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Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein

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Repression of transcription from densely methylated genes can be mediated by the methyl-CpG binding protein MeCP-1 (Boyes and Bird, 1991). Here we have investigated the effect of methylation on genes with a low density of methyl-CpG. We found that sparse methylation could repress transfected genes completely, but the inhibition was fully overcome by the presence in *cis* of an SV40 enhancer. Densely methylated genes, however, could not be reactivated by the enhancer. *In vitro* studies showed that the sparsely methylated genes bound weakly to MeCP-1 and that binding interfered with transcription. In the absence of available MeCP-1, methylation had minimal effects on transcription. From these and other results we propose that sparsely methylated genes form an unstable complex with MeCP-1 which prevents transcription when the promoter is weak. This complex can be disrupted by a strong promoter, thereby allowing the methylated gene to be transcribed.

Key words: CpG density/DNA methylation/MeCP-1/transcription repression

Introduction

Transcription of genes by RNA polymerase II can be inhibited by methylation at CpG dinucleotides. For example, CpG methylation contributes to the stable repression of genes on the inactive X chromosome of mammalian females (reviewed by Grant and Chapman, 1988) and also to the repression of many genes in cultured cell lines (Harris, 1982; Holliday, 1987; Antequera *et al.*, 1990). The promoters which are strongly repressed in these cases have many CpGs since they are within CpG islands. The latter are stretches of DNA which are normally free of methylation (apart from the exceptional cases mentioned above) and are therefore exempt from the depletion of CpG which affects the vast majority of the mammalian genome (Bird *et al.*, 1985; Bird, 1986). We have presented evidence that the inactivation of CpG island-associated genes by DNA methylation is mediated by the methyl-CpG binding protein, MeCP-1 (Boyes and Bird, 1991). This protein binds to DNA containing multiple symmetrically methylated CpGs (Meehan *et al.*, 1989) and is thought to cause transcriptional repression by excluding transcription factors, since addition of excess methylated competitor DNA reactivates methylated genes in *in vitro* transcription extracts, and to some extent *in vivo*. Moreover, cells that are depleted in MeCP-1, and extracts derived from them, permit transcription of methylated genes (Boyes and Bird, 1991). Although MeCP-1 has not been tested in a purified form, the weight of evidence suggests that it plays a key role in the suppression of methylated genes.

The experiments implicating MeCP-1 in transcriptional repression involved promoters that are rich in methyl-CpG. The known preference of MeCP-1 for multiple methyl-CpGs on a single DNA molecule fits well with the idea that the protein binds to clustered methylated sites in genes of this kind. What of genes that are not associated with clustered CpGs? The genes in this category (i.e., genes lacking CpG islands) are expressed in a tissue-specific manner, and have an average frequency of one CpG per 100 bp. A number of previous studies have established that DNA methylation can inhibit transcription of CpG-poor genes under certain circumstances. For example, artificial methylation of the human γ-globin (Busslinger *et al.*, 1983), β-globin (Yisraeli *et al.*, 1988) or the rat α-actin gene (Yisraeli *et al.*, 1986), all of which have few CpGs, could inhibit transcription. Similarly, the adenovirus E2A promoter is repressed even when relatively few methyl groups are added, for example by methylating only *Hpa*II (CCGG) sites (Vardimon *et al.*, 1980; Langner *et al.*, 1984). The common feature of these experiments is that inhibition of the sparsely methylated promoters was observed in cell types that express the gene concerned sub-optimally. Contrasting results were obtained when the genes were tested in efficiently expressing cell types. The methylated α-actin gene was activated normally upon myoblast differentiation (Yisraeli *et al.*, 1986) and the methylated E2A promoter was fully active in the presence of the adenovirus transactivator E1A (Langner *et al.*, 1986). The results suggest that low levels of methylation can inhibit transcription only when the promoter is weak.

It was not obvious that MeCP-1 could be responsible for repressing genes with few methyl groups. We had observed, however, that when a CpG-rich gene (α-globin) was partially methylated, a weakened complex with MeCP-1 was formed and inhibition *in vivo* was somewhat less severe (Boyes and Bird, 1991). Significantly, this inhibition could now be overcome by co-transfection of methylated competitor DNA, suggesting that repression of this sparsely methylated promoter was in fact mediated by a methyl-CpG binding protein. We decided therefore to study further the effect of DNA methylation on transcription of CpG-deficient genes, using promoters of different strengths to gauge the severity of repression. The results show that MeCP-1 can interact with sparsely methylated genes, but the complex is weak. This suggests a general explanation for the observation that low levels of methylation repress transcription from a weak promoter, but cannot repress when the promoter is strong.

Results

To study the effect of low density CpG methylation on expression, three different genes were chosen: the human γ-globin gene, the mouse α-globin gene and the human α-globin gene. The density of CpGs in these genes is shown in Figure 1. The two genes without CpG islands, human γ-globin and mouse α-globin, have average CpG densities of 1 per 126 bp (26 CpGs over 3.3 kb) and 1 per 50 bp (28 CpGs over 1.4 kb) respectively. The human α-globin gene, on the other hand, has a high average density of 1 CpG per 10 bp (141 sites over 1.5 kb). This promoter can, however, be methylated to a lower density of 1 methyl-CpG per 62 bp (24 methyl CpGs over 1.5 kb) by using the bacterial methyltransferase M.*HhaI*.

