Methylation of coding region alone inhibits gene expression in plant protoplasts

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ABSTRACT Derivatives of the cauliflower mosaic virus 35S promoter lacking CG and CNG methylation targets were constructed and used to direct transcription of reporter gene constructs in transiently transformed protoplasts. Such methylation-target-free (MTF) promoters, although weaker than the 35S promoter, retain significant activity despite mutation of the as-1 element. The effect of methylation on gene expression in MTF- and 35S-promoter-driven constructs was examined. Even when the promoter region was free of methylation targets, reporter gene expression was markedly reduced when cytosine residues in CG dinucleotides were methylated in vitro prior to transformation. Mosaic methylation experiments, in which only specific parts of the plasmids were methylated, revealed that methylation of the coding region alone has a negative effect on reporter gene expression. Methylation nearer the 5′ end of the coding region was more inhibitory, consistent with inhibition of transcription elongation.

Plant genomes are, in general, more extensively methylated than those of animal cells; up to 30% of cytosine residues can be methylated (1). Cytosine residues are methylated in the 5-position by DNA methyltransferase (reviewed in ref. 2). The target sequences for methylation are CG and CNG, and two distinct methyltransferases have been identified in pea nuclear extracts (3). As in animal cells (4, 5), there is much evidence to suggest that cytosine methylation plays a role in the regulation of gene expression in plants (reviewed in refs. 6 and 7), and that methylation is involved in at least some examples of plant transgene silencing (for reviews see refs. 8–10). Although DNA methylation can be correlated with the inactive state in many cases (11–20), it remains unclear whether methylation is a cause or a consequence of silencing, leading to a need for further investigation into the mechanistic links between DNA methylation and transcriptional activity in plants. DNA methylation can affect transcription initiation in at least two ways. Methylated cytosine residues might directly block the binding of specific transcription factors to their cognate binding sites (21–23). Alternatively, inhibition could be indirect, with access of transcription factors being blocked by nonspecific methylated DNA-binding proteins or by the inability to penetrate a particular chromatin conformation formed by the methylated sequences and associated proteins. The presence of DNA binding proteins and structured chromatin will also affect transcriptional elongation, thus compounding the negative effect.

Transient expression studies, in which DNA can be methylated in vitro prior to being introduced into cells, have shown that genes with methylated promoter regions are inactive when transformed into plant protoplasts (24, 25), and avian (26) or mammalian cells (27). Hemi-methylated genes were also inactive in transformed plant protoplasts (28, 29) and this inactive state persisted in transgenic plants regenerated from such protoplasts (30).

This study reports the construction of plant promoters deficient in, or completely lacking, CG and CNG methylation targets. The effect of DNA methylation on the expression of the β-glucuronidase (GUS) reporter gene in transiently transformed plant protoplasts was investigated. The lack of methylation sites in the promoter did not provide complete protection from suppression of reporter gene expression by in vitro methylation. We show that methylation of the coding region alone can also lead to a reduction in gene activity in plant cells.

MATERIALS AND METHODS

Plasmid Constructions. The basic methylation-target-free (MTF) promoter was constructed by annealing pairs of synthetic oligonucleotides, creating fragments with cohesive ends (XbaI-NcoI, NcoI–SpI, SpI–HindIII, HindIII–BglII, BglII–KpnI, and KpnI–NcoI). The sequences of the sense strand oligonucleotides can be seen in Fig. 1 (MTF sequence). The MTF promoter/leader region was assembled by stepwise insertion of these oligonucleotide pairs into the vector pUC19. The 35S′ promoter was constructed by replacing an NcoI–KpnI fragment of the MTF promoter with the corresponding sequence from the original 35S promoter (strain CM4-184). The appropriate 35S promoter sequence was amplified by PCR to create an NcoI–KpnI fragment.

Plasmids pMTF-GUS and p35S′-GUS were constructed by inserting the GUS coding region from plasmid pRAJ275 [NcoI–BamHI; ref. 31] and the cauliflower mosaic virus (CaMV) polyadenylylation signal from plasmid R-CAT [BamHI–EcoRI; ref. 32], downstream of the MTF or 35S′ promoter, resulting in promoter-GUS-poly(A) signal cassettes cloned between the XbaI–EcoRI sites of pUC19.

