Isolation and characterization of the *M.EaeI* modification methylase

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The modification enzyme (*M.EaeI*) corresponding to the restriction endonuclease *EaeI* was partially purified from *Enterobacter aerogenes* PW201. The *M.EaeI* enzyme methylates the innermost cytosine residue in each strand of the family of related sequences that constitute the *EaeI* recognition site to give:

where 5^mC is 5-methylcytosine. *M.EaeI* protects these sites against cleavage by *HaeIII*, and protects overlapping $5'-C-C-G-G-3'$ sites against cleavage by both *HpaII* and *MspI*.

INTRODUCTION

Restriction endonucleases have found wide use in the analysis and restructuring of DNA molecules and as model systems for studying the interactions of proteins and DNA. Many such activities have been characterized from a large number of bacterial genera (Roberts, 1985), and in virtually all cases examined a corresponding methylase is present that protects the bacterial DNA from cleavage (McClelland & Nelson, 1985). These modification methylases have been less well-studied, presumably because they are of less importance as tools in the manipulation of DNA *in vitro*. However, such methylases can be used to protect specific sites against cleavage by the cognate endonuclease, and they have been used to alter the number of target sites for non-cognate restriction endonucleases on DNA to generate novel specificities of cleavage (McClelland *et al.*, 1984).

We now report the specificity of the methylase *M.EaeI* corresponding to the endonuclease *EaeI* from *Enterobacter aerogenes* PW201 (Whitehead & Brown, 1983). We show that the methylase can be used to protect a subset of *HaeIII* recognition sequences from *HaeIII* cleavage and *HpaII*/*MspI* sites from *HpaII* or *MspI* cleavage. Such protection may be useful in genetic manipulations.

MATERIALS AND METHODS

Bacteria, DNA and enzymes

Enterobacter aerogenes PW201 was maintained and grown as described previously (Whitehead & Brown, 1983). Cell paste was stored at -20°C until use. Bacteriophage λ DNA, ϕX174 replicative-form (RFI) DNA and plasmid pBR322 were prepared as described previously (Whitehead & Brown, 1982). Restriction endonucleases other than *EaeI* were purchased from Anglian Biotechnology (Colchester, Essex, U.K.) or Boehringer Corp. (Lewes, East Sussex, U.K.), and were used as suggested by the manufacturers.

Restriction endonuclease *EaeI* was prepared as described by Whitehead & Brown (1983), with the modifications that 0.02 mM-phenylmethanesulphonyl fluoride and 0.05 mM-benzamidine were present as proteinase inhibitors throughout the ultrasonication and centrifugation steps, and that chromatography was successively on phosphocellulose P11, hydroxyapatite and DEAE-cellulose, rather than phosphocellulose, DEAE-cellulose and heparin-Sepharose. The conditions for phosphocellulose and DEAE-cellulose chromatography were as described before, and those for hydroxyapatite were as described below for purification of the methylase activity.

Methylase assay

The methylase preparation was incubated with $0.5\text{ }\mu\text{g}$ of bacteriophage λ DNA in $25\text{ }\mu\text{l}$ of $50\text{ }\mu\text{M}$ -*S*-adenosyl-methionine/ 5 mM -EDTA/ 5 mM -2-mercaptoethanol/ 50 mM -Tris/HCl buffer, pH 7.5, containing $100\text{ }\mu\text{g}$ of autoclaved gelatin/ml at 37°C for 1 h. (The methylase preparation was less than 10% of the total assay volume.) MgCl_2 was then added to 25 mM , followed by approx. 10 units of endonuclease *EaeI*, and incubation was continued for a further 30 min. The samples were analysed by gel electrophoresis in 1% agarose in 2.5 mM -EDTA/ 90 mM -Tris/borate buffer, pH 8.3, containing $0.5\text{ }\mu\text{g}$ of ethidium bromide/ml. Samples in which no methylase was added before *EaeI* digestion, or in which *EaeI* was not added after methylation, were routinely run as controls. Samples fully or partially protected against *EaeI* cleavage could readily be identified.

One unit of *M.EaeI* activity is defined as that which in 60 min at 37°C in $25\text{ }\mu\text{l}$ of the above buffer will convert $1\text{ }\mu\text{g}$ of bacteriophage λ DNA into a form that is not cleaved by excess endonuclease *EaeI*.

Characterization of the product of methylation

Bacteriophage λ DNA ($2\text{ }\mu\text{g}$) was methylated by using 5 units of *M.EaeI* for 3 h at 37°C with *S*-adenosyl-L-[$\text{Me-}^3\text{H}$]methionine ($12.5\text{ }\mu\text{Ci}$; 77 Ci/mmol) in $50\text{ }\mu\text{l}$ of 5 mM -EDTA/ 5 mM -2-mercaptoethanol/ 50 mM -Tris/

Abbreviations used: representations of nucleotide degeneracies in enzyme recognition sites follow the IUB recommendations [Biochem. J. (1985) 229, 281–286]: R = either purine nucleotide; S = G or C; Y = either pyrimidine nucleotide.

