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The 5'-cytosine in CCGG[1] is methylated in two eukaryotic DNAs and Msp I is sensitive to methylation at this site

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ABSTRACT

Novikoff rat hepatoma and bovine liver DNAs were digested with *Msp I* or *Hpa II*. Restriction fragments were end-labeled using [α-^{32}P]-dCTP and the Klenow fragment of *E.coli* DNA polymerase I and then digested to 2'-deoxyribonucleoside-3'-monophosphates using micrococcal nuclease and spleen phosphodiesterase. Mononucleotides were separated by two-dimensional thin layer chromatography, localized by radioautography, and the [^{32}P]-label quantitated by scintillation spectrometry. This method, based on known specificities of *Msp I* and *Hpa II*, shows that CCGG, CMGG, and MCGG (M refers to 5-methylcytosine) occur at frequencies of 89.6%, 1.4%, and 9.0%, respectively, in the rat DNA and at 41.6%, 48.3%, and 10.0%, respectively, in the bovine DNA. [^{32}P] recovery in 3'-5-MedCMP from end-labeled *Msp I* digests was negligible compared to recovery from *Hpa II* digests. Hence, *Msp I* is sensitive to methylation at the 5' cytosine in the sequence CCGG.

INTRODUCTION

The sequence specificities of DNA methylation sites have recently been probed using bacterial restriction endonucleases which include the dinucleotide CpG in their recognition/cleavage sequences.[2-12] This approach is based upon studies that have been interpreted as showing that eukaryotic DNA methylation occurs predominantly on cytosines contained in the dinucleotide CpG.[13-15]

Comparisons of DNA restriction fragments generated by the isoschizomers *Hpa II* and *Msp I* have been particularly widely used to probe the methylation status of the sequence CCGG. In this case the rationale is that *Hpa II* will cleave CCGG and MCGG but not CMGG whereas *Msp I* will cleave CCGG and CMGG.[16-17] Although the original work of Waalwijk and Flavell did not address the question, their paper has been interpreted as showing that *Msp I* is <u>in</u>sensitive to methylation at either cytosine in CCGG.

In this paper I present an *in vitro* labeling method which directly probes the methylation of the 5' cytosine in CCGG and which also yields the extent of methylation of the internal cytosine in that sequence. Application of this

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method to bovine and rat DNAs clearly demonstrates that 1) significant levels of MCGG exist in these eukaryotic DNAs, 2) methylation patterns within the sequence CCGG differ considerably between rat and bovine DNAs, and 3) the enzyme *Msp I* is sensitive to methylation of the 5' cytosine in the CCGG sequence.

MATERIALS AND METHODS

Experimental Approach: Figure 1A illustrates the rationale for these experiments. A given DNA is separately digested with *Hpa II* or *Msp I*. The restriction fragments are labeled at the 3'-end by incubation with [α-^{32}P]-dCTP and the Klenow fragment of *E.coli* DNA polymerase I. The end-labeled fragments are successively digested with matrix-bound bacterial alkaline phosphatase, micrococcal nuclease, and bovine spleen phosphodiesterase to yield the 2'-deoxyribonucleoside-3'-monophosphates. The [^{32}P] is thus transferred to the 5' cytosine in the CCGG sequence. The digest is subjected to two-dimensional thin layer chromatography, the chromatogram autoradiographed, and the [^{32}P] in the separated 3'-dCMP and 3'-5MedCMP quantitated.

Since *Hpa II* is known to cleave CCGG and MCGG,[16] [^{32}P] label in the 3'-5MedCMP spot of the chromatographed *Hpa II* digestion mixture constitutes direct evidence for the methylation of the 5'-cytosine in CCGG. Conversely, *Msp I* is known to cleave CCGG and CMGG but it is not known if MCGG is resistant to *Msp I* cleavage.[17] If *Msp I* is insensitive to methylation at the 5'-cytosine, then the amount of [^{32}P] in the 3'-5MedCMP spot in a *Msp I* digest should be identical to the amount of label in the 3'-5MedCMP spot from a *Hpa II* digest of that same DNA. If MCGG is resistant to cleavage by *Msp I* , the 3'-5MedCMP spot should be labeled in the *Hpa II* but not the *Msp I* digest.

