DNA Methylation Inhibits Transcription Indirectly via a Methyl-CpG Binding Protein

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Summary

We have studied the mechanism by which DNA methylation inhibits transcription both in cell-free nuclear extracts and in the living cell. Repression of transcription in vitro for four different promoters was shown to be an indirect effect. The mediator of repression had properties indistinguishable from those of a methyl-CpG binding protein (MeCP-1) that has been previously identified. Use of differentially methylated promoters and methylated competitors in transient transfection assays suggested that indirect repression via MeCP-1 also occurs in the living cell. This was supported by the fact that MeCP-1–deficient cells showed much reduced repression of methylated genes.

Introduction

Mammalian DNA is heavily methylated at cytosine residues within the dinucleotide sequence CpG. The primary role of this modification remains a matter for debate, but several studies have established that methylation of the 5′ end of several genes is incompatible with their transcription. This was first suggested by an inverse correlation between DNA methylation and transcriptional activity (reviewed in Razin and Riggs, 1980) and was later confirmed by introduction of in vitro methylated genes into cells (Varshimon et al., 1982; Stein et al., 1982; Busslinger et al., 1983; Keshet et al., 1985; Yersa et al., 1988). The importance of DNA methylation as a repressor of transcription was reinforced by experiments with the demethylating agent 5-azacytidine (Jones and Taylor, 1980). Treatment of cells with this nucleoside analog often led to the reactivation of genes that were previously methylated and repressed (Mohandas et al., 1981; Venolia et al., 1982).

How does CpG methylation cause transcriptional repression? Two possible models can be envisaged. The “direct” model postulates that essential transcription factors see a methyl-CpG as a mutation in their binding site and so are unable to bind. The “indirect” model, on the other hand, hypothesizes that methylated DNA is bound by a nuclear protein(s), which secondarily prevents transcription factors from interacting with the gene. Circumstantial support for the direct model derives from the discovery of a number of transcription factors that are indeed sensitive to methylation. These include the cyclic AMP responsive element binding protein (CREB, Iguchi-Ariga and Schaffner, 1989) and two factors found in HeLa cell extracts that stimulate transcription from adenoviral promoters (Kovesdi et al., 1987; Watt and Molloy, 1988). However, not all transcription factors are methyl sensitive; for example, Sp1 can bind and activate equally well, regardless of methylation (Hoeller et al., 1988). Moreover, relatively few transcription factors contain the dinucleotide CpG in their recognition site.

Evidence for the indirect mechanism comes from several directions. Murray and Grosveld (1987) showed that the inhibition was not dependent on the methylation of specific sites in the γ-globin promoter, since the presence of methyl-CpGs in different regions of the promoter was sufficient to repress transcription. The authors concluded that a minimal methylation-free zone was required for expression, and they hypothesized that proteins with an affinity for methylated DNA were responsible for repression. Further evidence suggesting indirect inhibition derived from the observation that a methylated herpes simplex virus thymidine kinase (HSVtk) gene remained transcriptionally active for about 8 hr after injection into L cell nuclei before becoming repressed (Buschhausen et al., 1987). This result was incompatible with direct inhibition, and it implied that time was needed for the methylated DNA to interact with nuclear components before transcription was inhibited. Methylated DNA has indeed been shown to interact differentially with nuclear components. For example, upon transfection of different methylated and nonmethylated constructs into mouse L cells, the methylated constructs were shown to be preferentially assembled into relatively nuclease-resistant chromatin (Keshet et al., 1986). Furthermore, the methylated CpGs themselves are protected against nonspecific nucleases (Golage and Cedar, 1978) and against restriction enzymes that recognize CpG (Antequera et al., 1989). The data imply that there are factors in the nucleus that bind to methylated CpGs leading to the formation of an “inactive” chromatin structure. Recently, a factor of this kind that binds to methyl-CpGs regardless of sequence context has been identified (Meehan et al., 1989). In this paper we present evidence that this protein, MeCP-1, plays a role in the methylation-mediated repression of transcription both in vitro and in vivo.

Results

Inhibition by Methylation In Transient Assays

Figure 1 shows the promoters chosen for this study. Since we were keen to study promoters where the inhibition by natural methylation has been demonstrated, we included in the analysis an X-linked promoter derived from the mouse phosphoglycerate kinase (PGK) gene and a retroviral LTR derived from the mouse myeloproliferative sarcoma virus (PCMV; Hilberg et al., 1987). In the constructs that we used, the PGK promoter was placed upstream of a promoterless γ-globin gene, and the myeloproliferative

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Figure 1. Constructs Used to Study the Inhibitory Effects of Methylation

(A) PCMVjun: the promoter/enhancer region from the murine myeloproliferative sarcoma virus (dotted region, a) was used to promote transcription of a promoterless c-jun cDNA (region b; shaded region represents translated exons; nonshaded region represents the nontranslated sequences). The c-jun cDNA was, in turn, linked to an SV40 splice/poly(A) tail (region c, wavy lines). The dashed lines represent pUC16 sequences.

