Transcriptional repression by methylation: cooperativity between a CpG cluster in the promoter and remote CpG-rich regions

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Abstract Cytosine methylation of binding sites for transcription factors is a straightforward mechanism to prevent transcription, while data on an indirect mechanism, by methylation outside of the factor binding sites, are still scarce. We have studied the latter effect using a model promoter construct. For this, a 69 bp G + C rich DNA segment with a cluster of 14 CpG sites was inserted between upstream lexA sites and the TATA box. Transcription was measured in transient transfection assays with lexA-VP16 as an activating factor. When the entire plasmid was methylated at all CpGs before transfection, transcription was blocked (to 3% residual activity), whereas transcription was only mildly inhibited (to 60%) by methylation of a control plasmid that lacked the 69 bp CpG cluster. However, the effect could not simply be attributed to methylation of the CpG cluster: neither a methylated CpG cluster in an otherwise methylation-free reporter gene plasmid, nor the methylated plasmid with an unmethylated CpG cluster, inhibited transcription considerably (69% and 44% remaining activity, respectively). The data presented here suggest that a minimal length of methylated DNA in the promoter is required for repression, and imply that concomitant methylation of CpGs in the promoter region and in remote sequences can cooperatively block transcription, without the need to methylate any binding sites for transcription factors. We also note that the cooperation for a negative effect described here bears an analogy to transcriptional activation, where a promoter often cooperates with a remote enhancer.

Key words: Transcriptional silencing; CpG methylation; CpG islands; Cooperativity

1. Introduction

DNA methylation in vertebrates is an important mechanism to repress undesired expression of genes [1]. In particular, DNA methylation at CpG sites in promoter regions is negatively correlated with gene activity in mammals [2,3]. One obvious way in which repression by DNA methylation can be achieved is via direct inhibition of transcription factor binding to its methylated binding site. Indeed, we and others have demonstrated this mechanism to be valid ([4], for review see [5]). However, the situation is certainly more complex since CpG methylation outside of factor binding sites were also found to repress transcription [6,7].

The efficiency of repression by this indirect mechanism seems to be largely dependent on three parameters, namely position, size and density of methylated CpG sites. It has been reported that the most effective transcriptional repression by CpG methylation is observed when the promoter/leader region is methylated [2,3,8]. On the other hand, in a transient transfection experiment using regionally methylated plasmid, methylation of non-promoter regions were also reported to inhibit transcription of a reporter gene, depending on the size of the methylated sequence [9]. Also, there is the observation that the higher the density of CpGs, the stronger the suppressive effect on transcription [10,11].

These indirect repression processes seem to involve eventual packaging of the promoter into dense, inaccessible chromatin, as was suggested by Buschhausen et al. [6]. This effect may be mediated by histone H1 [12,13] and/or by specialized methyl CpG binding proteins including MeCP-1 [10,12,14,15]. Nucleosomes and non-histone proteins such as MeCP-1 might repress transcription independently of each other resulting in an increased efficiency of repression [1]. Interestingly, with regard to the density effect of CpG methylation on transcription, MeCP-1 requires at least 12 methyl CpGs for efficient DNA binding [14].

From this it seems reasonable to assume that the promoters embedded in a long (0.5–2 kb) CpG-rich stretch, a so-called CpG island [16,17], are particularly strongly repressed by methylation [7]. However, the majority of CpG islands are free of methylation throughout development and their active demethylation in embryonic stem cells seems to be associated with binding of transcription factors [18–20]. Methylated CpG islands are present in inactive female X chromosomes and apparently ensure stringent silencing of X-chromosome genes: Marsupials, which inefficiently methylate their inactive X chromosome, also appear to inactivate their X chromosomes less efficiently. This is suggested by the observation that inactive X chromosomes are spontaneously reactivated at a higher frequency in marsupial cultured cells than in eutherian cultured cells [21]. Imprinted genes [22] and certain tissue specific genes [23] also contain methylated CpG islands. However, to prevent mutational erosion of CpG islands by deamination of methylcytosine to thymine ([24]; see als [25–27]), the islands should be kept unmethylated in germline cells.