Finally, quantitation of the amount of [^{32}P] label in the 3'-dCMP spots of the *Hpa II* and *Msp I* digests and in the 3'-5MedCMP spot of the *Hpa II* digest can provide estimates of the percentages of CCGG sequences present as CCGG, CMGG, and MCGG [see Figure 1B].

Preparation of DNA Substrates: Nuclei were isolated from Novikoff rat hepatoma cells or from livers of freshly slaughtered cows using a previously described method.[18] DNAs were isolated from purified nuclei by treatment with sodium dodecyl sulfate (0.5%) and 2 M NaCl; incubation with nuclease-free Pronase (10 mg/ml) at 45°C for 2 hrs.; three deproteinizations with chloroform: isoamyl alcohol (10:1 v/v); incubation with DNAase-free RNAase (10 mg/ml) at 45°C for 2 hrs.; three deproteinizations with chloroform:isoamyl alcohol; and, spooling the DNA from solution after addition of two volumes of cold absolute ethanol. All buffers contained 10 mM disodium EDTA to minimize degradation of

FIGURE 1A

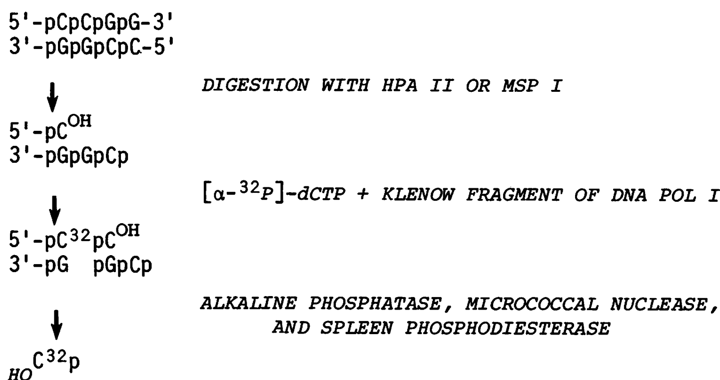

FIGURE 1B

HPA II	*MSP I*
C'CGG	*C'CGG*
M'CGG	*C'MGG*
	? M'CGG ?

GIVEN THE CLEAVAGE PATTERNS SHOWN ABOVE, THEN:

$$\text{Total CCGG's} = \text{cpm in dCMP}_{Msp} + \text{cpm in 5MedCMP}_{Hpa}$$

$$\text{\% as MCGG} = \frac{\text{cpm in 5MedCMP}_{Hpa}}{\text{Total CCGG's}}$$

$$\text{\% as CMGG} = \frac{\text{cpm in dCMP}_{Msp} - \text{cpm in dCMP}_{Hpa}}{\text{Total CCGG's}}$$

$$\text{\% as CCGG} = 100 - \text{\% as CMGG} - \text{\% as MCGG}$$

Figure 1. Experimental approach to determine the methylation status of the sequence CCGG. (A) Experimental protocol summarized. (B) Mode of treating experimental data to derive the methylation status of both the external and internal cytosine residues in CCGG. Cleavage sites indicated by apostrophes in the sequences. All sequences written in the 5' to 3' direction right to left. The known *Hpa II* and *Msp I* cleavage patterns are from [16-17].

the DNAs. DNAs thus prepared have no detectable contamination with RNA or proteins.