(B) tkneo: the same as the vector pMClNeo (Thomas and Capecchi, 1987) and has a polyoma enhancer (oblique shading, region a) linked to theHSVtk promoter (dotted region, b), which is used to drive the bacterial neo resistance gene (unshaded region). The dashed lines represent pUC9 sequences.

(C) p3EαGlob: a 1.5 kb piece of the human α-globin gene (section marked a) was cloned into pSP64. The stippled regions represent exons and the dashed lines pSP64 sequences. The checkered region (b) represents the SV40 enhancer at the 3' end of the gene.

(D) PGKγ: the promoter region of the mouse X-linked PGK gene (dotted region, a) was used to drive a promoterless human γ-globin gene (unshaded region, b) linked to the mouse PGK splice site/poly(A) tail (dotted region, c). The polyoma enhancer (d) from tkneo was cloned at the 3' end of the construct. Dashed lines represent pGEM11 sequences.

In all cases, an arrow marks the transcriptional start site. The plots beneath each construct show the CpG frequency versus the GpC frequency and indicate that, unlike the bulk genome, the promoters in this study were not CpG depleted.

Methylation has been shown to inhibit the expression of all these promoters when integrated into the chromosome, both in "natural" situations (PGK and retroviral LTRs, of which PCMV is an example) and, in the case of α-globin and the tk promoter, when methylation was added in vitro before stable transfection into cells (Busslinger and Flavell, 1983; Keshet et al., 1985).

We first asked if methylation at every CpG could inhibit expression from the four promoters in these constructs in transient transfection assays. This experiment was made feasible by the availability of SsI methyltransferase (Renbaum et al., 1990), which methylates every CpG and therefore has the same sequence specificity as the mammalian DNA methyltransferase. Following complete methylation using SsI methyltransferase, the plasmid was transiently transfected into HeLa cells. As a control, the human β-globin gene plus SV40 enhancer were cotransfected in a nonmethylated condition. RNAase protection mapping of the cytoplasmic RNA from the separate transfections showed that, for each of the four genes, no transcript was detectable from the methylated construct (Figures 2A and 2B). The nonmethylated constructs, on the other hand, were all active in the transfection assay.

Inhibition by Methylation In Vitro

To be able to study the mechanism of inhibition in more detail, we sought to reproduce this methylation-mediated inhibition in chromatin-free nuclear extracts. Extracts that are capable of supporting transcription have been used previously by Dobrzenski et al. (1988) to show that the presence of methyl-CpGs in the adenovirus E2A promoter can suppress transcription in vitro. We therefore prepared HeLa cell nuclear extracts and used them to transcribe methylated and nonmethylated α-globin genes, varying both the amount of added DNA and the amount of extract protein. The adenovirus major late promoter was included in all reactions as a nonmethylated control gene, and the transcription products of the control and test genes were mapped by S1 protection of the probes shown. Figure 3A shows that at high amounts of template (300 ng) no inhibition was seen. However, as the template concentration was lowered, with the extract concentration held constant, the inhibition became increasingly obvious until at 10 ng of DNA, transcription from the methylated α-globin
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HeLa cells were transiently transfected with either methylated (+) or nonmethylated (−) constructs (Figure 1) in the presence of the human β-globin gene (αglob) as a cotransfection control. RNAase protection mapping with the uniformly labeled probes shown give the protected regions indicated by the shaded block beneath the gene. The sizes of the protected regions are given in parentheses after each shaded block and are as follows: α-globin, 131 nt; PGKy, 100, 90, and 65 nt; PCMVjun, 364 nt; tkneo, 162 nt; and β-globin, 106 nt. Lane K represents hybridization to RNA from K562 cells that constitutively express α-globin (Dean et al., 1983) and acts as a positive control for the α-globin probe. Lane t shows hybridization of the probes to tRNA and lane C shows hybridization to mock-transfected HeLa cells. Some protection of the endogenous c-jun transcript in mock-transfected cells is evident for PCMVjun. The multiple transcriptional start sites for the mouse PGKy construct in HeLa cells reflect those already noted for the human PGK promoter (Gingor Sam et al., 1984).

