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Epigenetic Regulation of the Maize *Spm* Transposable Element: Novel Activation of a Methylated Promoter by TnpA

Michael Schläppi, Ramesh Raina, and Nina Fedoroff
Carnegie Institution of Washington
Department of Embryology
Baltimore, Maryland 21210

Summary

***Spm* is epigenetically inactivated by C-methylation near its transcription start site. We have investigated the interaction between TnpA, an autoregulatory protein that can reactivate a silent *Spm*, and the promoter of the element. The promoter undergoes rapid de novo methylation and inactivation in stably transformed plants, but only if it includes a GC-rich sequence downstream of the promoter. TnpA activates the inactive, methylated promoter and leads to reduced methylation. By contrast, TnpA represses the active, unmethylated *Spm* promoter. Only the internal DNA-binding and dimerization domains of the protein are required for repression, while activation requires an additional C-terminal sequence. TnpA is therefore a unique regulatory protein with a conventional transcriptional repressor activity and a novel ability to activate a methylated, inactive promoter.**

Introduction

Studies by McClintock (1954, 1955, 1956, 1957, 1959, 1961a, 1961b) on the genetic cross-talk between *Suppressor-mutator* (*Spm*) elements and their transposition-defective *Spm* (*dSpm*) derivatives constitute some of the earliest published genetic data on interactions between regulatory proteins and their target sequences. The existence of both negative and positive regulatory genes was first suggested by the ability of *Spm* to inhibit and activate expression of genes with *dSpm* insertions (McClintock, 1954, 1961b; Masson et al., 1987). McClintock later described the epigenetic inactivation of the *Spm* element and reported that an active *Spm* can override the inactivation mechanism, transiently reactivating a silent element (McClintock, 1957, 1958, 1959, 1971). Active elements also promote heritable reactivation of inactive elements (Fedoroff and Banks, 1988; Banks et al., 1988; Fedoroff, 1989). Thus, *Spm* is regulated by an epigenetic inactivation mechanism and *Spm*-encoded autoregulatory functions.

Both positive and negative regulatory functions of *Spm* have been attributed to the *Spm*-encoded TnpA, a protein that also participates directly in transposition (Grant et al., 1990; Frey et al., 1990; Masson et al., 1991; Cook and Fedoroff, 1992; Schläppi et al., 1993). However, the regulatory target sequence of TnpA within the element has not been identified, nor is the mechanism of regulation understood. A single promoter sequence has been identified for the *Spm* element, whose multiple protein-coding sequences are derived by alternative splicing of its primary transcript (Masson et al., 1989; Raina et al., 1993). The 0.2 kb sequence immediately upstream of the transcription start site at nucleotide 209 (the upstream control region [UCR]) and the adjacent 0.35 kb GC-rich sequence (the downstream control region [DCR]) have been identified in both maize and tobacco as sites of altered C-methylation during epigenetic inactivation and reactivation of *Spm* (Banks et al., 1988; Schläppi et al., 1993). *Spm* promoters consisting of either the UCR or both the UCR and DCR were therefore used in the present study, although all promoter activity detectable in a transient assay resides in the UCR (Raina et al., 1993).

Transient assays in tobacco cells were used to analyze the effect of TnpA on the unmethylated promoter because they are of sufficiently short duration to preclude significant replication-associated DNA methylation. Stably transformed tobacco plants were used to study the effect of TnpA on the methylated *Spm* promoter. To identify parts of the protein required for regulation, coding sequences were deleted from (or stop codons introduced into) *tnpA* cDNAs to create plasmids coding for C- and N-terminally truncated proteins. We report that the ability to undergo rapid de novo methylation is a property of the *Spm* promoter. We show that the regulatory effect of TnpA on the *Spm* promoter is determined by promoter methylation. TnpA acts as a conventional repressor of the unmethylated promoter. TnpA has a different and novel regulatory effect on a methylated, inactive *Spm* promoter, both activating it and promoting reduced methylation. Only the DNA-binding and protein dimerization domains of TnpA are required for repression, while activation of the methylated promoter requires an additional C-terminal sequence, suggesting underlying differences in mechanism or cofactor requirements.

Results

The *Spm* Promoter Is Rapidly Inactivated and Methylated in Transgenic Plants Only If It Contains the DCR

The genetic inactivation of the *Spm* element in maize and transgenic tobacco plants is accompanied by methylation of C residues in both the UCR and DCR sequences adjacent to the transcription start site (Fedoroff and Banks, 1988; Banks et al., 1988; Banks and Federoff, 1989; Schläppi et al., 1993). To identify the *Spm* sequences required for inactivation and methylation of its promoter, we assessed the ability of stably transformed plants to inactivate a reporter gene expressed from *Spm* promoters comprising either the UCR alone or both the UCR and DCR. *Spm* promoter activity in stably transformed callus cell lines and plants regenerated from them was detected by monitoring expression of a luciferase (LUC) gene inserted into a mini-*Spm* element (see Experimental Procedures). The promoter of the LUC gene was either the 220 bp 5′ terminus (UCR only) of the element or a 560 bp 5′-terminal fragment containing both the UCR and the DCR (Figure

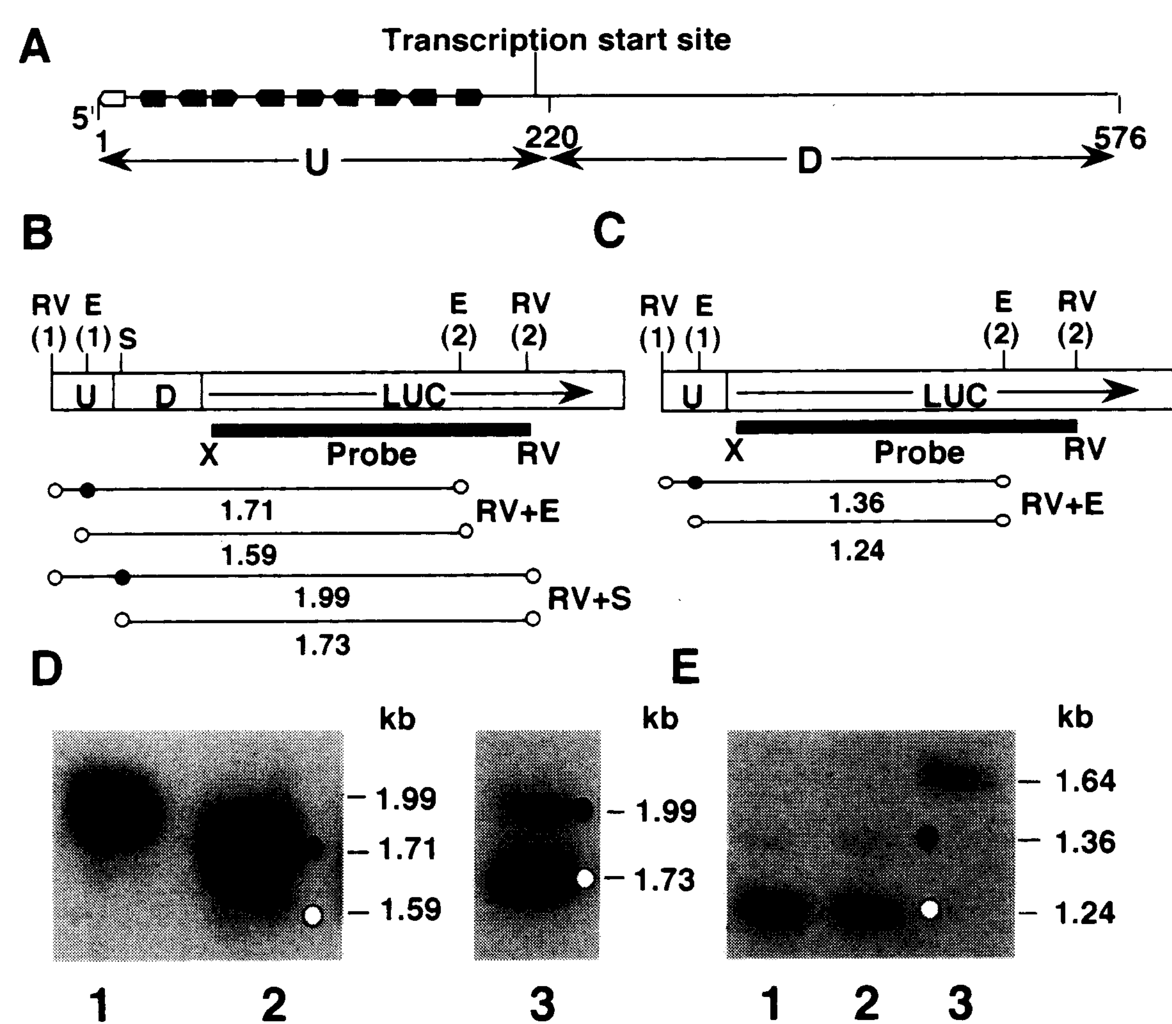

Figure 1. Southern Blot Hybridization Analysis of *Spm* Promoter Methylation

(A) A diagram of the 5'-terminal 576 bp of the *Spm* element. The open arrow represents the 13 bp terminal inverted repeat and closed arrows represent TnpA-binding sites.