Restriction Endonuclease Digestions: All restriction endonuclease digestions were done at 37°C for 2 hrs. Final reactant concentrations for *Hpa II* digestions were: DNA substrate, 0.1 mg/ml; Tris-Cl buffer (pH 7.4); 20 mM; $MgCl_2$, 10 mM; dithiothreitol, 1 mM; bovine serum albumin, 100 μg/ml; *Hpa II* (New England Biolabs.), 1 unit/ μg DNA substrate. Final reaction mixture volumes, 200 μl. Final concentrations for *Msp I* digestions were: DNA substrate, 0.1 mg/ml; Tris-Cl buffer (pH 7.4), 10 mM; $MgCl_2$, 10 mM; KCl, 6 mM; dithiothreitol, 1 mM; bovine serum albumin, 100 μg/ml; *Msp I* (New England Biolabs.), 1 unit/ μg DNA substrate. Final reaction mixture volumes, 200 μl. Final reactant concentrations for *Eco RI* digestions were: DNA substrate, 0.1 mg/ml; Tris-Cl buffer (pH 7.4), 100 mM; $MgCl_2$, 5 mM; NaCl, 50 mM; dithiothreitol, 2 mM; bovine serum albumin, 200 g/ml; *Eco RI* (Sigma Chemical Co.), 1 unit/μg DNA substrate. Final reaction mixture volumes, 200 μl. All enzyme units referred to in this and succeeding sections are as defined by the suppliers of the respective enzymes. Both the *Hpa II and Msp I* preparations used in these studies were free of detectable exonucleolytic and nonspecific endonucleolytic activities as certified by the supplier of these enzymes (New England Biolabs.) and independently verified in this laboratory in the following manner: there was no acid-solubilization of end-labeled DNA after 2 hrs. of incubation at 37°C which indicates absence of exonucleases. Nonspecific endonucleolytic activity was analyzed by incubating the enzymes with a *Pst I-Hind III* fragment (ca. 1000 base pairs) of *Acanthamoeba castellani* rDNA which contains one *Msp I* site ca. 400 base pairs from the 5'-end of the fragment (the rDNA fragment was kindly supplied by Dr. M. Paule). Only fragments of the expected size were noted on both neutral and alkaline gels.

End-labeling of Restriction Fragments: Each restriction enzyme digestion mixture was subsequently incubated with 10 μCi of [α-^{32}P]-deoxycytidine-5'-monophosphate (New England Nuclear Corp.) and 1.0 unit of the Klenow fragment of *E.coli* DNA polymerase I (PL Biochemicals) at 25° and 37°C for 5 minutes at each temperature [see RESULTS section]. The reactions were stopped by adding 4.5 μl of 0.5 M disodium EDTA. Unreacted [α-^{32}P]-dCTP was removed by dialysis against three changes of 4000 volumes of Tris-Cl buffer (pH 8.0) at 4°C over a 24 hour period using a multiple port microdialysis chamber.

Removal of 5'-phosphates: 5'-phosphate groups on the labeled restriction fragments must be removed to ensure completeness of digestion by the micrococcal nuclease-spleen phosphodiesterase steps [see below]. This was done by

incubating each dialyzed labeled restriction enzyme digest with 250 units of Sepharose-bound bacterial alkaline phosphatase (Bethesda Research Labs.) at 65°C for 60 minutes. The matrix-bound alkaline phosphatase was removed from the digests by filtration through a siliconized glass wool plug placed in a siliconized 0.5 ml glass tuberculin syringe. Recovery of the end-labeled restriction fragments was nearly 100% provided that the matrix-bound alkaline phosphatase retained on the column was rinsed 4 times (100 μl each time) with 10 mM Tris-Cl buffer (pH 8.0).

Nuclease Digestions to the Nucleoside-3'-monophosphates: The dephosphorylated restriction fragment digests were made 1.5 mM in $CaCl_2$ and incubated at 37°C for 60 minutes with 1.0 unit of micrococcal nuclease (Sigma Chemical Co.). The pH of the digestion mixture was then adjusted to 6.5 using 0.1 N HCl, 1.0 unit of bovine spleen phosphodiesterase (Sigma Chemical Co.) added, and reincubated at 37°C for 60 minutes. Following this step the reaction mixtures were shell-frozen and lyophillized to dryness in siliconized glass tubes.

Thin-layer Chromatography and Autoradiography: The lyophillized material was redissolved in 200 μl of water. A 20 μl aliquot was spotted 1.5 cm from the corner edges of a cellulose chromatography sheet (20 x 20 cm; Eastman Chemical Co.; Chromagram™ #13255). Saturated ammonium sulfate:isopropanol: 1 M sodium acetate (80:2:18 v/v; described in [19]) was used to develop the chromatogram in both dimensions. Average development time for each dimension was about 2 hours.

