

Correlation between DNA methylation and poly(ADP-ribosyl)ation processes

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Summary

The aim of this article is to show the close relationship between DNA methylation and poly(ADP-ribosyl)ation which are two important nuclear enzymatic mechanisms. An open question is to explain how some CpG dinucleotides, in particular those present into GpG islands, can maintain their unmethylated state in spite of the presence of active DNA methyltransferase in chromatin. This paper illustrates some data indicating that H1 histone is a possible *trans*-acting factor involved in protecting genomic DNA from full methylation and proposes that the somatic variant H1e, in its poly(ADP-ribosyl)ated isoform, is the protein capable of undertaking this role.

I. Introduction

A. Inhibitory effect of DNA methylation on gene expression.

DNA methylation is a specific post-synthetic modification of DNA that, in eukaryotic cells, appears to play an important role in the epigenetic modulation of gene expression. It is the major enzymatic DNA modification that transfers methyl groups from S-adenosyl methionine (S-AdoMet) to cytosine (C) and converts these residues into 5-methylcytosine (5mC) (Bestor and Ingram, 1983). Although in vertebrates the presence of 5mC has occasionally been reported to be found in dinucleotide sequences CpC, CpA and CpT (Woodcock et al., 1987, 1988; Toth et al., 1990; Tasheva and Roufa, 1994; Clark et al., 1995), the best substrate for DNA methyltransferase is cytosine located in the CpG dinucleotide (Gruembaun et al., 1981).

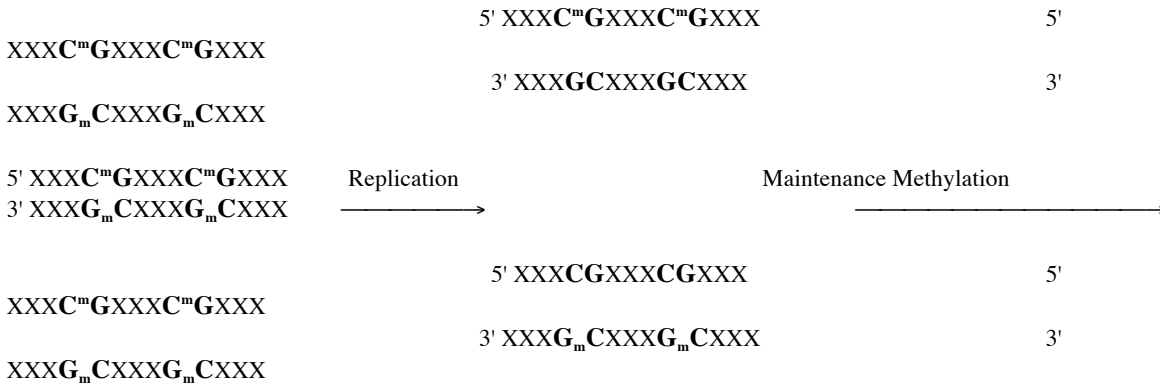
As for the distribution of 5mCs, evidence already existed (Yisraeli and Szyf, 1984) that they are distributed in a non-random fashion in genomic DNA. Successive studies have shown that the methylated cytosines are present in bulk DNA (Bloch and Cedar, 1976) while the unmethylated ones are essentially located within some particular DNA regions termed "CpG islands" (Bird et al.,

1985; Bird, 1986,1987), **Figure 1**. The specific DNA methylation pattern results from the combination of maintenance and *de novo* methylation and of demethylation processes, **Figure 2**. The maintenance methylase recognizes and modifies hemimethylated sites generated during DNA replication thus preserving the tissue-specific methylation pattern (Razin and Riggs, 1980). In higher eukaryotes this enzymatic process takes place within a minute or two after replication (Leonhardt et al., 1992).

The final "correct" methylation pattern is reportedly obtained, in somatic cells, during the early stages of embryonic development, through a combination of demethylation and *de novo* methylation steps (Brandeis et al., 1993). Demethylation occurs by an active reaction (Frank et al., 1991; Brandeis et al., 1993; Jost, 1993; Weiss et al., 1996) where a 5-methyldeoxycytidine excision repair system cleaves the DNA strand at 5mCpG sites, removes the methylcytosine from DNA and replaces it with cytosine. Subsequently, a burst of *de novo* methylation starts the differentiation process leading to a bimodal pattern of methylation in which the "CpG islands" at the 5' end of the housekeeping genes remain constitutively unmethylated, while other genomic sequences undergo a massive wave of *de novo*

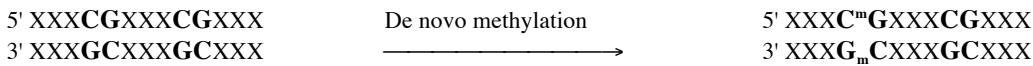
methylation. Demethylation of individual genes occurs also during tissue-specific differentiation (Razin et al.,

Maintenance DNA methylation



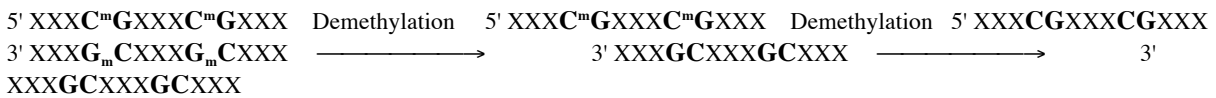
Substrate: hemimethylated DNA
Role: to preserve the tissue specific methylation pattern
When: 1-2 min. after replication.

De novo DNA methylation



Substrate: unmethylated CpG sequences.
Role: to define the final correct tissue specific methylation pattern involved in the differentiation process, or repress the active genes in somatic cells.
When: during the early stages of embryonic development, or during carcinogenesis.

Active DNA demethylation



Substrate: fully methylated DNA hemimethylated DNA .
Role: to define the final correct methylation pattern, or gene activation in somatic cells.
When: after replication, during the early stages of embryonic development (blastula stage), or during tissue specific differentiation.

Figure 1. Processes involved in defining the DNA methylation pattern.

Distribution of CpG and 5 ^h meCpG dinucleotides in eukaryotic DNA.	
Bulk	CpG island
<i>G+C content</i>	
40%	60%
<i>CpG level (CpG/GpC)</i>	
0.2	> 0.6

Methylation level	
High	Unmethylated

Figure 2. Non-random distribution of CpG and 5mCpG dinucleotides in genomic DNA.