(B and C) Diagrams of the *Spm* promoter–LUC reporter gene constructs used, showing the EcoRV (RV), EcoO109 (E), and SalI (S) sites in the UCR (U) and DCR (D), the XbaI (X)–EcoRV LUC fragment used as a probe, and the lengths of fragments expected from double digests depending upon whether the methylation-sensitive sites are uncleaved (methylated, closed circles) or cleaved (unmethylated, open circles; note that E[2] is not methylation sensitive).

(D and E) Restriction fragments detected by the LUC probe in DNA from plants containing the constructs shown in (B) and (C), respectively. (D) DNA was digested with EcoRV only (lane 1), EcoRV plus EcoO109 (lane 2), or EcoRV plus SalI (lane 3). (E) The DNA in lanes 1 and 2 were isolated from two different transformants and digested with EcoRV plus EcoO109, while the DNA in lane 3, which was from one of the same two plants, was digested only with EcoRV. The circles in (D) and (E) mark the fragments expected if the methylation-sensitive restriction site is unmethylated and cleaved (open circles) or methylated and uncleaved (closed circles).

1A, UCR–DCR promoter). The UCR comprises the *Spm* promoter, and its activity is unaffected by the presence of the DCR when measured in transient assays (Raina et al., 1993). The mini-*Spm*–LUC constructs were introduced into tobacco by Agrobacterium-mediated transformation, and transgenic plants regenerated from transformed calli were assayed for LUC activity (see Experimental Procedures).

Both the UCR and UCR–DCR sequences show promoter activity in transgenic plants, while the DCR sequence alone does not (Table 1). As commonly observed with transgenic plants, the amount of reporter gene activity varied considerably among transformants (Peach and Velten, 1991; Breyne et al., 1992). Nonetheless, among 10 transformants analyzed for each construct, both the mean and the highest level of LUC activity detected with the UCR promoter were approximately two orders of magnitude lower than the values obtained with the strong MAC promoter (a chimeric plant promoter) (Table 1; Comai et al., 1990), and this difference is comparable to that reported for the cauliflower mosaic virus (CaMV) 35S and

Table 1. *Spm* Promoter Strength in Transgenic Tobacco

	LUC Units per Microgram of Protein	
Promoter	Range	Average
MAC	1,555–1,242,871	306,288
DCR	10–73	18
UCR–DCR	118–630	438
UCR	141–7,517	2,662

Promoters were transcriptionally fused to the LUC gene. Ten plants regenerated from independent transformants were analyzed for each promoter. MAC is a constitutive chimeric promoter (Comai et al., 1990).

Spm promoters in a transient expression assay (Raina et al., 1993). However, LUC gene expression from an *Spm* promoter comprising both UCR and DCR was significantly lower than that observed with the UCR alone (Table 1), in sharp contrast with the observation that the DCR has no effect on *Spm* promoter activity in a transient assay (Raina et al., 1993).

Because inactivation of intact *Spm* elements in maize is accompanied by methylation of the UCR and the DCR, we asked whether the inactive *Spm* promoter in the transgenic plants was methylated (Banks et al., 1988; Schläppi et al., 1993). We used cleavability of the EcoO109 site at nucleotide 118 as diagnostic of UCR methylation and cleavability of the SalI site at nucleotide 266 as diagnostic of DCR methylation (Banks and Fedoroff, 1989; Schläppi et al., 1993). The EcoO109 site in the UCR is much more extensively methylated in DNA from a plant in which the *Spm* promoter contained the DCR sequence (>90% methylated; Figure 1D) than in DNA from a plant in which it did not (<10% methylated; Figure 1E). The SalI site in the DCR was also methylated, although less extensively (41% methylated; Figure 1D). Thus, lower expression of the LUC gene from the *Spm* promoter containing the DCR is associated with more extensive methylation of the promoter.

TnpA Activates the Methylated UCR-DCR *Spm* Promoter in Transgenic Tobacco

We have reported that TnpA activates an inactive, methylated *Spm* element in transgenic tobacco (Schläppi et al., 1993). To determine whether TnpA exerts its effect on the *Spm* promoter, we introduced a *tnpA* cDNA into plants containing the inactive, methylated UCR–DCR promoter–LUC gene and tested its ability to reactivate the reporter gene. To control internally for the variability in results obtained with stable transformants (Table 1), the assay constructs were modified to permit the identification of plants with different levels of TnpA activity by assessing its ability to activate transposition. Each *Spm* promoter–LUC construct was linked to an excision assay construct within the T-DNA (Figure 2A). The excision assay construct comprised a CaMV 35S promoter-driven β-glucuronidase (GUS) reporter gene disrupted by insertion of *dSpm* (Masson and Fedoroff, 1989; Schläppi et al., 1993). Tobacco leaf fragments were transformed with a plasmid containing the *Spm* promoter–LUC gene and the excision assay con-

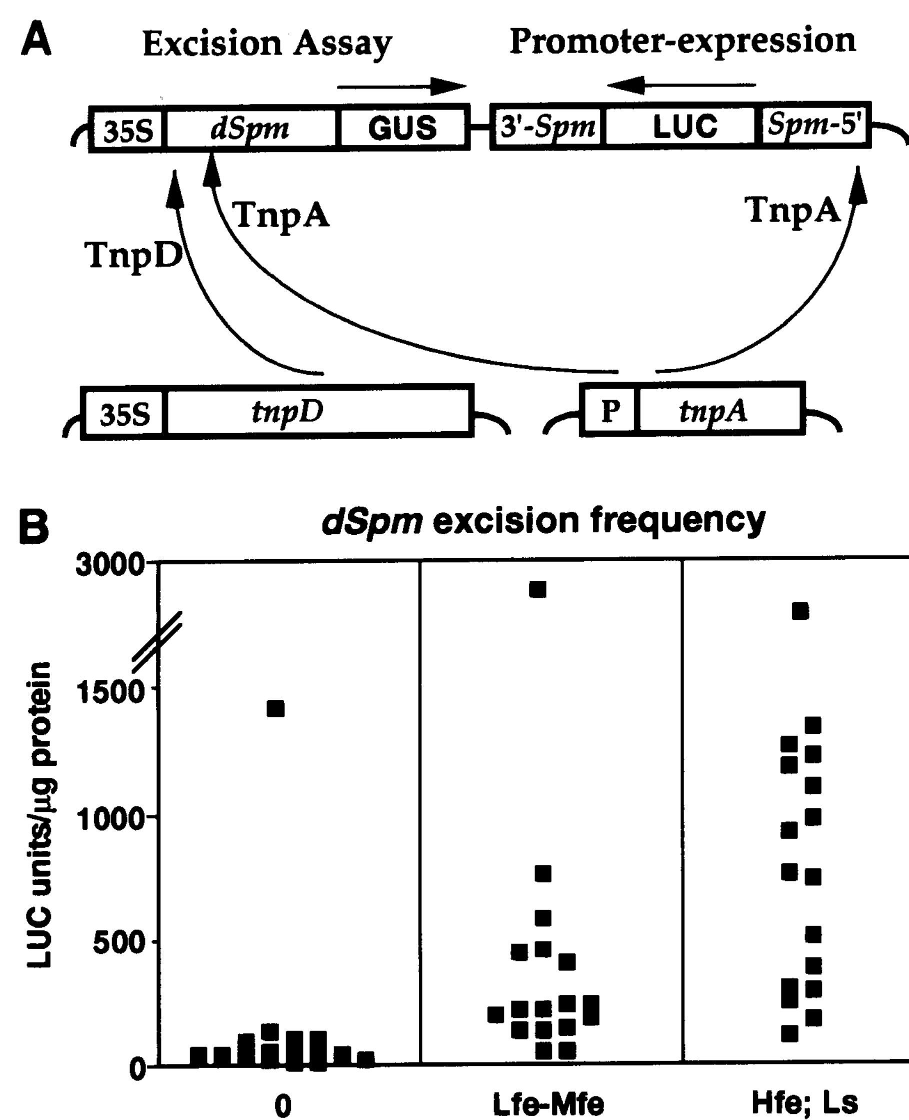

Figure 2. The Effect of TnpA on the Inactive, Methylated UCR–DCR *Spm* Promoter in Transgenic Plants

(A) A diagram of the constructs used to detect *dSpm* excision and TnpA effects on *Spm* promoter activity (pMS127 and pMS109).

(B) A plant containing a methylated UCR–DCR *Spm* promoter–*dSpm* excision construct (Figures 1B and 1D) and *tnpD* was retransformed with *tnpA* cDNAs (plasmids pMS114, pMS116, pMS118, pMS121, pMS122, and pMS124). Each point represents LUC gene expression and *dSpm* excision assayed in an independent transformant (see Experimental Procedures). LUC activity is shown in successive panels for plants that exhibit no *dSpm* excision (0), a low to medium frequency of excision (Lfe–Mfe), and a high frequency of excision (Hfe) or large sectors (Ls) indicative of early excision events.

