnpA* cDNA (see Figures 4B and 5B and Table 4). More than 1/3 of the resulting F_2 progeny exhibited the large GUS-positive sectors characteristic for early *dSpm* excision events in the

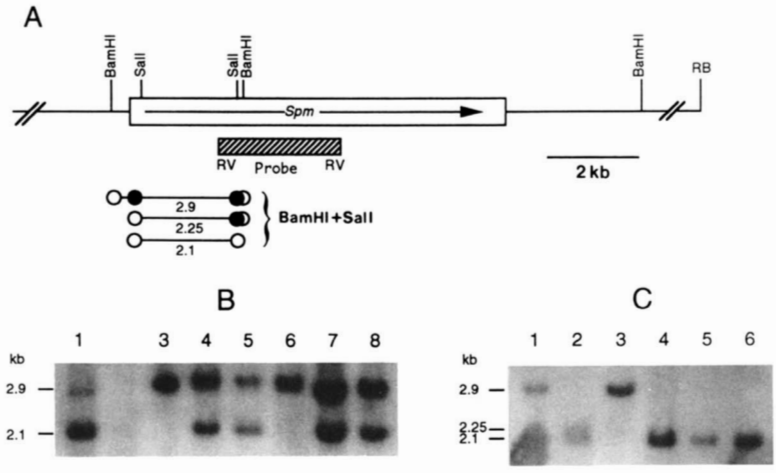

FIGURE 5.—Analysis of the methylation state of the *Sal*I sites at nucleotide 266 in the G + C-rich untranslated leader of *Spm* and at nucleotide 2372 in genomic DNA of plant *8*-49 and retransformed with CaMV 35S-*tnpA* or the control vector by Southern hybridization, and GUS staining patterns in leaves from the same plants. DNA was treated as indicated in Figure 3 and leaves as in Figure 2. (A) The restriction sites analyzed are shown in a partial restriciton map of *Spm* inserted in pMON754. Expected DNA fragments for unmethylated (○) and methylated (●) *Sal*I sites and the internal *Eco*RV (RV) probe specific for *Spm* but not for *dSpm* are shown. (B) Methylation analysis of the *Sal*I sites in DNA from plants without CaMV 35S-*tnpA*. Lanes 1, *8*-49; 2, *8*-49(ALS-3); 3 and 4, F1#15 [*8*-49(ALS-1) × SR1]; 5, F1#30 [*8*-49(ALS-1) × SR1]; 6, F1#42 [*8*-49(ALS-1) × SR1]; 7, F1#52 [*8*-49(ALS-1) × SR1]; 8, F1#62 [*8*-49(ALS-1) × SR1]. DNA in lane 3 was digested with *Bam*HI and in lanes 1 and 4–8 with *Bam*HI and *Sal*I, respectively. (C) Methylation analysis of the *Sal*I sites in DNA of plants retransformed with CaMV 35S-*tnpA*. Lanes 1 and 2, *8*-49(*tnpA*-7); 3 and 4, F1#3 [*8*-49(*tnpA*-7) × SR1]; 5, F1#60 [*8*-49(*tnpA*-7) × SR1]; 6, F1#68 [*8*-49(*tnpA*-7) × SR1]. DNA in lanes 1 and 3 was digested with *Bam*HI, and DNA in lanes 2, 4–6 with *Bam*HI and *Sal*I, respectively. For terminology concerning transgenic plants refer to MATERIALS AND METHODS.

presence of the CaMV 35S-*tnpA* cDNA. This indicates that *tnpA* can still reactivate *Spm* elements after a second generation in which the excision activity had decreased and the extent of methylation had increased.

