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DNA methylase from HeLa cell nuclei

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ABSTRACT

A DNA methylase has been purified 270-fold from HeLa cell nuclei by chromatography on DEAE-cellulose, phosphocellulose, and hydroxyapatite. The enzyme transfers methyl groups from S-adenosyl-L-methionine to cytosine residues in DNA. The sole product of the reaction has been identified as 5-methylcytosine. The enzyme is able to methylate homologous (HeLa) DNA, although to a lesser extent than heterologous DNA. This may be due to incomplete methylation of HeLa DNA synthesized *in vivo*. The HeLa enzyme can methylate single-stranded DNA, and does so to an extent three times greater than that of the corresponding double-stranded DNA. In single-stranded *M. luteus* DNA, at least 2.4% of the cytosine residues can be methylated *in vitro* by the enzyme. The enzyme also can methylate poly (dG-dC·dG-dC) and poly (dG, dC). Bilateral nearest neighbors to the 5-methylcytosine have been determined with *M. luteus* DNA *in vitro* and HeLa DNA *in vivo*. The 5' neighbor can be either G or C while the 3' neighbor is always G and this sequence is, thus, p(G/C)pmCpG.

INTRODUCTION

The methylated bases N-6-methyladenine and 5-methylcytosine occur in many species of bacteria.[1] These bases are not incorporated as such during DNA replication, but are the product of DNA methylases which transfer methyl groups from S-adenosyl-L-methionine to specific sites in DNA.[2] Some of these DNA methylases function as "host-specific modification" and thus protect the host DNA from degradation by its own restriction enzymes (for recent reviews see Arber[3] and Meselson *et al.*).[4] Both N-6-methyladenine[5-8] and 5-methylcytosine[9] occurring at specific base sequences ("recognition sites") in DNA can serve this function. However, the majority of methylated bases in *E. coli* are unrelated to modification of restriction sites.[10,11]

The nuclear DNA of several types of animal cells contains 5-methylcytosine.[12] However, no restriction enzymes have been reported in animal cells, and the biological function of 5-methylcytosine in these cells remains unknown. Partially purified DNA methylases have been prepared from rat spleen and liver[13,14] and the mechanism of action of the latter enzyme has been studied[15-17] by Morris and his co-workers. HeLa cell nuclear DNA is known to

contain 5-methylcytosine[18-20] and it was of interest to examine the enzymology of DNA methylation in this cell type in some detail. As a first step in this direction we describe the purification and properties of a DNA methylase from HeLa cell nuclei. A preliminary report has been published.[21]

MATERIALS AND METHODS

Enzymes and chemicals

Pancreatic DNase (electrophoretically pure, 3750 units/mg, 1 mg/ml, RNase A (electrophoretically pure, 6100 units/mg), venom phosphodiesterase (potency 0.43, 5 mg/ml) and E. coli alkaline phosphatase (32 units/mg, 8 mg/ml) were obtained from Worthington Biochemical Corp. The deoxynucleosides dA, dC, dG and dT were obtained from Sigma Chemical Co.

Radiochemicals and scintillation media

S-adenosyl-L-[methyl-^{3}H] methionine (8.5 Ci/mmole, 0.56 mCi/ml) was obtained from New England Nuclear Corp. and diluted to 1.0 Ci/mmole, 0.5 mCi/ml with unlabeled S-adenosyl-L-methionine (Sigma) and 1 mM H_2SO_4. L-[methyl-^{3}H] methionine (2.6 Ci/mmole, 1.0 mCi/ml) was obtained from Schwarz/Mann. LSC-acetic acid scintillation medium was LSC (Yorktown) to which was added 2 ml/liter of glacial acetic acid. Aquasol scintillation fluid was obtained from New England Nuclear.

DNA and synthetic substrates

M. luteus DNA was obtained from Miles Laboratories, Inc. The DNA (25 mg) was dissolved in 50 ml of 20 mM Tris-Cl, 10 mM EDTA, pH 8.5, then treated with pancreatic RNase (20 µg/ml) for 30 min at 37°, extracted three times with equal volumes chloroform - isoamyl alcohol (24:1) and precipitated with 100 mg of 95% ethanol. The DNA was wound on a glass rod and dissolved in 50 ml of 15 mM NaCl, 1.5 mM sodium citrate. Sodium acetate (5 ml of 3 M) was added and the DNA precipitated with 30 ml of isopropanol. The DNA was again collected by winding and dissolved in 15 mM NaCl, 1.5 mM sodium citrate to a final concentration of 0.7 mg/ml. Denatured M. luteus DNA was prepared by heating the DNA to 100° for 5 min followed by quenching on ice. E. coli B DNA was obtained from Grand Island Biological Company and further purified as above. HeLa DNA was prepared from nuclei by suspending HeLa nuclei from 11.8 g of cells in 120 ml of 20 mM Tris-Cl, 10 mM EDTA, pH 8.5, adding 1.2 ml of 10% sodium dodecyl sulfate and 2.4 ml pronase (50 mg/ml) and digesting for 12 hours at 37°. Ten percent sodium dodecyl sulfate (14 ml) and 5 M NaClO4 (28 ml) were added and the solution was extracted twice with equal volumes of chloroform - isoamyl alcohol (24:1) after which the DNA was precipitated with 170 ml of

