Biochemistry

 Volume 36, Number 26 July 1, 1997

Articles

Does Poly(ADP-ribosyl)ation Regulate the DNA Methylation Pattern? †

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Received January 31, 1997; Revised Manuscript Received April 14, 1997⊗

ABSTRACT: The existence of a possible correlation between poly(ADP-ribosyl)ation and DNA methylation processes was investigated. *In vivo* and *in vitro* experiments were carried out on L929 mouse fibroblasts preincubated for 24 h with or without 3-aminobenzamide, a well-known inhibitor of poly(ADP-ribose) polymerase. Both experimental approaches evidenced a close relationship between these two important nuclear enzymatic mechanisms, suggesting that the poly(ADP-ribosyl)ated isoform of H1 histone and/or long and branched protein-free ADP-ribose polymers could act as protecting agents against full methylation of the CpG dinucleotides in genomic DNA.

Poly(ADP-ribose) polymerase (EC 2.4.2.30) and DNA methyltransferase (EC 2.1.1.37) are two nuclear enzymes that have been implicated in a number of important biological processes (Jacobson & Jacobson, 1989; Jost & Saluz, 1993; Eden & Cedar, 1994; Adams, 1995; 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 & Alvarez-Gonzalez, 1993), has three domains which play specific roles in the poly(ADP-ribosyl)ation 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; Ikejma et al., 1990; de Murcia & 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, to which ADP-ribose polymers can bind both in a covalent and in a noncovalent 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

† This work was supported by the Italian Ministry of University and Scientific and Technological Research (60% Progetti di Ateneo and 40% Progetti di Interesse Nazionale), by the Fondazione "Istituto Pasteur-Fondazione Cenci Bolognetti", and by the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.).

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⊗ Abstract published in *Advance ACS Abstracts*, June 15, 1997.

S0006-2960(97)00241-9 CCC: $14.00

histone itself (Naegeli & Althaus, 1991), while long branched polymers of ADP-ribose are capable of forming noncovalent adduct(s) with chromatin proteins, primarily with H1 histone (Panzeter et al., 1992).

Several authors have stressed the importance of H1 histone poly(ADP-ribosyl)ation in the excision repair mechanism (Boulikas, 1989; Realini & Althaus, 1992; Malanga & Althaus, 1994) as well as in chromatin decondensation (Poirier et al., 1982; Aubin et al., 1983; D'Erme et al., 1996). We have recently shown by *in vitro* experiments that—among the several roles attributed to this protein—the H1 histone is the unique chromatin protein that, through its H1e variant (Santoro et al., 1995; Zardo et al., 1996), is able to cause a marked inhibition of DNA methylation. 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 whereby the regulatory "CpG-rich 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., 1986; Brandeis et al., 1993; Jost & Jost, 1994), this process being probably required for gene activation.

Since demethylation introduces some breaks on DNA, the poly(ADP-ribosyl)ation of H1 histone, induced by these breaks (Ménissier de Murcia et al., 1989; Gradwohl et al., 1990; Ikejma et al., 1990; de Murcia & Ménissier de Murcia, 1994), could regulate the *de novo* remethylation that defines the methylation pattern that maintains the CpG islands in an unmethylated state.

The aim of this paper will be to examine, *in vivo* and *in vitro*, the possible correlations between DNA methylation and poly(ADP-ribosyl)ation, and in particular whether the inhibition exerted by H1 histone on enzymatic DNA methylation can be ascribed essentially to the poly(ADP-ribosyl)ated isoform of this histone.

MATERIALS AND METHODS

Chemicals and Materials. L929 mouse fibroblast cells were a kind gift of Roger L. P. Adams (Institute of Biomedical and Life Sciences, University of Glasgow). All materials for cell culture were obtained from GIBCO BRL Life Technologies; [^{32}P]NAD (specific activity 800 Ci/mmol), *S*-adenosyl-L-[*methyl*-^{3}H]methionine (specific activity 70−80 Ci/mmol), and [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) were from Du Pont−New England Nuclear; proteinase K (EC 3.4.21.14), trypsin (EC 3.4.21.4), and polynucleotide kinase (EC 2.7.7.7) were from Boehringer Mannheim; *SssI* methylase was from New England BioLabs; 3-aminobenzamide and *S*-adenosyl-L-methionine were from Sigma; MNNG[1] and BrdUrd were from Fluka; anti-BrdUrd (IgG clone BU5.1) and fluoresceinated anti-IgG antibodies were from Ylem; Sephadex G-50 was from Pharmacia LKB Biotechnology; and aminophenylboronate was from Amicon Division. All other chemicals used were of the highest purity commercially available.

