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Purification and characterization of two new modification methylases; MClaI from Caryophanon latum L and MTaqI from Thermus aquaticus YTI

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ABSTRACT

A method for detecting Type II modification methylases and determining their methylation site by assaying the ability of methylated DNA to be cleaved by heterologous restriction enzymes is described and applied to the isolation of the restriction modification methylases from Thermus thermophilus HB8, Thermus aquaticus YTI and Caryophanon latum L. M.TaqI is shown to have a methylation specificity identical to M.TthI (TCG^{me}A). M.ClaI methylates at adenine and protects a subset of TthI sites indicating that it methylates the sequence ATCG^{me}AT. Methylation by M.TthI also protects against cleavage by SalI, XhoI and at some HindII, AccI and MboI sites.

INTRODUCTION

As postulated by Arber (1), site specific enzymes that protect bacterial DNA from endogenous type II restriction endonucleases have been found to be methylases in all the species so far examined. Type II modification methylases require S-adenosylmethonine (SAM) but not Mg^{2+} for their activity (2). These methylases have been found to methylate within the sequence recognized by the corresponding endonuclease at either adenine or cytosine. Methylation at guanine and thymine has not been detected (3).

Type II methylases will almost certainly become useful tools to the molecular biologist. EcoRI methylase is currently being used in the preparation of recombinant DNA libraries (4). Other methylases will be useful in studies of the effect of in vitro cytosine methylation on gene expression in eucaryotes (5,6).

In this study I present a simple and rapid method for the detection and assay of the type II methylases from Caryophanon latum L, Thermus aquaticus YTI and Thermus thermophilus HB8 which contain the type II restriction endonucleases ClaI (ATCGAT) (7), TaqI (TCGA) and TthI (TCGA) (8), respectively. I also show how the sites of methylation by these enzymes can be determined by analyzing the ability of the enzymes to protect cleavage sites

recognized by heterologous endonucleases.

MATERIALS AND METHODS

Thermus aquaticus YTI (9) and *Thermus thermophilus* HB8 (8) (10) were kindly provided by S. Sato. The cells were grown to stationary phase in 10 liters of medium containing 0.5% Bactotryptone (Difco), 0.3% yeast extract (Difco), 0.2% NaCl at 75°C using a 14-liter Chemapac fermenter. The cells were harvested by centrifugation and frozen as a paste (8).

Caryophanon latum L was a gift of H. Mayer. The bacteria was grown to late log phase by a procedure described by H. Mayer (7): 5 liters of media was prepared; cow dung was homogenized in an equal volume of distilled water and centrifuged at 25,000 g for one hour. The supernatant was added to the same volume of an enriched nutrient broth; 8g nutrient broth (Difco), 5g Bactopeptone, 5g NaCl, 3.5g Na_2HPO_4, 1.5g KH_2PO_4, 1g glucose, 5mg thiamine HCl per 100 ml adjusted to pH 7. The cells were harvested by centrifugation and frozen at -20°C.

Phage λ DNA was prepared after induction of *E. coli* C600 *dam*$^+$ and *dam*$^-$ strains containing λ $C_{1857}S_{am}$ lysogens. pBR322 was isolated from C600 *dam*$^+$.

Assay for M.TthI and M.TaqI

The assay for methylase activity was performed in the following manner. Reaction mixtures contained 0.75 µg of phage λ DNA or 0.5 µg of pBR322 DNA, 0.1 mM S-Adenosyl methionine (Sigma), 50 mM Tris-HCl, pH 7.5, 10 mM Na_2 EDTA, 10 mM β-mercaptoethanol and 10 µl sample aliquots in a total volume of 50 µl. The assays were carried out for 60 minutes at 70°C, then extracted with an equal volume of phenol. 5 µl of 3M sodium acetate and 200 µl of ethanol were added. After one hour at -20°C the tubes were spun in an eppendorf centrifuge for 5 minutes and the DNA was resuspended in *TthI* endonuclease buffer; 6 mM Tris-HCl, pH 7.5, 6 mM β-mercaptoethanol, 6 mM $MgCl_2$ (8); and digested with one unit of *TthI* for one hour at 70°C. (One unit digests one µg of λ in one hour.)