ethanol. The DNA was collected by winding and dissolved in 20 mM Tris-Cl pH 8.5, 10 mM EDTA to a concentration of 0.5 mg/ml and further purified as described above for M. luteus DNA. Vaccinia DNA was prepared from vaccinia virus as described by LaColla and Weissbach.[22] Herpes simplex type 1 DNA was prepared by the method of Kieff et al.[23] Phage Qβ was obtained from Dr. A. Ramel of the Research Division of Hoffmann-La Roche, Inc. Cytoplasmic polyhedrosis virus (CPV) RNA was obtained from Dr. Y. Furuichi of the Roche Institute of Molecular Biology.

The synthetic copolymers $(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$, $(A)_n \cdot (dT)_n$, $(dC)_n$ and $(dG)_n \cdot (dC)_n$ were obtained from Miles Laboratories, Inc. The copolymers $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ and $(dG, dC)_n$ were obtained from P-L Biochemicals, Inc.

Standard assay for DNA methylase activity

Reaction mixtures (200 µl) contained 100 mM imidazole buffer (pH 6.5), 0.5 mM dithiothreitol, 20 mM EDTA (pH 6.5) 35 µg of DNA (M. luteus unless otherwise noted), 5 µCi of S-adenosyl-L-[methyl-^{3}H] methionine (final concentration 25 µM), and up to 50 units of DNA methylase (20-40 µl).

The mixtures were incubated at 37° for 20 min. Acid precipitation of the reaction products and preparation for counting were done as previously described[24] except that the counting was done in LSC-acetic acid scintillation medium.

One unit of DNA methylase is defined as that amount of enzyme which, under the standard assay conditions, catalyzes the incorporation of 1 pmole of methyl groups into M. luteus DNA in 1 hour.

Growth of cells

HeLa S-3 cells were grown in suspension cultures at 37° in F-13 medium (Gibco) supplemented with 5% fetal-calf serum (Gibco), 4 mM glutamine, 50 units per ml of penicillin and 50 µg per ml of streptomycin. The cells were harvested at a density of 5.0×10^5 cells per ml by centrifugation at 1000 x g and washed as described by Berkowitz et al.[25]

Preparation of nuclei

HeLa S-3 cells were suspended in 10 ml of 10 mM NaCl, 1 mM KPO_4 (pH 7.4) per g of cells and after 20 min at 0° were broken in this buffer with a Dounce homogenizer. The nuclei were separated from the cytoplasm by centrifugation (10 min at 800 x g) and the nuclear pellet was washed with a Triton N-101 solution as described by Berkowitz et al.[25] The nuclei were resuspended in Berkowitz buffer I (3 ml per g of cells) and frozen at -70° C until used.

Purification of DNA methylase

All purification steps were carried out at 0-4°. Frozen nuclei from 32 g of cells were thawed and 4 M NaCl was added to a final concentration of 0.3 M. After standing on ice for 15 min the nuclear extract was centrifuged at 150,000 x g for 3 hours. The supernatant fluid ("soluble" nuclear fraction) was dialyzed for 4 hours against two changes of 1 liter of 0.02 M Tris-HCl, pH 7.4, 0.02 M NaCl, 0.5 mM dithiothreitol buffer.

The dialyzed soluble nuclear fraction (276 mg total protein) was adsorbed onto a 90 ml DEAE-cellulose (Whatman DE-52) column, previously equilibrated with 0.02 M Tris-HCl pH 7.4, 0.02 M NaCl, 0.5 mM dithiothreitol. After the dialyzed soluble nuclear fraction was loaded on the column, the column was washed with one column volume of the above buffer and eluted with 8 column volumes of a linear gradient from 0.02 to 0.40 M NaCl containing 0.02 M Tris-HCl pH 7.4 and 0.5 mM dithiothreitol. DNA methylase activity eluted at 0.16 M NaCl.