To test if methylation can inhibit transcription when the

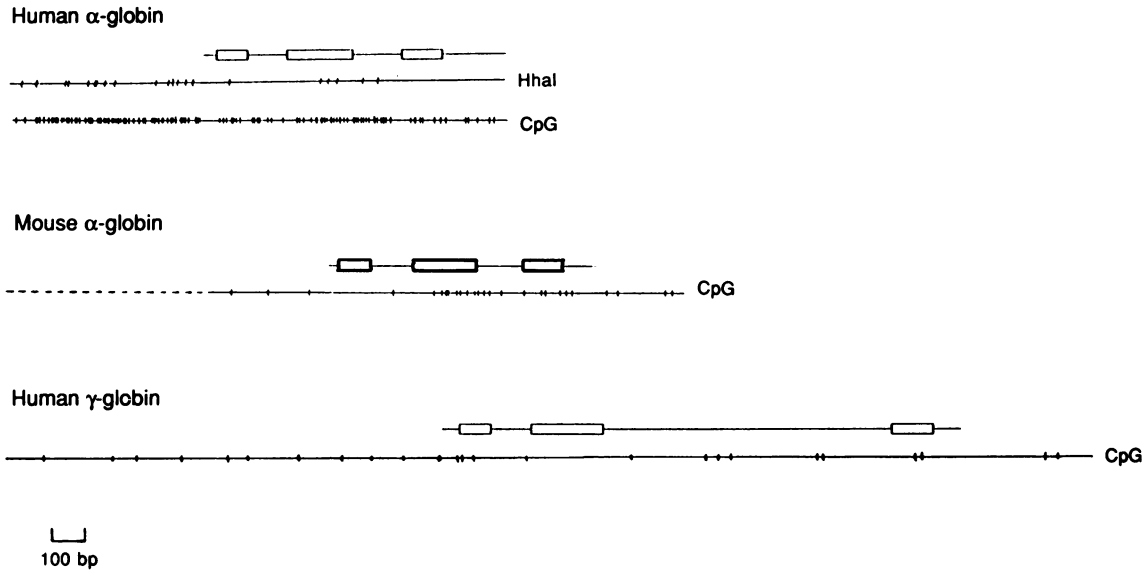

Fig. 1. CpG density of the genes used in this study. The transcribed regions of the three genes are pictured below the title of each gene; the open boxes represent exons and the thin lines represent transcribed but non-translated sequences. The plots below each gene show the CpG dinucleotides which can be methylated by M.*SssI* methyltransferase. In the case of the human α-globin gene the subset of CpGs that can be methylated by M.*HhaI* methyltransferase is also shown. The CpG plots cover the regions of genomic DNA which were subcloned to give the constructs described (see text and Materials and methods). For the mouse α-globin gene, the broken line shows DNA that was part of the construct but whose sequence was not available. Vector sequences are not shown.

