**In vitro methylation of CpG-rich islands**

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**ABSTRACT**

CpG islands are distinguishable from the bulk of vertebrate DNA for being unmethylated and CpG-rich. Since CpG doublets are the specific target of eukaryotic DNA methyltransferases, CpG-rich sequences might be expected to be good methyl-accepting substrates in vitro, despite their unmethylated in vivo condition. This was tested using a partially purified DNA-methyltransferase from human placenta and several cloned CpG-rich or CpG-depleted sequences. The efficiency of methylation was found to be proportional to the CpG content for CpG-depleted regions, which are representative of the bulk genome. However, methylation was much less efficient for CpG frequencies higher than 1 in 12 nucleotides, reaching only 60% of the expected level. That suggests that the close CpG spacing typical of CpG-islands somehow inhibits mammalian DNA methyltransferase. The implications of these findings on the in vivo pattern of DNA methylation are discussed.

**INTRODUCTION**

The regulation of enzymatic DNA methylation in higher eukaryotes has been widely investigated in the last decade (1–8). DNA methyltransferases have been purified to homogeneity from several sources (9–11) and molecular cloning of the methylase gene has recently been achieved (12). Biochemical studies have highlighted some common features shared by all methyltransferases: hemimethylated CpG doublets are the preferred substrate (13), particularly when located within a C+G-rich region (3) at a specific distance from another CpG doublet (4). However the in vivo control of the enzymatic activity probably involves more specific mechanisms capable of accounting for the tissue-specific establishment of methylation patterns. In addition, the DNA of vertebrates shows a strikingly uneven distribution of methylated/unmethylated CpGs within the same cell type: in contrast with the overall rarity of CpGs, which occur at only one fifth of the expected frequency, there are regions in which CpGs are not suppressed and are as a rule unmethylated (14,15). These regions are known as CpG-rich islands and are assuming an increasing importance for being gene-associated. All housekeeping genes so far examined, as well as some tissue-specific genes, have CpG-rich islands at their 5' ends, which usually include the region where RNA transcription is initiated. Lack of methylation at assayable sites is found in every tested tissue irrespectively of gene expression, except for CpG islands linked to the inactive X chromosome in female cells (15, 17). CpG islands are putative good substrates for DNA methyltransferase, given their abundance in G+C (60 to 80%) and the high density of CpG doublets, which are the specific target of the enzyme; this contrasts dramatically with their constitutive absence of methylation in vivo. The mechanism by
which the action of DNA methyltransferase is escaped in vivo remains to be clarified.

It has been hypothesized that methylation of CpG islands is simply antagonized by the permanent binding of nuclear factors in vivo (15). However, no nuclear activity has been reported so far to selectively bind to unmethylated DNA. Indeed, the only proteins that are known to discriminate the methylated vs. the unmethylated status of DNA have been shown to preferentially bind to methylated DNA (18, 19).

In this work, we have studied the in vitro methylation of several genomic clones containing either a CpG island or a non island-associated gene. In all clones discrete stretches of DNA with widely different CpG frequencies can be distinguished. We have tested the methylation capability of a eukaryotic DNA methyltransferase in relation to the CpG frequency, with the aim of assessing whether the intrinsic characteristics of the DNA substrate are involved in regulating the enzyme activity.

MATERIALS AND METHODS

Materials. S-adenosyl-L-(methyl-³H)methionine (55–85 Ci/mmol) was purchased from New England Nuclear. S-Adenosyl-L-methionine (AdoMet) was from Sigma. Restriction enzymes were from Bio Labs. Proteinase K was from E. Merck. Both pH9.2 and pL9.2 are pUC subclones of the mouse CpG-rich sequence HTF9 (14). The 3.8 kb EcoRI fragment in pL9.2 includes non-suppressed sequences on either side of HTF9, while the 2.6 kb HindIII fragment in pH9.2 contains HTF9 only. pEB9 was a gift from Dr. D. Toniolo. It is a 5.9 kb EcoRI-BamHI fragment cloned in pBR322 and contains the islands associated to the human GdX and P3 genes (20). pHb/31S is a 3.4 kb PstI fragment containing the human ß globin locus (sequence in 21) cloned into pBR322.

DNA methyltransferase from human placenta at term was prepared as previously described (7) with the addition of an initial batch adsorption on phosphocellulose, from which the enzyme was released by 0.4 M NaCl. DNA methyltransferase assays were performed as previously described (7) at 37°C in 50 mM Tris-HCl, pH7.8, in the presence of 5 mM EDTA and 0.5 mM DTT. Small aliquots were withdrawn at several time intervals for kinetic measurements or at the end of the incubation when the final percentage of CpG methylation was to be measured. An enzyme unit was defined as in (7).

