Inducible DNA Demethylation Mediated by the Maize Suppressor-mutator Transposon-Encoded TnpA Protein

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Heritable epigenetic inactivation of the maize Suppressor-mutator (Spm) transposon is associated with promoter methylation, and its reversal is mediated by the transposon-encoded TnpA protein. We have developed an assay that permits demethylation of the Spm sequence to be controlled by inducing the expression of TnpA in plant cells. Using this assay, we show that demethylation is a rapid, active process. TnpA is a weak transcriptional activator, and deletions that abolish its transcriptional activity also eliminate its demethylation activity. We show that cell cycle and DNA synthesis inhibitors interfere with TnpA-mediated Spm demethylation. We further show that TnpA has a much lower affinity for fully methylated than for hemimethylated or unmethylated DNA fragments derived from Spm termini. Based on these observations, we suggest that TnpA binds to the postreplicative, hemimethylated Spm sequence and promotes demethylation either by creating an appropriate demethylation substrate or by itself participating in or recruiting a demethylase.

INTRODUCTION

Methylation is an almost universal epigenetic modification of DNA in organisms ranging from bacteria to humans (Bestor, 1990). Although both adenosine and cytosine residues are methylated in prokaryotic cells (Palmer and Marinus, 1994), DNA methylation in eukaryotes is confined to cytosine residues, primarily CG dinucleotides and CNG trinucleotides (Hohn et al., 1996; Lorincz and Groudine, 2001). In mammals, DNA methylation is implicated in a variety of epigenetic phenomena, including parental imprinting (Reik and Walter, 1998), X-inactivation (Csankovszki et al., 2001), and allelic exclusion of gene expression (Mostoslavsky et al., 1998). DNA methylation is important in plants as well. Mutants with reduced levels of cytosine methylation display highly abnormal developmental phenotypes (Finnegan et al., 1996; Kakutani et al., 1996; Ronemus et al., 1996), and DNA methylation is a key component of the mechanism that regulates transposition (Chandler and Walbot, 1986; Chomet et al., 1987; Brutnell and Dellaporta, 1994; Fedoroff et al., 1995; Wang and Kunze, 1998; Singer et al., 2001; Lisch et al., 2002). Because the abundant viral, transposon, and retroposon sequences in higher eukaryotes often are heavily methylated (Kochanek et al., 1993; Fedoroff et al., 1995; Wang et al., 1996; Yoder et al., 1997), it also has been suggested that DNA methylation is important in the maintenance of genome stability (Yoder et al., 1997; Fedoroff, 2000).

Some of the earliest evidence for epigenetic regulation of gene expression came from McClintock’s elegant genetic studies of the maize Suppressor-mutator (Spm) transposable element. McClintock reported that an active Spm could undergo what she termed a “change of phase,” by which she meant a heritable, but reversible, genetic inactivation (McClintock, 1958, 1963). In further studies, she identified epigenetic variants of the Spm transposon exhibiting different developmental patterns of activation and inactivation (McClintock, 1957, 1958, 1961, 1968, 1971). Importantly, she deduced that an active Spm element could transiently reactivate an inactive one when they were brought together by a genetic cross (McClintock, 1958, 1959, 1971), suggesting that Spm encodes a trans-acting factor that can reactivate a genetically silent transposon.

The genetic activity of Spm is correlated with DNA methylation of the transposon’s 5′ end, which comprises its promoter and the adjacent GC-rich sequence, which is termed the downstream control region (DCR) (Banks et al., 1988). Neither is methylated in an active transposon, and both become progressively methylated as the heritability of the inactive state increases (Banks and Fedoroff, 1989). A moderately methylated Spm is reactivated readily by the introduction of an active Spm, whereas a heavily methylated “cryptic” Spm is extremely resistant to both spontaneous and Spm-mediated reactivation (Fedoroff, 1989). An active

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Spm promotes the heritable reactivation of both inactive and cryptic Spm transposons, although the heritable activation of the latter is a slow process that occurs over several plant generations (Banks and Fedoroff, 1989). Trans-activation of an inactive Spm by an active one is correlated with demethylation of its 5′ terminal sequence (Banks et al., 1988). Transgenic tobacco lines containing an inactive, methylated Spm have been used to identify TnpA as the Spm-encoded protein necessary for Spm activation and demethylation (Schläppi et al., 1993, 1994).

DNA methylation in eukaryotes interferes with transcription, either by reducing the binding affinity of transcription factors for their target promoter sequences (Bednarik et al., 1991; Kochanek et al., 1995) or through the assembly of a repressive chromatin structure (Kass et al., 1997). A family of methyl-DNA binding domain proteins has been identified, and there is evidence that they repress transcription by promoting chromatin modification (Hendrich and Bird, 1998; Ballestar and Wolff, 2001).

DNA methylation is both heritable and reversible. It is maintained by DNA methyltransferases, which catalyze the remethylation of newly replicated, hemimethylated DNA (Adams, 1995). Genome-wide DNA demethylation occurs in mammalian germ cells as well as in early developing embryos (Dean et al., 2001; Reik et al., 2001). Loss of DNA methylation in the paternal genome after fertilization is rapid and independent of DNA replication (Mayer et al., 2000; Oswald et al., 2000; Santos et al., 2002). Early embryonic demethylation is followed by de novo methylation (Reik et al., 2001). Establishment of the adult methylation pattern results in the tissue-specific hypomethylation of some genes (Wilks et al., 1984; Migeon et al., 1991; Grange et al., 2001; Thomassin et al., 2001). The new methylation pattern is propagated stably through development, except for the selective demethylation of genes in particular tissues, at certain developmental stages, or in response to hormone treatment (Brunk et al., 1996; Kirillov et al., 1996).

DNA demethylation in mammalian cells is promoted by trans-acting factors, among them the transcription factors Sp1 (Silke et al., 1995), NF-κB (Kirillov et al., 1996), and the EBNA-1 protein (Hsieh, 1999) and the glucocorticoid receptor (Grange et al., 2001; Thomassin et al., 2001). There is evidence that these proteins promote active DNA demethylation rather than interfering with maintenance methylation (Wilks et al., 1984; Matsuo et al., 1998; Hsieh, 1999; Grange et al., 2001; Thomassin et al., 2001). The target specificity of demethylation appears to be determined by the DNA binding domain, but transcription activation also may play a role (Matsuo et al., 1998). Studies of the NF-κB factor suggest that additional proteins are required for demethylation (Matsuo et al., 1998).

