Binding of histone H1e-c variants to CpG-rich DNA correlates with the inhibitory effect on enzymic DNA methylation

Raffaella SANTORO,* Maria D'ERME,* Stefania MASTRANTONIO,† Anna REALE,† Stefania MARENZI,* Hans-Peter SALUZ,§ Roberto STROM†‡ and Paola CAIAFA*‡||

Departments of *Biochemical Sciences 'A. Rossi Fanelli' and †Human Biopathology, University of Rome 'La Sapienza', Rome, Italy, and ‡C.N.R. Centre for Molecular Biology, Rome, Italy, and §Hans Knöll Institut für Naturstoff-Forschung, Jena, Germany

Within the H1 histone family, only some fractions enriched in the H1e-c variants are effective in causing a marked inhibition, *in vitro*, of enzymic DNA methylation and, in gel retardation and Southwestern blot experiments, in binding double-stranded (ds) CpG-rich oligonucleotides. Both the 6-CpG ds-oligonucleotide and the DNA purified from chromatin fractions enriched in 'CpG islands' are good competitors for the binding of H1e-c to 6-meCpG ds-oligonucleotide. Because of their ability to bind any DNA sequence and to suppress the enzymic methylation in any sequence containing CpG dinucleotides, these particular H1 variants could play some role in maintaining linker DNA at low methylation levels and even in preserving the unmethylated state of the CpG-rich islands which characterize the promoter regions of housekeeping genes.

INTRODUCTION

H1 histone protein(s) modulate(s), in chromatin structure, the DNA methylation process. Within the 'physiological' range (0.3:1, w/w) of histones:DNA ratios, only H1 has been found [1] to exert a severe (90%) inhibition on methylation of double-stranded (ds)-DNA *in vitro*, catalysed by human placenta DNA-methyltransferase. Because of the specific localization of H1 histone in linker DNA, we have previously verified whether the known hypomethylation of linker DNA sequences [2–5] could be due to an intrinsic deficiency in CpG dinucleotides, which are usually methylated by the mammalian enzyme. Our experiments [6] have shown however the presence of CpG dinucleotides in linker regions, suggesting that the association of H1 histone with linker DNA causes a local inhibition of the DNA methylation process. Addition of 'physiological' amounts of H1 to H1-depleted oligonucleosomes markedly reduced their methyl-acceptance to a level similar to that occurring in native oligonucleosomal particles.

The H1 histone family consists however of several different somatic variants: H1a, H1b, H1c, H1d and H1e [7]. All these genic H1 subtypes possess three domain structures: an N-terminal tail (about 40 amino-acid residues), a globular region (80 amino-acid residues) and a C-terminal tail (about 100 amino-acid residues). Studies on the protein sequence of the H1 domains showed that the globular region is highly conserved, with 98% identity [7] among the different somatic variants. This genetic microheterogeneity, further increased by post-translational modifications [8], could be essential for their various structural [9–12] and functional [13–17] roles. The number and relative amounts of these variants differ in various tissues and species (reviewed in [7,8]) throughout the development stages of the organism and in neoplastic systems [18–21]. They may play different roles in chromatin organization, with a non-random distribution [20,22–24]: H1c is more abundant in the 'soluble' chromatin fraction [22], while the efficiency in causing chromatin condensation progressively decreases from H1a to H1bde and to H1c [25–27].

Previous experiments have shown that enzymic methylation of native oligonucleosomes [6] and of native chromatin [28] is not entirely suppressed by the intrinsic presence of H1 histone. Three different hypotheses can account for this result: (1) competition between the enzyme and histone H1 for some common DNA binding site(s); (2) the presence of DNA regions escaping the negative control of H1 histone; and (3) the existence of some particular variant(s) more or less capable of inhibiting enzymic DNA methylation. The first hypothesis has been disproved by experimental evidence indicating that enzymic DNA methylation *in vitro* is independent of the H1:enzyme ratio [29]. The aim of the present work was to test the two other hypotheses by assaying the effect of the different H1 variants on enzymic DNA methylation *in vitro* as well as their affinity for various synthetic oligonucleotides of known sequence.

MATERIALS AND METHODS

Separation and analysis of H1 histone variants

The usual starting material was calf thymus H1 histone purchased from Boehringer (Mannheim, Germany). H1 histone variants were separated, according to Quesada et al. [30], on a reverse-phase RPC4-300 Å column (5 mm × 250 mm Vydoc) using a Perkin-Elmer 410 apparatus equipped with a diode array 235 u.v. detector. The crude H1 preparations were dissolved, for loading on the column, in 0.1% (v/v) trifluoroacetic acid (TFA) in H_2O. Elution was performed at room temperature at a flow rate of 1 ml/min, using a linear gradient (0.1% TFA in H_2O–0.1% TFA in 95% acetonitrile). Protein fractions were collected, freeze-dried and analysed by SDS/PAGE (15% acrylamide + 0.4% methylene bisacrylamide). A more accurate characterization of the protein components was obtained by two-dimensional electrophoresis on 15% polyacrylamide, with the first run in 5% acetic acid + 2.5 M urea [31] and the second one in 0.1% SDS. Protein concentrations were determined by a commercial adaptation of Bradford's procedure [32].