The pooled peak fractions (#38-48; 130 ml) from the DEAE cellulose column were dialyzed for 2 hours against 1 liter of 0.02 M KPO_4, pH 8.0, 0.5 mM dithiothreitol. The dialyzed material (33 mg total protein) was loaded on a 20 ml phosphocellulose column (Whatman P-11) previously equilibrated with the above buffer, and the column was washed with one column volume of this buffer. The column was eluted with 10 column volumes of a linear gradient from 0.02 M to 0.50 M KPO_4 (pH 8.0) in 0.5 mM dithiothreitol. The enzyme eluted at 0.23 M KPO_4.

The pooled peak fractions (#50-62; 56 ml) from the phosphocellulose column were dialyzed for 2 hours against 1 liter of 0.02 M KPO_4, pH 8.0, 0.5 mM dithiothreitol. Glycerol was added to a final concentration of 20% and the enzyme (6.4 mg total protein) loaded onto a 3 ml hydroxyapatite column equilibrared with 0.02 M KPO_4, pH 8.0, 0.5 mM dithiothreitol, 20% glycerol. The column was washed with one column volume of this buffer and eluted with 30 ml of a linear gradient from .02 to .50 M KPO_4 (pH 8.0) in 0.5 mM dithiothreitol, 20% glycerol. The enzyme eluted as a single peak at 0.27 M KPO_4. The peak fractions (#47-56; 6.9 ml) were pooled and stored at -70° until used.

Methyl-labeled DNA's

HeLa DNA was methylated *in vivo* as follows: HeLa cells were grown as described above. Sodium formate (10 ml of 2 M) was added to one liter of cells (2 x 10^5/ml). After 2 hours of incubation, L-[methyl-^{3}H] methionine (2 mCi in 2 ml) was added and the incubation continued for 24 hours. The

cells were then harvested, the nuclei prepared, and the DNA extracted as described above for unlabeled HeLa DNA.

M. luteus DNA was methylated in vitro as follows: Reaction mixtures (0.6 ml) containing 100 mM imidazole·HCl (pH 6.5), 1 mM dithiothreitol, 20 mM EDTA (pH 6.5), 100 µg M. luteus DNA (double-stranded or single-stranded), and 50 units of DNA methylase were incubated at 37° for 20 minutes. The mixtures were collected on glass filters (Whatman GF/C) and washed with 25 ml of 5% trichloroacetic acid, followed by 10 ml of 95% ethanol. After counting in LSC scintillation fluid, the filters were washed with toluene and dried under an infrared lamp. The DNA was recovered by macerating the filters in 2 ml of 0.2 M NH_4OH and then filtering the resulting suspensions through semi-micro, medium-sintered glass funnels. After washing the funnels twice with 1 ml of 0.2 M NH_4OH, the filtrates containing the dissolved DNA were placed at 37° and evaaporated to about 1.0 ml under a directed air jet.

Nucleosides

Digestion of [methyl-^{3}H] DNA to nucleosides was carried out by consecutive hydrolysis with pancreatic DNase I, snake venom phosphodiesterase, and E. coli alkaline phosphatase.[26] A reaction mixture (1.0 ml) containing about 20 to 30 µg of the labeled DNA, 10 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, and 20 µg pancreatic DNase I/ml, was incubated at 37° for 30 min to yield oligonucleotides. The oligonucleotides were digested to 5'-mononucleotides by adding 50 µl of 1 M glycine buffer (pH 9.0) and 50 µl of snake venom phosphodiesterase (5 mg/ml) and incubating at 37° for 30 min. Five µl (1.3 units) of alkaline phosphatase was added and the incubation continued for another 30 min. The mixture was extracted 4 times with equal volumes of chloroform: isoamyl alcohol (24:1) and evaporated to 100 µl. Twenty-five µl of dT, dC, dG, and dA (20 mg/ml) were added and the mixture chromatographed on a Dowex 50 (NH_4^+ form) column by the method of Lawley et al.[19]

Dinucleoside monophosphates

Digestion of [methyl-^{3}H] DNA to short oligonucleotides was carried out by hydrolysis with pancreatic DNase I under the conditions described by Bollum.[27] Reaction mixtures contained 100 µg of [methyl-^{3}H] DNA, 10 mM Tris·HCl (pH 7.5) 8 mM $MgCl_2$, 2 mM $CaCl_2$, and 50 µg of pancreatic DNase I. The mixtures were incubated at 37° for 16 hours. Glycine buffer, pH 9.0, (50 µl of 1 M); and 5 µl of E. coli alkaline phosphatase were added and the mixture incubated at 37° for 30 min. The mixtures were made 7 M in urea by addition of solid urea and Tris·HCl (pH 7.5) was added to 10 mM. The mixtures were chromatographed on DEAE-cellulose-7 M urea by the method of Tomlinson and

