CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation

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In mammals and other vertebrates, cytosine methylation in CpG sites is often negatively correlated with gene activity. Because methylation of the promoter region is most crucial for this effect, the simplest hypothesis is that CpG methylation interferes with the binding of specific transcription factors. We have examined this hypothesis with two different transcription factor-binding sites that contain a CpG dinucleotide, namely the cAMP-responsive element (CRE; 5'-TGACGTCA) and the Sp1-binding site (5'-$^G_T$$^G_A$GGCG$^G_T$$^G_A$$^C_T$). We have reported previously that CpG methylation of the Sp1-binding site affected neither factor binding nor transcription in HeLa cells, which may be related to the fact that Sp1 is typically associated with promoters of housekeeping genes. In contrast, CREs are often associated with promoters of cell type-specific genes. A synthetic oligonucleotide containing two tandem CREs derived from the gene encoding the human glycoprotein hormone α-subunit was cloned upstream of a reporter gene. Transcription of this gene was dependent on the CRE sequences in both PC12 and HeLa cells. Bandshift and methylation interference assays show that similar, if not the same, factor(s) bind to the CRE in both cell lines, even though induction by cAMP was only observed in PC12 cells. CpG methylation of the CRE consensus sequences (TGACGTCA) resulted in loss of specific factor binding, as well as loss of transcriptional activity in vitro and in vivo, in both cell types. This suggests that the inactivity of methylated promoters can, at least in some cases, be explained by their inability to bind specific transcription factors.

Methylation of cytosine residues in mammalian DNA is thought to be an important component of a multilevel control mechanism for gene expression. Approximately 3–4% of all cytosine residues in mammalian DNA are methylated. CpG dinucleotides, which are underrepresented in the DNA of higher eukaryotes, are the preferential site for cytosine 5-methylation. Specific methylation at CpG sites is apparently involved in inactivation of gene expression and seems to play a role in tissue-specific and developmental stage-specific gene expression by providing additional genetic information (for reviews, see Dörfler 1983; Cristy and Scangos 1986; Cedar 1988). The simplest model for gene inactivation by methylation is that methylation affects protein–DNA interactions required for efficient transcription, either directly, by steric hindrance of factor binding, or indirectly, by altering the chromatin structure of the methylated DNA. There is limited evidence for both the first (Kovesdi et al. 1987; Watt and Molloy 1988) and second (Buschhausen et al. 1987; Cedar 1988) mechanisms.

Among various transcriptional regulatory sequences so far reported, the Sp1-binding site (5'-$^G_T$$^G_A$GGC-G$^G_T$$^G_A$$^C_T$) and the cAMP-responsive element (CRE; 5'-TGACGTCA) have a CpG dinucleotide in the center of their consensus sequences. Recently, several groups, including ours, have independently reported that CpG methylation does not affect the binding of Sp1 to its recognition sequence (Ben-Hattar and Jiricny 1988; Höller et al. 1988; Harrington et al. 1988). In parallel with binding studies using HeLa and B-cell extracts, we have performed transcription assays in vitro and in vivo (Höller et al. 1988). These assays showed that transcription was not affected by CpG methylation of the Sp1 site. Because Sp1 factor often is associated with promoters of housekeeping genes, and methylation-free islands are typically found in the promoter–leader regions of those genes (for reviews, see Bird 1986, 1987), we suggested that constitutive binding of Sp1 may prevent de novo methylation of the DNA, which would be important for maintaining expression of those genes (Höller et

al. 1988).

CRE DNA sequence motifs, on the other hand, have been identified in 5′-flanking regions of various cell type-specific genes, including hormone-coding, and also viral genes (for review, see Roesler et al. 1988). Expression of these genes is modulated by cAMP in the appropriate cells, apparently by at least two independent mechanisms. Phosphorylation of a preexisting binding factor specific for CRE (CREB) by cAMP-dependent protein kinase (protein kinase A) activates transcription (Montminy and Bilezikjian 1987; Nakagawa et al. 1988; Yamamoto et al. 1988). Furthermore, phosphorylation of CREB by protein kinase C at a different site stimulates dimer formation, increasing the binding affinity of the protein for CRE (Yamamoto et al. 1988). A common cellular transcription factor, ATF, probably identical to CREB, binds to CRE and was found to be regulated in vivo by either cAMP or adenovirus E1A protein (Hurst and Jones 1987; Lin and Green 1988). Another possible mechanism for the modulation of cAMP-regulated gene expression, namely by differential DNA methylation, has not been addressed so far. Therefore, we have examined the effect of CpG methylation on protein binding and promoter activity of CRE, both in cAMP-responding cells (PC12 cells) and nonresponding cells (HeLa cells). Our results show that CpG methylation of CREs interferes with factor binding and abolishes transcriptional activity. This suggests that inactivation of gene expression by CpG methylation can, at least in some cases, be explained by the inability of the methylated DNA to bind specific transcription factors.

