Investigation of restriction-modification enzymes from *M. varians* RFL19 with a new type of specificity toward modification of substrate

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ABSTRACT

The characterization of MvaI restriction-modification enzymes, isolated from *Micrococcus varians* RFL19, is reported. Both enzymes recognize the 5'CC⁺(A/T)GG nucleotide sequence. The endonuclease cleaves the sequence at the position indicated by the arrow, whereas the methylase modifies the internal cytosine, yielding N⁴-methylcytosine. This type of modification protects the substrate from R.MvaI cleavage. 5-Methylcytosine in the same position of the recognition sequence does not protect the substrate from R.MvaI cleavage. R.MvaI proved to be the first example of a restriction endonuclease differentiating the position of the methyl group in the heterocyclic ring of cytosine, located in the same site of the recognition sequence. M.MvaI modifies DNA dcm⁺ in vitro yielding N⁴,5-dimethylcytosine. N⁴-methylcytosine cannot be differentiated from cytosine using the Maxam-Gilbert DNA sequencing procedure.

INTRODUCTION

For a long time since the discovery of restriction-modification enzymes it was generally accepted that methylases protect DNA from restriction endonuclease action by methylating either adenine to N⁶-methyladenine or cytosine to 5-methylcytosine (m⁵C) ¹. Recently the first example of an unusual methylation was proved to exist in the R.-M.BcnI system where the action of the methylase on the substrate results in N⁴-methylcytosine (m⁴C) ². This discovery brings forth many questions to be answered, among them the following: (i) the occurrence of methylases yielding m⁴C in various taxonomic groups of microorganisms; (ii) the effect of the m⁵C change to m⁴C (and vice versa) on the action of restriction and modification enzymes in the same site of the recognition sequence.

The methylase from *Micrococcus*, producing N⁴-methylcytosine, is described. Substrate specificity of MvaI restriction-

modification enzymes, both isolated from *M. varians* strain, was investigated together with the ability of *R. MvaI* to differentiate the nature of methylcytosine (m^4C and m^5C) in the recognition sequence.

MATERIALS AND METHODS

Materials. Sephadex G-50 (fine) was purchased from Pharmacia; agarose from Calbiochem; acrylamide, N,N-(bis)-methylacrylamide, TEMED from Fluka; dC, dpC, S-adenosyl-L-(3H -methyl)-methionine (3H -SAM), 15 Ci/mmol from Amersham; SAM from Serva; α - ^{32}P -dCTP and γ - ^{32}P -ATP from Isotope (Tashkent). dCpC, dCpA, dCpT and dCpG were synthesized by the modified phosphotriester method ³. N^4 -methyl- and $N^4,5$ -dimethyldeoxycytidines were synthesized by a newly developed procedure ⁴. Other reagents were analytical grade commercial materials.

R. MvaI was purified to electrophoretic homogeneity by two chromatographic steps ⁵. *M. MvaI* was isolated in the following way: 15 g of frozen cells were suspended in 60 ml of buffer A (10 mM potassium phosphate, pH 7.0, 150 mM NaCl, 10 mM EDTA, 7 mM 2-mercaptoethanol) containing 1 mg/ml lysozyme, incubated overnight at 4°C, disrupted by sonication and centrifuged for 60 min at 48 000 x g. The supernatant was applied on a phosphocellulose column (1.5 x 20 cm) and chromatographed with a 320 ml linear gradient of KCl (0.15-0.7 M) in buffer B (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 7 mM 2-mercaptoethanol). The fractions, which eluted at 0.34-0.40 M KCl and contained methylase *MvaI* activity, were pooled and dialyzed against buffer B, containing 0.2 M KCl. The dialysate solution was chromatographed on a 1 x 8 cm column of heparin-sepharose using a 100 ml linear 0.2-1.0 M KCl gradient in buffer B. *M. MvaI* activity eluted at 0.40-0.48 M KCl. The active fractions were pooled and concentrated by dialysis against 10 mM Tris-HCl buffer, pH 7.5, 200 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 200 μ g/ml albumin and 50% glycerol (v/v), and then stored at -20°C.