Cell Cultures and FACS Analysis. L929 mouse fibroblasts were grown in BHK21 medium with the addition of 10% newborn calf serum and 2 mM glutamine in a humidified 5% CO_2 atmosphere at 37 °C. For FACS analysis, the cells were grown in a medium containing 0.5% newborn calf serum, added after 48 h, with newborn calf serum to 10% final concentration with or without 8 mM 3ABA and collected at 15 and 24 h in order to monitor the effects of 3ABA on the cell cycle. To analyze the G1 to S progression, the cells were exposed to 45 mM BrdUrd for 15 min at 37 °C, collected by trypsinization, and fixed with a 1:5 (v/v) acetone/methanol solution. The fixed cells were then centrifuged, washed in PBS + 0.5% Tween 20, incubated in 1 N HCl for 45 min, neutralized, exposed to a monoclonal anti-BrdUrd antibody for 30 min in the dark, washed, and exposed to a fluoresceinated secondary anti-IgG antibody. Propidium iodide (50 μg/mL) was then added, and FACS analysis was performed after 30 min in the dark.

Isolation and Purification of Poly(ADP-ribosyl)ated H1 Histone Isoform. At a cell density of 20×10^6 cells/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. Cells were permeabilized at 4 °C for 30 min in 10 mM Tris-HCl buffer (pH 7.8) containing 5 mM DTT, 4 mM $MgCl_2$, and 1 mM Na-EDTA and collected by centrifugation at 2500 rpm for 10 min at 5 °C in a Sorvall centrifuge. The cells were then incubated for 10 min with 500 μM NAD in 50 mM Tris-HCl buffer (pH 7.8) containing 10 mM $MgCl_2$, 45 mM KCl, 5 mM DTT, and 0.1 mM PMSF. After precipitation with 20% TCA on ice, cells were washed twice with 5% TCA, twice with ethanol, and once with diethyl ether. The entire H1 histone fraction was isolated by overnight extraction in 0.2 M H_2SO_4 followed by a second extraction in 10% (w/v) PCA (Johns, 1977).

The poly(ADP-ribosyl)ated H1 histone isoform was purified by affinity chromatography on aminophenylboronate column (1 × 20 cm) equilibrated in the 50 mM morpholine hydrochloride buffer (pH 8.5) containing 6 M guanidine hydrochloride. To this purpose, the entire H1 histone fraction was dissolved and eluted in the same buffer, and the poly(ADP-ribosyl)ated H1 histone was then eluted with 0.5 M sodium phosphate buffer, pH 4.5, containing 6 M guanidine hydrochloride (Okayama et al., 1978; Adamiezt et al., 1979). The elution profile was monitored by the absorbance at 230 nm. The fractions corresponding to the poly(ADP-ribosyl)ated H1 histone were subjected to progressive dialyses against 5 mM Tris-HCl buffer (pH 7.8) *plus* 0.1 mM PMSF to remove the salts. The unlabeled purified poly(ADP-ribosyl)ated H1 histone, obtained from 1.3×10^9

[1] Abbreviations: 3ABA, 3-aminobenzamide; BrdUrd, 5′-bromo-2′-deoxyuridine; BBF, bromophenol blue; PBS, phosphate-buffered saline; DTT, dithiothreitol; FACS, fluorescence-activated cell sorter; MNNG, *N*-methyl-*N*′ nitro-*N*-nitrosoguanidine; Na-EDTA, ethylenediaminetetraacetate sodium salt; PMSF, phenylmethanesulfonyl fluoride; PCA, perchloric acid; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; S-AdoMet, *S*-adenosyl-L-methylmethionine; TBE, Tris−borate−EDTA; XC, xylene cyanol.

mouse fibroblasts with a recovery of 0.08−0.1% relative to total H1 histone, was used in the *in vitro* methyl-accepting ability and in gel retardation assays.

We checked that the fraction collected as unlabeled purified poly(ADP-ribosyl)ated H1 had been correctly identified by carrying out parallel preparations with 50 μM [^{32}P]-NAD. The comparison showed that both the elution profile obtained by following the radioactivity associated to the ADP-ribose polymers and the absorbance at 230 nm were superimposable. Before fractionating on the aminophenyl-boronate column, the labeled preparation of H1 histone was analyzed as previously described (D'Erme et al., 1996) by 15% SDS−polyacrylamide gel electrophoresis (data not shown).