Results

Protein binding studies in vitro

The synthetic oligonucleotides designated 1 × CRE and 1 × CREmet contain one CRE. In 1 × CREmet, the cytosine residue in the center of the CRE in each strand is methylated, as shown in Figure 1. These oligonucleotides were end-labeled, annealed, and used as probes in bandshift assays. As shown in Figure 2A, 1 × CRE and the 1 × CREmet gave different shift patterns after incubating with nuclear proteins extracted from PC12 cells, a rat pheochromocytoma cell line known to contain CRE-binding proteins (CREB) (Montminy and Bilezikjian 1987). Several retarded bands could be seen with both 1 × CRE and 1 × CREmet, but the patterns were quite different (Fig. 2A). The faint band below complexes 6 and f is likely to be a complex between a single-stranded oligonucleotide and a cellular protein(s), because addition of excess single-stranded DNA eliminated this band. This methylated probe 1 × CREmet gave fewer, weaker bands. Notably, three bands observed with the 1 × CRE probe were missing, namely the major band 3 and the minor bands 2 and 4 (Fig. 2A, lanes 1 and 7). When 1 × CRE was used as probe, all three related complexes, 2, 3, and 4, could be competed with a large excess of unlabeled unmethylated oligonucleotide (1 × CRE) but not with methylated oligonucleotide (1 × CREmet), even at a 1000-fold excess. In that compe-

1×CRE

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     5' CGAGACTGTATTGACGTCATGGTACTGTG 3'
     3' TCGAGCTCTGACATAACTGCAGTACCATGACACAGCT 5'
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1×CREmet

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                      CH3
     5' CGAGACTGTATTGACGTCATGGTACTGTG 3'
     3' TCGAGCTCTGACATAACTGCAGTACCATGACACAGCT 5'
                      CH3
```

2×CRE

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     5' CGAGATTGACGTCATGGTAAAAATTGACGTCATGG 3'
     3' TCGAGCTCTAACTGCAGTACCATTTTTAACTGCAGTACCAGCT 5'
```

2×CREmet

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            CH3                    CH3
     5' CGAGATTGACGTCATGGTAAAAATTGACGTCATGG 3'
     3' TCGAGCTCTAACTGCAGTACCATTTTTAACTGCAGTACCAGCT 5'
            CH3                    CH3
```

Figure 1. Oligonucleotides used in this study containing either one or two CRE consensus sequences (TGACGTCA, boxed) were synthesized and designated 1 × CRE or 2 × CRE, respectively. Oligonucleotides 1 × CREmet and 2 × CREmet have exactly the same sequences as 1 × CRE and 2 × CRE, but the CpG sites in the CRE consensus are methylated. (CH₃) 5-Methyl group.

tition, the minor complexes 2 and 4 disappear before complex 3, as seen in Figure 2A (lanes 4–6). As is evident from their identical methylation interference pattern (see below), the proteins of bands 2, 3, and 4, are closely related or even variants of the same protein. These results suggest that several proteins in the PC12 nuclear extract can bind to the 1 × CRE and 1 × CREmet oligonucleotides, different proteins bind to 1 × CRE and 1 × CREmet, and the binding affinity of the proteins that bound specifically to 1 × CRE is stronger than that of the proteins that bound to both 1 × CRE and 1 × CREmet.

Similar results also were obtained with HeLa cell nuclear extract (Fig. 2B). A clear difference was observed between the patterns of 1 × CRE and 1 × CREmet. The position of the major shifted band with the 1 × CRE probe (Fig. 2B, lane 1, band *) indicates a protein of similar size to that of complexes 2–4 with PC12 extract. This band also behaved like complexes, 2–4 of PC12 cells: It was missing with the methylated 1 × CREmet probe and could be competed with excess 1 × CRE oligonucleotide but not with 1 × CREmet.

Methylation interference experiments, the results of which are summarized in Figure 3, revealed that the shifted bands of labeled 1 × CRE that remain after addition of excess 1 × CREmet oligonucleotide (Fig. 2) are due to specific binding to the CRE consensus sequences. Because these bands were missing in the shift patterns with 1 × CREmet probe, it is suggested that the specific CRE-binding proteins cannot bind to CRE when the CpG site at the center is methylated, although other proteins can still bind to a wider region overlapping the CRE.