T4 DNA polymerase, alkaline phosphatase, T4 polynucleotide kinase, restriction endonucleases *Cfr*10I, *Pst*I, *Eco*RII, methylase *Eco*RII, phage λ DNA *dcm*⁻ and pBR322 DNA *dcm*⁻ were

isolated in our laboratory.

Methods

DNA cleavage with endonucleases. DNA cleavage with R.MvaI was performed in a reaction mixture (40 μ l): 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin and 50 μ g/ml phage λ DNA dcm⁻ for 1 hour at 37°C. Double digests were carried out by simultaneous addition of R.MvaI and R.EcoRII to the above mentioned reaction mixture. DNA fragments were subjected to analysis on a 1.4% agarose gel.

DNA methylation. Methylation of substrate (phage λ DNA dcm⁻ or pBR322 DNA dcm⁻) with M.MvaI or M.EcoRII was performed at 37°C in a reaction mixture (40 μ l): 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 3 mM EDTA, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 50 μ g/ml DNA and either 100 μ M SAM or 2 μ M ³H-SAM. One arbitrary unit of methylase activity is the amount required to incorporate 1 pmol (17 000 cpm) of methyl groups in 1 μ g of phage λ DNA dcm⁻ within 1 hour at 37°C.

Determination of R.MvaI substrate specificity. For the determination of the cleavage site within the recognition sequence, pBR322 DNA (50 μ g) was digested with R.Cfr10I and subjected to electrophoresis on a 0.8% horizontal agarose gel containing ethidium bromide. The desired Cfr10I fragment (1076 bp) was isolated by electroelution ⁶. The pooled solution of DNA was concentrated with a rotor evaporator under reduced pressure, extracted with an equal volume of butanol, desalted on a Sephadex G-50 column in 10 mM triethylammonium bicarbonate (TEAB) (pH 8.5), 0.1 mM EDTA, and DNA precipitated with ethanol. The precipitate was dissolved in water (20 μ l) and an aliquot (3 μ l) was 3' labeled with T4 DNA polymerase and γ -³²P-dCTP ⁷. An equal amount of the sample was dephosphorylated with alkaline phosphatase, and then 5' labeled with polynucleotide kinase and γ -³²P-ATP ⁸. Both DNAs were cleaved with PstI in separate reactions, the single-end-labeled subfragments were separated by electrophoresis on a 5% polyacrylamide gel and extracted from the gel ⁶. Sequence analysis of isolated subfragments was performed according to Maxam-Gilbert

procedure ⁹. The respective labeled PstI-Cfr10I subfragments were digested with R.MvaI and subjected to electrophoresis alongside with the products of chemical reactions.

Determination of M.MvaI specificity. Isolation of methylated DNA, hydrolysis to bases and chromatographic identification of the modified base was performed as described earlier ^{2,10}. For the identification of modified deoxynucleoside, ³H-DNA was hydrolyzed with pancreatic DNase and VPDE, dephosphorylated with alkaline phosphatase, and HPLC analysis with standards was performed on a Nucleosil C-8 column using 20 mM KH₂PO₄ (pH 4.25) in a 1% solution of acetonitrile.

To identify the position of the methylated base in the recognition sequence, ³H-DNA was subjected to hydrolysis with pancreatic DNase and dephosphorylated with alkaline phosphatase. A dinucleoside monophosphate fraction was isolated after high-voltage electrophoresis on Whatman 3 MM in a 50 mM TEAB (pH 8.5) buffer. Individual cytidine-containing dinucleoside monophosphates were separated by electrophoresis on Whatman 3 MM paper in 0.05 M formic acid (pH 2.7). Each of the labeled nucleotides was subjected to hydrolysis in parallel probes with spleen and snake venom phosphodiesterases (SPDE and VPDE) ¹¹. Electrophoretic separation of the resulting radioactive products was performed in a mixture with standards dC, dpC, dCpA, dCpG, dCpC and dCpT on Whatman 3 MM paper in a 50 mM TEAB buffer (pH 8.5).