Assay for M.ClaI

Methylase activity was assayed in 40 mM Tris HCl (pH 8.0), 5 mM EDTA, 1 mM 2-mercaptoethanol, 10 µg/ml BSA, 0.75 µg phage λ DNA, 1 mM S-Adenosyl-methionine (Sigma) with 5 µl of sample aliquots in a total volume of 50 µl. The mixture was incubated at 37°C for 2 hours. Assays using S-Adenosyl [^{3}H-methyl]-methionine (Amersham) substituted 15 pmoles of 15Ci/mmol ^{3}H-SAM per µg of DNA. After phenol extraction and ethanol

precipitation the DNA was resuspended in 20 µl of 20 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$ and digested with 1 µl of 2 units/µl ClaI restriction endonuclease for one hour.

For assays using other restriction endonucleases, the methylated DNA was resuspended in the appropriate restriction buffer (New England Biolabs) and digested with an excess of restriction enzyme to ensure complete digestion. TthI and ClaI restriction endonucleases were prepared as described above, all other enzymes were purchased from New England Bio-labs.

Determination of methylated base

The base methylated by M.ClaI was determined as follows: 30 µg of phage λ DNA was methylated with 15 pmoles of ^{3}H-SAM and either M.ClaI, M.TaqI, or M.TthI; phenol extracted twice; ethanol precipitated; resuspended in 100 µl of 88% formic acid and hydrolysed in a sealed tube at 170°C for 2 hours. The samples were dried and resuspended in 10 mM Tris HCl pH8, 1 mM EDTA. Aliquots were spotted on Whatman 3MM chromatography paper along with authentic bases (Sigma) and developed in Butanol:Methanol:H_2O:Ammonia (sp. gr. 0.90) (60:20:20:1) and methanol:HCl (sp. gr. 1.18):water (70:20:10 v/v) (12). The position of tritiated compounds was determined by counting 1 cm^2 pieces of the chromatographs in Toluene based scintillation fluid.

RESULTS AND DISCUSSION

15g of cells were resuspended in "Taq extract buffer" (10mM K_2 HPO_4 -KH_2PO_4, pH 7.0, 0.2 M NaCl, 7 mM β-mercaptoethanol, 1 mM Na_2 EDTA and 20 µg/ml phenylmethylsulfonyl fluoride). The suspension was sonicated using a Bronwill sonifer at maximum output in 1 minute pulses for a total of 10 minutes. The lysate was then centrifuged for 10 minutes at 2000 X g and the supernatant centrifuged for 45 minutes at 100,000 X g in a Beckman Type 45 rotor at 0°C.

The cleared lysate was applied to a phosphocellulose (Whatman P11) column and eluted with linear gradient of 0.1 to 1.0 M NaCl in "Taq extract buffer". The restriction endonuclease TaqI (TCGA) eluted at about 0.4 M NaCl. The methylase M.TaqI activity, detected by the assay procedure described below, eluted at 0.7 M NaCl. Fractions containing methylase activity were pooled, dialyzed against extreact buffer, applied to a DEAE-Sephacel (Pharmacia) column and eluted with a 0.1 to 1.0 M NaCl gradient. The methylase activity eluted at 0.17 M NaCl. Fractions containing significant activity were pooled, dialyzed against extract buffer made up in 50% glycerol, and stored at -20°C.

The yield of M.TaqI obtained from 15g of cells was more than 15,000 units as determined by the ability of the enzyme to protect TaqI sites and by the rate of incorporation of ^{3}H-methyl groups from S-Adenosyl-[^{3}H-methyl]-methionine (data not shown). One unit is the amount of methylase required to protect one pmole of TaqI recognition sites in one hour at 70°C. The methylase and endonuclease remained stable at -20°C in 50% glycerol for over two years.