Mutations in the as-1 element were constructed by replacing the HindIII–BglII fragment of pMTFΔ6-GUS (see below) with synthetic oligonucleotides carrying the desired mutations (sequences can be seen in Fig. 2C). The TATA-box mutation [TATAATA → TAGCTAA; pSan 9 (T–)] was made in MTF-derivative pSan 9 (Fig. 2C) by replacing the SpII–KpnI fragment of pSan 9 with a PCR-derived fragment containing the desired mutation.

pMTFΔ6-GUS and p35S′Δ6-GUS were derived by deletion of sequences between the BamHI and NcoI sites defining module 6 of MTF and 35S′. In pMTF2-GUS, promoter sequences upstream of the BglII site were deleted and in pMTFD-GUS, the BamHI–BglII promoter fragment was duplicated. pMT34P-GUS, pMT4P-GUS, and pMT3P-GUS were constructed by replacing the appropriate modules in the MTF promoter with the corresponding sequences from the 35S′ promoter.

In Vitro Methylation. Cytosine residues in CG dinucleotides were methylated by treatment of plasmids in vitro with the CG methylase M.SstI (New England Biolabs). Routinely, plasmid DNA (10.5 μg) was methylated by incubation overnight at

Abbreviations: GUS, β-glucuronidase; MTF, methylation-target-free; CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyltransferase

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Sequence of the MTF promoter. The nucleotide sequence of the MTF promoter/leader region is shown in the upper line and is aligned to the corresponding sequence of the 35S' promoter (the name 35S' is used to indicate that the sequence of the control promoter differs very slightly from the wild-type 35S promoter (strain CM4-184), as noted in the figure. The six modules (6, 5, 4, 3, 2, L) referred to in the text are indicated here. Restriction sites are named and the respective sequences underlined, and the TATA box and the repeated TGACG motif in the as-l element are double underlined. The transcription start site is shown with an arrow. CG and CNG motifs are shown as white letters on black. The AUG translation initiation codon is in boldface type.

37°C in 50 μl of NEB buffer 2 containing 160 μM S-adenosylmethionine and 9 units of M-SssI. The total number of methylatable sites in p35S'-GUS is 328, of which 20 (6%) are within HpaII sites. We routinely checked completeness of methylation by HpaII digestion only. However, in some cases M-SssI-treated plasmids were additionally restricted with CfoI and FnuDII (also sensitive to CG methylation). This raises the number of sites tested to ~20% of the total. In all cases, we observed complete resistance to digestion in methylated plasmids.

Mosaic Methylation. Appropriate restriction fragments (methylated or unmethylated) were prepared for mosaic methylation experiments by excision from agarose gels followed by purification using a QIAEX gel extraction kit (Qiagen, Basel). In cases where the enzymes used were inhibited by prior methylation of the plasmid, M-SssI treatment was carried out after digestion. Ligations were carried out overnight at 16°C in a final volume of 100 μl containing 6 units of T4 DNA ligase (New England Biolabs) in the buffer supplied with the enzyme. Each ligation contained a total of 5 μg of DNA. The ligation mix was used directly for transformation of protoplasts. In all cases control plasmids were restricted and religated in parallel with experimental mixtures.

Protoplast Preparation and Transformation. Conditions for growth of suspension cultures of Orychophragmus violaceus, preparation of protoplasts, and transformation by electroporation have been described (33). Routinely, 5–10 μg of plasmid DNA were used per transformation and an internal control plasmid expressing chloramphenicol acetyltransferase (CAT) was cotransformed in all experiments.

Enzyme Assays. Determination of GUS and CAT activities in protoplast extracts prepared after overnight incubation was carried out as described (34). GUS activities were calculated relative to the internal control in all cases. Activities cited are an average of at least three independent transformations, unless otherwise stated.