Abbreviations used: DTT, dithiothreitol; TFA, trifluoroacetic acid; ds, double-stranded; r.p., reverse-phase.

|| To whom correspondence should be addressed.

DNA methyltransferase assay

DNA methyltransferase (EC 2.1.1.37) was purified from human placenta nuclei [33] and assayed according to Caiafa et al. [34] in a 50 mM Tris/HCl buffer (pH 7.8) containing 10% (v/v) glycerol, 5 mM EDTA, 0.5 mM DTT, 10 Ci/ml *S*-adenosyl-L-[methyl-^{3}H]methionine (New England Nuclear; specific activity 70 Ci/mmol–80 Ci/mmol) and 30 μg/ml of either *Micrococcus luteus* DNA or synthetic ds-oligonucleotides. Assay mixtures were incubated at 37 °C for 1 h and the reaction was terminated by heating to 60 °C for 20 min in the presence of 1% (w/v) SDS and 0.2 μg/ml of proteinase K. After cooling on ice, 300 μg of salmon sperm DNA was added to serve as carrier and the DNA was precipitated at 0 °C with 10% (w/v, final concentration) trichloroacetic acid. The pellets, washed again with trichloroacetic acid, were resuspended in 0.5 ml of 0.5 M NaOH and heated to 60 °C for 20 min so as to remove, by alkaline hydrolysis, any contaminating RNA. After cooling, DNA was precipitated again at 0 °C with 2 ml of 15% trichloroacetic acid and collected on a glass-fibre paper (GF/C, Whatman), repeatedly washed with 5% trichloroacetic acid and then with 95% ethanol. The radioactivity was then measured in a Beckman LS6800 liquid scintillation spectrometer. Previous experiments [33] had shown that with ds-DNA the reaction is linear for at least 80 min at 37 °C.

Before enzymic assays, all H1 histone protein fractions, eluted from h.p.l.c., were resuspended in 30 mM EDTA buffer to induce their renaturation [1]. Their effect on DNA methylation was tested using a 0.2 protein:DNA ratio (w/w).

DNA purification from 'CpG islands' chromatin fractions

Chromatin fractions enriched in CpG islands were obtained from human placenta according to Tazi and Bird [14]. After digestion with *Msp*I (700 units/mg of DNA), nuclei were layered on the top of 25 ml of 15–30% sucrose gradients and centrifuged for 15 h in a SW27 Beckman rotor at 25000 rev. min^{-1} (82500 *g*) and 4 °C. The fractions were analysed and 200 bp purified DNA fragments were used as competitor in gel retardation assays.

Gel retardation and Southwestern-blot assays

Oligonucleotides were synthesized by the phosphoamidite method on a DNA synthesizer (Applied Biosystems).

Single-stranded oligonucleotides were end-labelled at their 5′ ends with [γ-^{32}P]-ATP (specific activity 3000 Ci/mmol) using polynucleotide kinase (EC 2.7.1.78) under the conditions recommended by the manufacturer. The oligonucleotides (44 bp long) were purified on a 3 mm thick 15% polyacrylamide gel containing 1 × TBE [89 mM Tris/89 mM boric acid/2 mM EDTA (pH 8)] and 7 M urea. The band containing the probe was excised from the gel, crushed and soaked overnight, at room temperature, in 0.5 M ammonium acetate + 0.5 mM Na-EDTA, pH 7.8 [35]. The oligonucleotides were recovered by ethanol precipitation in the presence of 10 μg of tRNA as carrier and then annealed according to Kadonaga and Tjian [36].

End-labelled oligonucleotides were mixed with the whole H1 histone or with the different h.p.l.c. fractions in a 20 μl volume of binding buffer [50 mM Tris/HCl, pH 7.5/5 mM Na-EDTA/0.5 mM dithiothreitol (DTT)] using as a rule a protein:DNA ratio of 5:1 (w/w). Incubation was performed at 37 °C for 1 h and samples were loaded on a 6% polyacrylamide gel (at a 30:0.8 acrylamide:methylene bisacrylamide ratio) in 0.25 × TBE buffer, pH 8.3. The gels were run for about 2 h at 30 mA, fixed in 10% acetic acid and 10% methanol for 15 min, dried and autoradiographed.

Binding of DNA by the various fractions was evaluated by densitometric scanning (Bio Image, Millipore) of the autoradiograms, the percentage of free DNA being given relative to the DNA of samples incubated in the absence of protein. Due to the highly cationic charge of H1 histone and to the limited size of our synthetic oligonucleotides, the actual DNA–protein complex(es) could only be evidenced by running bidirectional gels.