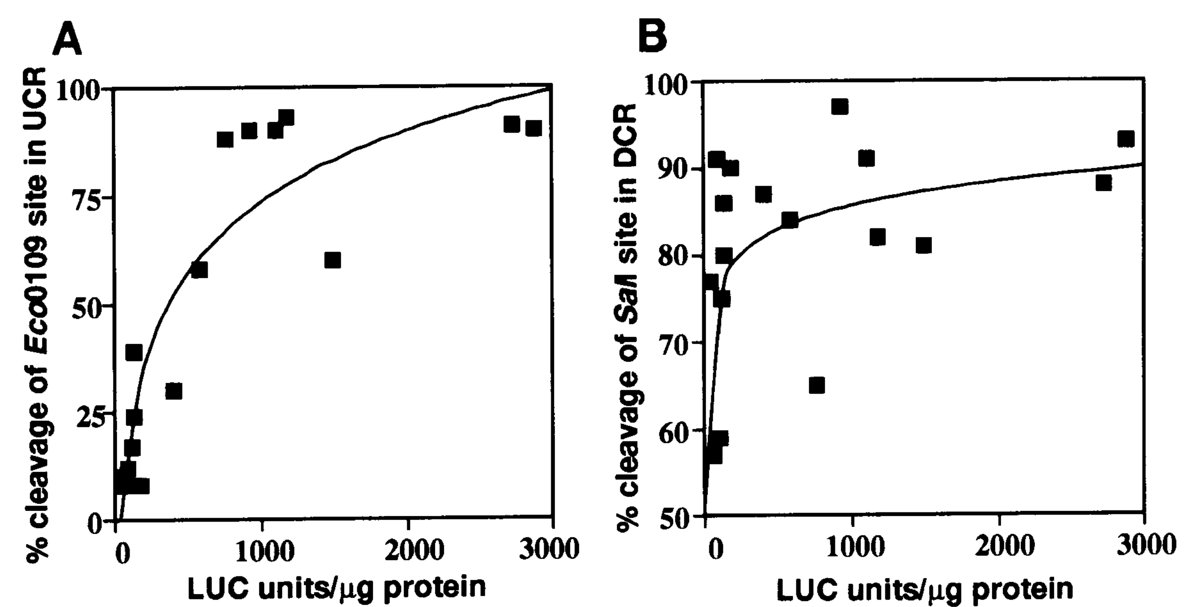

Figure 3. The Relationship between *Spm* Promoter Methylation and Expression of the LUC Gene in Transgenic Plants

DNA was extracted from plants transformed with pMS127 and pMS109, and the extent of methylation was determined for the EcoO109 site in the promoter (A) and the SalI site in the DCR (B) as described in Figure 1. The results are represented as percent (%) cleavage at each restriction site. LUC activity was measured for each transformant (see Experimental Procedures). Logarithmic curves were generated using CA-Cricket Graph III and gave regression values of 0.913 and 0.470 for (A) and (B), respectively.

struct, as well as one carrying a CaMV 35S–*tnpD* cDNA (Masson et al., 1991; Schläppi et al., 1993). Because TnpD alone is not sufficient for transposition, the *dSpm* element is stable in such transformed plants (Frey et al., 1990; Masson et al., 1991). The plants were then retransformed with *tnpA* cDNA plasmids also carrying an herbicide-resistance marker (see Experimental Procedures). Transformants expressing TnpA were then identified by the presence of GUS+ sectors resulting from excision of the *dSpm* element from the GUS gene (Figure 2A). Transformants were classified into three groups reflecting the transposition activity of TnpA: no *dSpm* excision, low to medium frequency of *dSpm* excision, and high frequency of *dSpm* excision or large sectors resulting from early *dSpm* excision (Schläppi et al., 1993). Each group was assayed for LUC activity to determine the effect of TnpA on the *Spm* promoter.

There is a clear positive correlation between the level of TnpA expression assessed by the *dSpm* excision assay and activation of the *Spm* promoter detected by LUC gene expression (Figure 2B). More than half of the plants showing no GUS+ sectors had LUC activity levels of less than 50 U/µg protein, while all of the plants with either many or very large GUS+ sectors had LUC levels above 50 U/µg protein and a third of them gave values higher than 500 U/µg protein (Figure 2B). Plants showing a low to intermediate frequency of GUS sectoring gave intermediate LUC values. Similar results were obtained when the *tnpA* cDNA was introduced by a genetic cross (data not shown). Thus, TnpA activates LUC gene expression from an inactive, methylated UCR–DCR *Spm* promoter.

The TnpA-Activated UCR-DCR Promoter Is Demethylated

To determine whether the TnpA-activated UCR–DCR *Spm* promoter is demethylated, DNA from *tnpA* cDNA-retransformed plants was analyzed with the methylation-sensitive restriction enzymes EcoO109 and SalI, diagnostic for the UCR and DCR, respectively, as described above. The extent of cleavage at each site corresponds to the fraction of *Spm* promoters not methylated at that site within the population of DNA molecules extracted from each plant. There is a positive correlation between the cleavablity of both diagnostic sites and the LUC activity in the plant from which the DNA was isolated (Figure 3). Because there is a positive correlation between *Spm* promoter expression as detected by LUC activity and TnpA as detected by transposition (see Figure 2B), it follows that plants with high levels of TnpA exhibit low levels of *Spm* promoter methylation. Hence, expression of TnpA results in *Spm* promoter demethylation.

TnpA Trans-Activates the Methylated *Spm* Promoter in a Transient Assay

While the foregoing experiments show that TnpA activates and promotes demethylation of the *Spm* promoter, they provide no information about mechanism. TnpA may activate the methylated *Spm* promoter indirectly by stimulating demethylation. Alternatively or additionally, TnpA may activate the methylated promoter directly. We therefore asked whether TnpA can activate a methylated *Spm* promoter in transient assays. The experiments were done both in suspension cell cultures derived from a stably transformed plant with a methylated, inactive UCR–DCR

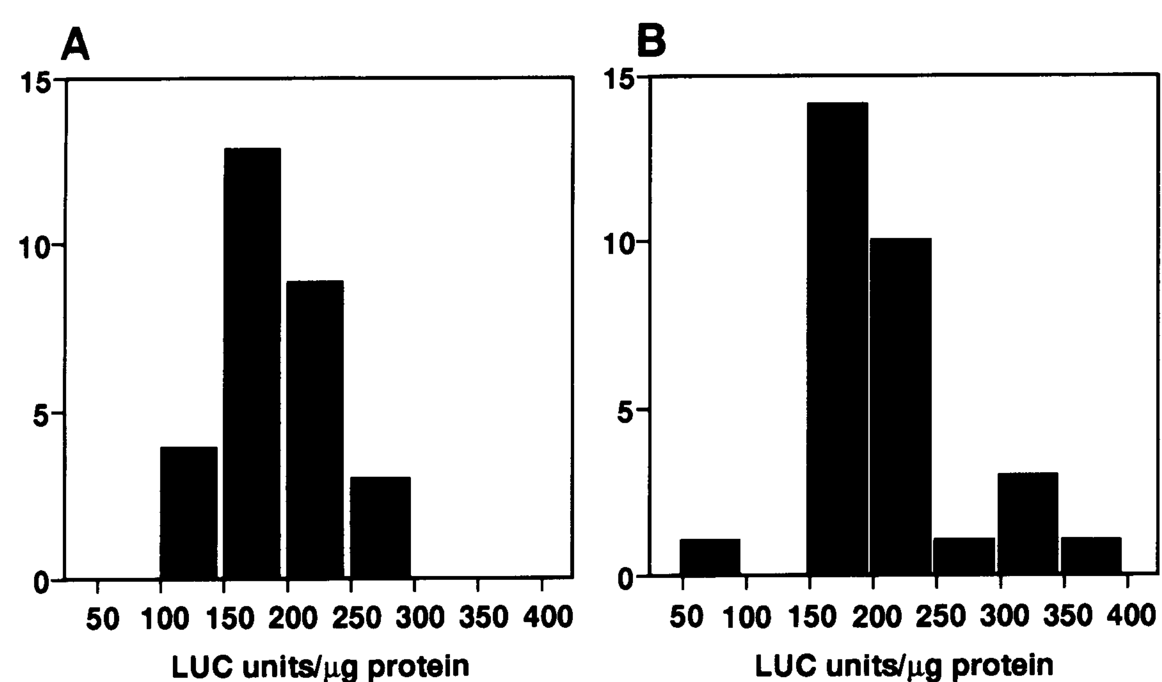

Figure 4. The Effect of TnpA on LUC Gene Expression from a Methylated UCR–DCR *Spm* Promoter in a Transient Assay

Suspension culture cells derived from stable transformants with a methylated *Spm* promoter were transiently transformed with a control plasmid (pUC18) (A) and a *tnpA* cDNA-containing plasmid (pMS163) (B). Y axis values correspond to the number of independently transformed samples giving LUC values within the indicated range.