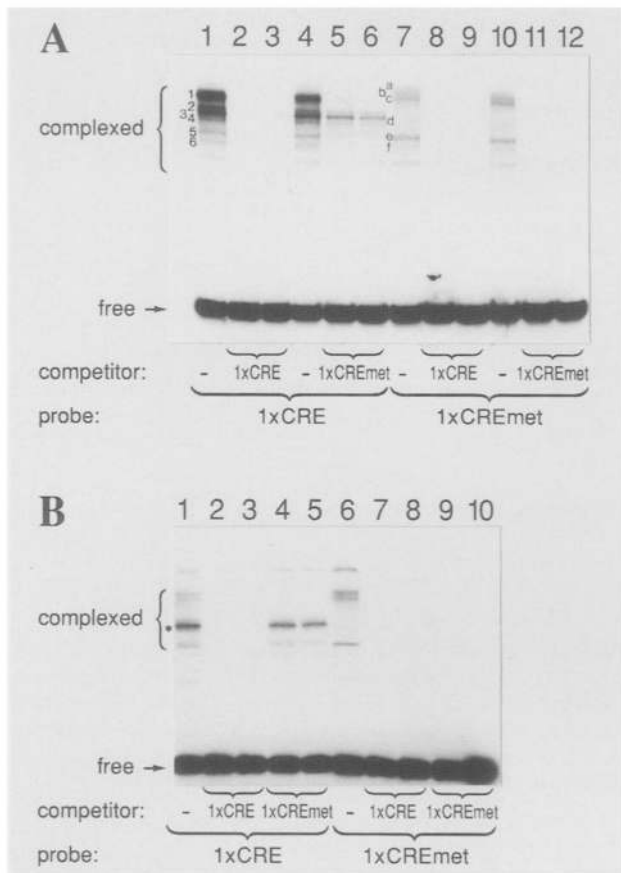
Figure 2. Binding of specific nuclear proteins to methylated and unmethylated CRE sequences in vitro. (A) Bandshift analysis with a PC12 cell nuclear extract. The end-labeled oligonucleotides $1 \times CRE$ (lanes 1–6) or $1 \times CREmet$ (lanes 7–12) were incubated with a PC12 cell nuclear extract in the absence (lanes 1,4,7,10) or presence of nonlabeled competitor oligonucleotides, either $1 \times CRE$ (lanes 2,3,8,9) or $1 \times CREmet$ (lanes 5,6,11,12). Molar ratios of the probe and the competitor were 1 : 500 (lanes 2,5,8,11) and 1 : 1000 (lanes 3,6,9,12). The numbers beside the shifted bands correspond to those in Fig. 3. (B) Bandshift analysis with a HeLa cell nuclear extract. End-labeled oligonucleotides $1 \times CRE$ (lanes 1–5) or $1 \times CREmet$ (lanes 6–10) were incubated with a HeLa cell nuclear extract in the absence (lanes 1–6) or presence of nonlabeled competitor oligonucleotides, either $1 \times CRE$ (lanes 2,3,7,8) or $1 \times CREmet$ (lanes 4,5,9,10). Molar ratios of the probe and the competitor were 1 : 500 (lanes 2,4,7,9) and 1 : 1000 (lanes 3,5,8,10). Asterisk (*) corresponds to that in Fig. 3.

In vitro transcription

Because we observed that specific binding to CRE in vitro was inhibited by CpG methylation of the consensus sequence, we also examined whether or not transcriptional activation due to CRE would be affected by methylation. The nonmethylated $1 \times CRE$ oligonucleotide was cloned immediately upstream of the TATA box and coding sequence of the rabbit β-globin gene in the OVEC expression vector (Westin et al. 1987). This reporter gene contains a truncated promoter with convenient cloning sites for cis-acting DNA segments from

enhancers or promoters. Reporter gene transcription can be assayed both in vivo, after transfection into cells, and in vitro, with cell-free extracts. Promoter activity in vitro in PC12 cell nuclear extract was assayed by S1 nuclease mapping of the transcripts. $p1 \times CRE-OVEC$ gave a very low level of transcription, equivalent to that of the negative control clone pOVEC-1, which contains a TATA box only (Fig. 4A). We then synthesized another oligonucleotide, $2 \times CRE$, containing two CRE consensus motifs in tandem, as in the upstream region of the α-subunit of the human gonadotropin gene (Delegeane et al. 1987; Jameson et al. 1987; see Fig. 1). This was also cloned into OVEC and examined for promoter activity in vitro. $p2 \times CRE-OVEC$ was transcribed efficiently, suggesting that two tandem repeats of CRE can work well as a promoter element in an in vitro transcription system. $2 \times CRE$ and $2 \times CREmet$, in which the two CpG sites in both CREs are methylated (see Fig. 1),
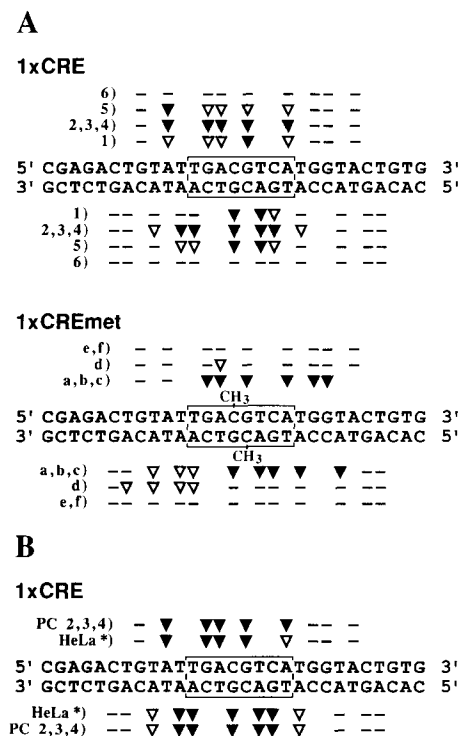

Figure 3. Determination of the region of protein–DNA contact by a methylation interference assay. Both strands of the $1 \times CRE$ and $1 \times CREmet$ oligonucleotides were end-labeled and analyzed for protein-binding sites by a chemical modification assay (methylation interference; Siebenlist and Gilbert 1980), using PC12 cell nuclear extract. The data are summarized in A, where the numbers correspond to shifted bands in Fig. 2A. Symbols above the sequence represent purines whose methylation interferes with protein–DNA interaction: (▼) Strong interference; (▽) weaker interference. Boxed sequences are CRE consensus sequences. (CH₃) 5-Methyl group. The same analyses were performed on the shifted bands of the $1 \times CRE$ probe, which cannot be competed by addition of a large excess of the $1 \times CREmet$ oligonucleotide, using nuclear extracts of either PC12 or HeLa cells. These results are summarized in B, where the numbers and the asterisk (*) correspond to shifted bands in Fig. 2A,B.