The data are consistent with the indirect model described above. At low amounts of template (or high amounts of extract) there would be sufficient inhibitor in the extract to bind all of the methylated template and prevent transcription. When, however, the amount of added template exceeded the amount of inhibitor in the extract, transcription could occur. If this explanation is correct, we reasoned that any methylated DNA added to the in vitro transcription reaction should be able to compete out the inhibitor and restore expression from the methylated gene. To test this prediction, two different competitor plasmids were chosen: the first, pMlys, contains a 1 kb random piece of Micrococcus lysodeikticus (G+C rich) DNA cloned into pSVOCAT; the other, pCG11, contains a CpG-rich 135 bp sequence cloned into pUC19. Neither of these plasmids has any promoter activity as assayed by the CAT assay (data not shown) and so should not compete for transcription factors from the test plasmids. As can be seen in Figure 4A, the addition of a 30-fold excess of methylated pMlys or pCG11 completely restored transcription from the methylated α-globin gene. The same competitors were unable to alleviate the inhibition when added in a nonmethylated form. Similar loss of inhibition in the presence of methylated competitor was seen for the other three constructs used in this study: PGKy (Figure 4B), PCMVjun, and tkneo (not shown).

It is notable in Figure 4 that the addition of methylated competitor restored transcription from the methylated α-globin and PGKy constructs to the same level as that from the nonmethylated genes. Complete restoration of transcription was also seen for methylated tkneo. If methylation was able to inhibit transcription by direct interference...
Methylated (+) or nonmethylated (−) p3'Ea-glob (A) and PCMVjun (B) constructs were transcribed in HeLa cell nuclear extracts at the amounts in nanograms shown above each pair of lanes. The adenovirus major late promoter was included in all reactions as a control. The resulting RNA was mapped using the 51 probes shown beneath each gel and gave the protected sizes (shown in parentheses beside the hatched protected region) of 40 nt for the α-globin construct (αglob), 334 nt for the PCMVjun construct, and 197 nt for the adenovirus major late construct (Adeno). The right side of (A) shows a transcription of 100 ng of methylated (+) or nonmethylated (−) p3'Ea-glob where the amount of extract (in microliters) used in the in vitro transcriptions was increased as shown above each pair of lanes. Lane I shows hybridization of the probes to tRNA and lane N shows hybridization to a transcription reaction carried out in the absence of DNA. Lane K shows hybridization to RNA from the K562 cell line and lane M shows the markers whose sizes are given at the side of each gel.

with the binding of transcription factors, we would have expected some inhibition to persist under these conditions. Thus, in HeLa extracts we were unable to detect any evidence for a direct effect of DNA methylation on transcription of these three promoters. In the case of PCMVjun, transcription could only be restored to about 50% of the control level by addition of methylated competitor (data not shown). We conclude that methylation of this promoter had a small but significant direct effect on the transcription machinery.

Involvement of a Methyl-CpG Binding Protein

The first protein to be identified that will bind to any DNA that is symmetrically methylated at multiple CpGs, MeCP-1, was detected in extracts from mouse cells (Meehan et al., 1989). It seemed possible that the presence of a similar protein in our transcription extracts could account for the indirect inhibition that we observed. We tested for the presence of an MeCP-1-like protein in the HeLa extracts by means of a bandshift assay using methylated and nonmethylated forms of the probe CG11 (a 135 bp oligonucleotide that can be methylated at 27 CpGs, see Experimental Procedures). Figure 5A shows that a protein–DNA complex similar to that of mouse MeCP-1 was obtained with the methylated, but not with the nonmethylated, oligonucleotide. Additional experiments with alternative competitors confirmed that the binding properties of the HeLa protein were indistinguishable from those of mouse MeCP-1 (see below and data not shown).

We next asked whether HoLa MeCP-1 was able to bind to the methylated constructs whose expression was inhibited in the extracts. Figure 5B shows that all of these constructs when methylated are bound by MeCP-1 since they competed effectively for the bandshift activity. The nonmethylated forms of the constructs did not compete. Furthermore, pMlys and pCG11, the plasmids used to compete away the inhibition from the methylated template, also competed for the bandshift activity when methylated, but not when nonmethylated (data not shown).

To further determine if MeCP-1 is responsible for the inhibition in vitro, two additional methylated competitors were tested for their ability to alleviate the inhibition of transcription from methylated templates. Productive binding of mouse MeCP-1 was previously shown to require
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Figure 4. The Repression Seen In Vitro Can Be Overcome by the Addition of Methylated, but Not Nonmethylated, Competitors

Ten nanograms of the methylated (+) or nonmethylated (−) p3Eaglob (A) and PGKy (B) constructs were transcribed in HeLa cell nuclear extracts in the presence of either no added competitor (−), a 30-fold excess of nonmethylated competitor (Mlys or CGII), or a 30-fold excess of the same methylated competitor plasmids (MeMlys or MeCGII). The adenovirus major late promoter was included as a transcription control and the resulting RNAs were mapped using the S1 probes shown in Figure 3 for p3Eaglob and Adeno. The S1 probe used to map the PGKy RNA is shown beneath the mapping of the gene and gave a protected species of 65 nt. The other start sites seen in vivo for PGKy seem not to be active in vitro. Lanes t, nD, K, and M are the same as in Figure 3.

