In vitro* DNA cytosine methylation of *cis*-regulatory elements modulates c-Ha-ras promoter activity *in vivo

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Received March 1, 1989; Revised May 4, 1989; Accepted June 1, 1989

ABSTRACT

The effect of DNA cytosine methylation on promoter activity was assessed using a transient expression system employing pHrasCAT. This 551 bp Ha-ras-1 gene promoter region is enriched with 84 CpG dinucleotides, six functional GC boxes, and is prototypic of many genes possessing CpG islands in their promoter regions. Bacterial modification enzymes HhaI methyl transferase (MTase) and HpaII MTase, alone or in combination with a human placental DNA methyltransferase (HP MTase) that methylates CpG sites in a generalized manner, including asymmetric elements such as GC box CpG's, were used to methylate at different types of sites in the promoter. Methylation of HhaI and HpaII sites reduced CAT expression by ~70%–80%, whereas methylation at generalized CpG sites with HP MTase inactivated the promoter by >95%. The inhibition of H-ras promoter activity was not attributable to methylation-induced differences in DNA uptake or stability in the cell, topological form of the plasmid, or methylation effects in non-promoter regions.

INTRODUCTION

The distribution of cytosine methylation in vertebrate genomes into tissue- and gene-specific patterns has suggested an underlying involvement in differentiation and gene regulation (reviewed in 1). This has been broadly supported by numerous correlative studies using methyl-sensitive restriction enzymes to evaluate the methylation status of genes with their functional activity (reviewed in 2,3).

Studies initiated by Bird and colleagues have revealed that the organization of the majority of the CpG dinucleotide-containing sequences is clustered or enriched in particular regions of DNA, the so-called CpG islands (reviewed in 4,5). Such CpG islands can occur in the body, and in 3' and 5' flanking region of certain genes (6). However, there is a paucity of information on the methylation status of these islands, except for housekeeping genes where they appear to be under- to unmethylated (6). Interestingly, CpG islands occur in the 5' flanking and regulatory region of several cellular oncogenes, including the c-myc, c-fos, c-sis, and c-ras family, leading to speculation that DNA methylation in these regions might be intimately involved in their regulation. We chose the Ha-ras-1 gene, by virtue of its containing a high frequency of clustered CpG's in its promoter region, for a functional study on the effect of cytosine methylation in a transient expression assay.

In this paper, we examine the functional activity of the human H-ras gene promoter in a transient expression assay with respect to cytosine methylation produced by bacterial modification enzymes and in combination with human placental DNA methyltransferase (HP MTase). The promoter region of the Ha-ras-1 gene from positions 116–666 with

respect to the Bam-HI site at position 1 has been mapped and the cis-acting elements necessary for its promoter activity have been determined (7,8). It contains a CpG island in its 5' flanking region overlapping the promoter as well as a very high frequency of CpG sites, 15.2% versus 1.2% in the gene itself (6). This high frequency of CpG includes 12 HpaII and 12 HhaI sites, which, owing to their tight clustering, are not amenable to Southern blot analysis with cognate methyl-sensitive restriction enzymes. For this reason, correlative study results to date on the effect of cytosine methylation on the Ha-ras-I gene activity are inconclusive.

Previous studies on the pattern of methylation in the non-promoter region using methyl-sensitive restriction enzymes have shown an inverse correlation of genetic activity of the H-ras-I gene with degree of methylation (9–11). In addition, the activity of the Ha-ras-I gene has shown a concordance with the methylation status of a polymorphic VTR region, which lies 1 kb downstream from the polyadenylation site (12). Borrello et al. (13) have demonstrated that after cytosine methylation of HpaII and HhaI sites, the transforming potential of the activated EJ (gly→val) Ha-ras-I gene was reduced by about 80% in transfection assays in the NIH3T3 cell. Reactivation experiments with 5-azacytidine gave rise to transformed foci and re-expression of mutated p21 species characteristic of EJ mutation. Probing with HpaII and HhaI restriction enzymes also showed that inactivation of the trans-gene correlated with methylation of these sites from the nonpromoter region.

