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Effects of DNA Binding Proteins on DNA Methylation in Vitro†

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ABSTRACT: The inheritance of DNA methylation patterns may play an important role in the stability of the differentiated state. We have therefore studied the inhibitory effects of DNA binding proteins on DNA methylation in vitro. Mouse L1210 cells grown in the presence of 5-azacytidine acquire hemimethylated sites in their DNA. Purified hemimethylated DNA accepted methyl groups from *S*-adenosyl-L-methionine in the presence of a crude maintenance methylase more readily than purified DNA isolated from cells not exposed to 5-azacytidine. On the other hand, chromatin fractions isolated from cells grown in the presence or absence of 5-azacytidine were poor substrates for the maintenance methylase irrespective of the number of hemimethylated sites present in the DNA. Inhibition of DNA methylation was shown to be associated primarily with chromatin proteins bound to DNA, and trypsinization of nuclei increased their methyl accepting abilities. Methyl acceptance was increased by salt extraction of chromosomal proteins. These data suggest that association of histones with DNA may play a role in the modulation of methylation patterns.

A eukaryotic cell must accurately replicate both its genotype and its phenotype when traversing S phase. Newly replicated DNA is rapidly organized into nucleosomes by the association of proteins with DNA (DePamphilis & Wassarman, 1980; McGhee & Felsenfeld, 1980; Igo-Kemenes et al., 1982), and nucleic acid–protein interactions are likely to be important in controlling gene expression. However, the processes by which cellular phenotypes are maintained or altered are still unclear. Evidence indicating that the 5-methylcytosine (5-MeCyt)[1] pattern present in DNA is a component of the multilevel control of gene expression has accumulated in many systems (Razin & Riggs, 1980; Ehrlich & Wang, 1981; Doerfler, 1983). The correlation between hypomethylation of specific gene sequences and the ability of these genes to be expressed has been shown (Busslinger et al., 1983; Lieberman et al., 1983; Stein et al., 1983), and agents which induce DNA hypomethylation can induce changes in gene expression (Jones & Taylor, 1980; Venolia et al., 1982). Thus, there may be a cause and effect relationship between losses of 5-MeCyt residues at specific sites and cellular differentiation. The copying of 5-MeCyt patterns in DNA may depend upon a "maintenance methylase" (Riggs, 1975; Holliday & Pugh, 1975). This enzyme would be responsible for maintaining the methylation pattern over many generations (Stein et al., 1982) and would transfer methyl groups from SAM to hemimethylated sites, consisting of a cytosine residue opposite a methylated CG doublet in duplex DNA.

Since interactions between the enzyme and the DNA substrate are necessary for methylation to occur, we have studied the effects of DNA binding proteins on the ability of hemimethylated DNA to accept methyl groups from SAM in a test

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[1] Abbreviations: 5-MeCyt, 5-methylcytosine; SAM, *S*-adenosyl-L-methionine; 5-aza-C, 5-azacytidine; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid; SSC, 0.15 M NaCl and 0.015 M sodium citrate; SDS, sodium dodecyl sulfate.

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tube reaction catalyzed by a crude maintenance methylase. These studies demonstrated that purified hemimethylated DNA was a good substrate for the maintenance methylase, while chromatin fractions were poor substrates. Native chromatin could be transformed into a better methyl acceptor by removal of histone proteins from DNA using either trypsinization or salt extraction.

Materials and Methods

Materials. Both [*methyl*-^{3}H]SAM and Biofluor were obtained from New England Nuclear (Boston, MA). All other materials used were obtained from Sigma (St. Louis, MO).