Sequencing of DNA containing N⁴-methylcytosine. Two aliquots of the pBR322-Cfr10I 1076 bp fragment (5 µg) were methylated in separate reactions with M.MvaI and M.EcoRII in the presence of SAM. The unreacted SAM was removed by gel-filtration on Sephadex. Both DNAs were labeled with α -³²P-dCTP and DNA-polymerase (Klenow fragment), cleaved with PstI, and, single-end-labeled PstI-Cfr10I subfragments, containing MvaI or EcoRII methylated recognition sequences, were isolated from the polyacrylamide gel. The isolated subfragments were treated by the base specific chemical procedures determined by Maxam and Gilbert ⁹.

Simultaneous methylation of DNA with M.EcoRII and M.MvaI. Methylation of phage λ DNA dcm⁻ (50 µg) was performed for 5

hours in separate reaction mixtures (1 ml) with 0.1 mM SAM and 200 units of methylase (M.EcoRII or M.MvaI). Unreacted SAM was removed by gel-filtration on Sephadex, and DNA was precipitated with ethanol. Methylation with a second methylase was performed for 30 min. in 100 μ l reaction mixture, containing 5 μ g of methylated DNA, 0.5 nmol 3 H-SAM and 20 units of the second methylase (M.EcoRII or M.MvaI). After enzymatic hydrolysis of methylated DNA, 3 H-labeled deoxynucleoside was identified by HPLC analysis in a mixture with standards.

RESULTS AND DISCUSSION

In all systems so far studied, bacterial cells produce a modification methylase in addition to the sequence-specific endodeoxyribonuclease; both enzymes recognize the same specific DNA sequence ¹². As was shown, the R.-M.MvaI system is not an exclusion to this rule.

Determination of R.MvaI specificity. The comparison of R.MvaI and R.EcoRII substrate cleavage specificity is presented in fig.1 . As can be seen, the R.MvaI cleavage pattern is identical to that of R.EcoRII, which is known to recognize the

FIGURE 1. Comparison of cleavage patterns on agarose gel
(a) λ DNA dcm⁻(DNA); (b) DNA+R.MvaI;
(c) DNA+R.MvaI+R.EcoRII; (d) DNA+
+R.EcoRII.

5'CC(A/T)GG nucleotide sequence ¹¹.

A pBR322 Cfr10I DNA fragment (fig.2.) was used as a substrate to determine the exact cleavage site of R.MvaI. Results are presented in fig.3. From fig. 3-B it is evident that the mobility of MvaI-Cfr10I restriction fragment, labeled with T4 DNA polymerase, coincide with one of the fragments in the sequence ladder. Following interpretation rules¹³ it may be suggested that R.MvaI cleaves the recognition sequence between C and T residues as follows: 5'CC[↓]TGG. The mobility of the same restriction fragment, bearing a 5'-³²P-kinase label, does not coincide with that of any fragment of similar length in the sequence ladder (fig. 3-A). This is most likely due to the presence of additional phosphate at the 3'termini in products of DNA chemical degradation as compared to that of MvaI-Cfr10I restriction fragment.

Data available on the increased electrophoretic mobility of short DNA fragments, containing one additional terminal phosphate ¹⁴, allows us to maintain that R.MvaI cleaves the 5'CC[↓]AGG sequence between C and A residues, yielding 3' hydroxyl and 5' phosphate termini (fig. 4-A). It should be mentioned that if R.MvaI cleavage resulted in 5'hydroxyl and 3'phosphate termini, the restriction fragment would not align with a respective fragment in the sequence ladder in the first case (analysis of 3'end-labeled fragment) and would align in the second case (analysis of 5'end-labeled fragment) (fig. 4-B).