1986; Brandeis et al., 1993; Jost and Jost, 1994), this process being probably required for gene activation. To explain the demethylation process two different mechanisms have been described.

The first one involves a proteic factor 5-methylcytosine endonuclease activity that is able to remove the 5-methylcytosine and to substitute it with cytosine (Jost, 1993; Jost and Jost, 1994; Jost et al., 1995). The second one involves the presence of a ribozyme or maybe a ribozyme associated with a proteic factor that is able to remove the mCpG dinucleotide and to substitute it with CpG dinucleotide (Weiss et al., 1996). The fact that CpG dinucleotides are present in an unmethylated state in "CpG islands" is of interest since their frequency in them is five times more than in bulk DNA, **Figure 1**.

As far as the correlation between DNA methylation and gene expression is concerned, the "CpG islands", that go from 500-2000 base pairs in size, are usually found in the 5' promoter region of housekeeping genes and overlap genes to variable extents (Bird, 1986). There is evidence that transcription of genes, correlated with "CpG islands", is inhibited when these regions are methylated (Keshet et al., 1985).

That the "CpG islands" are not by themselves unmethylable is demonstrated by *in vitro* experiments (Carotti et al., 1989; Bestor et al., 1992). A great deal of investigation has been and is performed in order to clarify why the "CpG islands" remain untouched by the action of DNA methyltransferase (Ysraeli and Szyf, 1984) in spite of their localization on promoter region of housekeeping genes which are, in decondensed chromatin, permanently accessible to the transcriptional factors.

A question yet to be solved is to identify different *cis*-acting signals and *trans*-acting protein factors that may play a key role in defining the bimodal pattern of methylation involved in cell differentiation and gene expression. It has been suggested that the density of CpG dinucleotide inside "CpG-islands" could be *per se* a signal involved in protecting the unmethylated state of these DNA regions (Frank et al., 1991) but further experiments suggest that there are some sequence motifs that are intrinsically protected against *de novo* methylation (Szyf et al., 1990; Christman et al., 1995; Tollefsbol and Hutchinson, 1997) and/or that there are some *cis*-acting "centers of methylation" capable of preventing the methylation pattern of flanking DNA sequences (Szyf et al., 1990; Szyf, 1991; Mummaneni et al., 1993; Brandeis et al., 1994; Hasse and Schultz, 1994; MacLeod et al., 1994; Magewu and Jones, 1994; Mummaneni et al., 1995). The simple possible explanation that there are

trans-acting protein factors associated with "CpG islands" which prevent access to those DNA regions, has been difficult to demonstrate up to now. Research on the identification of factors able to link methylated DNA has met with greater success. The first protein identified as able to bind methylated DNA sequences is the methylated DNA binding protein (MDBP) a ubiquitous family of closely related proteins in vertebrates (Huang et al., 1984; Zhang et al., 1993). Its consensus sequence is composed of 14 bp and has a substantial degree of degeneration. MDBP sites can have up to 3 CpG's and generally the degree of binding increases when more of these are methylated and when they are methylated on both strands. In spite of the high level of degeneracy of the consensus sequence, MDBP can be considered sequence-specific in its binding because mutations in inopportune positions cause this protein to lose its ability to link the sequence. This protein can also link the consensus sequence independently of its methylated level provided the C is substituted by T and T is present in TpG or TpA dinucleotides (Zhang et al., 1986; Khan et al., 1988). It is possible that the consensus methylation independent sequences could derive from the spontaneous deamination of 5mC in T. From transfection experiments a role of down-regulation of gene expression has been proposed for this protein (Asiedu et al., 1994; Zhang et al., 1995). The MDBP-2-H1 is another protein that binds itself preferentially to certain DNA sequences containing a simple mCpG pair (Pawlak et al., 1991). Although the protein is not sequence specific, its affinity for the consensus sequence is highest in the promoter region (+2 +32) of vitellogenin II gene where it plays a down-regulatory role of gene expression (Pawlak et al., 1991). Further investigations have shown (Jost and Hofsteenge, 1992) that this protein - identified as H1 histone-like - must undergo phosphorylation before to its interaction with the methylated DNA sequence (Bruhat and Jost, 1995). Two other proteins named MeCP1 and MeCP2, that have the ability to link DNA regions in which the CpG dinucleotides are methylated to higher or lower levels, have been proposed as proteins involved in the silencing of gene expression (Meehan et al., 1989; Boyes and Bird, 1991; Lewis et al., 1992). In particular MeCP1, whose molecular weight is of about 800 KDa, is suggested to be involved in a mechanism through which its association with methylated DNA could prevent the linkage of transcription factors in these DNA regions. This protein binds sequences containing about 12 or more methylated CpGs and the enrichment in CpG dinucleotides argues that these DNA regions are "CpG island-like" (Meehan et al., 1989).

The strength of promoter and the density of mCpGs (Boyes and Bird, 1992) are two factors which regulate the association of MeCP1 with DNA. It is clear that low levels of methylation can repress transcription of a weak promoter but not of a strong promoter. In fact, sparsely methylated genes bind MeCP1 weakly and the transcription is partially repressed if the gene promoter is weak while if the promoter is strong the gene is expressed (Boyes and Bird, 1992). The MeCP2 factor is able to bind DNA that contains a single mCpG pair (Lewis et al., 1992; Meehan et al., 1992). MeCP2, for which a transcriptional repressor role has been described, is very abundant in bulk vertebrate genomic DNA - 100 times more abundant than MeCP1 - where it is in competition with H1 histone. This result supports the hypothesis that MeCP2 is involved in condensing chromatin structure (Nan et al., 1997).

Although these proteins play an important role in mediating the methylation-dependent repression of genes, an open question to answer is how the CpG moieties of the "CpG islands", become vulnerable or resistant to the action of DNA methyltransferase and can thus lose or maintain their characteristic pattern of methylation.

This is the goal of our research: our aim is to identify and pinpoint a nuclear protein *trans*-acting factor directly involved in maintaining the unmethylated state of "CpG islands".

II. H1 histone and DNA methylation.

A. Methylation-dependent binding of H1 histone to DNA.

An attractive hypothesis to explain the repressive effects of DNA methylation on gene expression is that H1 histone binds itself preferentially to DNA sequences containing mCpG dinucleotides.