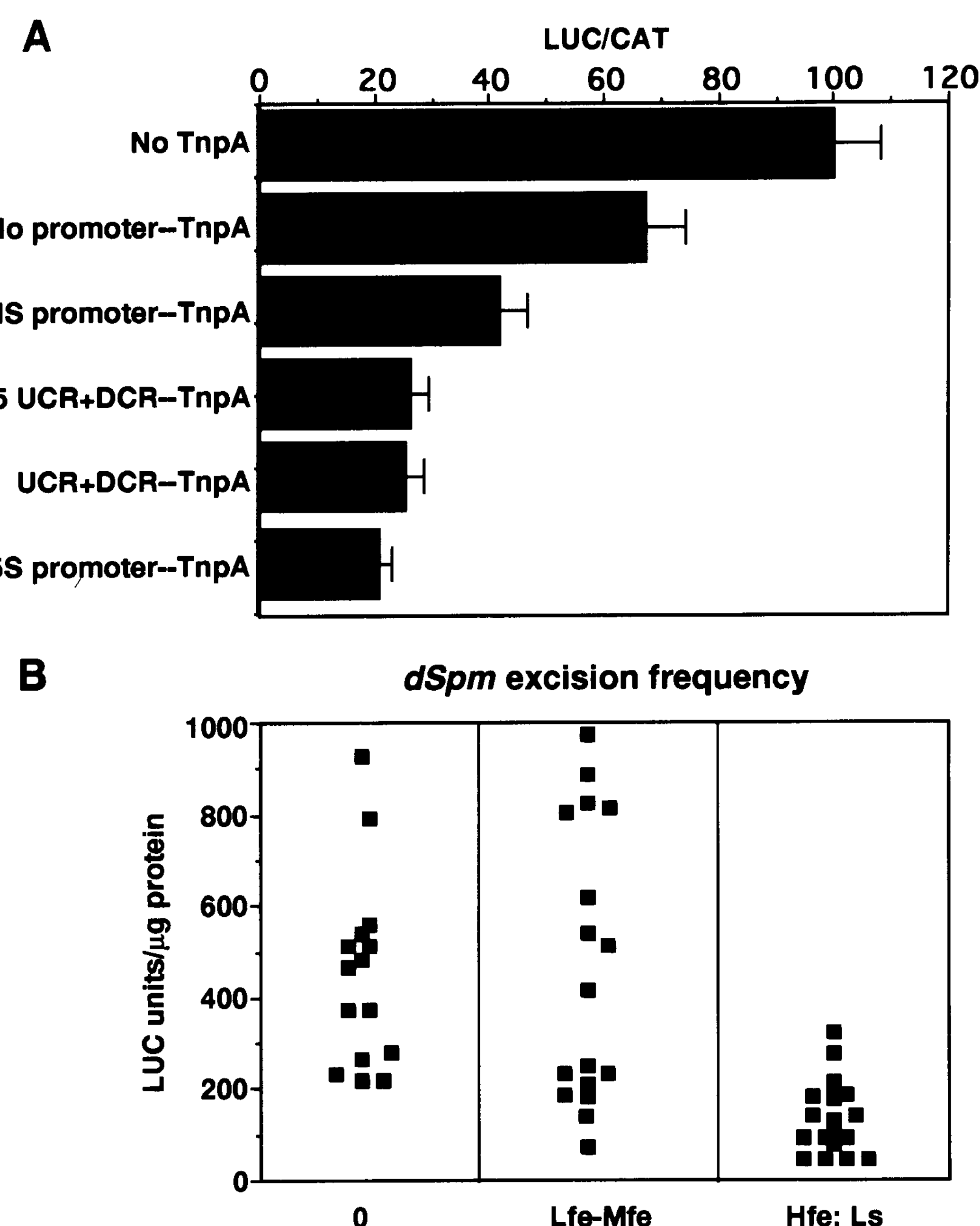

Figure 5. The Effect of TnpA on LUC Expression from the Hypomethylated *Spm* Promoter in Transient Assays and Stably Transformed Plants

(A) Each transient assay was done with a plasmid containing the UCR–DCR *Spm* promoter–LUC gene fusion (pDC120), with a TnpA plasmid (or a control plasmid) with either a promoterless *tnpA* cDNA (pRR434) or a *tnpA* cDNA expressed from the promoter indicated below each column (plasmids pRR432, pRR433, pRR435, and pRR438), and with a 35S-chloramphenicol acetyltransferase (CAT) reference plasmid. The activity of the reporter gene is expressed as the ratio of LUC to CAT activity (Raina et al., 1993).

(B) A plant containing a hypomethylated UCR *Spm* promoter–*dSpm* excision construct (Figures 1C and 1E) and *tnpD* was retransformed with *tnpA* cDNAs (plasmids pMS114, pMS116, pMS118, pMS121, pMS122, and pMS124). LUC activity is shown for independent transformants exhibiting no *dSpm* excision (0), a low to medium frequency of excision (Lfe–Mfe), and a high frequency of excision (Hfe) or large sectors (Ls) indicative of early excision events.

promoter–LUC gene and leaves from such plants. The cell culture, derived from the plants used for the TnpA retransformation experiment described in Figure 2B, had a doubling time of 2 days in the presence of the growth regulator auxin. Assays were done 18 hr after bombardment, minimizing DNA replication-associated changes in DNA methylation and permitting detection of short-term changes in promoter activity.

LUC gene expression was stimulated by introduction of a *tnpA* cDNA, but not by a plasmid lacking the cDNA (Figure 4). Suspension cells containing the LUC gene under the control of the methylated *Spm* promoter show a low level of LUC activity. The mean value in assays of plasmid-bombarded controls was 197 U/μg protein and the range was 132–259 U/μg protein. The same general distribution was observed in identical assays of cells bombarded with a *tnpA* cDNA-containing plasmid. However, 13% of the assays gave values higher than 300 U/μg protein. Similar experiments done using two halves of single leaves taken from plants with an inactive, methylated *Spm* promoter–LUC gene gave similar results. In three independent experiments, LUC values averaged 11%–33% higher after 18 hr in leaf halves bombarded with a *tnpA* cDNA plasmid than in the halves bombarded with a control plasmid (data not shown).

Although the difference between experimental and control values was not large in either type of experiment, it was invariably positive. This is in marked contrast with repression by TnpA of the unmethylated *Spm* promoter (vide infra). Because only a small fraction of cells receive and express the *tnpA* cDNA plasmid, while the LUC gene is expressed at a low level in all cells, we cannot assess the magnitude of the direct activation by TnpA of the methylated promoter in such experiments. Nonetheless, the small positive effect on LUC gene expression from the methylated *Spm* promoter observed in the short-term experiments suggests that TnpA directly activates either the methylated or hemimethylated promoter. However, because we cannot assess the magnitude of the effect, it is possible that full TnpA-mediated activation of the methylated *Spm* promoter also requires DNA replication.

TnpA Represses the Unmethylated *Spm* Promoter

Expression of a GUS reporter gene is inhibited by TnpA if a TnpA-binding site is inserted in its promoter (Grant et al., 1990). We have reported that TnpA represses the *Spm* promoter in transient expression assays, as well as *Spm* transcription in transgenic tobacco (Cook and Fedoroff, 1992; Schläppi and Fedoroff, 1992; Raina et al., 1993). Both observations suggest that TnpA is also a negative regulator. To ascertain whether the ability of TnpA to activate or repress the *Spm* promoter depends on its level of expression, we used *tnpA* cDNA expressed from promoters of different strength. Those tested were the CaMV 35S promoter, both an intact and a deleted *Spm* promoter with about 10% of the intact promoter strength (Raina et al., 1993), and an uninduced soybean heat shock promoter (Ainley and Key, 1990). A promoterless *tnpA* gene was also used. Regardless of promoter strength, expression