Figure 5. MeCP-1 Is Present in HeLa Extracts and Can Bind to the Constructs That Show Indirect Inhibition of Transcription In Vitro

(A) About 0.1 ng of the methylated (MeCGII) or nonmethylated (CGII) radiolabeled oligonucleotide probe was incubated with 5 μg of HeLa cell nuclear extract in the presence of increasing amounts of M. lysodeikticus nonspecific competitor DNA (shown in micrograms above each lane). Lane C shows a control bandshift done in the absence of extract. The prominent band present only in the CGII lane is due to an unknown protein that binds nonmethylated CGII. In some experiments this band is also present in the MeCGII lanes, suggesting that the protein also binds the methylated probe, but perhaps less strongly than MeCP-1.

(B) The bandshift to MeCGII was performed as described in (A) in the presence of 2 μg of M. lysodeikticus to give the bandshift shown (−). The constructs shown in Figure 1 (100 ng of each) were tested for their ability to compete for this bandshift in either their methylated (+) or nonmethylated (−) form.

at least 15 methyl CpGs on an oligonucleotide template (Meehan et al., 1989). Neither W17, an 18-mer with only three methyl CpGs, nor MeED89, a 34-mer with five methyl CpGs, is bound by mouse or HeLa MeCP-1 in their unligated form. Both are bound, however, when ligated to average lengths above 100 bp (i.e., above 15 CpGs). Unligated MeED89 and W17, as well as ligated W17, were tested as competitors for the inhibition in the in vitro transcription reactions. Figure 6 shows that unligated, methylated W17 does not alleviate the inhibition, while its ligated form does. Similarly, the unligated MeED89 is inactive in removing the inhibition. The effectiveness of these oligonucleotides in alleviating the inhibition correlates very well
Figure 6. Only Known Ligands for MeCP-1 Can Overcome Repression of Methylated Constructs

Ten nanograms of the methylated (+) or nonmethylated (−) p3E’aglob construct was transcribed in HeLa cell nuclear extracts with no added competitor (−), with unligated, methylated W17 (W17), with methylated W17 ligated to an average size of 150 bp ((W17)n), with unmethylated ED89 (ED89), or with methylated ED89 (MeED89). Each of these competitors was added to give a 30-fold excess; the sequences of these oligonucleotides are given in the Experimental Procedures. The adenovirus major late promoter was included as a transcription control and the transcripts were mapped by the probes shown in Figure 3. Control lanes t and nD are as described in Figure 3. Incomplete restoration of transcription from the methylated template in the presence of ligated W17 is probably due to a lower affinity of MeCP-1 for short oligonucleotides compared with longer heavily methylated plasmids with their effectiveness in binding MeCP-1, suggesting strongly that HeLa MeCP-1 is responsible for inhibiting transcription in the extracts.

Reduced Inhibition In MeCP-1-Deficient Extracts

If MeCP-1 is involved in methylation-mediated repression, we would expect that extracts deficient in MeCP-1 would be unable to repress methylated genes efficiently. Meehan et al. (1989) reported that two mouse embryonal carcinoma cell lines (PC13 and F9) contain barely detectable levels of MeCP-1. Figure 7A compares the ability of nuclear extracts from the embryonal carcinoma cell line F9 and from HeLa to bind to the methylated probe CG11. F9 extracts showed negligible binding, thereby confirming previous results. We next carried out in vitro reactions in the F9 extract using methylated and nonmethylated constructs as before. In contrast to previous results with HeLa extracts, very little inhibition could be seen even at low template concentrations. To test if the reduced inhibition was due to some modification (for example, demethylation) of the methylated template by the F9 extracts, the template DNA was recovered from an in vitro transcription in an F9 extract and used as the template in a HeLa cell extract. Figure 7B shows that the same templates that were only weakly inhibited in F9 extracts could be inhibited strongly in HeLa nuclear extracts. Thus, the methylated template is not incapable of being inhibited per se, but is expressed owing to the shortage of a mediator of repression in F9 extracts. The bandshift result indicates that the limiting mediator is most probably MeCP-1.

Is MeCP-1 Involved in the Inhibition In Vivo?

Having already established that transcription from the four methylated constructs was inhibited in living cells (see Figures 2A and 2B), we wished to find out whether this in vivo inhibition was also mediated by MeCP-1. Three different approaches were taken. In the first of these, we made a series of increasingly methylated constructs that would bind with different affinities to MeCP-1 in vitro. The purpose of the experiment was to ask if the level of inhibition in vivo corresponded to the affinity of the construct for MeCP-1. The p-glob construct was methylated with HpaI methyltransferase alone, HhaI methyltransferase alone, both methyltransferases together, or completely methylated with SssI methyltransferase. The number and position of methyl groups was determined using a methylation-dependent complex in the bands in the assay is reduced in the F9 extract compared with the HeLa extract. Approximately 0.1 ng of radiolabeled methylated (MeCG11) or nonmethylated (CG11) was incubated with either no extract (−) or with 10 μg of HeLa (H) or F9 nuclear extract in the presence of 4 μg of M. lysodeikticus nonspecific competitor DNA.