Purification of MClaI

M.ClaI was simultaneously purified with ClaI in a manner analogous to that used for M.TthI and TthI (10). Frozen cells were resuspended in "Cla extract buffer", and (10 mM Potassium phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol pH 7.5) and sonicated using a Branson sonifier until the $O.D._{578nm}$ had dropped by 50%. The homogenate was centrifuged at 45,000g for 20 min. at 2°C (7). The extract was eluted from a phosphocellulose (Whatman P11) column using a linear gradient of 0.0 to 1.0 M NaCl in Extract Buffer. The restriction endonuclease ClaI (ATCGAT) eluted just after the methylase which eluted at about 0.4 M NaCl as determined by conductivity (fig. 1). The active fractions were dialysed against 50% glycerol, then loaded onto a

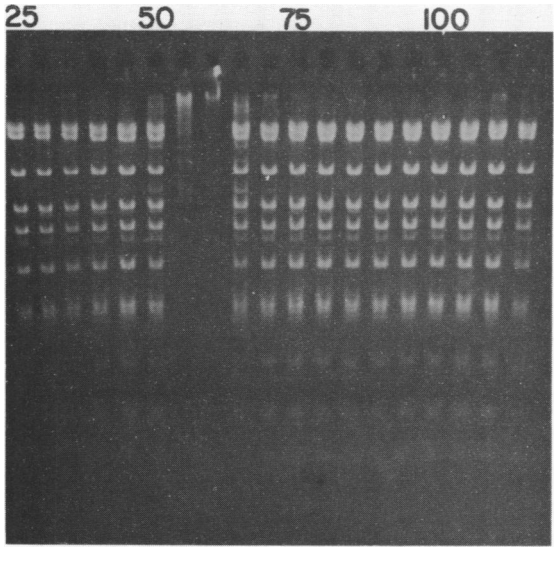

Fig. 1: Assay for M.ClaI off the phosphocellulose column. The gradient was 500ml of 0.0 to 1.0 M NaCl between fractions 26 and 115. See "methods" for details.

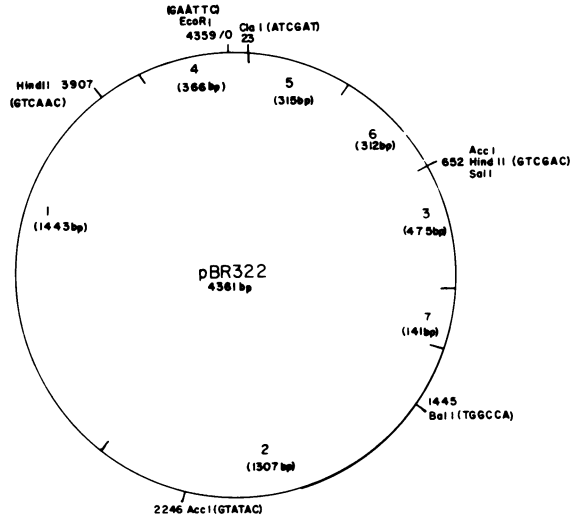

Fig. 2: Restriction map of pBR322. TaqI sites are given inside the circular map. The recognition sequences of other endonucleases and their cleavage sites relative to EcoRI are given outside the circle (13).

DEAE Sephacel (Pharmacia) Column and eluted with a linear gradient of 0.1 to 1.0 M NaCl. The methylase eluted at 0.2 M NaCl and the endonuclease at 0.25 M NaCl. Active fractions were dialysed separately against 10 mM Potassium phosphate, 100 mM KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.5 in 50% glycerol and stored at -20°C. Fractions from column chromatography were assayed for specific methylase activity as described in Materials and Methods (fig. 1). Cleavage of protected DNA occurs when the methylation reaction is carried out in the absence of SAM. (fig. 3, lane A and B). Thus, the methylase had the properties required of a restriction modification methylase. Approximately 50,000 units of ClaI methylase were recovered from 50g of cells after phosphocellulose and DEAE chromatography followed by dialysis.