RESULTS

Transient Expression of GUS Driven by a MTF Promoter. A promoter based on the CaMV 35S promoter, but lacking CG and CNG methylation targets, was designed. The MTF promoter (269 bp) and leader (59 bp) were assembled using synthetic oligonucleotides (Fig. 1). Restriction sites define six DNA modules termed 6, 5, 4, 3, 2, and L (leader), ranging from 5' to 3'. The core promoter, containing the TATA box, is within module 2; enhancer elements (35) are located in module...
3 and further upstream. The 3'-proximal NcoI restriction site and its preceding A residue constitute an ATG start codon in optimal context (ACCATGG; ref. 36). The control promoter used in this study (35S'; aligned to MTF in Fig. 1) differs only very slightly from the wild-type 35S promoter. 35S' was constructed by replacing modules 5 through 2 of MTF with the corresponding original CaMV (strain CM4-184) sequence, which contains a total of 12 CG and CNG motifs. Two of the CGs are part of the TGACG doublet, which constitute the as-1 (37) or oec element (38) that is the target for the nuclear factor ASF-1 and a CREB-like transcription factor, TGA1a (ASF-1 and TGA1a might be identical; refs. 39 and 40).

The leader regions of MTF and 35S' have very few G residues on the coding strand (only within the KpnI and Bst1107I sites, see Fig. 1) and, accordingly, lack CG and CNG methylation targets. A consequence of this lack of G residues is that the leader region of the resulting mRNA will be relatively unstructured, favoring efficient translation (41, 42).

Plasmids pMTF-GUS and p35S'-GUS were assembled by combining the promoter/leader fragments described above with the GUS reporter gene coding region (31) and the CaMV poly(A) signal (32) in the vector pUC19. Transformation of protoplasts prepared from O. violaceus (a host of CaMV) with p35S'-GUS gave rise to high GUS activities (set to 100%; Fig. 2A). Values obtained were much higher than with the standard GUS expression plasmid, pBI221.1 (31), probably due to the optimal AUG context and the favorable leader sequence. GUS activities obtained with pMTF-GUS, on the other hand, were comparable to that of pBI221.1. A derivative of p35S'-GUS containing a much longer 35S enhancer and promoter fragment (531 bp) and the same leader was not more active than the original 35S' plasmid (p35SSL-GUS; Fig. 2A). Furthermore, module 6 of both promoters could be removed without compromising activity (pMTFΔ6-GUS and p35SΔ6-GUS; Fig. 2A). These results indicate strongly that the MTF and 35S' promoters are complete, at least in the transient expression systems used here. Deletion of sequences upstream of module 2 in MTF (pMTF2-GUS) nearly abolished activity, showing that MTF modules 3–5 still retain some enhancer activity. In contrast to expectations from the literature (43, 44) duplication of module 6 through 3 of MTF did not increase promoter strength (pMTFD-GUS; Fig. 2A).

Alteration of as-1 Elements in Module 3. The fact that pMTF-GUS still retains considerable activity shows that none of the CG and CNG motifs present in the 35S promoter is absolutely required for promoter activity, although one or more of them obviously contributes strongly to full activity. To further define the relative importance of individual CG/CNG motifs, we replaced some of the restriction fragments in the MTF promoter in pMTFΔ6-GUS with the corresponding original CaMV 35S promoter sequences (Fig. 2B). Replacing promoter modules 3 and 4 together restored activity to 60% of that of p35S'-GUS. Replacing these modules individually showed that restoration of activity depended almost entirely on the presence of sequences within module 3. In the original 35S promoter this module contains 1 CNG and 3 CG sequences. Two of the latter motifs are within the as-1 element. Restoration of one or both of these motifs in the context of pMTFΔ6-GUS (pCC-GUS, pCT-GUS, and pTC-GUS; Fig. 2C), was sufficient to restore activity to 50% of the level of 35S', whereas replacement of both TGACGs with either TACG, TACA, or TGACG (pTT-GUS, pPA-GUS, and pGG-GUS) did not restore activity, confirming the importance of the cytosine residue within the TGACG sequence for as-1 element function. None of these mutations restored promoter activity without reintroducing a CG motif. The standard MTF promoter was therefore used in all subsequent experiments described in this work.