Little is known about DNA demethylation in plants, but there is evidence that DNA is demethylated actively during pollen development (Oakeley et al., 1997). In addition to TnpA-mediated Spm promoter demethylation, gene-specific demethylation also has been reported to occur during vernalization (Sheldon et al., 1999). To investigate the mechanism of TnpA-mediated demethylation of the Spm sequence, we developed a novel assay system in which Spm demethylation can be controlled by inducing the expression of TnpA using a glucocorticoid-inducible promoter. We show that Spm promoter demethylation is rapid, suggesting that it occurs by an active process rather than by interference with remethylation. We show that TnpA is a weak transcriptional activator of the Spm promoter and that C-terminal deletions that eliminate transcriptional activity also eliminate the ability of TnpA to promote Spm demethylation. We report that inhibitors of cell cycle progression and DNA replication inhibit TnpA-mediated Spm demethylation. Finally, we show that TnpA binds much more strongly to unmethylated and hemimethylated than to fully methylated Spm promoter fragments. These observations suggest that TnpA-mediated demethylation occurs when TnpA binds to the newly replicated, hemimethylated promoter and either recruits or facilitates the access of demethylases to the Spm promoter.

**RESULTS**

**Inducible TnpA-Mediated Demethylation of the Spm Sequence**

To study the mechanism of TnpA-mediated Spm demethylation, we need to be able to examine the early events in TnpA–DNA interactions. Therefore, we expressed the TnpA cDNA from a glucocorticoid-inducible promoter (Aoyama and Chua, 1997) in transgenic tobacco lines containing a methylated Spm sequence in a luciferase reporter construct, designated Spm-LUC (Schläppi et al., 1994) (Figures 1 and 2A). To facilitate protein monitoring, we added a FLAG epitope tag to TnpA’s N terminus based on the previous observation that an N-terminal 120-amino acid deletion did not affect its ability to mediate Spm demethylation (Schläppi et al., 1994). To determine whether the FLAG-tagged TnpA protein promotes demethylation, we crossed a plant expressing the fusion protein from a 35S promoter of Cauliflower mosaic virus (CaMV) to a plant containing a heavily methylated Spm-LUC transgene and monitored the methylation of the Spm sequence. The Spm sequence was demethylated in all six progeny plants examined (data not shown), indicating that the FLAG–TnpA retains its demethylation activity.

We transformed the inducible TnpA construct, designated pTAA-FLAG–TnpA, into an Spm-LUC transgenic tobacco line, SR1-83, in which the Spm sequence showed ~50% methylation. The Spm sequence was demethylated in the resulting transgenic calli, and methylation was restored to a very low level even in the second generation of regenerated plants (data not shown). Therefore, we screened lines containing only the Spm-LUC construct, selected the line in which the Spm sequence was the most heavily methylated (SR1-1; ~90 and 70% methylation at the sites monitored in the pro-
EcoO109I and SalI, are located in the methylated and unmethylated fragments. The two monitored sites, EcoO109I and SalI, are used to monitor the methylation status, and a representation of the expected band sizes for the methylase sites used to monitor its methylation status, and a diagram of the transgenic pTA-FLAG-TnpA plant. The Spm promoter was methylated in leaves of the F1 plants, but methylation was unaffected by dexamethasone (data not shown).

Because cell division had ceased in leaves and continuing cell division may be necessary for demethylation, callus cultures were established from leaves of 1-month-old F1 progeny plants carrying both pTA-FLAG-TnpA and Spm-LUC constructs. These also maintained a high level of Spm methylation in the absence of dexamethasone but exhibited dexamethasone-inducible demethylation. Figure 2A shows a diagram of the Spm promoter region in the Spm-LUC reporter gene construct that was tested for methylation, the locations of the methylation-sensitive restriction endonuclease sites used to monitor its methylation status, and a representation of the expected band sizes for the methylated and unmethylated fragments. The two monitored sites, EcoO109I and Sall, are located in the Spm promoter and the DCR, respectively, the latter of which encodes the 5’ untranslated region of the single Spm transcript. Both sites in the Spm sequence became fully sensitive to restriction in some calli after 3 weeks of induction with dexamethasone, but not in others. Figure 2B shows a DNA gel blot of genomic DNA digested with EcoRV and either EcoO109I or Sall. In Figure 2C, the data obtained from replicates of assays on the same calli are expressed as the percentage of the total signal in the band corresponding to the methylated fragment (percent methylation). The extent of Spm methylation observed in the absence of dexamethasone showed some variation among independently derived callus lines (Figure 2C). This variation may be attributable to small differences in the background expression levels of the TnpA transgene, because the prolonged maintenance of callus lines containing the gene resulted in the gradual disappearance of methylation. Sibling lines lacking the TnpA gene maintained Spm methylation levels. Nonetheless, as is evident in Figure 2C, replicate measurements made on an individual callus line at a similar growth stage, both with and without dexamethasone induction, were highly reproducible. One of the three calli assayed in Figure 2 showed no decrease in DNA methylation upon dexamethasone induction. To determine whether the TnpA gene was expressed in all induced calli, the TnpA mRNA level was monitored by reverse transcriptase–mediated (RT) PCR. As shown in Figure 2D, TnpA mRNA was detectable in the two calli that showed demethylation after dexamethasone induction but not in the callus that did not show demethylation of the Spm sequence. The data in Figure 2C represent replicates of induction experiments performed with three independent calli derived from a single cross. Similar results were obtained with two to three independent callus lines derived from four different crosses between SR1-1 plants and plants containing the inducible TnpA construct. Callus lines were checked by PCR to verify that both the Spm-LUC and dexamethasone-inducible TnpA constructs were present. Approximately half (55%) of the callus lines containing both constructs exhibited dexamethasone-inducible demethylation, and all of these also showed dexamethasone-inducible TnpA mRNA expression, detectable by RT-PCR, as shown in Figure 2D. In the remaining lines, dexamethasone was unable to induce either demethylation or TnpA mRNA expression. It follows that demethylation of the Spm sequence can be controlled by inducing the expression of TnpA.

**TnpA Promotes Spm Demethylation by an Active Mechanism**

The complete loss of Spm methylation observed after 20 days of dexamethasone induction in the foregoing experiments suggests that TnpA-mediated DNA demethylation is...
The extent of demethylation should depend on the number of cell divisions and some methylation should still be detectable at 20 days, the precise amount depending on the exact doubling time. We compared the extent of Spm DNA demethylation after treatment of calli with 5-aza-cytosine or 5-aza-deoxycytosine (aza-dC), both of which are incorporated into DNA in place of cytosine but cannot be methylated (Bender et al., 1999). Little effect on Spm methylation was observed after 10 days of treatment with either compound at concentrations of either 10 or 50 μM. Even after 20 days of treatment, methylation was reduced by 50% at the higher concentration of aza-dC (Figure 3A). By contrast, no methylation was detected in calli treated with dexamethasone, either in the presence or the absence of aza-dC. Thus, TnpA-mediated demethylation is either more rapid or more efficient than the demethylation resulting from the incorporation of aza-dC.

