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Specific Inhibitory Effect of H1e Histone Somatic Variant on *in Vitro* DNA-Methylation Process

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H1e and H1c histone variants were purified from mouse L929 fibroblasts using a reverse phase HPLC, and their effect on *in vitro* DNA methylation was investigated, together with their ability to bind unmethylated or methylated CpG-rich 44bp oligonucleotides. In a "physiological" range of H1:DNA ratios, only H1e, at variance from H1c, was found to cause a marked inhibition of *in vitro* enzymic DNA methylation. It was also shown that both variants have a similar affinity in binding a methylated CpG-rich oligonucleotide, but that the binding to the same oligonucleotide in the unmethylated form occurs preferentially with H1e rather than with H1c. H1e is therefore likely to be directly involved in maintaining CpG-rich sequences in the unmethylated state.

Although the DNA methylation process is known to play, in eukaryotic cells, an important role in the modulation of gene expression [1, 2] no protein specific for CpG-rich unmethylated DNA stretches has been so far reported—and only proteins exhibiting some level of preferential binding to DNA sequences containing meCpG moieties or methylated bases have been described [3–5]. In particular, the existence of a "ubiquitous nuclear factor," capable of preventing the interaction of DNA methyltransferase with these regions, has been hypothesized, but never proven. The "reluctance" of CpG-rich islands to be substrate for the methylating enzyme has therefore been attributed to the presence of "poison sequences" [6] within the DNA itself, or even to an intrinsically high CpG density [7–10]. Two independent series of experimental data [11, 12] have suggested a critical role of Sp1-binding sequences in preventing the *de novo* methylation of CpG-rich islands in the developing embryo—although a direct active role of the Sp1 protein itself has not been unequivocally demonstrated.

As shown by Davis and co-workers [13], the activity of the mouse ascites cells DNA methyltransferase can be almost completely inhibited by addition of a crude histone preparation. Conversely, Kautiainen and Jones [14] have reported that the methyl-accepting ability of native chromatin undergoes an almost 20-fold increase upon extraction with 2 M NaCl. Ample experimental evidence points therefore, in eukaryotic cells, to an inhibitory role of H1 histone on the enzymatic DNA methylation process [15]. Previous experiments have shown however that, upon addition of mammalian DNA methyltransferase to native or to H1-depleted oligonucleosomes, most of the methyl-accepting ability is located in the linker DNA region [16]. Although this methyl-accepting ability is decreased by the presence of H1 in native oligonucleosomes or by addition of H1 to the reconstituted particles, hardly more than 50% suppression can be attained [16, 17]. These results can be accounted for by assuming that only some particular H1 variants are able to play a specific

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Abbreviations used: DTT, dithiothreitol; PBS, phosphate-buffered saline (pH 7.2); TBE, 89 mM Tris–89 mM borate–2mM EDTA buffer, pH 8.0; TFA, trifluoroactic acid.

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regulatory role in the formation of methylcytosine residues, these variants being involved in the non-random distribution of methylated or unmethylated CpG's in chromatin structure.

We have already shown that only the fractions enriched in H1e-c variants, prepared from H1 histone from calf thymus [18] or from human placenta (unpublished data), were able to exert a severe inhibition on *in vitro* enzymatic DNA methylation and on the binding of double-stranded CpG-rich oligonucleotides—these combined effects suggesting a specific role in preserving the unmethylated state of the CpG-rich islands which characterizes the promoter regions of house-keeping genes [19].

In this paper we managed to purify H1c and H1e from L929 mouse fibroblasts in order to individuate which variant is really involved in the inhibition of DNA methylation process and to evaluate whether they have a different affinity for CpG-rich oligonucleotides in their unmethylated or methylated form.

METHODS

Separation and analysis of H1 histone variants. L929 mouse fibroblasts were grown in a BHK21 medium with the addition of 10% newborn calf serum in a humidified 5% CO_2 atmosphere at 37°C. At cell density of $20\times10^6/175$ cm^2 flask, cells were washed with PBS pH 7.2, removed from flasks by treatment with trypsin and collected by low-speed centrifugation. H1 histone was prepared from these cells according to Johns (20) and fractionated in its somatic variants according to Quesada et al. (21), on a reverse phase RPC4-300 Å column (5 × 250 mm Vydoc), using a Perkin-Elmer 410 Apparatus equipped with a Diode-Array 235 ultraviolet 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 to 0.1% TFA in 95% acetonitrile]. Protein fractions of 0.5 ml were collected, lyophilised and analysed by SDS-polyacrylamide (15% acrylamide + 0.4% methylene bisacrylamide) gel electrophoresis. Protein concentrations were evaluated by densitometric scanning (BioImage, Millipore) of silver-stained electrophoretic bands and/or by a commercial adaptation of Bradford's procedure [22].

