Volume 161, number 1 FEBS 0779 September 1983

Cytosine modification in DNA by *Bcn*I methylase yields N^4-methylcytosine

A. Janulaitis, S. Klimašauskas, M. Petrušyte and V. Butkus

Institute of Applied Enzymology, 232028 Vilnius, Fermentu 8, Lithuanian SSR, USSR

Received 11 July 1983

Abstract and keywords not received

1. INTRODUCTION

The means by which bacteria protect their own DNA from their restriction enzymes have not been fully investigated. In all systems that have been studied, cells produce a modification methylase in addition to the restriction endonuclease. Both enzymes recognize the same specific DNA sequence. Not many DNA methylases were studied in detail, but all of those studied methylate either adenine to N^6-methyladenine (m^6A) or cytosine to 5-methylcytosine (m^5C) [1].

Recently a site-specific endonuclease and methylase *Bcn*I, both of which recognize the sequence 5′CC(C/G)GG, have been isolated from *Bacillus centrosporus* strain RFLI [2,3]. We here describe the property of M*Bcn*I to methylate cytosine residues in DNA in vitro at the N^4 position yielding N^4-methylcytosine (m^4C). The same minor base in DNA isolated from *B. centrosporus* was detected. Such an unusual DNA modification is described for the first time.

2. MATERIALS AND METHODS

The following commercial materials have been used: *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]-SAM), 65 Ci/mmol (Amersham, Bucks), SAM (Serva), VPDE (Merck), pancreatic DNase, alkaline phosphatase (Sigma, St Louis MO), Sephadex G-50 f. (Pharmacia), thin-layer chromatography (TLC)-cellulose Filtrak, ion-exchange TLC sheets Fixion 50 × 8 (Hungary), cellulose acetate strips (Schleicher und Schüll), m^5C (Chemapol), 3-methyl-cytosine (m^3C) and 5-hydroxymethylcytosine (Hm^5C) (Sigma). N^4-methylribocytidine was kindly supplied by D.Yu. Jakovlev (Moscow), m^4C was prepared by treatment of N^4-methylcytidine with 60% $HClO_4$ (1 h, 100°C). Methylase M*Bcn*I was isolated from *B. centrosporus* and from *Escherichia coli* (pBCl) cells harboring a cloned M*Bcn*I gene [4] as in [3]. *E. coli* B and *B. centrosporus* DNA were isolated by the method resulting in DNA essentially free of RNA [5].

For DNA methylation in vitro 5 μg of *E. coli* B DNA, 200 pmol [^{3}H]SAM and 20 units of M*Bcn*I were incubated for 5 h at 37°C in a 100-μl solution containing 25 mM Tris–HCl (pH 8.0), 25 mM NaCl, 1 mM EDTA and 5 mM 2-mercaptoethanol. After gel-filtration on Sephadex G-50, labeled DNA was precipitated with ethanol.

Preparative DNA methylation: 32 mg *Eco*B DNA, 40 μM SAM and 6000 units of M*Bcn*I were incubated in 50 ml of the above mentioned methylation buffer for 10 h at 37°C. Methylated DNA was precipitated with 2% $HClO_4$.

DNA hydrolysis to bases was performed in 60% $HClO_4$ (1 h, 100°C). [^{3}H]Nucleoside was obtained after treating labeled DNA with DNase, VPDE and alkaline phosphatase [6].

Descending chromatography on Whatman I was performed in a solution containing *n*-butanol–water–ammonia, 84:16:0.1 (solvent A). TLC on cellulose was performed in a solution of isopropanol–0.1 M sodium acetate–concentrated ammonium sulfate, 2:19:79 (solvent B) [7]. TLC on Fixion 50 × 8 was carried out as in [8]. To evaluate the relative mobility of the [^{3}H]-methylated base it was mixed with standards and then

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

separated under various conditions. Standard spots were located under UV light, and the position of tritiated compounds was determined by counting 1 cm^2 pieces of the chromatograms or electrophoretograms in toluene-based scintillation fluid. Electrophoresis of the bases was carried out on Whatman I paper in ammonium formate (pH 4.3). Electrophoresis of the nucleosides was carried out on cellulose acetate in two solutions: ammonium formate (pH 3.5) and sodium borate (pH 8.5). The unlabeled methylated minor base was preparatively isolated on Dowex 1 × 8 in 0.02 M $HCOONH_4$, followed by paper chromatography on Whatman I (solvent A) and then high pressure liquid chromatography (HPLC) (Du Pont 8800, Zorbax-C8 4.6 × 250 in 0.02 M KH_2PO_4 or 0.1 M CH_3COONH_4). UV spectra were performed on Hitachi 330 at pH 1.0 (HCl), pH 7.0 (Tris–HCl) and pH 13.0 (NaOH).