To determine how rapidly the Spm sequence was demethylated after the induction of TnpA expression relative to the cell doubling time, we first determined the callus growth curve under our experimental conditions (Figure 3B). There was an initial lag phase, giving an initial doubling time of 6 to 7 days, followed by an exponential growth phase, with a doubling time of ~2 days. We examined the methylation of the Spm sequence under precisely these growth conditions 1.5 and 6 days after transfer to dexamethasone-containing medium. As shown in Figure 3C, the Spm DNA was demethylated almost completely after 6 days of dexamethasone treatment, and significant loss of methylation could be detected by 36 h. If demethylation is a consequence of interference with the remethylation of newly replicated DNA, the Spm sequence should be hemimethylated after one cycle of DNA replication. If the hemimethylated DNA is still resistant to restriction by the methylation-sensitive enzymes SalI and EcoO109I, no change in methylation is expected after a single doubling time unless the DNA is actively demethylated.

Although it has been reported that the fully hemimethylated SalI site with both of its C residues methylated is resistant to cleavage, there is no information regarding either the sensitivity of the fully hemimethylated EcoO109I site or either site when less than fully methylated (Nelson et al., 1993). Therefore, we first methylated a DNA fragment containing the Spm promoter and DCR using the bacterial methyltransferase SssI, which methylates only the cytosine (C) residue in CpG dinucleotides. The sequence of the EcoO109I cleavage site in the Spm promoter is GGGTCCC, and the last C is followed by a G, placing it in the CG dinucleotide context. The Sall site sequence is GTCGAC. Thus, SssI methylates only one of the three C residues in the EcoO109I site and one of the two C residues in the Sall site. As shown in Figure 3D, double-stranded DNA methylated by SssI was completely resistant to Sall but was still sensitive to EcoO109I. Because genomic Spm DNA was largely resistant to EcoO109I in these experiments, it is likely that the EcoO109I sequence of the 3' end of Spm of the restriction sites used to monitor its methylation status. The chart at right shows the sizes of the genomic DNA fragments expected using the LUC gene probe. Spm 3' is sequence of the 3' end of Spm. Luc5' and Luc3' are primers for RT-PCR analysis. E' and S' are methylation-sensitive sites (EcoO109I and Sall) in the Spm promoter (Spm P) and DCR. The following restriction fragments are indicated: B, BamHI; E, EcoRV; O, EcoO109I; S, Sall; m, methylated; u, unmethylated.

(B) DNA gel blot of genomic DNA extracted from transgenic tobacco calli containing the constructs shown in Figure 1 and (A) before induction (control [Ctl]) and after induction (Ind) with 10 μM dexamethasone for 20 days.

(C) Data obtained from replicate experiments on the same callus lines used in (B) were quantified using a phosphorimager and expressed as percentages of the total signal (mean and standard error) in the band migrating at the position of the uncleaved fragment.

(D) RT-PCR assay of the FLAG-TnpA transcript in the calli used in (B) and (C) before and 48 h after dexamethasone induction.
site contains more than one methylated C residue (5mC), although it is possible that resistance is conferred by the methylation of an internal C but not the site’s terminal C. To methylate all of the C residues in one strand, we prepared hemimethylated DNA by incorporating 5-methyl-dCTP into one of the two strands, as described in Methods. Hemimethylated DNA prepared in this way is completely resistant to both EcoO109I and Sall (Figure 3E). To determine the resistance of a singly methylated hemimethylated Sall site, we used SssI-methylated DNA to prepare hemimethylated DNA. Singly hemimethylated DNA was more resistant to Sall than is unmethylated DNA, but it could be digested when a large excess of Sall was used (data not shown). To determine whether the singly hemimethylated Sall site is digested
under the conditions used to analyze Spm sequence methylation in genomic DNA, we prepared 32P-labeled singly hemimethylated Spm DNA using SssI methylation and added a trace amount of it to a genomic DNA digest (1 ng of labeled DNA per 6 μg of genomic DNA). As shown in Figure 3F, the singly hemimethylated DNA was almost completely resistant to Sall digestion, regardless of the strand into which the methyl group was introduced.

To examine the sensitivity of hemimethylated DNA with fewer 5mC residues per site to EcoO109I, we prepared hemimethylated DNA using a mixture of 5-methyl-dCTP and dCTP at ratios of 3:1 and 2:1. From the ratio of methylated to unmethylated dCTP, we calculated the expected frequencies of trimethylated, dimethylated, monomethylated, and unmethylated EcoO109I sites (see Methods). From this calculation, we predicted the extent of digestion if just unmethylated and monomethylated sites are cut (15.6% at 3:1 and 25.9% at 2:1; Table 1) and if dimethylated sites are cut as well (57.8% at 3:1 and 70.4% at 2:1; Table 1). The observed extent of EcoO109I cleavage, also given in Table 1, was very close to that predicted if only the unmethylated and monomethylated sites are cleaved in hemimethylated DNA (15.3 and 22.4%; Table 1). We conclude that hemimethylated DNA with any two of its three C residues methylated at the EcoO109I site is resistant to digestion. Because genomic Spm DNA is highly resistant to EcoO109I and therefore is likely to have at least two methylated C residues in its EcoO109I site, it follows that the newly replicated, hemimethylated site also will be resistant to cleavage by the enzyme.

These results clearly show that the hemimethylated EcoO109I and Sall sites are resistant to enzymatic digestion when methylated at all internal C residues. Furthermore, they provide evidence that under the conditions used in the present experiments, both sites are resistant even when less than fully hemimethylated. The initial doubling time for the callus cultures under our conditions was ~6 days, with subsequent doubling times of ~2 days. If Spm DNA is demethylated by TnpA’s interference with the remethylation of hemimethylated DNA, it should be fully resistant to restriction by Sall and EcoO109I after one doubling time (day 6), 50% resistant after two doubling times (day 8), and 25% resistant after three doubling times (day 10). Because methylation was reduced by ~40% after 36 h, which is much less than the initial doubling time, and was virtually gone by 6 days, we conclude that TnpA-mediated DNA demethylation is an active process.