Because of the important role played by the H-ras gene in oncogenesis, elucidation of regulatory signals that participate in its transcriptional activation are important. In this paper, we demonstrate that a transient expression assay for promoter function can be utilized to address questions of regulatory effects of cytosine methylation. Cytosine methylation of the H-ras gene promoter with bacterial methylases of different specificities modulated its activity, whereas extensive methylation by HP MTase suppressed promoter function. This downregulation was not mediated by other possible trivial effects including the topological status of the transfected plasmid or its stability and uptake in the cell. Furthermore, this inactivation was pinpointed to promoter region methylation and not to any nonpromoter effects of methylation in the plasmid.

MATERIALS AND METHODS

In Vitro Methylation

pHrasCAT methylated at HhaI and HpaII sites were prepared by incubating 20 µg of plasmid in 50 mM tris-HCl pH 7.4, 10 mM EDTA, 5 µM 2-mercaptoethanol, 100 µM S-adenosyl-methionine, 10 µg BSA, 3 units HpaII MTase/µg DNA, 5 units HhaI MTase/µg DNA in 200 µl at 37°C for 3 h. The units of HpaII and HhaI MTase (from New England Biolabs) are defined as the amount of enzyme incubated at 37°C for 1 h required to completely block cleavage of lambda DNA with cognate restriction enzyme. The methylated plasmids were deproteinized by phenol extraction, recovered by ethanol precipitation and resuspended in 0.1 XTE (1 mM tris HCl pH 7.4, 0.1 mM EDTA) prior to transfection.

For introduction of m⁵C at generalized CpG sites, the plasmids were first methylated with the HpaII and Hha MTase for 3 h as before and then incubated with an additional 200 µl of fresh buffer containing 100 µM fresh S-adenosylmethionine and 20 µl of HP MTase (fraction IV specific activity 14,000 poly(dC-dG) units/mg protein) 50 U/µg DNA. The HP MTase was purified starting from placental nuclear extract as described in Yoo et al. (14) and further purified as with rat MTase over DEAE-Affigel Blue (Bio-Rad) and Mono-Q (Pharmacia) and concentrated as described in Ruchirawat et al. (15). The eukaryotic

Figure 1. Kinetics of methylation of pH-rasCAT plasmid by bacterial HpaII and Hha MTase and eukaryotic HP MTase. The bacterial enzymes were incubated with an initial SAM concentration of 100 μ M in 400 μ l. and 40- μ l aliquots corresponding to 1 μ g of plasmid (kinetics denoted by $\circ - \circ$) were withdrawn. After 2 h, 250 U of purified HP MTase were added, and 21- μ l aliquots corresponding to 1 μ g of plasmid withdrawn and kinetics of methylation and of premethylated plasmids plotted ($\bullet - \bullet$) and corrected with a paired duplicate-incubated Hha + HpaII MTase. Kinetics of HP MTase enzyme alone under same initial conditions is shown by ($\square - \square$) for aliquots corresponding to withdrawal of 1 μ g of plasmid.

unit is defined as the amount of enzyme that transferred 1 pmol CH₃ at 37°C in 1 h to a given substrate. Poly(dC-dG) was used to titrate mammalian DNA MTase activity as it is a uniform substrate of juxtaposed unmethylated CpG sites. The plasmids were then purified as described above and quantitated by UV absorbance measurements.

Methylation of DNA was confirmed by analyzing restriction patterns of plasmid DNA with methyl-sensitive endonucleases and their methyl-sensitive isoschizomers. This panel included: HhaI, HpaII, MspI, FnuDII, XhoI, PaeR71, and Aval enzymes (from New England Biolabs); 0.5 μ g of methylated DNA was incubated with 10 U of enzyme under manufacturer's suggested conditions for 2 h and the restriction pattern analyzed following electrophoresis in 1% agarose gels.