The dried chromatogram was marked with [^{32}P] spots along two edges (to aid in later alignment of the autoradiogram and the chromatogram), placed between glass plates along with a 20 x 20 cm sheet of Kodak XR5 X-ray film, and exposed for 24 or 48 hours. After film development, the autoradiogram spots were traced onto transparent plastic sheets, the transparencies aligned on top of the chromatogram, and the radioactive spots located and cut from the chromatograms. Radioactivity was quantitated using a Packard TriCarb liquid scintillation spectrometer. The radioactivity in the 3'dCMP and 3'-5MedCMP nearly, but not exactly, cochromatographed with authentic unlabeled 5'-dCMP and 5'-5MedCMP which were also spotted onto each chromatogram as cold markers.

RESULTS

Analyses of the Labeling Protocol: Incubation of *Msp I* or *Hpa II* restriction fragments of Novikoff rat hepatoma cell DNA with [α-^{32}P]-dCTP and the Klenow fragment of *E.coli* DNA polymerase I resulted in immediate, extensive end-labeling (about 5 minutes at 25°C was required to add the [α-^{32}P]-dCTP

and enzyme to all reaction tubes). Maximal incorporation occurred after an additional 10 minutes at 37°C. In contrast, end-labeling of *Eco RI* fragments was initially 15,000 at "zero" time (compared to 222,200 cpm for the *Hpa II* fragments) but linearly increased with time such that the incorporation into *Eco RI* fragments was 14% of the incorporation into maximally labeled *Hpa II* fragments (41,100 cpm *vs* 290,600 cpm for *Hpa II* fragments after 10 minutes of incubation at 37°C). Subsequent nuclease digestions, chromatography, and autoradiography of *Eco RI* digests revealed transfer of the [α-^{32}P] to at least five different 2'-deoxyribonucleoside-3'-monophosphates including 3'-5MedCMP. Since *Eco RI* cleaves the sequence 5'-G'AATTC-3', transfer of the [α-^{32}P] from dCTP should not have been observed. The *Eco RI* used was a cheaper grade enzyme and may have been contaminated with nonspecific endo- and exonucleolytic activities which could account for the unexpected labeling since guanine residues opposite nicks or gaps would direct nonspecific incorporation of dCTP. A better control experiment involved the incubation of both rat and bovine nonrestricted DNAs with [α-^{32}P]-dCTP and the Klenow fragment of DNA pol I followed by nuclease digestions, chromatography, and autoradiography. Only three major radioactive 2'-deoxyribonucleoside-3'-monophosphate spots were noted (3'-dCMP, 3'-5MedCMP, and an as yet unidentified component), all of which had relatively low activity [see Table 1A]. This "background labeling" is presumed to occur at nicks and gaps introduced into the DNAs in the course of DNA isolation and purification and is subtracted from activities in 3'-dCMP and 3'-5MedCMP spots in the *Hpa II* and *Msp I* restriction fragment analyses.

Interestingly, the amount of label in 5-MedCMP in the nonrestricted rat and bovine DNAs is 60% and 43%, respectively, of the incorporation into 3'-dCMP. This disproportionate labeling of 3'-5MedCMP compared to 3'-dCMP could perhaps be explained as an artifact in which the 3'-5MedCMP spot was contaminated with 3'dCMP that tracked into the 3'-5MedCMP spot. However, this explanation is weak. The chromatographic separations of 3'-5MedCMP from 3'dCMP are quite good and show only minimal tailing, probably insufficient for the 60% and 43% referred to above. Second, analysis of the *Msp I*-digest should be equivalent to analysis of nonrestricted DNA with respect to the 3'-5MedCMP spot since *Msp I* is sensitive to MCGG [this paper]. If ca. 40-60% of the label in 3'-5MedCMP were simply due to contamination from 3'dCMP, then 10,000 to 15,000 cpm should have been noted in the 3'-5MedCMP spots in analyses of the *Msp I*-treated DNAs. In fact, however, the amount of label in 3'-5MedCMP in *Msp I*-treated DNAs is almost the same as that found in the nonrestricted DNAs (950 cpm *vs* 1010 cpm for rat DNA; 660 cpm *vs* 380 cpm for bovine DNA). This "background labeling" of

3'-5MedCMP (and 3'-dCMP) in the nonrestricted DNAs may therefore be real. The disproportionate labeling of 3'-5MedCMP relative to 3'-dCMP in the nonrestricted DNAs might indicate that nicking/gapping occur preferentially 5' to some 5-methylcytosine residues but further studies will be needed to test this thesis.

