Unmethylated thyroglobulin promoter may be repressed by methylation of flanking DNA sequences

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The thyroglobulin gene, like many other tissue-specific genes, appears to be specifically less methylated in the differentiated cell type where it is transcribed. The thyroglobulin gene promoter elements themselves are highly CG-deficient and do not contain any *HpaII*/*MspI* sites. In this study, using DNA constructs that were methylated *in vitro* with *HpaII* or *MspI* methylases, we show that DNA methylation of vector sequences is sufficient to repress the activity of the thyroglobulin gene promoter in transient transfection experiments. Reporter-gene expression from a plasmid containing only the proximal thyroglobulin gene promoter is sensitive to DNA methylation even in fully differentiated thyrocytes. Transcription from methylated plasmids containing the thyroglobulin gene enhancer and proximal promoter is also clearly reduced when the transfected cells are

maintained under less-differentiated conditions. These results indicate that DNA methylation can influence, from a distance, the activity of an unmodified promoter. Our results also agree with the view that loss of DNA methylation does not constitute a prerequisite for thyroglobulin gene expression in differentiated thyrocytes, where the thyroglobulin gene enhancer and promoter are activated. However, the production of thyroglobulin transcripts could be severely impaired when this activation is not maximal, as is the case in less-differentiated cells or when the enhancer element is lacking. We suggest that DNA methylation helps to maintain the thyroglobulin gene in an inactive state unless all of the conditions required for its expression are fulfilled, and that the thyroid-specific demethylation events are a consequence of the activation state of the gene.

INTRODUCTION

DNA modification by methylation, essentially on cytosine residues within CG dinucleotides, is found throughout most of the vertebrate genome (see [1] for a recent review). Such a modification of the genome appears to be required to sustain normal development of the individual, as targeted mutation of the DNA methyltransferase gene, which encodes the enzyme that maintains this modification of the DNA, causes abnormal development and embryonic death in the mouse [2]. However, although the overall trend supports the view that a high content of methylated bases can be associated with gene silencing, the precise role of DNA methylation in the control of expression of individual genes remains a controversial issue.

Like many other tissue-specific genes, that for thyroglobulin displays a lower content of 5-methylcytosine in thyroid cells which express this gene compared with cells which do not [3]. Moreover, a transgene containing the thyroglobulin gene promoter sequences has been shown to undergo a specific demethylation event in the thyroids of transgenic mice [4]. In both cases, the changes in DNA methylation have been detected by using the *HpaII* and *MspI* isoschizomers. No *HpaII*/*MspI* site was contained in the known regulatory elements of the thyroglobulin gene themselves. As the corresponding methylases were available and as the main regulatory elements of the thyroglobulin gene, the proximal promoter [5] and the enhancer [6], had been cloned, we decided to investigate the role of DNA methylation at *HpaII*/*MspI* sites on the activity of reporter constructs containing these control elements. The transcriptional activities of methylated and unmethylated DNA were assayed by transient transfection in primary-cultured thyrocytes, in which the expression of differentiated functions, including thyroglobulin gene transcription, is under tight control by cyclic AMP [7]. Our results

show that DNA methylation at CG dinucleotides in the flanking sequences may repress the activity of the thyroglobulin gene promoter when it is poorly activated, but that it has little effect on full promoter activity. Interestingly, the effect was thus observed even though methylation did not involve the regulatory elements themselves, which demonstrates that DNA methylation is able to act on the activity of the promoter from a distance.

EXPERIMENTAL

DNA methylations

Standard DNA manipulations were conducted according to published protocols [8].

The DNA constructs used in this work have been described previously [6] (see also Figure 1 of text). The enhancerless construct was pbTgCAT20MO, which contains the 240-bp-long bovine thyroglobulin gene proximal promoter fused to the chloramphenicol *N*-acetyltransferase (CAT) coding sequences; the constructs containing the thyroglobulin gene enhancer were either pbTgCAT20MA, pbTgCAT20MB or pbTgCAT21A (these constructs only differ in the position of the bovine thyroglobulin enhancer element relative to the reporter gene within the plasmid, which are as follows: in 5' from the 20MO fusion and forwards in 20MA, and in the reverse orientation in 20MB, and in 3' from the 20MO fusion in 21A).