For Southwestern-blot analysis [37], calf thymus H1 histone (about 6 μg) was separated in its variants by one- or two-dimensional electrophoresis and was transferred by simple diffusion, according to Bowen et al. [38], to nitrocellulose filters (Bio-Rad, Richmond, CA, U.S.A.). After equilibration for 2 h in Denhardt's solution [39] supplemented with 10 mM Tris/HCl buffer (pH 7.8), 15 mM Mg acetate, 7 mM KCl, 10 mM 2-mercaptoethanol and 0.1 mM Na-EDTA, the filter was transferred to a 'binding buffer' (50 mM Tris/HCl, pH 7.8, containing 25 mM NaCl, 2 mM 2-mercaptoethanol and 1 mM Na-EDTA) and incubated for 1 h at room temperature with 15 ml of herring sperm DNA solution (0.75 μg/ml) and for a further 2 h with the desired ^{32}P-labelled oligonucleotide (0.13 ng/ml). The filter was then washed with the binding buffer and, after air-drying, superimposed on an X-ray film and exposed for 10–18 h.

RESULTS

Calf thymus H1 histone somatic variants were purified by reverse-phase (r.p)-h.p.l.c. (Figure 1a), the protein components in the effluent composition being characterized by SDS/slab gel electrophoresis in 15% (w/v) polyacrylamide (Figure 1b). Every 1 ml fraction was assayed at a constant protein:DNA ratio of 0.2 (w/w) for its possible inhibitory effect in an assay of enzymic DNA methylation *in vitro*. As shown in Figure 1c, only a restricted number of fractions, eluting as a single peak ('p3'), was able to cause over 80% inhibition, the other fractions, namely 'p1' and 'p2', being in fact totally ineffective. In no case was there any evidence (checked by absorption and c.d. spectroscopy) of precipitation of histone–DNA complexes. SDS/PAGE characterization of the various fractions indicated, according to Lennox et al. [40] and to Lindner et al. [41], the presence in p1 of H1a, in p2 of H1d and in p3 of H1e and H1c. Preferential binding of H1-variants to different labelled synthetic oligonucleotides was explored by gel-retardation assays, using a series of synthetic oligonucleotides (Table 1) which varied in terms of their sequence and of the relative abundance in methylated or unmethylated CpGs with respect to NpGs (i.e. to all dinucleotide sequences having G as their second moiety). As a representative of genomic DNA we also used a 145 bp DNA prepared by digestion of human placenta chromatin with *Staphylococcus aureus* nuclease (EC 3.1.31.5).

As preliminary steps for the definition of the most suitable conditions, different salt concentrations [100, 50, 30, 15 mM NaCl in 10 mM Tris/HCl buffer (pH 7.5) with 1 mM EDTA and 1 mM DTT] were used in the binding reaction, using a protein:DNA ratio of 100:1 for total H1 histone and 50:1 for histone variants. Under all the conditions tested only total H1 histone and the H1e-c were able to bind the 44 bp synthetic oligonucleotides containing 6-CpG dinucleotides (results not shown). In subsequent experiments a 50 mM Tris/HCl (pH 7.5)/5 mM EDTA/0.5 mM DTT buffer (methylation assay buffer) without any added NaCl was used, with a protein:DNA ratio of 5:1. This protein:DNA ratio, which is high enough to eliminate any possible non-specific binding, is still above the so-called

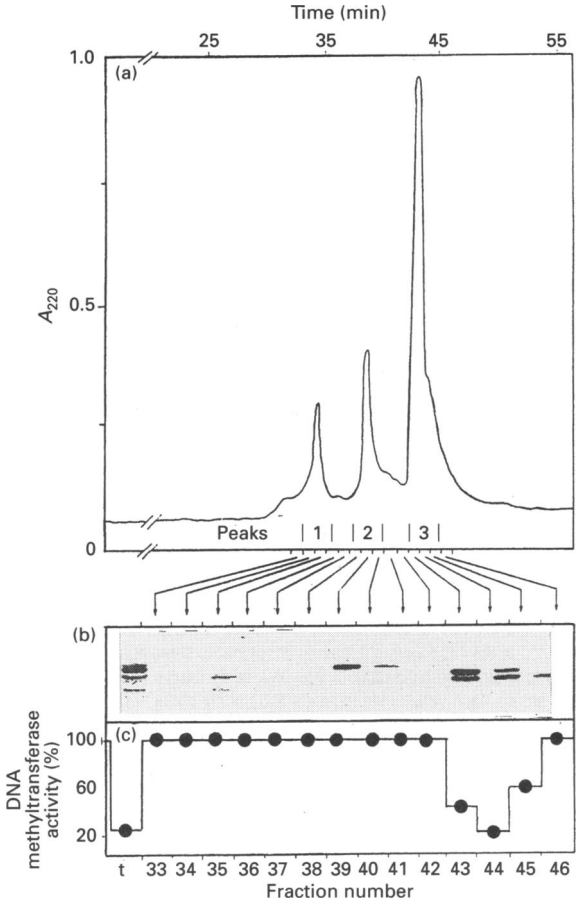

Figure 1 Separation and characterization of calf thymus H1 histone variants

(**a**) Elution profile from the r.p.-h.p.l.c. column; (**b**) SDS gel electrophoresis of all protein fractions, evidenced by Coomassie Brilliant Blue; (**c**) effect of total H1 histone ('t') and of the various fractions eluted from the r.p.-h.p.l.c. column, at a protein:DNA ratio of 0.2 (w/w), on the activity of human placenta DNA methyltransferase *in vitro*. Each point represents the average results of ten different separations by r.p. h.p.l.c.