Methylation of clones. All clones (DNA final concentration 20 µg/ml) were methylated in the presence of varying placenta DNA methyltransferase concentrations (ranging from 37 to 400 U/ml) at 37°C for 6 h in assay medium containing 16 µM (methyl-³H)-AdoMet (3500–4000 dpm/pmol). In order to reach a high methylation level an equivalent amount of fresh enzyme and of fresh (methyl-³H)-AdoMet, at the same specific activity, were added after 6h incubation, and the incubation was carried out for 16 further hours. Reactions were always terminated by digestion with 250 µg/ml proteinase K in 0.1% (w/v) SDS for 60 min at 37°C. Proteins were extracted with phenol/chloroform (1:1, v:v); after addition of 0.3 M sodium acetate and 10 mM spermidine, DNA was ethanol precipitated. Radiolabeled DNA was collected by centrifugation and washed twice with cold 70% ethanol. Analysis of methylation patterns. Methyl-³H-labeled DNAs were digested with restriction endonucleases (2–10 units/µg DNA) as recommended by the supplier. Restriction fragments were separated by electrophoresis on agarose gels. After electrophoresis each lane was cut in 2 mm-wide slices and each gel slice was redissolved in 1.5 ml of water for 10 min at 100°C. Radioactivity was measured after addition of 12 ml of Aquasol (NEN).

Gel shift assays. The CpG-rich regions from pL9.2 and pEB9 were digested with suitable restriction enzymes, gel-purified, internally cleaved with HpaII to obtain fragment sizes

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Fig. 1. (A) CpG frequency in pL9.2 subclone. Frequency was calculated as in (23) (window 200 bp, moving across the sequence at 50 bp interval). Vertical bars represent restriction sites while a to o indicate the resulting fragments. The straight line at 18 CpG/200bp marks the CpG frequency above which the \textit{in vitro} methylation is inefficient (see Results). (B) and (C). \textit{In vitro} methylation pattern in pL9.2. Each point represents a restriction fragment (or the sum of comigrating fragments) as generated by type I) restriction (B) and type II) restriction (C), respectively. (D) Methylation pattern in pH9.2. In all plots the amount of methylation is expressed for each fragment as the percentage of the total counts recovered from the gel and CpG are considered on a single strand basis (see Methods for details).

RESULTS

\textit{Methylation of pL9.2 and pH9.2.} HTF9 is a typical CpG-rich island originally isolated from the mouse genome and found to be unmethylated in all tested tissues by MspI/HpaII
**TABLE I** RESTRICTION ANALYSIS OF *IN VITRO* METHYLATED CLONES

<table>
<thead>
<tr>
<th>fragment</th>
<th>bp</th>
<th>CpG</th>
<th>bp/CpG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pL9.2 (6443 bp)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>type I restriction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>E396–D830</td>
<td>830***</td>
<td>9</td>
</tr>
<tr>
<td>b</td>
<td>D830–P2472</td>
<td>1642</td>
<td>149</td>
</tr>
<tr>
<td>c</td>
<td>D2544–P2876</td>
<td>332</td>
<td>18</td>
</tr>
<tr>
<td>d</td>
<td>P2876–E3757</td>
<td>881***</td>
<td>8</td>
</tr>
<tr>
<td>e</td>
<td>P306–D2276</td>
<td>716***</td>
<td>46</td>
</tr>
<tr>
<td>f</td>
<td>D2276–D1584</td>
<td>692***</td>
<td>38</td>
</tr>
<tr>
<td>g</td>
<td>D1563–P628</td>
<td>935</td>
<td>68</td>
</tr>
<tr>
<td>h</td>
<td>P628–E396</td>
<td>232</td>
<td>13</td>
</tr>
<tr>
<td><strong>pEB9 (9999 bp)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>E396–A1237</td>
<td>1237</td>
<td>29</td>
</tr>
<tr>
<td>j</td>
<td>A1237–A2015</td>
<td>778</td>
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<td>k</td>
<td>A2015–A2616</td>
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<tr>
<td>l</td>
<td>A2616–E3757</td>
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<td>20</td>
</tr>
<tr>
<td>m</td>
<td>E396–A429</td>
<td>2653</td>
<td>160</td>
</tr>
<tr>
<td><strong>pHbβ1S</strong>** (8921 bp)**</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>n</td>
<td>B375–S983</td>
<td>983</td>
<td>69</td>
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<tr>
<td>o</td>
<td>S983–S1291</td>
<td>308</td>
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<td>S1291–S2168</td>
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<td>E1629–B375</td>
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<tr>
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<td>P2068–Ps3613</td>
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<td>102</td>
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<tr>
<td>x</td>
<td>Ps3613–Sp42430</td>
<td>1778</td>
<td>10</td>
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<tr>
<td>y</td>
<td>Sp42430–Ps3613</td>
<td>2780</td>
<td>16</td>
</tr>
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*(A=AccI, Av=Aval, B=BamHI, D=DraI, E=EcoRI, H=HindIII, P= PvulI, Ps= PstI, S= SacI, Sp= SpHI)*
* fragments not present in pH9.2
** fragments comigrating in the gel
*** fragments comigrating in the gel
**** insert site numbering is as in (21)