In vitro methylations were performed on 15 µg of plasmid DNA, using 40 units of either *HpaII* methylase or *MspI* methylase in the conditions described by the manufacturer (Biolabs). The unmethylated control DNAs were incubated under the same conditions, but without addition of the enzyme. The reactions were terminated by heating at 65 °C for 20 min. The completeness, or the absence (in the controls), of DNA methylation was assayed by digesting 0.3 µg aliquots with the *HpaII* and

Abbreviation used: CAT, chloramphenicol 3-O-acetyltransferase (EC 2.3.1.28).

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MspI restriction enzymes. In all preparations, methylation of the DNA resulted in a complete modification at all *HpaII/MspI* sites. The remaining DNA was then extracted with phenol and ethanol-precipitated.

Transfections and CAT assays

The DNA preparations were used to transfect duplicate dishes (about 5 µg of DNA per dish) of primary-cultured dog thyrocytes as described previously [5]. Aliquots corresponding to about 200 ng of DNA from the individual preparations were analysed on agarose gels to monitor the amount and the quality of the transfected plasmids.

CAT assays were performed 3 days after transfection as described in ref. [6]. In each experiment, serial dilutions of purified CAT enzyme (Pharmacia) were used to determine the linear range of the assay. The background values of the CAT assays (deduced) were in the range of 450–750 c.p.m.

As individual batches of primary-cultured cells were used, each experiment was repeated at least three times to correct for possible batch-to-batch variations. The results shown are the mean values $\pm$ S.D. of the independent experiments.

Analysis of transfected DNA

A particulate fraction was prepared from the cell lysates used to assay CAT activity (see [5]). The crude nuclei were resuspended in 250 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) containing 120 µg of proteinase K and 50 µg of ribonuclease A and digestion was allowed to proceed for 90 min at 37 °C. SDS was then added to a final concentration of 0.2% and incubation was followed at 56 °C for a further 30 min. After extraction with phenol, the DNA was precipitated with ethanol.

The total DNA was restricted with *HpaII* (30 units) and *MspI* (30 units) separately and the fragments corresponding to the transfected sequences were visualized on Southern blots [8], using labelled pbTgCAT20MA plasmid DNA as probe.

RESULTS

Cytosine methylation in the flanking DNA reduces the activity of the transfected thyroglobulin gene promoter

The bovine thyroglobulin gene regulatory elements identified so far exhibit overall a strong CG-suppression and do not contain a single *HpaII/MspI* site [5,6]. To investigate the effect of methylation of the surrounding DNA on thyroglobulin gene promoter function, two constructs, containing either the thyroglobulin gene proximal promoter alone or the thyroglobulin gene promoter and enhancer (see Figure 1), were methylated *in vitro* using *HpaII* methylase (which methylates the internal cytosine residue at CCGG sites). These methylated plasmids, and the corresponding unmethylated constructs, were transfected in primary-cultured thyrocytes, and transient expression of the reporter CAT gene was analysed. After transfection, the cells were maintained continuously in the presence of 10 µM forskolin, to provide maximal stimulation of the differentiated functions [7]. As shown in Table 1, no significant differences could be seen in the activity of the methylated and unmethylated constructs containing both the thyroglobulin gene enhancer and promoter elements (pbTgCAT20MA, pbTgCAT20MB and pbTgCAT21A constructs). The absence of a significant effect of methylation *in vitro* was thus observed independently of the position of the enhancer element relative to the reporter gene in the transfected

plasmid. But, when only the proximal promoter was used (pbTgCAT20MO construct) the transcriptional activity of the methylated DNA appeared significantly reduced ($\pm 50\%$) when compared with that of the unmethylated construct. As neither the proximal thyroglobulin gene promoter, nor the enhancer element itself, contained a modified CG dinucleotide (a single *HpaII/MspI* site was present in the insert bearing the enhancer element, but clearly outside the sequences required for enhancer activity [6]), this implies that the observed effect of *in vitro* methylation on the activity of the promoter resulted indirectly