Tener.[28] The dinucleoside monophosphate peak (-1 charge) was pooled and the dinucleoside monophosphates recovered by adsorption on partially inactivated Norit and subsequent elution as described by Roy and Smith.[7]

The eluates were evaporated to dryness and resuspended in 10 µl H_2O. GpC and CpC (1 µl each of 10 mg/ml) were added as absorbance markers and the mixtures spotted onto a thin-layer cellulose plate and electrophoresed on a cooled-flat-plate apparatus for 3.5 hours at 25 V/cm using 75 mM ammonium formate (pH 3.5) buffer. The electropherograms were cut into 0.5 cm strips and counted in LSC.

The strips containing the dinucleoside monophosphate peaks were washed in toluene and evaporated to dryness. To elute the radioactivity, the strips were suspended in 0.2 M NH_4OH, the plastic backing removed, the cellulose macerated and removed by centrifugation, and the supernatant again evaporated to dryness. The dinucleoside monophosphates were resuspended in 10 µl of 10 mM Tris HCl (pH 7.4); 1 µl pGpC and 1 µl snake venom phosphodiesterase were added and the mixture digested at 37° for 30 minutes. A 1 µl aliquot was chromatographed on PEI-cellulose with 1 M sodium acetate (pH 3.5) buffer to monitor digestion of the absorbance marker. The remainder was chromatographed on PEI-cellulose with water, dried, and counted in LSC. In this system the nucleoside (from the 5' side of the dinucleoside monophosphate) chromatographs at the solvent front while the nucleotide (from the 3' side of the dinucleoside monophosphate) remains at the origin.

RESULTS

Assay for DNA methylase activity

HeLa DNA is known to contain 5-methylcytosine and the methyl groups are presumably incorporated by one or more specific DNA methylases. Although eukaryotic DNA methylases can methylate homologous DNA to some degree[13] the majority of potential DNA methylase sites are probably already methylated *in vivo*. A foreign DNA, *M. luteus*, was chosen since it contains no detectable methylated bases.[1] The conditions for the methylase reactions were adapted from those used by Roy and Smith[26] for the *H. influenzae* DNA methylases and by Morris and Pih[14] for rat liver DNA methylase. The reactions required *S*-adenosyl-L-[*methyl*-^{3}H] methionine (25 µM) as a methyl group donor and were carried out in 100 mM imidazole-HCl buffer (pH 6.5), 0.5 mM dithiothreitol, and 20 mM EDTA (pH 6.5). The latter was present in order to inhibit deoxyribonuclease activity.

DNA methylase activity was found in nuclear extracts of HeLa cells but not in cytoplasmic extracts. In assays of crude extracts it was necessary

to use parallel controls lacking added substrate DNA to discriminate between DNA methylation and methylation of RNA and/or protein in the crude extracts.

Purification of DNA methylase

HeLa cell nuclei were extracted with 0.3 M NaCl followed by centrifugation at 150,000 x g for 3 hours. The methylase activity was found as a soluble form in the supernatant, and is thus separated from the majority of nucleic acids and nucleoprotein by centrifugation at 150,000 x g. The nuclear supernatant fluid was then dialyzed and the methylase purified by successive chromatography on DEAE-cellulose (Figure 1A), phosphocellulose (Figure 1B), and hydroxyapatite (Figure 1C). The methylase elutes as a single peak in each of these steps. Glycerol is added before hydroxyapatite chromatography in order to stabilize the enzyme. The total purification of the pooled hydroxyapatite peak from nuclei is 273-fold and the purification is summarized in Table 1. The hydroxyapatite step is a convenient method of concentration as well as purification, and the hydroxyapatite-purified enzyme is stable for at least several weeks when stored at -70°. The subsequent characterization of the enzyme was carried out with this fraction.