(Fragments below 90 bp, not detectable on the gel, have been omitted)

The unmethylated domain spans roughly 2 kb which are on average 60% G+C-rich, with a central region in which the G+C content is as high as 80%. The region contains a bidirectional promoter shared by two housekeeping genes (23). The subclones used in this study contain either the island sequence only (pH9.2) or the island flanked by non-island genomic DNA sequences (pL9.2). Sequence analysis of pL.9.2 showed 173 CpG dinucleotides clustered over 2686 bp. As shown in Fig. 1, the high CpG density within HTF9 stands out in comparison to that found in the flanking genomic regions, while the vector sequence (pUC19) has an intermediate level.

Both pL9.2 and pH9.2 clones, as well as the pUC vector alone, were used as substrates for a partially purified DNA methyltransferase from human placenta. The initial rate of the methylation reaction was very similar for all three clones. No difference was seen between the circular and the linear form, even after prolonged incubation (not shown).

To analyse the *in vitro* methylation pattern of cloned HTF9, both pL9.2 and pH9.2 were methylated to different extents by varying the incubation time and/or the enzyme digestion (14). The unmethylated domain spans roughly 2 kb which are on average 60% G+C-rich, with a central region in which the G+C content is as high as 80%. The region contains a bidirectional promoter shared by two housekeeping genes (23). The subclones used in this study contain either the island sequence only (pH9.2) or the island flanked by non-island genomic DNA sequences (pL9.2). Sequence analysis of pL.9.2 showed 173 CpG dinucleotides clustered over 2686 bp. As shown in Fig. 1, the high CpG density within HTF9 stands out in comparison to that found in the flanking genomic regions, while the vector sequence (pUC19) has an intermediate level.
Fig. 2. Methyl-\(^3\)H incorporation in individual fragments from pL9.2 versus the methylation extent of the whole subclone. The extent of in vitro methylation in each single fragment was comparable even upon a 100-fold variation in the total methylation reached in different experiments. ▲, △, □, ■, ●, ○ represent fragments h, a+d, c, g, e+f and b (HTF9) in Fig. 1, respectively.

concentration. This was done to minimize the effect of any inhibitor or activator which might have been present in the enzyme preparation. Radiolabeled DNA was digested with restriction endonucleases so as to separate HTF9 from the vector (in the pH9.2 subclone) as well as from the flanking regions (in the pL9.2 subclone). Fig. 1 show the fragments resulting from restriction endonuclease digestion (coordinates for each clone are given in Table I). Following agarose gel electrophoresis, the radioactivity present in each lane was measured as described in Materials and Methods. Lanes were sliced throughout their length to detect any product of partial digestion. Partial digestion, if any, accounted for a very small percentage of the total counts loaded on the gel.

A summary of the results obtained with pL9.2 is shown in Fig. 1-B. A very good correlation between the extent of methylation and the number of CpGs was observed for most fragments, with the only exception of the b fragment corresponding to HTF9 and containing 149 CpGs, which incorporated significantly fewer counts than expected. A statistical analysis was carried out using a bestfit algorithm. The linear proportion between radioactivity incorporation and CpG content was striking if only points in a 2-sigma range around the fitting curve were retained in a two-step approach. We wish to emphasize that only the point corresponding to the island was discarded by that selection criterion. These results were confirmed by digesting pL9.2 with a different set of restriction enzymes that cleaved within the island region (Fig. 1-C). The resulting j and k fragments, although differing in their individual CpG content, both consisted of non CpG suppressed DNA and both showed hypomethylation. Fig. 2 shows that the level of hypomethylation of island DNA (about 40%) was consistent at every tested percentage of in vitro methylation (ranging between 0.3 and 27%). To compare results deriving from different amounts of radioactivity loaded on the gel, due to the different extent of methylation reached, data were expressed for each restriction fragment as the percentage of total counts recovered from the gel. The 16 CpG-point, corresponding to the comigrating a and d fragments (i.e. to the non-
Fig. 3. (A) CpG frequency in pEB9 subclone. Frequency was calculated as in Fig. 1. Vertical bars represent restriction sites while p to u indicate the resulting fragments. The straight line at 18 CpG/200 bp marks the CpG frequency above which the *in vitro* methylation is reduced (see Results). (B) *In vitro* methylation pattern in pEB9. Data are expressed as in Fig. 1.

island regions flanking HTF9) is worth noticing: the CpG content is low and doublets are very far apart from each other (see Table I), yet both fragments appear to be methylated as efficiently as any vector derived fragment, if not better.