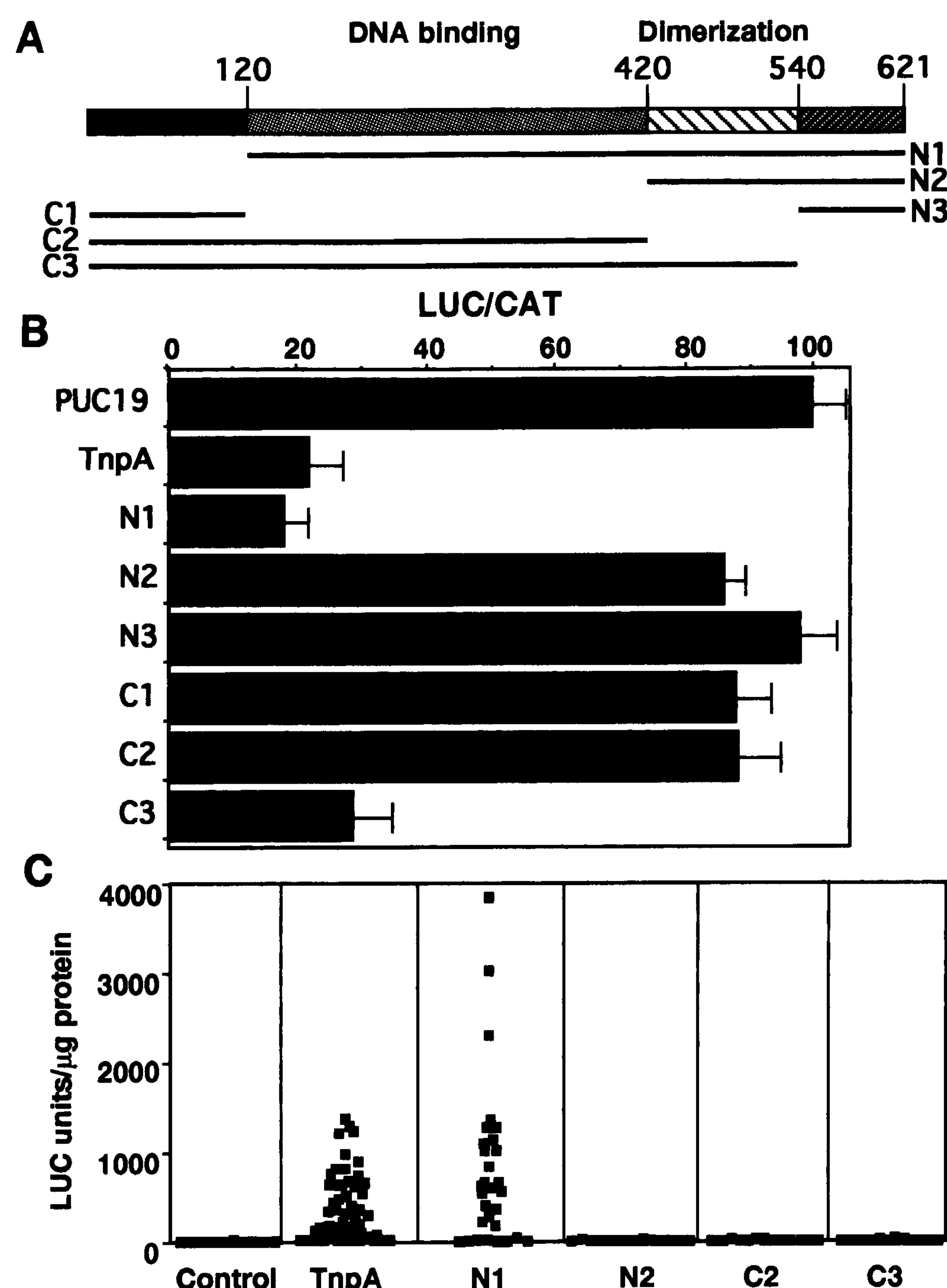

Figure 6. The Effect of Deletions on the Ability of TnpA to Affect Expression of the Unmethylated and Methylated *Spm* Promoter

(A) A diagram of the TnpA coding sequence showing the DNA-binding and dimerization domains of the protein and the endpoints of the deletions used here (see Experimental Procedures), with lines representing the remaining coding sequence.

(B) Repression of the unmethylated UCR–DCR *Spm* promoter in a transient expression assay by intact and deleted TnpA. Experiments were performed as described in Figure 5, using plasmids pDC120, pMS163, pMS194, pMS195, pMS196, pMS197, pMS198, and pMS199.

(C) Activation of the methylated UCR–DCR *Spm* promoter by intact and deleted TnpA in stably transformed cells. Experiments were performed as described in the legend to Figure 2B, using pCGN1548/ALS (control), pMS169 (intact *tnpA* cDNA), and pMS200, pMS201, pMS202, pMS203, pMS204, and pMS205 (*tnpA* cDNA deletions).

of TnpA repressed the *Spm* promoter in transient assays (Figure 5A). The extent of repression ranged from more than 80% for a CaMV 35S promoter-driven *tnpA* cDNA to 30% for a promoterless *tnpA* cDNA, declining with decreasing promoter strength. These results suggest that the different abilities of TnpA to activate and repress the *Spm* promoter cannot be explained by differences in the amount of the protein.

Transient assays are sufficiently brief to preclude extensive methylation of the introduced DNA, suggesting that TnpA may represses the *Spm* promoter in the transient assay because it is not methylated. To determine whether TnpA can also repress a hypomethylated *Spm* promoter stably integrated into the tobacco genome, we used leaves from a plant containing an active, hypomethylated UCR LUC gene and the CaMV 35S-*tnpD* cDNA that were retransformed with *tnpA* cDNAs (or control plasmids lacking the cDNA). Regenerated plants were scored for *Spm* promoter activity by the LUC assay and TnpA activity by the *dSpm* excision assay. Plants with high TnpA levels had low levels of LUC activity (Figure 5B). Thus, TnpA represses the active, hypomethylated UCR *Spm* promoter. This contrasts sharply with its ability to activate the inactive, methylated UCR–DCR *Spm* promoter in identical experiments (see Figure 2B). It is perhaps significant that low LUC activity was observed only in the plants with the highest levels of TnpA activity, as judged by the frequency of *dSpm* excision. While the UCR promoter is much less methylated at the EcoO109 site than the UCR–DCR promoter, it is not entirely unmethylated (see Figure 1). Thus, some promoter methylation may be compatible with expression, and promoter repression in vivo may occur only at TnpA concentrations high enough to achieve complete promoter demethylation.

The Repressor Function of TnpA Requires Only the DNA-Binding and Dimerization Domains

Deletions and translational termination mutations were introduced into the TnpA coding sequence to identify the parts of the TnpA protein required for its autoregulatory functions. A DNA-binding domain between amino acids 122 and 427 and a protein dimerization domain between amino acids 428 and 542 had been identified in oligonucleotide binding studies (Trentmann et al., 1993). Based on these results, we constructed cDNAs coding for the N- and C-terminally truncated TnpA proteins, designated N1–N3 and C1–C3 respectively, shown in Figure 6A (see Experi-

Table 2. The Effect of TnpA Deletions on *dSpm* Excision Frequency and *Spm* Promoter Methylation

TnpA Construct	Percent GUS+ Plants[a]	Total Analyzed[b]	Percent EcoO109 Cleavage in the UCR[c]	Percent SalI Cleavage in the DCR[c]
Control vector	0	56	11 ± 4	11 ± 0
Wild-type TnpA	62	90	88 ± 12	97 ± 2
N1 deletion	(5)[d]	56	98 ± 3	79 ± 20
N2 deletion	0	51	15 ± 7	ND
N3 deletion	0	10	ND	ND
C1 deletion	0	10	ND	ND
C2 deletion	0	69	13 ± 3	9 ± 6
C3 deletion	(1)[d]	83	18 ± 2	19 ± 17

[a] Plants with GUS+ sectors resulting from *dSpm* excision.
[b] Each plant represents an independent transformant.
[c] DNA from three or four independent transformants was analyzed for each TnpA construct.
[d] Very low frequency of *dSpm* excision and very small GUS+ sectors (one to two small spots).
ND, not determined.

mental Procedures). To identify the protein domains required for *Spm* promoter repression, mutant *tnpA* cDNAs expressed from the CaMV 35S promoter were tested in both transient assays and stably transformed plants. Deletion of sequences coding for the N-terminal 120 (N1) or the C-terminal 81 (C3) amino acids had no effect on the ability of TnpA to repress the UCR–DCR *Spm* promoter in the transient assay (Figure 6B). Longer deletions eliminating either the DNA-binding domain (N2) or the dimerization domain (C2) or both (C1 and N3) eliminated the repressor activity of TnpA (Figure 6B). Nonsense mutations were as effective as deletions, showing that repressor activity resides in the TnpA protein. These results suggest that only the DNA-binding and dimerization domains of the TnpA protein are required for *Spm* promoter repression in the transient assay.

The same mutant cDNAs were stably transformed into plants with an active, hypomethylated UCR LUC gene. Because these plants also contain an excision assay plasmid (see Figure 2A), both the transpositional and regulatory activities of the TnpA derivatives can be assessed simultaneously. The N1 and C3 deletion derivatives repressed LUC gene expression by 70%–80%, whereas mutants lacking either the DNA-binding domain (N2) or the dimerization domain (C2) or both (C1 and N3) had no effect (data not shown), results identical to those obtained in the transient assay (Figure 6B). None of the mutant *tnpA* cDNAs complemented the resident TnpD to promote frequent *dSpm* excision (Table 2). Some of the plants transformed with N1 and C3 deletions had GUS^+ sectors, but these were few and small, indicating that the excision activity of TnpA is severely curtailed by even short N-terminal and C-terminal deletions. We conclude that while most or all of the sequence of the TnpA protein is required for transposition, the DNA-binding and dimerization domains suffice for repressing the *Spm* promoter.