'physiological' value, which, for histone H1, can be considered to be around 0.2:1, although this ratio should probably be

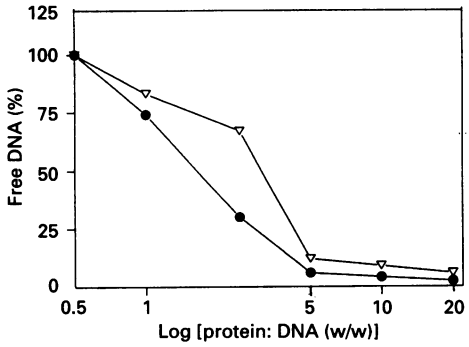

Figure 2 Evaluation of the binding of r.p.-h.p.l.c. protein-fraction 43 to 6-CpG and 6-meCpG oligonucleotides

Increasing aliquots of h.p.l.c. fraction 43 were incubated at 37 °C for 1 h in 50 mM Tris/HCl/5 mM EDTA/0.5 mM DTT (pH 7.5) with constant amounts of 6-CpG (●) or 6-meCpG (▽) end-labelled oligonucleotides. The binding was evaluated in terms of fractional recovery of unbound DNA.

corrected so as to take into account the paucity of CpG-rich sequences in genomic DNA. Our experimental conditions were stringent enough to allow comparison of the binding affinities of the different protein fractions to the various oligonucleotides. By titrations of 6-CpG and 6-meCpG oligonucleotides with various amounts of the H1e-c-containing fraction, the mid-point in binding corresponded to a protein:DNA ratio of approximately 2:1 with 6-CpG and 3:1 with 6-meCpG (Figure 2).

Table 2 shows the results of gel-retardation autoradiograms where the fractions eluted from r.p.-h.p.l.c. in p1, p2 and p3 were incubated with a 44 bp duplex DNA containing 6-CpG dinucleotides in the unmethylated or methylated form respectively. On the basis of the disappearance of the electrophoretic band corresponding to the free oligonucleotide [42] it was evident that the fractions containing the H1e-c variants were, together with the unfractionated H1, the only ones able to bind these particular sequences. The unmethylated oligonucleotide specifically binds fraction 43 of p3, which is enriched in the H1e variant, while the methylated oligonucleotide also binds fraction 44 where H1c is more abundant.

None of the other fractions could bind oligonucleotides containing either 6-CpGs or 6-meCpGs. They efficiently inter-

Table 1 Structural characteristics of the synthetic oligonucleotides assayed for their ability to bind the H1 variants

Oligonucleotide	Sequence	bp	CpG	meCpG	CpG/NpG (%)
ds-DNA 6-CpG	GTCAA**CG**AGGGAGC**CG**ACTGC**CG**A**CG**TG**CG**CTC**CG**GAGGCTTGC CAGTT**GC**TCCCTCG**GC**TGACG**GC**T**GC**AC**GC**GAG**GC**CTCCGAACG	44	6	0	41.37
ds-DNA 6-meCpG	GTCAA**CG**AGGGAGC**CG**ACTGC**CG**A**CG**TG**CG**CTC**CG**GAGGCTTGC CAGTT**GC**TCCCTCG**GC**TGACG**GC**T**GC**AC**GC**GAG**GC**CTCCGAACG	44	0	6	41.37
ds-DNA 3-CpG	TCCAGGGAGAAATAT**CG**CTT**CG**TCCTCAC**CG**AAGCCTGATTCTG AGGTCCCTCTTTATA**GC**GAA**GC**AGGAGTG**GC**TTCGGACTAAGAC	44	3	0	25.00
ds-DNA 3-meCpG	TCCAGGGAGAAATAT**CG**CTT**CG**TCCTCAC**CG**AAGCCTGATTCTG AGGTCCCTCTTTATA**GC**GAA**GC**AGGAGTG**GC**TTCGGACTAAGAC	44	0	3	25.00
ds-DNA 2-CpG	GTCAA**CG**AGGGAGGGCACTGGCCAGCTGCCCTC**CG**GAGGCTTGC CAGTT**GC**TCCCTCCCGTGACCGGTCGACGGGAG**GC**CTCCGAACG	44	2	0	15.38
Genomic ds-DNA	–	145	–	–	4.23*

* See [58].

Table 2 Binding of the main H1 fractions to 44 bp ds-oligonucleotides with different CpG content

End-labelled ds-oligonucleotides, all 44 bp long but with a different content in CpG or meCpG, were incubated with total H1 histone and with the various protein fractions obtained by r.p.-h.p.l.c. separation. The binding is expressed as the percentage of free DNA remaining in the assay, relative to DNA incubated in the absence of protein, estimated by densitometric scanning (Bio Image, Millipore) of the autoradiograms. Each value is the mean (±S.D.) of 15 distinct experiments.