Table 1

Purification of HeLa DNA Methylase

Step	Total Units (pmoles/ hr)	Volume (ml)	Total Protein (mg)	Specific Activity (units/mg protein)	Purification (fold)	Recovery
Nuclei	3200	98	1065	3.00	1.0	---
High salt extract supernatant-dialyzed	5540	122	276	20.1	6.7	100
DE 52 peak dialyzed	8100	135	52.6	154	51.5	146
P 11 peak dialyzed	6450	56	33.1	195	65.1	116
Hydroxyapatite peak	5220	6.9	6.4	816	273	94

The purification was carried out as described in Materials and Methods. Methylase activity was assayed in standard reactions incubated for 20 minutes. Proteins were assayed by the method of Lowry et al.[29]

General properties of DNA methylase

The requirements for activity of the enzyme are shown in Table 2. The enzyme required no cofactors other than S-adenosyl-L-methionine and a sulf-

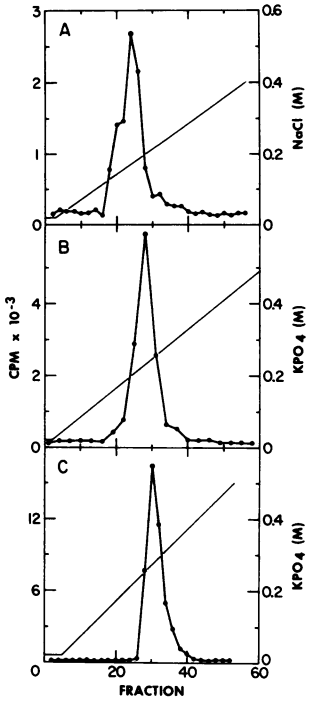

Fig. 1. DEAE-cellulose (A), phosphocellulose (B), and hydroxyapatite (C) chromatograms of the HeLa cell DNA methylase. Elutions were carried out as described in "Materials and Methods" and fractions of 40 µl (DEAE and phosphocellulose) or 20 µl (hydroxyapatite) were assayed under the standard conditions as described in "Materials and Methods".

hydryl reagent (such as dithiothreitol). It is inhibited by the sulfhydryl inhibitor, iodoacetamide. The optimal pH for the activity is 6.5. The product of the reaction is DNase sensitive and RNase resistant. To determine its molecular weight, the enzyme was sedimented on a 20-40% glycerol gradient using bovine serum albumin (4.3 S) and rabbit muscle aldolase (7.35 S) as markers. The HeLa DNA methylase activity was recovered in a single symmetrical peak at 6.3 S. If one assumes a spherical configuration and a partial specific volume of 0.73, the molecular weight may be estimated to be about 120,000.

Substrate specificity of the reaction

HeLa DNA methylase was assayed with several natural and synthetic substrates and the rates of reaction in a 20 minute incubation are summarized in Table 3. The enzyme is active on single-stranded as well as double-stranded

Table 2

Requirements of DNA Methylase Assay

	cpm	% of Complete
Complete	16,740	100
- Enzyme	145	1
- DNA	165	1
+ RNase	15,336	92
+ DNase	205	1
+ Iodoacetamide	2,005	12

Reactions were standard assays and contained 50 units of enzyme. The RNase reaction contained 120 µg (730 units) pancreatic RNase. The DNase reaction contained 5 µg (18 units) DNase, 12.5 mM $MgCl_2$ and no EDTA. The iodoacetamide reaction contained no added dithiothreitol, 0.5 mM iodoacetamide, and 0.1 mM residual dithiothreitol from the enzyme preparation.

DNA. E. coli DNA, which has a lower GC content than M. luteus, and contains methylated bases, is methylated less than half as rapidly as M. luteus DNA. Vaccinia virus and herpes simplex virus DNA's obtained from viruses grown in HeLa cells are similarly poorly methylated. A small amount of methylation was found with Qβ RNA, but this may be due to a contaminating RNA methylase activity in our preparations. A terminally methylated double-stranded RNA, purified from cytoplasmic polyhedrosis virus,[30] was not further methylated by the enzyme. The synthetic copolymers $(A)_n \cdot (dT)_n$, $(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$, $(dC)_n$, and $(dG)_n \cdot (dC)_n$ were not methylated. However, both the alternating, double-stranded $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ and the random, single-stranded $(dG,dC)_n$ were methylated at a rate comparable to M. luteus DNA.

Kinetics of the reaction

The standard reaction (35 µg of M. luteus DNA) is linear for 20 minutes, continues at a slower rate for an additional 40 minutes, and then essentially stops. Addition of more DNA at 60 minutes causes no resumption of the reaction, while addition of enzyme causes the reaction to resume, but at a rate 40% slower than the initial reaction. Therefore, cessation of the initial reaction is due to loss of enzyme activity rather than saturation of available sites on DNA. This may represent inactivation of the enzyme during