**Table 1. Expected and Observed Extent of EcoO109I Cleavage of Hemimethylated DNA with Different Numbers of Methylated Cytosine Residues (5mC) at the Recognition Site**

<table>
<thead>
<tr>
<th>Ratio of 5-Methyl-dCTP:dCTP</th>
<th>Expected DNA&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>5mC (1) + 5mC (0) (%)</th>
<th>5mC (2) + 5mC (1) + 5mC (0) (%)</th>
<th>DNA Cleaved (%)</th>
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<tbody>
<tr>
<td>3:1</td>
<td>5mC (3): 42.2</td>
<td>15.6</td>
<td>57.8</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>5mC (2): 42.2</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5mC (1): 14.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5mC (0): 1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:1</td>
<td>5mC (3): 29.6</td>
<td>25.9</td>
<td>70.4</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>5mC (2): 44.5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5mC (1): 22.2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5mC (0): 3.7</td>
<td></td>
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</tr>
</tbody>
</table>

The hemimethylated DNA was generated by copying unmethylated single-stranded DNA using Taq DNA polymerase and a mixture of dCTP and 5-methyl-dCTP.

<sup>a</sup>EcoO109I recognition sequence in the Spm promoter.

<sup>b</sup>The ratio of 5-methyl-dCTP to dCTP used in preparing hemimethylated DNA.

<sup>c</sup>The calculated percentage of DNA, with the indicated number (n) of 5mC residues in the EcoO109I site (see Methods for calculation).
tein strongly activated the transcription of both the His synthase (data not shown) and the β-galactosidase (Figure 4B) reporter genes. Gal4 fusion proteins expressing truncated versions of TnpA lacking the C-terminal 80 or 200 amino acids did not significantly activate transcription in yeast (Figure 4B), although the proteins were expressed (Figure 4C). The slight transcriptional activation observed with the constructs expressing the shorter proteins in yeast is likely attributable to translation in yeast cells through the stop codons used to truncate the proteins (Figure 4A), as indicated by the small amount of full-length TnpA detected in cells expressing the deletion constructs (Figure 4C). These observations suggest that TnpA, but not its deletion derivatives, is a transcriptional activator.

Analysis of the TnpA sequence using a motif-recognition program (http://www.motif.genome.ad.jp/) revealed the presence of a Gln-rich motif, a common transcription activation domain motif, within the C-terminal Leu-zipper domain (Figure 5A). To assess the transcriptional activity of TnpA on an unmethylated Spm promoter, we used a suspension culture derived from a tobacco callus line containing a dexamethasone-inducible TnpA gene and an Spm-LUC gene in which the Spm promoter was not methylated. We generated similar suspension cultures carrying dexamethasone-inducible genes encoding the FLAG-TnpA540 and FLAG-TnpA420 proteins as well as a FLAG-TnpA540-VP16 fusion protein (Figure 5A). Dexamethasone induction of the full-length and truncated TnpA genes peaked at 24 to 48 h (Figures 5B-1 and 5C-1). Expression of the tagged proteins peaked at ∼72 h (Figures 5B-3 and 5C-2). Induction of the full-length TnpA enhanced the transcription of the luciferase gene, albeit weakly (Figure 5B-1). Because the strong TnpA induction could artfactually enhance the LUC mRNA signal, the blot was stripped and reprobed with just a LUC probe, confirming the increase in the LUC transcript level (Figure 5B-2).

Induction of the FLAG-TnpA540 protein (Figure 5C-2) and the FLAG-TnpA420 protein (data not shown) had no effect on luciferase gene expression. By contrast, although induction of the FLAG-TnpA540-VP16 gene was very slight, it substantially enhanced the transcription of the LUC gene from the Spm promoter (Figures 5B-1 and 5B-2). We conclude that TnpA can stimulate transcription from the unmethylated Spm promoter and therefore is a transcriptional activator, albeit a much weaker one than the FLAG-TnpA540-VP16 fusion protein.

Truncated TnpA Derivatives Lack Both Transcription Activation and Demethylation Activities

The finding that TnpA is a transcriptional activator prompted us to ask whether this activity is required for the ability of TnpA to promote Spm demethylation. We crossed SR1-1 plants carrying the methylated Spm-LUC construct to plants carrying the FLAG-TnpA540 or the FLAG-TnpA420 construct under the control of the CaMV 35S promoter. The extent of Spm methylation in the F1 plants expressing either truncated protein was almost the same as that in parental SR1-1 plants, confirming our previous report that the C terminus is essential for TnpA-promoted demethylation (data not shown) (Schläppi et al., 1994). We also expressed the truncated proteins in the dexamethasone-inducible system. Callus tissue derived from transgenic F1 plants from a cross between

Figure 4. TnpA Is a Transcriptional Activator in Yeast.

(A) Constructs used for the expression of Gal4-TnpA fusion proteins in yeast cells. The closed circles indicate the positions of the stop codons introduced into the TnpA cDNA to truncate the proteins at amino acids 420 and 540. The full-length protein is 621 amino acids long. (B) Quantitative β-galactosidase assay in transformed yeast cells. SNF1/SNF4 cells express the Gal4 binding domain (BD)–SNF1 and SNF4-Gal4 activation domain fusion proteins and serve as weak positive controls. Values shown are means and standard errors from three replicate experiments. (C) Protein gel blot analysis of Gal4-TnpA fusion proteins in yeast using anti-Gal4 binding domain antibodies. The arrow marks a nonspecific band that reflects total protein loading.
SR1-1 plants and transgenic plants with inducible expression of the truncated FLAG-TnpA<sub>540</sub> or FLAG-TnpA<sub>420</sub> protein was treated with dexamethasone for 15 days. Although the FLAG-TnpA<sub>420</sub> gene was expressed in many calli, its expression had no effect on Spm methylation (Figure 6A), whereas expression of the FLAG-TnpA<sub>540</sub> gene resulted in a small decrease in Spm methylation (Figure 6B). After a comparable period of induction, calli expressing the full-length FLAG-TnpA showed complete loss of methylation (Figures 2B and 3C). Analysis of proteins by protein gel blotting using anti-FLAG antibodies showed that TnpA and both of its truncated derivatives were expressed at comparable levels from the CaMV 35S promoter in the transgenic plants that did not show changes in DNA methylation (Figure 6C). Neither truncated derivative activated transcription significantly in yeast cells (Figure 4B), and loss of just the C-terminal 80 amino acids eliminated TnpA’s ability to activate the Spm promoter in plant cells (Figure 5C). We conclude that the C-terminal domain of TnpA is necessary for its ability to activate transcription and promote demethylation.