Figure 7. The Reduction of MeCP-1 in F9 Nuclear Extracts Is Paralleled by a Reduced Repression of Transcription from Methylated Constructs

(A) Formation of a methylation-dependent complex in the bands for hybridization to RNA in lane nD to a transcription reaction performed in the absence of DNA.
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Figure 8. Transcriptional Repression In Vivo of Various Methylated Forms of the Human α-Globin Gene Matches the Respective Affinity of Each Form for MeCP-1

(A) Computer plot showing the sites methylated by the bacterial methyltransferases HpaII, HhaI, HpaII+HhaI, and SssI (the latter methylates all CpGs) for the human α-globin gene. In this experiment the α-globin construct is without the SV40 enhancer; an arrow indicates the transcriptional start site.

(B) The methylated radiolabeled oligonucleotide probe MeG11 (0.1 ng) was incubated in the presence of 5 μg of HeLa cell nuclear extract protein and 2 μg of M. lysodeicticus nonspecific competitor DNA to give the bandshift shown by the minus sign. The methylated constructs shown in (A) (50 ng) were added to this mix to test their ability to compete for the bandshift.

(C) The methylated constructs shown in (A) (2 μg) were transiently transfected into HeLa cells in the presence of 0.5 μg of the human β-globin gene (βglob) cotransfection control. The RNA was harvested after 30 hr and mapped by the RNAase protection assay using the probes shown in Figure 2. Lane Pure, RNA from cells transfected with CsCl gradient purified DNA; lane tRNA, hybridization of the probes to tRNA; lane Control, RNA from mock-transfected HeLa cells; lane K562, hybridization of the probes to K562 RNA as a positive control for the α-globin probe.

tion of the methylated CpG pairs in each case is indicated in Figure 8A. When added as competitors to the bandshift reaction using methylated G11 as probe in a HeLa extract, it was found that the nonmethylated and HpaII methylated constructs competed indetectably for binding of MeCP-1. Increased levels of methylation at HhaI sites or HpaII + HhaI sites gave strongly competing molecules, and methylation of all CpGs gave a molecule that competed still more strongly for MeCP-1 binding (Figure 8B). Transfection of these molecules into HeLa cells showed that expression of the variously methylated forms matched exactly their affinity for MeCP-1. Nonmethylated and HpaII-methylated genes were expressed at the same high level; methylation of HhaI sites and HpaII + HhaI sites caused strong inhibition although a small amount of expression was detected; and methylation of all sites effectively abolished expression (Figure 8C). The close correspondence between MeCP-1 affinity and degree of transcriptional inhibition is most easily explained if MeCP-1 is the agent of repression in vivo.
gene. In the final series of experiments to test if MeCP-1 could not be able to repress the transcription of a methylated site.

Some upon transfection with potentially strong binding sites.

The cells, the pool of unbound MeCP-1 (or other inhibitor) was prepared after 30 hr and mapped using the RNAase protection assay. Either no competitor DNA (--), 10 μg of unmethylated competitor (Mlys), or 10 μg of methylated competitor (MeMlys) was added. Cytoplasmic RNA was prepared after 30 hr and mapped using the RNAase protection probes shown in Figure 2. Lanes 1, C, and K are as for Figure 2.

The second approach to determine if MeCP-1 was responsible for the inhibition in vivo derived from the finding that addition of methylated competitors to in vitro transcription extracts could remove the inhibition of transcription from a methylated template (see above). We attempted to achieve the same removal of inhibition in vivo by cotransfecting methylated constructs with the methylated pMlys or pCG11 competitors. Methylated and nonmethylated p3En-glob constructs were transiently transfected into HeLa cells together with a 10-fold excess of the methylated or nonmethylated competitors. In this experiment, α-glob was methylated with the bacterial methyltransferases Hpall and Hhal, which gave the level of methylation seen in Figure 8A. Both competitor plasmids, pMlys (Figure 9) and pCG11 (not shown), when methylated, could alleviate the inhibition of the methylated α-globin. Although reactivation was reproducibly observed, the extent of the phenomenon was variable between experiments and was not always as complete as seen in Figure 9. Moreover, it was not possible to reactivate a fully methylated α-globin template by cotransfection with these competitors. This may mean that there is some "direct" component to the inhibition seen in vivo. Alternatively, it may be explained by a number of unknown parameters in the in vivo system, in particular, the amount of competitor that actually enters the cells, the pool of unbound MeCP-1 (or other inhibitor) present in the cells, and the ability of MeCP-1 to redistribute from some of its weaker binding sites on the chromosome upon transfection with potentially strong binding sites.