Although H1 histone is mainly present in highly methylated condensed chromatin there is ample disagreement in the scientific literature - at variance from the other above mentioned proteins - as to whether or not its presence is dependent on the methylated state of DNA. A preference of H1 histone for double-stranded DNA with a relatively high abundance of methylated CpGs has however been recently shown by McArthur and Thomas (1996), who have suggested that the condensing ability of H1 histone could thus be favored by the higher level of DNA methylation existing in transcriptionally inactive chromatin. Parallel experiments (Caiafa et al., 1995; Reale et al., 1996) have been performed in order to examine whether in oligonucleosomal DNA, purified from inactive chromatin fraction, an increased methylation of CpG residues would interfere with the formation of the appropriate H1-H1 interactions critical for attainment of folded chromatin structures. Conflicting

results respect to those of McArthur and Thomas (1996) were obtained since the introduction of new methyl groups into oligonucleosomal DNA was surprisingly found to decrease its ability to allow these H1-H1 interactions (**Figure 3**), suggesting that, *in vivo*, the presence of some unmethylated CpGs in linker DNA is likely to be an important prerequisite for chromatin compaction. These differences could be explained by differences in the DNAs selected for the two experiments as, despite the common aim of avoiding sequence-specific effects in H1-DNA binding, there are indeed considerable differences in terms of CpG frequency and of the overall methylation level of the DNAs. The DNA sequences used by McArthur and Thomas (1996), chosen as representative of a large region of the sea urchin genome, are essentially obtained from unmethylated CpG-rich DNA regions, while our oligonucleosomal DNA was extracted from human placenta inactive chromatin fraction whose relatively scarce CpG moieties have a rather high basal methylation level.

The band shift assays did not solve the problem of methylation dependent binding of H1 histone to DNA. In fact experiments carried out using DNA fragments with different amounts of CpGs dinucleotides, failed to show any effect of CpG methylation on H1 histone binding since H1 histone has shown an identical affinity for either methylated or non-methylated DNA (Campoy et al., 1995). It may be recalled that while Higurashi and Cole (1991) have also found that the interaction of H1 histone with CCGG is independent of the methylation level, Levine et al. (1993) have shown a preferential binding of total H1 histone to plasmid methylated DNA.

B. Inhibitory effect of H1 histone on *in vitro* DNA methylation.

In our research on a nuclear proteic factor involved in DNA methylation process, we focused our attention on histone proteins since previous papers have reported a possible inhibitory role played by histones on DNA methylation (Kautiainen and Jones, 1985; Davis et al., 1986).

Our experiments (Caiafa et al., 1991) have shown that the ability of total histones to affect *in vitro* enzymatic DNA methylation was essentially due to a single H1 histone that, in the "physiological" range (0.3:1, w/w) histone:DNA ratio, was the only one able of exerting a consistent (90%) inhibition on methylation of double stranded DNA, catalyzed by human placenta DNA methyltransferase. Neither H1-depleted preparations of "core" histones nor, separately, any other single histone (H2a, H2b, H3) were able to affect the methylation process, **Figure 4**.

Since H1 is known to be preferentially associated to linker DNA (van Holde, 1988) its ability to suppress *in vitro* DNA methylation is consistent with previous

Figure 3A: SDS-PAGE patterns of H1 histone after treatment, in the presence of native (lanes 1, 2, 3) or of artificially overmethylated oligonucleosomal DNA (lanes 4, 5, 6), with dithiobis-(succinimidylpropionate) at different H1:DNA ratios -- 0.1, 0.3, 0.5 (w/w) -- in 40 mM NaCl. In lane 7, H1 histone treated with DSP in the absence of DNA; in lane 8, untreated H1 histone. **(B):** Electrophoretic patterns, in 1% agarose stained with ethidium bromide, of glutaraldehyde-fixed H1-DNA complexes, formed in 40 mM NaCl at H1:DNA ratios ranging from 0.1 to 0.9 (w/w), using native oligonucleosomal DNA (left panel) or artificially overmethylated DNA (right panel). DNA molecular marker III from Boehringer is in lane III. Naked DNA controls (native in the left panel, artificially overmethylated in the right one) are in lanes C and C₁. "Reprinted from **Biochem. Biophys. Res. Comm.** **227**, Reale et al.. H1-H1 Cross-linking efficiency depends on genomic DNA methylation, 768-774, (1996) with kind permission of Academic Press, Inc."

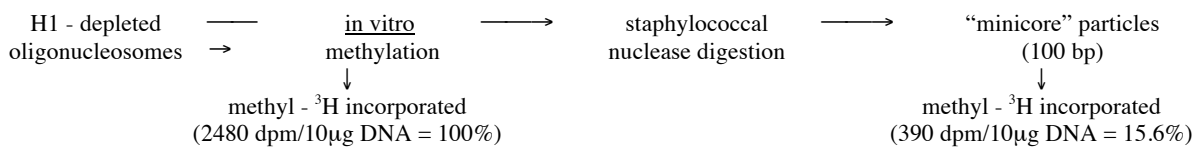
Figure 4. Effect, on the *in vitro* activity of human placenta DNA methyltransferase, of histones H1, H2a, H2b and H3 (from calf thymus) renaturated by progressive dialysis at decreasing urea and NaCl concentrations in the presence (closed triangles) or absence (closed circles) of 5 mM EDTA. Each point represents the mean result of at least five different experiments in triplicate, S.D. "Reprinted from **Biochim. Biophys. Acta** **1090**, Caiafa et al. *Histones and DNA methylation in mammalian chromatin. I° Differential inhibition by histone H1*, 38-42, (1991) with kind permission of Elsevier Science Publishers - NL Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands".

findings of higher 5mC levels in nucleosomal core DNA as compared to linker DNA (Razin et al., 1977; Solage and Cedar, 1978; Adams et al., 1984; Caiafa et al., 1986). Some experiments were carried out to assess whether the observed hypomethylation of linker DNA sequences reflect an intrinsic deficiency in CpG dinucleotides or whether the well-documented association between DNA and H1 histone causes a local inhibition of enzymatic DNA methylation process (D'Erme et al., 1993).