Figure 1 Schematic representation of the DNA constructs

These constructs have been described previously ([6]; see also Experimental section). pbTg, proximal promoter element (–239 to +9) from the bovine thyroglobulin gene; enhancer, bovine thyroglobulin upstream enhancer element (–1906 to –1744; the arrow indicates the natural orientation). The bars indicate the positions of *HpaII/MspI* (CCGG) sites. The enhancerless pbTgCAT20MO plasmid (a) and the enhancer-containing pbTgCAT20MA (b), 20MB (c) and 21A (d) constructs harbour 13 and 14 methylatable positions respectively. The numbers identify the fragments that correspond to the signals on Figure 2.

Table 1 Transient assay of *HpaII*-methylated thyroglobulin/CAT constructs in forskolin-treated thyrocytes

The values shown are the mean $\pm$ SD of six independent results. CAT expression from the unmethylated pbTgCAT20MA construct was taken as reference (100%), it corresponded to 10–12% acylation (butyryl-) of [3 H]chloramphenicol (values measured in the assays varied from 59746 to 71461 c.p.m.).

Construct	CAT expression (%)	
	Unmethylated	Methylated
Promoter alone		
pbTgCAT20MO	33 $\pm$ 8	14.5 $\pm$ 4
Promoter + enhancer		
pbTgCAT20MA	100	84 $\pm$ 7
pbTgCAT20MB	94 $\pm$ 6	109 $\pm$ 20
pbTgCAT21A	76 $\pm$ 17	61 $\pm$ 11

from the presence of 5-methylcytosine residues in the surrounding DNA.

As reporter gene expression was much higher in the presence of the thyroglobulin gene enhancer, the possibility remained that we failed to detect a difference between the methylated and unmethylated constructs in this case because of the existence of a saturating level of CAT expression in the transfected cells. To check this point, the cells were transfected with decreasing amounts of the enhancer-containing construct, methylated and unmethylated. No significant differences between the two DNAs could be detected even at low levels of CAT expression (results not shown).

Effect on promoter activity results specifically from methylation at CG base pairs

To verify that the observed effect of *in vitro* methylation on the expression of the enhancerless construct was specific for methylated CG dinucleotides, we compared the transcriptional activities of the same construct methylated with *HpaII* or *MspI* methylase (which methylates the external cytosine residue at CCGG sites). It is worth noting that the two methylated constructs harboured exactly the same number of modified residues, as both methylases only act at CCGG sites. The results shown in Table 2 demonstrate that *in vitro* methylation of the plasmid with *MspI* methylase did not result in a decrease in reporter gene expression, as was observed when the *HpaII* methylase was used. Expression from a construct containing the thyroglobulin gene enhancer element showed little change in both cases.

To check that the methylation pattern, in particular the methylation at CC dinucleotides (which appears to be quite uncommon in the eukaryotic genome [1]) resulting from the action of the *MspI* methylase, was preserved in the DNA taken up by the transfected thyrocytes, total DNA was extracted from the cells 3 days after transfection and the methylation status of the plasmid DNA was analysed on Southern blots after digestion with *HpaII* or *MspI*. As shown on Figure 2, both *HpaII* and *MspI* methylation patterns were preserved in all the constructs tested. In consequence we must conclude that DNA methylation at CG dinucleotides exerts a specific negative effect on the expression of a gene under the control of the proximal thyroglobulin gene promoter, and that this effect is largely reduced in the presence of the enhancer element.

