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EFFECT OF H_1 HISTONE ISOFORMS ON THE METHYLATION OF SINGLE- OR DOUBLE-STRANDED DNA

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SUMMARY: The loosely and tightly bound H1 histone isoforms were shown to exert, on the *in vitro* methylation of linker DNA in H1-depleted oligonucleosomes, inhibitory or activating effects respectively similar to those previously shown in the methylation of *Micrococcus luteus* dsDNA. When assayed on the enzymic methylation of *Micrococcus luteus* ssDNA, addition of the tightly bound one resulted in a stimulation similar to that exerted on double-stranded bacterial DNA or on linker DNA from mammalian chromatin, while the loosely bound isoform had no effect whatsoever. The transformation of the "typical" loosely bound H1 isoform into its tightly bound counterpart can be visualized as being an essential event in the modulation of DNA methylation process in eukaryotic chromatin.

Only H1 histone has been found to exert a specific *in vitro* inhibitory effect on enzymic DNA methylation when added in a "physiological" histone-to-DNA ratio (1). *In vitro* methyl-accepting experiments have also shown that, in the nucleosomal structure, the lower methylation levels of linker DNA (2-5) are not due to an intrinsic deficiency in CpG dinucleotides (which are the typical methyl-accepting sequences when the mammalian enzyme is used), but can rather be ascribed to the inhibitory effect specifically exerted by H1 on the DNA region to which it is associated in eukaryotic chromatin (6).

Among the "tightly-bound" proteins of human placenta chromatin, that resist extraction at high ionic strength, we have described a component which is quite similar to the "typical" H1 histone in terms of acid solubility, of apparent molecular mass and of specific immunoreactivity (7). This "tightly-bound" H1 histone can be prepared by performing, on the high-salt "stripped" loops fraction (7), the classical procedure (8,9) used for the preparation of the "loosely-bound" H1 histone from whole chromatin. Its amount in placenta chromatin is

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approximately 2% with respect to its loosely-bound counterpart. Preliminary experiments (7) showed that, in an *in vitro* assay of human placenta DNA methyltransferase acting on *Micrococcus luteus* double-stranded DNA, it had a stimulatory, rather than inhibitory, effect on the methylation process. The aim of present work was to clarify the possible role played by the "loosely-bound" and "tightly-bound" isoforms of H_1 histone on the enzymic methylation of linker DNA.

METHODS

Preparation of H_1-depleted oligonucleosomes.

Oligonucleosomes (containing 5-20 nucleosomes each) were prepared by controlled digestion of human placenta chromatin with *Staphylococcus aureus* nuclease (EC 3.1.31.5), followed by separation on a 5-20% (w/v) linear sucrose gradient. When required, they were deprived of their H_1 content by exposure to 0.6 M NaCl and subsequent centrifugation (twice) on a 5% (w/v) sucrose cushion in 0.6 M NaCl buffered with 10 mM Tris-HCl (pH 7.5), in the presence of 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The absence or presence of H_1 histone was checked by gel electrophoresis in 13.5% (w/v) polyacrylamide in the presence of 0.1% (w/v) SDS. The size of DNA was estimated by gel electrophoresis in 1.8% (w/v) agarose.

Isolation and renaturation of the tightly bound H_1 histone isoform from human placenta chromatin.

After removal of histones and of other loosely-bound proteins by exposure to 2 M NaCl, a "stripped chromatin preparation" was obtained, which could also be fractionated in "stripped loops" and "stripped matrix" by use of differential density centrifugation, as described elsewhere (10). The tightly-bound H_1 histone was prepared (7) by performing, on the stripped loops fraction, the classical procedure for the preparation of the loosely-bound isoform from whole chromatin, i.e., extraction with 0.2 M H_2SO_4 (8), dialysis against H_2O and precipitation in 9 volumes of cold acetone, followed by extraction with $HClO_4$ (10%, w/v), according to Johns (9).

The tightly-bound H_1 isoform was solubilized by addition of 0.001% (w/v) dodecyl-β-D-maltoside in H_2O (Calbiochem-Behring Corp.). Prior to enzymatic assays, histones were usually renaturated by progressive dialyses in the presence of 10^{-2} M EDTA (1,7). The presence of 0.001% dodecyl-β-D-maltoside caused apparently no interference with the enzymatic assays and no distortion of the circular dichroism spectra.

Circular dichroism (CD) spectra, at histone concentration usually around 10^{-5} M, were used to verify protein renaturation (1).