Enzyme Assay. Hemimethylated DNA was prepared from mouse L1210 cells injected intraperitoneally (10^5 cells/mouse) into CD2F1 mice (Charles River Labs, Wilmington, MA) as previously described (Wilson et al., 1982). Mice were injected with 1 mg/kg body weight 5-aza-C and the ascites cells harvested after 18 h. Mouse spleen DNA methyltransferase assays were performed as previously described (Taylor & Jones, 1982; Jones & Taylor, 1981). Samples of 1.0 μg of hemimethylated DNA, unless otherwise specified, were assayed in a final volume of 80 μL containing 50 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5 mM dithiothreitol, 5 μM [*methyl*-^{3}H]SAM (0.5–1.0 μCi per assay), and partially purified methyltransferase enzyme (approximately 10 μg of protein per assay). Assays were incubated for 30 min at 37 °C, and reactions were terminated by addition of self-digested Pronase (60 μg/assay) at 60 °C for 20 min. Carrier salmon sperm DNA (20 μg/assay) and Cl_3CCOOH were added to a final concentration of 10% (w/v). The precipitated DNA was redissolved in 0.5 mL of 0.5 M NaOH and incubated for 20 min at 65 °C. The DNA was again precipitated by the addition of Cl_3CCOOH and washed extensively with 5% Cl_3CCOOH prior to hydrolysis of the DNA in 5% Cl_3CCOOH at 100 °C for 30 min. The hydrolyzed samples were centrifuged, and the radioactivities of the supernatants were determined by liquid scintillation counting in Biofluor (New England Nuclear, Boston, MA).

Methyl Accepting Abilities of Chromatin Fractions and DNAs. Chromatin fractions were prepared by the method of Gottesfeld et al. (1974). L1210 nuclei from approximately 10^9 cells grown in the presence or absence of 5-aza-C were suspended in 25 mM sodium acetate, pH 6.6, to a concentration of 2 mg of DNA/mL as determined by the method of Burdon (1956). The solution was brought to 24 °C for 4 min and 100 units/mL DNase II added. The reaction was terminated after 15 min by addition of 50 mM Tris-HCl, pH 11.0, to a final pH of 7.5 and left to stand on ice for 10 min. The solution was centrifuged for 10 min at 5000*g* to sediment the first chromatin fraction referred to as residual nuclei, and the supernatant was centrifuged for 30 min at 14000*g* to sediment the nucleosomal fraction. Both of these fractions were resuspended in 0.1 SSC, and DNA was extracted from portions of residual nuclei and nucleosomal fractions by a modification (Jones & Taylor, 1980) of techniques previously described (Marmur, 1961). Nucleosomes and residual nuclei and their respective purified DNAs isolated from cells grown in the presence or absence of 5-aza-C were added to the enzyme assay (0.5–1 μg of DNA/assay), and the DNA concentrations of chromatin fractions were determined (Burton, 1956).

Trypsinization of Nuclei. L1210 nuclei (10^8) containing hemimethylated DNA were resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.8, and 3.3 mM $CaCl_2$ to a DNA concentration of 460 μg/mL as determined by the method of Burton (1956). To each assay tube was added 5 μL of nuclei (2.3 μg of DNA/assay) followed by 5 μL of trypsin at various concentrations, and tubes were incubated at 0 °C. Reactions were terminated by the addition of 5 μL of soybean trypsin inhibitor (150 μg/mL) after 0 and 30 min and left to stand on ice for 20 min followed by the addition of all necessary components of the DNA methyltransferase reaction. The reaction was then carried out at 37 °C for 30 min, and the activities of each sample were determined. The progress of trypsinization was followed by gel electrophoresis on SDS–18% polyacrylamide gels (Laemmli, 1970).

Salt Extraction of Nuclei. Aliquots of L1210 nuclei (10^8 cells) isolated from cells grown in the presence of 5-aza-C were suspended (600 μg of DNA/mL) in RS buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 3 mM magnesium acetate containing 35% glycerol). These nuclei were divided into four fractions and brought to final salt concentrations of 0, 0.4, 0.6, and 2 M (Senear & Palmiter, 1981). Samples were incubated on ice with occasional mixing for 1 h and centrifuged in a Beckman SW 50.1 rotor at 38000*g* for 1 h. This procedure was repeated, and samples were dialyzed 1 h against 0.1 SSC. Samples were sheared 15 times through a 25 gauge needle and added (0.4–0.8 μg of DNA/assay) to the enzyme assay. The abilities of these substrates to accept methyl groups from SAM in the presence of a crude maintenance methylase were determined.