were ligated directly into OVEC, and the uncloned, co-valently closed circular DNAs, designated p*li*2 × CRE-OVEC and p*li*2 × CREmet-OVEC, respectively, were sep-arated from linear and nicked DNAs by extraction with acid phenol (Zasloff et al. 1978). As shown in Figure 4A,
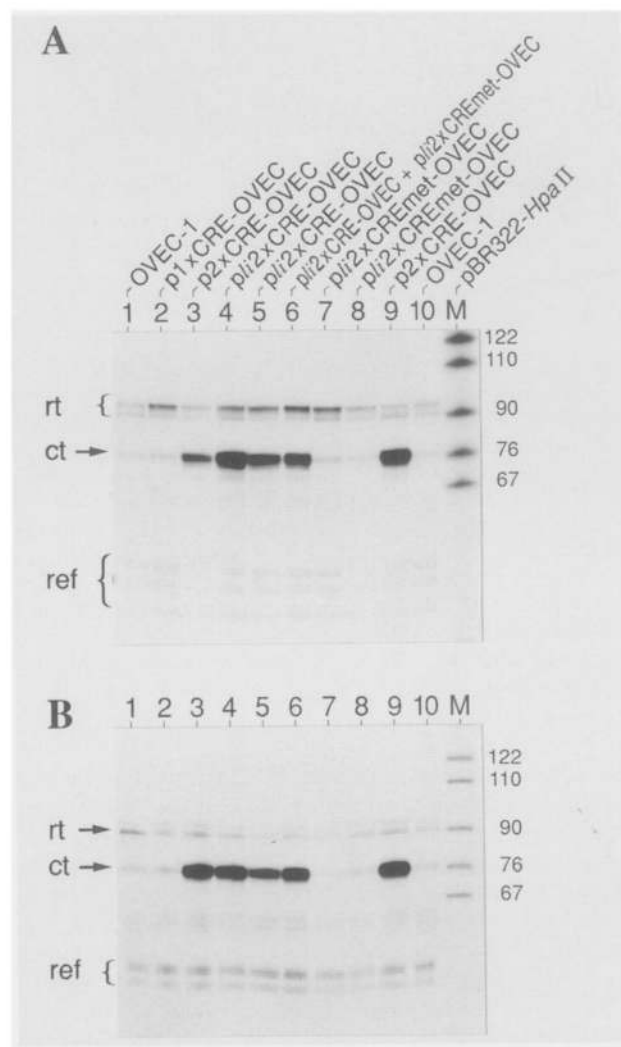

Figure 4. A promoter with CpG methylated CREs is not active in vitro. The oligonucleotides containing CREs were cloned or ligated into the *Sac*I and *Sal*I sites upstream of the rabbit β-globin gene TATA box in the OVEC expression vector (Westin et al. 1987), and the resultant plasmids were used as templates in an in vitro transcription assay with nuclear extract of either PC12 cells (*A*) or HeLa cells (*B*). Transcripts synthesized from circular DNA templates were analyzed by quantitative nuclease S1 mapping. (Lane *1*) 100 ng of OVEC-1; (lane *2*) 100 ng of p1 × CRE-OVEC; (lane *3*) 100 ng of p2 × CRE-OVEC; (lane *4*) 100 ng of p*li*2 × CRE-OVEC; (lane *5*) 50 ng of p*li*2 × CRE-OVEC; (lane *6*) 50 ng of p*li*2 × CRE-OVEC and 50 ng of p*li*2 × CREmet-OVEC; (lane *7*) 50 ng of p*li*2 × CREmet-OVEC; (lane *8*) 100 ng of p*li*2 × CREmet-OVEC; (lane *9*) 100 ng of p*li*2 × CRE-OVEC; (lane *10*) 100 ng of OVEC-1. OVEC-REF DNA (100 ng) was added in all lanes. (*M*) pBR322 fragments digested with *Hpa*II as size marker. (ct) Position of correctly initiated transcripts; (rt) position of read-through transcripts; (ref) position of RNA synthesized from the truncated β-globin gene of OVEC-REF.