	Protein fractions						
Oligonucleotide	Total H1	35	39	40	43	44	45
6-CpG	28±3	100±1	100±1	100±1	29±2	100±1	100±1
6-meCpG	16±2	100±1	100±1	100±1	22±2	32±3	100±1
3-CpG	15±2	100±1	82±1	72±1	8±1	4±1	65±1
3-meCpG	3±1	100±1	65±2	100±1	3±1	5±1	30±2
2-CpG	3±1	100±1	30±3	37±4	5±1	7±2	27±3

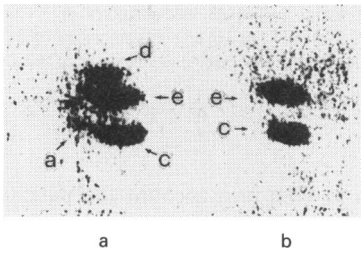

Figure 3 Two-dimensional gel electrophoresis of H1 variants from total calf thymus H1 histone (a) and of the protein fraction that shows an inhibitory effect on enzymic DNA methylation *in vitro* (b)

Electrophoresis was run in acid-urea gels in the first dimension and in SDS gels in the second dimension. After electrophoresis, proteins were detected using Coomassie Brilliant Blue. H1 variants were identified according to Lennox et al. [40].

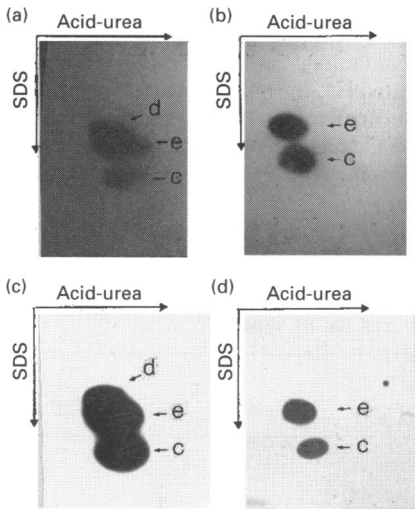

Figure 4 Southwestern-blot assays between two-dimensional gel electrophoresis of H1 histone variants and 6-CpG or 6-meCpG ds-oligonucleotides

End-labelled ds-oligonucleotides containing 6-CpG (**a, b**) or 6-meCpG (**c, d**) were used as probes, in the absence (**a, c**) or presence (**b, d**) of herring sperm DNA as competitor, to assay their binding to H1 histone variants.

acted instead with genomic DNA (results not shown) or with 2-CpG oligonucleotides and, even if rather poorly, also with 3-CpG- or 3-meCpG-containing oligonucleotides.

Table 3 Competition assay for the binding to r.p.-h.p.l.c. fraction 43 of DNA (200 bp) purified from a chromatin fraction enriched in 'CpG islands' or of 6-CpG and 6-meCpG 44 bp ds-oligonucleotides

The fraction eluted from r.p.-h.p.l.c. as number 43 of peak p3 was incubated with labelled 6-CpG or 6-meCpG ds-oligonucleotides, at a protein:DNA ratio of 5 (w/w), in the presence, as competitor DNAs, of increasing amounts of DNA (200 bp) purified from chromatin fraction enriched in 'CpG islands' or of the unlabelled 6-CpG or 6-meCpG ds-oligonucleotides. The values reported in the Table were obtained by densitometric scanning (Bio Image, Millipore) of the autoradiograms, the percentage of free DNA being relative to DNA incubated in the absence of protein. Each value is the mean (±S.D.) of six distinct experiments.

		Competitor:probe ratio (w/w)			
		0	2.5	5	10
Probe	Competitor	Value			
6-CpG oligonucleotide	6-meCpG oligonucleotide	3±1	8±1	10±2	15±1
	CpG-rich genomic DNA (200 bp)	0	5±2	30±5	18±4
6-meCpG oligonucleotide	6-CpG oligonucleotide	4±2	6±1	100±1	100±1
	CpG-rich genomic DNA (200 bp)	18±3	5±2	46±5	86±4

In the single-stranded form, the same sequences failed to bind any H1 variant (results not shown).

H1 histone preparation containing all the somatic variants (Figure 3a) and the h.p.l.c. fractions which had been found to be the only ones able to bind CpG-rich oligonucleotides (Figure 3b) and to inhibit DNA methylation were subjected to two-dimensional gel electrophoresis. By transferring total H1 histones to nitrocellulose membrane and by using end-labelled 6-CpG or 6-meCpG oligonucleotides as probes in the Southwestern assay, we observed, at a very high protein:DNA ratio (around 3×10^3:1, w/w) and in the absence of any competitor DNA (Figures 4a and 4c), an aspecific binding of both oligonucleotides to all the H1 variants except H1a, this variant (as shown in Figure 3a) being a minor component in total calf thymus H1 histone, but still in high excess with respect to probe DNA. We could show, however, that this 'non-specific' binding was an artifact of the very high protein excess (needed to allow protein staining): addition of herring sperm DNA as competitor, in a 1000-fold excess (w/w) with respect to probe DNA, allowed identification of H1e and

H1c as the only CpG-binding components (Figures 4b and 4d), with practically no difference between them.

Further gel-retardation competition experiments showed a higher affinity of fraction 43 for the 6-GpC oligonucleotide and for the 200 bp DNA purified from the CpG-rich chromatin fraction than for the 6-meCpG oligonucleotide; results are given in Table 3.