Table 3

Substrate Specificity of HeLa DNA Methylase

Substrate	Double Stranded		Single Stranded	
	cpm	% of *M. luteus*	cpm	% of *M. luteus*
M. luteus DNA	12487	100	25174	100
E. coli B DNA	4588	37	12302	49
HeLa cell DNA	1101	8.8	2239	8.9
vaccinia DNA	1643	13	---	---
herpes simplex DNA	664	5.3	---	---
Qβ RNA	---	---	773	3.0
CPV RNA	0	0	---	---
$(A)_n \cdot (dT)_n$	0	0	---	---
$(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$	0	0	---	---
$(dC)_n$	---	---	0	0
$(dG)_n \cdot (dC)_n$	0	0	---	---
$(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$	11003	88	---	---
$(dG, dC)_n$	---	---	18666	74

Standard reaction mixtures containing 35 µg of the substrate indicated and 40 units of DNA methylase were incubated for 20 minutes. For the RNA and $(A)_n \cdot (dT)_n$ substrates the acid precipitable material was washed with 0.5 M Tris-HCl, pH 9.8, instead of 0.2 M NaOH. The specific activity of the S-adenosyl-L-[methyl-^{3}H] methionine used in the standard reaction mixture was 1000 cpm/pmole.

the prolonged incubation, or a very long turnover time such as that exhibited by the *E. coli* B and P1 modification methylases.[31,32]

The extent of methylation of double-stranded and single-stranded *M. luteus* DNA was determined by using reaction mixtures containing 0.5 µg of DNA and adding fresh enzyme at 40, 80, and 120 minutes (Figure 2). By 180 minutes, the reaction has reached a plateau of 3.50 pmoles of methyl groups per 0.5 µg of DNA for double-stranded DNA and 13.0 pmoles for single-stranded DNA. This is equivalent to one methyl group per 430 bases and per 116 bases, respectively. In single-stranded *M. luteus* DNA, this represents 2.4% of the total cytosine residues. Extents were also determined on $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ (1 µg), and $(dG,dC)_n$ (0.5 µg). The plateau values are equivalent to one methyl group per 1800 bases for the alternating copolymer and one methyl group per 200

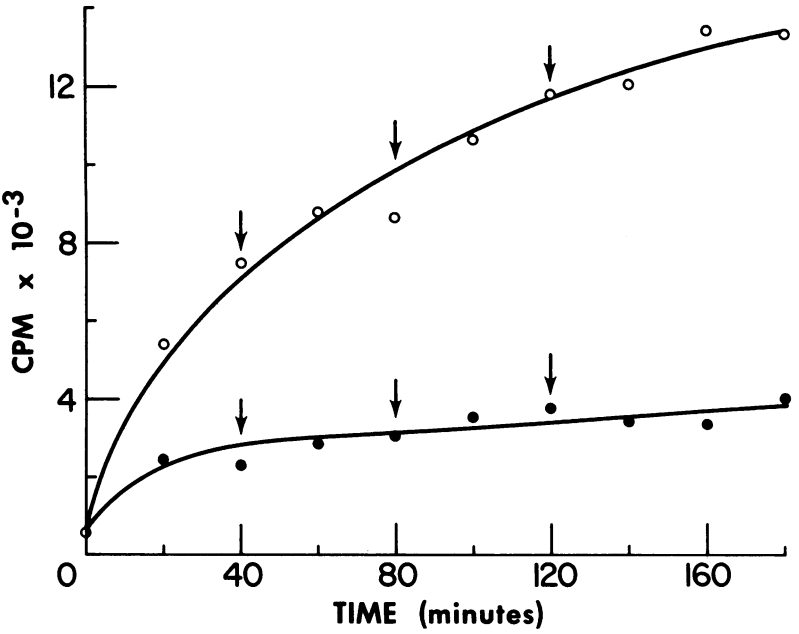

Fig. 2. Kinetics of the DNA methylase reaction. Assay mixtures contained 0.5 μg of M. luteus double-stranded or single-stranded DNA and 20 units of DNA methylase. At 40 min., 80 min., and 120 min., 10 units of DNA methylase was added and the incubation continued as indicated. Acid-precipitable radioactivity was determined as described in "Materials and Methods". ●——● = double-stranded DNA; o——o = single-stranded DNA.

bases for the random copolymer. A similar experiment using 5 μg of HeLa cell double-stranded and single-stranded DNA's yielded incorporations of one methyl group per 15,100 and 7,550 bases, respectively. This is 2.8% and 1.5% of the extent of reaction for M. luteus double-stranded and single-stranded DNA.

The S-adenosyl-L- methionine and DNA concentration dependence of the reaction were studied and yielded linear reciprocal plots (1/V vs. 1/S). The observed K_m values were 3.25 μM for S-adenosyl-L-methionine and 86 μM for M. luteus DNA.