Since *E. coli* lacks a system for maintenance methylation of CpG dinucleotides, regionally methylated DNA cannot be amplified in *E. coli*. Therefore, for almost all repression experiments, entire recombinant plasmids are methylated in vitro just before transfection into mammalian cells [6,8,10,14,15,23]. To analyze the effect of methylating specific DNA segments on transcription, several different methods have been developed to obtain regionally methylated plasmid DNAS [2,4,9,28]. We have devised a method to efficiently ligate a methylated oligonucleotide into the plasmid backbone by using non-palindromic restriction enzyme sites. With this ligation method, we have analyzed the effect of methylation on transcriptional repression with a short 69 bp CpG rich fragment. Our data suggest that a relatively short CpG cluster that does not include

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transcription factor binding sites, even when located in a proximal promoter position cannot by itself repress transcription. Such a cluster can however cooperate with methylated CpGs at more remote positions, in our case CpG-rich plasmid sequences, to abrogate transcription.

2. Materials and methods

2.1. Plasmid construction

2L-OVEC was constructed by inserting and multimerizing twice the oligonucleotide 5′-GAGCTCGAGTGCTGTATATAAAACGAGTGGTTATATGTACAGTAGTCGAC-3′ containing the lexA binding site from ColE1 (underlined, [29]) between the *Sac*I and *Sal*I sites upstream of the TATA box of OVEC [30]. 2L-Met5-OVEC was constructed by insertion of the Met5 oligonucleotide into 2L-OVEC between the two lexA sites and the TATA box. The sequence of the Met5 oligonucleotide is 5′-CGAGCCGGTAAGCGCACCGGCTCCGGATCGATCCGGTGTGCCGGAGCGCGTCGACGCCTCAACGGCTCG-3′ (underlined CpG sites methylated or unmethylated). The region between the lexA oligonucleotide and the β-globin TATA box (underlined) has the following sequence: GCTAG (69 bp Met5 oligonucleotide)ATTGGGCCTCGACCTTGGGCATAAAA). The expression vector for lexA-VP16 contains the cDNA coding for the lexA DNA binding domain (amino acids 1 to 202) fused to the cDNA coding for the 77 C-terminal amino acids of the VP16 activation domain (amino acids 413 to 490) driven by the cytomegalovirus enhancer/promoter (−522 to +72).

2.2. DNA methylation

Full CpG methylation of plasmids was performed with *Sss*I methylase [31] according to the manufacturer (New England Biolabs). Mock methylation was performed by excluding *S*-adenosylmethionine to obtain a similar topological form of plasmid [32]. Complete methylation was verified by extensive digestion with the methylation sensitive restriction enzyme *Hpa*II. CpG methylation of Met5 oligonucleotides was done by incorporating methyl cytosines during synthesis.

2.3. Ligation of oligonucleotide into the vector backbone

In large scale ligations, the following four template DNAs were generated: Met5 oligonucleotide, either methylated or unmethylated, was ligated into *Sss*I methylated or mock methylated 2L-OVEC plasmid. To ensure unidirectional, single copy insertion of the Met5 oligonucleotide we used plasmids and oligonucleotides with overhangs generated by the restriction enzymes *Nhe*I (upstream of the Met5 oligonucleotide) and *Sfi*I, which has a non-palindromic recognition sequence (downstream of the Met5 oligonucleotide). Both closed circular and nicked circular forms were produced by the ligation reaction. We tested the closed circular form after separation of the two forms by preparative agarose gel electrophoresis.

2.4. Transfection and S1 mapping

HeLa cells were grown in DMEM supplemented with 2.5% fetal calf serum, 2.5% newborn calf serum and penicillin/streptomycin. Transfections and S1 mappings were performed as described [30]. Briefly, the calcium phosphate method was used to transfect 0.5–1 μg of 2L-Met5-OVEC reporter plasmid, 1 μg lexA-VP16 transactivator plasmid, 0.01 μg reference plasmid, and 18 μg of carrier DNA (5 μg sonicated calf thymus DNA and 13 μg pSP64 carrier plasmid) into HeLa cells. RNA was isolated 48 hours after rinsing CaP precipitates from the cells. Quantification of the signals was performed by PhosphoImager-analysis (Molecular Dynamics) or by laser densitometric scanning of different film exposures. Transcription signals were normalized using the internal reference gene signal.