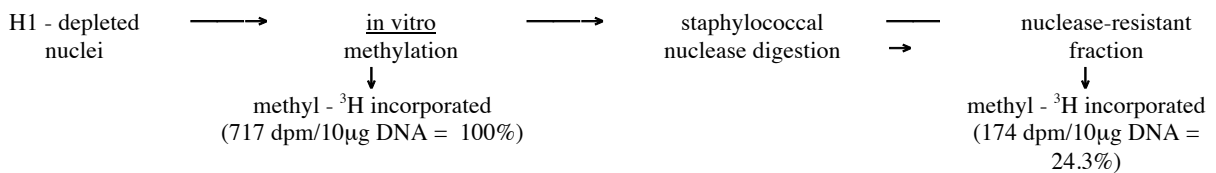
The net level of methyl-accepting ability of CpG dinucleotides in linker DNA - defined as the DNA region which can be hydrolyzed by staphylococcal nuclease digestion of H1-depleted oligonucleosomes - was evaluated by making use of a number of distinct experimental strategies in order to minimize possible artefacts. Since the removal of H1 histone by two alternative procedures yielded quite similar results, it is

unlikely that artefactual nucleosome sliding may have significantly altered the regions of chromatin DNA accessible to methylation. In the first set of experiments we measured the proportion of labelled methyl groups remaining in the "100 bp minicore particles" upon extensive staphylococcal nuclease digestion of *in vitro* methylated H1-depleted oligonucleosomes. As shown in **Figure 5a,b** - where H1 had been taken away, respectively from oligonucleosomes and from nuclei by two alternative procedures - nuclease treatment removed the majority (85% in one case, 75% in the other) of the labelled 5-methylcytosine residues. By contrast, nuclease digestion removed from native oligonucleosomes (H1-containing) only a relatively small portion of the 5-methylcytosine residues which had been inserted by *in vitro* enzymatic DNA methylation, **Figure 5c**.

a- Residual methyl groups in 100 bp "minicore" particles after nuclease digestion of methylated H1-depleted oligonucleosomes (treated with 0.6 M NaCl):



b- Residual methyl groups in the nuclease-resistant fraction from methylated H1-depleted nuclei (treated at low pH):



c- Residual methyl groups in 145 bp "core" particles after nuclease digestion of methylated native oligonucleosomes:

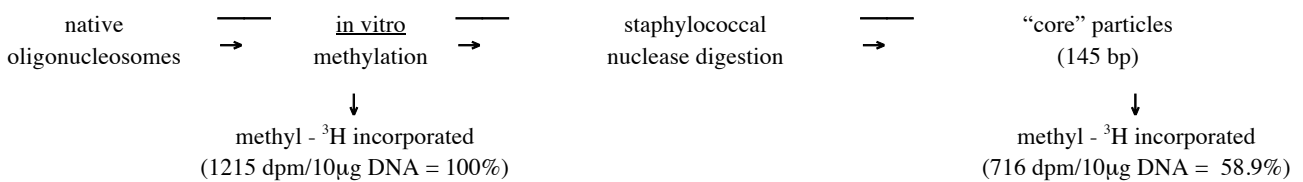
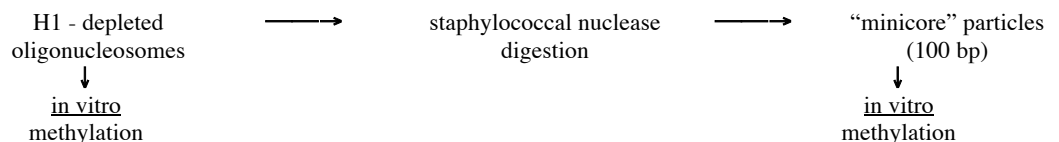


Figure 5. Evaluation, by three distinct experimental strategies involving nuclease digestion after *in vitro* methylation, of the distribution of methyl-accepting CpGs in the nuclease-sensitive fraction. The data obtained refer to a similar set of experiments run in parallel, so as to obtain comparable values. Five other similar experiments gave slightly different results in terms of absolute incorporation of methyl groups, but almost identical as percent radioactivity values remaining in the nuclease-resistant fractions. "Reprinted from *Biochim. Biophys. Acta* 1173, D'Erme et al., *Inhibition of CpG methylation in linker DNA by H1 histone*, 209-216, (1993) with kind permission of Elsevier Science Publishers - NL Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands".

a - Direct methylation of 100 bp "minicore" particles vs H1 - depleted oligonucleosomes:



