Different Effects of Histone H1 on *de Novo* DNA Methylation *in Vitro* Depend on both the DNA Base Composition and the DNA Methyltransferase[†]

Daniela Carotti,*[,‡,§] Salvatore Funiciello,[‡,§] Patrizia Lavia,[||] Paola Caiafa,[⊥] and Roberto Strom[§,▽]

Departments of Biochemical Sciences 'A. Rossi Fanelli' and of Human Biopathology, University of Rome 'La Sapienza', Department of Biomedical Sciences and Technologies, University of L'Aquila, and CNR Centers of Molecular Biology and Evolutionary Genetics, Rome, Italy

Received March 11, 1996; Revised Manuscript Received May 16, 1996[⊗]

ABSTRACT: We have characterized the inhibition exerted by histone H1 on the activity of human placenta DNA (cytosine-5-)-methyltransferase. Our experiments demonstrate that the extent of inhibition depends on the DNA base composition, AT-rich substrates being more severely affected than GC-rich substrates and CpG-rich islands. With bacterial *Sss*I methylase, the effect is completely reversed since its activity on AT-rich substrates undergoes a 4−5-fold stimulation upon the addition of H1. Poly(L-lysine) mimicks H1 effects, suggesting an essential role of lysine residues in both the inhibitory and stimulatory effects of H1. By comparison of the different behaviors of the two enzymes, the inhibitory effect over the eukaryotic enzyme might be accounted for by hypothesizing a competition between minor groove-binding motifs (SPKK-like) present in placenta methylase as well as in histone H1.

H1 histone is regarded as a major chromatin protein involved in the formation and maintenance of different levels of chromatin structure (Thoma et al., 1979; Garrard, 1991). Its DNA binding ability has been reported to depend, to a certain extent, on the base composition (Renz & Day, 1976; Izaurralde et al., 1989), on the DNA structure (Singer & Singer, 1978; Varga-Weisz et al., 1993), and also on DNA methylation (Higurashi & Cole, 1991). In the last few years, reports have been accumulating which indicate an active and specific role of H1 histone in modulating chromatin functions, such as gene expression. In support of a regulatory role are the findings of a nonuniform distribution of this histone along chromatin (Huang & Cole, 1984; Mohr et al., 1989) and of its ability to inhibit *in vitro* transcription initiation (Wolffe, 1989; Croston et al., 1991) as well as *in vitro* DNA methyltransferase activity (Caiafa et al., 1991). It is therefore important to establish which DNA features can induce or prevent the interaction with histone H1. This issue is far from having an unambiguous answer.

In spite of a recognized general preference for AT-rich over GC-rich DNA stretches (Sponar & Sromova, 1972; Renz & Day, 1976), it has been recently shown that the H1e variant preferentially binds to GC-rich DNA (Wellman et al., 1995). In addition, although H1 is generally thought to have a low DNA sequence-binding specificity, both the rat albumin (Berent & Sevall, 1984) and the mouse α-globin genes (Yaneva & Zlatanova, 1992), as well as restriction fragments from pBR322 and pUC19 plasmids (Yaneva et al., 1995), have been found to contain sequences of preferential binding, while fragments from SV40 DNA have shown different degrees of protection from H1 binding (Hendrickson & Cole, 1994).

The effect of methylation at CpG dinucleotides on H1 binding is also controversial. *In vitro* transcription initiation is more severely inhibited by H1 on methylated than on unmethylated templates (Levine et al., 1993; Johnson et al., 1995). Higurashi and Cole (1991) have suggested a preferential binding of H1 to methylated DNA, on the basis of its resistance to *Msp*I digestion after H1 binding, but failed to confirm this conclusion by gel retardation experiments. More recently, Campoy et al. (1995), in a detailed study, could not find any significant difference in the binding of chicken erythrocyte histone H1 to a variety of DNAs in the methylated or the unmethylated form. However, most of these studies have been performed with mixtures of histone variants that are differently represented in various tissues (Cole, 1984) and might contribute differently to the phenomena under investigation. In fact, Santoro et al. (1995) have shown, in binding competition assays, a higher affinity of a histone H1e-c fraction for unmethylated vs methylated GC-rich oligonucleotides and only the H1e variant is capable of exerting a severe inhibition on *in vitro* methylation catalyzed by the human placenta DNA methyltransferase (Zardo et al., 1996).

In light of this emerging functional role of H1, we focused our attention on the possible effect exerted by histone H1 on *in vitro* DNA methylation, using DNA substrates differing in their base composition and/or CpG dinucleotide content. The substrates were either purified DNAs of genomic and synthetic origin or plasmids containing different types of eukaryotic sequences. Among the latter, we analyzed the methylation of sequences known as CpG-rich islands (Bird,

[†] This work was supported by grants from Ministero per l'Università e la Ricerca Scientifica e Tecnologica, from Istituto Pasteur-Fondazione Cenci Bolognetti, and from Associazione Italiana per la Ricerca sul Cancro (AIRC).

* Corresponding author: Dr. D. Carotti, Dipartimento di Scienze Biochimiche 'A. Rossi Fanelli', Università di Roma 'La Sapienza', piazzale A. Moro 5, 00185 Roma, Italy. Telephone: +39.6.491198. Fax: +39.6.4440062.

[‡] Department of Biochemical Sciences 'A. Rossi Fanelli', University of Rome 'La Sapienza'.

[§] CNR Center for Molecular Biology.

[||] CNR Center of Evolutionary Genetics.

[⊥] University of L'Aquila.

[▽] Department of Human Biopathology, University of Rome 'La Sapienza'.

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1996.