DNA methyltransferase assay. DNA methyltransferase (EC 2.1.1.37) was purified from human placenta nuclei (23), and assayed according to Caiafa et al. (24) in a 50 mM Tris-HCl buffer (pH 7.8) containing 10% (v/v) glycerol, 5 mM EDTA, 0.5 mM DTT, 10 mCi/ml S-adenosyl-L-[methyl-^{3}H]methionine (New England Nuclear, specific activity 70–80 mCi/mmol) and 30 μg/ml of *Micrococcus luteus* DNA.

Prior to enzymatic assays, all H1 histone protein fractions, eluted from reverse phase HPLC, were resuspended in 30 mM EDTA buffer to induce their renaturation (15). Their effect on DNA methylation was usually tested using a 0.1÷0.3 protein/DNA ratio (w/w).

Oligonucleotides. A "CpG-rich" sequence of 44 bp oligonucleotides was synthesized, in its unmethylated and methylated forms, by the phosphoamidite method, on a DNA synthesizer (Applied Biosystem). The sequence was the following:

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

Gel retardation. Double-stranded oligonucleotides were end-labelled at their 5′ ends with [γ-^{32}P]-ATP (specific activity 3000 Ci/mmol) using T_4 polynucleotide kinase (EC 2.7.7.7) under the conditions recommended by the manufacturer.

End-labelled oligonucleotides were mixed with either the H1e or the H1c variant in a 20μl volume of binding buffer (50 mM Tris-HCl pH 7.5, 5 mM Na-EDTA, 0.5 mM DTT), using generally a protein/DNA (w/w) ratios ranging from 1 to 10. Incubation was performed at 37°C for 1 hour 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 1 hour at 200 V, fixed in 10% acetic acid and 10% methanol for 15 minutes, dried and autoradiographed.

Binding of DNA by the various fractions was evaluated by densitometric scanning (BioImage, Millipore) of the autoradiograms, the percentage of free DNA being referred to DNA 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.

RESULTS

H1 histone somatic variants from L929 mouse fibroblasts were purified by reverse-phase HPLC. The elution pattern (Fig. 1A) showed the presence of four peaks. Because of the closeness of the last three peaks the effluent was fractionated in 0.5 ml aliquots in order to obtain protein fractions containing only one H1 somatic variant.

The SDS gel electrophoretic pattern of the collected fractions corresponding to the four major

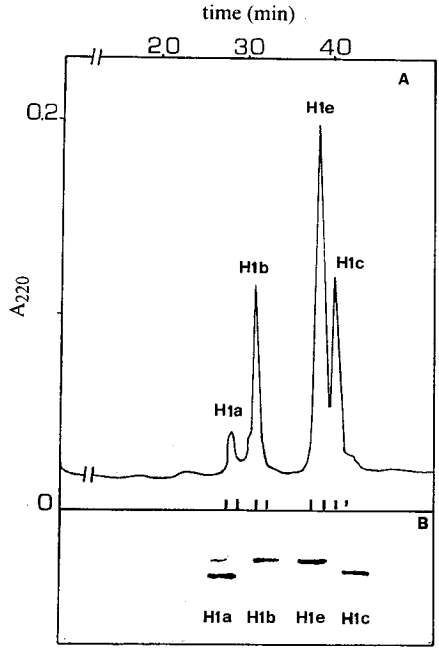

FIG. 1. HPLC separation and characterization of H1 histone variants from L929 fibroblasts. (A) HPLC elution profile; (B) electrophoretic pattern, in 12% SDS-polyacrylamide gel of the eluted fractions (upon visualization by silver staining).

peaks is shown in Fig. 1B. The HPLC retention time of each peak, combined with the as well as the electrophoretic mobility of various bands, allowed us to identify the H1a, H1b, H1e and H1c variants.

When the H1e *vs* H1c variants were assayed for their effect on *in vitro* DNA methyltransferase activity, only H1e was effective in causing a marked inhibition, at a H1:DNA "physiological" ratio (Fig. 2).

Our further aim was to assess whether H1e and H1c differed also in their ability to bind a

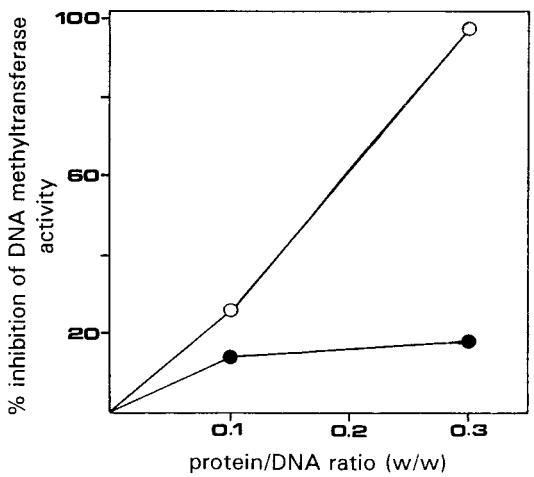

FIG. 2. Inhibition of DNA methyltransferase activity by H1e (open circles) or H1c (closed circles), at different protein-to-DNA ratios. Experimental details are reported in Methods.

synthetic CpG-rich oligonucleotides, similar to the CpG-rich islands which characterize the promoter regions of housekeeping genes.