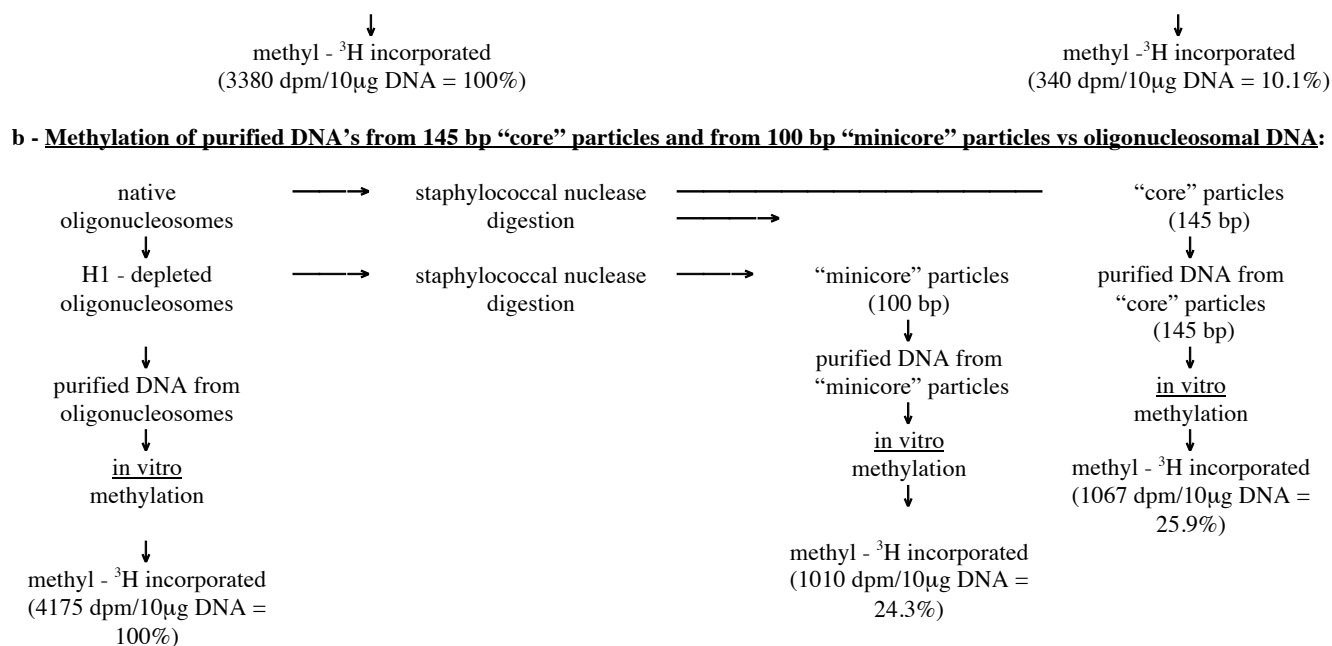


Figure 6. Evaluation, by two distinct experimental strategies involving nuclease digestion before *in vitro* methylation, of the distribution of methyl-accepting CpGs in the nuclease-sensitive fraction. "Reprinted from *Biochim. Biophys. Acta* **1173**, D'Erme et al.. *Inhibition of CpG methylation in linker DNA by H1 histone*, 209-216, (1993) with kind permission of Elsevier Science Publishers - NL Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands".

	Histone proteins added			
	none	H1 (0.3 mg/mg DNA <i>n</i> =6	"core" histones (1.0 mg/mg DNA <i>n</i> =3	H _{2a} (1.0 mg/mg DNA <i>n</i> =3
Number of experiments:	<i>n</i> =6			
Native oligonucleosomes	48.4±0.7	-	-	-
H1-depleted oligonucleosomes	100.0	58.0±1.3	101.4±3.5	102.6±2.7
Purified DNA from oligonucleosomes	155.0±2.1	41.6±0.8	153.8±5.8	-

Table 1. Inhibition by H1 of the methyl-accepting ability of oligonucleosomal DNA. The incorporation of labeled methyl groups in the DNA of H1-depleted oligonucleosomes is made equal to 100 and all the other results obtained in a same set of experiments are referred to this value.

In a complementary approach, when the "100 bp minicore particles" obtained by digestion with staphylococcal nuclease of H1-depleted oligonucleosomes were used as substrates for subsequent *in vitro* methylation, their methyl-accepting ability was found to be, on a DNA basis, only one-tenth of that of the original H1-depleted oligonucleosomes, **Figure 6a**. By assaying the susceptibility to methylation of the purified DNAs from the same particles, the methyl-accepting ability of oligonucleosomal DNA was four times larger than that of either the "145 bp core particles" or of the "100 bp minicore particles" **Figure 6b**.

These data (D'Erme et al., 1993) have shown that the lower level of DNA methylation in linker regions than in

"core" particles (Razin and Cedar, 1977; Solage and Cedar, 1978; Adams et al., 1984; Caiafa et al., 1986) was not due to an intrinsic CpG deficiency of linker DNA, which was, in H1-depleted oligonucleosomes, susceptible to extensive *in vitro* methylation, but can rather be ascribed to the inhibition exerted by H1 histone on the process of enzymatic DNA methylation (Caiafa et al., 1991), which would occur in these linker DNA regions because of their preferential association with H1 histone.

The ability and the specificity of H1 histone to inhibit CpG methylation in linker DNA were assayed by re-adding purified H1 to H1-depleted oligonucleosomes or to the DNA purified from them, **Table 1**. H1-depletion doubled the methyl-accepting ability of

oligonucleosomes, with a further 50% increase as the remaining proteins were also removed. Addition of H1, in a protein-to-DNA (w/w) ratio of 0.3, reduced the incorporation of labelled methyl groups in H1-depleted oligonucleosomes and in the purified oligonucleosomal DNA to the same level occurring in native oligonucleosomal particles. This inhibition was paralleled by a re-condensing effect occurring upon addition of H1 to H1-depleted oligonucleosomes, as shown in **Figure 7**. Both phenomena are apparently specific to H1 histone, since they could not be obtained by addition of other histones or of serum albumin up to a 1:1 protein/DNA (w/w) ratio.

These experiments and others previously performed by Davis et al. (1986) have shown that enzymatic DNA methylation is not entirely suppressed by the intrinsic presence of H1 histone.

The hypothesis of a competition (Santoro et al., 1993) between the enzyme and histone H1 for some common DNA negative control of H1 histone was investigated and disproved 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 H1 histone, the H1/DNA ratio being fixed to its "physiological" value of 0.3. As shown in **Figure 8**, the enzymatic DNA methylation *in vitro* was independent of the H1 to enzyme ratio. It seems therefore unlikely, at least in our experimental conditions, that competition between the histone and enzyme for some common DNA binding site(s) is the main mechanism regulating the incorporation of methyl groups in the CpG sequences of chromatin. The methyl-accepting ability of intact oligonucleosomes was, on the other hand, far from negligible. Although it underwent a two-fold increase upon H1 histone 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 histone was added to H1-depleted oligonucleosome preparations (**Table 1**).

Other two hypotheses can account for these results: the presence of some particular variant(s) more or less capable of inhibiting enzymatic DNA methylation and/or the presence of DNA regions escaping the negative control of H1 histone.

Figure 7. CD spectra, in the region of DNA chromophores, of native (—) and H1-depleted oligonucleosomes (---) and re-condensing effect occurring upon addition to the H1-depleted oligonucleosomes of "core" histones (protein/DNA ratio, w/w=1) or of H1 histone (protein/DNA ratio, w/w = 0.1: - - - ; w/w = 0.2: -o-o-o-). Oligonucleosomes were suspended, at a DNA concentration of 60 mg/ml, in a 60 mM NaCl, 5 mM Tris-HCl buffer (pH 7.4). "Reprinted from **Biochim. Biophys. Acta 1173**, D'Erme et al.. Inhibition of CpG methylation in linker DNA by H1 histone, 209-216, (1993) with kind permission of Elsevier Science Publishers - NL Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands".

III. H1 histone somatic variants.

The hypothesis that some particular variant could be specifically involved in the *in vitro* inhibition of enzymatic DNA methylation stems from the fact that H1 histone is composed of a family of different somatic variants termed H1a, H1b, H1c, H1d and H1e (Cole, 1987). They all have a three domain structure, with a highly conserved central globular domain (98% identity in 80 aa sequence). The differences between the variants are located in the N-terminal and C-terminal tails, which consist of about 40 and 100 amino acids respectively (Cole, 1987), with the overall variation in molecular mass being approx 1.0-1.4 kDa.