The Activator Function of TnpA Requires the C-Terminus in Addition to the DNA-Binding and Dimerization Domains

The activator function of TnpA deletion mutants was tested by introducing them into plants with an inactive, methylated UCR–DCR *Spm* promoter. Because all of the deletions drastically reduce the ability of TnpA to participate in excision (Table 2), transformants that do not express TnpA protein cannot be independently identified. Nonetheless, most plants transformed with wild-type *tnpA* cDNA had substantially higher levels of LUC activity than control vector-transformed plants (Figure 6C). The N1 mutant is an equally good or better activator of the methylated *Spm* promoter than the complete TnpA protein. Mutants missing the DNA-binding domain (N2) or the dimerization domain (C2) do not activate the methylated promoter, nor does the C3 mutant lacking the C-terminal 81 amino acids. Transient assays in cultured cells derived from stably transformed lines with an inactive, methylated UCR–DCR *Spm* promoter–LUC gene gave similar results (data not shown). Moreover, only those proteins of mutants that activate the methylated *Spm* promoter result in its demethylation (Table 2). Thus, none of the mutants tested abolish the ability of TnpA to promote demethylation without disrupting its ability to activate the methylated promoter. We conclude that the ability of TnpA to activate the methylated *Spm* promoter requires a C-terminal sequence not required for the repressor function of TnpA.

Discussion

Spm Is Regulated by Interacting Epigenetic and Autoregulatory Mechanisms

Proteins with both repressor and activator functions have been identified in eukaryotic cells (Yang-Yen et al., 1990; reviewed by Schüle and Evans, 1991). The properties of such proteins are generally influenced by interactions with other regulatory proteins, but not by methylation of the binding site, although suppression of gene expression has been attributed to competition between protein factors with different affinities for a methylated binding site (Boyes and Bird, 1991; Jost and Hofsteenge, 1992; Jane et al., 1993). There is correlative evidence that methylation changes affect gene expression in eukaryotes, but little is known about mediating regulatory proteins (reviewed by Razin and Cedar, 1991). In Escherichia coli, DNA adenine methylation contributes to regulation of *Tn5* and *Tn10* transposition (Roberts et al., 1985; Yin et al., 1988), as well as expression of the phage Mu *mom* gene (Bölker and Kahmann, 1989), the *dnaA* gene (Braun and Wright, 1986), and the pyelonephritis-associated pilus operon (Nou et al., 1993; Braaten et al., 1994). Although these systems generally involve methylation at a small number of sites, methylation within protein-binding sites can exert both positive and negative regulatory effects.

The results of the present and earlier genetic studies on *Spm* define a novel system of epigenetic regulation in which the *Spm*-encoded autoregulatory protein TnpA interacts differentially with the promoter of the element, depending on its methylation state to determine the level, as well as the developmental pattern, of element transcription and transposition. A striking and unique characteristic of *Spm* regulation is that the element can be maintained in a heritably inactive state, in a heritably active state, or in one of a variety of heritable states that cause different developmental patterns of *Spm* expression (reviewed by Fedoroff and Chandler, 1994). *Spm* elements exhibit different levels of methylation at different times in development, suggesting that part of the patterning mechanism resides in the differential expression or activity of plant factors that alter element methylation (Fedoroff and Banks, 1988; Banks and Fedoroff, 1989). In addition, an inactive element can be activated transiently by introduction of an active element (McClintock, 1957, 1958, 1959, 1971). However, in maize plants, the heritability of the inactive state can change, and as it increases, the capacity of the element to be trans-activated by an active *Spm* decreases (Banks et al., 1988; Banks and Fedoroff, 1989). Spontaneous reactivation of extremely inactive elements, which have been termed cryptic *Spm* elements, occurs at a very low frequency (Fedoroff, 1989). Curiously, both establishment of the cryptic state and the full reactivation of a cryptic *Spm* are slow processes in maize, requiring

multiple plant generations and therefore many rounds of DNA replication (Fedoroff and Banks, 1988; Fedoroff, 1989). Reactivation of cryptic *Spm* elements is enhanced by the introduction of an active element (Fedoroff, 1989). These genetic observations suggested the existence of an element-encoded regulatory gene product capable of reactivating an inactive element both transiently and heritably. The present observations clarify the underlying molecular mechanisms and show that the *Spm* element encodes an autoregulatory protein with unique properties.

The *Spm* Promoter Is the Target of Epigenetic Inactivation

Epigenetic inactivation of the *Spm* element is accompanied by methylation of sequences at its 5′ end in both maize and transgenic tobacco plants (Banks et al., 1988; Schläppi et al., 1993). The present experiments show that the 5′ end of the *Spm* element suffices for de novo promoter methylation in transgenic tobacco cells. Strikingly, the UCR promoter was much more extensively methylated within the timeframe of these experiments if the promoter fragment included the DCR sequence than if it did not. A promoter lacking the DCR was only slightly methylated and supported levels of reporter LUC gene expression comparable to those seen in a transient expression system, which measures the activity of the unmethylated promoter (Raina et al., 1993). If the DCR was present, both UCR and DCR were methylated and the LUC reporter gene showed very little activity. The DCR is contained within the sequence coding for the untranslated first exon of all of the *Spm* transcripts. It is 80% GC and internally repetitive and contains many methylatable C residues (Masson et al., 1987; Banks et al., 1988). The DCR has no effect on *Spm* promoter activity in a transient assay (Raina et al., 1993). However, the DCR makes the *Spm* promoter quite insensitive to an upstream enhancer, and it is also an effective silencer of both a complete and a minimal CaMV 35S promoter (Raina et al., 1993).

Thus, the DCR is a sequence with a unique role in regulation of *Spm* expression. It not only buffers the promoter of the element from the influence of external promoters and enhancers, but is central to its capacity for epigenetic inactivation. The propensity for rapid methylation may be an inherent property of the 5′-terminal sequence of the element. Alternatively, methylation may be mediated by the DCR-containing transcript, as recently reported for genomic copies of replicating viroid RNA (Wassenegger et al., 1994). It appears that a low level of methylation is compatible with *Spm* promoter expression, suggesting that some threshold level of methylation is required to inactivate the promoter (Figure 7). Extensive methylation significantly represses the *Spm* promoter, although we have observed neither complete methylation at either restriction site examined nor complete inhibition of LUC reporter gene expression in either transgenic or transiently transformed tobacco cells. How methylation inhibits *Spm* promoter expression is not understood. Among the possibilities are interference with the binding of transcription factors (Iguchi-Ariga and Schaffner, 1989), changes in DNA or chromatin conformation (Lewis and Bird, 1991), and binding of inhibitory proteins to methylated sequences (Meehan et al., 1989; Boyes and Bird, 1991).