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HCl buffer, pH 7.5, containing 100 μ g of autoclaved gelatin/ml. The procedure described by Butkus *et al.* (1985) was followed for the precipitation, gel-filtration chromatography and HClO₄ hydrolysis of the methylated DNA, and for the isolation of free bases. The nucleic acid bases were analysed by ascending paper chromatography on Whatman no. 1 paper in the solvents described in Table 1. *S*-Adenosyl[Me-³H]methionine that had been through the hydrolysis procedure and untreated 5-methylcytosine were run as markers; the latter marker was located under u.v. light, and was mixed with the sample.

RESULTS AND DISCUSSION

Preparation of modification methylase *M.EaeI*

Cell paste (5 g) was resuspended in 30 ml of buffer A [10% (v/v) glycerol/10 mM-2-mercaptoethanol/0.1 mM-EDTA / 100 mM-KCl / 10 mM-potassium phosphate buffer, pH 7.4) and sonicated at 60 W for 10 $\times$ 30 s, with intermittent cooling. The sonicated mixture was centrifuged at 50000 *g* for 90 min, and the supernatant was applied to a phosphocellulose P11 column (2 cm $\times$ 10 cm) and eluted with buffer A modified to contain a linear gradient of 0.1–1.0 M-KCl. The active fractions were eluted at 0.2–0.35 M-KCl, and were dialysed against buffer A. The methylase activity was then purified on a hydroxyapatite column (1 cm $\times$ 5 cm) eluted with buffer A modified to contain a linear gradient of 0.01–0.3 M-potassium phosphate. Active fractions (0.1–0.2 M-potassium phosphate) were dialysed against buffer A and purified on a CM-cellulose column (1 cm $\times$ 5 cm) eluted with buffer A modified to contain a linear gradient of 0.1–1.0 M-KCl. The final preparation (eluted with 0.1–2 M-KCl) was dialysed against buffer A modified to contain 50% glycerol and stored at –20 °C. The preparation is stable for several months, and the total yield of *M.EaeI* was approx. 800 units/g of cell paste. The preparation contained no detectable endonuclease *EaeI* or non-specific nucleases.

Characterization of the *M.EaeI* modification activity

The restriction endonuclease *EaeI* recognizes the family of related sequences 5'-Y-G-G-C-C-R-3', cleaving the DNA to give 5'-tetranucleotide extensions (Whitehead & Brown, 1983). We assumed that *M.EaeI* recognized and modified the same sequence. The optimum ranges of pH (6.5–8.5), temperature (30–42 °C) and concentrations of univalent cation (below 150 mM-NaCl) for protection of DNA against endonuclease *EaeI* were determined, and resulted in the choice of assay buffer described in the Materials and methods section. The methylase does not require Mg²⁺ for activity, whereas the endonuclease does. We demonstrated that the modification was likely to be methylation by showing that protection against cleavage by *EaeI* was dependent on *S*-adenosylmethionine (results not shown). Methylase activities in other bacteria, and in eukaryotes, methylate cytosine or adenine residues. Thus the likely site of *M.EaeI* modification is one of the two residues at the fourth and fifth positions of the site, as these are the only cytosine or adenine residues common to all *EaeI* sites.

The majority of cytosine methylases so far characterized methylate at the 5-position of the pyrimidine ring. However, Janulaitis *et al.* (1983) and Butkus *et al.* (1985)

report methylation of the N-4-position of cytosine by *M.BcnI*, *M.Cfr6I* and *M.Cfr9I*. This means that the methylated nucleotide cannot be identified simply from the effect of methylation on cleavage by restriction endonucleases with overlapping sites unless the nature of the modification is known. Methylation of cytosine residues at the 4-position may have effects very different to those of methylation at the 5-position. In order to determine whether *M.EaeI* methylation gave 5- or 4-methylcytosine, bacteriophage λ DNA was methylated with *M.EaeI* and *S*-adenosyl[Me-³H]methionine, and the methylated base was characterized as described in the Materials and methods section. The results of chromatography of the methylated base are shown in Table 1. The radioactivity in the samples co-migrated with the 5-methylcytosine marker in both solvents used, in which 4- and 5-methylcytosine have different *R_F* values, thus showing that *M.EaeI* methylates cytosine at the 5-position.

