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Methylation of cytosines in nonconventional methylation acceptor sites can contribute to reduced gene expression

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Abstract Epigenetic silencing of gene expression is often correlated with extensive DNA methylation at cytosine residues in the promoter and the coding region of silenced genes. Increasing evidence indicates that, in such cases, DNA methylation can also occur in sequence contexts other than CG and CNG, resulting in genomic regions with almost complete modification of cytosines. Whether this nonconventional methylation at CNN sites also contributes to gene repression is not known. We constructed genes with a promoter and a coding region devoid of the conventional methylation acceptor sites CG and CNG in addition to constructs with the corresponding wild-type sequences containing these sites. We generated unmethylated and completely methylated DNA by the polymerase chain reaction and performed expression assays in plant protoplasts. Quantification of transcript levels by RNase protection assay demonstrated that DNA methylation at positions other than CG or CNG sites contributes to the reduction in gene expression.

Key words DNA methylation · Gene silencing · Gene expression

Introduction

DNA methylation in eukaryotes is found almost exclusively at position 5 of cytosine residues. In many organisms, this modification is so prevalent that 5-methyldeoxycytidine (mC) has been considered the fifth nucleotide (Doerfler 1991). Numerous studies have shown that the presence of methylcytosine in nuclear DNA is correlated with reduced gene activity (Desrosiers et al. 1979; Mandel and Chambon 1979; McGhee and Ginder 1979; Stuhlmann et al. 1981). In several cases, such as embryogenesis, tissue-specific expression, X-chromosome inactivation, genomic imprinting and transgene silencing, methylation patterns are stably maintained through cell divisions, suggesting that methylation is involved in epigenetic gene regulation (for review see, e.g., Jost and Saluz 1993). To confirm that DNA methylation not only correlates with but is indeed the cause of gene repression, a frequently used experimental approach is to investigate gene expression from reporter genes methylated in vitro. Such in vitro methylation is catalyzed by DNA methyltransferases (MTases) that modify specific cytosine residues on both strands within their recognition sequence (Stein et al. 1982; Hershkovitz et al. 1990), or can be accomplished by second-strand synthesis in the presence of dmCTP of genes cloned in M13 vectors, resulting in hemimethylated DNA (Hershkovitz et al. 1989; Weber and Graessmann 1989; Weber et al. 1990). These studies have provided evidence for a significant reduction in rates of gene expression following methylation of DNA templates.

In animals, the vast majority of mC is found at CG dinucleotides (Doerfler 1983), with the rare exceptions occurring in CNG sequences (Clark et al. 1995). In plants, methylation at both CG and CNG sequences is common (Gruenbaum et al. 1981). The specificity of CG and CG/CNG methylation in animals and plants, respectively, is attributed to the properties of the MTases that create and maintain the pattern of methylation throughout DNA replication. The purified vertebrate MTases show a strong preference in vitro for methylation of CG dinucleotides, as compared with CA and CT (Adams et al. 1993). In plants, two distinct DNA MTases were purified from *Pisum sativum*, one specific

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for CG dinucleotides, the other for the trinucleotides CAG and CTG (Pradham and Adams 1995). In vitro, most MTases act preferentially on hemimethylated DNA, which is the intermediate during replication of a fully methylated template. Together with the symmetry of CG and CNG sequences, this could explain the maintenance of a given methylation pattern.

However, methylcytosine occurs in nuclear DNA outside the context of CG and CNG sequences. In fungi, the methylation of repeated sequences that have undergone repeat induced point mutations (RIP) in *Neurospora crassa* (Selker and Stevens 1985) or methylation induced premeiotically (MIP) in *Ascobolus immersus* (Goyon et al. 1994) also modifies cytosines followed by other bases. Such "nonconventional" methylation has recently received more attention since it has been observed in epigenetically silent transgenes in tobacco (Ingelbrecht et al. 1994), *Petunia* (Meyer et al. 1994) and *Arabidopsis* (O. Mittelsten Scheid, personal communication), as well as in hypermethylated regions of the *Ac* transposon in maize (Wang et al. 1996). The overall degree of cytosine methylation can reach almost 100% for certain regions of the genes (Selker et al. 1993; Goyon et al. 1994; Meyer et al. 1994; Wang et al. 1996). This methylation density in the case of epigenetic repression of gene activity is surprisingly high when compared with methylation of single cytosine residue(s) at particular position(s) that alter(s) the binding of a transcription factor (Saluz et al. 1988).