Table I: Methyl Accepting Abilities of Naked DNA and Chromatin Fractions[a]

treatment	substrate	methyl acceptor ability [pmol of CH_3 (μg of DNA)$^{-1}$ h^{-1}]	
		naked DNA	native chromatin
none	nucleosomes	0.17	0.02
	residual nuclei	0.27	0.01
5-aza-C	nucleosomes	1.13	0.01
	residual nuclei	1.81	0.11

[a] Nuclei prepared from L1210 cells grown in the presence or absence of 5-aza-C (1 mg/kg) were digested with DNase II to yield two fractions, nucleosomes and residual nuclei, upon centrifugation. DNA was isolated from both fractions, and the abilities of these naked DNAs, as well as their native chromatin counterparts, to accept methyl groups from SAM in the presence of crude maintenance methylase were determined under conditions where DNA was limiting (0.5–1 μg of DNA/assay). The levels of activity represent the total activity minus the endogenous activity [<0.08 pmol of CH_3 (μg of DNA)$^{-1}$ h^{-1}]. Results are given for one of three separate determinations yielding similar values.

Results

DNA isolated from cells treated with low levels of 5-aza-C contains many hemimethylated sites (Jones & Taylor, 1981) which are efficiently methylated in a test tube reaction catalyzed by a crude extract of mouse spleen maintenance methylase in the presence of SAM (Taylor & Jones, 1982). We therefore first determined whether chromatin fractions isolated from L1210 cells grown in the presence or absence of 5-aza-C could also act as substrates for the crude maintenance methylase.

DNase II digestion of nuclei prepared from control or 5-aza-C-treated cells yielded two chromatin fractions following centrifugation: residual nuclei consisting of bulk chromatin not significantly cleaved by the enzyme and nucleosomes which were liberated from bulk chromatin by the nuclease. DNA purified from these chromatin fractions isolated from control cells showed a low methyl accepting ability (Table I). However, hemimethylated DNAs purified from chromatin fractions isolated from cells grown in the presence of 5-aza-C were able to act as efficient substrates for the maintenance methylase. Thus, 5-aza-C treatment generated a large number

Table II: Effect of Nuclei on the Methyl Accepting Ability of Hemimethylated DNA[a]

substrate	methyl acceptor ability [pmol of CH_3 (μg of DNA)$^{-1}$ h^{-1}]
hemimethylated DNA	1.09
L1210 nuclei	0
hemimethylated DNA + L1210 nuclei	0.94

[a] L1210 nuclei (2 μg of DNA/assay) and DNA isolated from L1210 cells (1 μg/assay) grown in the presence of 5-aza-C (1 mg/kg) were tested separately and in combination for their abilities to accept methyl groups from SAM in the presence of a crude maintenance methylase. Levels of activity represented the total activity minus the endogenous activity [<0.08 pmol of CH_3 (μg of DNA)$^{-1}$ h^{-1}], the percent activity of the assays relative to assays containing only hemimethylated DNA was determined. Results given are for one of two separate determinations yielding similar values.