Hence, it can be concluded that R.MvaI cleaves the recognition sequence, as indicated by arrows, leaving 3'hydroxyl and 5'phosphate ends.

Up till recently it was widely accepted that DNA cleavage with restriction endonucleases leaves 5'phosphate and 3'hyd-

FIGURE 2. Partial restriction map of the pBR322 Cfr10I fragment used for determining R.MvaI cleavage site. Coordinate of the base corresponds to the 5'nucleotide of each recognition sequence.

FIGURE 3. Determination of the exact cleavage site by R.MvaI on Cfr10I 1076 bp fragment of pBR322 DNA. As described in the text, the fragment was labeled with T4-polynucleotide kinase or T4 DNA polymerase and cleaved with PstI. The labeled subfragments, containing R.MvaI site in the vicinity of the labeled end, were isolated from PAAG gel and sequenced by the Maxam-Gilbert method. MvaI line represents R.MvaI digest of the same subfragment.

A - 5'kinase labeled strand.

B - 3'polymerase labeled strand.

roxyyl termini. Therefore, in most studies no special experiments were performed to exclude some other possible type of phosphodiester bond cleavage, namely that, which would result in 3'phosphate and 5'hydroxyl termini. After an endonuclease NciI acting in such a way was discovered (cited in ref.¹⁵

FIGURE 4. Two possible modes of cleavage of the phosphodiester bond by *R.MvaI*:

A - generating 3'OH and 5'P-termini.

B - generating 3'P and 5'OH-termini.

Cleavage sites marked by arrows. Asterisk indicates labeled phosphate.

as an unpublished observation by Hu.A.W., Marschell A.H.), it became desirable to determine the phosphate location in the termini of the DNA fragment, produced by restriction enzymes.

Conclusions on 5' termini structure have been mostly drawn from the difference in kinase labeling of dephosphorylated and non-dephosphorylated restriction fragments, obtained with the endonuclease under investigation^{16,17}. Our results indicate that in some cases the type of phosphodiester bond cleavage may be established on the basis of the non-identity of electrophoretic mobilities of the aligned restriction fragment as compared to a DNA fragment of the same length, obtained after chemical reactions. However, the cleavage point of the restriction endonuclease under investigation must be within 15 to 40 nucleotides of the labeled end. It is the range where the mobility shift of oligonucleotides, differing merely in the phosphate group may be interpreted unequivocally^{13,14}.

R.MvaI cleaves DNA at all *R.EcoRII* sites (fig. 1). However, *R.MvaI*, in contrast to *R.EcoRII*, is insensitive to DNA modification with dcm methylase (see below).

Three specificity manifestations, namely: identical sequence, identical cleavage site and insensitivity to dcm modification, allow us to assume that *R.MvaI* like *TaqXI*¹⁸, *AorI* and *ApyI* (cited in ref.¹⁵ as an unpublished observation by Seurinck J., van Montagu M. and DiLauro R.) is an isoschizomer of *BstNI* (cited in ref.¹⁵ as an unpublished observation

FIGURE 5. Protection of DNA from endonuclease MvaI cleavage by pre-treatment with M.MvaI. (a) phage λ DNA dcm⁻ (DNA); (b) DNA methylated with methylase MvaI (M.MvaI DNA); (c) M.MvaI DNA+R. MvaI; (d) DNA+M.MvaI DNA+R.MvaI; (e) DNA+R.MvaI; (f) M.MvaI DNA+R. BcnI.

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Determination of M.MvaI substrate specificity. It was demonstrated that chromosomal DNA, isolated from *M. varians* RFL19, is resistant to R.MvaI (not shown). This indicates an *in vivo* modification of DNA sites recognized by R.MvaI.