3. Results

To study the effect of DNA methylation on transcription, we used derivatives of the plasmid OVEC, which contains the rabbit β-globin gene and its minimal promoter [30]. The 4 kb β-globin gene region, like large segments of the mammalian genome, is depleted in CpG dinucleotides having only 0.54 CpGs per 100 bp on average (G + C% = 40%, CpG/GpC = 0.13), i.e. a typical non-CpG island region, whereas the remote 2.6-kb prokaryotic part of the plasmid contains CpGs at a high density of 6.4 CpGs per 100 bp (G + C% = 51%, CpG/GpC = 0.84). The reporter plasmid 2L-OVEC contains two lexA-binding sites [29] immediately upstream of the TATA box of OVEC (Fig. 1). Since the lexA-binding site lacks any CpG dinucleotide, binding of chimeric lexA transcription factors cannot be directly affected by CpG methylation. As expected, methylation of CpGs present around the lexA sites did not interfere with binding of lexA-VP16 fusion activator in electrophoretic mobility shift experiments (data not shown). We initially methylated in vitro the entire 2L-OVEC plasmid DNA at the cytosines of all 206 CpG dinucleotides, including those in the prokaryotic part of the plasmid. When the plasmid was transiently transfected into HeLa cells together with an expression plasmid for a lexA-VP16 fusion transactivator, the CpG methylated 2L-OVEC was transcribed about 30–70% (60%, an average of 4 experiments) as strongly as the mock methylated control plasmid, as judged by S1 mapping analysis (Fig. 2, lanes 2 and 3). Therefore, 2L-OVEC was relatively insensitive to CpG methylation of the entire plasmid. It should be noted that any change in methylation state during cell trans-

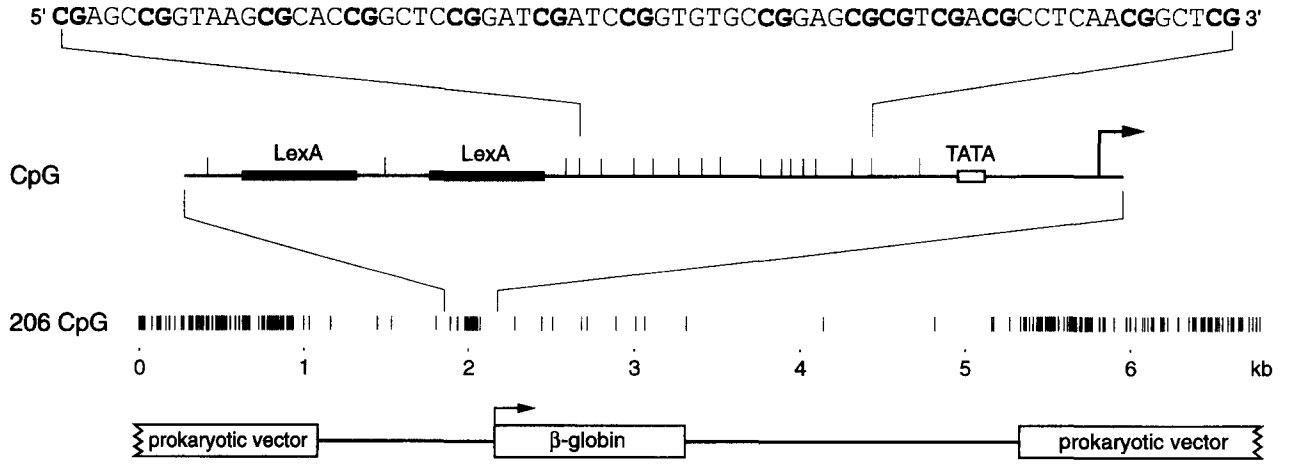

Fig. 1. Schematic representation of the 2L-Met5-OVEC plasmid. The sequence of the 69 bp of Met5 oligonucleotide is shown above, with synthetically methylated CpG sites in bold letters. The lexA sites are indicated by filled boxes. Each vertical line indicates the position of a CpG dinucleotide in the recombinant plasmid sequence, except in regions of high CpG density where one vertical line may represent more than one CpG.