^{3}H labelling studies using ^{3}H-methyl SAM indicated a pH optimum 7.5 to 8.0 and a slow decrease in activity as the salt concentration was increased between 0.0 and 200 mM NaCl. The temperature optimum was 37-40°C. (unpublished observations)

Site of methylation by M.TaqI

M.TthI is known to methylate at TCG^{me}A (10). DNA methylated with M.TthI is protected against both TthI and TaqI (T↓CGA) (fig. 3 lane H). M.TaqI

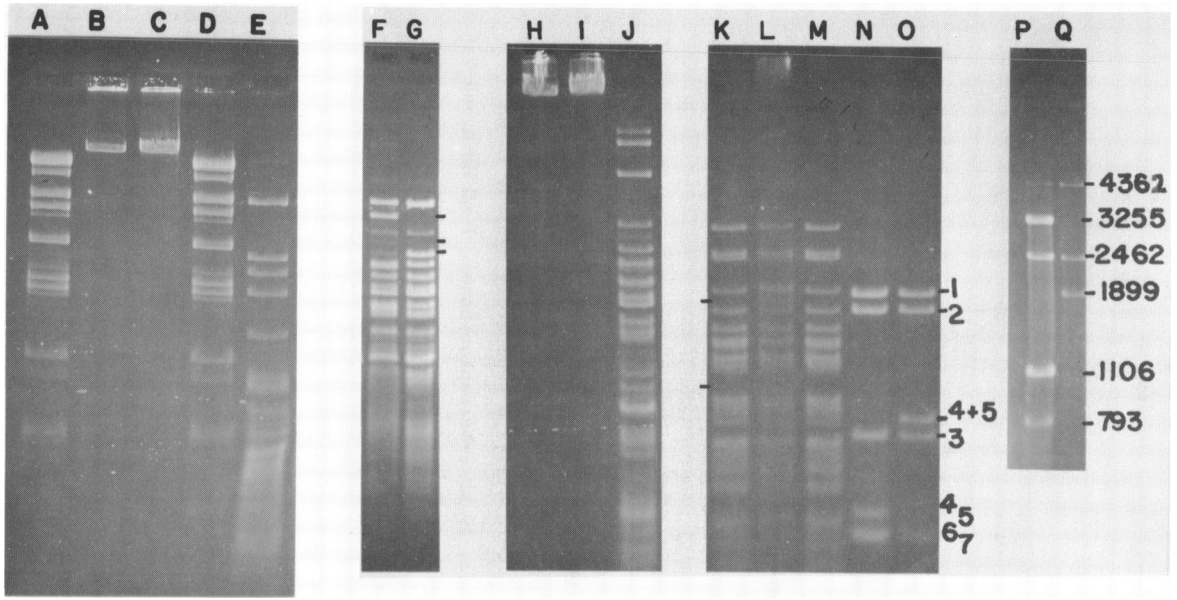

Fig. 3. 1% agarose TBE gel electrophoresis; all lanes contain one μg of DNA. (A) λ DNA+MClaI (no SAM), then ClaI digestion. (B) λ DNA+MClaI+SAM, then ClaI digestion. (C) λ DNA+MTthI+SAM, then ClaI digestion. (D) λ DNA, ClaI digestion. (E) λ DNA+SAM, then HAEIII digestion. (F) λ dam⁻ DNA+MClaI+SAM, then TthI digestion. (G) λ dam⁻ DNA TthI digestion. (H) λ DNA+MTthI+SAM, then TaqI digestion. (I) λ DNA+MTaqI+SAM, then TthI digestion. (J) λ dam⁺ DNA+MTthI (no SAM), then TaqI digestion. (K) λ dam⁻ DNA+MboI digestion. (L) λ dam⁻ DNA+MTthI+SAM, then MboI digestion (M) λ dam⁻ DNA+MTthI+SAM, then Sau3A digestion. (N) pBR322+TaqI digestion. (O) pBR322+MClaI+SAM then TaqI digestion. (P) pBR322+MClaI(no SAM), then BalI partial and HincII total digestion (Q) pBR322+MClaI+SAM, then BalI partial HincII total digestion. Refer to Fig. 2 for fragment size designation.

methylation also protects against cleavage by both TthI and TaqI (fig. 3 lane I). Since TaqI is known to cut $T^{me}CGA$ (11) this suggests that M.TaqI methylates at $TCG^{me}A$. Base hydrolysis of M.TaqI methylated DNA followed by paper chromatography shows that M.TaqI methylates at adenine (Table I).