A wider consensus sequence of the as-1/oec element with a high degree of symmetry (TGACGTCAANNNNTGAGCTCA) has been proposed (38). This sequence contains a total of four TGACG motifs (two forward and two reverse). In later experiments, we explored whether conservation of symmetry of the two subelements in all bases but the CG cores would improve the strength of these elements (pSan7, pSan8, and pSan9 in Fig. 2C). Although not generally the case, in one example this tactic led to a 3-fold increase in activity (compare pSan9 with pTT-GUS).

Effect of CG Methylation on Expression of pMTF-GUS and p35S'-GUS. To analyze the effect of DNA methylation on gene expression from pMTF-GUS and p35S'-GUS, cytosine residues in the target sequence were methylated in vitro by treating plasmids with the CG methylase M-SSlI prior to transformation of O. violaceus protoplasts. Completeness of CG methylation was verified by restricting with HpaII (methylated plasmids being fully resistant to HpaII digestion; data not shown). As expected, M-SSlI treatment of p35S'-GUS resulted in a drastic reduction of GUS activity, with activities being reduced by around 150-fold (unmethylated = 100; methylated = 0.65 ± 0.2). Despite the lack of methylation targets in the promoter, an ~60-fold decrease in activity was also seen with pMTF-GUS (unmethylated = 4 ± 1; methylated = 0.07 ± 0.01). The drop in activity was not specific to the GUS ORF, since it was also observed with pMTF-CAT, which expresses CAT activity (not shown); it was not due to plasmid degradation before transformation, as monitored by gel electrophoresis of the M-SSlI-treated plasmid; or to the presence of the substrate 3-adenosylmethionine (SAM), as shown by control incubation of the plasmid with this reagent. In addition, incubation of plasmids with M-SSlI, but without SAM, did not affect activity, thus ruling out an effect of a contaminating activity in the enzyme preparation. We considered three possibilities to explain the sensitivity of pMTF-GUS to methylation: (i) C residues not in a CG context could have been methylated; (ii) we might have created a novel, methylation-sensitive promoter with a transcription start site further downstream; or (iii) methylation of CG motifs in regions of the plasmid other than the promoter/leader could affect the production of functional mRNA. The first possibility is considered unlikely as the recombinant SSLI methylase from Spiroplasma is absolutely specific for cytosines in the target sequence 5'-CG-3' (45). The second possibility can also be ruled out; mutation of the TATA box inactivated the San9 version of the MTF promoter (see Fig. 2), indicating that we are not dealing with a novel or cryptic promoter. In addition, RNase A/T1 protection analysis confirmed that the same transcription start site was used in pMTF-GUS and p35S'-GUS and that the amounts of GUS mRNA from methylated and unmethylated plasmids correlated with activity in each case, indicating that GUS activities in fact reflect steady state RNA levels (data not shown). The third possibility is addressed in detail in the following section.

To obtain an indication that the methylation pattern conferred on the plasmids in vitro was stable in protoplasts, GUS activities were measured at different times after transformation of protoplasts with methylated or unmethylated pMTF-GUS/p35S'-GUS. In all cases, GUS activity increased to a maximum level 3 days after transformation. The ratio of activities obtained from the four transformations remained constant over this period (data not shown). Most importantly, there was no dramatic increase in the GUS activity in extracts of protoplasts transformed with methylated plasmids. This result, indirect evidence that the methyl pattern was stable within the cells, as demethylation would be expected to result in a concomitant rise in GUS activity. Although the high stability of the GUS protein in transformed cells makes it difficult to judge when the plasmid DNA loses activity, the constant ratio of activities obtained from the four transformations argues against differential degradation rates for methylated and unmethylated DNA. In the experiments described...
in this paper, GUS activity was measured in extracts of protoplasts harvested 20–24 hr after transformation.