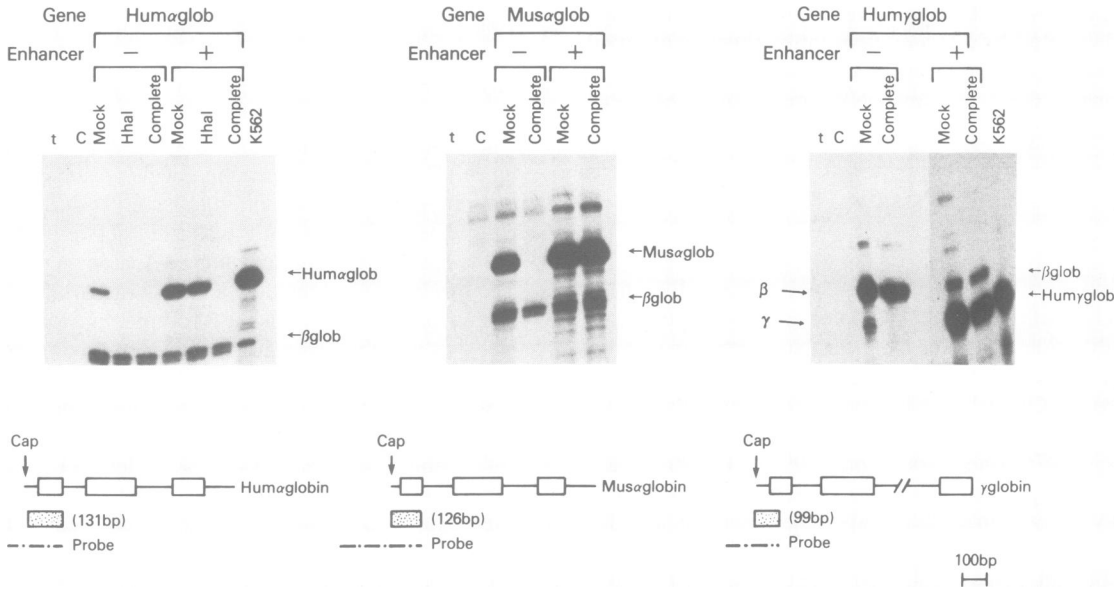

Fig. 2. Effect of low density methylation on transcription in the presence and absence of an enhancer. The human α-globin gene (p3′Eαglob, pαglob), mouse α-globin gene (Mus3′Eαglob2, Musαglob2) and human γ-globin gene (p3′E^{A}γglob, p^{A}γglob) were mock methylated and methylated to completion with M.*SssI* in the presence (+) and absence (−) of the SV40 enhancer. In addition the human α-globin gene constructs were methylated with M.*HhaI* methyltransferase. 2 μg of each of these constructs was then transiently transfected into HeLa cells in the presence of 0.5 μg of the p3′Eβglob construct as a cotransfection control. The cells were harvested after 30 h and the cytoplasmic RNA was mapped using RNase protection probes shown below the figure. Lanes 't' show hybridization of the probes to tRNA; lanes 'C' show hybridization of the probes to RNA from mock transfected HeLa cells. Lane K562 shows hybridization of K562 RNA with the human α- and γ-globin probes for which it acts as a positive control. The figure is a composite of different exposures of the same autoradiograph, the last three lanes are from the shorter exposure.

number of modified CpGs in the promoter region is low, constructs containing the human γ-globin ($p^A\gamma glob$) and mouse α-globin (Musαglob2) genes were methylated to completion using *SssI* methyltransferase (M.*SssI*) and the human α-globin gene (pαglob) was methylated with *HhaI* methyltransferase (M.*HhaI*). As a control, pα-glob was methylated at all CpGs with M.*SssI*. The methylated and non-methylated constructs were then transiently transfected into HeLa cells in the presence of the human β-globin gene as a cotransfection control. RNA was prepared from the cells after 30 h and was mapped using the RNase protection assay. As can be seen in Figure 2 (lanes 'enhancer −'), transcription of all methylated genes was inhibited regardless of methyl-CpG density.

We next tested if the inhibitory effects of low density methylation could be overcome by addition of an enhancer. The SV40 enhancer was therefore placed at the 3′ end of the gene (to give $p3'E^A\gamma glob$, Mus3′Eαglob2 and p3′Eαglob) and the constructs were methylated in the same way. Surprisingly, the fully methylated γ-globin, mouse α-globin and M.*HhaI*-methylated human α-globin genes were now transcribed at the same level as non-methylated controls upon transient transfection (Figure 2, lanes 'enhancer +'). Thus, the inhibitory effects of methylation could be overcome for three different promoters by the presence of the SV40 enhancer. This was not the case, however, for the completely methylated human α-globin construct, which remained fully repressed in the presence of the SV40 enhancer. This last result was not due to inactivation of the enhancer by complete methylation of CpGs since there are no methylatable CpG dinucleotides in the SV40 enhancer sequence. Furthermore, complete methylation of the γ-globin and mouse α-globin constructs with the same methyltransferase clearly did not interfere with the function of the enhancer. If the enhancer had been inactivated in these cases, the level of transcription would not have been higher than the enhancerless non-methylated controls (3- to 10-fold higher in the case of the mouse α-globin gene, and 20- to 50-fold higher in the case of the γ-globin gene). Thus when the density of methyl-CpGs is high, the α-globin promoter remains repressed even when a functional enhancer is present.

The above phenomena could be explained by a simple hypothesis. Sparsely methylated genes may bind weakly to MeCP-1 to give a complex that will suppress transcription when the promoter is weak (i.e. enhancerless), but not when the promoter is strong. Densely methylated genes, on the other hand (for example, the human α-globin promoter methylated with M.*SssI*), may form a strong complex with MeCP-1 which cannot be disrupted even by a strong promoter. The hypothesis predicts that the sparsely methylated test genes will have a relatively low affinity for MeCP-1. To test this, we compared the ability of the various methylated templates (in the absence of plasmid sequences) to compete with the radioactively labelled probe MeCG11 for binding to MeCP-1 (Figure 3). Addition of non-methylated competitor DNA to the incubation mix had no effect on the amount of complex (lanes '−' and 'Mock'), whereas addition of the fully methylated α-globin gene abolished the complex (lane 'complete'). Competition with the three sparsely methylated genes reduced the level of complex significantly, but did not abolish it. These constructs therefore displayed a lower affinity for MeCP-1 than the fully methylated α-globin construct, as required by the hypothesis. It is of interest that the M.*HpaII*-methylated gene (lane HpaII) did not compete at all for MeCP-1 binding, and has been shown previously to be expressed at the same level as the non-methylated gene (Boyes and Bird, 1991).