Succeeding incubation with phage λ DNA dcm⁻ and SAM, methylase MvaI, isolated from *M. varians* RFL19, was observed to render the substrate resistant to restriction enzyme MvaI cleavage (fig. 5. track c). Resistance to R.MvaI cleavage of modified DNA should not be interpreted as an experimental artefact, since the same DNA can serve as a substrate for restriction endonuclease BcnI, recognizing the 5'CC(C/G)GG sequence (fig. 5. track f) with specificity not overlapping with that of R.MvaI (see below) and furthermore, unmodified DNA

TABLE 1. Distribution of ^3H -methyl label among cytidine-containing dinucleotides in M.MvaI methylated DNA

Type of modification		Ratio of radioactivity content in			
		(C,C)*	(C,A)*	(C,T)*	(C,G)*
Calculated	5'CC(A/T)GG	14	1	5	4
	5'CC*(A/T)GG	2	1	1	-
Experimental	5'CC*(A/T)GG	1.8	1.3	1	-

shows no resistance to R.MvaI cleavage in a mixture of modified and unmodified DNAs (fig. 5. track d).

From the obtained results it can be assumed that M.MvaI recognizes either the whole R.MvaI recognition sequence or part of it. To test the identity of recognition sequences of both enzymes, pBR322 DNA dcm^- was digested by R.MvaI, and the resulting fragments were probed as a substrate for MvaI methylase. No methylation was observed in R.MvaI fragments, whereas untreated DNA incorporated ^3H -methyl groups effectively (not shown). It can therefore be concluded that the substrate for MvaI methylase is 5'CC(A/T)GG.

In order to determine the position of the modified base in the recognition sequence, 5' and 3'neighbours of the methylated cytidine residues were established. For this purpose the cytidine-containing labeled dinucleotides, isolated from the enzymatic hydrolysate of DNA premethylated with M.MvaI, were analyzed.

^3H -Radioactivity was found in dinucleotides (C,C)*, (C,A)* and (C,T)* in the ratio 1.8:1.3:1. This distribution is in close agreement with one of the two values, both of which were calculated for two possible modes of site modification (Table 1), taking in consideration the known pBR322 DNA sequence in the vicinity of MvaI recognition sites and statistically random substrate cleavage by pancreatic DNase. From this it seems probable that the internal cytosine residue in CC(A/T)GG is methylated. This preliminary assumption was further confirmed by establishing the position of the modified base within each labeled dinucleotide.

TABLE 2. Localization of modified cytosine($\overset{*}{C}$) within dinucleotides by exonuclease digestions

Dinucleotide	Exonuclease	Distribution of radio-activity between, %			Deduced structure, 5'...3'
		dN	dNpN	dpN	
(C,C)*	VPDE	4	-	96	Cp $\overset{*}{C}$
	SPDE	48	50 ^a	2	
(C,A)*	VPDE	100	-	-	$\overset{*}{C}$ pA
	SPDE	1	-	99	
(C,T)*	VPDE	100	-	-	$\overset{*}{C}$ pT
	SPDE	1	1	98	

^a- possibly due to incomplete digestion with SPDE

As shown in Table 2, M.MvaI methylation produces ...Cp $\overset{*}{C}$..., ... $\overset{*}{C}$ pA... and ... $\overset{*}{C}$ pT... .These results indicate that the methylated cytosine is located in the center of the 5'Cp $\overset{*}{C}$ p(A/T) sequence. The partially determined site $\overset{*}{C}$ C(A/T) is consistent with the recognition sequence as follows: $\overset{*}{C}$ C(A/T)GG.

M.MvaI methylated DNA base analysis shows that the methylase produces N⁴-methylcytosine (not shown).This unusual type of cytosine modification in DNA was observed in B.centrosporus for the first time ². Recent investigations show methylases yielding m⁴C in DNA to exist in Citrobacter freundii ¹⁹. M.MvaI contributes yet another addition to the list of methylases with N⁴-methylcytosine specificity.