p*li*2 × CRE-OVEC gave a signal as strong as that of cloned p2 × CRE-OVEC (both the transcripts from p2 × CRE-OVEC and OVEC-REF in lane 3 are weaker, probably because of loss of the material during experiments, but the transcripts are comparable between lanes 4 and 9), whereas transcription from p*li*2 × CREmet-OVEC was undetectable. This result is not due to some inhibitory substance in the p*li*2 ×·CREmet-OVEC preparation, because transcription was readily detectable when a mixture of p*li*2 × CRE-OVEC and p*li*2 × CREmet-OVEC (1 : 1) was used as template (Fig. 4A).

The same in vitro transcription assays were carried out using HeLa cell nuclear extract. The results, shown in Figure 4B, were quite similar to those with the PC12 nuclear extract. p1 × CRE-OVEC was not transcribed significantly, whereas p2 × CRE-OVEC gave a clear signal. Here, also, p*li*2 × CRE-OVEC was transcribed, as well as cloned p2 × CRE-OVEC, whereas p*li*2 × CREmet-OVEC did not give detectable transcription. Therefore, we conclude that the 2 × CRE oligonucleotide, which acts as an efficient promoter component for transcription in vitro, is no longer active when the CpG sites at the center of the CREs are specifically methylated.

In vivo transcription

To examine the effect of CpG methylation on transcription in vivo as well as in vitro, the template OVEC DNAs were transfected into PC12 cells and HeLa cells. Cytoplasmic RNA was extracted from the transfected cells, and the transcripts were mapped with an SP6 RNA probe. In PC12 cells, transcription of p1 × CRE-OVEC was undetectable and that of the cloned p2 × CRE-OVEC was very low unless induced by treatment of the cells with forskolin, a putative activator of adenylate cyclase, or dibutyryl cAMP (dBucAMP), a stable cAMP analog (Fig. 5A). Uncloned p*li*2 × CRE-OVEC and p*li*2 × CRE-met-OVEC were transfected to examine the effect of methylation on expression in vivo. After treatment of the transfected cells with either forskolin or dBucAMP, both cloned p2 × CRE-OVEC and uncloned p2 × CRE-OVEC gave strong signals. However, transcription of uncloned p*li*2 × CREmet-OVEC could be detected neither before nor after treatment with forskolin or dBucAMP. In PC12 cells, the internal control OVEC-REF, the expression of which depends on the SV40 enhancer, did not give any signal, probably because the SV40 enhancer is inactive in these cells. This conclusion is supported because p2 × CRE-OVEC-SV, which contains the SV40 enhancer, did not produce more transcripts than p2 × CRE-OVEC (data not shown).

In HeLa cells, transcription of both p1 × CRE-OVEC and p2 × CRE-OVEC was undetectable (Fig. 5B). We could detect the transcripts only when we transfected the cells with p2 × CRE-OVEC-SV, in which the p2 × CRE oligonucleotide was cloned into OVEC-SV, which has the SV40 enhancer sequences inserted downstream of the β-globin reporter gene (Fig. 5B). Treatment of the cells with forskolin or dBucAMP did not give a significant increase in expression (data not shown). It is possible that the specific binding protein responsible for
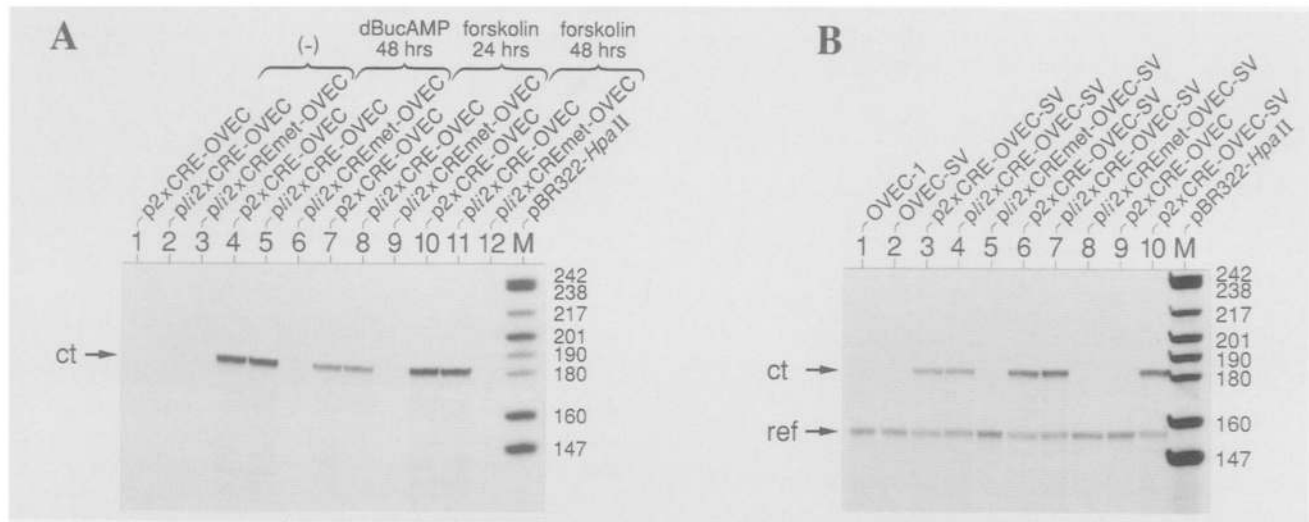