The presence of mC outside the context of CG or CNG sequences raises questions concerning its generation and maintenance, and whether it contributes to the regulation of gene expression. To address the last question, it is necessary to use a system in which the effect of nonconventional C methylation can be separated from the methylation at CG and CNG sequences. For this purpose, we created a CG/CNG-free version of a transgene consisting of the Cauliflower Mosaic Virus (CaMV) 35S promoter and the coding region of a mutant mouse dihydrofolate reductase (DHFR) gene resistant to methotrexate. We generated both methylated and unmethylated template DNAs by the polymerase chain reaction (PCR) and compared their transcriptional activities in plant protoplasts using RNase protection assays. The results presented here provide evidence that cytosine methylation in contexts other than that of the conventional CG and CNG influences transcriptional activity.

Materials and methods

Plasmid constructs

Site-specific mutations eliminating the CG/CNG sequences were created using a combination of synthetic oligonucleotides, PCR amplification and standard cloning techniques. Some mutations in the promoter region introduced new restriction sites. In the DHFR coding region, when elimination of CG and CNG sequences was not possible by conservative changes, amino acid replacements were chosen that retained the protein structure. In addition, the codon usage frequency was adapted to that found in plant genes (Wada et al. 1992). The modified nucleotide sequence was confirmed by sequencing. A comparison of wild-type and modified sequences and the numbers of C residues in different sequence contexts are shown in Fig. 1A for the promoter and in Fig. 1B for the coding region.

The CG/CNG-free version of the CaMV 35S promoter (termed F) was inserted as an *Eco*RI-*Bam*HI fragment into the plant expression vector pDH51 (Pietrzak et al. 1986), replacing the resident wild-type CaMV 35S promoter (termed W), and resulting in plasmid pFvec. To construct plasmids Wbar and Fbar, a *Bam*HI fragment containing the coding region of the phosphinotricin (PPT) acetyl transferase gene (*bar*) from *Streptomyces viridochromogenes* (Wohlleben et al. 1988) was inserted in the sense orientation into pDH51 and pFvec, respectively. The control construct Δbar contains the *bar* coding region and the 35S terminator, but lacks a promoter.

The CG/CNG-containing version (D1) of a mouse gene encoding a mutant DHFR resistant to methotrexate (MTX, Simonsen and Levinson 1983) and the corresponding CG/CNG-free version (D2) were cloned as *Bgl*II-*Sal*I and *Bam*HI-*Sal*I fragments, respectively, into the *Bam*HI-*Sal*I sites of the polylinker of both pDH51 and pFvec, generating the plasmids WD1, WD2, FD1 and FD2, shown schematically in Fig. 2A.

The plasmid p35nos (F. Nagy, personal communication) consists of the wild-type CaMV 35S promoter linked to the nopaline synthase (*nos*) region containing polyadenylation signals (Bevan et al. 1983).

Preparation of DNA for transformation

The DNA used for transformation was synthesized by the PCR with *Nde*I-linearized plasmids WD1, WD2, FD1 and FD2 as templates. The 1.5 kb PCR products comprised the CaMV 35S promoter, the DHFR coding region and the CaMV 35S polyadenylation signals, flanked by approximately 260 and 100 bp of pUC18 sequences at the 5′ and 3′ end, respectively. As a negative control, we used a PCR product consisting of the D2 gene (starting 16 bases upstream of the initial ATG), but lacking a promoter (termed ΔD2).