Transfections

CaPO₄ transfections were performed essentially as described by Wigler et al. (16) with CV-1 cells maintained in DMEM-10% fetal bovine serum in a 5% CO₂ humidified incubator at 37°C. On day 1, 24 h before transfection, 2×10^6 cells were plated in a T75 flask that was refed 1–3 h prior to transfection on day 2. DNA in 0.1 TE was combined with an equal volume of 0.5 M CaCl₂, followed by slow addition of an equal volume of $2 \times$ Hepes buffered saline. The precipitate was allowed to stand 15 min prior

Figure 2. Restriction analysis for methylation by HP MTase. *Panel A.* Lane 1, HindIII size marker; Lane 2, HP MTase pHrasCAT, lower band is form I, upper band is form II; Lane 3, HP MTase pHrasCAT linearized with NdeI; Lane 4, HP MTase pHrasCAT cut with AvaI; Lane 5, pHrasCAT cut with AvaI; Lane 6, HP MTase pHrasCAT cut with XhoI; Lane 7 pHrasCAT cut with XhoI; Lane 8, HP MTase pHrasCAT cut with PaeR71.

Panel B. Lane 1, HP MTase pHrasCAT, middle band is linearized form III; Lane 2, unmethylated HrasCAT; Lane 3, HP MTase pHrasCAT cut with HpaII; Lane 4, unmethylated cut with HpaII; Lane 5, HP MTase pHrasCAT cut with MspI; Lane 6, unmethylated cut with MspI; Lane 7, HP MTase pHrasCAT cut with HhaI; Lane 8, unmethylated cut with HhaI; Lane 9, HP MTase pHrasCAT cut with FnuDII; Lane 10, unmethylated cut with FnuDII.

to direct addition to the cell monolayer. Four hours later, cells were glycerol-shocked for 2–3 min after removal of the CaPO_4 /DNA coprecipitate and refed with DMEM.

CAT Assay

The basic procedure of Gorman et al. (17) was followed. At 48 h after transfection, the monolayers were processed to make a cell extract. CAT activity was assayed by incubating 100 μl of cell extract normalized to equal protein concentration using BioRad Bradford assay with 25 μl H_2O , 0.5 μl of [^{14}C]chloramphenicol (40 Ci/mmol) (Amersham) and 20 μl of 4 mM acetyl CoA at 37°C for 60 min or up to 4 h for linearized plasmids. The reaction was stopped by extraction with 400 μl of ethyl acetate, which was evaporated to dryness in a Speed Vac and dissolved in 10 μl of ethyl acetate. Equal aliquots were spotted on silica gel G (Kodak) TLC plates, which were developed in 95% chloroform/5% methanol. For quantitation, the acetylated forms of [^{14}C]chloramphenicol (AcCm) were cut from the thin layer chromatography plate and the conversion made to the enzymatic activity present in the cell extracts.

Figure 3. Methylation target sites in 551 bp promoter region in pHrasCAT. GC boxes are underlined and denoted by Roman numerals. Potential CpG methylation target sites are denoted by ·; HpaII sites are denoted by P, and HhaI sites by h, FnuDII by f, and AvaI by a, over respective cytosines.

RESULTS

In Fig. 1, the kinetics of methylation of pHrasCAT is shown for a combination of HpaII and Hha MTases at 5 U/μg and 3 U/μg DNA, respectively, or in conjunction with HP MTase at 50 U/μg plasmid (1 eukaryotic unit being 1 pmol CH₃/h). The HpaII and Hha MTase enzymes at this enzyme/DNA ratio saturated HpaII and Hha sites by 3 h as shown by restriction analysis (data not shown). When the HP MTase was added at this point, its initial rate of methylation was nearly doubled compared with the kinetics of the unmethylated plasmid. This rate then tapered off and the kinetics in the de novo mode paralleled the solo addition, which is linear for over 6 h (15). This stimulatory effect enhanced the efficiency of the HP MTase enzyme at other target sites, including FnuDII, AvaI, and Xho sites, as shown in Fig. 2, and other asymmetric CpG sites. This premethylation permits overcoming the problem of slow kinetics in de novo mode, and consequently, inactivation of HP MTase that occurs during long term incubation due to its lability.