Note that label appears in two extra spots in the *Msp I* and *Hpa II* digests of rat hepatoma DNA [Figure 2A and B] and three extra spots in *Msp I* and *Hpa II* digests of bovine liver DNA [Figure 2C and D]. Technical problems (lack of resolution of the spots in the rat DNAs, solvent erosion and flaking of the thin layer cellulose layer of the lower quarter of the chromatograms which contained these spots) precluded quantitation of the amount of label therein. These spots presumably reflect incorporation into A, G, and T residues 3' to G residues on the opposite strand of DNA at nicks and gaps in the DNA. Examination of the X-ray films qualitatively show this "background labeling" to be somewhat greater than was the case with "background labeling" of the nonrestricted DNAs. This discrepancy is not presently explicable but is not likely to be due to exo/endonucleolytic contaminants of the restriction enzymes since two independent testings [see METHODS] detected no such contaminants.

Another control experiment [data not shown] demonstrated the complete acid solubilization of end-labeled *Hpa II* restriction fragments after even short incubations at 37°C with micrococcal nuclease and spleen phosphodiesterase. However, chromatography and autoradiography of these digests showed that not all fragments were reduced to the 3'-monophosphate level. If the end-labeled fragments were dephosphorylated with matrix-bound bacterial alkaline phosphatase prior to nuclease digestions, complete digestions were then obtained. The data in Table 1 and Figure 2 are therefore based on micrococcal nuclease/spleen phosphodiesterase digestions of 5'-<u>de</u>phosphorylated restriction fragments.

<u>Occurrence of 5-methylcytosine in CCGG</u>: The experimental protocol involves insertion of [α-^{32}P]-5'-dCTP at free 3'-hydroxyl sites opposite guanine residues on the other strand of duplex DNA or duplex DNA restriction fragments. Subsequent digestion of the DNA to 2'-deoxyribonucleoside-3'-monophosphates effects a transfer of [^{32}P] label to the 5' neighbor of the cytosine residue. Since *Hpa II* is known [16] to be able to cleave 5'-MCGG-3', occurrence of label in 3'-5MedCMP spots of nuclease/phosphodiesterase digests of end-labeled *Hpa II* restriction fragments proves that 5-methylcytosine occurs in the dinucleotide CpC. This is the result obtained with both bovine and rat DNAs [see Table 1A and Figure 2].