S0006-2960(96)00605-8 CCC: $12.00

1986), 1−2 kb in length, where the CpG dinucleotide is not under-represented, as is the case for the bulk of vertebrate genomic DNA. *In vivo*, CpG islands are positioned mainly at the 5′ end of all housekeeping and certain tissue-specific genes and are constitutively unmethylated in somatic cells, except for those linked to the inactive X chromosome in female cells; they are however *in vitro* substrates for the DNA methyltransferase enzyme, although with a somewhat reduced efficiency as compared to what might have been expected from their high CpG content (Carotti et al., 1989; Bestor et al., 1992). It is not entirely clear, as yet, what prevents CpG-rich DNA from being methylated *in vivo*. Recent reports have suggested a role for Sp1 elements in preventing methylation of the CpG island associated with the *aprt* gene (Brandeis et al., 1994; Macleod et al., 1994). However, this may not represent a universal mechanism, given that not all CpG islands harbor Sp1 recognition sites. The intricacy of these results prompted us to investigate the possibility that histone H1, and particularly the H1e variant in view of its preference for GC-rich sequences, might be involved in inhibiting methylation of CpG dinucleotides.

In the present work, we used these various types of DNAs as substrates of a partially purified DNA methyltransferase from human placenta as well as bacterial *Sss*I methylase. With the eukaryotic enzyme, H1 was inhibitory for DNA methylation, the inhibition being less marked for DNAs of higher G+C content. Consistently, methylation of CpG-rich island-derived fragments was less inhibited than that of any other DNA fragment from the same plasmid. The effect of H1 was exactly reversed with the bacterial enzyme, the histone showing a stimulatory, rather than an inhibitory, activity when AT-rich DNAs were used as substrates. We believe that the different effects displayed by H1 may help to depict some general features of the methylation reaction driven by the eukaryotic enzyme that are not shared by the prokaryotic methylase.

MATERIALS AND METHODS

Materials. Calf thymus histone H1 was from Boehringer Mannheim. Placenta H1 was prepared by standard procedures (Johns, 1987) from nuclei obtained from fresh human placentas. Histone H1 variants were separated according to Quesada et al. (1989) and Santoro et al. (1995) on a reverse phase RPC 4−300 Å column. All histone proteins were resuspended in 30 mM EDTA to induce renaturation. Protein concentrations were evaluated by Bio-Rad protein assay. Poly(L-lysine), 15−30 kDa, and poly(L-arginine), 17 kDa, were purchased from Sigma. *Sss*I methylase was from New England BioLabs. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (55−85 Ci/mmol) was purchased from New England Nuclear and *S*-adenosyl-L-methionine from Sigma. Poly(dC-dG)•poly(dC-dG) was purchased from Pharmacia Biotech, and all other DNAs were of commercial origin. The DK21/22 oligonucleotide from the Htf9a/*RanBP-1* promoter had the sequence 5′-GGCCGGCATCGCCGCGGGCGTTTTGGCGG-GAAGCGCGAG-3′. Both complementary strands were synthesized by the phosphoramidite method, on an Applied Biosystems DNA synthesizer.

Plasmids. pL9.2 is a pUC subclone of the mouse CpG-rich sequence Htf9 (3.8 kb-long insert, accession number X50830; Lavia et al., 1987). pH9.2 is a shorter subclone of the same genomic region (2.6 kb-long insert). pEB9, a kind gift from Dr. D. Toniolo, is a 5.9 kb-long genomic fragment, derived from the human X chromosome and cloned in pBR322.

In Vitro Methylation. Partially purified DNA methyltransferase (EC 2.1.1.37) from human placenta was prepared as previously described (Carotti et al., 1989). Assays were performed with 5−10 units of enzyme [as defined in Carotti et al. (1986)] in a 100 μL volume at 37 °C for 2−3 h, in 50 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol, and 0.2 mM PMSF, in the presence of 16 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (10−15 μCi/mL, 1000−2000 dpm/pmol), as previously described (Carotti et al., 1986), with a final DNA concentration of 20 μg/mL and various H1/DNA ratios (w/w). *Sss*I methylase was assayed in the same conditions with 0.4 unit per test. Plasmid DNAs were methylated as previously described (Carotti et al., 1989). After *in vitro* methylation reaction, purified DNA was digested with suitable restriction endonucleases. Restriction fragments were separated by agarose gel electrophoresis; each lane was then cut in 2 mm-wide slices, and each gel slice was redissolved in 2 mL of water for 10 min at 100 °C, for radioactivity measurements. In some experiments, plasmid DNA was first methylated using the bacterial *Sss*I methylase and, after heat inactivation of the enzyme, treated with placenta methylase in the conditions described above.

Gel Shift Assays. A 306 bp-long *Xma*I fragment and a 314 bp-long *Hind*III-*Eco*RI fragment were respectively purified from the most CpG-rich and from the CpG-suppressed region of pL9.2. Both fragments were end-labeled using the Klenow fragment of DNA polymerase I and [α^{32}P]dCTP or [α^{32}P]dATP. End-labeled fragments (0.3−0.6 ng) were mixed with H1 histone in 20 μL of binding buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 500 μg/mL acetylated BSA, and 10% glycerol] at varying protein/DNA ratios (w/w). Incubation was carried out on ice for 2 h, and samples were loaded on a 6% polyacrylamide gel in 0.25 × TBE buffer.

RESULTS

Effect of Histone H1 on in Vitro M*ethylation of CpG-Rich Genomic Sequences.* Plasmid pL9.2 (Lavia et al., 1987) contains a 2 kb-long CpG-rich island containing the bidirectional promoter shared by the Htf9a/*RanBP-1* gene (Coutavas et al., 1993; Di Matteo et al., 1995) and by a divergently transcribed gene whose product is as yet unidentified. The island is surrounded on both sides by CpG-depleted genomic sequences. In this construct, therefore, three types of DNAs can be distinguished on the basis of their sequence features: island-derived DNA, whose G+C content oscillates between 60 and 80% and where CpG dinucleotides are present at the expected frequency; CpG-depleted DNA, with the typical base composition of eukaryotic genomic DNA, i.e. 60% A+T, and CpG occurring at roughly 20% of the theoretical frequency; and, finally, vector DNA, where A+T equals G+C and CpG is present at the theoretical frequency. The different types of DNA are present on the same molecule and can be separated by digestion with the appropriate restriction enzymes.