Figure 4. Effect of plasmid topology on promoter function. Lane 1, superhelical (form I) pHrasCAT; Lane 2, Hha + HpaII MTase form I pHrasCAT; Lane 3, HP MTase form I pHrasCAT; Lane 4, NdeI linearized (form III) pHrasCAT; Lane 5, HpaII and Hha MTase form III pHrasCAT; Lane 6, HP MTase form III pHrasCAT.

Using oligonucleotide substrates, it has been observed that HP MTase can be stimulated by m^5C in nonhemimethylated configuration on C-rich strands containing a GC box element (e.g., CCCGCC), which occurs within this H-ras promoter sequence (Rachal, Serface and Lapeyre, in preparation). Although not all possible sites are saturated, extrapolation from the number of HpaII and HhaI CpGs in the promoter region (12 each) to the 84 total remaining potential CpG target sites, shown in Fig. 3 for the 551 bp NaeI fragment, and the kinetics of incorporation by HP MTase suggest that maximum methylation achievable by HP MTase has been approached by overnight incubation (see Aval, XhoI, and FnuDII site analysis in Fig. 2). The incomplete methylation, e.g., non-saturability of all CpG sites, appears to be a reflection of the fact that HP MTase operating in de novo mode exhibits neighboring sequence effects (Rachal, Serface and Lapeyre, in preparation) in accord with recent results with HeLa DNA MTase for non-stoichiometric methylation at given sites (18,19). Under these conditions, approximately 65% of total CpG sites are methylated. It is not clear whether all sites are partially methylated (non-stoichiometric) since CpG-sensitive endonuclease cannot cleave hemimethylated sites, or certain sites are completely excluded. However, under these conditions of HP MTase incubation, we have verified, using oligonucleotide substrates containing GC box elements, that their CpG sites are methylated (Rachal, Serface, Lapeyre, in preparation).

Evaluation of in vitro Methylation Reactions

The degree of enzymatic methylation in the reactions was evaluated using restriction digestion by methyl-sensitive endonucleases and their methyl-resistant isochizomers, followed by agarose gel electrophoresis. By analyzing the degree of resistance to restriction digestion with HhaI (GCGC) and HpaII (CCGG) endonuclease, which cleave their recognition sites provided the internal cytosines are not methylated, the degree of enzymatic methylation of the sites can be evaluated. Methylation at generalized CpG sites by HP MTase was analyzed in an analogous manner using the methyl-sensitive enzyme Aval (CPyCGPuG), XhoI and its methyl-insensitive isoschizomer PaeR71 (CTCGAG) (which recognizes a subset of Aval specific for site at position 195), and FnuDII (CGCG). The pHrasCAT promoter contains 12 HhaI, 12 HpaII, 18 FnuDII, and 10 Aval restriction sites; and an additional 13 HpaII and 13 Hha sites which occur in nonpromoter portions of the plasmid. The results of such analysis is shown in Fig. 2 for HP MTase methylated pHrasCAT.

When the plasmid was methylated with the HP MTase alone for 24 h, it showed resistance

Table 1.

Exp.	DNA source	[¹⁴ C]-AcCm dpm	Relative CAT Activity (%)
1	pSVOCAT	116	0.0
1	pHrasCAT	9,618	100.0
1	HpaII and Hha MTase pHrasCAT	2,556	27.8
1	HP MTase pH-rasCAT	326	3.0
2	pSVOCAT	80	0.0
2	pHrasCAT	12,129	100.0
2	HpaII + Hha MTase pHrasCAT	3,555	28.6
2	HP MTase pHrasCAT	680	4.9

to cleavage by HhaI and HpaII restriction enzymes (Fig. 2B). However, with respect to MspI, partial protection was achieved; we attribute that to methyl modification of 5' prime cytosine in CCGG, an infrequent modification in higher eukaryotes. Most important, after HP MTase reaction, the methylated plasmids were partially resistant to cleavage at AvaI and FnuDII sites (Fig. 2A). Given that 10 AvaI sites are located within this 551-bp promoter region, including an overlap with a XhoI site, which appears to be partially methylated compared to more extensive methylation at other AvaI sites, either conformational or neighboring sequence effects must render certain sites refractory to HP MTase action.