To synthesize unmethylated PCR products, 1 ng of linearized plasmid template was used in a 50 µl reaction volume containing 0.5 µM of each primer, 2.5 U of *Taq* polymerase (Boehringer), 0.25 mM of each dNTP Pharmacia and 1 ×*Taq* amplification buffer. The cycling program consisted of 30 cycles of 30 s at 94° C, 30 s at 55° C and 30 s at 72° C. Methylated PCR products were produced according to Colassanti and Sundaresan (1991), with minor modifications. Amplification was performed with a linearized plasmid template (80 ng) in a 100 µl reaction volume containing 0.01% gelatin, 0.25 µM of each primer, 0.8 U of *Taq* polymerase, 1 ×*Taq* amplification buffer and 0.2 mM each of dATP, dGTP, dTTP and 5-mdCTP (Boehringer). The amplification program was 30 cycles of 30 s at 94° C, 30 s at 55° C and 5 min at 72° C. The methylated PCR products were examined for mutations introduced during the incorporation of dmCTP by reamplification in the presence of nonmethylated nucleotides and digestion with several restriction enzymes. For each experiment, ten independent PCRs were performed with each template and pooled, to avoid an accumulation of a particular pattern of mutations. The complete methylation of the PCR product was assessed by digestion with methylation-sensitive restriction enzymes. DNA was precipitated with ethanol and resuspended in sterile water prior to transformation.

Protoplast transformation

For stable transformation, aliquots of 1.2×10^6 leaf protoplasts of *Nicotiana tabacum* cv. Petit Havana line SR1 (Maliga et al. 1973) were transformed by the polyethyleneglycol method (Negrutiu et al. 1987) with 5 µg linearized plasmid DNA supplemented with 25 µg