Figure 8. Variations in the extent of *Micrococcus luteus* ds DNA methylation *in vitro*, as a function of added DNA methyltransferase, in absence (open circles) or presence (open triangles) of H1 histone at a constant histone-to-DNA ratio equal to 0.3 (w/w). "Reprinted from **Biochem. Biophys Res. Comm.** **190**, Santoro et al.. Effect of H1 histone isoforms on the methylation of single- or double-stranded DNA, 86-91, (1993) with kind permission of Academic Press, Inc."

The number and relative amounts of these variants differ in various tissues and species throughout the development stages of the organism and in neoplastic systems (Liao and Cole, 1981a,b; Pehrson and Cole, 1982; Lennox and Cohen, 1983; Huang and Cole, 1984; Lennox, 1984; Cole, 1987; Davie and Delcuve, 1991; Baubichon-Cortay et al., 1992; Giancotti et al., 1993; Schulze et al., 1993; De Lucia et al., 1994), so that they may play different roles in chromatin organization, with a non-random distribution.

A. Tight correlation between H1e variant and the inhibition of DNA methylation.

Some experiments were performed to verify whether or not these variants could differ from each other in their ability to exert a negative control on DNA methylation. Calf thymus H1 histone somatic variants were purified by reverse phase HPLC, the protein components in effluent composition being characterized by SDS/slab gel electrophoresis in 15% (w/v) polyacrylamide. As shown in **Figure 9**, only a restricted number of fractions, eluting as a single peak ("p3") was able to cause over 80% inhibition while the other fractions, namely "p1" and "p2" were totally ineffective (Santoro et al., 1995). The SDS/PAGE characterization of the various fractions indicated, according to Lennox et al. (1984) and to Lindner et al. (1990) the presence in "p1" of H1a, in "p2" of H1d and in "p3" of H1e and H1c.

Having not yet achieved a satisfactory separation of H1e and H1c, we managed to purify H1c and H1e (Zardo et al., 1996) in order to individuate which variant is really involved in the inhibition of DNA methylation process. A good separation in four peaks was obtained when H1 histone from L929 mouse fibroblasts was purified. The HPLC retention time of each peak, combined with 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 H1:DNA "physiological" ratio, **Figure 10**, so that it can be concluded that H1e is the unique variant involved in the inhibition of the DNA methylation process.

B. H1e: the only one variant able to bind the "CpG-rich" sequences.

Gel retardation assays were carried out in order to test the affinity of the different H1 variants for various synthetic oligonucleotides 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.

Experiments have shown (Santoro et al., 1995) 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 CpGs, while the fraction H1e-c was unique in binding CpG rich DNA sequences. Later, using H1e and H1c purified variants, we assessed that H1e variant binds itself better than H1c to the 6CpG oligonucleotide, **Figure 11**.

Our experimental data underline two important characteristics of H1e variant: this is the only variant which suppresses enzymatic DNA methylation and it is the only variant able to bind itself to CpG-rich sequences.

Figure 9. Separation and characterization of calf thymus H1 histone variants and their effect on *in vitro* DNA methylation: **a)** elution profile from the RP-HPLC 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 RP-HPLC column, at a protein/DNA ratio equal to 0.2 (w/w), on the *in vitro* activity of human placenta DNA methyltransferase. Each point represents the average results of ten different separations by RP-HPLC. "Reprinted from **Biochem. J.** 305, Santoro et al.. *Binding of histone H1e-c variants to CpG-rich DNA correlates with the inhibitory effect on enzymatic DNA methylation*, 739-744, (1995) with kind permission of Portland Press"

Figure 10. Separation and characterization of H1e and H1c variants from L929 fibroblasts and their effect on *in vitro* DNA methylation: **a)** HPLC separation of H1 histone variants and electrophoretic pattern, in 12% SDS-polyacrylamide gel of the eluted fractions (upon visualization by silver staining). **b)** Inhibition of DNA methyltransferase activity by H1e (open circles) or H1c (closed circles), at different protein-to-DNA ratios. "Reprinted from **Biochem. Biophys Res. Comm.** 20, Zardo et al.. *Inhibitory effect of H1e histone somatic variant on *in vitro* DNA methylation process*, 102-107, (1996) with kind permission of Academic Press, Inc."

Figure 11. Binding of H1e (open circles) and H1c (closed circles) to 44 bp synthetic 6CpG duplex oligonucleotide with the cytosines in the CpG moieties in unmethylated state. The binding was evaluated by gel retardation after incubation of the H1e and the H1c variants with the appropriate oligonucleotide, the relative amount of free DNA being measured by densitometric scanning of the autoradiograms. "Reprinted from **Biochem. Biophys Res. Comm.** 20, Zardo et al.. *Inhibitory effect of H1e histone somatic variant on *in vitro* DNA methylation process*, 102-107, (1996), with kind permission of Academic Press, Inc."

IV. Why poly(ADP-ribosyl)ation was selected out of all H1 histone post-synthetic modifications.

To explain how H1 histone could be involved in playing many multiplex very important structural and functional roles in chromatin it is important to remember that everyone of the genetic somatic variants can be dynamically modified by different post-synthetic enzymatic reactions (Wu et al., 1986; Davie, 1995) and sometimes the same protein can be substrate for more than one modification. Only in this way can we consider H1 histone as a protein characterized by a big

macroheterogeneity that allows different possible interactions with DNA or with other proteins. Some experimental data have led us to focus our attention on the poly(ADP-ribosylation) process.

A starting point derived from our results showing that when H1e is poly(ADP-ribosylated) it loses its condensing effect on chromatin structure even though it remains associated with linker DNA (D'Erme et al., 1996), **Figure 12**. Taking into account polyADP-ribose dependent chromatin decondensation (Poirier et al., 1982; Aubin et al., 1983; D'Erme et al., 1996), we considered the possibility that this modification may alter the interaction of H1 histone with linker DNA, causing a change in the methyl-accepting ability of CpG dinucleotides present essentially in their unmethylated form on linker DNA. Our aim was, therefore, to compare the methyl-accepting ability of native nuclei with that of nuclei in which chromatin decondensation was induced by poly(ADP-ribosylation). **Figure 13A** shows the incorporation of ADP-ribose polymers into H1 histone during the experimental time and that at the same time the methyl-accepting ability was not increased in the decondensed chromatin structure induced by the poly(ADP-ribosylation) process, **Figure 13B**. These data suggest that the poly(ADP-ribosylated) H1 histone has not been removed from linker DNA, despite possible alterations in the H1-DNA interactions and that, even if poly(ADP-ribosylation) decrease the H1e-H1e interactions that are essential for the formation of the higher levels of chromatin structure, the poly(ADP-ribosylated) isoform of H1e could be present in decondensed chromatin structure where the housekeeping genes are located.