Effect of Cytosine Methylation on Ha-ras-1 Promoter Activity

The plasmid pHrasCAT features a NaeI excised 551 bp fragment containing all cis-acting sequence elements of the human c-H-ras promoter linked to the CAT gene (7). The H-ras promoter is a possible candidate for regulation by cytosine methylation since it contains an overlapping CpG island that is 72% G+C rich and has a CpG dinucleotide frequency of 15.2%. The promoter element lacks canonical TATA and CAAT motifs present in many eukaryotic promoters and features 6 active GC box elements (GGGCGG and the inverted repeat CCCGCC) that have been shown to bind ubiquitous spl transcriptional factor (20,21). This region contains 84 potential methylation target sites (CpG dinucleotide sequences) 60 of which are non-assessable by methyl-sensitive CpG restriction enzymes as shown in Fig. 4.

Utilizing the bacterial HhaI MTase (CGm⁵CCG) and HpaII MTase (CCm⁵CCG) alone or in combination to stimulate HP MTase isolated in our laboratory, pHrasCAT was enzymatically methylated. The resulting methylated plasmids and unmethylated controls were transfected into CV-1 cells and the CAT activity assayed 48 h post-transfection as suggested from the work of Buschhausen et al (22). Methylation at specific HhaI and HpaII sites decreased CAT expression by ~70%, whereas following treatment with HP MTase, which methylates the a large proportion of the remainder of the CpG sites, including GC box CpGs and other asymmetrical sites, essentially abolished CAT activity (>95% inhibition). These results are summarized in Table 1.

Although enzymatic methylation of pHrasCAT appears to reduce CAT expression, several problems inherent to the transient expression assay system must be ruled out in order to assert causality. These are: (1) methylation effects in nonpromoter regions; (2) methylation-induced differences in DNA uptake and stability of methylated plasmids; and (3) methylation-induced changes in plasmid topology as factors responsible for the apparent decreases in CAT activity.

Table 2.

Exp.	DNA source	[¹⁴ C]-AcCm (dpm)	Relative CAT Activity (%)
1	pSVOCAT	1,023	0
1	Form I pRSVCAT	49,491	100
1	HpaII + Hha MTase Form I pRSVCAT	37,305	75
2	pSVOCAT	630	0
2	Form I pRSVCAT	24,085	100
2	HpaII + Hha MTase Form I pRSVCAT	25,972	107
2	HP MTase Form I pRSVCAT	23,749	98
*2	Form III pRSVCAT	28,701	100
*2	HpaII + Hha MTase Form III pRSVCAT	19,320	66
*2	HP MTase Form III pRSVCAT	16,220	57

* CAT assays performed for 4 h with NdeI linearized plasmids; otherwise, all other assays performed for 2 h with 10 μ g transfected plasmid.

Methylation of Nonpromoter Regions Does Not Affect Levels of CAT Expression

Since methylase enzymes do not discriminate between the promoter region, reporter gene, or bacterial sequences present in pHrasCAT, we determined whether DNA methylation of nonpromoter regions was responsible for the decrease in CAT expression. This issue was examined utilizing the plasmid pRSVCAT, which is identical to pHrasCAT except for the promoter region, which features the RSV long terminal repeat (LTR) instead of the 551 bp NaeI fragment from the H-ras-1 gene (17).

A total of 13 HhaI and 13 HpaII sites are featured in pRSVCAT, none of which reside in the RSV LTR promoter region. Four HpaII sites occur in the CAT cartridge, with the remaining sites being distributed in the bacterial and SV40 sequences. In contrast, pHrasCAT features 12 HhaI, 12 HpaII, and 10 Aval sites in its promoter region alone. Unmethylated, HpaII and Hha MTase treated, and HP MTase treated, supercoiled or linear pRSVCAT constructs were introduced into CV-1 cells and CAT activity assayed 48 h after transfection. The results, summarized in Table 2 for two independent experiments, demonstrate that the levels of CAT activity are independent of methylation status with superhelical pRSVCAT. Despite an effect of methylation on CAT expression observed for NdeI linearized LTR-containing plasmids (~50 % reduction), it is insufficient to explain the striking level of suppression (>95%) in both linearized and superhelical pHrasCAT treated with HP MTase. Since pHrasCAT and pRSVCAT are identical constructs, save for their promoters, we conclude that DNA methylation in nonpromoter regions is not responsible for inhibition of CAT activity seen in methylated pHrasCAT plasmid.