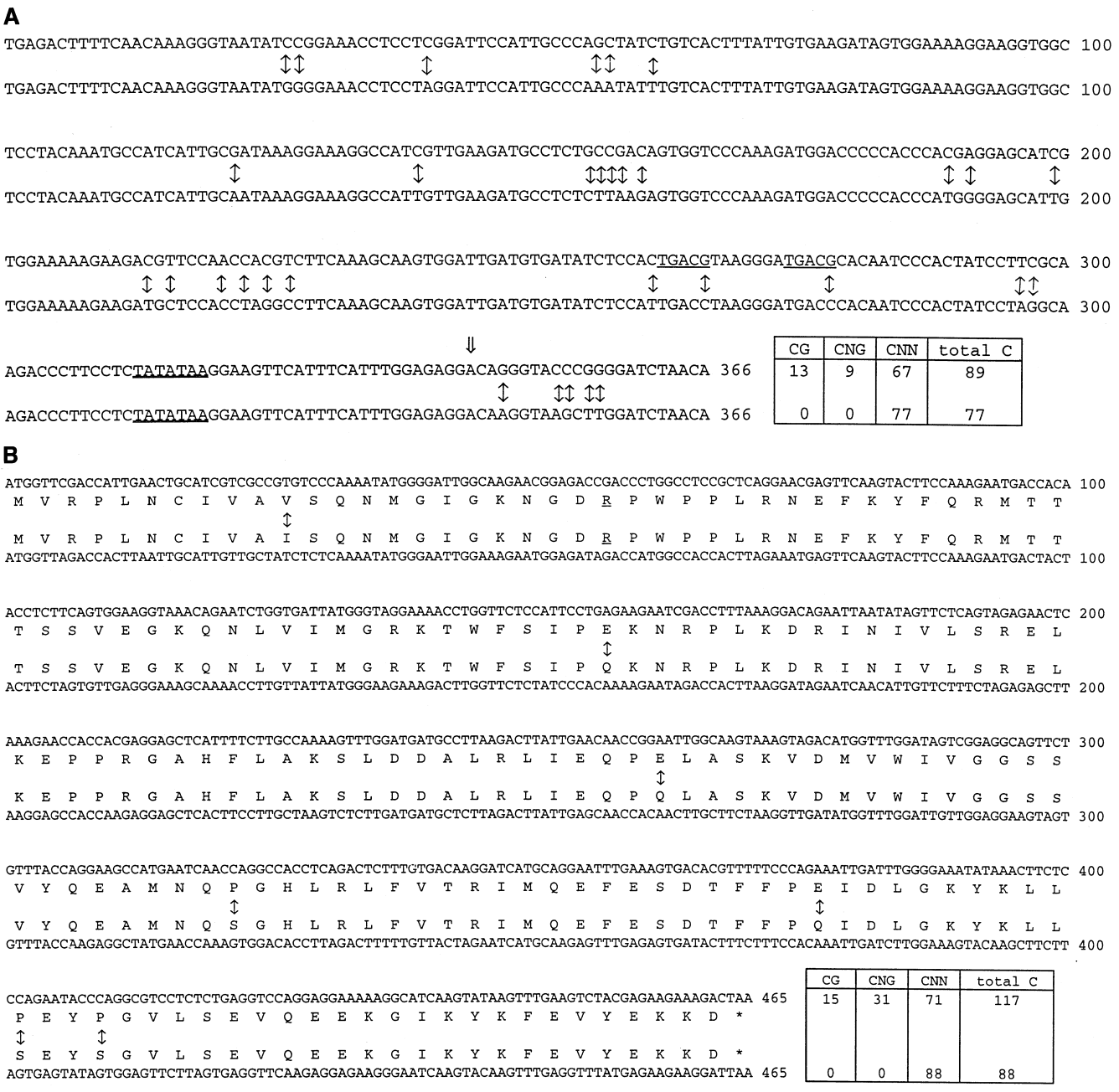

Fig. 1A, B Comparison of the wild-type (*upper line*) and CG/CNG-free sequences (*lower line*). The number of CG dinucleotides, CNG and CNN trinucleotides and the total number of Cs is shown. **A** Cauliflower mosaic virus (CaMV) 35S promoter corresponding to −343 to +23 in the wild-type sequence (Pietrzak et al. 1986) modified to introduce an optimal sequence context for the AUG initiation codon (Lütcke et al. 1987). The TGACG motifs (binding sites for the ASF1 transcription factor) are *underlined*. The TATA box is indicated by *double underlining*. The *open arrow* marks the RNA start site. The *double-headed arrows* denote the positions where the sequences differ. **B** Nucleotide sequence of the methotrexate (MTX)-resistant dihydrofolate reductase (DHFR) gene with its deduced amino acid sequence. The *underlined* arginine, replacing the original leucine, confers MTX resistance. The seven amino acid changes needed to generate the CG/CNG-free version are indicated by *double-headed arrows*.

calf thymus DNA as carrier. After 1 week of culture, transformed clones were selected for resistance to 5, 10 and 20 mg/l PPT or 0.2 and 0.5 mg/l MTX. The number of resistant clones was scored after 4 weeks. Resistant clones were not observed in controls when only carrier DNA was used.

For transient transformation, aliquots of 6×10^5 leaf protoplasts of *Nicotiana plumbaginifolia* were transformed in duplicate as described (Goodall et al. 1990) with 1.1 μg of PCR product supplemented with 12 μg of linearized pUC18 DNA as carrier. Parallel samples were transformed with 10 μg of circular p35nos plasmid. After 6 h incubation at 26° C in the dark, protoplasts from duplicated samples were combined. Cells transformed with p35nos were pooled and 1.5×10^5 of these protoplasts were added to every other transformed sample (except the mock-transformed sample) as an internal control. This procedure was chosen to avoid extrachromosomal recombination between control and tested templates.