Sequencing of DNA containing N⁴-methylcytosine. The identification of m⁴C in DNA put forth a number of questions, among them the question about the ability of different methods, used for m⁵C identification in DNA, to discriminate m⁴C and m⁵C. Recently we investigated TLC systems most often used in the analysis of minor components of nucleic acids ¹⁰, and found that not all of them can discriminate m⁴C and m⁵C. The Maxam-Gilbert DNA sequencing procedure is successfully used in discriminating m⁵C and unmethylated cytosine ²⁰. We decided to test whether the Maxam-Gilbert procedure was suitable in

FIGURE 6. Sequencing of the PstI-Cfr10I pBR322 DNA fragment 5'-³²P-labeled at Cfr10I cleavage site with T4-polynucleotide kinase (for details see text).
 A - unmethylated fragment (*dcm*⁻);
 B - fragment methylated with *M.EcoRII*;
 C - *M.MvaI* methylated fragment.

the detection of m^4C .

In our sequencing experiments single-end labeled Cfr10I-PstI pBR322 DNA *dcm*⁻ fragments, containing either m^4C (*MvaI* methylation) or m^5C (*EcoRII* methylation) in the 5'CC(A/T)GG site, were used. As expected, the sequence ladder of DNA methy-

lated with M.EcoRII displayed no zone in the position corresponding to m⁵C in the Cm⁵C(A/T)GG sequence (fig.6).

In contrast, the ladder of DNA, methylated with M.MvaI and containing m⁴C in the same position of the sequence, has a zone of similar intensity, as compared to the cytosine of unmethylated DNA (fig.6). The resistance of DNA, methylated with M.MvaI, to R.MvaI cleavage may be due to hemimethylation of the target sequence. Thus the zone in the sequencing ladder, attributed to m⁴C, may be derived from the unmethylated strands. The possibility of hemimethylation was excluded after melting-annealing experiments of the DNA under investigation were performed together with the same unmethylated DNA fragment. The obtained "hybrid" DNA was also resistant to R.MvaI cleavage (not shown). These experiments confirmed that the DNA under analysis was completely methylated with M.MvaI at both strands and, in addition, the hemimethylated recognition sequence was not cleaved by R.MvaI.

Therefore, it can be concluded that N⁴-methylcytosine, unlike 5-methylcytosine, reacts with hydrazine under chemical reaction conditions ⁹, and resembles unmethylated cytosine in the sequencing ladder. Similar results were obtained when ribosomal RNA, containing N⁴-methylcytosine, was sequenced after chemical reactions ²¹.

Restrictase susceptibility to 5- and N⁴-methylcytosine interchange at identical position in the recognition sequence.

It was demonstrated that M.MvaI methylates the internal cytosine of the recognition sequence 5'CC(A/T)GG, producing N⁴-methylcytosine. The same cytosine is methylated by dcm ²² and EcoRII ¹¹ methylases. However, the product of the reactions was shown to be 5-methylcytosine. The MvaI-EcoRII restriction-modification enzyme system presented us with the unique possibility of investigating restriction endonuclease susceptibility to the interchange of 5- and N⁴-methylcytosines in the recognition sequence. Experimental results are presented in fig.7. As can be seen, DNA methylated with M.EcoRII is resistant to R.EcoRII cleavage, but it is readily cleaved with R.MvaI. In contrast, DNA methylated with M.MvaI is resistant

FIGURE 7. Cleavage of differently methylated λ DNA with *MvaI* and *EcoRII* restriction endonucleases: (a) DNA dcm^- (DNA); (b) *M. MvaI* methylated DNA (*M. MvaI* DNA); (c) *M. MvaI* DNA+*R. EcoRII*; (d) DNA+*M. MvaI* DNA+*R. EcoRII*; (e) DNA+*R. EcoRII*; (f) DNA; (g) *M. EcoRII* methylated DNA (*M. EcoRII* DNA); (h) *M. EcoRII* DNA DNA+*R. MvaI*; (i) *M. EcoRII* DNA+*R. EcoRII*; (j) *M. EcoRII* DNA+DNA+*R. EcoRII*; (k) DNA+*R. EcoRII*.

to the action of both restriction endonucleases under investigation.