The observed correlation between the loss of TnpA’s capacity to stimulate transcription and demethylation in the deletion derivatives suggests a causal relationship. Therefore, we investigated whether the addition of a transcriptional activation domain affects the ability of the truncated proteins to promote Spm demethylation. Irrespective of the initial methylation level, the Spm sequence is completely unmethylated in all calli and regenerated plants expressing the FLAG-TnpA<sub>540</sub>-VP16 fusion protein (data not shown). We expressed the TnpA<sub>540</sub>-VP16 fusion protein from the dexamethasone-inducible promoter in transgenic tobacco lines with high levels of Spm methylation, as we did for TnpA. None of the lines containing the inducible construct was able to maintain methylation of the Spm sequence (Figure 6D). Because transgenic tobacco lines containing an inducible TnpA gene can maintain Spm methylation for a long period of time in the absence of dexamethasone, we infer that the consistent absence of Spm methylation in lines expressing the FLAG-TnpA<sub>540</sub>-VP16 fusion protein is attributable to its much greater strength as a transcriptional activator of the Spm promoter compared with intact TnpA. Thus, the low level of FLAG-TnpA<sub>540</sub>-VP16 expression in the absence of inducer probably suffices for DNA demethylation. As noted above, even lines containing the inducible TnpA construct lose Spm methylation after several months in culture. These observations suggest that the ability to activate transcription is essential for TnpA’s ability to promote Spm demethylation.

Figure 5. TnpA Is a Weak Transcriptional Activator in Tobacco Cells. (A) The top diagram shows the domain structure of the TnpA protein and the positions at which proteins were truncated. The bottom diagram shows the structure of the FLAG-tagged TnpA-VP16 fusion protein. (B-1) and (C-1) Analysis of TnpA and LUC transcripts after induction of the FLAG-TnpA-VP16 and FLAG-TnpA genes (B-1) and the FLAG-TnpA<sub>540</sub> gene (C-1) in suspension-cultured transgenic tobacco cells. RNA was amplified by RT-PCR (15 cycles) followed by DNA gel blot analysis for signal detection. Equal amounts of TnpA and LUC probes were used. The 18S rRNA was amplified as a loading control. Ctl, control; Ind, induced. (B-2) To better reveal the change in LUC transcript level in FLAG-TnpA-expressing cells, the membrane used in (B-1) was stripped and reprobed with only the LUC probe. The TnpA bands represent residual label from the initial hybridization. (B-3) and (C-2) Protein gel blot analyses of proteins in the corresponding cultures using anti-FLAG antibodies.
Inhibitors of DNA Replication and Cell Division Interfere with TnpA-Mediated Demethylation

Although TnpA expression in tobacco cells is nearly maximal at 36 h after induction, Spm demethylation is far from complete at that time. This could be the result of a slow demethylation process, the lack of TnpA expression in some cells, or a requirement that DNA replicate before demethylation can occur, as suggested by the observation that demethylation can be induced by dexamethasone in callus cultures but not in the leaves from which they were derived. Therefore, we asked whether interfering with DNA synthesis and cell division interferes with Spm demethylation. We treated calli containing a methylated Spm-LUC transgene and a dexamethasone-inducible FLAG-TnpA gene with aphidicolin, a DNA synthesis inhibitor (Sala et al., 1980), and olomoucine, an inhibitor of the Cdc2/Cdk2 kinase that blocks the G1-to-S cell cycle transition (Glab et al., 1994). The efficiency of DNA replication inhibition was examined by monitoring the incorporation of bromodeoxyuridine into newly replicated DNA using anti-bromodeoxyuridine antibody. At the concentration used in our experiments, DNA replication was arrested completely (Figure 7A). We then compared the levels of Spm methylation after 2 days of treatment with the inhibitors, with dexamethasone alone, and with a combination of all three compounds. Treatment of calli with aphidicolin and olomoucine slightly enhanced Spm methylation, whereas treatment with dexamethasone alone resulted in
Spm demethylation (Figure 7B). Treatment of calli with aphidicolin and olomoucine prevented dexamethasone-induced TnpA-mediated Spm demethylation. These findings suggest that DNA replication is a rate-limiting step in TnpA-mediated demethylation.

TnpA Binds More Strongly to Unmethylated and Hemimethylated DNA Than to Methylated DNA

It has been reported previously that methylation reduces the affinity of TnpA for oligonucleotides containing its consensus 12-bp binding site, CCGACACTCTTA (Gierl et al., 1988). To assess the effect of methylation on the binding affinity of DNA fragments derived from the Spm promoter, we compared TnpA binding to unmethylated, hemimethylated, and fully methylated synthetic oligonucleotides and DNA fragments derived from the 5′ end of the Spm transposon (Raina et al., 1998). We used an electrophoretic mobility shift assay to compare the binding of DNA fragments containing one, two, and six TnpA binding sites. We prepared fully methylated derivatives as well as both hemimethylated substrates (see Methods).

TnpA bound strongly to unmethylated and hemimethylated DNA fragments, irrespective of which strand was methylated. For the fragment containing one binding site, the amount of bound protein was slightly greater for the unmethylated fragment than for the hemimethylated fragments, but the difference was not large (Figure 8A). Similar results were obtained with synthetic oligonucleotides (two binding sites) and with Spm-derived DNA fragments (six binding sites) (Figures 8B and 8C). These observations are consistent with the previous report that TnpA's affinity for fully methylated oligonucleotides containing one or two TnpA binding sites is less than that for hemimethylated or unmethylated oligonucleotides (Gierl et al., 1988). However, more of the promoter-derived fragment containing six TnpA binding sites was in DNA-protein complexes than the DNA fragments containing one or two binding sites. Spm promoter-derived fragments with multiple binding sites formed large complexes, spreading the labeled DNA over a large fraction of the gel (Figure 8C). We reported previously the formation of such large complexes, noting that the concentration of TnpA required for binding decreases with increasing numbers of binding sites per DNA fragment (Raina et al., 1998). In the present experiments, fully methylated DNA fragments bound TnpA less effectively than hemimethylated DNA fragments in all cases. However, methylated DNA fragments containing six TnpA binding sites bound a larger fraction of the input DNA than those with fewer binding sites. Nonetheless, the TnpA-DNA complexes that formed with methylated DNA migrated faster than those formed with unmethylated DNA.
or hemimethylated fragments, suggesting that they are smaller. This finding indicates that fewer binding sites per methylated DNA molecule were occupied by TnpA. These results are consistent with the observation that DNA replication and cell cycle inhibitors interfere with TnpA-mediated demethylation and support the interpretation that DNA replication is necessary to produce the hemimethylated Spm sequence to which TnpA can bind.

DISCUSSION

An Inducible DNA Demethylation Assay

To study the mechanism of TnpA-mediated demethylation of Spm, we developed an assay in which demethylation of the Spm’s 5’ terminus is controlled by inducing the expression of TnpA from a glucocorticoid-inducible promoter. The assay permitted us to characterize the initial stages of DNA demethylation through several cell division cycles in the native chromatin context. This has not been possible previously because methylation was studied either in transformed cells or in regenerated plants many cell generations after the introduction of a TnpA gene or Spm transposon by transformation. The ability to induce DNA demethylation will facilitate the further molecular analysis of TnpA-mediated DNA demethylation.