A preparation of H1 histone "enriched" in its poly(ADP-ribosyl)ated form was carried out with the aim of controlling the size of ADP-ribose polymers covalently associated to H1 histone. This preparation was obtained by following the above described procedure, except for the addition to the fibroblasts of 30 μM MNNG in the incubating buffer in order to cause DNA fragmentation (Malanga & Althaus, 1994) and to stimulate the poly(ADP-ribosyl)ation process. As shown by Naegeli and Althaus (1991), treatment with this drug does not affect the size of the polymers covalently bound to H1 histone since it is established by the protein itself.

Isolation of H1 Histone in Its Poly(ADP-ribose)-Free Isoform. To obtain the poly(ADP-ribose)-free isoform of H1 histone, mouse fibroblasts (at a cell density of 20×10^6 cells/175 cm^2 flask) were preincubated for 24 h with 3ABA, and the H1 histone was isolated by overnight extraction of cells in 0.2 M H_2SO_4 followed by a second extraction in 10% (w/v) PCA (Johns, 1977).

Purification of Total ADP-Ribose Polymers from Mouse Fibroblasts and from H1 Histone. Cells, previously permeabilized and preincubated with 50 μM [^{32}P]NAD for 10 min, were precipitated with 20% TCA in ice. Labeled ADP-ribose polymers were purified according to Malanga and Althaus (1994). ADP-ribose polymers were extracted by overnight digestion of the pellet with proteinase K (0.2 mg/mL) at 37 °C in 10 mM Tris-HCl, 1 mM Na-EDTA (pH 8.0). An equal volume of 1 M KOH *plus* 100 mM Na-EDTA was added, and the incubation was prolonged for 2 h at 37 °C. After the pH was adjusted to a value of 9 by addition of 6 M HCl, the solution was centrifuged at 3500 rpm for 10 min at 25 °C.

The supernatant was repeatedly extracted with equal volumes of chloroform/isoamyl alcohol 24:1 (v/v). ADP-ribose polymers were subjected to a preliminary fractionation on a Sephadex G-50 column (0.5 × 12 cm) and eluted in water. Each fraction (0.4 mL), monitored by radioactivity and absorbance at 230 nm, was dissolved in 50% (w/v) urea, 25 mM NaCl, and 4 mM Na-EDTA and then loaded on a 20% polyacrylamide gel [bis(acrylamide):acrylamide ratio = 1:19] in 0.09 M Tris−borate buffer, pH 8.3. The gels were run for 5 h at 400 V in the same buffer, then dried, and autoradiographed on Kodak X-ray film.

Poly(ADP-ribose) chain lengths were calculated on the basis of their mobility *vs* that of the dyes bromophenol blue (BBF) and xylene cyanol (XC) (Alvarez-Gonzalez & Jacobson, 1987).

ADP-ribose polymers covalently bound to H1 histone were purified from a labeled H1 histone preparation "enriched" in its poly(ADP-ribosyl)ated isoform. Their size was determined as previously described (D'Erme et al., 1996).

DNA Methyltransferase Assay. DNA methyltransferase was purified from human placenta nuclei (Carotti et al., 1989). Its activity was assayed at 37 °C for 1 h, by using 5 units of enzyme [defined according to Caiafa et al. (1991)] in 100 μL of 50 mM Tris-HCl buffer (pH 7.8) containing 10% (v/v) glycerol, 5 mM Na-EDTA, 0.5 mM DTT, 30 μCi/mL [^{3}H]-S-AdoMet, and 30 μg/mL single- or double-stranded DNA from *Micrococcus luteus*. Single-stranded DNA has been obtained by heating the double-stranded DNA at 100 °C for 10 min, followed by rapid cooling on ice. The effect of polymers of different sizes on DNA methylation was tested using polymer:DNA ratios in the 0.25−1.0 (w/w) range.

The same procedure was used to test the effect, on DNA methylation, of poly(ADP-ribosyl)ated H1 histone *vs* H1 histone in its poly(ADP-ribose)-free isoform.