3. RESULTS

Relative chromatographic and electrophoretic mobilities of the isolated [^{3}H]-methylated base and standards of methylated cytosines are listed in table 1. Radioactivity in the spot of m^4C was found to be not less than 90% of the input value; 95% of the radioactivity input was found at the m^4C peak in the HPLC analysis of the mixture of the [^{3}H]methylated base, m^3C, m^4C and m^5C (fig.1). HPLC analysis of the minor base isolated from *B. centrosporus* DNA or from *Eco*B DNA methylated in vitro with M*Bcn*I obtained from *B. centrosporus* and *E. coli* (pBCI) cells was also performed. In all 3 cases m^4C was detected. UV spectra of the isolated minor base and standard m^4C coincided at different values of pH and were in agreement with [9]: pH 1.0, λ_{max} = 213,278 nm, λ_{min} = 240 nm; pH 7.0, λ_{max} = 267 nm; pH 13.0, λ_{max} = 284 nm, λ_{min} = 254 nm.

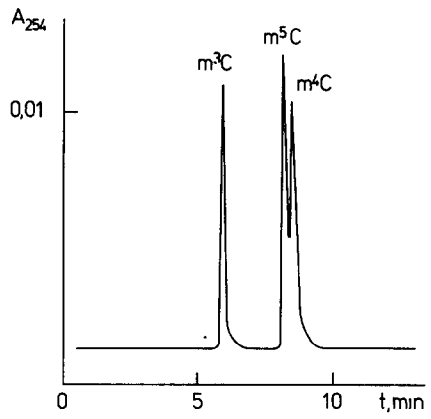

Fig.1. High pressure liquid chromatography analysis of the methylated cytosines.

While investigating the electrophoretical mobility of synthetic N^4-methylribocytidine and isolated [^{3}H]methylated minor nucleoside it was found that both of them showed the same mobility in an ammonium formate buffer. Their mobility differed in a sodium borate buffer, and only N^4-methylribocytidine was negatively charged.

Table 1

No.	Separation method	R_f-value				
		[^{3}H]Methylated base	m^4C	m^5C	m^3C	Hm^5C
1.	Descending chromatography on Whatman 1 (solvent A)	0.41	0.41	0.29	0.39	
2.	TLC on Fixion 50 × 8[a]	1.05	1.05	0.70	0.20	0.95
3.	TLC on cellulose					
	(solvent A)	0.30	0.30			
	(solvent B)	0.57	0.57	0.53	0.78	
4.	Electrophoresis (pH 4.3)	0.82	0.82	0.97	1.44	0.75

[a] Represented R_c-values, relative mobility of the base to the cytosine

4. DISCUSSION

From the results it can be concluded that M*Bcn*I modifies cytosine residues in DNA both in vitro and in vivo yielding N^4-methylcytosine. Although various unusual bases occur in some types of bacteriophage DNA [10–13] generally the only modified bases formed in eukaryotic and bacterial DNAs are m^5C or m^6A [9,14–16]. As far as we know it is the first time that m^4C occurrence in DNA was detected. Until now this minor base was found only in RNA [17]. Control experiments were performed although it was quite improbable that m^4C in our experiments could have been derived from some RNA contaminants. These experiments showed that ^3H-modified nucleoside (isolated from a DNA sample methylated with M*Bcn*I in vitro) moved during electrophoresis in borate buffer as deoxyribonucleoside.

As M*Bcn*I is a counterpart of the restriction modification system found in *B. centrosporus* [2,3], which renders DNA resistant to R*Bcn*I cleavage, the participation of m^4C in DNA protection from endogenous sequence-specific endonuclease is proved for the first time. Bacterial DNA can be protected from endogenous restriction endonucleases not only by methylation of adenosine to m^6A or cytosine to m^5C [1] but also, as we see, by methylation of cytosine to m^4C.

We here demonstrated that the cloned M*Bcn*I gene is expressed in vivo in *E. coli* [4] and that the enzyme isolated from these cells yields m^4C in DNA. No deleterious effect of such unusual DNA methylation on *E. coli* cells was observed. These observations encouraged us to think that m^4C occurrence in bacterial DNA is not restricted to the *B. centrosporus* strain used. This assumption was confirmed by the discovery of methylases yielding m^4C in two other strains (*C. freundii* and *M. varians*), producing restriction modification enzymes (in preparation).

The determination of the effects of methylation on the ability of sequence-specific endonucleases to cleave DNA is an important characteristic per se, and a prerequisite for its use in various experiments, such as:

(i) Detection of methylase modification sites [18];
(ii) Evaluation of the methylation pattern in eukaryotic [19] and prokaryotic DNA [20].