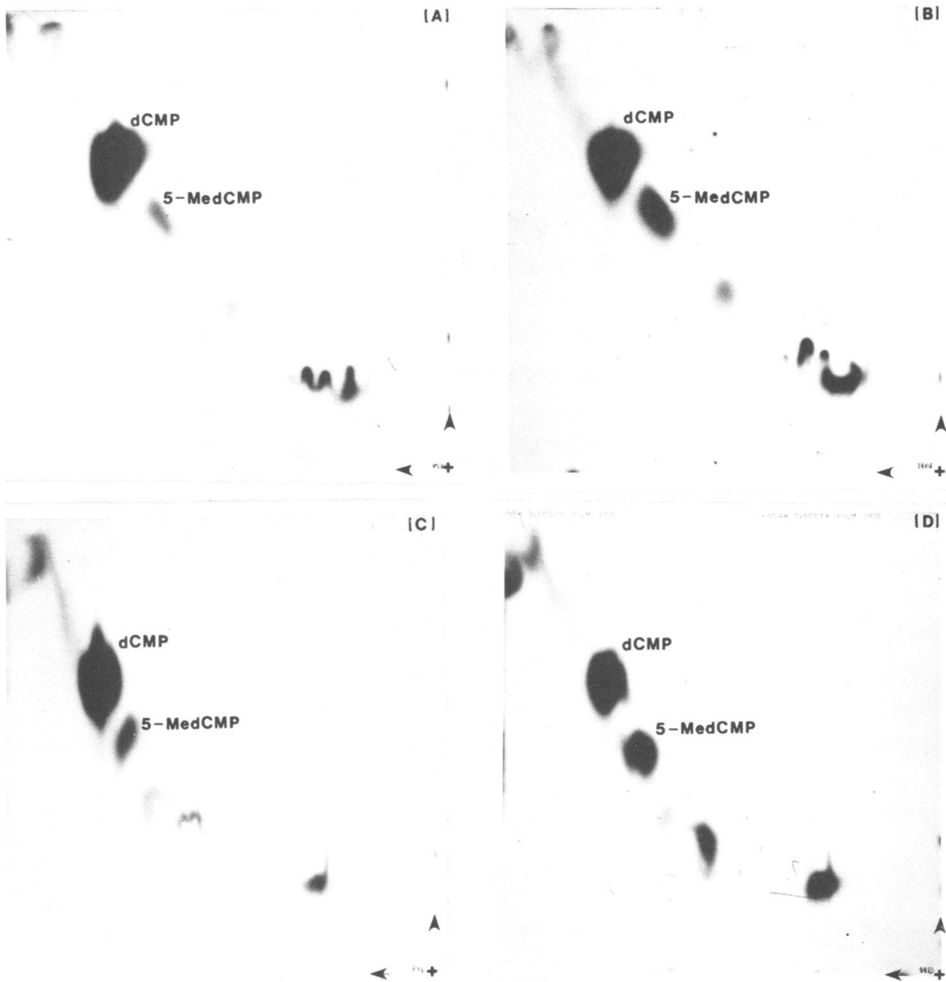

Figure 2. Autoradiograms of end-labeled nuclease digested *Msp I* or *Hpa II* DNA restriction fragments. Equal quantities of either Novikoff rat hepatoma or bovine liver DNAs were digested with *Msp I* or *Hpa II* or were not restricted at all. Following end-labeling and nuclease digestions [see text], aliquots were spotted onto thin layer cellulose sheets, developed in two dimensions, and autoradiographed for 24 (rat DNA digests, A and B) or 48 (bovine DNA digests, C and D) hours. Autoradiograms [not shown] of digests of nonrestricted DNAs had faint 3'-dCMP and 3'-5MedCMP spots which, when quantitated, yielded the data in Table 1. Additional spots on autoradiograms, most clearly seen in (D), are 3'-monophosphates of deoxyadenosine, deoxyguanosine, and deoxythimidine. Resolution of these spots is not consistently obtained due to solvent erosion of the lower ¼ of the chromatogram in the second dimension with the lot of TLC sheets used in these studies. In all cases, the origin is at the lower right corner. (A) *Msp I* and (B) *Hpa II* digests of Novikoff rat hepatoma cell DNA; (C) *Msp I* and (D) *Hpa II* digests of bovine liver DNA.

TABLE 1A. NEAREST NEIGHBOR ANALYSES OF THE INTERNAL CYTOSINE IN CCGG [a]

DNA SOURCE	ENZYME TREATMENT	cpm in dCMP	cpm in 5MedCMP	net cpm in dCMP	net cpm in 5MedCMP
Novikoff rat hepatoma cell	nonrestricted	1670	1010	-	-
	Msp I	25720	950	24050	0
	Hpa II	25360	3380	23690	2370
Bovine liver	nonrestricted	880	380	-	-
	Msp I	21900	660	21020	280
	Hpa II	10600	2740	9720	2360

TABLE 1B. METHYLATION STATUS WITHIN THE SEQUENCE CCGG [b]

DNA SOURCE	PERCENT OF CCGG SEQUENCES PRESENT AS CCGG	CMGG	MCGG
Novikoff rat hepatoma cell	89.6 %	1.4%	9.0%
Bovine liver	41.6	48.3	10.1

[a] See text and Figure 1A for details.
[b] Calculated from the data in Table 1A using the relationships shown in Fig.1B.

Since *Msp I* can cleave CMGG and *Hpa II* can cleave MCGG, the methylation status at both the internal and external cytosines of CCGG can be estimated from the relationships noted in Figure 1B. As shown in Table 1B, CCGG in a DNA of rat origin is predominantly nonmethylated (ca. 90% of total CCGG sequences) or exists as MCGG (9% of total CCGGs). Almost no CMGG is found. In contrast, CCGG in bovine liver DNA exists predominantly as CMGG (48% of total CCGGs), in nonmodified form (42%), or as MCGG (10% of total CCGGs).