To determine which of the two cytosine residues in each strand is the site of modification, we used the restriction endonuclease *HaeIII*. This enzyme recognizes and cleaves the sequence 5'-G-G-C-C-3' to leave flush termini. The central tetranucleotide of the *EaeI* site is an *HaeIII* site, and *HaeIII* is known to cleave the sequence 5'-G-G-C-^{5m}C-3, but it does not cleave the sequence 5'-G-G-^{5m}C-C-3' (Mann & Smith, 1977). We therefore modified ϕ X174 RFI DNA with *M.EaeI* and compared the *HaeIII*-cleavage pattern of the modified DNA with that of the unmodified DNA (Fig. 1). Modification of DNA with *M.EaeI* protected *EaeI*-cleavage sites against cleavage by *HaeIII*. The two *EaeI*-cleavage sites correspond to the *HaeIII*-cleavage sites Z2/Z6b (position 4835; Sanger *et al.*, 1978) and Z6a/Z9 (position 552); the fragments that disappear (Z2, Z9 and both fragments in the Z6 doublet) and the sizes of the product fragments (approx. 1350 and 390 base-pairs) agree with this assignment. The larger of the product fragments (Z2 + Z6b) co-migrates with the largest *HaeIII*-cleavage fragment (Z1). The protection appears to be absolute, as we have been unable to detect cleavage of these sites even with a large excess of *HaeIII*. As *HaeIII* will cleave its site

Table 1. Paper chromatography of the base methylated by *M.EaeI*

For experimental details see the Materials and methods section. *S*-Adenosylmethionine that had been through the DNA hydrolysis procedure of Butkus *et al.* (1985) had *R_F* values of 0.0 and 0.25 in solvents 1 and 2 respectively. Radioactivity was detected by cutting the chromatogram into about 30 strips for scintillation counting.

	Observed <i>R_F</i>		Expected <i>R_F</i> *	
	³ H	^{5m} C	³ H	^{5m} C
1: Butanol/water/ conc. NH ₃ (84:15:0.2, by vol.)	0.30	0.30	0.41	0.29
2: Butanol/methanol/ water/conc. NH ₃ (60:20:20:1, by vol.)	0.48	0.48	0.58	0.47

* From Butkus *et al.* (1985).

Fig. 1. Gel electrophoresis of the products of *HaeIII* or *HpaII* cleavage of DNA before and after modification with *M.EaeI*

Lane a, ϕ X174 RFI DNA cleaved with *HaeIII*; lane b, ϕ X174 RFI DNA methylated with *M.EaeI* and then cleaved with *HaeIII*; lane c, pBR322 DNA cleaved with *HpaII*; lane d, pBR322 DNA methylated with *M.EaeI* and then cleaved with *HpaII*. The samples were analysed on an 8% polyacrylamide gel and stained with ethidium bromide after electrophoresis. The arrows show the fragments that are only present in the absence of *M.EaeI* methylation (the upper fragment marked in the *HpaII* digest is one of two co-migrating fragments).

if the second cytosine residue is methylated at the 5-position, the first cytosine residue must be that methylated by *M.EaeI*.

Effect of *M.EaeI* methylation on the cleavage of DNA by other restriction endonucleases

DNA of known sequence were fully modified by *M.EaeI* and were incubated with restriction endonucleases whose cleavage sites overlapped with one or more *EaeI*-cleavage sites on that DNA. In addition to protection against *HaeIII* cleavage, described above,

methylation by *M.EaeI* prevented cleavage of the site 5'-Y-G-G-C-C-G-G-3' at position 533 in pBR322 DNA (Sutcliffe, 1979) by the enzymes *MspI* and *HpaII*, which recognize 5'-C-C-G-G-3' (Fig. 1). [Note that, although *MspI* will normally cleave 5'-C-^{5m}C-G-G-3' but not 5'-^{5m}C-C-G-G-3', whereas *HpaII* will cleave neither, in the case of the sequence 5'-G-G-C-C-G-G-3', such as in *EaeI/MspI* overlapping sequences, *MspI* will not cleave if either cytosine residue is methylated (Keshet & Cedar, 1983). The lack of *MspI* cleavage at methylated *EaeI*-cleavage sites cannot therefore be used to confirm the site of methylation.] Fig. 1 shows protection at one (position 533) of the two *EaeI/HpaII*-cleavage sites on pBR322 DNA; the other site (position 402) involves fragments of nine and 15 nucleotide residues length and cannot be detected on this gel. Methylation by *M.EaeI* has no effect on the cleavage of sites 5'-Y-G-G-C-C-G-G-G-3' by the isoschizomers *CauII* and *NciI*, which have the recognition site 5'-C-C-S-G-G-3' (results not shown).

Whitehead & Brown (1983) showed that *dcm* modification of pBR322 DNA at the sequence 5'-T-G-G-C-^{5m}C-A-G-G-3' prevents cleavage by *EaeI*. Thus modification of either conserved cytosine residue in the *EaeI* recognition sequence is sufficient to prevent cleavage by *EaeI*.

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