Table 3. Recovery of pHrasCAT from Hirts Supernatants 48 h Post-transfection.

Exp.	Unmethylated (ng)	HpaII + Hha MTase (ng)	HP MTase (ng)
1	1200	1000	1000
2	1000	1200	1000
3	800	1500	1500
average	1000 $\pm$ 200	1233 $\pm$ 251	1167 $\pm$ 288

Table 4. Effect of Plasmid Topology on Promoter Activity.

DNA Source	[¹⁴ C]-AcCm (dpm)	Relative CAT activity (%)
Unmethylated Form I H-rasCAT (superhelical)	8380	100.0
Hha and HpaII MTase Form I pHrasCAT	1408	16.8
HP MTase Form I pHrasCAT	389	4.6
Unmethylated Form III pHrasCAT (NdeI linearized)	3680	43.9
Hha and HpaII MTase Form III pHrasCAT	706	8.4
HP MTase Form III pHrasCAT	418	5.0

Uptake and Stability of Methylated Plasmids

To examine whether DNA methylation affect either plasmid uptake or stability in the cell, the transfected monolayer was processed by Hirt's procedure 48 h post-transfection (23). This technique allows for selective isolation of extrachromosomal DNA, which was dot-blotted to nitrocellulose, probed with radio-labeled pSVOCAT, and autoradiographed. The signal intensity yields quantitative data regarding the amount of uptake of transfected DNA that survives in the cell 48 h after transfection, which is summarized in Table 3 for transfected CV-1 cells with unmethylated, HpaII MTase plus Hha MTase, and HP MTase methylated pHrasCAT, respectively. These data from three independent experiments indicate comparable levels of recovered pSVOCAT plasmid sequences, from which we conclude that cytosine methylation does not impair the uptake or stability of methylated pHrasCAT in CV-1 cells over the initial 48 h post-transfection period.

Methylation Effect is Independent of Plasmid Topology

Weintraub et al. (24) have provided evidence that suggests that DNA topology influences gene expression. Form I (super-helical) plasmids were shown to yield higher levels of expression than their form III (linear) counterparts in transfection experiments. This effect was most pronounced for plasmids containing both enhancer and promoter elements. A close examination of the untreated and MTase-treated pHrasCAT on agarose gels revealed minor effects on plasmid topology as shown in Fig.s 2. HpaII and Hha MTase treatment resulted in a very slight increase in form II (nicked circular) molecules, whereas HP MTase resulted in a minor increase in form III (linear) molecules with concomitant increase in form II molecules.

Accordingly, we investigated the effects of topology on H-ras promoter activity. This analysis employed supercoiled and NdeI linearized methylated and unmethylated pHrasCAT plasmids. These DNAs were transfected into CV-1 cells and CAT activity assayed 48 h after transfection. The results are shown in Fig. 4 and summarized in Table 4. Lanes 1–3 represent unmethylated, HpaII and Hha MTase methylated, and HP MTase methylated, principally form I supercoiled plasmids (containing about 10% form II), respectively, and lanes 4–6 represent their respective linearized counterparts (containing >99% form III molecules).

With regard to unmethylated controls, supercoiling enhanced the activity over their linear counterparts by 60%, in accordance with the results of Weintraub et al. (24). In accord with the data shown in Table 1, the effect of methylation of generalized CpG sites with HP MTase drastically inhibited promoter activity (<5% activity remaining). After NdeI linearization at 530 bp upstream from the promoter insert, CAT activity was similarly inhibited by site-specific methylation of HhaI and HpaII sites, and almost completely

inhibited by methylation with the HP MTase (Fig. 4, lanes 4–6). These data indicate that the inhibitory effect of methylation on H-ras promoter activity is independent of plasmid topology. We conclude that topological changes in the small portion of plasmid DNA which arise from in vitro enzymatic methylation are not responsible for the decreases in CAT activity.