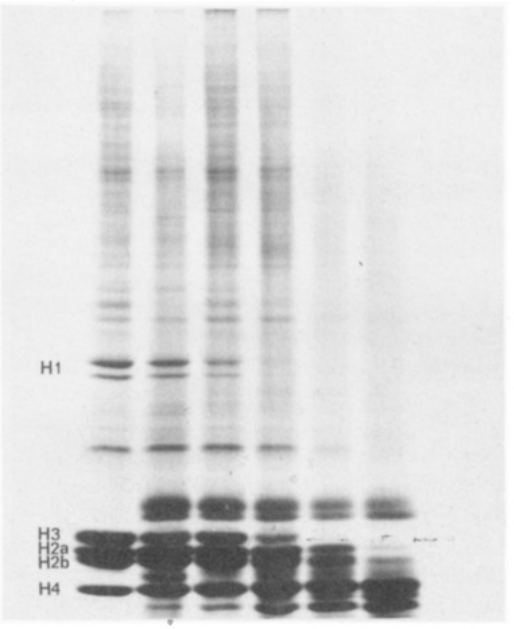

FIGURE 1: Digestion of histone proteins from nuclei by trypsin. Nuclei isolated from L1210 cells (40 μg of DNA/mL) grown in the presence of 5-aza-C (1 mg/kg) were digested on ice for 30 min with various concentrations of trypsin. The reactions were terminated by the addition of excess soybean trypsin inhibitor (150 μg/mL) and precipitated in 10% Cl_3CCOOH. The pellets were washed with ethanol and run on an SDS–18% polyacrylamide gel, yielding five major bands corresponding to histones H1, H3, H2a, H2b, and H4, respectively, in descending order. From left to right, lane 1 shows histones isolated from native L1210 nuclei, and lanes 2, 3, 4, 5, and 6 show digestions of 0, 0.33, 1.0, 3.3, and 10 μg of trypsin/mL, respectively.

of hemimethylated acceptor sites within the DNA as found earlier (Jones & Taylor, 1981). In marked contrast, nucleosomes or residual nuclei were both poor substrates for the maintenance methylase irrespective of the number of hemimethylated sites present in the DNA. Thus, chromatin proteins associated with the DNA strongly inhibited the maintenance methylase.

To determine if soluble chromatin proteins inhibited maintenance methylation, nuclei which were poor substrates for the maintenance methylase (Table I) were added to purified hemimethylated DNA and the methyl accepting activities of the mixture measured (Table II). Nuclei caused only a 14% inhibition of the methylation of the purified hemimethylated DNA when both substrates were present in the reaction mixture. Thus, diffusible chromatin proteins were not responsible for the large differences in methyl accepting abilities between purified DNA and chromatin.

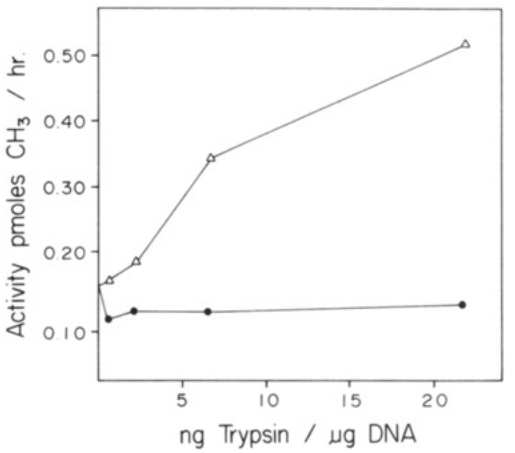

FIGURE 2: Methyl accepting abilities of trypsinized nuclei containing hemimethylated DNA. Nuclei isolated from 5-aza-C-treated L1210 cells (2.3 μg of DNA/assay) were digested with various concentrations of trypsin at 0 °C for 0 (●) and 30 min (△). The abilities of these nuclei to accept methyl groups from SAM in the presence of crude maintenance methylase were determined. The levels of activity represent the total activity minus the endogenous activity [<0.8 pmol of CH_3 (μg of DNA)$^{-1}$ h^{-1}]. Results given are for one of two separate determinations, yielding similar values.

Table III: Methyl Accepting Abilities of Salt-Extracted Nuclei[a]

NaCl concn (M)	methyl acceptor ability [pmol of CH_3 (μg of DNA)$^{-1}$ h^{-1}]	NaCl concn (M)	methyl acceptor ability [pmol of CH_3 (μg of DNA)$^{-1}$ h^{-1}]
0	0.07	0.6	0.25
0.4	0.17	2.0	1.23

[a] Nuclei isolated from L1210 cells grown in the presence of 5-aza-C were extracted with various concentrations of NaCl. The extracted nuclei were tested for their abilities to accept methyl groups from SAM in the presence of a crude maintenance methylase under conditions of limiting DNA (0.4–0.8 μg/assay). Levels of activity represent the total activity minus the endogenous activity [<0.08 pmol of CH_3 (μg of DNA)$^{-1}$ h^{-1}]. Results are given for one of two separate determinations yielding similar values.

Nuclei isolated from L1210 cells grown in the presence of 5-aza-C were digested for 30 min with increasing concentrations of trypsin to determine whether limited proteolysis could increase the accessibility of hemimethylated sites for the methylase. The residual proteins from each digestion were analyzed by polyacrylamide gel electrophoresis (Figure 1). A sequential loss of histone proteins was observed as the concentration of trypsin per assay increased, and the linker histone H1 was more sensitive to trypsin digestion than core histones as previously reported (Weintraub & Van Lente, 1974). The core histones H3 and H2a were sensitive to trypsin digestion at higher enzyme concentrations.