Similar results were obtained with pH9.2. The b fragment corresponding to HTF9 was
hypomethylated as compared to the vector DNA (see Fig.1-D). No CpG-suppressed regions are present in this subclone, which suggests that hypomethylation of HTF9 was not affected by and did not depend on the CpG content of the DNA sequences flanking it.

A local inhibitory effect of supercoiling was ruled out by the analysis of the EcoRI linearized pH9.2 subclone: the methylation pattern obtained with the linear form of the
Subclone was identical to that of native supercoiled DNA (data not shown). *Methylation of pEB9.* The only *in vivo* examples of methylated CpG islands are found on the inactive X chromosome in females. We were interested to know whether X-linked islands differ from autosomal ones in their ability to undergo methylation. Since the methyltransferase used in our studies was purified from human placenta, islands of human origin were preferred for this analysis. We examined the *in vitro* methylation pattern of a genomic subclone containing part of the coding sequences of the human X-associated gene *GdX* which codes for a housekeeping protein highly homologous to ubiquitin (20). A large CpG cluster extends from the 5'-flanking region into the gene as far as the second intron. The cloned insert also contains a second island, associated to another X-linked gene of unknown function, called *P3.* Fig 3-A shows the CpG distribution in the subclone: it is possible to see that CpG doublets are clustered in two regions separated by an area of low CpG frequency. Methylation and restriction analysis were carried out as described above. Restriction was designed to separate the two CpG clusters (see Table I). The methylation pattern obtained for this clone is shown in Fig.3-B. As previously observed with *pL9.2* and *pH9.2*, the fragments with a high CpG frequency were significantly hypomethylated with respect to the CpG-depleted fragments.

*Methylation of β-globin gene.* Methylation of isolated CpGs had been reported to be more difficult than that of clustered CpGs (5). As a control of the results obtained for the methylation of CpG-suppressed regions in the previous experiments, we studied the human β-globin gene using the same approach. The pBR322-derived clone *pHbβ1S* contains the 5'-flanking and coding sequences of the β-globin gene. CpG suppression extends over the entire 4.4kb-long genomic insert, which only has 26 CpGs scattered throughout its length (Fig.4-A). The *in vitro* methylation results showed that isolated CpGs were efficiently methylated and confirmed the linear trend described above (Fig.4-B). Interestingly, the only point below the curve in this experiment corresponds to a pBR322-derived region whose CpG content is similar to that of a CpG island (y fragment).

Data from all experiments were summarized in Fig.5. To compare the results obtained with different clones, the CpG content of single fragments was expressed as the percentage of total CpGs in each clone. The plotted points fell onto two lines with different slopes, reflecting the different alignment of island and non-island sequences respectively. The smaller slope of the island sequence line makes the hypomethylation effect readily apparent.

The possibility did however exist that protein(s) present in the enzyme preparation might somehow ‘mask’ CpGs in the island sequences, while having little or no effect on CpG-suppressed regions. Therefore we assayed the ability of the methylase fraction to bind to island DNA in gel retardation experiments. Short CpG-rich probes were generated by HpaII digestion of all three HTF9, *GdX* and *P3* islands. End-labeled, fragments were incubated with the methyltransferase preparation using various competitor DNAs—i.e. double stranded poly(dl-dC), poly (dG-dC), poly(d5meC-dG), or the gel-purified HpaII-resistant fraction of mouse genomic DNA which is virtually devoid of CpG-rich sequences (see 14). No specific interaction was formed between any of the tested island-derived probes and the protein fraction (not shown).

**DISCUSSION**

*De novo* methylation is currently regarded as a secondary activity of mammalian DNA methyltransferases, as compared to the predominantly ‘maintenance’ type of activity, by which methyl groups are added to the 5 position of a cytosine included in the sequence
Fig. 5 Island-like fragments show the same extent of hypomethylation. Data from all experiments have been combined in one plot. The CpG content of single fragment is expressed as the percentage of the total CpGs in each clone. ○ island-like fragments, ● non island-like fragments.