Figure 5. A promoter with CpG methylated CREs is also not active in vivo. PC12 cells (*A*) and HeLa cells (*B*) were transfected by the calcium phosphate coprecipitation method, with the OVEC plasmids cloned or ligated with oligonucleotides carrying CREs. Cytoplasmic RNA was extracted from the cells after 24 or 48 hr and analyzed by SP6 RNA mapping. PC12 cells were treated with forskolin or dBucAMP 18 hr after transfection or left untreated as a control. (*A*) Mapping of the transcripts from transfected PC12 cells. Cytoplasmic RNA was extracted 24 or 48 hr after treatment with cAMP activators as indicated. (Lanes *1,4,7,10*) p2 × CRE)-OVEC; (lanes *2,5,8,11*) p*li*2 × CRE-OVEC; (lanes *3,6,9,12*) p*li*2 × CREmet-OVEC. OVEC-REF was added in all lanes in a ratio of 1 : 4 to the test DNAs. (Lanes *1–3*) No treatment (RNA was extracted 48 hr after transfection); (lanes *4–6*) 48 hr after treatment with 1 mM dBucAMP; (lanes *7–9*) 24 hr after treatment with 10 μM forskolin; (lanes *10–12*) 48 hr after treatment with 10 μM forskolin. (*B*) Mapping of the transcripts from the transfected HeLa cells. Cytoplasmic RNA was extracted 48 hr after transfection. (Lane *1*) 5 μg of OVEC-1; (lane *2*) 5 μg of OVEC-SV; (lane *3*) 2.5 μg of p2 × CRE-OVEC-SV; (lane *4*) 2.5 μg of p*li*2 × CRE-OVEC-SV; (lane *5*) 2.5 μg of p*li*2 × CREmet-OVEC-SV; (lane *6*) 5 μg of p2 × CRE-OVEC-SV; (lane *7*) 5 μg of p*li*2 × CRE-OVEC-SV; (lane *8*) 5 μg of p*li*2 × CREmet-OVEC-SV; (lane *9*) 5 μg of p2 × CRE-OVEC; (lane *10*) 5 μg of p2 × CRE-OVEC-SV. OVEC-REF was added in all lanes. (ct) Position of correctly initiated transcripts; (ref) position of RNA synthesized from the truncated β-globin gene of OVEC-REF.

transcriptional stimulation by the CRE may already be activated in HeLa cells, so that further induction is not seen after cAMP treatment. The 2 × CRE and 2 × CRE-met-oligonucleotides were then directly ligated into OVEC-SV, and the resulting uncloned p*li*2 × CRE-OVEC-SV and p*li*2 × CREmet-OVEC-SV transfected into HeLa cells. As shown in Figure 5B, p*li*2 × CRE-OVEC-SV gave a signal as strongly as that from cloned p2 × CRE-OVEC-SV, whereas the transcription of p*li*2 × CREmet-OVEC-SV was undetectable, as is the case with OVEC-SV, which has no promoter element except the TATA box. These observations lead us to conclude that specific CpG methylation not only strongly inhibits the promoter activity of CRE in an in vitro transcription system but also in an in vivo transient expression assay.

Discussion

In cell lines such as PC12, the cellular enhancer–promoter motif TGACGTCA (CRE) is known to be activated by the transcription factor CREB in response to cAMP (Montminy and Bilezikjian 1987). The CRE motif contains a CpG site that is a potential methylation site. We have examined the effect of CpG methylation on binding of factors in vitro and on transcription in vitro or in transfected cells. The data show that protein factors specific for CRE cannot bind to the sequence when the CpG site is methylated. Methylation of the CpG site in

the CRE also prevents transcriptional activation by this sequence, both in vitro and in vivo, most probably by inhibiting the binding of factors to the DNA. This inhibition is most likely a result of direct steric hindrance by the methyl groups, although it seems that CpG methylation, even at sites that may not bind transcription factors, can induce an altered chromatin structure (Keshet et al. 1986; Buschhausen et al. 1987).