DISCUSSION

Our previous results had shown that enzymic methylation of native oligonucleosomes [6] and of native chromatin [28] was not entirely suppressed by the intrinsic presence of H1 histone. The possibility of competition between this histone and the enzyme for common DNA binding site(s) had however been disproved by previous experimental evidence indicating that enzymic DNA methylation was independent of the H1:enzyme ratio [29]. In this paper we examine whether this effect is due to the existence of H1 variants that differ in their ability to inhibit enzymic DNA methylation, and/or to the presence of DNA regions that escape the negative control of H1 histone.

Our present experimental evidence indicates that the inhibitory effect exerted by the H1 histone protein(s) on the methylation of ds-DNA *in vitro* [1] is strictly related to the presence of some particular somatic variants of this histone, but depends also, in terms of affinity for these variants, on the number of CpG and/or meCpG moieties present in the DNA sequence. It may be recalled that Levine et al. [43] have shown a preferential binding of total H1 histone to plasmidic methylated DNA as compared with unmethylated DNA, while Higurashi and Cole [42] as well as Lewis et al. [37] have found that the interaction of H1 histone with CCGG repeats or with oligonucleotides having a very high CpG content (12 CpGs in 40 bp) is independent of the methylation level.

In our system, Southwestern-blot assays indicate that among the H1 histone components, all of which are able to interact with genomic DNA, only the H1e and H1c variant(s) can bind ds-DNA sequences with 6-CpGs out of 44 nucleotides. (After this work had been completed, Wellman et al. [44] have reported evidence of a preferential binding of pure H1e to the 'GC-rich region' of pBR322 DNA.)

Gel mobility-shift assays, performed with a limited (5-fold) excess (w/w) of protein with respect to DNA, have shown that the other H1 histone variants, despite their ability to bind aspecifically a genomic DNA, exhibit an affinity for DNA oligonucleotides which is inversely related to the CpG content. The h.p.l.c. fractions 43 and 44 of p3 are, in particular, the only ones able to bind the 6-meCpG oligonucleotide, while only fraction 43 can bind the same oligonucleotide sequence in the unmethylated form. Indeed, competition experiments showed a higher affinity of fraction 43 for the unmethylated oligonucleotide than for the methylated one. Our gel-electrophoresis analyses, confirming data from other authors [41,45], indicated that fractions 43 and 44 are enriched in H1e and in H1c, respectively. Having not yet achieved a satisfactory separation of these two variants, the suppression of DNA methylation by some h.p.l.c. fractions can be ascribed to either variant or to the concomitant presence of both of them. We may visualize the inhibitory variant(s), that we will uncommitedly indicate as H1e-c, as denying access of the DNA methyltransferase to CpG dinucleotides through its high affinity for these moieties. Since H1 is unable to bind single-stranded oligonucleotides or to inhibit their methylation, the inhibitory effect of H1e-c on the methylation of ds-DNA could be due to the 'sealing' of two complementary strands [29].

The exclusive ability of H1e-c to bind CpG-rich sequences and to inhibit their methylation could obviously account for their maintenance in an unmethylated condition, as occurs in the CpG-rich unmethylated DNA domains of eukaryotic chromatin, known as CpG islands [46,47], which are localized in the promoter regions of housekeeping genes and are considered to be gene markers [48]. In fact DNA purified from CpG-rich chromatin fractions exhibits a very high affinity for fraction 43 of p3 and competes efficiently for the binding of this fraction to our synthetic 6-meCpG oligonucleotide. The H1e-c variant(s) could thus be the 'ubiquitous nuclear factor' which has been hypothesized [48] to be responsible for inducing an 'available' chromatin structure and of denying access to DNA methyltransferase. This conclusion, which would point to H1e-c as the specific protein factor(s) responsible for the unmethylated condition of CpG islands, should be considered together with other proposed mechanisms (which may however be compatible with it) such as the intrinsic effect exerted by the distance between CpGs on the recognition between DNA methyltransferase and its substrate [49–52], the presence of some poison sequence able to prevent DNA methylation [53], or the existence of other proteins interacting preferentially with methylated DNA sequences [54,55]. Among these regulatory proteins, we might also include the histone variant contained in the r.p.-h.p.l.c. fraction 44, which possesses the ability to bind the 6-meCpG oligonucleotide and can be shown to be enriched in H1c. r.p.-h.p.l.c. fraction 43, which is enriched in H1e, appears instead to be the only one able to interact with unmethylated CpG-rich sequences, and is indeed the first report of a protein having this property.

The hypothesis that some H1 variant(s) are involved in the maintenance of the unmethylated state of CpG islands does not necessarily contradict the finding by Tazi and Bird [14], of low amounts of H1 histone (< 10%) in the chromatin structure at CpG islands. Other authors [15] have reported that the housekeeping α-actin gene is only partially depleted of H1, and we may therefore hypothesize an actual association of some specific histone variant(s), such as H1e-c, with the CpG islands. H1e-c may even play a role in maintaining the fixed pattern of methylation determined in the various tissues by the combination of demethylation and methylation *de novo* occurring during early embryonal development [56].