Identification of the methylated base

In contrast with an earlier report by Culp, Dore, and Brown,[33] in which several methylated bases were reported to exist in HeLa cell DNA, Lawley et al.[19] reported that the sole methylated base in HeLa DNA is 5-methylcytosine, and we have confirmed their results. While in vivo labeling of cells with [methyl-^{3}H] or [methyl-^{14}C] methionine may lead to some incorporation (via cytoplasmic pathways) into purines and thymine, in vitro

labeling with S-adenosyl-L-[methyl-^{3}H] methionine and DNA methylase should yield only the product of methylation of the polymerized DNA, in this case, 5-methylcytosine.

M. luteus DNA was methylated by HeLa DNA methylase using S-adenosyl-L-[methyl-^{3}H] methionine and hydrolyzed to nucleosides by the consecutive action of pancreatic DNase, venom phosphodiesterase, and alkaline phosphatase. The digest was deproteinized and analyzed by chromatography on Dowex 50 (NH_4^+ form). The result, shown in Figure 3, indicates that 95% of the

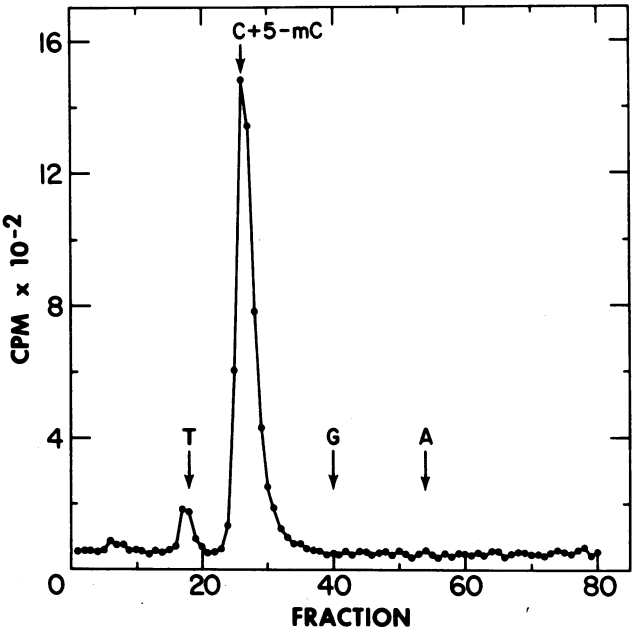

Fig. 3. Identification of the methylated base. M. luteus DNA was methylated in vitro, digested to nucleosides and chromatographed on Dowex 50 (NH_4^+ form) as described in "Materials and Methods".

radioactivity chromatographs with 5-methylcytosine. However, a few percent of the radioactivity is found associated with the thymine peak and its origin is uncertain but it may be the result of an artifactual deamination during the digestion process.

Bilateral nearest neighbors to 5-methylcytosine

Digestion of methyl-labeled DNA (HeLa cell DNA labeled in vivo or M. luteus double-stranded or single-stranded DNA labeled in vitro) with pancreatic DNase under the conditions described by Bollum[27] resulted in digests

containing 40-50% dinucleotides, the remainder being mostly trinucleotides with some mononucleotides. After treatment with alkaline phosphatase, the digests were chromatographed on DEAE-cellulose - 7 M urea[28] and the dinucleoside monophosphates recovered. Since pancreatic DNase does not cleave DNA randomly, this procedure does not allow precise quantitation of the dinucleoside monophosphates containing ^{3}H-5-methylcytosine. However, by separation of the dinucleoside monophosphates with electrophoresis and subsequent cleavage with snake venom phosphodiesterase (which produces the cleavage XpY → X + pY), the polarity of each species and thus the 5' and 3' nearest neighbors to ^{3}H-5-methylcytosine can be qualitatively determined.

The dinucleoside monophosphates from each pancreatic DNase - alkaline phosphatase digest were resolved by thin-layer electrophoresis. The patterns for each of the digests were identical and 90% of the label migrated as (mC,G) while 10% migrated as (mC,C). When recovered and digested with snake venom phosphodiesterase, the label from (mC,G) chromatographed as 60% mC and 40% pmC (thus, 60% mCpG, 40% GpmC), while the digest of (mC,C) chromatographed quantitatively as pmC (thus, all CpmC). These results indicate that the 5' neighbor to mC may be either G or C, while the 3' neighbor is always G. Also, the nearest neighbors observed in M. luteus DNA labeled in vitro by the action of the DNA methylase are identical to those occurring in HeLa cell DNA in vivo.

DISCUSSION

We have purified a DNA methylase over 270-fold from HeLa cell nuclei. This activity behaves as a single enzyme during purification and transfers methyl groups from S-adenosyl-L-methionine to cytosine residues in DNA.