Recently, several reports also have suggested that CpG methylation can be involved in the regulation of gene expression by affecting DNA–protein interactions. Binding of unidentified proteins to sequences 2.5 and 1 kb upstream of the tyrosine aminotransferase gene was found to be inhibited by CpG methylation of the binding site (Becker et al. 1987). Methylation of a single CpG site in the E2 promoter or the major late promoter of adenovirus was shown to prevent binding of the cellular transcription factors E2F or MLTF, respectively. Inhibition of gene expression was demonstrated in vivo for the E2 promoter and in vitro for the major late promoter (Kovesdi et al. 1987; Watt and Molloy 1988).

In contrast, methylation of the Sp1-binding sequence has no effect on factor binding (Ben-Hattar and Jiricny 1988; Harrington et al. 1988; Höller et al. 1988) or on activation of transcription in vivo or in vitro (Höller et al. 1988). These contrasting results may reflect different roles of methylation in the transcriptional regulation of housekeeping genes and tissue-specific and/or inducible

genes. Most housekeeping genes (for review, see Parker et al. 1986) contain so-called methylation-free islands (for review, see Bird 1986, 1987) that typically span a region of ~1 kb around the transcription initiation site and are rich in nonmethylated CpG dinucleotides. As we discussed in a previous paper (Höller et al. 1988), constitutive binding of Sp1 factor may result in methylation-free CpGs in Sp1-binding sites that are frequently present in methylation-free islands (Gardiner-Garden and Frommer 1987). For housekeeping genes, it might be imperative that Sp1 can bind to its recognition site even if the site becomes methylated by chance. However, for the genes that are expressed tissue specifically or are inducible by biological factors such as cAMP and viral proteins, methylation of the promoter−enhancer sequences with consequent inhibition of the binding of transcription factors would provide an efficient regulatory mechanism. Tissue-specific genes often are found in the CpG-deficient, methylated portion of the genome. During development and cell differentiation, gene expression may be switched off and on by methylation and demethylation at specific sites or over more extensive regions. This idea is supported by the observation of transcription activation by site-specific demethylation in promoter regions and estradiol/glucocorticoid−receptor binding sites (Saluz et al. 1986; Yisraeli et al. 1986; Shimada et al. 1987). Demethylation may not only be involved in switching on a silent gene but may also facilitate maintenance of the transcriptionally active state (Kelly et al. 1988).

Here, we show that methylation of the single CpG in a CRE sequence inhibits transcription, most probably by direct interference with the binding of a transcription factor. This indicates that methylation at specific CpG sites can contribute to the regulation of gene expression.

Finally, an interesting aspect is that expression of our reporter gene with CRE motifs in the promoter region was induced dramatically by cAMP-activating agents in PC12 cells, even in the absence of an SV40 enhancer. In contrast, in HeLa cells, constitutive expression was observed in the presence of an SV40 enhancer. Despite the different responsiveness to cAMP, the same factor may be responsible for CRE-mediated transcription in both cell lines, as is suggested by the methylation interference patterns of protein−DNA interaction (Fig. 3). Several CRE-binding proteins, ranging from 30 to 76 kD have been reported recently for HeLa cells (Hurst and Jones 1987; Cortes et al. 1988). The most prominent of these, termed ATF, is similar or identical to the CREB factor of PC12 cells, a 43-kD protein (Hurst and Jones 1987; Montminy and Bilezikjian 1987; Lin and Green 1988). As discussed above, there are apparently at least two regulatory mechanisms for the activation of the CREB factor: phosphorylation by cAMP-dependent protein kinase (protein kinase A) (Montminy and Bilezikjian 1987; Nakagawa et al. 1988; Yamamoto et al. 1988) and dimerization of the protein, promoted by phosphorylation at a different site, via protein kinase C (Yamamoto et al. 1988). Because HeLa cells are derived from a cervix carcinoma that contains a human papilloma

virus, expression of a viral transforming gene might convert CREB from an inactive state into a constitutively active form. This explanation seems reasonable because cellular transformation often is associated closely with disorders in signal transduction through the protein kinase A-dependent pathway and/or the protein kinase C-dependent pathway (for review, see Edelman et al. 1987).

Materials and methods

Purification and annealing of oligonucleotides

Oligonucleotides were synthesized on a Pharmacia gene assembler and purified on a 12% polyacrylamide gel containing 7.5 M urea. The DNAs were eluted from the gel into distilled water and absorbed onto a Sep-pak column (Waters Associates, Millipore Corp.) equilibrated with 50% acetonitrile in 100 mM sodium acetate (pH 4.5). The DNAs were then eluted with 30% acetonitrile in distilled water, lyophilized, and redissolved in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. After kinasing with T4 polynucleotide kinase, complementary oligonucleotides were denatured together and annealed. For the bandshift probes, one oligonucleotide was kinased with [α-^{32}P]ATP before annealing with the complementary oligonucleotide.