The hypothesis depended on the idea that repression of the methylated constructs is mediated by MeCP-1. In the case of the partially methylated human α-globin gene, previous work has provided support for this view (Boyes and Bird, 1991). It was shown that the affinity of the M.*HhaI* plus *M.HpaII*-methylated gene for MeCP-1 closely matched the level of repression seen *in vivo*. Moreover, addition of methylated competitor DNA to cells transfected with the M.*HhaI*-methylated gene could alleviate repression. In order to determine if the inhibition of the mouse α-globin and human γ-globin promoters is also consistent with MeCP-1-mediated repression, we assayed transcription of methylated and non-methylated gene constructs in an *in vitro* transcription extract. Transcription of the fully methylated mouse α-globin gene was inhibited at low concentrations of added template (3 ng; Figure 4A). This inhibition could be overcome by adding more template (Figure 4A), or by adding methylated DNA of unrelated sequence (Figure 4B) suggesting that the mediator of repression is present in limiting amounts in the extract. The results parallel those obtained previously with CpG-rich promoters (Boyes and Bird, 1991), and they likewise implicate MeCP-1 in the inhibition of transcription from this gene in extracts. In the absence of MeCP-1 (i.e. in the presence of high concentra-

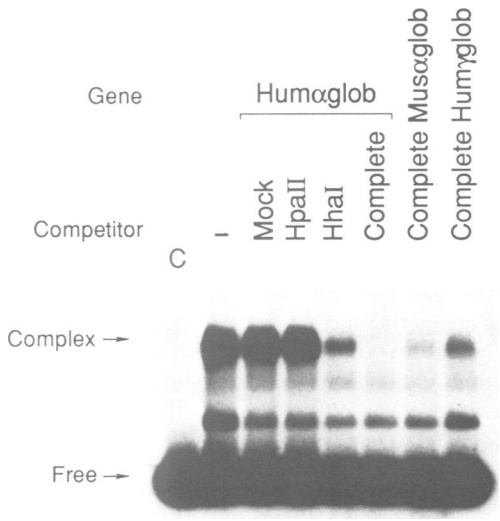

Fig. 3. Promoters with a low density of methyl-CpGs bind MeCP-1 less strongly than those with a high density of methyl-CpGs. 0.1 ng of the methylated oligonucleotide probe MeCG11 was incubated with 5 μg of HeLa cell nuclear extract in the presence of 2 μg of *Micrococcus lysodeikticus* non-methylated competitor DNA to give the bandshift shown (−). The gene inserts (300 ng), of the human α-, mouse α- and human γ-globin genes, methylated as shown, were isolated and tested for their abilty to compete for this bandshift. The mock methylated form of the human α-globin gene was included as a control. The M.*Hpa*II methylated human α-globin gene has been shown previously to have no affinity for MeCP-1 (Boyes and Bird, 1991) and was also included as a control. Lane C shows the probe incubated in the absence of extract. The major slow migrating complex was absent when the same probe was used in a non-methylated form (not shown; see Boyes and Bird, 1991). The fast-migrating complex that is common to all extract-containing lanes is non-specific, as it was also seen with non-methylated DNA probe (data not shown).