As far as we know, restrictase *MvaI* is the first example of a sequence-specific endodeoxyribonuclease, which is sensitive to the position of the methyl group in the cytosine residue, located in the same modification site of the recognition sequence (i.e. cleaves DNA bearing internal m^5C in the recognition sequence and does not digest the substrate when the $4NH_2$ group is methylated in the same cytosine). The manifestation of this type of specificity of restriction endonucleases is described for the first time. It is not excluded that an identical type of specificity may also be characteristic of *BstNI* restriction endonuclease (see Discussion in ² and ²³).

FIGURE 8. HPLC identification of ^3H -deoxynucleoside obtained after enzymatic hydrolysis of ^3H DNA, premethylated with EcoRII and MvaI methylases. 5 μl of hydrolysate (15000 cpm) was mixed with standards and then analyzed. Peaks were collected and radioactivity measured in a dioxane-based scintillation cocktail. Chromatographic conditions: apparatus Gilson; column Nucleosil C8, 250x4.6; mobile phase, 0.02 M KH_2PO_4 (pH 4.25) in 1% acetonitrile solution, flow rate 1.5 ml/min.; detector Kratos, 254 nm; peak analysis was performed using Appligratation II data analysis system. A - 5-methyldeoxycytidine; B - N^4 -methyldeoxycytidine; C - $\text{N}^4,5$ -dimethyldeoxycytidine. Radioactivity found in peaks B(15%) and C(85%).

Until now, only the position of the modified base in the recognition sequence and its influence on restriction endonuclease action on DNA has been considered ¹. It was determined that restriction endonucleases differ in susceptibility to the modification pattern within their recognition site. Alongside enzymes, specifically inhibited by methylation anywhere

within the recognition sequence, there are enzymes, which are insensitive to modification at non-cognate sites ¹, ¹⁵.

In the light of recent developments it is essential to determine enzyme sensitivity to both types of cytosine modification (m^5C and m^4C) in order to produce a more detailed characterization of a given restriction enzyme. This aspect should be particularly emphasized when a restriction enzyme is used for the studies of DNA methylation.

Simultaneous methylation of substrate with EcoRII and MvaI methylases. The existence of two methylases capable of modifying the same cytosine base within the same sequence, yet at different positions of the cytosine ring raised the question of methylase susceptibility toward some other type of methylation in their recognition sequence. To investigate this possibility, phage λ DNA dcm^- was exhaustively methylated in two separate reactions with M.EcoRII and M.MvaI in the presence of SAM. Complete methylation at the 5'CC(A/T)GG sites was proved on the basis of two different approaches: (i) the resistance of methylated DNA to cognate restriction endonuclease cleavage; (ii) the absence of radioactivity incorporation when using the same methylase and 3H -SAM. Both completely methylated DNAs were further methylated with an equal amount of another methylase in the presence of 3H -SAM. 3H -Radioactivity was incorporated only when M.EcoRII was used for the first methylation and M.MvaI for the second.

It is interesting to notice that the methylation rate using M.MvaI was almost the same when either DNA dcm^- or DNA methylated with M.EcoRII was used (not shown). The inverse order of methylase treatment gave negative results even when a ten-fold amount of M.EcoRII was used.

The labeled DNA was hydrolyzed to deoxynucleosides and the radioactive products were identified by HPLC (fig.8). At least 85% of input radioactivity co-chromatographed with the peak, corresponding to $N^4,5$ -dimethyldeoxycytidine.

We have already demonstrated the possibility to produce $N^4,5$ -dimethylcytosine in vitro. The occurrence of this unusual base in vivo cannot be excluded. Possible biological implications of the occurrence of $m^4,5C$ in bacterial cells

will most likely be revealed after the cloning of MvaI methylase gene into E.coli.