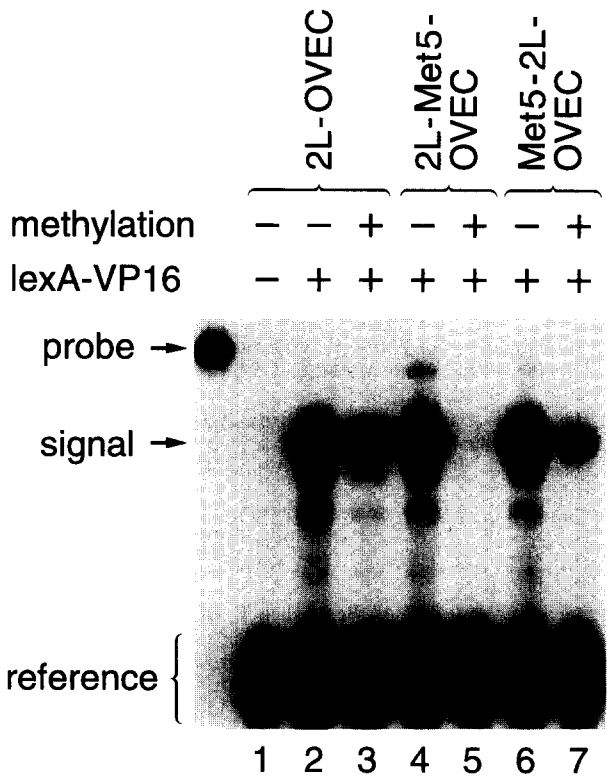

Fig. 2. Expression levels of entirely methylated reporter plasmids measured by S1 nuclease mapping. Names and methylation status of transfected plasmids, and the presence of the co-transfected expression vector for lexA-VP16 transactivator are indicated above. 'Signal' and 'reference' denote transcripts from reporter gene and internal transfection standard, respectively.

fection was not addressed in the experiments here since we used HeLa cells which apparently lack demethylation activity [33].

Since it is known that CpG methylation of the promoter region can dramatically repress transcriptional activity (e.g. [2,3,8,34]; reviewed in [5]), we intended to convert the methylation insensitive promoter into a sensitive one. For this, we inserted a 69 bp CpG-rich oligonucleotide, designated Met5, between the two lexA sites and the TATA box of the backbone plasmid 2L-OVEC (called 2L-Met5-OVEC, Fig. 1). This model oligonucleotide, which is not meant to be related to any known DNA sequence except for high G + C content, contains 14 CpG dinucleotides. The transcriptional activity of the CpG methylated 2L-Met5-OVEC plasmid was tested in transient transfection experiments in HeLa cells using lexA-VP16 as the coactivator (Fig. 2). The methylated 2L-Met5-OVEC showed only 0–6% (3%, an average of 4 experiments) of the level of transcription obtained from mock-methylated 2L-Met5-OVEC (Fig. 2, lanes 4,5). Unmethylated 2L-Met5-OVEC was transcribed to the same level as unmethylated 2L-OVEC, confirming that the Met5 oligonucleotide per se has little effect on transcription (Fig. 2, lanes 2,4). We also inserted the CpG cluster oligonucleotide upstream of the lexA sites but the transcriptional repression due to methylation was much less dramatic (Fig. 2, lanes 5 and 7). Thus, insertion of a CpG cluster downstream of the lexA sites in the promoter was critical for transcriptional repression of 2L-OVEC by methylation.

To see whether the methylated CpG cluster in the proximal position downstream of lexA sites is sufficient to repress transcription, we ligated methylated Met5 oligonucleotide in the promoter region of unmethylated 2L-OVEC plasmid. The ligated DNA was tested for transcriptional activity with cotransfection of lexA-VP16 activator expression vector in HeLa cells. Unexpectedly, the cluster of 14 methylated CpG dinucleotides embedded in unmethylated flanking DNA was unable to repress transcription efficiently. The methylated CpG cluster showed 41–93% (69%, an average of 5 experiments) of the level of transcription compared to the level from a control unmethylated CpG cluster inserted into an unmethylated plasmid (Fig. 3, lanes 3 and 4).

The lack of efficient repression by inserted methylated CpG cluster into unmethylated 2L-OVEC backbone prompted us to test the remaining two combinations. Firstly, the unmethylated CpG cluster oligonucleotide was ligated into a fully methylated 2L-OVEC plasmid. This again repressed transcription only mildly. The unmethylated CpG cluster inserted in the fully methylated 2L-OVEC plasmid gave 30–58% (44%, an average of 4 experiments) of the transcripts obtained from the constructs containing an unmethylated CpG cluster inserted in the unmethylated 2L-OVEC plasmid (Fig. 3, lanes 3 and 5).

Furthermore, the level of transcription from the methylated 2L-OVEC containing the unmethylated oligonucleotide insert was similar to that obtained from methylated 2L-OVEC alone, thus further confirming that the CpG cluster in itself has little effect on transcription (see Fig. 2). Finally, we also inserted methylated CpG oligo into methylated backbone, which reconstructs the situation of complete CpG methylation of the plasmid containing the cloned CpG rich DNA fragment. As expected, and consistent with the above result obtained with entire plasmid methylation, transcription was severely repressed, namely to 1–9% (4%, an average of 4 experiments) compared to unmethylated CpG cluster in the unmethylated 2L-OVEC plasmid. (Fig. 3, lanes 3 and 6). In conclusion, a dramatic inhibition of transcription was observed only when both proximal CpG cluster and distal CpGs in 2L-OVEC were methylated.