**Coding Region Methylation Inhibits Expression.** To determine the effect of DNA methylation of only selected parts of our test constructs, we developed a mosaic methylation assay. For these experiments, we used versions of pMTF-GUS and p3SS'-GUS in which for technical reasons the NcoI site within the promoter was removed and a KpnI site between the poly(A) signal and the vector was changed to an SnaBI site (Fig. 3). These modifications did not significantly affect GUS activity in transformed protoplasts. These plasmids were methylated in vitro using MmSI. Aliquots of methylated and unmethylated plasmids were then cleaved with pairs of restriction enzymes, such that one cut occurred within the vector and the second within the transcription unit (in cases where the enzymes used were inhibited by prior methylation of the plasmid, M-SstI treatment was carried out after digestion). The resulting pairs of restriction fragments (ScaI–KpnI, ScaI–NcoI, ScaI–MunI, EcoRI–NspV; SnaBI–SnaBI; AlwNI–AlwNI; see Fig. 3) were gel-purified. Upon religation of pairs of unmethylated fragments and transformation of *O. violaceus* protoplasts, considerable GUS activity was restored, ranging from about 1:30 to 1:3 compared with the original plasmid and depending on whether blunt end or cohesive end cuts were involved (not shown). Although religation of pairs of linearized fragments might also generate ligation products that would be expected not to be competent to express GUS activity, the proportion of productive/nonproductive products is assumed to be the same for ligations in which both partners are unmethylated and those in which one of the ligation partners is methylated. Visual inspection of ethidium bromide-stained gels revealed no observable difference between ligation products arising from different combinations of methylated and unmethylated fragments. Thus, a ligation of the corresponding pair of unmethylated fragments carried out in parallel was used as the control value in each case (set to 100). Methylation 5' to the NcoI site confirmed that the 3SS' promoter is inactivated by methylation and that the MTF promoter is insensitive to treatment with MmSI (Fig. 3). The lack of influence of methylated vector sequences upstream of the MTF promoter was also demonstrated (methylation of ScaI–KpnI or ScaI–NcoI had no effect; Fig. 3). Not surprisingly, because methyl- ation 5' to NcoI already reduces activity to the detection limit, extending the methylated region to sites within the GUS coding region had no detectable effect on GUS expression from p3SS'-GUS. In the case of pMTF-GUS, however, progressive extension of the methylated region to the MunI, SnaBI, AlwNI, and NspV sites in the GUS sequence resulted in a progressive reduction of GUS activity.

There are two possible explanations for this result: (i) methylation of the first part of the coding region inhibits expression, or (ii) increasing the extent of the methylated region results in the overall density of methylation on the plasmid reaching a critical threshold level above which transcription can no longer be supported. To distinguish between these possibilities, a second series of mosaic methylation experiments was performed in which increasing portions of the 3' end of the transcription unit were methylated. The results of this series support the conclusion that methylation of 5' proximal parts of the coding region is sufficient to inhibit expression. There is no direct relationship between the fraction of the plasmid methylated and the extent of the inhibition, thus excluding the threshold theory. Methylation of the 3' end of the GUS gene, the polyadenylation signal, and most of the

![Figure 3](image)

**Fig. 3.** Effect of mosaic methylation on expression from pMTF-GUS and p3SS'-GUS. A linear representation of pMTF/p3SS'-GUS showing the relative positions of the promoter (MTF/3SS'), leader (L), coding region (GUS), polyadenylation signal (polyA), and pUC19 (vector) is shown at the top of the figure. Restriction sites used to generate the pairs of fragments used in mosaic methylation experiments are indicated (see text for details of specific pairs: A, AlwNI; B, BamHI; E, EcoRI; K, KpnI; M, MunI; N, NcoI; Ns, NspV; Sc, ScaI; S, SnaBI). The single ScaI site is indicated at both ends of this linear map. Thin vertical lines represent the positions of CG dinucleotides (the target for MmSI), with arrows below indicating HpaII restriction sites (CCGG). MmSI-treated or untreated plasmids were restricted as described in the text at one site within the promoter or transcription unit and a second site within the vector. Isolated methylated and unmethylated fragments were ligated together prior to transformation of *O. violaceus* protoplasts. Thick lines represent the part of the plasmid that was methylated in each case. GUS activities are expressed relative to controls in which both partner fragments were unmethylated, but otherwise identically treated (set to 100 in each case). nd = not determined. Values obtained from individual experiments are given.