DISCUSSION

Considerable evidence in higher eukaryotes implicates DNA cytosine methylation in the regulation of transcriptional activity. Much of this evidence derives from studies demonstrating an inverse correlation between the methylation status at 5' flanking regions and transcriptional activity (1–3).

Despite the inverse correlation between levels of methylation and transcriptional activity, functional studies are required to prove that methylation of selected regions in DNA is necessary and sufficient to modulate gene activity. To address this question, several experimental methods (none in themselves an ideal approach) for altering methylation patterns have been employed to test the effect of cytosine methylation on promoter function.

Previously, methylation by HpaII MTase at Cm⁵CGG sites suppressed adenine phosphoribosyl transferase (APRT) gene was shown to suppress its activity in transfection assays (25). Similar functional studies on the methylation of HpaII sites from several proviral genes have been reported to produce a selective inactivation (26,27). Since in these studies the regulatory regions could not be manipulated specifically, the question whether cytosine methylation can effect selected regions in constructs is of practical importance.

Cytosine methylation of the HpaII or HhaI sites in adenovirus E1a and protein IX promoter blocked chloramphenicol acetyltransferase (CAT) expression in their respective promoter-reporter constructs (28). This effect was limited to sequences restricted to the regulatory region defined for these two genes.

Despite the ability to methylate a selected subset of potential sites with bacterial modification enzyme, no information is obtainable on the effect of other methylated CpG sequences that are not recognized by bacterial enzymes. In the case of the MuLV proviral genome, whose activity is correlated with methylation status deduced from methyl-sensitive restriction enzymes, its biological activity was inhibited only with eukaryotic (rat) MTase and methylation of HpaII sites alone was insufficient to block activity (29).

Because of a lack of suitable large-scale preparations of stable eukaryotic MTase to examine these questions, a number of investigators have relied on primer extension with dm⁵CTP in M13 constructs, thereby replacing all cytosines with m⁵C prior to transfection. With this approach, Busslinger et al. (30) showed that methylation of the upstream region between –790 and +92 of the gamma globin gene was necessary for the suppression of activity, whereas methylation of the coding region of the gene itself was not sufficient. By isolation and religation of the smaller primer-extended hemi-methylated restriction fragments and deletion of the particular CpG sites in these fragments, Murray and Grosveld (31) pinpointed particular the CpG dinucleotides in the –210 to +100 bp region for methylation suppression of expression. These results suggested the operation of region-specific rather than site-specific regulatory signals, which is compatible with the findings of regional effects of cytosine methylation on structural changes and reorganization of chromatin into inactivated domains (32,33).

Primer extension protocols, where all cytosines are replaced in a hemimethylated configuration with m⁵C, thus have the drawback stemming from the formation of an

unnatural, if not aberrant, copy of the *in vivo* methylation pattern. Presently, a complete picture of *in vivo* methylation pattern in genes has not been defined, with the exception of the vitellogenin gene, where genomic sequencing has been applied to map all m⁵C sites in its regulatory region (34). The genomic sequencing data indicated that CpG sites are variably methylated and as a response to induction are found in a hemimethylated state as a consequence of different rates of site-specific demethylation in each DNA strand. Similar demethylation events have been postulated to take place during gene activation in a number of other systems (35–37). Since these changes in cytosine methylation cannot be assessed using methyl-sensitive restriction endonucleases, site-specific methylation with bacterial methylases may fail to reveal the potential role of cytosine methylation on promoter activity.