The second starting point was the observation that the demethylation process utilizes an excision-repair mechanism to remove 5-methylcytosine. Since it is known that the poly(ADP-ribosylation) of H1 histone plays a relevant role in the repair mechanism (Boulikas, 1989; Realini and Althaus, 1992; Malanga and Althaus, 1994) H1 histone in its poly(ADP-ribosylated) isoform could indeed, following the demethylation process, remain bound to demethylated regions and regulate the *de novo* re-methylation process that defines the methylation pattern where the "CpG islands" are in an unmethylated state.

V. Correlation between DNA methylation and poly(ADP-ribosylation) processes.

A. Poly(ADP-ribosylation) process.

Poly(ADP-ribose) polymerase (EC 2.4.2.30) is a nuclear enzyme that has been implicated in a number of important biological processes (Jacobson and Jacobson, 1989; de Murcia et al., 1995). Although poly(ADP-ribose) polymerase is able to bind undamaged DNA, it needs DNA strand breaks for its activation. Each monomer of this enzyme, which is a dimer in its catalytic form (Mendoza-Alvarez and Alvarez-Gonzales, 1993), has three domains which play specific roles in the poly(ADP-ribosylation) process. The zinc finger motifs in the N-terminal domain are responsible for the DNA recognition site, taking advantage of DNA strand breaks rather than of specific polynucleotide sequences (Ménissier de Murcia et al., 1989; Gradwohl et al., 1990; Ikejima et al., 1990; de

Figure 12. A) Cross-linking analysis to investigate the role played by each H1 histone variant on the formation of H1-H1 polymers: SDS-PAGE patterns of H1 histone variants, at 30% (w/w) H1:DNA ratio, incubated with 1.2 kb oligonucleosomal DNA in 40 mM NaCl for 1 hour at room temperature and then treated with dithiobis(succinimidyl)propionate (DSP 0.2 mg/ml) for 20 min: H1a, H1b, H1e, H1c (lane1-4). In lanes 5 and 6, untreated histone H1 and histone H1 treated with DSP were run as controls in the absence of DNA and **B)** the effect of the "enriched" poly-ADP-ribosylation of H1e variant, vs the native one, on the formation of H1-H1 polymers: SDS-

PAGE patterns of the product of cross-linking of the H1e histone isoforms at different (w/w) H1:DNA ratio, incubated with 1.2 kb oligonucleosomal DNA, in 40 mM NaCl for 1 hour at room temperature and then treated with dithiobis(succinimidyl)propionate (DSP 0.2 mg/ml) for 20 min: 30%, 20% and 10% (w/w) of H1e:DNA (lane 1-3); 30%, 20% and 10% (w/w) of "enriched" poly(ADP-ribosyl)ated H1e:DNA (lane 4-6). "Reprinted from **Biochem. J.** 316, D'Erme et al.. *Co-operative interactions of oligonucleosomal DNA with the H1e histone variant and its poly(ADP-ribosyl)ated isoform*, 475-480, (1996) with kind permission of Portland Press".

Figure 13. Methyl-accepting ability as assay to study the interactions of H1 histone to linker DNA in native nuclei vs poly(ADP-ribosyl)ated ones. **A):** time course of incorporation of [³²P] ADPribose polymers associated to H1 histone extracted by 10% PCA (w/v) from nuclei incubated with 50 μM [³²P]-NAD; **B):** methyl-accepting ability of native nuclei (open circles) vs poly(ADP-ribosyl)ated ones (closed circles). "Reprinted from **Biochem. J.** 316, D'Erme et al.. *Co-operative interactions of oligonucleosomal DNA with the H1e histone variant and its poly(ADP-ribosyl)ated isoform*, 475-480, (1996) with kind permission of Portland Press".

Murcia and Ménissier de Murcia, 1994). The C-terminal domain contains the catalytic site (de Murcia et al., 1995). As for the central domain, it undergoes automodification upon binding of the enzyme on the damaged DNA by introducing ribose polymers -- up to 200 residues according to Alvarez-Gonzalez and Jacobson (1987) -- on 28 automodification sites (Kawaichi et al., 1981; Desmarais et al., 1991) which are essentially localized in this domain.

The active enzyme can then start a series of heteromodification reactions that modulate the functions of chromatin proteins (Ferro et al., 1983; Yoshihara et al., 1985; Boulikas, 1989; Scovassi et al., 1993).

In vitro experiments have shown that this poly(ADP-ribosyl)ation mechanism can involve H1 histone binding polymers both in a covalent and in a non-covalent manner. The covalent modification introduces in the C and N-terminal tails of this histone short polymers (8-10 units), whose sizes are specifically defined by the histone itself (Naegeli and Althaus, 1991), while long branched polymers of ADP-ribose are able to form non-covalent interactions with this chromatin protein (Panzeter et al., 1992).

B. Effect of poly(ADP-ribosyl)ated H1 histone on *in vitro* DNA methylation.

The aim of these experiments was to examine, *in vitro* the possible correlation between DNA methylation and poly(ADP-ribosyl)ation processes and, in particular, whether or not the inhibitory effect exerted by H1 histone on *in vitro* enzymatic DNA methylation (Caiafa et al., 1991) could be essentially due to the poly(ADP-ribosyl)ated isoform of this protein.

In order to verify this hypothesis the poly(ADP-ribosyl)ated and the poly(ADP-ribose)-free H1 histone isoforms were purified. The modified protein was purified by affinity chromatography on an aminophenylboronate column of H1 histone obtained from permeabilized L929 mouse fibroblasts (Zardo et al., 1997) incubated for 10 min with 500 μM NAD, **Figure 14A**, while the unmodified one was obtained from mouse fibroblasts preincubated for 24 hours with 8 mM 3-aminobenzamide, a well-known inhibitor of the poly(ADP-ribosyl)ation process (Griffin et al., 1995). In both preparations the entire H1 histone fraction was isolated by overnight extraction in 0.2 M H₂SO₄ followed by a second extraction in 10% (w/v) PCA (Johns, 1977). DNA methyltransferase assays, performed in presence of 5 units DNA methyltransferase purified from human placenta nuclei and using as methyl donor 16 μM SAM plus 50

$\mu\text{Ci/ml } ^3\text{H-SAM}$, have shown that the poly(ADP-ribose)-free isoform of H1 histone failed to inhibit *in vitro* DNA methylation when added up to a protein/DNA ratio of 0.25 (w/w) while the poly(ADP-ribosyl)ated one was, instead, highly inhibitory under the same condition, **Figure 14B**.