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vector, had no effect on expression of pMTF-GUS (seen most clearly with the large \textit{Awh}NI-$Awh$NI fragment; Fig. 3). Only when the methylated region was extended to include the sequence between the \textit{Sna}BI site and the $Awh$NI sites did expression drop, with further extension toward the 5' end causing activity to drop still further.

**Coding Region Methylation Is More Inhibitory in Constructs with Weak Promoters.** To examine the effect of coding region methylation with promoters of different strengths, mosaic methylation experiments were also carried out with pMT4P-GUS and pMT3P-GUS (see Fig. 2). Methylated or unmethylated plasmids were restricted with \textit{Kpn}I (at the transcription start site) and \textit{Sac}I (within the vector) and fragments were isolated and religated as described above prior to transformation of \textit{O. violaceus} protoplasts. Values for GUS activities are given relative to the same pair of religated fragments where both partners were unmethylated (set to 100 for each case). As before, pMTF-GUS was unaffected by promoter methylation, whereas expression from p35S'-GUS and pMT3P-GUS was severely inhibited (Fig. 4), due to cytosine methylation within the CG motifs remaining in these promoters. Promoter methylation had much less effect on pMT4P-GUS. This is consistent with the results presented in Fig. 2, which showed that module 4 makes only a modest contribution to promoter strength suggesting that there are no major targets for transcription factors in this region. Consequently, methylation of these sequences would not be predicted to have a major effect. Specific differences in the effects of coding region methylation were also observed between strong and weak promoters. As seen in the previous assay, coding region methylation in the context of the weak MTF promoter resulted in a dramatic reduction in GUS activity. Similarly, methylation of the transcribed region of pMT4P-GUS greatly reduced activity. With the stronger promoters (35S' and MT3P), there was only about a 2-fold reduction in activity. Thus, coding region methylation was much more inhibitory in the context of weak promoters than with strong promoters.

**DISCUSSION**

MTF, a promoter based on the CaMV 35S promoter, but completely free of CG and CNG methylation targets, was constructed. When transiently expressed in plant protoplasts, the activity of the MTF promoter is around 5% of that of the 35S' promoter used in the same context. Since in our test plasmids this context was optimized by using a favorable leader sequence and translation start codon context, GUS expression driven by MTF could be measured with accuracy, despite relatively low values. Constructs driven by the MTF and 35S' promoters were used to evaluate the effect of CG methylation on gene expression. As expected, methylation dramatically reduced expression from p35S'-GUS, an effect which is probably largely due to direct inhibition of transcription factor-promoter interactions. However, in pMTF-GUS, despite the lack of CG and CNG sites in the entire promoter/leader region, GUS activity was also considerably reduced upon methylation by M-$\text{Ss}$I. Selective methylation of different regions of pMTF-GUS showed that methylation of the coding region alone could inhibit GUS expression. The negative effect of coding region methylation was strongest close to the 5' end of the GUS ORF and was markedly reduced if central or 3' end regions were methylated.

Coding region methylation has also been shown to inhibit gene expression in animal cells (46, 47). The results presented here suggest that similar mechanisms operate in plant cells. Taken together, these findings suggest the possibility that DNA methylation can regulate gene expression by means of mechanisms other than direct effects on the promoter.

How might methylation of the coding region affect expression? There are a number of possible explanations. The methylated DNA might adopt a local chromatin conformation that inhibits either transcription initiation, elongation, or both. There is evidence both for and against this model in mammalian cells. In studies on episomal minichromosomes, methylated regions "seeded" spreading of inactive chromatin to surrounding unmethylated sequences (48), and the degree of transcriptional repression was found to depend on the overall density of CpG methylation (49). In contrast, when plasmids were integrated into host chromosomes, even dense methylation of vector sequences had no effect on reporter gene expression in certain constructs (46).