Sensitivity of *Msp I* to Methylation at the 5'-cytosine in CCGG: The occurrence of 5'-methylcytosine at the 5' position in CCGG was directly demonstrated by the finding of [^{32}P] label in the 3'-5MedCMP spot of the *Hpa II* digests of both bovine and rat DNAs. If *Msp I* is insensitive to methylation at that site, the radioactivity recovered from the 3'-5MedCMP spots of *Msp I* digests of the DNAs should be identical to that recovered from the 3'-5MedCMP spots of the *Hpa II* digests. The data in Table 1A show that, after correction for label in 3'-5MedCMP of nonrestricted DNAs, either no (Novikoff rat hepatoma cell DNA) or little (bovine liver DNA; 12% of the amount of label found in *Hpa II* digests) label is found in 3'-5MedCMP spots of *Msp I* digests. These results

strongly suggest that *Msp I* is sensitive to methylation at the 5'-cytosine in the sequence CCGG.

DISCUSSION

The method described here to quantitate methylation in CCGG in DNAs is a modified nearest neighbor analysis [20] of restriction fragments generated by *Msp I* or *Hpa II* and uses [α-^{32}P]-dCTP and the Klenow fragment of *E.coli* DNA polymerase I. The addition of [α-^{32}P]-dCTP to ends of *Hpa II* or *Msp I* restriction fragments is rapidly completed. Degradation of the fragments to the 2'-deoxyribonucleoside-3'-monophosphates is readily accomplished provided that 5-phosphoryl groups are first removed by incubation with matrix-bound bacterial alkaline phosphatase. To use the method quantitatively [see Fig.1B], end-labeling of nonrestricted DNAs must be included to correct for insertion of labeled dCTP at nicks/gaps in DNA that may arise during DNA isolation and purification. Moreover, the *Hpa II* and *Msp I* digests must be complete so as to end-label the totality of CCGG fragments and the *Hpa II* and *Msp I* must be free of nonspecific endo- and exonucleolytic contaminants.

Application of this technique to DNAs of both rat and bovine origin clearly demonstrates significant occurrence of 5-methylcytosine at the 5' end of CCGG. These results confirm an earlier conclusion, based on an indirect approach [18], that CCGG in Novikoff rat hepatoma DNA is predominantly methylated as MCGG but not CMGG. The present data, showing that 5-methylcytosine occurs in the dinucleotide MpC in both rat and bovine DNAs, are also consistent with earlier analyses of 5-methylcytosine in pyrimidine isostichs in several DNAs.[21-23] The currently held concept that 5-methylcytosine in eukaryotic DNAs occurs only or predominantly in the dinucleotide CpG is based on experiments of Sinsheimer [13], Doskocil and Sorm [14], and Grippo et al.[15] However, the previous pyrimidine isostich analyses on DNAs of rat and mouse origin [21-23] and the present results indicate that that concept may not necessarily be valid for all higher eukaryotic DNAs.

The present results show a striking difference in the methylation pattern of CCGGs between DNAs of rat and bovine origin. Although both DNAs show about the same level of methylation of MCGG (ca. 10%), the internal cytosine is extensively methylated in bovine DNA (48%) but almost nonmethylated in rat hepatoma DNA (ca. 1%). Since the present method probes only CCGG sequences, and since CCGG sequences are but a fraction of total DNA cytosine residues, it will be of considerable interest to extend these studies with different restriction endonucleases to examine methylation of additional sequences in rat *vs* bovine

DNAs. Such analyses may help clarify the question of the putative evolutionary conservation of DNA methylase sequence specificities in higher eukaryotic organisms.[24] The method described in this paper may also, when applied to DNAs from differentiated *vs* undifferentiated tissues of a given organism, shed some light on a role for DNA methylation in specific gene activation.[5,7,8,12]

Finally, the paper of Waalwijk and Flavell [17] has often been interpreted as showing that *Msp I* is insensitive to <u>any</u> methylation in the CCGG sequence. This interpretation, coupled with the view that 5-methylcytosine occurs only as MpG, has led to the belief that simple comparison of *Hpa II vs Msp II* restriction fragment patterns totally defines the methylation status in CCGG sequences. The present experiments show that *Msp I* is sensitive to methylation at the 5'-cytosine in CCGG. Therefore *Msp I* restriction fragment patterns may not necessarily reflect the totality of CCGG sequences in a probed DNA.