We have previously shown (Carotti et al., 1989) that this plasmid can be efficiently methylated *in vitro* by a partially purified DNA methyltransferase from human placenta. The

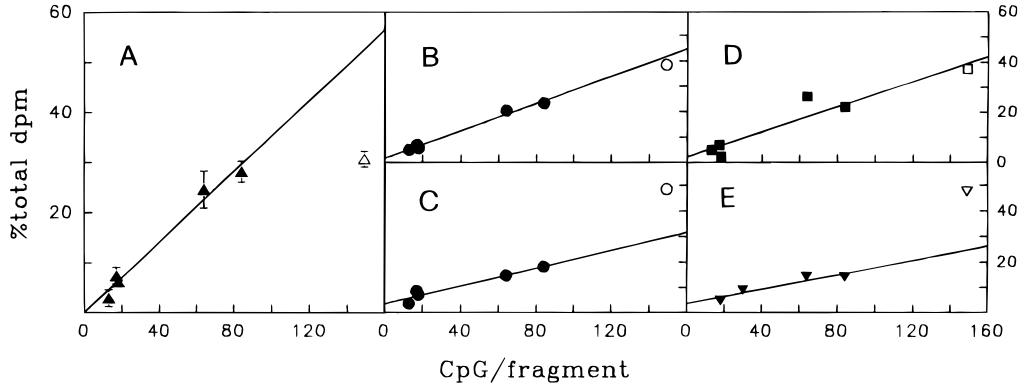

FIGURE 1: *In vitro* methylation pattern of pL9.2 in the absence (A) or presence of calf thymus H1 histone (B and C), of human placental H1 (D), and of the H1e-c fraction of calf thymus histone (E). Each point represents a restriction fragment or the sum of comigrating fragments. Methylation data are expressed for each fragment as the percentage of the total counts recovered from the gel. In A, each point represents the mean value calculated from at least three different experiments performed with three different enzyme preparations. In B and C, two different experiments are shown. Open symbols indicate CpG-rich island-derived restriction fragments.

extent of methylation was proportional to the CpG content for most fragments, except for the one corresponding to the CpG island, which was found to be significantly hypomethylated. This construct therefore provided a useful system to investigate the effect of histone H1 on *in vitro* methylation of DNAs with different base compositions. We found that the overall level of methylation of pL9.2 dropped from 2.3−10.3 to 1.3−6.4% of the CpGs being methylated, upon addition of calf thymus H1 to the incubation mixture at a protein/DNA ratio of 0.2/1 (w/w). These results therefore indicate that H1 consistently inhibited the reaction by 40% at this protein/DNA ratio, regardless of the total level of methylation obtained in the control reaction. After digestion of the methylated plasmid with *Dra*I, *Pvu*II, and *Eco*RI, the methylation pattern was analyzed by agarose gel electrophoresis and expressed for each fragment as the percentage of total counts recovered from the gel *vs* the number of CpGs in the fragment. An identical methylation pattern was obtained in the presence or absence of H1, except for the point corresponding to the Htf9 CpG island (149 CpGs), which was found to be less inhibited than expected (Figure 1, compare A with B and C). The methylation pattern was similar when the plasmid was used in the linear form and was independent of the degree of total inhibition (data not shown). In a separate experiment, purified H1 from human placenta was used; a 0.15 H1/DNA ratio induced 30% total methylation inhibition, and methylation was again found to be less inhibited in the Htf9 CpG-rich region than elsewhere in the plasmid, suggesting a general effect exerted by H1 proteins from different sources (Figure 1D).

The same results were observed when a HPLC-purified fraction containing both the H1e and H1c variants from calf thymus was used (Figure 1E). This fraction was previously shown to contain the only H1 variants capable of inhibiting DNA methylation and binding to CpG-rich oligonucleotides (Santoro et al., 1995) or to GC-rich DNA fragments (Wellman et al., 1994). Using this fraction, the overall methylation was inhibited by approximately 70%, at a protein/DNA ratio of 0.4.

Very similar results were obtained with pH9.2 and pEB9 plasmids. The former contains almost exclusively the Htf9 CpG-rich sequences and no surrounding CpG-depleted regions, while the latter carries two human islands in a 5.9 kb-long insert, respectively associated with the *GdX* and *P3* genes (Toniolo et al., 1988), and separated by a 2.6 kb-long stretch of CpG-depleted DNA. As previously found with pL9.2, fragments with a high CpG frequency were hypomethylated after *in vitro* methylation, as compared to CpG-depleted fragments (Carotti et al., 1989), but were much less sensitive to inhibition of methylation by calf thymus histone H1 (Figure 2). These results suggest a general effect of H1 on CpG-rich or GC-rich DNAs, rather than a sequence-specific effect which might have casually occurred with pL9.2.

Using a partially purified enzyme preparation, it might have been possible that an unrecognized component could induce the observed effects. We previously investigated the existence of sequence-specific DNA-binding activities in the methylase fraction, by gel retardation analysis, and detected no specific binding to the Htf9 island (Carotti et al., 1989). In addition, we specifically tested the presence of the Sp1 protein, because of its suggested role in preventing CpG island methylation; again, no specific binding to characterized Sp1 binding sites was observed (data not shown). Thus, the effects of H1 cannot be ascribed to the presence of protein(s) cofractionating with the methylase, which might mask specific DNA regions.

We next investigated whether the inhibition of methylation by H1 was altered by DNA premethylation. For this purpose, plasmid pL 9.2 was methylated *in vitro* at about 30% of the CpG sites using the *Sss*I methylase, which recognizes the same CpG target sequence as the placental methylase, but methylates island-derived DNA as efficiently as any other DNA. The premethylated DNA was then subjected to further methylation by placenta methylase in the presence of calf thymus H1. After hydrolysis by restriction endonucleases, the methylation pattern was found to be identical to that obtained with fully unmethylated DNA, suggesting that the effect exerted by H1 histone was insensitive to preexisting methylation (data not shown).