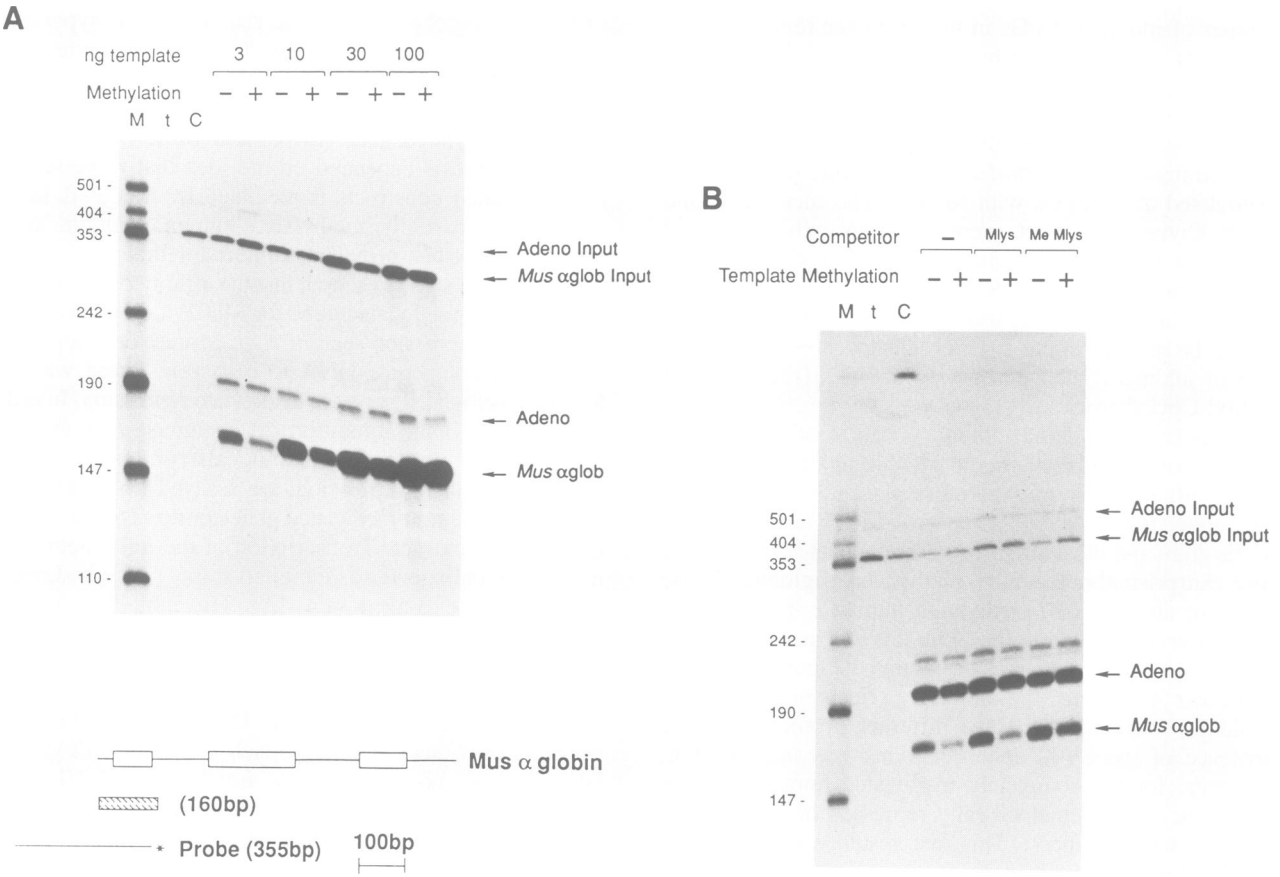

Fig. 4. Inhibition of transcription *in vitro* by methylation of the mouse α-globin gene. Inhibition of mouse α-globin gene transcription can be overcome by increased concentrations of template or addition of methylated competitor DNA. (**A**) Methylated (+) or non-methylated (−) Musαglob2 constructs were transcribed in HeLa nuclear extracts at the amounts in ng shown above each pair of lanes. The adenovirus major late promoter construct was included in all reactions as a non-methylated control and the transcripts were mapped with the S1 probes shown below the figure (see Materials and methods). The mouse α-globin S1 probe maps to within the first intron of the gene and, since splicing reactions do not occur efficiently *in vitro* in nuclear extracts alone (Hernandez and Keller, 1983), gives a protected species of 160 bp. Lane M shows pUC19 DNA cut with *Hpa*II as a size marker. Lanes C and t show an S1 analysis of an *in vitro* transcription performed in the absence of DNA, and of purified tRNA respectively. Identical results to the above were obtained with the SV40 enhancer-containing construct (not shown). (**B**) 3 ng of methylated (+) or non-methylated (−) Musαglob2 constructs were transcribed in HeLa nuclear extracts in the presence of either no added competitor (−), a 30-fold excess of non-methylated competitor (Mlys); or a 30-fold excess of methylated competitor (MeMlys). Control lanes are labelled as in panel A. Identical results were obtained with the Mus3′Eαglob construct.

tions of competing methylated DNA), CpG methylation has a negligible direct effect on the transcription machinery.

Attempts to duplicate these experiments with the γ-globin gene were only partially successful. This gene has the lowest density of methylation among the test genes (see Figure 1), and, even at low concentrations of methylated template, the level of transcription was >70% of the non-methylated control (Figure 5, see quantification below lanes). Nevertheless, slight inhibition was reproducibly seen, and this was overcome at high template concentrations. Thus only weak indirect (MeCP-1 mediated) effects could be detected. Once again, the full expression of the gene at high template concentrations indicates that there is no significant direct effect of complete CpG methylation on transcription *in vitro*. Fortunately, in the case of γ-globin the basis of inhibition has also been studied *in vivo* (Murray and Grosveld, 1987). Mutagenesis of CpGs near the promoter suggested that no specific methyl-CpGs were responsible for repression. The mere presence of methylated sites near the promoter seemed to be sufficient to block transcription, regardless of their position. These results are compatible with MeCP-1-mediated repression, but not with a direct mechanism of repression.

Discussion

Our results confirm that transcription can be inhibited by methylation of relatively few CpGs, and suggest that this inhibition is not due to direct interference of methylation with the binding of transcription factors. Instead we find that all three methylated genes can complex with the protein MeCP-1, leading to impaired transcription. The complexes are less stable than those with densely methylated DNA as judged by bandshift competition experiments, and the inhibition of transcription *in vitro* is correspondingly weaker (being <30% in the case of human γ-globin). The data are compatible with the hypothesis that MeCP-1 or a similar protein is responsible for the observed repression of sparsely methylated genes. Other data in the literature are also compatible with this hypothesis. In particular, *in vivo* studies have already suggested that two of the genes that we studied are inhibited indirectly (see Results). Thus, the partially methylated human α-globin gene can be reactivated by cotransfection of heavily methylated competitor DNA (Boyes and Bird, 1991) and the γ-globin gene is inactivated by CpG methylation in the promoter region regardless of the precise position of the methylated sites (Murray and Grosveld, 1987). Neither of these results would be expected if site-