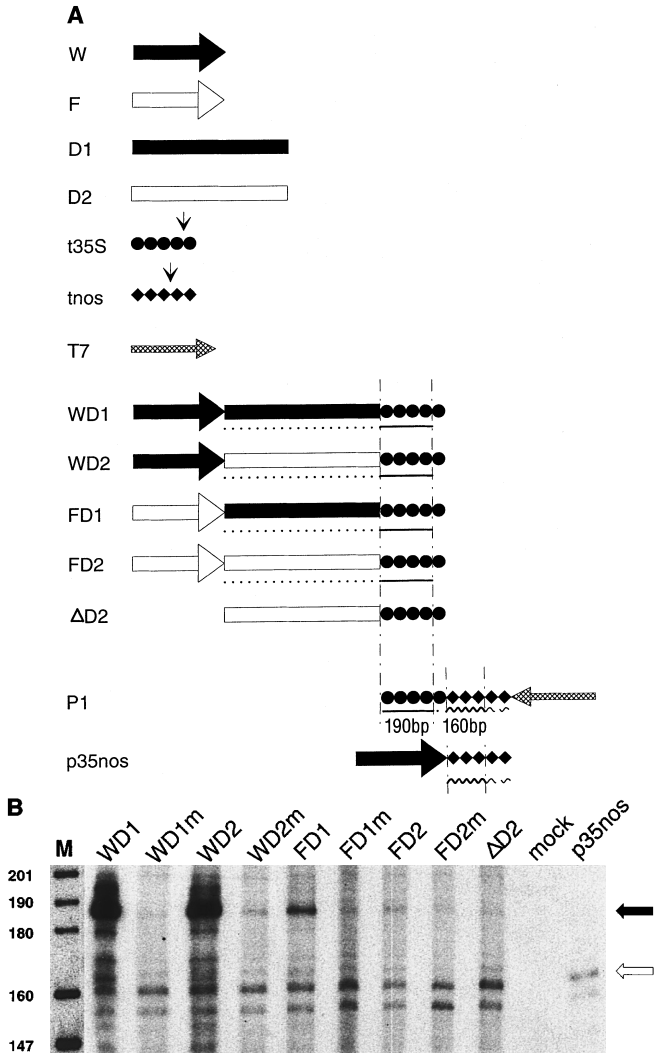

Fig. 2A Schematic representation of the gene constructs used in expression assays and fragments protected in the RNase protection assay. Promoters are drawn as *arrows* and coding regions as *rectangles*. Sequences containing CG/CNGs are displayed as *filled figures*, while CG/CNG-free sequences are *open*. The *vertical arrows* show the RNA processing site in the terminator sequences. Below each construct, the expected transcript is shown as a *horizontal line*. *Continuous lines* indicate the region protected by the probe in the RNase protection assay. *Dotted lines* indicate the part of the transcript digested in the assay. (*W* wild-type CaMV 35S promoter, *F* CG/CNG-free CaMV 35S promoter, *D1* wild-type mouse MTX-resistant DHFR, *D2* CG/CNG-free MTX-resistant DHFR, *t35S* polyadenylation signal of CaMV 35S transcript, *tnos* polyadenylation signal of nopaline synthase transcript, *T7* phage T7 promoter, *P1* plasmid used for synthesis of the probe for the RNase protection assay, *p35nos* plasmid used as internal control.) **B** Autoradiography of one RNase protection assay. The *filled arrow* denotes the position of the protected fragment for 35S-terminated transcripts, and the *open arrow* marks that of nos-terminated transcripts. [*M* size marker (the size of the fragments, in bases, is shown in the left margin), *m* methylated, ΔD2 promoterless D2 gene, *mock* no DNA and no aliquot of p35nos-transformed protoplasts added (see Materials and methods)]

Protoplasts were washed twice by sedimentation (5 min centrifugation at 115 *g* at room temperature) in W5 solution (Goodall et al. 1990). The protoplast pellets were resuspended in 0.5 ml W5 solution, transferred to Eppendorf tubes and collected by 4 min centrifugation at 1500 *g* at room temperature.

RNA analysis

The pelleted protoplasts were lysed in 600 µl of solution D (Goodall et al. 1990) by vigorous vortexing and stored frozen at − 80° C until RNA extraction. RNA isolation and the RNase protection assay were performed essentially as described (Goodall et al. 1990). The labeled probe (Fig. 2A) was synthesized by in vitro transcription in the presence of [α-^{32}P]UTP using the T7 promoter of the *Pst*I-linearized plasmid P1 (Sanfaçon and Hohn 1990) and purified by electrophoresis (Goodall et al. 1990). Protected fragments were separated on denaturing 6% polyacrylamide gels and visualized by autoradiography. The dried gel was exposed to a Phosphor Imager screen (Molecular Dynamics) and the radioactivity protected by the transcripts was quantified using Image Quant software. The total counts in the protected fragments of the expected size were determined and corrected for counts within a rectangle of the same size in the background of the same lane. The values obtained for the 35S terminated transcripts (190 base signal) were normalized by calculating the ratio to the counts of the nos terminated transcript (160 base signal) to avoid differences arising from RNA quality, handling during RNase protection assay and/or electrophoresis.