Enhancer-containing constructs are clearly sensitive to DNA methylation when transfected in unstimulated thyrocytes

Maximal expression of the thyroglobulin gene, as with most of the other differentiated functions of the thyrocyte, relies on the continuous stimulation of the cyclic AMP second-messenger pathway by thyrotropin [9]. Likewise, in primary-cultured cells, the presence of a cyclic AMP agonist in the culture medium (e.g. forskolin) is required to sustain expression of these differentiated functions. In primary-cultured thyrocytes maintained in the absence of cyclic AMP stimulation, thyroglobulin gene transcription is very low, and depends on the presence of insulin in the culture medium [10]. We asked whether in such conditions DNA methylation would also affect significantly the function of the thyroglobulin gene enhancer-promoter combination.

The methylated and unmethylated enhancer-containing constructs were transfected in primary-cultured thyrocytes maintained either in control medium or in medium supplemented with

Table 2 Transient assay of thyroglobulin/CAT constructs after *in vitro* methylation with *MspI* or *HpaII* methylases

The values shown are the mean $\pm$ S.D. of four independent results. CAT expression from the unmethylated pbTgCAT21A construct was taken as reference (100%), it corresponded to 9.5–11% acylation (butyryl-) of [3 H]chloramphenicol (values measured in the assays ranged from 57 899 to 67 096 c.p.m.).

Construct	CAT expression (%)		
	Unmethylated	<i>HpaII</i> -methylated	<i>MspI</i> -methylated
Promoter alone pbTgCAT20MO	39 $\pm$ 6	17 $\pm$ 4	44 $\pm$ 12
Promoter + enhancer pbTgCAT21A	100	78 $\pm$ 4	137 $\pm$ 20

Figure 2 Southern-blot analysis of the methylation status of the transfected DNA

(a) DNA from cells transfected with unmethylated (–ME) or *HpaII*-methylated (+ME) plasmids. The total DNA was cleaved with *HpaII* and *MspI* (uncut, unrestricted control DNA). The enhancerless plasmid was pbTgCAT20MO and the enhancer-containing plasmid was pbTgCAT20MB. (b) DNA was from cells transfected with unmethylated (–ME) or *MspI*-methylated (+ME) pbTgCAT20MO (enhancerless) plasmid. The total DNA was cleaved with *MspI* (uncut, unrestricted control DNA). Both marker lanes contained the 1 kb-ladder standard from Bethesda Research Laboratories (BRL). The 1.636 kb, 517 bp, 506 bp and 396 bp fragments that correspond to pBR322 plasmid DNA were recognized by the vector sequences contained in the probe. The numbers on the right allow identification of the fragments of the constructs that correspond to the signals with reference to Figure 1.

Table 3 Transient assay of *HpaII*-methylated enhancer-containing construct in forskolin-stimulated and unstimulated thyrocytes

The values shown are the mean $\pm$ S.D. of four independent results. The cells were maintained in the presence (stimulated) or the absence (unstimulated) of 10 μ M forskolin for 3 days after transfection. CAT expression from the unmethylated construct in forskolin-treated cells was taken as reference (100%), it corresponded to 8–11% acylation (butyryl-) of [3 H]chloramphenicol (values measured in the assays varied from 48 111 to 67 868 c.p.m.).

	CAT expression (%) from construct pbTgCAT21A	
	Unmethylated	Methylated
Stimulated cells	100	81 $\pm$ 3
Unstimulated cells	3.4 $\pm$ 1.4	0.9 $\pm$ 0.1

10 μ M forskolin after the transfection step (the dishes were treated separately only after the contact with DNA to avoid differences in transfection efficiencies). As expected, transcriptional activity of the construct, whether methylated or not, was dramatically reduced in cells cultured in the absence of forskolin (see Table 3). Whereas methylation of the construct had little effect in forskolin-stimulated cells, it resulted in a clear reduction of CAT activity in unstimulated cells. This suggests that the negative effect of DNA methylation could be largely over-ridden only when the thyroglobulin gene enhancer–promoter combination was maximally activated.