4. Discussion

We have found that methylation of a small CpG-rich promoter region is able to repress gene transcription only in cooperation with methylation at remote plasmid sequences. In addition to their general biological implications (see below), our data are relevant in a technical context: mammalian promoters are usually cloned into conventional plasmid vectors, which inevitably contain CpG-rich prokaryotic segments. Therefore, when an inhibitory effect of CpG methylation on transcription is observed with plasmid DNA, it is possible that the prokaryotic part contributes to this inhibition. Regional methylation of eukaryotic segments excluding the prokaryotic part (this study) or devising vector DNAs which have CpG density similar to that of the generally CpG-depleted mammalian genome may allow for a more reliable evaluation of methylation effects.

The majority of CpG islands remain free of methylation throughout development. When methylated, however, CpG island promoters are severly repressed ([7]; see also Introduction). Our data suggest that promoter CpG islands require a minimum length to silence a gene when methylated, since methylation of a 69 bp CpG cluster, which could be regarded as a 'mini-CpG island', was not sufficient to block transcription.

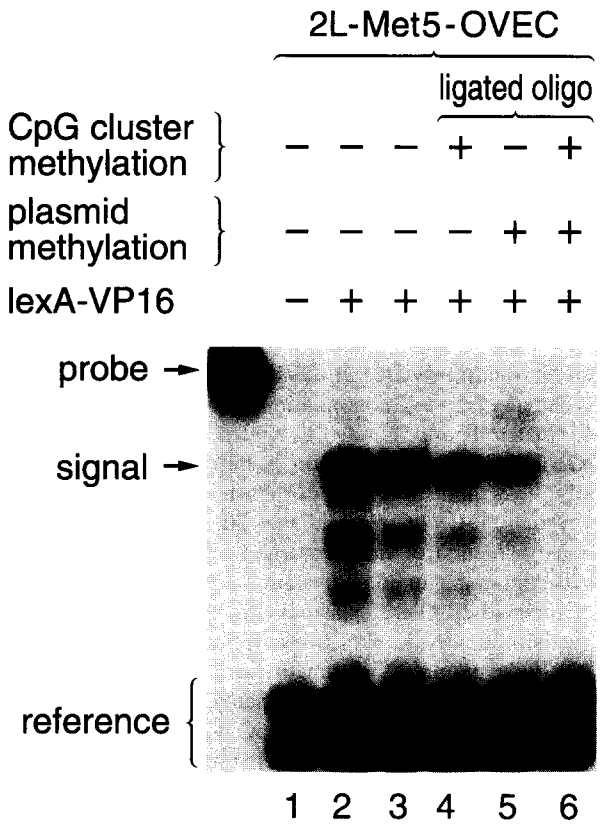

Fig. 3. S1 nuclease mapping of transcripts from partly methylated reporter plasmids. Four types of reporter genes were tested, containing an unmethylated or methylated CpG cluster inserted into unmethylated or methylated 2L-OVEC plasmid, using a novel joining procedure. Names and methylation status of the CpG cluster as well as the 2L-OVEC plasmid, and the presence of the co-transfected expression vector for lexA-VP16 transactivator are indicated above. 'signal' and 'reference' denote transcripts from reporter gene and internal transfection standard, respectively.

However, we also show that a region of high CpG density does not have to be contiguous for repression by DNA methylation, i.e. even a relatively small CpG cluster in a promoter region can repress transcription in cooperation with a high CpG density region that can be located more than one kilobase apart. Therefore a subset of tissue specific, non-CpG island promoters that contain a short CpG-rich segment, or CpG cluster, may be able to suppress transcription by methylation with the help of remote CpG-rich methylated regions. Short CpG clusters similar to the one tested by us are found in DNA database sequences. A striking example is the human interleukin 1α promoter, which is devoid of CpG sites except for a cluster of 12 CpG dinucleotides just upstream of the transcription start site [35]. This gene may therefore be similarly affected by methylation as our model construct.

It remains to be seen whether the cooperative inhibition of transcription by CpG methylation reported here is the result of cooperative binding of proteins, such as MeCP-1 [14] that specifically recognize methylated DNA. Finally, we note that the cooperation for a negative effect described here bears an analogy to transcriptional activation, where a promoter often cooperates with a remote enhancer [36,37].

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