Results

Biological activity of the reporter genes

The sequence modifications created in the CaMV 35S promoter and the coding region of DHFR could in themselves lead to a reduction or loss of gene activity. Therefore, we compared the activities of wild-type and modified promoter and coding regions in transformation assays.

Both promoter versions, the wild-type sequence (W) and that without CG/CNG (F), were linked to the *bar* gene, which confers on plant cells resistance to PPT, generating the plasmids Wbar and Fbar, respectively. These constructs were introduced into *N. tabacum* protoplasts. Fbar resulted in 49.3% PPT-resistant clones relative to the number obtained with the construct harboring the wild-type promoter (Table 1). A control plasmid lacking any promoter preceding the bar gene (Δbar) gave 0.4% of the number of resistant clones obtained with Wbar, probably by integrating into transcribed genomic regions. Therefore, most PPT-resistant colonies obtained with Fbar were derived from integrated intact copies of this construct. These results suggest that the activity of the hybrid gene containing the modified promoter is reduced, probably as a result of modification of transcription factor binding sites, e.g,

Table 1 Comparison of wild-type and CG/CNG-free 35S promoter in stable transformation. (*PPT* phosphinotricin.) Results from five independent transformations

Construct	Number of PPT-resistant colonies per 10^6 protoplasts	Ratio (%)
Wbar	1140	100.0
Fbar	562	49.3
Δbar	5	0.4
Mock	0	0.0

ASF1 (Lam et al. 1989). However, the remaining activity is clearly sufficient for efficient recovery of transformed clones.

A similar assay was performed to test the D2 coding region. Plasmids WD1 and WD2 (Fig. 2A) were introduced into *N. tabacum* protoplasts followed by the selection of MTX-resistant transgenic clones. The number of MTX-resistant clones given by WD2 was 53.6% that of the wild-type sequence (Table 2), showing that the gene product of the modified coding region with seven replaced amino acid residues conferred MTX resistance, although at a reduced level (Table 2).

Combination of the modified promoter with the modified DHFR gene (construct FD2, Fig. 2A) made selection of MTX-resistant colonies difficult. This, however, did not prevent further studies, since our experimental approach relied on direct detection of transcripts rather than selection of transgenic clones. Indeed, specific transcripts of FD1 and FD2 could be detected and clearly distinguished from the background (Fig. 2B, Table 2).

PCR products as transforming DNA

To ensure that DNA generated by PCR (PCR-DNA) was suitable for transformation, we compared its transformation efficiency with that of the corresponding template plasmid. A PCR-DNA synthesized from WD1 as template and transformed into *N. plumbaginifolia* protoplasts generated approximately 100-fold less transcript, as assayed by RNase protection, than an equivalent amount of circular plasmid WD1 (data not shown). However, the transcript level from the plasmid decreased to a similar degree when the DNA was cut to generate a fragment equivalent to the PCR-DNA. This suggested exonucleolytic degradation of the template as a possible cause of the lower transformation efficiency. Addition of 12 μg carrier DNA (either *Eco*RI-linearized pUC18 or calf thymus DNA sheared to approximately 5 kb) to the PCR product increased the transcript concentration to a level comparable to that observed with the circular plasmid (data not shown). Linear pUC18 as carrier DNA was therefore used routinely in all transformation experiments with PCR-DNA.

We also tested different amounts of PCR-DNA (molar equivalents to 3, 5 and 10 μg of plasmid DNA) and different duration of transient expression (3, 6 and 9 h). Since there was no significant difference between the various DNA amounts in the presence of carrier DNA, we routinely used 1.1 μg PCR product (the molar equivalent to 3 μg plasmid). Six hours incubation after transformation was determined to be optimal for transient expression (data not shown). Under these conditions, we detected transcripts derived from all PCR products corresponding to the plasmids WD1, WD2, FD1 and FD2 as RNase-protected fragments of the expected size of 190 bases (Fig. 2A). All signals were clearly distinguishable from the background and the control transformation with a promoterless PCR-DNA containing only the D2 gene (Fig. 2B, Table 2).