Methyl-Accepting Ability Assay. Experiments were carried out on nuclei obtained from 6.5×10^6 L929 mouse fibroblasts preincubated for 24 h with or without 8 mM 3ABA. Nuclei were prepared according to Davis et al. (1986), the final pellet being resuspended in 50 mM Tris-HCl buffer (pH 7.8), 1 mM DTT, 1 mM Na-EDTA, and 25−50% (v/v) glycerol. Endogenous methyl-accepting ability of nuclei was assayed, as described by D'Erme et al. (1993), by adding 16 μM [^{3}H]-S-AdoMet as methyl donor for 1 h at 37 °C. In nuclei obtained from cells where the poly(ADP-ribosyl)ation had been suppressed by preincubation with 8 mM 3ABA for 24 h, the same inhibitor concentration was present throughout all subsequent steps. In *in vitro* experiments, it was checked that, at the concentration used, 3ABA had no direct effect on DNA methyltransferase activity. The reaction was stopped by addition of 1% (w/v) SDS and 250 μg/mL proteinase K at 37 °C overnight. The total purified DNA was resuspended in 10 mM Tris-HCl (pH 7.8), 1 mM Na-EDTA, and the incorporation of labeled methyl groups was evaluated.

In parallel experiments, the endogenous methyl-accepting ability was saturated in the nuclei by adding 16 μM "unlabeled" S-AdoMet for 1 h at 37 °C. DNA purified from these nuclei (3 μg) was used as substrate for evaluating its residual methyl-accepting ability in a final volume of 100 μL in the presence of either 5 units of DNA methyltransferase purified from human placenta nuclei (using, in this case, as methyl donor 16 μM S-AdoMet *plus* 50 μCi/mL [^{3}H]-S-AdoMet) or 5 units of bacterial *SssI* methylase (using as methyl donor 80 μM S-AdoMet *plus* 50 μCi/mL [^{3}H]-S-AdoMet). The radioactivity was measured in a Beckman LS-6800 liquid scintillation spectrometer.

Band Mobility Shift Assay. Oligonucleotides were synthesized by the phosphoramidite method on a DNA synthesizer (Applied Biosystem).

Single-stranded oligonucleotides were annealed according to Kadonaga and Tjian (1986), and the double-stranded oligonucleotides (44 bp long) were purified on a 3 mm thick 15% polyacrylamide gel containing 1 × TBE [89 mM Tris, 89 mM boric acid, and 2 mM Na-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 *plus* 0.5 mM Na-EDTA, pH 7.8 (Saluz & Jost, 1990). The ds-oligonucleotides were recovered by ethanol precipitation in the presence of 10 mg/mL

tRNA as carrier and end-labeled at their 5′ ends with 50 μCi of [γ-^{32}P]ATP using polynucleotide kinase under the conditions recommended by the manufacturer. End-labeled oligonucleotides, purified through a G-50 Sephadex column, were mixed with the poly(ADP-ribosyl)ated H1 histone or with H1 histone, at a 2.5−10 protein:DNA ratio (w/w) in 20 μL of binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM Na-EDTA, and 0.5 mM DTT).

After 1 h incubation at 37 °C, the samples were loaded on a 6% polyacrylamide gel in 0.02 M TBE buffer, pH 8.3. The gels were run in the same buffer for 1 h at 30 mA, fixed in 10% acetic acid and 10% methanol for 15 min, dried, and autoradiographed on Kodak X-ray film.

Due to the high cationic charge of H1 histone and to the limited size of our synthetic oligonucleotides, the actual DNA−protein complex could not be evidenced in this kind of gel electrophoresis (Zardo et al., 1996). Binding of DNA to the two isoforms of H1 histone 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.

RESULTS

Methyl-Accepting Ability Assay on Isolated Nuclei and on Purified DNA from L929 Mouse Fibroblasts. The methyl-accepting ability assay on isolated nuclei and/or purified DNA from L929 mouse fibroblasts was used to study the correlation between poly(ADP-ribosyl)ation and DNA methylation.

A series of preliminary controls were carried out in order to ensure the correct experimental conditions. The results of these controls are briefly summarized without actually reporting the data. FACS analysis on synchronized cultures of fibroblasts incubated for 24 h with or without 8 mM 3ABA—which is, as already mentioned, an inhibitor of poly(ADP-ribose) polymerase (Griffin et al., 1995)—indicated that the cell cycle is not perturbed by the presence of 3ABA, the percentages of G1, S, and (G2+M) being, in both cases, 46%, 42%, and 12%, respectively. We also tested whether or how incubation of L929 fibroblasts with 3ABA for 24 h could influence the amount and the size of ADP-ribose polymers in the cells. A drastic decrease (by approximately 80%) of the ADP-ribose polymers was observed, and electrophoretic analysis on a 20% polyacrylamide gel evidenced, according to Malanga and Althaus (1994), that this decrease affected essentially the longest and more branched polymers. Furthermore, we checked that the incubation with 3ABA for 24 h did not introduce an abnormal DNA fragmentation in the cells. Finally, 3ABA by itself did not affect, *in vitro*, the DNA methyltransferase activity.