Purification of covalently closed circular DNA

After ligation of the oligonucleotides into OVEC-1 or OVEC-SV, the DNAs were purified by two phenol extractions and precipitated by adding 0.6 volumes of isopropanol at room temperature. DNA was resuspended in 1 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM sodium acetate (pH 4.0), and 75 mM NaCl and extracted twice with phenol saturated with 50 mM sodium acetate (pH 4.0) at 4°C. The pH of the DNA samples was adjusted to neutral with 1 M Tris (pH 9.0), and the DNAs were concentrated by ethanol precipitation. During the acid phenol extraction, linear and nicked DNA accumulate in the phenol layer, whereas the closed circular DNA remains quantitatively in aqueous phase (Zasloff et al. 1978).

Bandshift assay

Nuclear extracts of HeLa cells and PC12 cells were prepared as described previously (Dignam et al. 1983), with come minor modifications (Westin et al. 1987). After ammonium sulfate precipitation, the extract was resuspended in and dialyzed against 20 mM HEPES−KOH (pH 7.9), 20% glycerol, 20 mM KCl, 2 mM MgCl$_2$, 0.2 mM EDTA, and 0.5 mM dithiothreitol (DTT). For bandshift assay, 2–4 fmoles of end-labeled DNA (5000 cpm) were incubated with 2–3 μg of nuclear proteins at room temperature for 15 min in a buffer containing 0.5 μg poly[d(I-C)], 15 mM HEPES (pH 7.9), 1 mM EDTA, 4% Ficoll 400, 1 mM DTT, 4 mg/ml bovine serum albumin, and 50 mM KCl in a final volume of 15 μl and were electrophoresed on a 4% polyacrylamide (19 : 1) gel in 0.25 × TBE buffer. For competition experiments, 2–5 pmoles of double-stranded, annealed oligonucleotide were added to the reaction mixture prior to the addition of nuclear extracts.

Methylation interference

The labeled probes for bandshift assay were partially methylated for 12 min at 20°C by dimethylsulfate (DMS) (Siebenlist and Gilbert 1980) before using in a binding reaction for bandshift scaled up 10- to 20-fold with respect to the amount of labeled DNAs, nonspecific competitor DNAs, and nuclear ex-

tract. After electrophoresis and autoradiography, complexed and free DNA fragments were excised and eluted from the gel, purified by DEAE 52 cellulose chromatography, and ethanol precipitated. The pellets were resuspended in 20 μl of 10 mM sodium phosphate (pH 7.2) containing 1 mM EDTA, heated to 90°C for 10 min, followed by addition of 100 μl of 0.1 M NaOH containing 1 mM EDTA, and heated to 90°C for 30 min. This treatment provides both methylated G and A residues. The pH of the samples was adjusted to neutral with 25 μl of 1 M Tris (pH 7.5), 25 μl of 2 M sodium acetate (pH 7.0), and 100 μl of sodium acetate (pH 4.8). The samples were precipitated with ethanol and analyzed on an 8% polyacrylamide gel containing 7.5 M urea.

In vitro transcription assay

The 1×CRE and 2×CRE oligonucleotides with SacI and SalI protruding ends were cloned into SacI and SalI sites, respectively, of the OVEC vector in front of the TATA box at position −42 relative to the transcription initiation site (Westin et al. 1987) or the OVEC-SV vector, which has the SV40 enhancer in the EcoRI site located 1890 bp downstream of the β-globin gene cap site. The conditions for the in vitro transcription assay were as described previously (Westin et al. 1987). The synthesized RNA was analyzed by quantitative S1 nuclease mapping using 98-nucleotide single-stranded synthetic oligonucleotide, spanning positions −10 to +75 of the rabbit β-globin gene with respect to the transcription initiation site, as a probe. The samples were electrophoresed on a 6% polyacrylamide (19 : 1) gel containing 7.5 M urea. In all transcription initiation reactions, the OVEC-REF plasmid, with a deletion of 28 bp around the transcription initiation site, was included as an internal standard.

In vivo transcription assay

PC12 cells, a rat pheochromocytoma cell line, were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% horse serum, 5% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin. HeLa cells were grown in DMEM supplemented with 2.5% fetal calf serum, 2.5% newborn calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Transfection of DNA into cells was carried out using the calcium phosphate coprecipitation method as described (Graham and van der Eb 1973). Together with 2 μg of OVEC-REF, 8 μg of the test DNA was used to transfected 1×10^5 to 2×10^5 cells. After 24 or 48 hr of incubation, the cells were harvested and the cytoplasmic RNA was extracted as described (de Villiers and Schaffner 1983). After digestion of residual input plasmid DNA by RNase-free DNase, the RNA was analyzed with an SP6 polymerase-generated RNA probe. The probe was prepared using plasmid pSP6βTS, a pSP64 clone containing a 223-bp TaqI–SalI fragment of OVEC downstream of the SP6 promoter in an inverted orientation (Westin et al. 1987).

Acknowledgments

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CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation.

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