Table 2 Comparison of the constructs in stable transformation and transient expression

Construct	Stable transformation		Transient expression
	Number of MTX-resistant colonies per 10^6 protoplasts[a]	Ratio (%)	Relative amount of transcript (%)[b]
WD1	248	100.0	100.0
WD2	133	53.6	55.7
FD1	nd		8.3
FD2	nd		6.2
ΔD2	nd		0.9
Mock	0	0.0	0.0

[a]Results from four independent transformations
[b]Results from three independent transformations

Effect of in vitro DNA methylation on gene expression

Transient transformation experiments were performed with PCR-DNA amplified in the presence of either dCTP or dmCTP. Transcript levels were determined by RNase protection assays (Fig. 2B). The quantified signals were corrected by subtracting the background of the lane and normalized by calculating the ratio relative to the 160 bases transcript derived from the p35nos plasmid. In vitro methylation of all templates inhibits their transcription to the same low level, regardless of the variations in expression from the unmethylated templates (Fig. 3). Importantly, this is also true for the gene construct lacking conventional methylation ac-

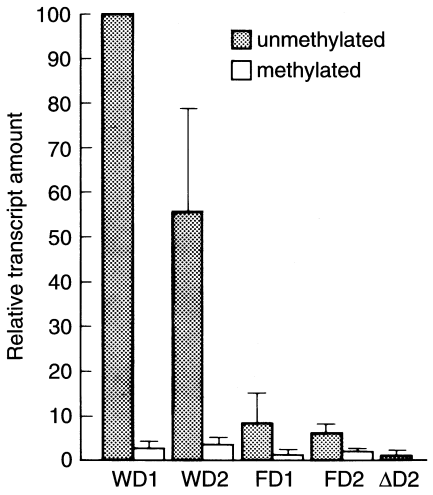

Fig. 3 Effect of in vitro DNA methylation on gene expression. The values are the means from three independent experiments; standard deviation is represented by the *error bars*. The amount of transcript, quantified as described in Materials and methods, is expressed as a percentage of the activity from the unmethylated wild-type construct (WD1) in the same experiment

ceptor sites. The pairwise comparison between unmethylated and methylated templates of individual constructs revealed the following results. For the wild-type gene, which contains CG/CNG sites in the promoter and in the coding region, methylation reduced the average level of expression to 2.7%. The gene with a wild-type promoter but a CG/CNG-free coding region, upon methylation, expressed on average 6.2% as much transcript as the unmethylated template, while methylation reduced average expression from the combination of a CG/CNG-free promoter with a wild-type coding region to 13.7%. Of most interest, the gene without CG/CNG sites in either the promoter or the coding region was also expressed at a lower level when methylated (31.4%). Since methylated cytosines in this case are incorporated only at nonconventional sites, this provides evidence that methylation at these positions indeed reduces gene expression.

Discussion

Since the stability of methylated transforming DNA does not differ from that of unmethylated templates (Hershkovitz et al. 1990), differences in expression from unmethylated and methylated genes in transformation assays indicate different transcriptional activities. Numerous studies have provided evidence that expression from in vitro methylated DNA is strongly reduced when assayed in animal cells (Wigler et al. 1981; Keshet et al. 1985; Murray and Grosveld 1987; Rachal et al. 1989; Götz et al. 1990; Levine et al. 1991; Sandberg et al. 1991; Graessmann et al. 1994; Hsieh 1994) and in plant cells (Hershkovitz et al. 1989, 1990; Weber and Graessmann 1989; Weber et al. 1990; Hohn et al. 1996). There are, however, exceptions, where methylation either does not influence (Graessmann et al. 1983; Götz et al. 1990) or is probably required for expression (Tanaka et al. 1983).