Our experimental data underline three important characteristics of the H1e-c variants: (1) they bind any DNA sequence; (2) they are the only variants which suppress enzymic DNA methylation; (3) they are the unique variants able to bind CpG-rich sequences.

In linker DNA, which contains only a few CpG dinucleotides, there will be some competition between the different H1 variants, interaction with H1e-c being the only one which inhibits the methylation process. A non-uniform localization of H1e-c within chromatin structure would be compatible with our previous finding [6] that methylation of native oligonucleosomes is not entirely suppressed by the intrinsic presence of any H1 histone. Native oligonucleosomes would then possess a relatively high methyl-accepting ability (approximately half of that found after removal of H1) because a relevant portion of their internucleosomal DNA, being localized on the outside of the solenoid structure, would interact only with other, non-inhibitory, somatic variants of this histone.

Experiments in progress (A. Reale, S. Marenzi, R. Santoro, M. D'Erme, R. Strom and P. Caiafa, unpublished work) indicate indeed that hypomethylation of linker DNA can play a critical role by inducing cooperative H1–H1 interactions and, as a consequence, the attainment of the highest levels of chromatin organization.

It may be worth considering that, upon replication, deposition of H1 histone on newly-synthesized DNA is known to occur [57] simultaneously with, or only slightly after, the deposition of core histone. Supposing that all the somatic variants of this histone are present and potentially available for DNA binding during the replicative process, the effective interaction between a given variant and the newly synthesized nucleosomal structure will depend on the availability of an appropriate linker sequence. Depending on the relative CpG abundance, H1e-c can therefore be expected to maintain a significant hypomethylation of linker DNA and, as a distinct functional role, the unmethylated state of CpG-rich islands.

These two distinct hypothetical functional roles seem to raise an apparent paradox, indicating that multiple strategies affect the regulation of transcription through DNA methylation. If the linker hypomethylation is an important condition for chromatin condensation, H1e-c variants are involved in gene inactivation, while these same variants, if effective in maintaining the unmethylated state of CpG islands present in decondensed chromatin, would favour gene expression.

This work was supported by the Italian Ministry of University and Scientific and Technological Research (60%, Progetti di Ateneo) and by the Fondazione 'Istituto Pasteur-Fondazione Cenci Bolognetti'.