In Vitro Binding Studies. DNA methyltransferase is thought to modify DNA with a processive mechanism, possibly involving a strand separation step (Smith, 1994); thus, the inhibitory effect of H1 on methylation might have reflected a steric hindrance for the methylase, induced by the DNA/H1 interaction. We therefore wanted to ascertain whether H1 had a different affinity for the various DNA fragments as a function of their CpG content. Two 300 bp-long fragments were isolated from pL9.2, respectively derived from the central part of the Htf9 island (a 314 bp-

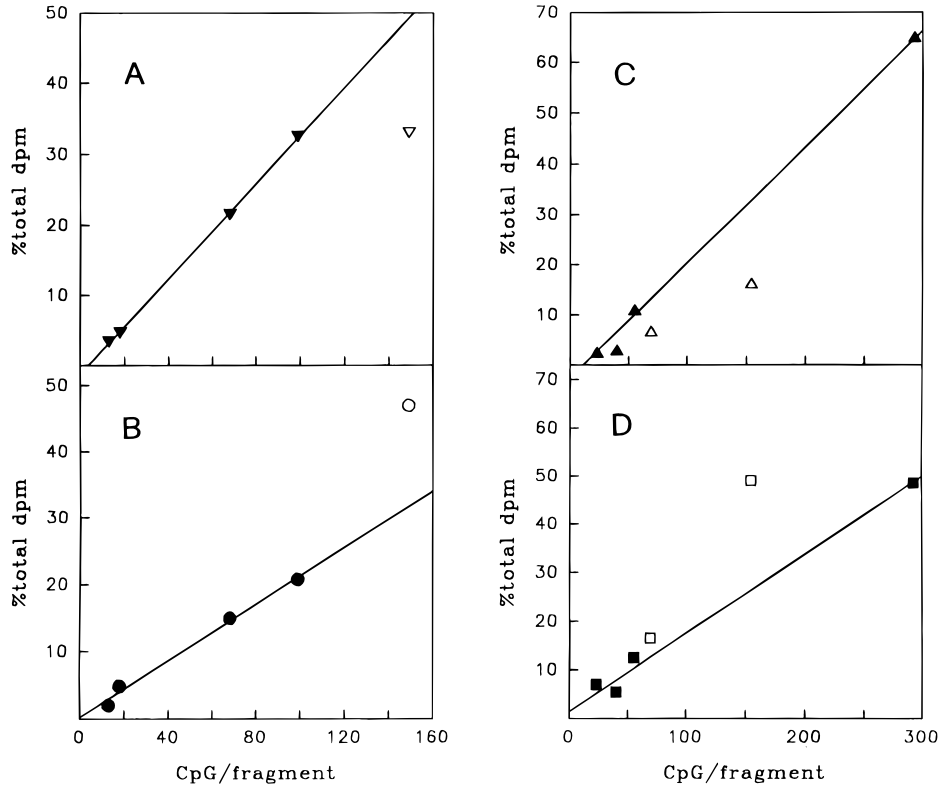

FIGURE 2: *In vitro* methylation pattern of pH9.2 in the absence (A) or presence (B) of calf thymus H1 histone and of pEB9 in the absence (C) or presence (D) of H1 histone. Data are expressed as in Figure 1. Open symbols indicate CpG-rich island-derived restriction fragments.

long *Xma*I fragment, 72% G+C, 44 CpGs) and from a CpG-depleted flanking genomic region (a 306 bp-long *Hind*III-*Eco*RI fragment, 46% G+C, 2 CpGs). Both fragments were used in gel shift experiments, with H1/DNA ratios ranging from 4/1 to 32/1. Basically, the experiments confirmed a somewhat higher affinity of H1 for CpG-rich DNA compared to CpG-depleted DNA, with both calf thymus H1 (Figure 3A,B) and human placenta H1 (Figure 3C,D). In this experiment, the whole H1 was used; the H1e-c variants, previously reported to prefer CpG-rich DNA (Santoro et al., 1995), represent approximately 60% of the total calf thymus and of human placenta H1. Therefore, the results of the binding experiments suggest that the inhibitory effect of H1 on DNA methylation does not simply reflect a different binding affinity of H1 for DNA regions with varying base compositions.

Effect of Histone H1 on in Vitro Methylation of DNAs with Homogeneous Base Composition. The simultaneous presence of three different classes of DNA sequences on the same molecule might have been the reason for the apparently contradictory result, that methylation of fragments with the highest H1-binding affinity showed the lowest inhibition by H1. In order to verify this, DNAs of various origin and with homogeneous base composition were methylated in the presence of H1, with H1/DNA ratios ranging from 0.2 to 1 (w/w). *Micrococcus luteus* DNA (72% C+G) and poly(dC-dG)·poly(dC-dG) were both found to be less sensitive to methylation inhibition by H1 than either *Escherichia coli DNA* (53% G+C) or calf thymus DNA (42% G+C) at every tested H1 concentration, confirming therefore the results previously obtained with pL9.2 (Figure 4A). Isolated fragments from the Htf9 island were also assayed. A 1.6 kb *Dra*I-*Pvu*II fragment, excised from pL9.2 (coordinates 830−2472 in the X05830 sequence, 70% G+C), corresponding to the point at 149 CpGs in Figure 1, and a 42 bp-long synthetic oligonucleotide (coordinates 1681−1722, 74% G+C, 8 CpGs), corresponding to a promoter element essential for cell cycle regulation of the Htf9a/*RanBP-1* gene,