Transcriptional Activation and Repression by TnpA

We reported previously that TnpA activates transcription from the methylated Spm promoter but represses transcription from the unmethylated Spm promoter (Schläppi et al., 1994). In the present study, we show that TnpA is a weak transcriptional activator of the unmethylated Spm promoter in plant cells, although its transcription activation domain functions as a strong transcriptional activator when fused to a heterologous DNA binding domain in yeast. DOPA, the TnpA homolog encoded by the Spm-like maize Doppia transposon, also has been reported to be a transcriptional activator (Bercury et al., 2001). The resolution of the apparent paradox that TnpA functions as both an activator and a repressor may lie in the protein concentration–dependent competition between TnpA functions in transcription and transposition. TnpA is a bifunctional protein, and the Spm sequence that constitutes its promoter likewise has two functions. TnpA is required for both transcription from the transposon’s promoter and the transposition of Spm (Masson et al., 1991; Schläppi et al., 1993). There are multiple TnpA binding sites at both transposon ends (Masson et al., 1987). Those at the 5’ end are required for promoter function, and the TnpA binding sites at both ends are involved in transposition (Masson et al., 1987; Raina et al., 1993).

Previous experiments to assess the ability of TnpA to activate transcription from an unmethylated Spm promoter were performed in transient transfection assays at high DNA concentrations (Schläppi et al., 1996). The experiments described here were performed by inducing the expression of a TnpA gene in vivo and measuring transcription from an integrated chromosomal copy of the Spm-LUC reporter gene. Hence, the concentrations of both the target promoter and the transcription factor are likely to have been much lower in these experiments than in the previous transient expression assays in biolistically bombarded cells (Schläppi et al., 1996). Because the Spm promoter region is coextensive with the TnpA binding region at the 5’ terminus (Raina et al., 1993), the most likely explanation is that the TnpA-mediated association of Spm termini competes with other types of protein–protein interactions involving TnpA, including transcription activation. Evidence that it is TnpA and not the inherent weakness of the Spm promoter that limits transcriptional activity is provided by the observation that the TnpA540-VP16 fusion protein is a strong transcriptional activator of the Spm promoter (Schläppi et al., 1996). The observation that TnpA is a much weaker transcriptional activator of the Spm promoter than might be anticipated from the ability of the Gal4-TnpA fusion protein to activate transcription in yeast is consistent with the interpretation that TnpA is a transcriptional activator at low concentrations but becomes a repressor by homodimerization as its concentration increases. However, we cannot exclude the possibility that TnpA’s transcriptional activity is modulated by corepressors present in plant cells but not in yeast.

DNA Replication and Demethylation

DNA can be demethylated by interfering with maintenance methylation or by the active elimination of methyl groups, methylated bases, or methylated nucleotides (Li et al., 1992; Jost et al., 1995; Vairapandi and Duker, 1996; Bhattacharya et al., 1999). DNA demethylation by interference with the remethylation of newly replicated DNA is a slow process. It requires two rounds of replication to produce the first fully unmethylated daughter molecules and further reduces the amount of methylated DNA by just twofold at each subsequent replication. The results of the present study reveal that demethylation of the Spm sequence occurs much more rapidly than can be accounted for by interference with maintenance methylation. However, the inhibition of DNA replication and cell cycle progression interferes with the ability of TnpA to demethylate DNA. The likely resolution of this dichotomy lies in the much greater affinity of TnpA for unmethylated and hemimethylated DNA than for fully methylated DNA (Figure 8). Because demethylation commences soon after TnpA induction in unsynchronized cell cultures, it seems likely that TnpA binding to newly replicated molecules is followed rapidly by demethylation and that DNA replication is the rate-limiting step for TnpA-mediated demethylation. This
interpretation is consistent with our observation that TnpA expression has no effect on Spm methylation in leaves, where cell division has ceased.

Transcription Activation and DNA Demethylation

Although the ability of DNA methylation to repress transcription is well documented, the connection between transcription activation and DNA demethylation is poorly understood. The present results clearly show that deletion of the C-terminal 80 amino acids of TnpA abolishes both its transcriptional and demethylation activities, although the TnpA540 protein still is able to dimerize (Trentmann et al., 1993). We also observed that Spm methylation was lost in plant cells (and plants) containing a FLAG-TnpA540-VP16 gene, even when its fusion construct was expressed from an inducible promoter in the absence of inducer. This finding shows that the TnpA's transcription activation domain can be replaced by a heterologous domain and further suggests that the much stronger transcription activation capacity of the fusion protein compared with that of intact TnpA increases its demethylation activity.

An important aspect of the TnpA-mediated demethylation of the Spm sequence is that it is not confined to the promoter, which contains all of the TnpA binding sites, but also includes the GC-rich sequence that encodes the untranslated leader of the Spm transcript (Banks et al., 1988; Masson and Fedoroff, 1989). Because the chromatin remodeling attendant on transcription activation is not limited to the protein binding sites, the extended region of demethylation is consistent with the notion that demethylation is associated with the formation of a transcription initiation complex. All of these elements are shown in diagrammatic form in Figure 9. TnpA is depicted as binding to the hemimethylated Spm promoter but not to the fully methylated, prereplicative promoter. The binding of TnpA nucleates the assembly of a transcription initiation complex that either contains or attracts additional proteins required for demethylation. Although we have not definitively excluded the possibility that TnpA itself has demethylase activity, initial efforts to demonstrate such activity have not been successful (J. Chen and N. Fedoroff, unpublished data). Moreover, given the tight binding of TnpA to the promoter, but not to the DCR, it appears unlikely that the protein itself demethylates the DCR. Demethylation is depicted as preceding transcription in Figure 9, but we do not know whether TnpA can promote the transcription of the hemimethylated promoter or whether demethylation occurs before and is required for promoter activation. It remains to be determined whether TnpA recruits a demethylase (either directly or indirectly) or whether the changes in chromatin structure attendant on the formation of the transcription initiation complex simply increase the accessibility of the sequence to such proteins.