In Figure 1A is reported a first series of experiments on the endogenous methyl-accepting ability of isolated nuclei obtained from 6.5 × 10^6 fibroblasts preincubated for 24 h with or without 8 mM 3ABA. We compared, by incubating them for 1 h at 37 °C in the presence of [^{3}H]-S-AdoMet, their ability to incorporate labeled methyl groups in their DNA in the absence of any exogenous DNA methyltransferase (i.e., by a process catalyzed by the endogenous enzyme). The incorporation of methyl groups was found to be 60% higher in the DNA from 3ABA-treated cells than in DNA from control cells (whose incorporation was 2.8 ± 0.1 pmol and was taken as 100%).

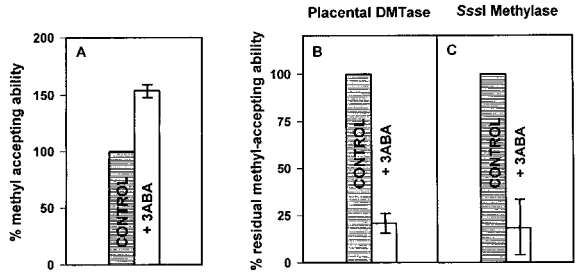

FIGURE 1: Methyl-accepting ability experiments. In panel A, the endogenous methyl-accepting ability of native nuclei, obtained from 6.5 × 10^6 L929 fibroblasts preincubated for 24 h without (control) and with 8 mM 3ABA, was performed in the presence of 16 μM [^{3}H]-S-AdoMet. The level of methyl groups has been evaluated on the total DNA purified from cells. Control DNA, whose incorporation was 2.8 ± 0.1 pmol of [^{3}H]-S-AdoMet, was considered as 100%. In panels B and C, DNA samples (3 μg each) purified from the nuclei [obtained from 6.5 × 10^6 L929 fibroblasts preincubated for 24 h without (control) and with 8 mM 3ABA and where the endogenous methyl-accepting ability had previously been saturated with 16 μM "unlabelled" S-AdoMet] 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 [^{3}H]-S-AdoMet in control DNA was 0.3 ± 0.02 pmol in panel B and 6 ± 0.2 pmol in panel C. Histograms, in which error bars have been included, represent the average value of three different experiments.

We also considered the possibility that a lower level of endogenous S-AdoMet, due according to Borek et al. (1984) to the interference of 3ABA in the metabolism of S-AdoMet, could have altered the incorporation of CH_3 groups in the previously described experiment. To clarify this point, endogenous methyl-accepting ability was saturated by adding 16 μM "unlabeled" S-AdoMet as methyl donor in the nuclei obtained from fibroblasts preincubated for 24 h with or without 8 mM 3ABA. The DNA (3 μg) purified from these nuclei was then used as substrate for the evaluation of its residual methyl-accepting ability by adding, as exogenous enzymes, 50 units/mL DNA methyltransferase from human placenta or 50 units/mL bacterial *SssI* methylase together with labeled S-AdoMet. The DNA purified from 3ABA-treated cells, whose available sites had previously been saturated with "unlabeled" S-AdoMet, showed a residual methyl-accepting ability, catalyzed by the exogenous methylases, of only 20−40% (Figure 1B,C) with respect to that of DNA from the control cells (corresponding to 0.3 ± 0.02 pmol with human DNA methyltransferase and to 6 ± 0.2 pmol with the bacterial enzyme). The results, shown in Figure 1, therefore support our working hypothesis of an *in vivo* relationship between poly(ADP-ribosyl)ation and DNA methylation suggesting a role of poly(ADP-ribosyl)ation in preserving a number of CpG dinucleotides from endogenous methylation, thus maintaining them in an unmethylated state.