Until now only the modified base (m^5C and m^6A) location in the recognition sequence affecting DNA cleavage by restriction endonucleases was considered [1]. It was determined that sequence-specific endonucleases differ in their sensitivity to the modification pattern within its recognition sequence. There are examples of enzymes specifically inhibited by modification anywhere within the recognition sequence as well as those which are not sensitive to methylation at non-cognate sites [1]. In this context, the discovery of m^4C protecting DNA from specific endonuclease cleavage deserves special consideration. There are some questions regarding the interaction of restriction modification counterparts to be answered; i.e., whether the m^5C change for m^4C in the cognate site of a recognition sequence can also prevent restriction endonuclease cleavage and vice versa and what the effects of m^5C and m^4C interchange at non-cognate sites would be. Elucidation of these questions would aid in determining whether restriction endonucleases are specific only to the location of modified cytosine in the recognition sequence (not discriminating m^5C and m^4C) or to the position of methyl groups in cytosine (4 or 5) or both.

There are some published experimental data which are difficult to interpret relying on cytosine modification only at position 5. For example, it was reported that *Bst*NI cuts $^{5'C^mCA\ GG}_{G\ GT^mCC5'}$, $^{5'^mCCAG\ G}_{GGTC^mC5}$ and DNA hemimethylated at both cytosines in one strand $5'\,^mC^mC(A/T)GG$ [1]. It is obvious that the occurrence of m^5C at any possible position in the recognition sequence does not render DNA resistant to *Bst*NI cleavage. It could not be excluded that methylation in *B. stereothermophilus* N cells results in cytosine modification at position 4. If the former assumption is true, then R*Bst*NI is an example of an endonuclease specific for the position of the methyl group in cytosine in cognate methylation site.

Some paradoxical observations dealing with bacterial genome modification profiles were difficult to interpret on the basis of known cognate modification patterns of cytosine residues in recognition sequences of some restriction endonucleases used (*Msp*I, *Hpa*II, *Sma*I, *Xma*I, *Nci*I) [20]. The discovery of m^4C should be borne in mind when analysing the nature of these paradoxical observations.

Research being carried out in this laboratory is

attempting to elucidate specificity toward m^4C and m^5C, and their location in recognition sequences of some restriction endonucleases.

ACKNOWLEDGEMENTS

The authors wish to thank Professor E.I. Budovsky for his advice and suggestions, Dr B.K. Chernov for his help in identifying and purifying m^4C by HPLC and Dr D.Yu. Jakovlev for his gift of N^4-methylribocytidine.

REFERENCES

[1] McClelland, M. (1983) Nucleic Acids Res. 11, r169–r173.
[2] Petrušyte, M.P. and Janulaitis, A.A. (1982) Bioorg. Khim. 7, 1885–1887.
[3] Janulaitis, A.A., Petrušyte, M.P., Jaskelevičiene, B.P., Krayev, A.S., Skryabin, K.S. and Bayev, A.A. (1981) Dokl. Akad. Nauk. SSSR 257, 749–750.
[4] Janulaitis, A., Povilionis, P. and Sasnauskas, K. (1982) Gene 20, 197–204.
[5] Saito, H. and Miura, K. (1969) Biochim. Biophys. Acta 72, 619–629.
[6] Wu, R. and Taylor, E. (1971) J. Mol. Biol. 57, 491–511.
[7] Markham, R. and Smith, J.D. (1951) Biochem. J. 49, 401–406.
[8] Tomasz, J. (1979) Chromatographia 12, 1, 100–105.
[9] Hall, R. (1971) in: The Modified Nucleosides in Nucleic Acids, Columbia University Press, New York.
[10] Simon, D., Grunert, F., Achen, U., Doring, H.P. and Kroger, H. (1978) Nucleic Acids Res. 5, 2153–2167.
[11] Warren, R.A.J. (1980) Annu. Rev. Microbiol. 34, 179.
[12] Nikolskaya, I.I., Lopatina, N.G. and Debov, S.S. (1976) Biochim. Biophys. Acta 435, 206–210.
[13] Hattman, S. (1982) Proc. Natl. Acad. Sci. USA 79, 5518–5521.
[14] Vanyushin, B.F., Mazin, A.L., Vasilyev, V.K. and Belozersky, A.N. (1973) Biochim. Biophys. Acta 229, 397–403.
[15] Vanyushin, B.F., Tkacheva, S.G. and Belozersky, A.N. (1970) Nature 225, 948–949.
[16] Bird, A.P. and Taggart, M.H. (1980) Nucleic Acids Res. 8, 1485.
[17] Cantoni, G.L., Gelbion, H.V., Luborsky, S.W. and Richards, H.H. (1962) Biochim. Biophys. Acta 61, 354–360.
[18] McClelland, M. (1981) Nucleic Acids Res. 9, 24, 6795–6804.
[19] Garfin, D.E. and Goodman, H.M. (1974) Biochem. Biophys. Res. Commun. 59, 108–116.
[20] Brooks, J.E. and Roberts, R.J. (1982) Nucleic Acids Res. 10, 913–934.