CONCLUDING REMARKS

As a DNA minor base, N⁴-methylcytosine was first detected in studies of DNA modification products by methylase BcnI from *Bacillus centrosporus* ². This led to the question of the occurrence of this minor base in DNA of other microorganisms. Our studies have demonstrated that several other site-specific methylases, isolated from *Citrobacter freundii* ¹⁹, *Proteus vulgaris* ²⁴ and *Micrococcus varians* (see RESULTS AND DISCUSSION), also yield m⁴C. Evidence of the wide spread of this novel base in microbial DNAs has recently been obtained by Ehrlich and co-workers ²³. According to these authors, about half of the examined thermophiles contained N⁴-methylcytosine in their DNA. It can thus be concluded that the presence of m⁴C in DNA is not an exotic phenomenon, and N⁴-methylcytosine should therefore be treated as a common minor base in bacterial DNA. This discovery raises the question of why different types of cytosine methylation are present in the genomes of different microorganisms.

We believe that the answer should be sought for in the analysis of possible effects caused by these minor bases upon DNA structure and functioning.

5-Methylcytosine is known to easily undergo spontaneous deamination (m⁵C→T) resulting in the substitution of the AT pair for the GC pair, which causes point mutations of DNA. According to Ehrlich et al. ²³, this process may be a substantially frequent event in extreme thermophiles and, consequently, m⁴C would evolutionarily be more preferable in order to suppress heat-induced spontaneous mutations of methylated DNA.

It should be pointed out, however, that our data also provides evidence about the wide occurrence of m⁴C in mesophiles. This fact implies that the cultivation temperature is not the sole factor determining the type of methylation in a given microbial species. It would be fruitful in this connection to discuss the differences in effects, caused by the two

methylated cytosines on DNA structure.

Methylation increases the hydrophobicity of the B-DNA major groove, which is known to be a contact surface for numerous regulatory proteins and enzymes, including restriction endonucleases²⁵. Both of the discussed methyl groups are located in this groove: 5-methyl group occupies the outer, whereas N⁴-methyl rests in the central position of the groove²⁶. Furthermore, N⁴-methylation sterically blocks the amino function of cytosine, which is considered part of the important portion of the C:G identification system, located in the major groove²⁶. It can thus be suggested that m⁴C provides a stronger signal for protein-DNA interaction, as compared with m⁵C.

This is in concordance with the data obtained in our study on DNA protection against cleavage with MvaI and EcoRII restriction endonucleases caused by the different types of cytosine methylation. Experiments on methylation of premethylated DNA, using M.MvaI and M.EcoRII, also agree with this view.

Methylation not only protects DNA from restriction endonucleases, but also plays a role in the interaction of other proteins with DNA. One can expect that different regulatory proteins respond in different ways to the presence of either m⁴C or m⁵C in DNA when sites of site-specific methylation overlap with DNA regulatory regions. On the other hand, there are numerous data indicating that m⁵C is a factor initiating the B-Z transition in certain DNA sequences under physiological conditions^{27,28}. Local Z-DNA regions are found in DNA from natural sources^{29,30}, and regulatory proteins and enzymes appear to strictly differentiate between the B and Z forms of DNA³¹⁻³³. The regulatory role of m⁵C was confirmed in a number of studies³⁴⁻³⁷; it is not excluded that such regulation operates via B-Z transitions. There is no experimental data, however, on the capability of m⁴C to potentiate the B-Z transition. At present we have synthesized oligonucleotides³⁸, containing m⁴C, and are investigating this and related questions.

In conclusion, we may assume that the type of cytosine modification is largely predetermined by the genomic structure and its functional peculiarities in a given microorganism or

taxonomic group, e.g. how a certain microorganism uses the above mentioned and possibly yet unknown features of m^4C or m^5C and adapts itself to the possible mutagenic effect of m^5C .

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