Do ADP-Ribose Polymers Play a Direct Role in the Modulation of DNA Methyltransferase Activity? ADP-ribose polymers, isolated from L929 fibroblasts incubated with 50 μM [^{32}P]NAD, were fractionated on a Sephadex G-50 column, and the chain length was analyzed by gel electrophoresis (Figure 2). Polymer fraction A differed from fraction B because the former also contained those long and branched polymers that did not enter the gel (Malanga & Althaus, 1994). Protein-free polymers caused a clear-cut inhibition of *in vitro* methylation of dsDNA but not of ssDNA (Figure 3). The extent of this inhibition was directly dependent on the size of the polymers, as compared to a

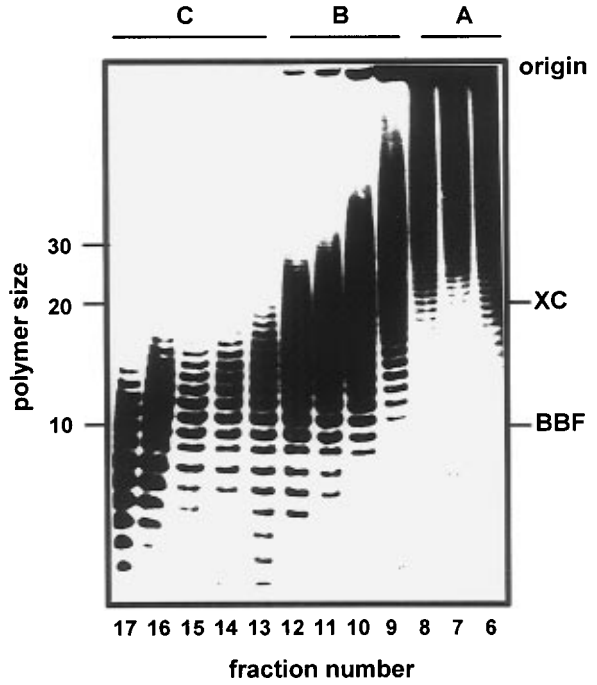

FIGURE 2: Autoradiography of gel electrophoresis of labeled ADP-ribose polymers extracted from L929 mouse fibroblasts and fractionated by a Sephadex G-50 column. The eluted fractions (0.4 mL) have been collected in three main groups containing polymers of different size: (A) $n > 40$; (B) $n\ 6 < n < 40$; (C) $n < 20$. Poly(ADP-ribose) chain lengths were calculated on the basis of their mobility relative to that of the dyes BBF and XC. The specific activity of polymers was as follows: fraction A, 37×10^3 cpm/mg; fraction B, 26×10^3 cpm/mg; and fraction C, 1.2×10^3 cpm/mg.

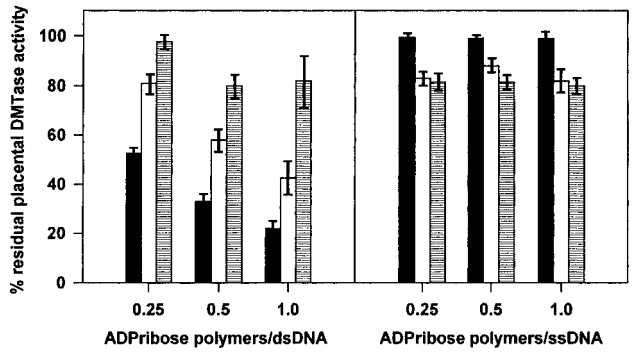

FIGURE 3: Effect of ADP-ribose polymers of different size (A: striped bars, $n > 40$; B: white bars, $n\ 6 < n < 40$; C: horizontally striped bars, $n < 20$) on *in vitro* DNA methylation. The control assay, taken as 100%, was performed in the absence of polymers. Different polymers:DNA ratios, ranging from 0.25 to 1.00, are indicated on 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 [^{3}H]-S-AdoMet as donor of methyl groups. The incorporation of [^{3}H]-S-AdoMet in control dsDNA was 4.1 ± 0.1 pmol and in control ssDNA 4.6 ± 0.3 pmol. Histograms, in which error bars have been included, represent the average value of three different experiments.

control assay in the absence of polymers considered as 100% (corresponding to 4.1 ± 0.1 pmol for dsDNA and to 4.6 ± 0.3 pmol for ssDNA). Since a high ADP-ribose polymers: DNA ratio did not affect methylation of ssDNA, the polymers, by themselves, could not be visualized as directly interacting with DNA methyltransferase.