REFERENCES

1 Caiafa, P., Reale, A., Allegra, P., Rispoli, M., D'Erme, M. and Strom, R. (1991) Biochim. Biophys. Acta **1090**, 38–42
2 Razin, A. and Cedar, H. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2725–2728
3 Solage, A. and Cedar, H. (1978) Biochemistry **17**, 2934–2938
4 Adams, R. P. L., David, T., Fulton, J., Kirk, D., Qureshi, M. and Burdon, R. H. (1984) Curr. Top. Microbiol. Immunol. **108**, 143–156
5 Caiafa, P., Attinà, M., Cacace, F., Tomassetti, A. and Strom, R. (1986) Biochim. Biophys. Acta **867**, 195–200
6 D'Erme, M., Santoro, R., Allegra, P., Reale, A., Marenzi, S., Strom, R. and Caiafa, P. (1993) Biochim. Biophys. Acta **1173**, 209–216
7 Cole, R. D. (1987) Int. Peptide Prot. Res. **30**, 433–449
8 Wu, R. S., Panusz, H. T., Hatch, C. L. and Bonner, W. M. (1986) CRC Crit. Rev. Biochem. **20**, 201–263
9 Thoma, F., Koller, T. and Klug, A. (1979) J. Cell Biol. **83**, 403–427
10 Allan, J., Hartman, P. G., Crane-Robinson, C. and Aviles, F. X. (1980) Nature (London) **288**, 675–679
11 Clark, D. J. and Thomas, J. O. (1986) J. Mol. Biol. **187**, 569–580
12 Izaurralde, E., Kas, E. and Laemmli, U. K. (1989) J. Mol. Biol. **210**, 573–585
13 Zlatanova, J. (1990) Trends Biochem. Sci. **15**, 273–276
14 Tazi, J. and Bird, A. (1990) Cell **60**, 909–920
15 Kamakada, R. T. and Thomas, J. O. T. (1990) EMBO J. **9**, 3997–4006
16 Laybourn, P. J. and Kadonaga, J. T. (1991) Science **254**, 238–245
17 Jost, J. P. and Hofsteenge, J. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 9499–9503
18 Pehrson, J. R. and Cole, R. D. (1982) Biochemistry **21**, 456–460
19 Lennox, R. W. and Cohen, L. H. (1983) J. Biol. Chem. **258**, 262–268
20 Lennox, R. W. (1984) J. Biol. Chem. **259**, 669–672
21 Giancotti, V., Bandiera, A., Ciani, L., Santoro, D., Crane-Robinson, C., Goodwin, G. H., Biocchi, M., Dolcetti, R. and Casetta, B. (1993) Eur. J. Biochem. **213**, 825–832
22 Huang, H.-C. and Cole, R. D. (1984) J. Biol. Chem. **259**, 14237–14242
23 Baubichon-Cortay, H., Mallet, L., Denoroy, L. and Roux, B. (1992) Biochim. Biophys. Acta **1122**, 167–171
24 Schulze, E., Trieschmann, L., Schulze, B., Schmidt, E. R., Pitzel, S., Zechel, K. and Grossbach, U. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2481–2485
25 Liao, L. W. L. and Cole, R. D. (1981) J. Biol. Chem. **254**, 6751–6755
26 Liao, L. W. L. and Cole, R. D. (1981) J. Biol. Chem. **256**, 10124–10128
27 Liao, L. W. L. and Cole, R. D. (1981) J. Biol. Chem. **256**, 11145–11150
28 Davis, T., Rinaldi, A., Clark, L. and Adams, R. L. P. (1986) Biochim. Biophys. Acta **866**, 233–241
29 Santoro, R., D'Erme, M., Reale, A., Strom, R. and Caiafa, P. (1993) Biochem. Biophys. Res. Comm. **190**, 86–91
30 Quesada, P., Farina, B. and Jones, R. (1989) Biochim. Biophys. Acta **1007**, 167–175
31 Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. **130**, 337–346
32 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
33 Carotti, D., Palitti, F., Mastrantonio, S., Rispoli, M., Strom, R., Amato, A., Campagnari, F. and Whitehead, E. P. (1986) Biochim. Biophys. Acta **866**, 135–143
34 Caiafa, P., Mastrantonio, S., Attinà, M., Rispoli, M. and Strom, R. (1988) Biochim. Biophys. Acta **951**, 191–200
35 Saluz, H. P. and Jost, J. P. (1990) A Laboratory Guide for *in vivo* Studies of DNA Methylation and Protein/DNA Interactions. Biomethods, vol. 3, Birkauser Verlag, Basel
36 Kadonaga, J. T. and Tjian, R. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5889–5893
37 Lewis, J. D., Meehan, R. R., Henzel, W. J., Maurer-Fogy, I., Jeppesen, P., Klein, F. and Bird, A. (1992) Cell **69**, 905–914
38 Bowen, B., Steinberg, J., Laemmli, U.K. and Weintraub, H. (1980) Nucleic Acids Res. **8**, 1–10
39 Denhardt, D. J., Dressler, D. and Ray, D. S. (1978) The Single-stranded DNA Phages, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
40 Lennox, R. W., Oshima, R. G. and Cohen, L. H. (1982) J. Biol. Chem. **257**, 5183–5189
41 Lindner, H., Helliger, W. and Puschendorf, B. (1990) Biochem. J. **269**, 359–363
42 Higurashi, M. and Cole, R. D. (1991) J. Biol. Chem. **266**, 8619–8625
43 Levine, A., Yeiven, A., Ben-Asher, E., Aloni, Y. and Razin, A. (1993) J. Biol. Chem. **268**, 21754–21759
44 Wellman, S. E., Sittman, D. B. and Chaires, J. B. (1994) Biochemistry **33**, 384–388
45 De Lucia, F., Faraone-Mennella, M. R., D'Erme, M., Quesada, P., Caiafa, P. and Farina, B. (1994) Biochem. Biophys. Res. Commun. **198**, 32–39
46 Bird, A. P. (1986) Nature (London) **321**, 209–213
47 Antequera, F. and Bird, A. P. (1993) in DNA Methylation: Molecular Biology and Biological Significance (Jost, J. P. and Saluz, H. P., eds.), pp. 169–185, Birkhauser Verlag, Basel
48 Bird, A. P. (1987) Trends Genet. **3**, 342–347
49 Bolden, A. H., Nalin, C. M., Ward, C. A., Pooniam, M. S., McComas, W. W. and Weissbach, A. (1985) Nucleic Acids Res. **13**, 3479–3494
50 Bolden, A. H., Nalin, C. M., Ward, C. A., Poonian, M. S. and Weissbach, A. (1986) Mol. Cell. Biol. **6**, 1135–1140
51 Ward, C. A., Bolden, A. H., Nalin, C. M. and Weissbach, A. (1987) J. Biol. Chem. **262**, 11057–11063
52 Carotti, D., Palitti, F., Lavia, P. and Strom, R. (1989) Nucleic Acids Res. **17**, 9219–9229
53 Szyf, M., Tanigawa, G. and McArthy, P. L. (1990) Mol. Cell. Biol. **10**, 4396–4400
54 Meehan, P. R., Lewis, J. D., McKay, S., Kleiner, E. L. and Bird, A. P. (1989) Cell **58**, 499–507
55 Boyes, J. and Bird, A. (1991) Cell **64**, 1123–1134
56 Frank, D., Keshet, I., Shani, M., Levine, A., Razin, A. and Cedar, H. (1991) Nature (London) **351**, 239–241
57 Bavykin, S., Srebreva, L., Banchev, T., Tsanev, R., Zlatanova, J. and Mirzabekov, A. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 3918–3922
58 Adams, R. P. L. and Burdon, R. M. (1985) in Molecular Biology of DNA Methylation (Rich, A., ed.), Springer-Verlag, New York

Received 14 March 1994/21 July 1994; accepted 26 July 1994