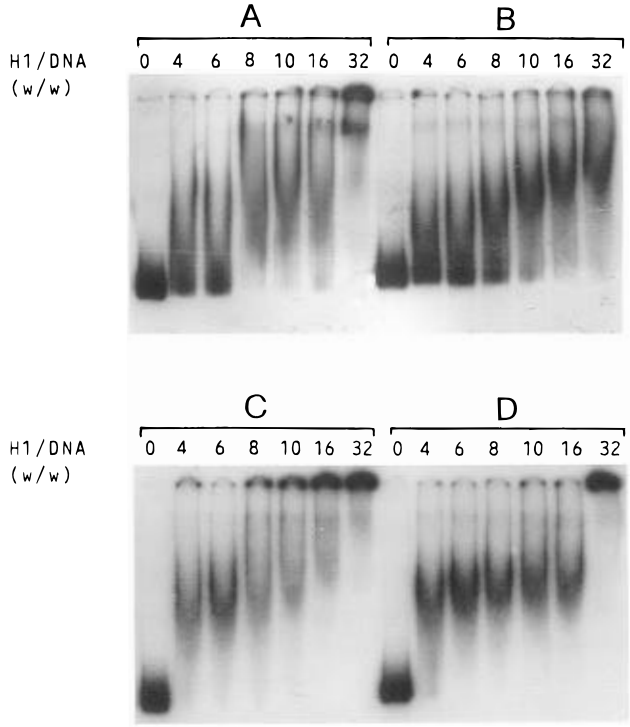

FIGURE 3: Gel shift assays with CpG-rich (A and C) and CpG-suppressed (B and D) DNA fragments, respectively, derived from the Htf9 CpG-rich island and from the flanking genomic sequence. Increasing amounts of H1 from calf thymus (A and B) and from human placenta (C and D) were used. The ratio of histone protein to DNA included in the binding reactions is indicated above each lane.

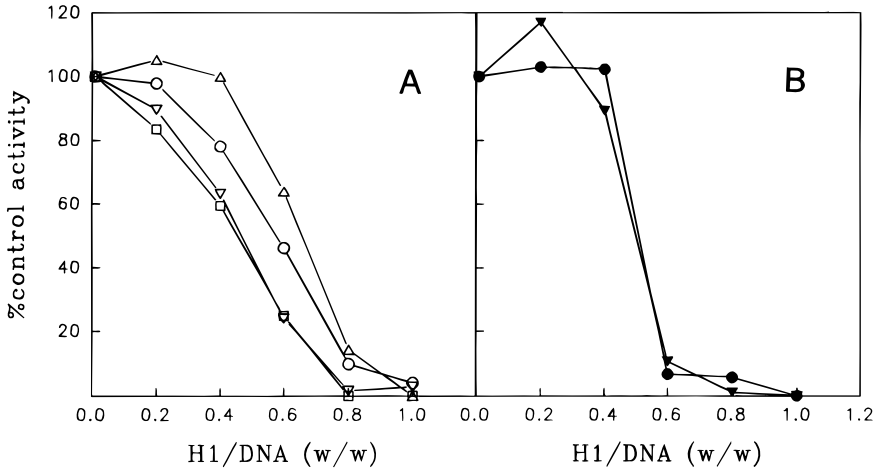

FIGURE 4: *In vitro* methylation of various DNAs by human placenta DNA methyltransferase in the presence of increasing amounts of calf thymus H1 histone: (A) (△) poly(dC-dG)·poly(dC-dG), (○) *M. luteus* DNA, (▽) *E. coli* DNA, and (□) calf thymus DNA; and (B) (●) Htf9 CpG-rich island fragment (1.6 kb), corresponding to the 149 CpGs point in Figure 1, and (▼) 42-mer (8 CpGs) from the Htf9a/*RanBP-1* promoter. Each point represents the mean value calculated from at least three different experiments, each performed in duplicate.

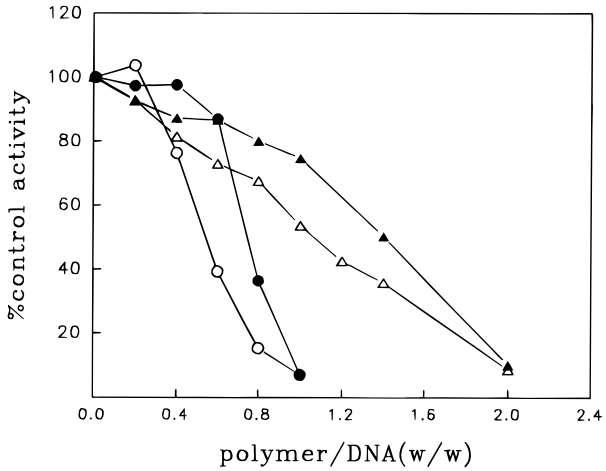

FIGURE 5: *In vitro* methylation of *M. luteus* DNA (closed symbols) or calf thymus DNA (open symbols) by human placenta DNA methyltransferase, in the presence of increasing amounts of poly(L-lysine) (circles) or of poly(L-arginine) (triangles).

were used in the same experiment. Both fragments behaved identically in spite of their widely different size (Figure 4B). These experiments again confirm the absence of specific DNA binding protein(s) in the enzyme preparation. In fact, when 97% of the island sequence was removed, the inhibitory effect of H1 was still observed. However, a peculiar response to the increase of the H1/DNA ratio was depicted; while methylation of most DNAs showed a steep yet gradual decrease, methylation of both island-derived fragments exhibited a sigmoidal dependency on H1-induced inhibition of *in vitro* methylation, with a sharp fall in the activity at H1/DNA ratios included between 0.4 and 0.6 (Figure 4). This behavior suggests a higher degree of cooperative inhibition for CpG-rich sequences.

Finally, we tested the effects of poly(L-lysine) and of poly(L-arginine) as substitutes of H1. Both polymers inhibited DNA methylation, but with a marked difference (Figure 5); poly(L-lysine showed a concentration dependency very similar to that of H1, although shifted toward higher polymer/DNA ratios, whereas poly(L-arginine) not only was much less effective in inhibiting methylation but also showed an almost linear concentration dependency. Nevertheless, both polymers showed a similar sensitivity to base composition as histone H1, methylation of calf thymus DNA being more inhibited than that of *M. luteus* DNA.