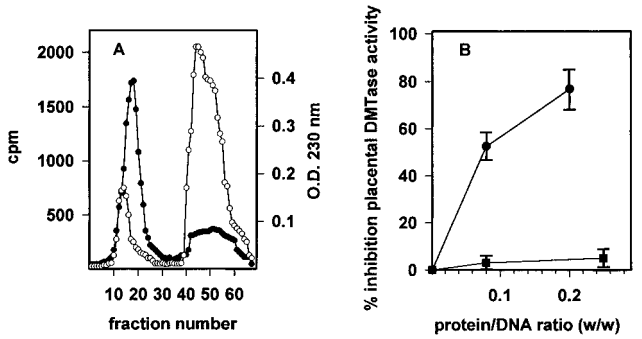

FIGURE 4: (A) Purification of poly(ADP-ribosyl)ated H1 histone isoform by aminophenylboronate column chromatography, monitoring the absorbance at 230 nm (●) or the radioactivity (○). (B) Comparison between poly(ADP-ribose)-free H1 histone (■) and the purified poly(ADP-ribosyl)ated isoform (●) for their inhibitory effect on *in vitro* DNA methylation, assayed as described under Materials and Methods. Each value is the average value of three different experiments.

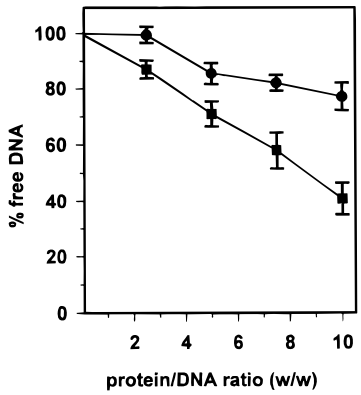

FIGURE 5: Band mobility shift assay of H1 histone (●) and of purified poly(ADP-ribosyl)ated isoform of H1 histone (■) incubated with the 44 bp synthetic "CpG-rich" ds-oligonucleotide. The binding was evaluated after incubation for 1 h at 37 °C and was reported as densitometric analysis (Bio Image, Millipore) of the autoradiograms. The percentage of free DNA was given relative to the DNA of samples incubated in the absence of protein (control, considered as 100%). Different protein:ds-oligonucleotide ratios—ranging from 2.5 to 10 (w/w)—are indicated on the abscissa.

Does Poly(ADP-ribosyl)ation of H1 Histone Modulate the DNA Methylation Process? In order to verify whether the inhibition 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, we have compared the effect of the poly(ADP-ribose)-free isoform of H1 histone *vs* the poly(ADP-ribosyl)ated one, using as enzyme the placental DNA methyltransferase.

The poly(ADP-ribose)-free form of H1 histone, obtained from fibroblasts preincubated for 24 h with 3ABA, failed to inhibit *in vitro* DNA methylation when added up to a protein: DNA ratio of 0.25 (w/w). The poly(ADP-ribosyl)ated isoform of H1 histone, purified by affinity chromatography on a phenylboronate column (Figure 4A), was instead highly inhibitory under the same condition (Figure 4B).

Unmodified H1 histone and the purified poly(ADP-ribosyl)ated isoform were compared, by band mobility shift assay, for their affinity for a 44 bp ds-oligonucleotide with CpG-rich sequence. The poly(ADP-ribosyl)ated isoform exhibited a higher affinity, as compared to the unmodified one, for this "CpG island-like" oligonucleotide (Figure 5).

DISCUSSION

Our *in vivo* experiments carried out on L929 mouse fibroblasts preincubated for 24 h with or without 8 mM 3ABA, a well-known inhibitor of poly(ADP-ribose) polymerase, indicate the existence of a negative correlation between poly(ADP-ribosyl)ation and DNA methylation: upon incubation with 3ABA, there is, in the isolated nuclei, a consistent increase of DNA susceptibility to be methylated by endogenous DNA methyltransferase. Subsequent methylation by exogenous enzymes is, as a consequence, severely reduced.

This increased DNA methyl-accepting ability cannot be ascribed to an increased demethylation occurring in cells preincubated with 3ABA, since Jost and Jost (1994) have shown that, *in vivo*, 3ABA blocks the 5-methyldeoxycytidine excision repair mechanism and the genome-wide demethylation linked to the onset of the differentiation process.

Poly(ADP-ribosyl)ation appears, therefore, as a mechanism capable of protecting CpG dinucleotides from full methylation in genomic DNA, although an effect of the mono(ADP-ribosyl)ation process cannot be excluded (Boulikas, 1993; Kurokawa et al., 1995).