DNA methyltransferase assay.

DNA methyltransferase (EC 2.1.1.37) was purified from human placenta nuclei according to Carotti *et al.* (11), and assayed, as described by Caiafa *et al.* (10), in a 50 mM Tris-HCl buffer (pH 7.8) containing 10% (v/v) glycerol, 5 mM EDTA and 0.5 mM dithiothreitol, using 15-50 μCi/ml of S-adenosyl-L-(methyl-^{3}H)-methionine (New England Nuclear; specific activity 70-80 Ci/mmol) as methyl donor. Tested methyl acceptors included the various chromatin preparations and *Micrococcus luteus* DNA in the double- and single-stranded forms. When required, appropriate amounts of H_1 histone (Boehringer) or of its tightly-bound isoform from human placenta, previously renaturated by progressive dialyses, were added to the reaction mixture. Quantitative determination of proteins was carried out by a commercial adaptation (Bio-Rad) of Bradford's method (12).

RESULTS

Addition of loosely-bound H_1 histone -- which had been shown to cause a quasi-complete inhibition of the ability of placental DNA methyltransferase to act on *Micrococcus luteus* double-stranded DNA (1) -- significantly depressed also the methyl-accepting ability of H_1-depleted oligonucleosomes or of purified oligonucleosomal DNA, although the residual methyl-accepting ability was never lower than that of native oligonucleosomes (6). By comparing, in parallel assays, the effects of the loosely-bound and tightly-bound H_1 isoforms, the latter was found to cause, as shown in Table 1, a significant stimulation of linker DNA methylation in H_1-depleted oligonucleosomes, similarly to what had been shown to occur (7) when *Micrococcus luteus* ds-DNA was used as substrate.

The results obtained with the loosely-bound isoform can be interpreted as suggesting the presence, in chromatin, of DNA sequences capable of being methylated even after addition of H_1 -- possibly because they do not bind this histone or because the histone can be displaced by the enzyme. The latter possibility was investigated -- and disproved, as shown in Figure 1 -- by performing experiments in which increasing amounts of purified DNA methyltransferase were added to *Micrococcus luteus* ds-DNA in the presence of a constant amount of H_1 (the H_1/DNA ratio being fixed to its "physiological" value of 0.3). Also the stimulation by the tightly-bound isoform was not modified by an increase in the amount of enzyme (Figure 1).

We also examined, as a working hypothesis, the possibility that occasional single-stranded regions be of importance in regulating the interference of either H_1 isoform with the DNA methylation process. Using *Micrococcus luteus* DNA as substrate, we found that, while addition of the loosely-bound H_1 histone was by no means inhibitory to ss-DNA methylation (Figure 2A), the stimulatory effect of the tightly-bound form was essentially identical on either single-stranded or double-stranded DNA (Figure 2B).

TABLE 1

Effect of loosely bound and tightly bound H_1 histone isoforms on DNA methylation in H_1-depleted oligonucleosomes as compared to *Micrococcus luteus* ds-DNA

	HISTONE PROTEIN ADDED		
	none	lb-H_1 (0.3 mg/ mg DNA)	tb-H_1 (0.3 mg/ mg DNA)
H_1-depleted oligonucleosomes	100.0	58.1	128.0
Micrococcus luteus ds-DNA	100.0	6.0	145.0

The values indicate the relative methyl-accepting ability of oligonucleosomal and micrococcal DNAs, obtained with a same preparation of placental DNA methyltransferase.

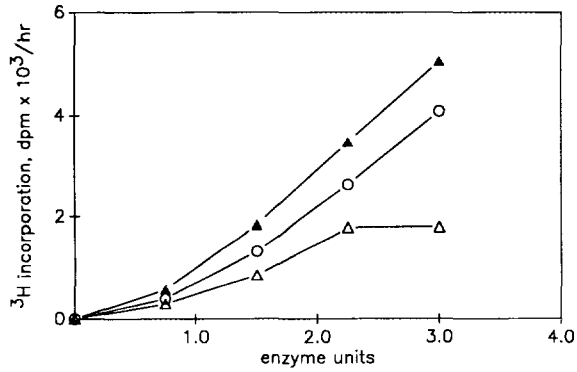

Figure 1. Variations in the extent of *Micrococcus luteus* dsDNA methylation *in vitro*, as a function of added DNA methyltransferase, in the absence (middle curve) or presence of loosely bound (lower curve) or tightly bound (upper curve) H1 histone isoforms at a constant histone-to-DNA ratio equal to 0.3 (w/w).