Alternatively, the presence of methylated C residues might impede the process of transcription elongation due to associated methylated DNA binding proteins. Again, there is conflicting evidence; in one case, methylation of a region downstream of the transcription start site had only a very slight negative effect on reporter gene expression (27), while in another, coding region methylation was completely inhibitory (47). It is likely that some of these effects are gene and/or promoter-specific, and may also depend on whether extrachromosomal versus integrated DNA is studied. Further studies will be required to resolve some of these apparent contradictions.

An effect of methylation on transcript elongation has been observed associated with methylation induced preemotively (MIP) in the filamentous fungus \textit{Asco}b\textit{olus im}\textit{mersus}. MIP results in the silencing of duplicated sequences (reviewed in refs. 50 and 51); stretches of methylated DNA within the coding region appear to block transcription elongation at a position corresponding to the 5' end of the methylated region (52).

In our experiments, the negative effect of coding region methylation was most apparent with the MTF promoter, which is intrinsically weak. The model presented in Fig. 5 illustrates why a weak promoter in combination with methylation of the coding region might result in a decrease in productive transcription events. Transcription complexes arising from a strong promoter are likely to be highly processive (i.e., less prone to stalling or pausing close to the transcription start site), and are able to read through obstacles such as DNA-binding proteins (reviewed in ref. 53). Thus, methylation of the coding region might not affect transcription from a strong promoter (Fig. 5). Transcription from a weak promoter like MTF might be expected to be less processive than activated transcription from a promoter with its full complement of enhancers (54). Thus, the presence of methylated DNA-binding proteins coating the coding region might be a significant obstacle to the few, and only weakly processive, transcription complexes arising from a weak promoter, with the result that the polymerase stalls or is released soon after initiation (Fig. 5). A methylation-free zone at the beginning of the transcribed region might alleviate the early block to transcription elongation, allowing sufficient buildup of polymerase complexes to enable the hindrance of the methylated region to be partially overcome (Fig. 5). It is becoming apparent that post-initiation modification of RNA
FIG. 5. A model explaining the inhibitory effect of coding region methylation in genes with weak promoters. The transcription initiation complex is schematically represented, with only RNA polymerase II (POL) and TFIID (TBP) named. Methylated cytosine residues in the DNA are indicated with an M, and their associated binding proteins are depicted as black ovals. RNA polymerase II is shown as large ellipses.

polymerase II complexes to convert from nonprocessive to processive forms plays an important role in transcriptional regulation (reviewed in ref. 55). The differential effect of methylation we have observed at different distances from the promoter may reflect these changes in the nature of the transcription complex.

The ultimate goal of this work is to investigate links between cytosine methylation and gene silencing in transgenic plants, with a view to developing strategies leading to stabilization of transgene expression. While it is clear that no single phenomenon is responsible for all instances of inhibition of gene expression, a broad distinction is emerging between transcriptional and post-transcriptional mechanisms (reviewed in ref. 8). DNA methylation has been shown to be a feature of both types of effect.

There are two features of the MTF promoter that might make genes under its control in transgenic plants less prone to inactivation. First, the lack of CG or CNG methylation targets may circumvent a promoter-methylation-mediated inactivation pathway. Second, the relatively low promoter strength may be advantageous as there is a clear correlation between high initial transcription levels and inactivation (see ref. 8).

Although using the MTF promoter may not be sufficient to avoid a methylated-DNA-mediated silencing mechanism in planta, our results show that the negative effect of coding region methylation decreased with the distance from the 5' end. This suggests that increasing the extent of the "nonmethylatable" region of pMTF-GUS might shield the coding region from the negative effects of methylation. We are currently investigating this approach.

Preliminary experiments show that the MTF promoter is active in transgenic Arabidopsis thaliana and Nicotiana tabacum and it will be interesting to see if use of this promoter confers any protection against transgene silencing.

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