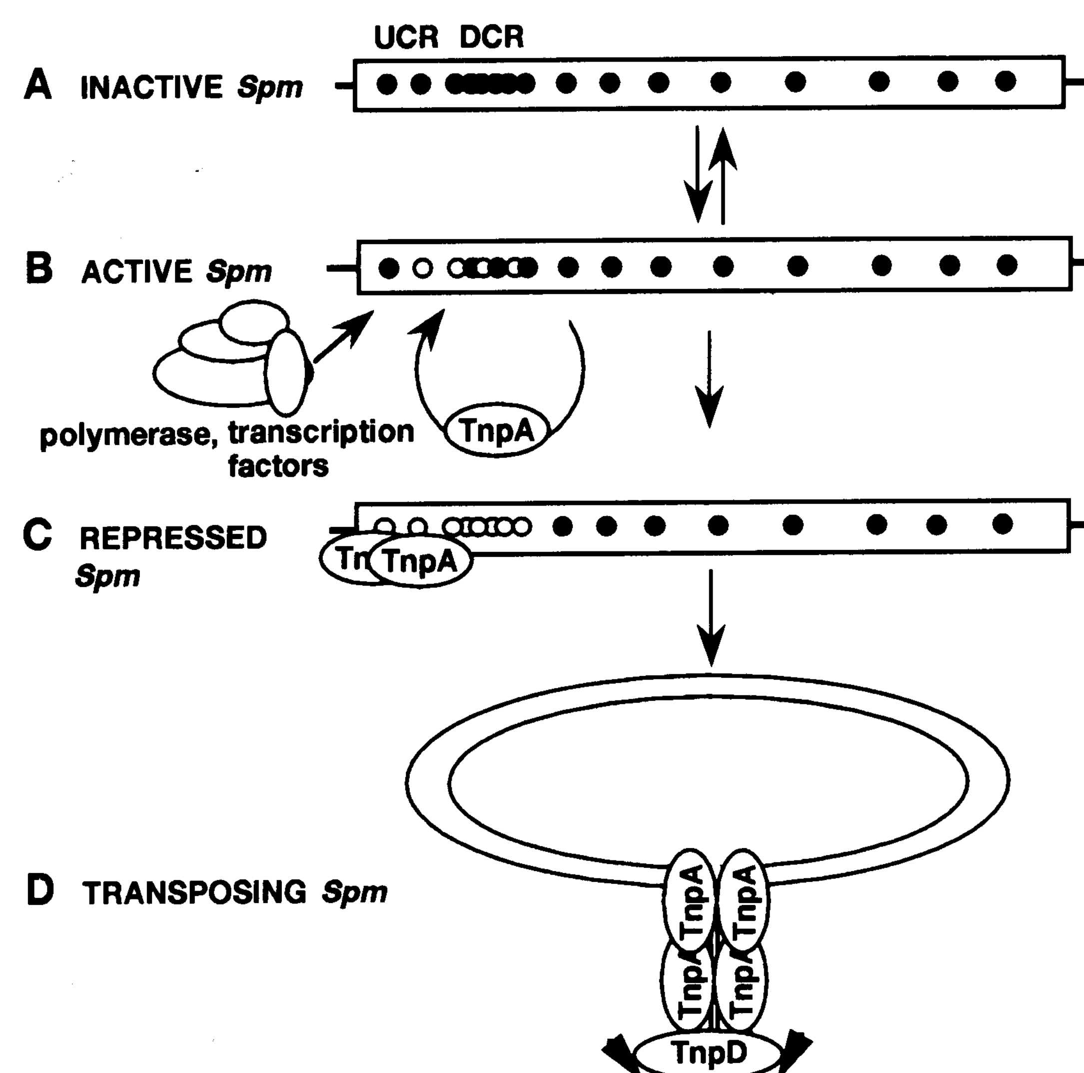

Figure 7. A Diagrammatic Representation of the Role of TnpA in Transcriptional Regulation and Transposition

Closed and open circles represent methylated and unmethylated C residues, respectively.

(A) Methylated elements are transcriptionally inactive and designated as cryptic *Spm* or inactive *Spm* elements, depending on the extent of C-methylation in the UCR and DCR and the heritability of the inactive state (Banks et al., 1988; Fedoroff, 1989). The extent of *Spm* promoter methylation changes during development and is at a minimum early in plant development (Banks and Fedoroff, 1989).

(B) Activation of an inactive *Spm* can occur spontaneously in development or be promoted by TnpA supplied either by an active *Spm* (Banks et al., 1988) or by a *tnpA* cDNA (Schläppi et al., 1993). *Spm* is transcriptionally activated in the presence of TnpA, and this is accompanied by a decrease in methylation.

(C) Accumulation of TnpA eventually results in complete promoter demethylation and TnpA-mediated repression of transcription.

(D) Transposition of *Spm* is postulated to occur when sufficient TnpA accumulates to saturate the binding sites at both element ends, whose proposed function in transposition is to bring element ends together. There is evidence that binding of TnpD to TnpA is necessary for its participation in transposition, and it is postulated to catalyze cleavage at element ends during transposition (Masson et al., 1991).

TnpA Is a Multifunctional Protein That Participates in Transposition, as Well as in Both Positive and Negative Regulation of *Spm* Promoter Expression

TnpA is a DNA-binding protein that recognizes and binds to 12 bp sequences present in multiple copies in each orientation at both ends of the *Spm* element (Gierl et al., 1988). It has been shown in oligonucleotide binding studies that the protein dimerizes and binds optimally as a dimer to a tail-to-tail dimeric binding site (Gierl et al., 1988; Trentmann et al., 1993). The protein is necessary for *Spm* transposition (Frey et al., 1990; Masson et al., 1991). Deletion of even some of its binding sites at either element end markedly delays transposition and reduces its frequency (Schiefelbein et al., 1985; Schwarz-Sommer et al., 1985). This suggests that transposition occurs only when most or all of the binding sites are present and occupied. It has

been postulated that TnpA multimerization functions to bring element ends together in transposition, as shown diagrammatically in Figure 7 (Frey et al., 1990; Masson et al., 1991). Our present results demonstrate that loss of any part of the TnpA sequence severely interferes with or completely abolishes its ability to participate in transposition. Preliminary observations suggest that TnpD, the other element-encoded protein required for transposition, interacts with TnpA bound to DNA (R. R., unpublished data). Although it has been postulated that TnpD is an element-encoded nuclease (Masson et al., 1991; Figure 7), the precise role of each protein in transposition remains to be elucidated.

TnpA is also an autoregulatory protein with novel properties: it represses its unmethylated promoter and activates its methylated promoter. The ability of TnpA to repress the unmethylated promoter declines with the decreasing strength of the promoter driving the *tnpA* gene, suggesting that the extent of promoter repression is governed by the supply of TnpA. If the promoter is sufficiently methylated to be inactive, TnpA activates it. Promoter activation increases in proportion to the amount of TnpA activity detected by an excision assay, suggesting that there is also a simple relationship between the availablility of TnpA and promoter activation. However, TnpA represses a hypomethylated, active *Spm* promoter only in transformed cells exhibiting high levels of TnpA activity. Expression of the hypomethylated promoter is about the same in lines expressing TnpA at relatively low levels as in lines showing no TnpA activity. Thus, whether TnpA activates or represses the *Spm* promoter in vivo appears to be determined by both the extent of promoter methylation and the availability of TnpA. As diagrammed in Figure 7, the ability of TnpA to repress its promoter may be an essential step in the transition from a transcriptionally active to a transpositionally active form.

There are indications that TnpA represses the unmethylated promoter by direct competitive binding. *Spm* promoter activity is confined to the UCR, which contains multiple TnpA-binding sites (Raina et al., 1993). Deletion of successive TnpA-binding sites gradually eliminates promoter activity, suggesting that there are redundant binding sites for transcription factors coincident with or overlapping TnpA-binding sites (Raina et al., 1993). Moreover, TnpA binds more strongly to unmethylated binding site oligomers than to either hemimethylated or fully methylated binding site oligomers (Gierl et al., 1988). Taken together with the evidence that only the DNA-binding and dimerization domains are required for repressor activity, these observations suggest that TnpA represses by competing directly with transcription factors for binding to the promoter.

It is likely that TnpA activates the methylated *Spm* promoter by a different mechanism than that involved in repression. Activation of the methylated promoter is invariably accompanied by a decrease in promoter methylation. However, we have detected TnpA-mediated promoter activation within a period of time too short to permit complete DNA demethylation, suggesting that TnpA may act initially in the direct transcriptional activation of the methylated or hemimethylated promoter. The observation that promoter activation requires a C-terminal sequence not required for repressor activity suggests that the C-terminus might either be the activator domain of the protein or serve indirectly to interact with other regulatory proteins required to activate the promoter. We cannot presently evaluate the relative importance of direct activation of the methylated promoter and indirect activation of the promoter as a consequence of reduced methylation. All mutant TnpA proteins that are capable of activating the promoter also support promoter demethylation. However, it appears likely that promoter demethylation is more complex than simple competitive binding of TnpA to methylation target sequences because C residues in sequences that do not bind TnpA show reduced methylation in its presence. This includes the DCR, which does not bind TnpA (R. R., unpublished data) and which contains 89 methylatable C residues per strand as well as C residues outside of TnpA-binding sites in the UCR. The UCR coding strand contains 16 potentially methylatable C residues (13 in the noncoding strand), of which 12 are within TnpA-binding sites (10 in the noncoding strand). Of the nine sequences in the UCR with at least 75% homology to the consensus TnpA-binding site, seven contain at least one methylatable C residue per strand and six contain two or three of them (Masson et al., 1987; Gierl et al., 1988). Thus, many of the 5′-terminal C residues that show methylation changes in the presence of TnpA are not in TnpA-binding sites, implying that the ability of the protein to reduce methylation is mediated either by changes in DNA (or chromatin) conformation or by additional proteins.

In summary, we have demonstrated that the molecular basis of the epigenetic regulatory system of the *Spm* element resides in the properties of its 5′-terminal sequence and an element-encoded protein with unique regulatory properties. The promoter of the element is a target for rapid inactivation by de novo C-methylation, a property attributable to the presence of the adjacent GC-rich DCR sequence. TnpA, the element-encoded regulatory protein, serves as a conventional repressor of the unmethylated promoter, but has the novel capacity to activate the methylated promoter. TnpA-mediated promoter activation is associated with reduced methylation of C residues in both the promoter and the adjacent DCR sequence that does not bind the protein. These properties define TnpA as a member of a novel class of eukaryotic epigenetic regulatory proteins.