If MeCP-1 mediates repression of methylated genes, we predicted that F9 cells, like F9 extracts (see above), would not be able to repress the transcription of a methylated gene. In the final series of experiments to test if MeCP-1 is responsible for the inhibition in vivo, we transfected methylated and nonmethylated tkneo constructs into F9 cells and analyzed the resulting cytoplasmic RNA after 30 hr by the RNAase protection assay. Control transfections into NIH 3T3 cells showed that, as in HeLa cells, methylating effectively abolishes expression. In F9 cells, however, significant levels of expression from the methylated tkneo gene were seen (Figure 10A). Analysis of the RNAase protection pattern showed that transcription initiated at four sites in the control nonmethylated construct. Initiation at three of these sites was reproducibly observed for the methylated plasmid at about 50% of the level of the unmethylated plasmid. Initiation at the fourth start site was absent in transcripts from the methylated construct in F9 cells. We do not know why one start site is affected in this way, but it is possible that direct interference with transcription factor binding is involved. The major conclusion of the experiment, however, is that the dramatic inhibition of transcription seen in fibroblasts is not seen in F9 cells, as predicted if MeCP-1 is normally involved in repression of methylated genes.

As a control for possible loss of methylation from the tkneo gene during the experiment, DNA was reisolated from a crude nuclear preparation from transfected cells and challenged with Hpall and Hhal (Figure 10B). The reisolated construct remained fully resistant to digestion. This result argues against significant demethylation of tkneo during the experiment, but it cannot be considered conclusive, as the amount of DNA involved in transcription is unknown and may be small. Site-specific demethylation in a very small proportion of transfected DNA molecules would not have been detected. It will be recalled, however, that F9 cell-free extracts were also able to transcribe fully methylated constructs and did not demethylate them in order to do so (see Figure 7B).

Discussion

Using different promoters, we have demonstrated that inhibition of transcription by DNA methylation is an indirect effect. The evidence for this came partly from in vitro transcription experiments in which transcriptional inhibition was seen at low template concentrations, but could be overcome either by increasing the concentration of methylated template or by addition of methylated competitor DNA. The latter result is as expected if the competitor DNA mops up the mediator of inhibition leaving the template free to interact with the transcription machinery. Strikingly, the level of transcription from methylated templates in the presence of methylated competitors was virtually the same as that from the unmethylated templates with three of the four constructs. In the fourth case a 50% reduction in transcription persisted in the presence of competitor. Taken together these results suggest that any direct effect of CpG methylation on the repression of transcription is relatively small.

Our ability to obtain inhibition of transcription in nuclear extracts allowed identification of the kind of molecule that mediates indirect inhibition. The properties of the mediator were indistinguishable from those of a previously identified
methyl-CpG binding protein MeCP-1 (Meehan et al., 1989), since only strong ligands for MeCP-1 acted as competitors for the indirect inhibition; nonligands did not. Furthermore, extracts from cells deficient in MeCP-1 were unable to efficiently repress transcription from methylated DNA. It is notable that the repression of methylated genes in vitro was rapid (much less than 1 hr) in an extract that contained very low amounts of histone. This contrasts with the delayed onset and chromatin dependence of inhibition reported by Buschhausen et al. (1987). We cannot at present explain this difference, but it may be related to the comparatively low levels of methylation applied to the tk constructs in the earlier in vivo analysis.

Three sets of experiments indicated that MeCP-1 may also be responsible for the methylation-mediated repression of transcription in vivo. First, the binding affinity of MeCP-1 for increasingly methylated forms of the human α-globin construct was found to match exactly the level of repression of these forms upon transient transfection. Second, it was possible to alleviate to some extent the in vivo repression of a partially methylated construct by cotransfection with methylated constructs. Finally, transfection into F9 cells, which are deficient in MeCP-1, showed reduced inhibition of transcription from methylated constructs. While none of these experiments alone constitutes proof that MeCP-1 is responsible for the inhibition in the living cell, taken together they strongly imply that MeCP-1 is involved in the repression.

Methylated DNA in the nucleus has been shown to have a reduced accessibility to nucleases. Indeed, the nonviral...
promoters used in this study, PGK and α-globin, have both been found to adopt an altered chromatin structure when methylated. Both genes have CpG islands and so are normally nonmethylated in cells of the animal. In the case of PGK, however, the island does become methylated on the inactive X chromosome (Hansen et al., 1988), and the α-globin gene has been found to become methylated in permanent cell lines (Antequera et al., 1990). In their methylated form, these genes were shown to become resistant to the methyl-insensitive restriction endonuclease MspI, implying that methylated sites in the nucleus are made inaccessible by association with nuclear proteins. Since, when methylated, transcription from these promoters also seems to be inhibited by the binding of a nuclear protein, it is possible that MeCP-1 (or similar protein) may be involved in maintaining both the inaccessibility and gene inactivity at methylated CpG islands.