METHODS

Plant Material and Treatments

Tobacco (Nicotiana tabacum cv Petite Havana line SR1) plants were used in this study. Transgenic plants were obtained by the Agrobacterium tumefaciens leaf-disc transformation method. Where indicated, callus cultures were established from leaves of 1-month-old F1 plants derived from crosses of Spm-LUC plants and plants carrying the dexamethasone-inducible FLAG-TnpA construct. Callus was

Figure 9. Model Depicting the Role of TnpA in DNA Demethylation.
(A) TnpA does not bind the fully methylated Spm sequence.
(B) In dividing cells, TnpA binds to newly replicated, hemimethylated DNA.
(C) TnpA assembles a transcription initiation complex and either actively recruits additional proteins necessary for demethylation directly (1) or indirectly (2) or restructures chromatin to increase the accessibility of the Spm sequence to demethylation (3).
plasmid by tripartite ligation into pBluescript II KS DNA fragment and a SplI-SpeI fragment from pRR483 then were SplI, the PCR products were resolved on a low-melting-point agarose blunt ended and ready for subsequent cloning. After restriction with Beverly, MA) was used for PCR so that the amplified product was CTTTACAGC-3 pFLAG-TnpA, pFLAG-TnpA540, and pFLAG-TnpA420 were cloned into pBluescript II KS (Stratagene) that was already cut with XhoI and EcoRV. The Vent DNA polymerase (New England Biolabs, Beverly, MA) was used for PCR so that the amplified product was blunt ended and ready for subsequent cloning. After restriction with SpII, the PCR products were resolved on a low-melting-point agarose gel, and the longer fragment (626 bp) was recovered and purified. This DNA fragment and a SpII-SpI fragment from pRR483 then were cloned by triplicate ligation into pBluescript II KS+ (Stratagene) that was cut with EcoRV and SpII. Clones with the full-length FLAG-TnpA cDNA insert (pFLAG-TnpA) were identified by PCR using the primer pair FLAG-1 and FLAG-2 and were confirmed by sequencing.

Inhibition of DNA replication in callus cells was achieved by treatment with a combination of aphidicolin and olomoucine (Sigma-Aldrich) at concentrations of 15 and 50 μM, respectively. The efficiency of DNA replication inhibition was evaluated by monitoring the incorporation of bromodeoxyuridine (Sigma-Aldrich) into DNA using a mouse monoclonal antibody to bromodeoxyuridine (Sigma-Aldrich), alkaline phosphatase-conjugated antibodies to mouse IgG (Sigma), and the WesternBreeze chemiluminescence immunodetection system (Invitrogen, Carlsbad, CA).

Cell doubling time was determined by weighing callus, transferring it onto fresh callus maintenance medium, and then reweighing it daily.

Plasmid Constructs

To clone the FLAG-TnpA cDNA, a 750-bp fragment containing the FLAG epitope sequence at the 5′ end was amplified by PCR from pRR483 (Raina et al., 1998) using the primer pair FLAG-1 (5′-ATC-TTATGACTACAAGGAGCAGC-3′) and FLAG-2 (5′-ATACATCATACC-CCTTACAGC-3′). The Vent DNA polymerase (New England Biolabs, Beverly, MA) was used for PCR so that the amplified product was blunt ended and ready for subsequent cloning. After restriction with SpII, the PCR products were resolved on a low-melting-point agarose gel, and the longer fragment (626 bp) was recovered and purified. This DNA fragment and a SpII-SpI fragment from pRR483 then were cloned by triplicate ligation into pBluescript II KS+ (Stratagene) that was cut with EcoRV and SpII. Clones with the full-length FLAG-TnpA cDNA insert (pFLAG-TnpA) were identified by PCR using the primer pair FLAG-1 and FLAG-2 and were confirmed by sequencing.

All other FLAG-tagged clones were derivatives of pFLAG-TnpA, pFLAG-TnpA400 and pFLAG-TnpA840 were cloned by ligating the XhoI-SpII fragment from pFLAG-TnpA and the SpII-SpI fragment from pMS190 or pMS198 (Schläppi et al., 1994) (the Salt site was filled in using the DNA polymerase Klenow fragment) together onto pBluescript II KS+ that was already cut with XhoI and EcoRV. pFLAG-TnpA400 and pFLAG-TnpA840 encode the N-terminal 400 and 840 amino acids of TnpA, respectively, as a result of a nonsense mutation introduced by site-directed mutagenesis at the 3′ end of the cDNA for TnpA (Schläppi et al., 1996). pFLAG-TnpA400-VP16 was obtained by ligating the XhoI-SpII fragment of pFLAG-TnpA and the SpII-Kdel fragment (the Salt site was filled in using the Klenow fragment) from pMS249 (Schläppi et al., 1996) together into the pBlueScript II KS+ vector cut with XhoI and EcoRV.

For inducible protein expression, the XhoI-SpII fragments from pFLAG-TnpA, pFLAG-TnpA400, and pFLAG-TnpA840 were cloned into pTA7002 (Aoyama and Chua, 1997), giving rise to pTA-FLAG-TnpA, pTA-FLAG-TnpA400, and pTA-FLAG-TnpA840, respectively. We cloned pTA-FLAG-TnpA400-VP16 by ligating a Clal to Spel fragment from pFLAG-TnpA400-VP16 and pTA7002 that was cut with XhoI and Spel, and the XhoI site was partially filled in to the adenine residue. For constitutive protein expression, the FLAG-TnpA cDNA and its deletion derivatives were first cloned as an XhoI-XbaI fragment from pFLAG-TnpA into pAVA120 (von Arnim et al., 1998), yielding pAVA-FLAG-TnpA, pAVA-FLAG-TnpA400, and pAVA-FLAG-TnpA840, respectively. The expression cassette then was subcloned as a HindIII fragment into pCGN1549 (McBride and Summerfelt, 1990) or pCam1300 (http://www.cambia.org.au/main/r_et_camvec.htm), giving rise to pCGN-35S-FLAG-TnpA, pCam1300-35S-FLAG-TnpA400, and pCam1300-35S-FLAG-TnpA840, respectively.

Constructs for the expression in yeast cells of fusion proteins composed of the Gal4 DNA binding domain and TnpA, as well as their deletion derivatives, were cloned as follows. The yeast expression vector pAS2 was cut with Ncol, filled in with Klenow, and then cut with SalI, pRR466 (Raina et al., 1993), pMS199, and pMS198 (Schläppi et al., 1994) were cut with BamHI, filled in with Klenow, and then cut with SalI to release the CDAs for TnpA and its deletion derivatives (TnpA400 and TnpA300), which then were cloned into pAS2, giving rise to pAS2-TnpA, pAS2-199, and pAS2-198, respectively.

Spm Methylation Analysis

Genomic DNA was prepared using a cetyl-trimethyl-ammonium bromide protocol as described previously (http://www.arabidopsis.org/info/Protocols_Mundy2.html). Six micrograms of genomic DNA was digested in a 40-μL reaction for 16 h with 20 units each of EcoRV and the methylation-sensitive enzyme EcoO109I or SalI (New England Biolabs). The digest was resolved on a 1.25% Seakem LE agarose gel (BioWhittaker Molecular Applications, Rockland, ME) and transferred to a Hybond N+ nylon membrane (Amersham). A BamHI-EcoRV fragment from pDC107, part of the luciferase gene, was used as the probe for subsequent DNA gel blot analysis. The signal was quantified using a phosphorimager (Amersham Pharmacia Biotech, Piscataway, NJ), and the extent of DNA methylation is represented as the percentage of the total signal in the methylated band.