Methylation with Bacterial SssI Methylase. Why should there be a bias in inhibition of methylation by H1 in favor of AT-rich sequences? Since a preferential H1 binding to AT-rich DNA does not appear to be involved (see Figure 3), this bias might reflect a feature of the methylase enzyme. To test this hypothesis, experiments performed with the human placental enzyme were repeated with the bacterial *Sss*I methylase. This enzyme was chosen because it recognizes the same CpG sequence as the eukaryotic enzyme (Renbaum et al., 1990). As shown in Figure 6A, results were completely reversed as compared to those obtained thus far with the mammalian methylase. Not only did calf thymus histone H1 fail to inhibit the enzyme, but it induced instead a 5-fold stimulation of methylation of calf thymus DNA. Methylation of both poly(dC-dG)·poly(dC-dG) and *M. luteus* DNA was affected only at very high protein/DNA ratios. Both poly(L-lysine) and poly(L-arginine) were also capable of stimulating the *Sss*I-catalyzed methylation of calf thymus DNA, while leaving *M. luteus* methylation unaffected; thus, basic residues of H1 have a crucial role also in the stimulatory effect. The stimulating effect on calf thymus DNA was dependent on the maintenance of the double-stranded structure, being practically absent when DNA in the single-stranded form was used (Figure 6B).

DISCUSSION

Histone H1 *in vitro* inhibits the *de novo* activity of human DNA methyltransferase to variable extents, depending on the base composition, and affects methylation of AT-rich DNA more severely than that of GC-rich DNA. Moreover, with CpG-rich island-derived DNAs, methylation inhibition appeared to be critically dependent on the amount of H1 in the methylation assay. H1 is the only histone capable of inhibiting DNA methylation (Caiafa et al., 1991), and among its somatic variants, only H1e has been shown to exert this effect (Zardo et al., 1996). Our experiments were mostly performed with a mixture of all somatic variants from calf thymus; however, when we used purified H1e-c, the results were very similar to those obtained with the unfractioned H1. Thus, the somewhat lower susceptibility to methylation of CpG islands as compared to nonisland DNA stretches

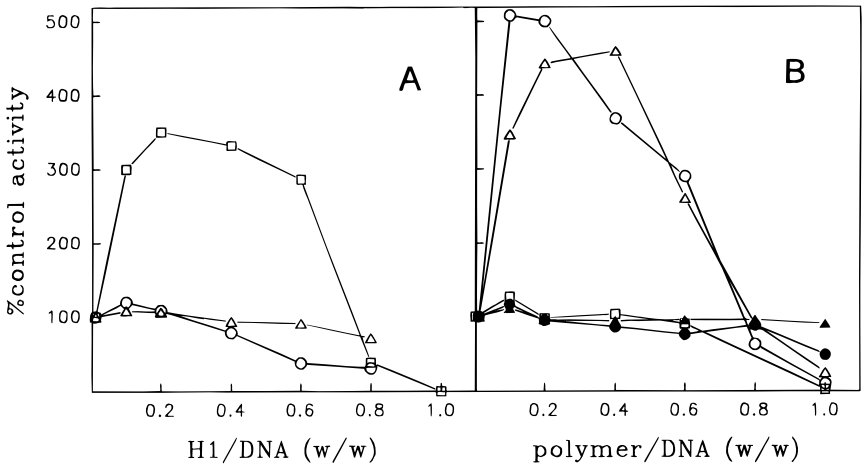

FIGURE 6: *In vitro* methylation of various DNAs by *Sss*I methylase in the presence of increasing amounts of calf thymus H1 histone (A) or synthetic polymers (B): (A) (□) calf thymus DNA, (△) poly(dC-dG)·poly(dC-dG), and (○) *M. luteus* DNA; and (B) poly(L-lysine) (circles) and poly(L-arginine) (triangles) incubated with calf thymus (open symbols) or *M. luteus* (closed symbols) DNA, (□) poly(L-lysine) with single-stranded calf thymus DNA.

cannot be ascribed, at least *in vitro*, to competition among the different variants. As already mentioned, histone H1e has been shown (Wellman et al., 1994) to bind preferentially a GC-rich region in a pBR322 fragment. In our experiments, CpG-rich sequences are favored by H1, as far as binding is concerned (Figure 3), but their methylation is less affected by the histone (Figure 4B). The highly cooperative dependency of methylation inhibition observed with CpG-rich fragments (Figure 4B) might actually reflect this preferential binding, although a precipitation of H1·DNA complexes at very high protein/DNA ratios cannot in this case be ruled out.

The effect of H1 was almost completely reversed with the bacterial *Sss*I methylase; in that case, the histone stimulated methylation of the AT-rich calf thymus DNA, while showing very little effect, if any, on both *M. luteus* DNA and synthetic poly(dC-dG)·poly(dC-dG). The experiments with the bacterial enzyme rule out, at least at relatively low H1/DNA ratios, i.e. in the physiological range, the possibility of a gross precipitation of H1·DNA complexes.

Taken together, the results obtained with the eukaryotic and the bacterial methylase are compatible with a preferential effect of H1 on the methylation of CpG moieties in AT-rich DNA. They also confirm, by a different assay, the results from previous reports indicating that H1 does not preferentially bind CpG-methylated DNA (Campoy et al., 1995). Finally, they may provide some insight into the mechanism of inhibition of eukaryotic DNA methyltransferase.

Enzymes with DNA (cytosine-5-)-methyltransferase activity are known in both prokaryotes and eukaryotes. The enzymes from both sources share several features (Smith, 1994; Adams, 1995). They have the same catalytic mechanism, involving the formation of a covalent adduct with a highly conserved cysteine residue (Wu & Santi, 1987; Smith et al., 1992). The mammalian enzyme is a large protein (190 kDa), and its 500 C-terminal amino acids are highly homologous to all known bacterial methylases (Bestor et al., 1988), showing a well-defined set of conserved sequence motifs, including the *S*-adenosyl-L-methionine binding site and the catalytic domain. The N-terminal domain contains regulatory regions, among which a putative metal binding site and a *de novo* activity regulatory domain (Bestor, 1992), as well as a nuclear localization signal (Leonhardt et al., 1992), have been identified.

Crystallographic evidence obtained with the *Hha*I methylase (Klimausaskas et al., 1994) has helped to elucidate the mechanism of the methyl transfer reaction, which requires a massive, very localized disruption of the DNA double helix allowing the target cytosine residue to swing out of the helix and to fit in the active site of the enzyme. Due to the sequence analogies, a similar mechanism can be hypothesized for the eukaryotic enzyme (Smith, 1994), consistent with the acceleration of the *de novo* reaction by mispaired and therefore loosely stacked cytosine residues, as demonstrated by experiments performed with DNA containing unusual structures (Smith et al., 1992).