Experimental Procedures

Plasmids for Tobacco Transformation

The *dSpm* excision construct (Figure 2A) has been described (Masson and Fedoroff, 1989; Masson et al., 1991; Schläppi et al., 1993). The LUC–mini-*Spm* constructs (Figure 2A) were assembled in pCGN1549 (gift of K. McBride, Calgene Incorporated; McBride and Summerfelt, 1990). The *Spm* 3′ end from pSpm(Xho) (Masson and Fedoroff, 1989) was inserted as a PstI–HindIII fragment (pMS66). A LUC reporter gene was used because the enzyme has a short half-life, permitting temporal changes in gene expression to be detected (Thompson et al., 1991). The LUC coding sequence was transferred as a BamHI–SacI fragment from pDC107 (Raina et al., 1993) into pCGN7334 (K. McBride, Calgene Incorporated) to give pMS68, and a BamHI–PstI fragment containing

LUC with the *mas* terminator was inserted into pMS66 to give pMS70. A 220 bp or 576 bp Asp718–BamHI 5′ *Spm* fragment (Figure 1A) from pDC121 or pDC105, respectively (Raina et al., 1993), was inserted into pMS70, yielding pMS83 and pMS73. The DCR and MAC promoter (Comai et al., 1990) controls contained the Asp718–BamHI fragments from pDC131 (Raina et al., 1993) and from pMS64 (which contains the BglII fragment of pCGN7334 in the BamHI site of pCGN1549) in pMS70 (pMS75 [DCR LUC] and pMS81 [MAC LUC] controls, respectively). HindIII mini-*Spm* fragments from pMS83 and pMS73 were inserted in the ClaI site of GUS4 (Masson and Fedoroff, 1989), giving pMS127 (UCR LUC–mini-*Spm*) and pMS109 (UCR-DCR LUC–mini-*Spm*). The 35S–*tnpD* cDNA binary vector has been described (Masson et al., 1991). The *tnpA* cDNA (in pSP64; Promega) was moved as a filled-in Asp718 fragment into the Asp718 site of pMA406 (Ainley and Key, 1990) to give pMS86 (*tnpA* expressed from the soybean 2019E heat shock promoter). 2019E–*tnpA* was inserted as a BglII fragment into the BamHI site of the binary vector pMS26, constructed by inserting the BglII fragment of pCGN1541 (from K. McBride) into the BamHI site of pCGN1532 (McBride and Summerfelt, 1990) to give pMS94. *tnpA* with the nopaline synthase (*nos*) terminator from pMS86 was inserted as a BamHI–BglII fragment into the BamHI site of pMS26 to give pMS93. Asp718–BamHI fragments from pDC105, pDC121, pDC227, pDC241 (Raina et al., 1993), and pMS64 were inserted into pMS93 to give pMS95, pMS105, pMS99, pMS101, and pMS103 to give *tnpA* expressed from the UCR–DCR, UCR, pMS–95 UCR–DCR, pMS–41 UCR–DCR, and MAC promoters, respectively. A mutated acetolactate synthase (ALS) gene from Arabidopsis thaliana that confers resistence to chlorsulfuron (Haughn et al., 1988) was used to select plants containing the *tnpA* cDNAs. It was inserted as an Asp718 fragment from pUC19 (from J. Mauvais, Du Pont Company) into pMS93, pMS94, pMS95, pMS105, pMS99, pMS101, and pMS103 to give pMS114, pMS116, pMS118, pMS121, pMS122, and pMS124, containing promoterless *tnpA*, *tnpA* expressed from heat shock 2019E, UCR–DCR, UCR, pMS–95 UCR–DCR, pMS–41 UCR–DCR, and MAC promoters, respectively. The plasmid containing the CaMV 35S–*tnpA* in pCGN1548/ALS has been described (Schläppi et al., 1993).

Plasmids Used in the Transient Expression Assay

pDC120, the UCR–DCR LUC construct, has been described previously (Raina et al., 1993). The *Spm* 3′ end was not added to *Spm* promoter–LUC constructs because it has no effect in the transient assay (Raina et al., 1993). pRR432, pRR433, pRR434, pRR435, and pRR438 contain the *tnpA* cDNA (EcoRI–HindIII fragment from pSP64) in the SmaI site of Bluescript II KS(+) followed by the *nos* terminator from pMA406 and expressed from UCR–DCR promoter (HindIII–BamHI fragment from pDC177), the CaMV 35S promoter (HindIII–BamHI fragment from pDC107), no promoter, the pMS–95-deleted UCR–DCR promoter (HindIII–BamHI fragment from pDC227), and 2019E soybean heat shock promoter (BglII–HindIII fragment from pMA406), respectively. The *tnpA* cDNA was transferred as a BamHI–SalI fragment from pMS86 into pMS161 to give pMS163. pMS161 was derived from pBIN-HYG-TX (from C. Gatz; Gatz et al., 1992) by filling in the Asp718 site of the plasmid and transferring the EcoRI–HindIII fragment (with a filled-in HindIII site) into EcoRI–SalI-cleaved Bluescript II KS(+) (BamHI and SalI sites removed).

Site-Directed Mutagenesis of *tnpA* cDNA

The *tnpA* cDNA was mutated by phagemid in vitro mutagenesis (Muta-Gene, version 2, Bio-Rad). The *tnpA* cDNA from pMS86 was inserted as a BamHI–SalI fragment into Bluescript II KS(+) to give pRR466. The *tnpA* cDNA 5′ end was subcloned as a BamHI–SphI fragment into the phagemid pTZ19 and the 3′ end as an SphI–SalI fragment into pTZ18 to give pMS174 and pMS175, respectively. The oligonucleotides 5′-GGCGTGCCAT**GG**GGTATCGTCG-3′, 5′-GGTGACGGTG**CC**-**ATGG**CCCGTCGGAG-3′, and 5′-ATAGGAGGGC**CC**ATG**G**TGCCGT-GGGC-3′ (mismatch in bold) were used to introduce NcoI sites into pMS174, pMS174, and pMS175, respectively, to give pMS176 (Ile-120 and Arg-121 changed to Met-120 and Gly-121), pMS178 (Val-422 and Pro-423 changed to Met-422 and Ala-423), and pMS177 (Met-543 changed to Val-543). The oligonucleotides 5′-TGCCATCAG**TT**G**A**CG-TCGGTCCC-3′, 5′-GGTGACGGT**TGAC**TCCCCCGTC-3′, and 5′-GAT-AGGAGG**TTGAC**TGATGCCGTG-3′ were used to introduce HincII sites into pMS174, pMS174, and pMS175 to give pMS179 (Tyr-122 to OPA-122), pMS180 (Val-421 to OPA-421), and pMS181 (Gln-541 to OPA-541). BamHI–SphI fragments from pMS176, pMS178, and pMS177 were cloned back into pRR466 to give pMS182, pMS183, and pMS184. SphI–BamHI fragments from pMS179, pMS180, and pMS181 were cloned into pRR466 to give pMS185, pMS186, and pMS187. 5′-terminal deletions of *tnpA* were made by blunt-end self-ligation of pMS182, pMS183, and pMS184, cut, and filled in at the BamHI and NcoI sites to give pMS188, pMS189, and pMS190, respectively. The mutated *tnpA* cDNAs were transferred as BamHI–SalI fragments from pMS188, pMS189, pMS190, pMS185, pMS186, and pMS187 into pMS161 to give pMS194, pMS195, pMS196, pMS197, pMS198, and pMS199, respectively, and XbaI–Asp718 fragments of these replaced the CaMV 35S–*tnpA* of pMS169 to give pMS200, pMS201, pMS202, pMS203, pMS204, and pMS205, containing N1 (Met-121), N2 (Met-422), N3 (Met-542), C1 (OPA-122), C2 (OPA-421), and C3 (OPA-541) deletions, respectively.

Transient Expression Assay and Tobacco Transformation

LUC and *tnpA* plasmids were introduced into midlog NT1 suspension cells (Russell et al., 1992) on DNA-coated 1.1 μm tungsten particles using the particle gun (Biolistic PDS-1000/He System, Bio-Rad). Leaves of transgenic plants were cut in half longitudinally, and each half was bombarded with either a *tnpA* cDNA or a control plasmid (no cDNA). LUC and CAT assays were done as previously described (Raina et al., 1993). Suspension cell lines were derived from young plants containing UCR–DCR LUC–mini-*Spm* and CaMV 35S–*tnpD* by first growing pith explants on agar-solidified standard medium (Schläppi et al., 1993) containing 50 mg/l kanamycin (Sigma), 20 mg/l hygromycin (Calbiochem), and 0.2 mg/l 2,4-dichlorophenoxyacetic acid (GIBCO). Selected white callus was then propagated in liquid medium of the same composition.

LUC–mini-*Spm* and *tnpA* constructs were transformed into the Nicotiana tabacum SR1 by the Agrobacterium leaf disc method (Rogers et al., 1988; Schläppi et al., 1993). The mini-*Spm* constructs in pMS109 and pMS127 were cotransformed with CaMV 35S–*tnpD* (all in Agrobacterium strain TiT37SE). Plants were regenerated from independently transformed callus lines and self-pollinated, and the number of T-DNA integration sites was determined as described (Schläppi et al., 1993). Leaves from regenerants were retransformed with the various *tnpA* constructs in Agrobacterium strain LBA4404. Small leaves of independent transformants were cut in half, and one half was assayed for LUC activity and the other half was stained for GUS activity, as described previously (Masson and Fedoroff, 1989; Schläppi et al., 1993). LUC units were normalized to the amount of total protein in the leaf extracts determined by the Bradford method as recommended by Bio-Rad.

Genomic DNA Analysis

Isolation and restriction of genomic DNA, Southern blot analysis, and determination of the percent cleavage of methylation-sensitive restriction sites by computing densitometry were done as described previously (Schläppi et al., 1993).

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