DISCUSSION

Genetic regulation of *Spm* can be monitored in transgenic tobacco: Using a transgenic tobacco system containing a full-length *trans*-activating *Spm* and an excision assay construct with a *dSpm*-disrupted GUS gene, we have shown that the *Spm* element undergoes the same type of epigenetic inactivation previously described in maize (MCCLINTOCK 1957–1959, 1971; MASSON *et al.* 1987; FEDOROFF and BANKS 1988). Transgenic plants containing multiple copies of the *Spm* element exhibited more *Spm* activity, as judged by the number and size of GUS-positive somatic sectors, than plants with just one or two copies of the element. When a CaMV 35S-*tnpA* cDNA was introduced, excision activity in the low copy-number lines increases, but was unaffected in the high copy-number lines. By contrast, introduction of either the CaMV 35S-*tnpB*, the CaMV 35S-*tnpC*, or the CaMV 35S-*tnpD* cDNAs had no effect on excision frequency. We infer that TnpA, the gene product encoded by the *tnpA* cDNA is rate-limiting for *dSpm* excision in the low copy-number lines, but is present at a sufficient concentration in the high copy-number lines. While we lack direct proof that the observed effect of the cDNA is directly attributable to the TnpA protein, its known DNA-binding properties support the present inference (GIERL, LÜTTICKE and SAEDLER 1988).

The low *Spm* copy-number lines often gave rise to regenerants that showed a decrease or disappearance of excision activity, as monitored by the presence of GUS-positive somatic sectors. *De novo* methylation of the *Spm* element was detected in DNA from plants with few or no GUS-positive sectors, but not in DNA from plants with high *Spm* activity, as judged by the GUS assay. Thus the type of epigenetic inactivation of the *Spm* element observed in maize also occurs in tobacco. Moreover, these observations suggested that TnpA supplied by the intact *Spm* element is not only rate-limiting for *dSpm* excision in the transgenic tobacco, but that an adequate supply might also be required to prevent inactivation of the *trans*-activating element.

TnpA is an element-encoded regulator of *Spm*: To determine whether element-encoded proteins can reactivate an inactive *Spm* element in transgenic tobacco, as they can in maize, we introduced CaMV 35S-promoted cDNAs encoding *tnpA*, *tnpB*, *tnpC* and *tnpD*, the four protein-coding sequences identified for *Spm*, into plants containing inactive *Spm* elements (MASSON *et al.* 1989). *Spm* reactivation was observed only when the CaMV 35S-*tnpA* cDNA was introduced. The other cDNAs had no effect. Similar results were obtained whether the cDNAs were introduced by Agrobacterium-mediated transformation or by genetic crosses. Moreover, reactivation of the resident inactive *Spm* element was accompanied by reduced

TABLE 4

Percent cleavage of methylation sensitive restriction sties in the *Spm* element

Plant genotype	Excision phenotype[c]	Upstream[a]	G + C-rich leader[b]		Coding region	
		*Eco*O109 118[d]	*Sal*I 266	*Ava*I 488	*Sal*I 2372	*Eco*O109 6385
8-47	Lfe	38	67	46	82	97
8-47(Als-1)[e]	vLfe	10	68	28	ND[f]	ND
F1#17[*8*-47(Als-2) × SR1]	vLfe	2	25	0	>95	99
F1#36[*8*-47(Als-2) × SR1]	vLfe	6	84	13	>90	96
8-49	Hfe	38	84	61	>95	87
8-49(Als-3)	Mfe	45	ND	ND	ND	ND
F1#15[*8*-49(Als-1) × SR1]	0	11	32	16	44	78
F1#30[*8*-49(Als-1) × SR1]	vLfe	6	30	16	36	92
F1#42[*8*-49(Als-1) × SR1]	vLfe	5	0	<1	24	90
F1#52[*8*-49(Als-1) × SR1]	Lfe	10	31	18	63	78
F1#62[*8*-49(Als-1) × SR1]	vLfe	ND	28	10	47	ND
8-47(*tnpA*-7)	L.s.	80	100	ND	90	84
F1#10[*8*-47(*tnpA*-0) × SR1][g]	vLfe	45	100	32	53	81
F1#46[*8*-47(*tnpA*-0) × SR1][g]	L.s.	92	99	94	43	81
F1#47[*8*-47(*tnpA*-0) × SR1]	Hfe	93	100	86	100	78
F1#53[*8*-47(*tnpA*-7) × SR1]	L.s.	90	100	96	78	88
F1#54[*8*-47(*tnpA*-7) × SR1]	Hfe	82	100	99	100	88
F1#55[*8*-47(*tnpA*-7) × SR1]	Hfe	90	100	85	100	90
8-49(*tnpA*-7)	L.s.	99	100	96	50	ND
F1#3[*8*-49(*tnpA*-7) × SR1]	Mfe	89	100	86	100	85
F1#60[*8*-49(*tnpA*-7) × SR1]	Hfe	78	100	84	100	89
F1#68[*8*-49(*tnpA*-7) × SR1]	L.s.	91	100	85	100	92