It is therefore conceivable that factors capable of facilitating or discouraging this local deformation would stimulate or inhibit methylase activity, respectively. In this regard, the frequent occurrence, in CpG-rich island DNA, of CpG dinucleotides surrounded by CG base pairs has been viewed as a possible factor leading to the low methyl-accepting capacity of these sequences (Bestor et al., 1992).

In both the N- and C-termini of histone H1, sequences containing (S/T)-P-basic-basic motifs are repeated several times (von Holt et al., 1984). SPKK peptides have been shown to preferentially bind to AT-rich sequences (Churchill & Suzuki, 1989), probably weakening the adenine-thymine base pairing hydrogen bonds and therefore loosening the double-helical structure (Takeuchi & Sasamori, 1995). This preference is likely to be due to the narrower width of the minor groove in AT-rich DNA and to differences both in the charge distribution and in the position of hydrogen bond acceptors or donors. In addition, AT-rich DNA lacks the 2-amino groups of guanine, known to protrude into the minor groove. Similar sequences are found in the N-terminal domain in murine as well in human DNA methyltransferase (Bestor et al., 1988; Chuang et al., 1996) but not in the prokaryotic enzymes, such as the *Sss*I methylase, which lack the regulatory domain present in the mammalian enzyme (Renbaum et al., 1990). These considerations taken together lead to the following hypothesis. Histone H1 might preferentially inhibit the activity of the human enzyme on AT-rich substrates possibly by competing with the enzyme for

binding to the minor groove of the double helix. This hypothesis is supported by our findings concerning the effect of H1 with the prokaryotic enzyme. The absence of competing SPKK peptides in this enzyme allows the SPKK peptides from the histone to interact with AT-rich regions, locally loosening the surroundings of CpG dinucleotides and therefore facilitating the activity of the methylase on calf thymus DNA, with which we indeed observed a 4−5-fold stimulation of the activity. Interactions of eukaryotic methylase with the DNA minor groove are also suggested by the inhibitory effect on *in vitro* (Tanaka et al., 1982) and *in vivo* (Hashimoto et al., 1979) methylation showed by compounds known to specifically bind to the minor groove in AT-rich DNA, such as distamycin A. The double-helical structure requirement was clearly indicated by the absence of either inhibition or stimulation, when histone H1 (Santoro et al., 1993) or poly(L-lysine) (Figure 6B) were used with single-stranded DNA with either enzyme.

The importance of basic residues in inducing the effect was also indicated by the experiments with synthetic polypeptides; both poly(L-lysine) and poly(L-arginine) mimicked the effect of histone H1 with both enzymes. In addition, poly(L-lysine) showed the same cooperative behavior as histone H1 with the placenta enzyme, while poly(L-arginine) was much less effective; this result emphasizes the role of lysine residues, as suggested by the lack of inhibition previously observed with arginine-rich histone H3 (Caiafa et al., 1991).

The question arises as to why only subtype H1e is effective in inhibiting the DNA methyltransferase. Presumptive DNA binding motifs of the SPKK type or related structures (Churchill & Travers, 1991) are present in all five major H1 variants. Two possible alternative explanations can be hypothesized. Subtle sequence differences not immediately recognizable can be of primary importance. It has been shown, for instance, that the minor groove-binding affinity of the 434 repressor is lowered by 200-fold by the substitution, in its basic DNA binding motif (KRPR), of one arginine by an alanine (Aggarwal et al., 1988). On the other hand, it is well known that C- and N-terminal tails of all subtypes are differentially subjected to post-translational modifications such as phosphorylation, acetylation, or poly(ADP-ribosyl)ation (van Holde, 1988); these processes either directly involve residues in the putative binding site or can easily alter the affinity and/or the sequence specificity for the DNA substrate.

The inhibition of the eukaryotic methylase by H1 observed in the experiments reported here is not an "all-or-nothing" effect. DNA methylation is known to be a very finely tuned phenomenon; for example, a 40% overall loss of methylation has been reported upon redifferentiation of Friend erythroleukemia cells (Razin et al., 1988). Similarly, methylation of genomic DNA has been shown to increase in specific loci, in tumor cell lines (Makos et al., 1992). Therefore, the effects that we observe are well within the range of variations in the pattern of DNA methylation found in biological systems.

ACKNOWLEDGMENT

We are grateful to Dr. Maria D'Erme, Dr. Stefania Marenzi, and Dr. Giuseppe Zardo for histone H1 fractions, to Vincenzo Peresempio for technical assistance, and to Graziano Bonelli for photography.