Since measurement of the extent of reaction required large amounts of enzyme, we chose first to compare the rates of methylation of a wide variety of substrates in standard 20-minute reactions with an excess of DNA substrate. (Subsequent comparison of extent and rate data for M. luteus and HeLa double-stranded and single-stranded DNA's shows that rates are qualitatively proportional to total available sites of methylation. The data in Table 3 show that M. luteus DNA (72% GC) was methylated more rapidly than E. coli B DNA (50% GC). This difference may reflect differences in the frequency of occurrence of recognition sites which could be related to GC content.

DNA's from vaccinia or herpes simplex viruses grown in HeLa cells are only slightly methylated. This may result from A) a previous host-directed methylation of the viral DNA in vivo or B) a lack of specific sites for the host methylase in the viral DNA's. The finding of Low, Hay, and Keir[34] that

herpes simplex virus DNA is not methylated in vivo (where the host DNA is methylated) suggests that the latter alternative is more likely.

We have compared the extents of the methylation reactions for M. luteus and HeLa cell DNA's (double-stranded and single-stranded) and for $(dG,dC)_n$ and $(dG-dC)_n \cdot (dG-dC)_n$. The maximum incorporation of methyl groups into M. luteus double-stranded DNA was one methyl group per 430 bases. This is considerably lower than the 0.7 mole % (1 methyl group per 140 bases) 5-methylcytosine content reported to exist in HeLa DNA.[20] This discrepancy could be due to a relatively specific recognition sequence which occurs more often in reiterated sequences in HeLa DNA than in the unique sequences of bacterial DNA's. This possibility has been suggested by the findings of Schneiderman and Billen[35] that rapidly reassociating DNA is enriched in 5-methylcytosine.

The HeLa DNA methylase can methylate single-stranded DNA. A similar result was observed by Drahovsky and Morris[16] for rat liver DNA methylase, and they suggest that this may represent an in vivo requirement for unwinding of double-stranded DNA in order for the methylase to "read" the base sequence. Our data indicate that the extent of methylation of single-stranded DNA is 3- to 4-fold higher than on double-stranded DNA. Assuming that the methylase recognition site occurs in a specific base sequence, it is possible that the recognition site on single-stranded DNA is less specific, perhaps by one base, than that on double-stranded DNA (e.g., a pentanucleotide vs. a hexanucleotide). However, our nearest neighbor data indicate that if such a difference in specificity does exist, it is not immediately adjacent to the 5-methylcytosine.

HeLa cell DNA is methylated by the enzyme, but only about 3% as much as M. luteus. This is to be expected since in bacterial systems homologous DNA's are not methylated at all because their methylation sites are presumably already occupied. However, in mammalian systems there may be a "lag" between DNA polymerase and methylase action, leaving a small number of potential methylation sites exposed on the progeny strands of newly replicated DNA. In this respect Adams[36] has found that in mouse L929 cells, short DNA chains are not methylated before being joined to higher molecular weight DNA.

A surprising result was that HeLa DNA methylase can methylate two synthetic copolymers: the double-stranded, alternating $(dG-dC)_n \cdot (dG-dC)_n$ and the single-stranded, random $(dG,dC)_n$. This result could be interpreted to mean that the DNA methylase recognition site consists only of G and C residues and Pu·Py ambiguities, such as the sites known for the Haemophilus

aphirophilus,[37] H. parainfluenzae,[38] H. aegyptius, H. haemolyticus, and Anabaena variabilis (R. Roberts, personal communication) restriction enzymes and, presumably, their modification methylases. This could also explain the preference of the enzyme for a high-GC DNA such as M. luteus. The extent data on the alternating copolymer indicate that the site is not simply GCGC or CGCG, and the methylation observed (1 methyl group per 1800 bases) may be due to some slight inhomogeneity of the copolymer.

Our nearest neighbor data indicate that 5-methylcytosine in HeLa DNA, as well as DNA methylated in vitro by the enzyme, occurs in either of two trinucleotides: pGpmCpG and pCpmCpG. It has not been determined whether this represents an ambiguity within the recognition site or whether the trinucleotides occur on opposite strands within a non-palindromic sequence. We are attempting to further elucidate the nature of the site by predigestion of substrate DNA with various restriction enzymes to determine if any overlaps in specificity occur.

Finally, the biological function of the HeLa DNA methylase remains unknown. Although no corresponding restriction enzymes have yet been found in eukaryotes, no evidence exists to rule out this possibility, and we are examining HeLa cells for such an activity.

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