For a better understanding of the biological meaning of our *in vivo* results, some *in vitro* experiments were carried out in order to investigate whether activation of poly(ADP-ribose) polymerase can influence the following *de novo* DNA methylation step, presumably through hetero(ADP-ribosyl)-ation (Ferro et al., 1983; Yoshihara et al., 1985; Boulikas, 1989; Scovassi et al., 1993) of some specific protein able, in its modified isoform, to maintain some CpG dinucleotides in the unmethylated state (Brandeis et al., 1993). Among chromatin proteins, H1 histone heteromodification appeared as a most interesting candidate, since this histone is, on the one hand, the unique chromatin protein that, through its H1e variant (Santoro et al., 1995; Zardo et al., 1996), is able to cause a marked inhibition on DNA methylation and, on the other hand, poly(ADP-ribosyl)ation of H1 histone has already been suggested to be involved in the excision repair mechanism (Boulikas, 1989; Realini & Althaus, 1992; Malanga & Althaus, 1994).

Our data show the following: (a) Protein-free ADP-ribose polymers inhibit *in vitro* enzymatic DNA methylation, their effect being dependent on polymer size and occurring only when dsDNA is used as substrate. Since ADP-ribose polymers do not affect methylation of ssDNA, they cannot be visualized as directly interacting with DNA methyltransferase. Although the polymer:DNA ratio used in *in vitro* experiments is definitely higher than that occurring in the physiological cellular environment, a possible *in vivo* mechanism does not call for a large amount of polymers as the few CpG unmethylated dinucleotides are predominantly located in the DNA regions denominated "CpG-islands" that represent only 1−2% of all genomic DNA. (b) The inhibitory effect of H1 histone on *in vitro* enzymatic DNA methylation is essentially due to the poly(ADP-ribosyl)ated isoform of this protein. Since, on the other hand, only relatively short poly(ADP-ribose) chains 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 by the poly(ADP-ribosyl)ated isoform of H1 histone on the methylation of dsDNA.

In the close relationship that exists between poly(ADP-ribosyl)ation and DNA methylation, the poly(ADP-ribosyl)-ation of H1 histone appears to play a key role. Since the association of H1 histone to ADP-ribose polymers can be of covalent (Naegeli & Althaus, 1991) or noncovalent (Panzeter et al., 1992) nature, further investigations are needed to ascertain whether noncovalent polymers can also be effective in maintaining CpG dinucleotides in their unmethylated state.

It cannot indeed be excluded that, at their physiological concentration, the "free" poly(ADP-ribose) polymers could play a role in maintaining the unmethylated state of CpG dinucleotides, either by themselves or by means of a noncovalent association to H1 histone in its ADP-ribosylated isoform or to some other proteic nuclear component.

In our nondifferentiating cellular system, the ADP-ribose polymers and/or poly(ADP-ribosyl)ated H1 histone can be supposed to interact with DNA during cell replication, exerting their inhibitory effect on the subsequent *de novo* enzymic DNA methylation. Since we could show, by a gel retardation assay, that poly(ADP-ribosyl)ated H1 histone has a higher affinity for a CpG-rich ds-oligonucleotide than the "native" H1 histone, this modified isoform could have a preferential location on genomic DNA. After DNA packaging into nucleosomes, the access to the DNA of a roving methyltransferase would then be limited by the presence of poly(ADP-ribosyl)ated H1 and/or by preferentially long and branched polymers linked in a noncovalent way to the histone, so as to afford considerable protection of the unmethylated state of those CpG-rich DNA regions (Bird, 1986, 1987; Antequera & Bird, 1993).

The presence of Sp1 binding elements has been reported to be critical in "protecting" the neighboring CpG-rich islands from *de novo* methylation (Brandeis et al., 1994; Macleod et al., 1994). A steric hindrance by some protein factors denying the accessibility of methyltransferase to the "CpG-rich islands" has been proposed (Meehan et al., 1989; Pfeifer et al., 1990; Boyes & Bird, 1992; Antequera & Bird, 1993; Stapleton et al., 1993; Macleod et al., 1994; Santoro et al., 1995; Zardo et al., 1996) as a mechanism involved in maintaining them in the unmethylated state, but the general mechanism has not yet been pinpointed.

ACKNOWLEDGMENT

This work is dedicated to Professor A. Ballio on the occasion of his 75th birthday. We thank Dr. Enrico Cundari and Dr. Simona Moretti for access to FACS analysis and Vincenzo Peresempio for skilled technical assistance in the preparation step.

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BI970241S