[a] Upstream of transcription start site, defining the *Spm* promoter (BANKS, MASSON and FEDOROFF 1988).
[b] Downstream of transcription start site, the first G + C-rich untranslated *Spm* exon (BANKS, MASSON and FEDOROFF 1988).
[c] Refer to Table 2 for definition of *dSpm* excision phenotype.
[d] The number below the restriction sites indicates the nucleotid number on the *Spm* map (PEREIRA *et al.* 1986).
[e] Numbers in parentheses indicate different callus lines obtained from retransformation with 35S-*tnpA* or the control vector. Refer to MATERIALS AND METHODS for definition of plant genotype.
[f] Not determined.
[g] CaMV 35S-*tnpA* segregated away.

methylation of methylation-sensitive restriction sites both upstream and downstream of the transcription start site at nucleotide 209, but not elsewhere in the element. Reactivation of the inactive *Spm* element, as judged by the assay for GUS-positive tissues and somatic sectors, was supported by molecular evidence for enhanced excision and transposition in the presence of the CaMV 35S-*tnpA* cDNA.

TnpA maintains *Spm* in a genetically active state: Progeny of low copy-number *Spm* plants in which the *Spm* showed little or no activity either resembled their parents or exhibited even less *Spm* activity, suggesting that the progressive inactivation of elements observed in maize also occurs in tobacco (FEDOROFF and BANKS 1988). Among the progeny of inactive *Spm* plants into which the CaMV 35S-*tnpA* cDNA had been introduced, reactivating the element, progeny plants that received the cDNA locus continued to show high *Spm* activity and low *Spm* methylation. Progeny plants that received the *Spm* and *dSpm* excision assay constructs, but not the CaMV 35S-*tnpA* cDNA, generally showed low *Spm* activity, as judged by the small size and paucity of GUS-positive sectors, and showed increased methylation of the element's 5′-end.

However, the number of progeny from a plant containing both a reactivated *Spm* and a *trans*-activating CaMV 35S-*tnpA* cDNA that showed many large GUS-positive sectors was higher than predicted based on the expected segregation ratios. Molecular analysis of some of these progeny plants showed that there were indeed plants that exhibited a high excision frequency, but lacked the CaMV 35S-*tnpA* cDNA locus. In these plants, the *Spm* element's 5′-end was less extensively methylated than in siblings showing a low excision frequency. A similar observation has been made in maize, in which a fraction of progeny kernels not receiving the *trans*-activating *Spm* produced on a plant containing both an inactive and a *trans*-activating *Spm* continue to exhibit *Spm* activity (FEDOROFF 1989b). In turn, this resembles the phenomenon MCCLINTOCK designated "presetting," in which a gene with a *dSpm* inserted in its promoter region and which is normally not expressed, can be expressed transiently after exposure to an active *Spm* in the parent plant. These effects of a *trans*-acting *Spm* element are both believed to reflect the ability of an active element to promote the heritable reactivation of an inactive ele-

ment (BANKS, MASSON and FEDOROFF 1988; BANKS and FEDOROFF 1989; FEDOROFF 1989b). Thus the present observations in transgenic tobacco bear the additional implication that TnpA is the element-encoded gene product that promotes the heritable reactivation of the *Spm* element.