In the experiments presented here, expression levels were clearly reduced after methylation, most drastically in the construct in which both parts of the gene had the wild-type sequence (WD1). The presence of mCG/mCNGs in the promoter sequence had a more pronounced inhibitory effect than methylation only in the coding part (comparison of WD2 with FD1), but if mCG/mCNGs were present exclusively in the coding region, their methylation also reduced expression (comparison between FD1 and FD2). These findings are similar to previously described observations (Keshet et al. 1985; Hohn et al. 1996). It should be emphasized that, in our case, the different degrees of reduction originate mainly from different absolute expression levels of the unmethylated templates, since all methylated genes are transcribed at a comparable, low level, regardless of the number of CG/CNG sites. Therefore, the degree of reduction of template activity due to methylation is not of primary importance, rather the occurrence of suppression of transcription to the basal levels per se is the salient observation. Such a methylation-dependent reduction of transcriptional activity observed for a gene devoid of all CG/CNG sites therefore revealed that methylation of cytosines at nonconventional positions also influences the transcriptional activity of the template.

The reduction in expression from methylated DNA may be achieved by several mechanisms. It may simply reflect an altered affinity for transcription factors. It is not surprising that the methylation of the wild-type promoter has a strong effect, since both binding sites of the ASF1 transcription factor contain a CG, and ASF1 binding is inhibited by methylation (Inamdar et al. 1991). Alternatively, it is also possible that the local density of methyl groups, independent of their sequence context, reduces the expression to the basal level. Indeed, it was reported that the repression is an exponential function of methylation, 23% CG modification being enough to reduce gene expression by 99% (Hsieh 1994). Transcriptional suppression of genes with a high density of mC could also be based on a change in the helical configuration of the DNA. Although synthetic polymers of mCG can indeed adopt Z-DNA conformation under physiological conditions (Zacharias 1993), it is difficult to extrapolate this observation to genes with mixed base composition. Thus, another more plausible possibility is that the repression may be mediated by a protein that binds to methylated DNA. The pea protein DBP-m recognizes mC in DNA without appreciable DNA sequence specificity (Zhang et al. 1989). In addition, methylation can provoke premature transcription termination, as suggested for MIP (Barry et al. 1993). We quantified only the full-length transcripts with the probe specific to the 3′ end of the mRNA, thus accumulation of shorter transcripts cannot be excluded. Finally, it has also to be considered that the short pUC18- derived sequences flanking the CG/CNG-free gene construct contained these motifs. It has been shown that methylation of such flanking sequences can repress transcription of neighboring genes (Kass et al. 1993). However, only methylation of flanking sequences longer than 500 bp was able to reduce reporter gene activity to less than 50% of the control. Methylation of short flanking regions (approximately 200-bp, as in our experiments), had only a minor effect on gene expression (about 10% reduction). In addition, this suppressive effect seemed to be mediated by the formation of inactive chromatin complexes and occurred only 48 h post-transfection. For plants, it has been shown that methylation of long flanking sequences has no effect on gene expression 18 h after transformation (Hohn et al. 1996). Our measurements were performed 6 h after transformation, therefore, it is rather unlikely that the suppression described here is mediated by methylation of CG and CNGs in flanking sequences.

Whatever the mechanism of methylation-mediated transcriptional suppression may be, the observation of nonconventional C methylation in endogenous and foreign genes and its influence on gene activity raises two important questions: how is this methylation generated

and how is it maintained. In sequences that have undergone MIP in *Ascobolus*, methylation at CG is maintained more precisely than that of other cytosines (Goyon et al. 1994). It was noticed that, upon transformation of tobacco with hemimethylated DNA, the methylation of non-CG/CNG cytosines was less stable than that at CG sites (Weber et al. 1990). In *Petunia* and *Arabidopsis* also, methylation of nonconventional cytosines in silent transgenes showed a high degree of variability (Meyer et al. 1994; O. Mittelsten Scheid, personal communication). In contrast, the maintenance of methylation of a CT sequence in the inactive late E2A promoter was reported for an integrated adenovirus over several cell generations (Toth et al. 1990). In addition, stable inheritance of the sequence GAGmCTC in a restriction fragment length polymorphism allele of maize (Timmermans et al. 1996) indicated that maintenance of methylation was not limited to mCG and mCNG symmetrical sites. However, it can still be restricted to a particular sequence. It is also conceivable that methylation kept faithfully in neighboring CGs and CNGs provides a signal for overall methylation in the region. Although an important question remains as to how such methylation patterns are maintained, the results presented here have revealed that nonconventional methylation contributes to a reduction in gene activity and thus may influence the epigenetic state.

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