Can MeCP-1 account for the inhibition of all methylated promoters? The promoters in this study are all CpG rich and therefore would provide strong ligands for MeCP-1. The role of MeCP-1 in repression of promoters that are not CpG rich is less certain. There are examples of genes that are repressed by the presence of only one or a small number of CpGs (Benson and Jiricny, 1988, Langner et al., 1984), and it seems unlikely that these are strong ligands for MeCP-1. It is, however, conceivable that MeCP-1 may also be responsible for the repression of these genes. For example, small numbers of methyl groups that have no detectable affinity for MeCP-1 in vitro may bind sufficiently strongly when integrated into the chromosome to prevent transcription. In this context, it is of interest that the inhibitory effects of methylation on transcription are not always equally strong. Genes with few methyl-CpGs can be reactivated in the presence of strong trans activators (Weisshaar et al., 1988; Bednarik et al., 1990), while genes with a high density of methyl-CpGs, such as methylated CpG islands, are stably locked into an inactive state. It is possible that this difference reflects differing affinities of the promoters for MeCP-1. Strong promoters may be able to overcome the weak binding of MeCP-1 to a poorly methylated gene, allowing some flexibility in the repression of these genes. When the gene is densely methylated, however, strong promoters may not prevail against the tight complex formed with MeCP-1.

Experimental Procedures

Vectors and Methylation

The plasmid PCMVjun was a gift from Frank Hilberg and had the PCMV enhancer/promoter region (Hilberg et al., 1987) from -340 to +30 linked to the human c-jun cDNA from positions +740 to +2365. This, in turn, was linked to an SV40 splice site/poly(A) tail. The plasmid tkneo is the same as pMC1 neo (Thomas and Capecchi, 1987) and was derived from a human α-globin genomic clone from positions -572 to +932. A 500 bp region of the human α-globin gene from positions +42 to +1502. A 500 bp region of the P-globin gene has been found to become methylated in transformed cells of the Sacl/Sall sites in the promoter of OVEC (Westin et al., 1987). The latter vector consists of the rabbit β-globin gene deleted of all upstream elements beyond the TATA box; an SV40 enhancer is at the 3' end of the gene. The PCMV enhancer/promoter was selected from a mutant mouse myeloproliferative sarcoma virus for its ability to be expressed in early embryonic cells (Hilberg et al., 1987); the plasmid PCMVvec was therefore used as the cotransfection control in F9 cells. The adenovirus major late construct, the control plasmid for the in vitro transcription, is the packaging plasmid described by Haiermann and Pongs (1985) and has a 3' deleted adenovirus 2 major late promoter cloned into pAT153.

The plasmids used to generate the antisense RNAase protection probes were constructed by cloning the following fragments downstream of the T7 promoter in pSP6B in an inverted orientation. T7PGKvlun (a gift from Frank Hilberg): a 517 bp fragment from PCMVjun, extending from -158 to -158 in the PCMV enhancer/promoter to 326 bp within the c-jun cDNA; this gives a region complementary to the transcribed RNA of 364 bp. T7tkneo: a 240 bp fragment from -78 in the tk promoter to 144 bp within the neo gene to give a region that is antisense to transcribed RNA of 162 bp. T7α-glob: a 244 bp fragment from -86 to +158 of the α-globin gene; the latter site falls 27 bp into intron 1 of the α-globin gene and so the region complementary to processed RNA is 131 bp. T7PGKY: a 510 bp fragment extending from -400 of the P-glob promoter (the most upstream start site is counted as +1 in this case) to 10 bp within the promoterless β-globin insert in PGKv. Depending on the start site used, fragments complementary to transcribed RNA of 100, 90, and 65 bp are obtained. T7β-glob: a 375 bp fragment from -267 to +108 of the β-globin gene; the latter site falls 27 bp into intron 1 of the β-globin gene and so the region complementary to processed RNA is 131 bp. T7ovec: a 223 bp fragment extending from the Sacl site at the 3' end of the inserted PCMV promoter to 186 bp within the rabbit β-globin gene. Since the PCMV enhancer/promoter overrides the rabbit β-globin promoter, the entire 223 bp is protected in RNAase mapping (Figure 10A).

Methyltransferases Sssl, HpaII, and HhaI were obtained from New England Biolabs. Prior to its commercial availability, samples of Sssl methyltransferase were kindly given to us by Aharon Razin. Methylation by Sssl was for 12 hr in 10 mM Tris (pH 7.9), 10 mM MgCl₂, 1 mM dithiothreitol, and 60 μM S-adenosylmethionine at 30°C with 10% of the number of the recommended units of Sssl methyltransferase. Completion was checked by digestion with an enzyme that linearized the plasmid and then by resistance of the plasmids to the methylnsensitive restriction enzymes HpaII and HhaI. Only plasmids that showed complete resistance were used as methylated constructs. Mock methylations were carried out in parallel but in the absence of methyltransferase. HpaII and HhaI methylations were carried out according to the manufacturer's instructions.