The abilities of trypsin-digested nuclei isolated from 5-aza-C-treated cells to accept methyl groups from SAM increased with the degree of proteolysis (Figure 2). The sequential loss of histone H1 and remaining core histones increased the ability of the maintenance methylase to act upon the hemimethylated substrate, while nondigested nuclei remained relatively poor substrates for the methylase. This method of analysis could not determine whether non-histone proteins played a role in the inhibition of DNA methylation but demonstrated that chromatin proteins could block methylation of hemimethylated sites.

Nuclear proteins can also be extracted by increasing salt concentrations (Ohlenbusch et al., 1967), and the methyl accepting abilities of nuclei containing hemimethylated sites could be improved substantially by increasing concentrations

of NaCl (Table III). Removal of non-histone proteins including high mobility group proteins from chromatin with 0.4 M NaCl produced a slight increase in the methyl accepting ability of the substrate. Extraction of histone H1 and the majority of non-histone proteins, but not the core histones with 0.6 M NaCl (Senear & Palmiter, 1981; Ohlenbusch, 1967), produced a moderate increase in methyl accepting ability relative to chromatin extracted with 2 M NaCl. Extraction of the remaining core histones with 2 M NaCl which leaves only tightly bound chromosomal proteins (Gates & Bekhor, 1980) caused a substantial increase in methyl acceptance. These results provided additional evidence that histone proteins were a major ingredient in chromatin responsible for the inhibition of maintenance methylation.

Discussion

Our experiments show that hemimethylated CpG sites, which are efficient methyl acceptors in purified DNA, are refractory to methylation when associated with chromosomal proteins. The inhibitory effect of chromatin proteins apparently required their close association with the acceptor sites, since nuclei containing nucleoprotein complexes did not inhibit the methylation of exogenously added purified DNA. Chromatin proteins bound to hemimethylated DNA isolated from Friend leukemia cells have similarly been shown to inhibit DNA methylation in vitro (Creusot & Christman, 1981).

The methyl accepting abilities of these cryptic sites were not restored when nucleosomes were prepared from nuclei, suggesting that the histones exhibited considerable inhibitory activity. Also, the partial restoration of substrate availability on limited trypsinization or salt extraction supports the idea that the inhibition is due to protein–DNA interactions and that histones may play a role in this inhibition.

The ability of histone proteins to act as inhibitors of the maintenance methylase may be important in the temporal sequence of events which occur during S phase. Maintenance methylation of hemimethylated CpG sites takes place soon after DNA synthesis (Burdon & Adams, 1969; Kappler, 1969). A temporal link between DNA synthesis and methylation is supported by a recent study which suggests that an enzyme complex found in nuclei called a "replitase" can catalyze both DNA synthesis and methylation (Noguchi et al., 1982). Higher rates and overall levels of DNA methylation during DNA repair have been reported in logarithmic phase cultures, relative to nondividing cultures (Kastan et al., 1982). Chromatin structures containing core histones have been shown to retard rates of DNA repair (Smerdon et al., 1978). From our results, histones may also alter rates of DNA methylation during repair by blocking potential modification sites.

Shortly after DNA replication, newly synthesized DNA is associated with histone proteins to form nucleosomal structures (Annunziato et al., 1981; Weintraub, 1973; Rassev & Hancock, 1982). If histone proteins bind to DNA before methylation, a loss in the fidelity of the enzymatic methylation at hemimethylated sites may occur. This could lead to a heritable loss of the methylation state at specific sites in the daughter strand of the DNA and could lead to a change in phenotype. We therefore have developed a more dynamic system to study the relationships between DNA synthesis and methylation in isolated nuclei. Results obtained from that system show that newly synthesized DNA in nuclei can be methylated in the presence of chromatin proteins in contrast to the present results with a static system (T. L. Kautiainen and P. A. Jones, unpublished results).

Registry No. DNA maintenance methylase, 9037-42-7.

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