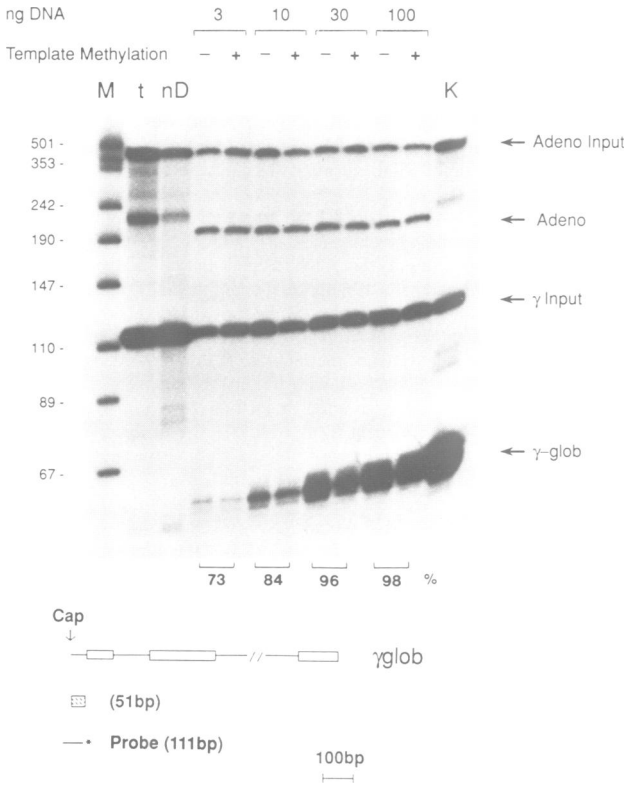

Fig. 5. Weak inhibition of transcription *in vitro* from the methylated human γ-globin gene at low concentrations of template. Methylated (+) and non-methylated (−) forms of the p3′E^{A}γglob were transcribed in HeLa cell nuclear extracts at the amounts shown in ng above each pair of lanes. The products were mapped by the S1 probes shown. RNA from the K562 cell line was used as a control for this probe (lane K), since these cells express the γ-globin gene constitutively. Control lanes are labelled as in Figure 4A. Numbers below the lanes show quantification of the level of expression of the methylated human γ-globin gene compared with the non-methylated gene. An autoradiograph of an equivalent experiment to that shown was scanned by densitometry (Materials and methods) and the areas under the peaks were measured. The non-methylated γ-globin signal was divided by the control adenovirus major late signal at each DNA concentration and this normalized value was taken as 100% expression. The equivalent ratio for methylated γ-globin was determined in the same way. The ratio of these two normalized values at each DNA concentration gives the per cent expression of the methylated construct relative to the non-methylated construct at this DNA concentration.

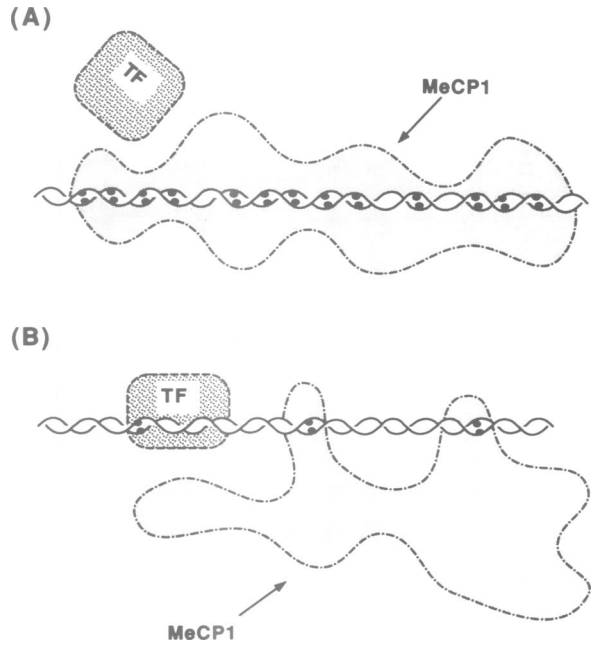

Fig. 6. A schematic interpretation of the interactions between MeCP-1 and regions of high or low density methyl-CpG, and their effects on transcription. (**A**) When many methyl groups are locally concentrated in the molecule, the sum of multiple weak interactions with MeCP-1 gives strong overall binding which prevents access to transcription factors. (**B**) When only a few methyl-CpGs are present, the DNA is weakly complexed with MeCP-1, and allows access to transcription factors with a high affinity for the DNA sequence. Assembly of the transcription machinery can lead to displacement of MeCP-1. Factors with a low affinity for the promoter are thought to be unable to displace the complex with MeCP-1. MeCP-1 is drawn as a large structure due to its high apparent molecular weight on gel filtration columns (~800 kDa; Meehan *et al.*, 1989). TF represents a transcription factor.

specific methylation was interfering with the binding of transcription factors to the promoter.