Cell Culture and Transfections

HeLa and NIH 3T3 tk- cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum; F9 cells were grown in DMEM supplemented with 10% fetal calf serum and 0.05% NaCl. Transfections were performed using the calcium phosphate method (Fraiman and van der Eb, 1973) using (unless otherwise indicated) 8 μg of test plasmid per transfection with either 0.5 μg of p-globin (for HeLa and NIH 3T3) or 2 μg of PCMVvec (for F9) as the cotransfection control. The total amount of DNA was made up to 10 μg using pUC19 as carrier. Glycerol shocks were performed for HeLa and NIH 3T3 cells. The cells were harvested 30 hr after transfection, and cytoplasmic RNA was prepared according to the method of Gilman (1987).
RNAse Protection Assay
Hessical plasmid DNA from the transient transfections was removed by treatment with RNAse-free DNase. Antisense radiolabeled RNA probes were generated by linearizing the T7 plasmids described above at a site beyond the cloned insert and transcribing them with T7 RNA polymerase as described in Cotten et al. (1989). Hybridization and digestion conditions were those described in Filman (1987b). The products were phenol–chloroform extracted, ethanol precipitated, redissolved in 80% formamide loading buffer, and separated on 7% polyacrylamide–urea gels.

In Vitro Transcription Assay and S1 Analysis
Nuclear extracts were prepared according to the method of Dignam et al. (1983) and in vitro transcriptions were essentially performed as described by West et al. (1987) except that the final reaction volume was 20 μl and the total DNA concentration was standardized to 300 ng with pUC19 in all reactions. The amount of protein was also standardized to 120 μg unless otherwise indicated. The reactions were allowed to proceed for 1 hr at 30°C and then stopped by addition of RNAse-free DNase. Following proteinase K treatment, phenol–chloroform extraction, and ethanol precipitation, the products were mapped using quantitative S1 analysis. S1 hybridization was carried out as for the RNAse protection (above), and digestion was carried out in a final volume of 370 μl at 200 mM NaCl, 30 mM NaOAc, and 2 mM ZnSO4 (pH 4.6) with 225 U of enzyme for 1 hr at 22°C. Products were analyzed on 4% (PCMVjun; tkneo) or 10% (α-glob; PGK) acrylamide–urea gels.

Oligonucleotides and Bandshift Assay
The oligonucleotides CGII, ED89, and W17 have already been described (Meehan et al., 1989) and have the following sequences:

CGI 1, GTACCCAAGCGCGCGCTGGCGCCCGGGCCGGCTCCC
CGII, CGGGCGTCACGGCGAT; W17, CGGGCGTCACGGCGAT.

The HeLa and F9 extracts were those prepared for the in vitro transcription assays. The bandshift assay was performed as described by Meehan et al. (1989) except that (unless otherwise indicated) 5 μg of nuclear extract was used. Electrophoresis was carried out at 4°C on a 1.5% agarose gel in 0.5 x TEE.

Preparation of DNA from Transfected Cells
DNA and RNA were prepared from the same transfected cells: the cells were washed three times in PBS, harvested, and then lysed in an ice-cold solution of 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl2, 0.5% NP-40. Following incubation on ice for 5 min, the cells were spun at 14,000 rpm in an Eppendorf centrifuge at 4°C for 2 min. RNA was prepared from the supernatant (see above), and DNA was prepared from the pellet by resuspending it in 400 μl of a solution: 100 mM Tris–HCl (pH 8.0), 150 mM NaCl, 20 mM EDTA. SDS and protease K were added to final concentrations of 0.5% and 0.5 mg/ml, respectively, and the reaction incubated at 37°C overnight with shaking. The following day the reactions were extracted twice with phenol and once with chloroform and ethanol precipitated. RNA was removed by treatment with RNAsa A, and this latter protein was removed by a second treatment with protease K. The preparation was then extracted twice with phenol, once with chloroform, ethanol precipitated, redissolved in TE, and then used for Southern analysis.

Southern Blotting
Five nanograms of plasmid DNA or 10 μg of DNA recovered from transfected or mock-transfected F9 cells was digested and electrophoresed on 0.7% agarose gels.Restriction fragments were blotted onto GeneScreen membrane and hybridized at 85°C in 0.5 M Na2HPO4, 7% SDS, and 1 mM EDTA. The probe was labeled according to the random priming method (Feinberg and Vogelstein, 1984). The filter was washed at 65°C in 40 mM Na2HPO4, (pH 7.9) and 1% SDS.

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