TnpA has been shown to be a DNA-binding protein that recognizes and binds to motifs, termed the "subterminal repeats" to distinguish them from the TIRs, that occur in both direct and inverted orientation near element ends (MASSON *et al.* 1987; GIERL, LÜTTICKE and SAEDLER 1988). It has been reported that TnpA binds less well to methylated, than to unmethylated, binding site-containing oligonucleotides (GIERL, LÜTTICKE and SAEDLER 1988). At the 5′ end of the element, the TnpA binding sites are confined to the 0.2-kb sequence between the TIR and the transcription initiation site at nucleotide 209. In previous studies in maize, however, it has been observed that *Spm* inactivation is associated with methylation of sites both upstream and downstream of the transcription initiation site (BANKS, MASSON and FEDOROFF 1988). The 0.35-kb sequence just downstream from the transcription initiation site and contained within the element's untranslated first exon is extremely GC-rich (80% G + C) and contains 11 direct repeats of a 17-bp GC-rich sequence that does not resemble the TnpA-binding sequence (MASSON *et al.* 1987; BANKS, MASSON and FEDOROFF 1988). The results of the present study strengthen the correlation between methylation of C residues near the transcription start site, both upstream and downstream, and element inactivation. They also support the conclusion that reactivation of an inactive element is correlated with a selective reduction in element methylation at sites in both the upstream TnpA-binding region and in the downstream, GC-rich first exon.

Taken together, these observations suggest that the *Spm* element is a target for *de novo* methylation and that methylation correlates with reduced ability of the element to be expressed. Maintenance of the element in an active form requires an adequate supply of TnpA protein, for which there are multiple binding sites at both ends of the element. The present study reveals that the introduction of an abundant TnpA supply into plants with methylated elements results in genetic reactivation and decreased methylation of sequences around the transcription start site. However, since there are no TnpA binding sites in the demethylated sequence downstream of the transcription start site, it appears that the TnpA-promoted decrease in methylation of the element's 5′ end must occur by a mechanism more complex than simple blocking of a methylation-sensitive site by direct binding of TnpA protein. Thus, the ability of TnpA to promote element reactivation and demethylation might involve stabilizing the element's 5′-end in a configuration that is not favorable for methylation of downstream sequences, either by virtue of DNA conformation or chromatin structure. Another possibility is that TnpA interacts with endogenous proteins that either protect DNA sequences from methylation in the vicinity of the TnpA binding site or that actively demethylate such sequences.

The role of TnpA in *Spm* transposition and regulation: The results of these and earlier studies show that TnpA is required to maintain the *Spm* element in a genetically active form (FEDOROFF and BANKS 1988; BANKS, MASSON and FEDOROFF 1988; KOLOSHA and FEDOROFF 1992). Although it has been shown that only active elements are transcribed, we do not yet know whether TnpA promotes element reactivation simply by interfering with methylation, permitting constitutive expression of its promoter, or whether TnpA plays a more active role, serving as a transcription factor for the *Spm* promoter in either or both methylated and unmethylated states. It has been reported that TnpA binds cooperatively as a dimer with the highest affinity for a tail-to-tail binding site pair located at −174 from the transcription start site (GRANT, GIERL and SAEDLER 1990). If this binding has physiological significance for the *Spm* element, its function may be to stabilize the transcription complex at low TnpA concentrations, promoting element transcription either directly or indirectly by interfering with methylation. This, in turn, would lead to the accumulation of more TnpA, as well as TnpD, both of which are required for transposition.

Based on the observation that there are multiple TnpA binding sites immediately adjacent to the TIRs at both element ends, it has been postulated that the role of TnpA in transposition is to bring the ends of the element together (FREY *et al.* 1990; MASSON, STREM and FEDOROFF 1991). At low concentration, TnpA might first bind to sites with higher affinity adjacent to the TIR (GRANT, GIERL and SAEDLER 1990). Based on the results of the present study that the TnpA supply is rate-limiting for transposition in tobacco, as the concentration of TnpA increases, it is likely to occupy binding sites with lower affinity, including the site overlapping the putative TATA box (PEREIRA *et al.* 1986), possibly inhibiting transcription (SCHLÄPPI and FEDOROFF 1992) and forming a transposition-competent complex (FREY *et al.* 1990; MASSON, STREM and FEDOROFF 1991; FEDOROFF *et al.* 1992). The reconstitution of the *Spm* regulatory system in transgenic tobacco will permit us to further investigate the molecular details of TnpA regulation and to demonstrate directly that the reactivation effect of the *tnpA* cDNA is mediated by the interaction of the TnpA protein with its target DNA binding sequence within the *Spm* element.

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