REFERENCES

Adams, R. L. P. (1995) *BioEssays 17*, 139−145.
Aggarwal, A. K., Rodgers, D. W., Drottar, M., Ptashne, M., & Harrison, S. C. (1988) *Science 242*, 899−907.
Berent, S. L., & Sevall, J. S. (1984) *Biochemistry 23*, 2977−2983.
Bestor, T. H. (1992) *EMBO J. 11*, 2611−2617.
Bestor, T., Laudano, A., Mattaliano, R., & Ingram, V. (1988) *J. Mol. Biol. 203*, 971−983.
Bestor, T. H., Gundersen, G., Kolsto, A.-B., & Prydz, H. (1992) *GATA 9*, 48−53.
Bird, A. P. (1986) *Nature 321*, 209−213.
Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A., & Cedar, H. (1994) *Nature 371*, 435−438.
Caiafa, P., Reale, A., Allegra, P., Rispoli, M., D'Erme, M., & Strom, R. (1991) *Biochim. Biophys. Acta 1090*, 38−42.
Campoy, F. J., Meehan, R. R., McKay, S., Nixon, J., & Bird, A. (1995) *J. Biol. Chem. 270*, 26473−26481.
Carotti, D., Palitti, F., Mastrantonio, S., Rispoli, M., Strom, R., Amato, A., Campagnari, F., & Whitehead, E. P. (1986) *Biochim. Biophys. Acta 866*, 135−143.
Carotti, D., Palitti, F., Lavia, P., & Strom, R. (1989) *Nucleic Acids Res. 17*, 9219−9229.
Chuang, L. S.-H., Ng, H.-H., Chia, J.-N., & Li, B. F. L. (1996) *J. Mol. Biol. 257*, 935−948.
Churchill, M. E. A., & Suzuki, M. (1989) *EMBO J. 8*, 4189−4195.
Churchill, M. E. A., & Travers, A. A. (1991) *Trends Biochem. Sci. 16*, 92−97.
Cole, R. D. (1984) *Anal. Biochem. 136*, 24−30.
Coutavas, E., Ren, M., Oppenheim, J., D'Eustachio, P., & Rush, M. (1993) *Nature 366*, 585−587.
Croston, G. E., Kerrigan, L. A., Lira, L. M., Marshak, D. R., & Kadonaga, J. T. (1991) *Science 251*, 643−649.
Di Matteo, G., Fuschi, P., Zerfass, K., Moretti, S., Ricordy, R., Cenciarelli, C., Tripodi, M., Jansen-Durr, P., & Lavia, P. (1995) *Cell Growth Differ. 6*, 1213−1224.
Garrard, W. T. (1991) *BioEssays 13*, 87−88.
Hashimoto, H., Simon, D. S., Grunert, F., & Kröger, H. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1509−1512.
Hendrickson, F. M., & Cole, R. D. (1994) *Biochemistry 33*, 2997−3006.
Higurashi, M., & Cole, R. D. (1991) *J. Biol. Chem. 266*, 8619−8625.
Huang, H. C., & Cole, R. D. (1984) *J. Biol. Chem. 259*, 14237−14242.
Izaurralde, E., Ks, E., & Laemmli, U. K. (1989) *J. Mol. Biol. 210*, 573−585.
Johns, E. W. (1987) *Methods Cell Biol. 16*, 183−203.
Johnson, C. A., Goddard, J. P., & Adams, R. L. P. (1995) *Biochem. J. 305*, 791−798.
Klimasauskas, S., Kumar, S., Roberts, R. J., & Cheng, X. (1994) *Cell 76*, 357−369.
Lavia, P., Macleod, D., & Bird, A. (1987) *EMBO J. 6*, 2773−2779.
Leonhardt, H., Page, A. W., Weier, H.-U., & Bestor, T. H. (1992) *Cell 71*, 865−873.
Levine, A., Yeivin, A., Ben-Asher, E., Aloni, Y., & Razin, A. (1993) *J. Biol. Chem. 268*, 21754−21759.
Macleod, D., Charlton, J., Mullins, J., & Bird, A. P. (1994) *Genes Dev. 8*, 2282−2292.
Makos, M., Nelkin, B. D., Lerman, M. I., Latif, F., Zbar, B., & Baylin, S. B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1929−1933.
Mohr, E., Trieschmann, L., & Grossbach, U. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9308−9312.
Quesada, P., Farina, B., & Jones, R. (1989) *Biochim. Biophys. Acta 1007*, 167−175.
Razin, A., Levine, A., Kafri, T., Agostini, S., Gomi, T., & Cantoni, G. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9003−9006.
Renbaum, P., Abrahamove, D., Fainsod, A., Wilson, G. G., Rottem, S., & Razin, A. (1990) *Nucleic Acids Res. 18*, 1145−1152.

Renz, M., & Day, L. A. (1976) *Biochemistry 15*, 3220−3228.
Santoro, R., D'Erme, M., Reale, A., Strom, R., & Caiafa, P. (1993) *Biochem. Biophys. Res. Commun. 190*, 86−91.
Santoro, R., D'Erme, M., Mastrantonio, S., Reale, A., Marenzi, S., Saluz, H. P., Strom, R., & Caiafa, P. (1995) *Biochem. J. 305*, 739−744.
Singer, D. S., & Singer, M. F. (1978) *Biochemistry 17*, 2086−2095.
Smith, S. S. (1994) *Prog. Nucleic Acid Res. Mol. Biol. 49*, 65−111.
Smith, S. S., Kaplan, B. E., Sowers, L. C., & Newman, E. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4744−4748.
Sponar, J., & Sormová, Z. (1972) *Eur. J. Biochem. 29*, 99−103.
Takeuchi, H., & Sasamori, J. (1995) *Biopolymers 35*, 359−367.
Tanaka, M., Hibasami, H., Nagai, J., & Ikeda, T. (1982) *Aust. J. Exp. Biol. Med. Sci. 60*, 223−229.
Thoma, F., Koller, T., & Klug, A. (1979) *J. Cell. Biol. 83*, 403−427.
Toniolo, D., Persico, M., & Alcalay, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 851−855.
van Holde K. E. (1989) in *Chromatin*, Springer Series in Molecular Biology, Springer-Verlag, Inc., New York.
Varga-Weisz, P., van Holde, K., & Zlatanova, J. (1993) *J. Biol. Chem. 268*, 20699−20700.
von Holt, C., de Groot, P., Schwager, S., & Brandt, W. F. (1984) in *Histone Genes* (Stein, G. S., Stein, J. L., & Marzluff, W. F., Eds.) pp 65−105, John Wiley, New York.
Wellman, S. E., Sittman, D. B., & Chaires, J. B. (1994) *Biochemistry 33*, 384−388.
Wolffe, A. P. (1989) *EMBO J. 8*, 527−537.
Wu, J. C., & Santi, D. V. (1987) *J. Biol. Chem. 262*, 4778−4786.
Yaneva, J., & Zlatanova, J. (1992) *DNA Cell. Biol. 11*, 91−99.
Yaneva, J., Schroth, G. P., van Holde, K. E., & Zlatanova, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7060−7064.
Zardo, G., Santoro, R., D'Erme, M., Reale, A., Guidobaldi, L., Caiafa, P., & Strom, R. (1996) *Biochem. Biophys. Res. Commun. 220*, 102−107.

BI9606051