C. Effect of ADP-ribose polymers on *in vitro* DNA methylation.

Other experiments were carried out in order to verify whether ADP-ribose polymers by themselves could play a direct role in the modulation of DNA methyltransferase activity. ADP-ribose polymers, isolated from L929 fibroblasts incubated with $50 \mu\text{M } ^{32}\text{P-NAD}$ were fractionated on Sephadex G-50. These protein-free polymers caused a clear-cut inhibition of *in vitro* methylation of dsDNA but not of ssDNA. The extent of this inhibition is directly dependent on the size of the polymers, as compared to a control assay in absence of polymers considered as 100%, **Figure 15**. Since a high ADP-ribose polymers/DNA ratio did not affect methylation of ssDNA the polymers can hardly be visualized as directly interacting with DNA methyltransferase.

In the close relationship existing between poly(ADP-ribosylation) and DNA methylation processes, the poly(ADP-ribosylation) of H1 histone appears to play a key role. Since the association of H1 histone with ADP-ribose polymers can be either covalent (Naegeli and

Althaus, 1991) or non-covalent (Panzeter et al., 1992), further investigations are needed to ascertain whether also the latter adduct is effective in maintaining CpG dinucleotides in their unmethylated state. To go into this question some *in vivo* experiments were performed in which the correlation between DNA methylation and poly(ADP-ribosylation) processes was investigated by using the methyl-accepting ability assay on isolated nuclei and/or purified DNA from L929 mouse fibroblasts. The results shown in **Figure 16**, support the working hypothesis of an *in vivo* relationship between the two nuclear processes suggesting a role of poly(ADP-ribosylation) in preserving a number of CpG dinucleotides from endogenous methylation, maintaining them in an unmethylated state. By gel retardation assay we could also show that poly(ADP-ribosyl)ated H1 histone has a high capacity of linking CpG-rich ds-oligonucleotide, so that it is possible to suppose that it has a preferential location on genomic DNA in regions rich in these nucleotides. Since, on the other hand, only relatively short poly-ADPribose chain(s) are bound to H1 histone (D'Erme et al., 1996), it is unlikely that they can be responsible by themselves for the intense inhibitory effect exerted on the methylation of ds DNA by the poly(ADP-ribosyl)ated isoform of H1 histone. In conclusion our hypothesis is that after DNA packaging into nucleosomes, the access to the DNA of a

Figure 14. **A)** Purification of poly(ADPriboseyl)ated H1 histone isoform on an aminophenylboronate column chromatography, monitoring the absorbance at 230 nm (closed circles), or the radioactivity (open circles). **B)** Comparison between poly(ADP-ribose)-free H1 histone (closed squares) and the purified poly(ADP-ribosyl)ated isoform (closed circles) for their inhibitory effect on *in vitro* DNA methylation. Each value is the average value of three different experiments. "Reprinted from **Biochemistry** 36, Zardo et al.. *Does*

poly(ADP-ribosyl)ation regulate the DNA methylation pattern?, 7937-7943, (1997) with kind permission of the American Chemical Society".

Figure 15. Effect of ADP-ribose polymers of different size (**A**: striped bars, $n > 40$; **B**: white bars, $n < 6n < 40$; **C**: horizontally striped bars, $n < 20$) on *in vitro* DNA methylation. Control assay, taken as 100%, was performed in absence of polymers. Different polymers/DNA ratios, ranging from 0.25 to 1.00, are indicated in the abscissa. The assay was carried out for 1 h at 37°C in the presence of 50 units/ml DNA methyltransferase purified from human placenta nuclei, using 30 µg/ml *Micrococcus luteus* dsDNA (left panel) and ssDNA (right panel) as substrates and 30 µCi/ml ³H-SAM as donor of methyl groups. The incorporation of ³H-SAM in control dsDNA was 4.1 ± 0.1 picomoles and in control ssDNA 4.6 ± 0.3 picomoles. Histograms, in which error bars have been included, represent the average value of three different experiments. "Reprinted from **Biochemistry** 36, Zardo et al.. *Does poly(ADP-ribosyl)ation regulate the DNA methylation pattern?*, 7937-7943, (1997) with kind permission of the American Chemical Society".

Figure 16. - Methyl-accepting ability experiments. In panel **A** the endogenous methyl accepting ability of native nuclei, obtained from 6.5×10^6 L929 fibroblasts preincubated for 24 hrs without (control) and with 8 mM 3ABA, was performed in the presence of 16 µM ³H-SAM. The level of methyl groups has been evaluated on the total DNA purified from cells. Control DNA, whose incorporation was 2.8 ± 0.1 picomoles of ³H SAM, was considered as 100%. In panels **B** and **C**, DNA samples (3 mg each) purified from the nuclei -- obtained from 6.5×10^6 L929 fibroblasts preincubated for 24 hrs without (control) and with 8 mM 3ABA and where the endogenous methyl accepting ability had previously been saturated with 16 µM "cold" SAM -- were used as substrates for evaluating their residual methyl accepting ability in the presence either of 50 units/ml human DNA methyltransferase or of 50 units/ml bacterial SssI methylase. The incorporation of ³H SAM in control DNA was 0.3 ± 0.02 picomoles in panel **B** and 6 ± 0.2 picomoles in panel **C**. Histograms, in which error bars have been included, represent the average value of three different experiments. "Reprinted from **Biochemistry** 36, Zardo et al.. *Does poly(ADP-ribosyl)ation regulate the DNA methylation pattern?*, 7937-7943, (1997) with kind permission of the American Chemical Society".

moving methyltransferase would then be limited by the presence of poly(ADP-ribosylated) H1 and/or by preferentially long and branched polymers linked in a non-covalent way to the histone, so as to afford protection of the unmethylated state of those CpG-rich DNA regions (Zardo et al., 1997).

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