The results also show that genes with a low density of methyl-CpG are repressed in the absence of an enhancer, but are fully active when an SV40 enhancer is present in *cis*. A more densely methylated gene, however, could not be reactivated even in the presence of an enhancer. Parallel with these results, it has been shown previously that the partially methylated human α-globin gene can be reactivated *in vivo* by co-transfection with methylated competitor DNA, whereas the fully methylated gene cannot be activated by this method (Boyes and Bird, 1991; Boyes, 1991). Thus, by two independent criteria, the intensity of repression is proportional to the density of methylation.

The ability of poorly methylated genes to overcome repression when the strength of the promoter is increased has been reported in several systems. For example, the E2A promoter of adenovirus when methylated at *Hpa*II sites and introduced into cells is transcriptionally inert (Langner *et al.*, 1984), but in the presence of either the transactivator E1A or the cytomegalovirus enhancer it is transcribed normally (Langner *et al.*, 1986; Knebel-Morsdorf *et al.*, 1988; Weisshaar *et al.*, 1988). Similarly, the long terminal repeat (LTR) of HIV is inactivated by *Hpa*II site methylation, but is reactivated in the presence of the transactivator *tat* (Bednarik *et al.*, 1990). In both of these cases the promoter is still methylated at the time of activation. Analogous results have been obtained for tissue-specific genes that are activated during differentiation. Methylation of the rat muscle α-actin gene at *Hpa*II sites represses transcription in fibroblasts, but has no effect on the high level of transcription which occurs in differentiating myoblasts (Yisraeli *et al.*, 1986), presumably under the influence of stronger transcriptional activators. Promoter efficiency may also explain reports that several methylated, CpG-deficient genes can be transcribed at high rates while in the methylated state. For example, the genes for chicken δ-crystallin (Grainger *et al.*, 1983), chicken vitellogenin (Wilks *et al.*, 1984) and mouse IgG κ light chain (Kelley *et al.*, 1988) are all transcribed while methylated, as expected if strong promoters are overriding the effects of methylation. Since all of these genes are CpG-deficient, the density of CpG methylation is necessarily low. Interestingly, expression of the methylated IgG κ chain gene is enhancer-dependent in pre-B cells, but upon differentiation to mature B cells, the gene loses its methylation, and expression is no longer enhancer-dependent (Kelley

et al., 1988). The parallel between these results and those shown in Figure 2 is striking.

The molecular mechanism by which strong promoters can prevail over methylation is unknown. One explanation relies on the idea that transcription is inhibited by direct interference of specific methyl-CpGs with the binding of transcription factors. According to this view, the block is overcome by the appearance during development of tissue-specific factors that are not sensitive to methylation (discussed by Cedar, 1988). In the case of the rat α-actin gene, the binding of such factors apparently leads to a demethylation event, without which the gene cannot be activated (Paroush *et al.*, 1990). Our results lead us to propose an alternative explanation based on MeCP-1 (see Figure 6). Since the stability of MeCP-1 complexes with DNA is proportional to the local density of methyl-CpGs (Meehan *et al.*, 1989), sparsely methylated genes would form a 'weak' complex (Figure 6B). We suggest that this complex is enough to prevent transcription when driven by a weak promoter, but would be disrupted by strong promoters, thus allowing normal rates of transcription. Conversely, genes that are densely methylated would form a tight complex with MeCP-1 which would resist both weak and strong promoter activity (Figure 6A). Therefore the activation of sparsely methylated genes during development need not depend on the appearance of methyl-insensitive factors. Rather, activation would result from the appearance of factors capable of displacing the weak complex with MeCP-1, for example by their higher affinity for promoter sequences. Implicit in the model is the idea that direct effects of DNA methylation on transcription factors are minor in comparison to the indirect effect mediated by MeCP-1. Evidence for this view has come from our work with six different promoters (Boyes and Bird, 1991; and this work) which has consistently failed to show a major inhibitory effect of DNA methylation on transcription unless free MeCP-1 is available.

In summary, we suggest that many of the observed effects of DNA methylation on transcription are consistent with the biochemical properties of MeCP-1. The affinity of MeCP-1 for methylated DNA depends upon the local concentration of methyl-CpGs. Since CpG is deficient in bulk mammalian DNA, it follows that binding to MeCP-1 in most of the genome is weak. Many tissue-specific genes are within this bulk CpG-deficient DNA, and the repressive effect of methylation on their transcription is therefore correspondingly weak. MeCP-1 mediated repression may be sufficient to prevent spurious transcription in inappropriate tissues, but is irrelevant in cells where the promoter is transcribed at maximum efficiency. On the other hand, strong repression by MeCP-1 will occur when methylated DNA is not CpG-deficient. Examples include methylated CpG islands on the inactivated X chromosome and in cultured cells, and certain viral genomes that are CpG-rich. According to this picture, methylation-mediated repression of genes is a function of the density of methyl-CpGs, their location relative to the promoter and promoter strength. By taking account of these variables, we are able to explain the apparently variable effects of DNA methylation on transcription.