DISCUSSION

The ability of the typical loosely-bound H1 histone to exert a strong inhibition on *in vitro* DNA methylation (1), and the presence, in mammalian chromatin, of a minor tightly-bound isoform of the same histone (7), could be strictly connected to the existence, *in vivo*, of different DNA methylation levels within chromatin structure and to their possible role in the dynamics of gene expression. Within the first level of chromatin organization, 5-methylcytosine residues are reportedly more abundant in "core" DNA than in the internucleosomal one (2-5). *In vitro* experiments by Davis *et al.* (13) have shown, on the other hand, that the distribution of newly-incorporated methyl groups catalysed by endogenous DNA methyltransferase was different from that obtained if the reaction was catalyzed by exogenously added enzyme -- linker DNA becoming the preferred acceptor only in the latter case. In our hands, the inhibition exerted by H1 on the *in vitro* enzymic DNA methylation was

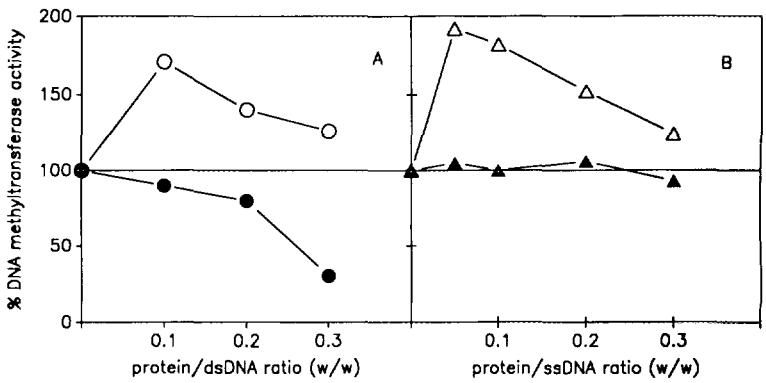

Figure 2. Effect of loosely bound (full symbols) and tightly bound (open symbols) H1 histone isoforms on the *in vitro* methylation of double-stranded (panel A) or single-stranded (panel B) *Micrococcus luteus* DNA.

found to be independent of the H1-to-enzyme ratio. It seems therefore unlikely, at least in our experimental conditions where the methylation reaction was catalysed by exogenous DNA methylase, that competition between the histone and the enzyme for some common DNA binding site(s) be the main mechanism regulating the incorporation of methyl groups in the CpG sequences of chromatin DNA. The methyl-accepting ability of intact oligonucleosomes was on the other hand far from negligible, although it underwent a two-fold increase upon H1 depletion (with a further 50% increment if also all other proteins were removed); it went back, indeed, to the same level as in native chromatin when excess H1 was added to any one of the above-mentioned preparations (6). At variance from the results obtained with *Micrococcus luteus* ds-DNA, enzymic methylation of chromatin DNA is therefore not entirely suppressed by the presence of H1 or by addition of an excess of this histone.

These results can be interpreted, in our opinion, as indicating the presence, in mammalian DNA, of regions which are not susceptible to negative control by the loosely-bound H1 histone. By contrast the tightly-bound isoform of H1 exerted on the methylation of linker DNA the same activating effect which had been found with *Micrococcus luteus* double-stranded DNA (7). Also in this case was the effect independent of the amount of enzyme catalysing the *in vitro* reaction.

As a further clue to the possible role played by the loosely- and tightly-bound isoforms on the enzymic methylation of linker DNA, we investigated their effect on *in vitro* DNA methylation using *Micrococcus luteus* single-stranded DNA as substrate. It was found that, while the loosely-bound H1 histone had no effect on the methylation of single-stranded DNA, the tightly-bound form had a stimulatory effect similar to that exerted on double-stranded DNA.

Since the typical H1 histone is reportedly bound to the exterior of each core particle just at the exit of the DNA filaments, it may be hypothesized that it inhibits the access of the enzyme to the CpG moieties by sealing the double-stranded DNA. This may also be important for the "new" functional roles, recently attributed to histones, in the regulation of gene transcription (14-18). The transformation of H1 histone from the loosely-bound form to the tightly-bound one could be an essential event, probably due to a change in the histone domains involved in the interaction with DNA, leading to a modulation of the DNA methylation process in eukaryotic chromatin.

It can be hypothesized that the activating effect exerted by the tightly-bound isoform on the methylation of both single- and double-stranded DNAs be due to a "bridging" role between DNA and the enzyme.

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