A 647-bp DNA fragment containing the Spm promoter and downstream control region, amplified by PCR from pDC105 (Raina et al., 1993) using primers KS and SK (see below), was used to determine the sensitivity of hemimethylated DNA to EcoO109I and SalI. DNA (0.2 μg) was digested for 1 h in a 20-μL reaction with 5 units of either enzyme, and the digest was resolved on a 1.8% MetaPhor agarose gel (BMA). The amount of DNA cleaved was quantified using the Alphalmager 2200 gel documentation and analysis system (Alpha Innotech Corp., San Leandro, CA). Methylated DNA was prepared either by using SssI methylase (New England Biolabs) or by incorporating 5-methyl-dCTP (Roche Applied Science, Indianapolis, IN) during PCR amplification. Hemimethylated DNA was prepared either by annealing complementary unmethylated and methylated single-stranded DNA or by incorporating 5-methyl-dCTP into the complementary strand of an unmethylated single-stranded DNA template. Less than fully methylated DNA was prepared by synthesizing DNA using a mixture of 5-methyl-dCTP and dCTP. The composition of DNA was calculated based on the random probability of dCTP and 5-methyl-dCTP incorporation from a substrate mixture. For example, at a 5-methyl-dCTP:dCTP ratio of 2:1, the probability of 5-methyl-dCTP incorporation at each cytosine residue is 2/3; thus, for the

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of the DNA, whereas those with two cytosines or one cytosine methylated would be 2/3 × 2/3 = 4/9 of the total DNA. Likewise, unmethylated DNA would be 1 × 1/3 = 1/3.

A biotin-streptavidin technique was used to prepare single-stranded DNA. Briefly, double-stranded DNA was amplified by PCR using one primer with 5′ biotin modification and the other without modification. After the double-stranded DNA was bound to streptavidin paramagnetic beads and washed thoroughly according to the vendor’s protocol (CPG, Lincoln Park, NJ), the unmodified single-stranded DNA was released by incubation in 0.1 M NaOH for 8 min. The eluate was neutralized by adding 0.1 volume of 1 N HCl and 1 M Tris-HCl, pH 8.0, and the single-stranded DNA was further desalted using the Qiagen PCR purification kit (Valencia, CA).

To examine enzyme sensitivity under the digestion conditions used for the analysis of genomic DNA, we added ~1 ng of 32P-labeled hemimethylated DNA to 6 μg of tobacco genomic DNA and digested it with 20 units of Sall in a 40-μL reaction for 16 h. One-fifth of the digest was resolved on a 2% agarose gel. After drying, the gel was exposed to x-ray film for visualization. DNA was labeled by preincubation with Klenow and dTTP at 30°C for 15 min, followed by the addition of dATP, dGTP, and 32P-dCTP (10 μCi in a 20-μL reaction) and an additional 15-min incubation. All DNA preparations were purified using Qiagen columns.

Transcription Assays

The plasmids pAS2-TnpA, pAS2-199, pAS2-198, and pAS2 were transformed into yeast strain Y190. Yeast transformation and the subsequent transcription assay were conducted according to the protocol described for the HybriZAP two-hybrid cDNA Gigapack cloning kit (Stratagene). Reverse transcriptase–mediated PCR was used to assay the transcriptional activation of the Smpluc gene by TnpA in plant cells. Total RNA was prepared using either the Qiagen RNeasy Plant Mini kit or the Gibco BRL TRizol reagent. To remove residual DNA, RNA was treated with DNase I for 5 min as recommended by the vendor (Life Technologies). For reverse transcription, 1 μL of 100 ng/μL total RNA was mixed with 3 μL of 100 ng/μL random primer and 8 μL of RNase-free water and then denatured at 70°C for 10 min. The solution was chilled on ice and collected by centrifugation. A premixed solution of the following reagents was added: 4 μL of 5× first-strand buffer (Life Technologies), 2 μL of 0.1 M DTT, 1 μL of 10 μM deoxy- nucleotide triphosphates, and 2 units of RNase block inhibitor. The solution was incubated at 25°C for 40 min, and then at 42°C for 2 min, followed by the addition of 1 μL of Superscript reverse transcriptase (Life Technologies) and incubation at 42°C for 50 min. The reaction was terminated by heating at 70°C for 15 min, and 1 to 2 μL was used in a 20-μL PCR procedure. Simultaneous amplification of the transcripts for the FLAG-TnpA gene and the LUC reporter gene was achieved by including both primer sets in the same reaction. For semiquantitative analysis, PCR was stopped after 15 cycles, and the product was fractionated on an agarose gel and transferred to a Hybond N+ nitrocellulose membrane (Amersham). The amplification products for the TnpA and LUC genes were detected by DNA gel blot analysis. To ensure that equal amounts of total RNA had been used in the reaction, the 18S rRNA also was amplified by reverse transcriptase–mediated PCR for 10 cycles using the primer pair 18S-5′ (5′-TACCGTCTAGTCTCAACCA-3′) and 18S-3′ (5′-AACATCTAA- GGGCATCAC-3′).

Gel Mobility Shift Assay

Gel mobility shift assays were performed as described previously (Raina et al., 1998). DNA sequences with different numbers of TnpA binding sites and differing methylation states were prepared by PCR amplification from plasmids containing either synthetic oligonucleotides or Smpluc promoter fragments (Raina et al., 1993). Primers KS (5′-TGAGGCTCGGATCT-3′) and downstream control region 3′ primer (5′-CACACCGGTGTGA-3′) were used for amplification of DNA fragments with one and six (derived from promoter deletion) or two (cloned synthetic oligonucleotide) TnpA binding sites. Unmethylated and fully methylated DNA sequences were prepared by PCR amplification with either dCTP (Life Technologies) or 5-methyl-dCTP (Roche Applied Science), respectively, in the reaction. Hemimethylated DNA fragments were prepared by annealing complementary single-stranded DNA with one unmethylated and the other fully methylated, generated by asymmetric PCR. Optimal single-stranded DNA amplification was achieved when asymmetric PCR was run for a total of 40 cycles with DNA template at a concentration of 1 ng/50 μL and primer concentrations of 2 μM for one primer and 0.2 μM for the other. NuSieve 3:1 agarose (3.5%); BMA) and 8% native PAGE gel were used for single-stranded DNA analysis and preparation, respectively. Radioactive labeling was performed as described above to test the sensitivity of hemimethylated DNA to restriction endonucleases.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

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