Materials and methods

Vectors and methylation

The plasmid pα-glob has been described (Boyes and Bird, 1991) and has a 1.5 kb region of the human α-globin genomic clone from positions −572 to +932 inserted into the polylinker of pUC19. The plasmid p3′Eαglob has the same region inserted into pSP64; in addition, a 196 bp fragment of the SV40 enhancer was cloned into the polylinker at the 3′ end of the α-globin gene. The plasmid Musαglob2 has a 2.2 kb region of the mouse α-globin genomic clone (a gift from Philip Leder) extending from positions −1000 to +1000 cloned into pUC18. The plasmid Mus3′Eαglob2 has the same region of the mouse α-globin genomic clone inserted into pSP64; a 196 bp fragment of the SV40 enhancer was cloned 3′ to the mouse α-globin insert.

The plasmid p^{A}γglob has a 3.3 kb fragment of the human ^{A}γglobin genomic clone (extending from −1348 to +1950) cloned into pUC18. The plasmid p3′E^{A}γglob (a gift from Giulio Superti-Furga) has the same region cloned into pSP64 and has a 196 bp fragment of the SV40 enhancer cloned at the 3′ end of the gene. The control plasmids, pβglob (Boyes and Bird, 1991) and pAdomal (Heiermann and Pongs, 1985) have been described.

The plasmids used to generate the antisense RNase protection probes were constructed by cloning the following fragments downstream of the T7 promoter in pSPT18 in an inverted orientation.

T7αglob: 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.

T7βglob: a 375 bp fragment from −267 to +108 of the β-globin gene; the region antisense to the β-globin RNA is 108 bp.

T7mαglob: a 355 bp fragment from −195 to +160 of the mouse α-globin gene; the latter site falls 34 bp into intron 1 of the mouse α-globin gene and so the region complementary to processed RNA is 126 bp.

T7γglob: a 152 bp fragment from −53 to +99 of the human ^{A}γglobin gene; the region antisense to RNA is 99 bp.

Constructs were methylated and checked for completion of the reaction as described (Boyes and Bird, 1991).

Cell culture and transfections

HeLa cells were grown in Dulbecco's modified Eagle Medium supplemented with 10% fetal calf serum. Spinner HeLa cells were grown in Joklik medium supplemented with 5% fetal calf serum. Transfections of the adherent HeLa cells were performed using the calcium phosphate method (Graham and van der Eb, 1973) using 2 μg of test plasmid and 0.5 μg of β-globin cotransfection control plasmid per transfection. The total amount of DNA was made up to 10 μg using pUC19 as carrier. Glycerol shocks were performed as described (Gorman, 1985). The cells were harvested after 30 h and cytoplasmic RNA was prepared according to the method of Gilman (1987a).

RNase protection assay

Residual plasmid DNA from the transient transfections was removed by treatment with RNase-free DNase. Antisense labelled 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 Gilman (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 Westin *et al.* (1987) except that the final reaction volume was 20 μl and the total protein concentration was standardized to 10 μg, unless otherwise indicated. The reactions were allowed to proceed for 1 h at 30°C and then stopped by incubation with RNase-free DNase for 15 min. Following subsequent treatment with proteinase K, phenol−chloroform extraction and ethanol precipitation, the products were mapped by quantitative S1 analysis. S1 probes were generated by isolating restriction fragments covering the following regions of the genes: mouse α-globin: −95 to +160; human γ-globin: −60 to +51 and adenovirus major late promoter −253 to +197. The restriction fragments were dephosphorylated with calf intestinal alkaline phosphatase and subsequently radiolabelled using T4 polynucleotide kinase (Boehringer Mannheim) to transfer the terminal phosphate from γ-labelled ATP (Amersham). S1 hybridization was carried out as for the RNase protection (above) and the digestion was carried out in a final volume of 370 μl in 200 mM NaCl, 30 mM NaOAc and 2 mM $ZnSO_4$ (pH 4.6) with 225 U of enzyme for 1 h at 22°C. Products were analysed on 10% (human γ-globin) or 6% (mouse α-globin) polyacrylamide−urea gels.

Quantification of S1 analysis

Autoradiographs were scanned on a Hoeffer Scientific Industries GS300 scanning densitometer and the areas under the peaks integrated for quantification. The percentage of inhibition at each amount of template of

the methylated DNA compared with the non-methylated DNA was then calculated from the relative intensities of the peaks.

Bandshift assay

The HeLa extract was that prepared for the *in vitro* transcription reaction. The bandshift assay was performed as described by Meehan *et al.* (1989) except that 5 μg of nuclear extract protein was used. Electrophoresis was carried out at 4°C on a 1.5% agarose gel in 0.5 × TBE. Competitor fragments were prepared by methylating the test plasmids with either *Sss*I, *Hpa*II or *Hha*I methyltransferases, as shown, and the gene region was excised from the remainder of the plasmid and gel purified. 300 ng of each gene insert were used as competitors for the MeCP-1 specific bandshift.

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