DISCUSSION

We have shown here that *in vitro* DNA methylation of vector sequences by the *HpaII* methylase reduces significantly the transcriptional activity of the transfected thyroglobulin gene promoter when it is not fully activated in the thyrocyte, or when an activating element, the thyroglobulin gene enhancer [6], is lacking. No clear effect was observed at maximal promoter activity, i.e. in stimulated cells, when both enhancer and promoter elements were present.

The methylation of the plasmid DNA at *HpaII*/*MspI* sites provides only a relatively low density of methylated cytosine residues, which is more or less comparable with that normally found at the level of many tissue-specific genes. The finding that such a methylation of the surrounding DNA is sufficient to reduce significantly the activity of the thyroglobulin gene promoter when it is not maximally activated, thus indicates that the presence of 5-methylcytosine residues may participate in the silencing of the gene. On the other hand, the absence of a similar effect on full promoter activity indicates that the negative effects of sparse DNA methylation can be over-ridden by an appropriate stimulation of promoter activity.

Because of the total absence of *HpaII*/*MspI* sites in the regulatory elements of the thyroglobulin gene used in this study, our results also demonstrate that DNA methylation can affect promoter function in an entirely indirect way. Although several recent studies [11–14] have inclined toward the prevalence of an indirect mechanism of action, as compared with the direct effect of methylation on the binding of transcription factors (see [15]), most, if not all, of the genes tested contain methylatable positions in their promoter regions. The results presented here nicely support the hypothesis [13,16] that the proteins that specifically bind to methylated DNA are the main effectors of the observed effects of DNA methylation on gene activity. If these methylated DNA-binding proteins are able to compete with the transcription machinery for binding to the methylated plasmid DNA, one would indeed expect that the fate of the transfected DNA will depend on the relative affinities of the protagonists for the DNA construct. Upon binding of the ubiquitous methylated CG-binding protein (MeCP-2) [17], which is dependent on the presence of isolated methylcytosine residues, it is conceivable that the transfected DNA is being condensed into a transcriptionally inactive chromatin structure. In the conditions used here, the establishment of the active transcription complex prevailed, unless some binding sites for transactivating factors were missing (enhancerless constructs) or some transcription factors were not activated (transfection in unstimulated cells). Such a capacity of the fully activated transcription complex to over-ride the effects resulting from the presence of 5-methylcytosine residues in the DNA has also been reported in the cases of other tissue-specific genes [18,19]. Due to their CG deficiency, most of these genes are only poorly methylated, and hence can be only weakly bound by some methylated-DNA-binding proteins,

such as MeCP-1 [13], or only contain a limited number of binding sites for others, such as MeCP-2 [17]. This situation may allow the displacement of these proteins when a strong transcription complex builds up on the DNA. However, the formation of weak transcription complexes on these sparsely methylated genes appears to be partially impaired.

Concerning the role of DNA methylation in the control of thyroglobulin gene expression, our results thus agree with the view that methylation of CG dinucleotides in the gene during development may help to maintain the gene in an inactive form, except in the differentiated follicular thyroid cells where it is committed to transcription. The transcription factor TTF-1, an activator of thyroglobulin gene transcription, is not restricted to the thyroid tissue, but has also been found in lung and brain [20]. The possibility thus exists that weak transcription complexes build up on the thyroglobulin promoter in these cells during a transient exposure of the DNA sequences to the nuclear environment. According to the results presented above, the presence of a few 5-methylcytosine residues in the DNA would already be sufficient to reduce significantly the consequences of such an event. On the other hand, within thyrocytes, the methylated state of the gene would most probably not constitute a block to its expression. It is conceivable that transcriptional activity per se could lead to the loss of this modification of the thyroglobulin sequences. A proposed mechanism for the specific demethylation of expressed genes involves the earlier replication of transcribed DNA, as compared with the inactive part of the genome, during the cell cycle, which possibly results in a lower methylation quotient [21].

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