Studies using eukaryotic DNA MTase may circumvent this problem, but are complicated by the fact that both maintenance and *de novo* activities are catalyzed by the same enzyme (14,38). Since there is evidence that the eukaryotic MTase(s) exhibit an methylation site preference, as shown from a sensitivity to the neighboring sequence environment (18,19), in addition to topological configuration effects (39), this may explain why it was not possible to saturate all potential CpG sites in the Ha-ras promoter region. On the other hand, this may be a property of methylation of clustered sites in CpG islands, as it has been observed that the Thy-1 CpG island could not be completely methylated *in vitro* (T. Bestor, personal communication). If such sequence-dependent effects are displayed by eukaryotic MTase (when operating in the *de novo* mode) for particular CpG sites, then it may prevent subsequent maintenance mode aberration, whereas it is not possible to abrogate this with hemimethylated substrates produced by primer extension where all cytosines have been completely replaced by m⁵C (15,30).

Previous functional tests to establish a relationship between methylation and gene activity relied either on *in vitro* methylation of the template with bacterial modification enzymes or primed synthesis in the presence of dm⁵CTP to replace m⁵C at all cytosine positions in M13 constructs, followed by transfection and assessment of transcriptional activity. In the former procedure only a small subset of possible m⁵C sites found in mammalian cells are methylated by the bacterial enzymes, whereas in the latter method, maintenance methylation of a completely hemimethylated substrate ensues, thereby establishing a methylation pattern of dubious nature stemming from the inappropriate replacement of all cytosines with m⁵C. The methylation system we employed relies on premethylation with bacterial modification enzymes of specific recognition sites that would be methylated by HP MTase alone, but which is stimulatory for the mammalian enzyme to accomplish efficiently the methylation of other generalized CpG sites that otherwise takes place in a kinetically slow reaction requiring long incubations (that lead to HP MTase inactivation). Despite the paucity of information about the normal *in vivo* methylation pattern of GC-rich promoter regions and its relation to transcriptional activity, this method for introducing a methylation pattern holds promise for reproducing an *in vivo* pattern. This promise is supported by recent evidence indicating mammalian MTase(s) have an intrinsic specificity for CpG sites mediated by neighboring sequences (18,19) or conformational status (39). In addition, protein 'determination factors' from nuclear extracts might be used to modulate methylation *in vitro* (36,40).

As a first step in this direction, the effects of cytosine methylation by a highly purified preparation of HP MTase on H-ras promoter function could be examined in an *in vitro* methylation/ transient transfection system. The results presented here demonstrate that

cytosine methylation in the H-ras promoter region modulates CAT expression. The apparent decreases in CAT activity are not attributable to methylation effects in nonpromoter regions, differences in DNA uptake or stability in the cell, nor methylation-induced changes in plasmid topology.

We have observed that generalized CpG methylation by HP MTase at about 65% saturation of the total CpG site, but not by HpaII and Hha MTase, induces a substantial local change in H-ras promoter conformation within the XhoI/SmaI portion of the regulatory region between positions 195 and 554 (Rachal and Lapeyre, in preparation), which is the most highly GC-rich region in the Ha-ras CpG island. Thus, mechanistically, cytosine methylation may modify the activity of CpG-rich promoters by stably altering local promoter conformation. Changes in promoter conformation would presumably affect the accessibility of cis-acting target sequences to trans-binding proteins required for gene expression. An extension of this argument may provide an explanation for our observation that clustered CpGs in the Ha-ras promoter region are not saturatable by HP-MTase in vitro.

Alternatively, unmethylated promoter regions may represent preferred sites of interaction between DNA and DNA-binding proteins where selective binding to unmethylated cis-regulatory sequences would lead to segregation of specific trans-acting factors to active regions of chromatin (4,32,33). By controlling these protein/DNA interactions, methylation and demethylation of specific upstream regulatory sequences could act as an off/on transcriptional switch. This has been observed at DNaseI footprinting level in the late adenovirus promoter for a protein factor binding to a HhaI site (41); but not in all cases as in the Ela promoter, which is inhibited by cytosine methylation (42).

ACKNOWLEDGMENTS

This investigation was supported by Grant CA31487 awarded by the National Cancer Institute, and by Grant No. BC-650 from the American Cancer Society. The stipend for MJR was provided by funding from the Kennerly Foundation. The authors wish to thank Dr. Glenn Merlino for providing the plasmid, pHRasCAT; Paula H. Holton for her technical assistance, and Marge Norton and Betty Martz for preparation of the manuscript.

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