Fig. 3 shows the results of gel retardation autoradiograms where the single variants H1e and H1c were incubated with a 44 bp duplex DNA containing 6 CpG dinucleotides in either the unmethylated or fully methylated forms. On the basis of the disappearance of the electrophoretic band corresponding to the free oligonucleotide it was evident that: a) the H1e variant binds preferentially to 6CpG rather than to 6mCpG oligonucleotides; b) the H1e and H1c variants show the same low affinity for 6mCpG oligonucleotide; c) the H1e variant binds better than H1c to the 6CpG oligonucleotide.

DISCUSSION

The H1 histone "family" is composed by a heterogeneous group of at least five different variants, generally expressed in the majority of tissues, designed as H1a, H1b, H1c, H1d and H1e and H1° [25]. In addition to these somatic variants, there are also some tissue-specific members of the H1 family, such as H5 from chicken erythrocytes [26], spH1 from sea urchin sperm [27], H1t from germ cells [28]. Several reports have shown that the relative amounts of these genic variants differ in various tissues and species, in condensed *vs* decondensed chromatin structure, in neoplastic systems, during the development stages of the organisms and through the cell cycle. The affinity of the purified H1 histone variants, for specific DNA sequences was investigated, on the assumption that differences in the flexibility and in the electrostatic properties of the peculiar DNA regions may induce a preferential protein-DNA binding.

AT-rich regions have been reported to be a preferential binding site for H1 histone (29–33). The binding of a pure H1e mouse variant to a homogeneous 214 bp fragment of DNA from pBR322 has been monitored, and it has been shown that this variant binds preferentially and cooperatively to the (G + C)-rich regions of DNA [34].

Previous results from this laboratory [18] have shown that, among H1 histone somatic variants, the H1a variant was able to bind a 145 bp genomic DNA fragment but was unable to bind 44 bp ds-oligonucleotides containing two or more CpG dinucleotides. The other variants were capable of binding sequences containing up to three CpG, while the fraction H1ec was unique in binding

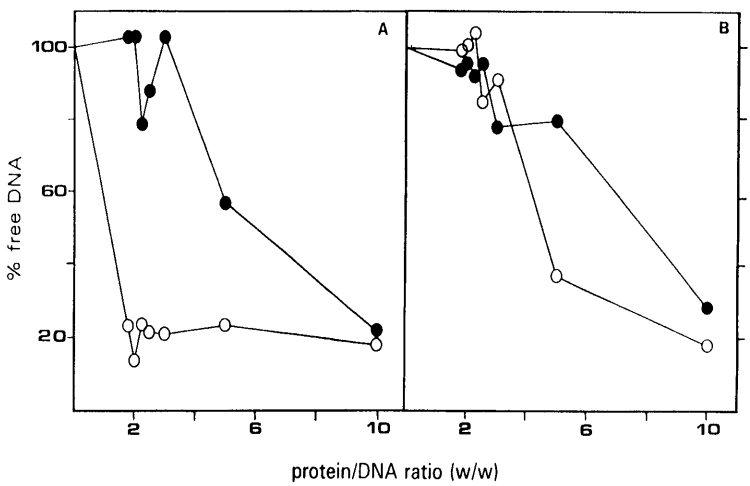

FIG. 3. Binding of H1e (open circles) and of H1c (closed circles) to the 44 bp synthetic 6CpG duplex oligonucleotide with the cytosines in the CpG moieties in the unmethylated (panel A) or fully methylated (panel B) form. The binding was evaluated by gel retardation after incubation of the H1e and H1c variants with the appropriate oligonucleotide, the relative amount of free DNA being measured by densitometric scanning of the autoradiograms.

CpG-rich DNA sequences. When the different variants were compared for their ability to inhibit *in vitro* DNA methylation, this property was restricted essentially to the same H1ec variant.

In this paper, we established H1e as the unique variant involved in the inhibition of the DNA methylation process. Being also the histone variant preferred by the unmethylated CpG-rich sequences, it is likely to be associated to the nucleosomal structure(s) of the CpG-rich islands [12, 35] which, as previously mentioned, are localized in promoter regions of housekeeping genes [19], and it could play a critical role in maintaining them in an unmethylated state. Genic microheterogeneity of H1 appears therefore as a mechanism by which this histone family can be involved in a number of fairly specific structural and/or functional roles, further modulation being possibly achieved [36] through post-synthetic modifications.

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