5'-mCpG-3'/3'-GpC-5'. In either type of reaction, the question is still open as to how the enzyme selects which cytosines are to be methylated and whether adjacent DNA sequences affect this choice. Previous work on 'de novo' methylation of synthetic oligomers has indicated some possible mechanisms driving the enzyme choice (3–5). The distance between CpGs appeared to be critical in determining the probability of methylation for any single CpG. Molecules having a spacing of 13–17 nucleotides between two CpG sites were reported to be the best substrates for the methylase (4), while larger and shorter distances seemed to somehow prevent methylation: in particular isolated CpGs were not efficiently methylated (5).

These results can however be hardly compared to the patterns observed in vivo. The bulk of mammalian genome is characterized by efficient methylation coupled with underrepresentation of CpG dinucleotides (1/5 of the expected frequency, which means an average CpG spacing of 100–150 nucleotides). These characteristics are completely reversed in CpG islands. In vivo, therefore, underrepresentation does not necessarily imply hypomethylation, whereas CpG clustering is associated to lack of methylation.

In the present work we have compared the enzymatic methylation of cloned mammalian DNA sequences significantly differing in their CpG content, i.e. with CpG frequencies ranging from 1:180 to 1:8 nucleotides. The cloned sequences were methylated proportionally to the amount of DNA methyltransferase in the reaction. The analysis of individual restriction fragments showed that the extent of methylation could be regarded as a linear function of the CpG density in CpG suppressed regions (i.e. with a typical CpG frequency below 1 in 12 nucleotides)—isolated CpG dinucleotides being efficiently methylated. In fragments whose CpG frequency was higher than 1 in 12 nucleotides, enzymatic methylation was significantly less efficient, reaching only about 60% of the expected level—though the linear dependence on the CpG content was still maintained. These results suggest that some minimum distance between CpGs may be required for optimal methylase activity.

Island hypomethylation might have arisen as an artefactual consequence of CpG masking.
if G+C-rich DNA-binding proteins were contaminating the enzyme preparation. However the amount of such contaminants would be expected to increase upon addition of increasing amounts of the enzyme to the methylation reaction; conditions which in fact did not affect hypomethylation (Fig.2). In addition, gel shift assays failed to detect any preferential binding to island-derived probes.

The DNA conformation is known to affect the DNA methyltransferase activity. Both DNA supercoiling and B/Z transitions have been shown to prevent methylation of specific DNA tracts (6). Such an effect can however be ruled out in our experiments. No difference in the methyl-accepting capability was detected between the native and the linear form of the plasmid subclones. Moreover the pattern of hypomethylation at CpG-rich regions was identical in a supercoiled and in a linear plasmid subclone. We conclude that in vitro methylation proceed in our system with characteristics similar to those occurring in vivo, at least as far as the relation between the CpG frequency and the probability of methylation is concerned.

All three islands examined in this study have been shown to be available in vivo to a variety of nucleases (24, 25), suggesting an accessible chromatin organization. Selective inaccessibility to the methyltransferase seems therefore unlikely. On the other hand, the binding of transcription factors whose target sequences occur within island promoter regions might be thought to antagonize the methylase. However, in HTF9 transcription factors only bind at two specific locations (P.L and P. Somma unpublished) and would therefore hardly be sufficient to prevent the further binding of the methylase over the whole 2kb-long CpG island. In our view, the inhibition of the methyltransferase activity by a high CpG frequency might be one element involved in the in vivo lack of methylation typical of CpG islands.

On the other hand, 'de novo' methylation is detected at all testable sites upon inactivation of the X chromosome (17). Some cofactor(s) may then be involved in making the islands accessible to methylation. Indications were recently obtained using an immunopurified preparation of DNA methyltransferase from a mastocytoma cell line and pBR322 DNA as the substrate (26). No correlation was apparent between the number of CpGs and the radioactivity incorporation in specific pBR322 fragments, however the relative ratio of radioactivity incorporation changed dramatically upon addition of a 29 kDa methyltransferase-stimulating protein to the purified enzyme. We noticed that the fragments in which the radioactivity incorporation was reversed, showed an average CpG spacing of 8–11 nucleotides, which is similar to that of CpG islands. Therefore 29 kDa-protein was suggested to elicit the dependence of the methylase activity on the CpG content: perhaps the protein stimulates the enzyme activity when methylating closely spaced CpGs. That hypothetical function could be of particular interest in relation to X chromosome inactivation. Other methylase stimulating proteins have been characterized in chromatin fractions among tightly-bound proteins (27), but no data are available as yet on their possible influence over the enzyme specificity.

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