ACKNOWLEDGEMENTS

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NOTE ADDED IN PROOF

After this paper was submitted for publication, van der Ploeg and Flavell reported (Cell <u>19</u>:947-958, 1980) that they will publish evidence showing that *Msp I* is sensitive to methylation at the 5'-cytosine in CCGG.

REFERENCES

1. Abbreviations used in this paper: CCGG, MCGG, and CMGG refer to the tetranucleotide DNA sequences recognized by *Hpa II* and *Msp I*. The sequences (written 5' to 3') show only one strand of the duplex DNA. The M is a nonstandard abbreviation which refers to the location of 5-methylcytosine in the sequence. 3'-dCMP, 2'-deoxyribocytosine-3'-monophosphate; 3'-5MedCMP, 2'-deoxyribo-5-methylcytosine-3'-monophosphate; 5'-dCMP, 2'-deoxyribocytosine-5'-monophosphate; 5'-5MedCMP, 2'-deoxyribo-5-methylcytosine-5'-monophosphate; dCTP, 2'-deoxyribocytosine-5'-triphosphate.
2. Gautier, F., Bünemann, H., and Grotjahn, L. (1977) Eur.J.Biochem. <u>80</u>: 175-183.
3. Bird, A.P. and Southern, E.M. (1978) J.Molec. Biol. <u>118</u>:27-47.
4. Bird, A.P. (1978) J.Molec. Biol. <u>118</u>:49-60.
5. Bird, A.P., Taggart, M.H., and Smith, B.A. (1979) Cell <u>17</u>:889-901.
6. Rae, P.M.M. and Steele, R.E. (1979) Nucleic Acids Res. <u>6</u>:2987-2995.

7. Waalwijk, C. and Flavell, R.A. (1978) Nucleic Acids Res. 5:4631-4641.
8. McGhee, J.D. and Ginder, G.D. (1979) Nature 280:419-420.
9. Singer, J., Robert-Ems, J.,and Riggs, A.D. (1979) Science 203:1019-1021.
10. Adams, R.L.P., McKay, E.L., Craig, L.M., and Burdon, R.H. (1979) Biochim. Biophys.Acta 563:72-81.
11. Browne, M.J., Cato, A.C.B., and Burdon, R.H. (1978) FEBS Letters 91:69-73.
12. Mandel, J.L. and Chambon, P. (1979) Nucleic Acids Res. 7:2081-2103.
13. Sinsheimer, R.L. (1954) J.Biol.Chem. 208:445-459.
14. Doskocil, J. and Sorm, F. (1962) Biochim.Biophys.Acta 55:953-959.
15. Grippo, P., Iaccarino, M., Parisi, E., and Scarano, E. (1968) J.Molec. Biol. 36:195-208.
16. Mann, M.B. and Smith, H.O. (1977) Nucleic Acids Res. 4:4211-4221.
17. Waalwijk, C. and Flavell, R.A. (1978) Nucleic Acids Res. 5:3231-3236.
18. Kaput, J. and Sneider, T.W. (1979) Nucleic Acids Res. 7:2303-2322.
19. Cedar, H., Solage, A., Glaser, G., and Razin, A. (1979) Nucleic Acids Res. 6:2125-2132.
20. Josse, J., Kaiser, A.D., and Kornberg, A. (1961) J.Biol.Chem. 236:864-875.
21. Sneider, T.W. (1971) J.Biol.Chem. 246:4774-4783.
22. Sneider, T.W. (1972) J.Biol.Chem. 247:2872-2875.
23. Salomon, R. and Kaye, A.M. (1970) Biochim.Biophys.Acta 204:340-351.
24. Browne, M.J. and Burdon, R.H. (1977) Nucleic Acids Res. 4:1025-1037.