Site of Methylation by M.ClaI

M.ClaI protects all 12 ClaI sites in phage λ DNA from cleavage by ClaI (fig. 3 lane B). A subset of TthI sites are also protected (fig. 3 lanes F and G). This indicates that M.ClaI methyaltes within some TthI recognition sequences at the central tetranucleotide of A(TCGA)T. Base hydrolysis of ^{3}H-methylated DNA followed by paper chromatography showed M.ClaI to methylate at adenine (Table I). Furthermore, because TthI is known to cut $T^{me}CGA$ (11) but not $TCG^{me}A$ (10) these experiments show that M.ClaI methylates at $ATCG^{me}AT$.

In order to demonstrate that the M.ClaI preparation was methylating at ATCGAT and did not contain additional specificities, pBR322 was methylated with M.ClaI and ^{3}H-SAM then cut with TaqI. The single ClaI site (see fig. 2) between TaqI fragments 4 (343bp) and 5 (315bp) was not cut and generated a new fragment (fig. 3 lane 0). Bands were cut out of the gel, crushed and suspended in Aquasol (New England Nuclear). Only the new 658bp fragment contained siginificant counts. (Table 2).

Table I

Each R_f is the average of 3 experiments. The range was less than 5%.

	Methanol:HCl (sp. gr.. 1.18): Water (70:20:10 v/v)		Butanol:Methanol:H_2O: Ammonia (sp. gr. 0.90) (60:20:20:1 v/v)	
	R_f	c.p.m.	R_f	c.p.m.
Adenine	35		56	
Cytosine	49		48	
Guanine	21		35	
Thymine	75		65	
Hydrolysed SAM (control)	65(a)		41(a)	
5 methylcytosine	63		58	
6 methyladenine	53		68	
M.ClaI methylated DNA	51(a)(b)	338	68(a)(b)	397
M.TaqI methylated DNA	50(a)(b)	894	68(a)(b)	1026
M.TthI methylated DNA	50(a)(b)	483	69(a)(b)	1054

(a) Peak radioactivity
(b) No counts above background in the region of 5 methylcytosine

Table II

Tritium incorporated into fragments of M.ClaI methyalted DNA (fig. 3 lane 0)			
	Fragment	c.p.m. above background	% of total counts
	1	7	4
	2	7	4
(new)	4 & 5	164	91
	3	3	2
	6 & 7	0	0

The effect of M.TaqI and M.TthI methylation on heterologous restriction endonucleases.

Endonucleases with recognition sequences overlapping that of M.TaqI might in some cases be inhibited by M.TaqI methylation. A number of endonucleases were tested and the data obtained is tabulated below (Table III). M.TthI and M.TaqI methylation gave identical results.

The SalI site at position 650 in pBR322 (see fig. 2) is protected by M.TaqI methylation (fig. 4 lanes A and C) indicating that SalI cannot cleave GTCG^{me}AC. Similarly the single XhoI site in λ which cleaves HindII fragment 3 (19.7 map units) to 12.2 map units and 7.5 map units is protected by M.TaqI methylation (fig. 4 lanes H and I). This implies that XhoI cannot cut CTCG^{me}AT. It is of interest to note that in addition neither SalI nor XhoI can cut DNA methylated at cytosine in the central CpG of their recognition sequence (14) (18).

One of two AccI sites in pBR322 (Fig.2), the GTCGAC sequence at position 650, is protected by M.TaqI methylation but the GTATAC site at 2246 is not (fig. 4 lanes D and F). Thus AccI does not cleave at GTCG^{me}AC and probably will not cut any GT$\binom{A}{C}\binom{T}{G}$^{me}AC sequence. Similarly HincII does not cut GTCG^{me}AC (fig. 3 lanes P and Q) which is consistent with the known specificity of MHindII (GTPyPu^{me}AC) (15).

MboI and Sau3A are isoschizomers, but, whereas Sau3A can cleave G^{me}ATC,

Table III

Restriction Endonuclease	Recognition Sequence	Figure	Effect of M.TaqI or M.TthI Methylation
SalI	GTCGAC	4;lanes A and C	protection
XhoI	CTCGAG	4;lanes H and I	protection
AccI	GT$\binom{A}{C}\binom{G}{T}$AC	4;lanes D and F	some sites protected
HindII	GTPyPuAC	3;lanes P and Q	some sites protected
MboI	GATC	3;lane L	some sites protected
Sau3A	GATC	3;lane M	no sites protected

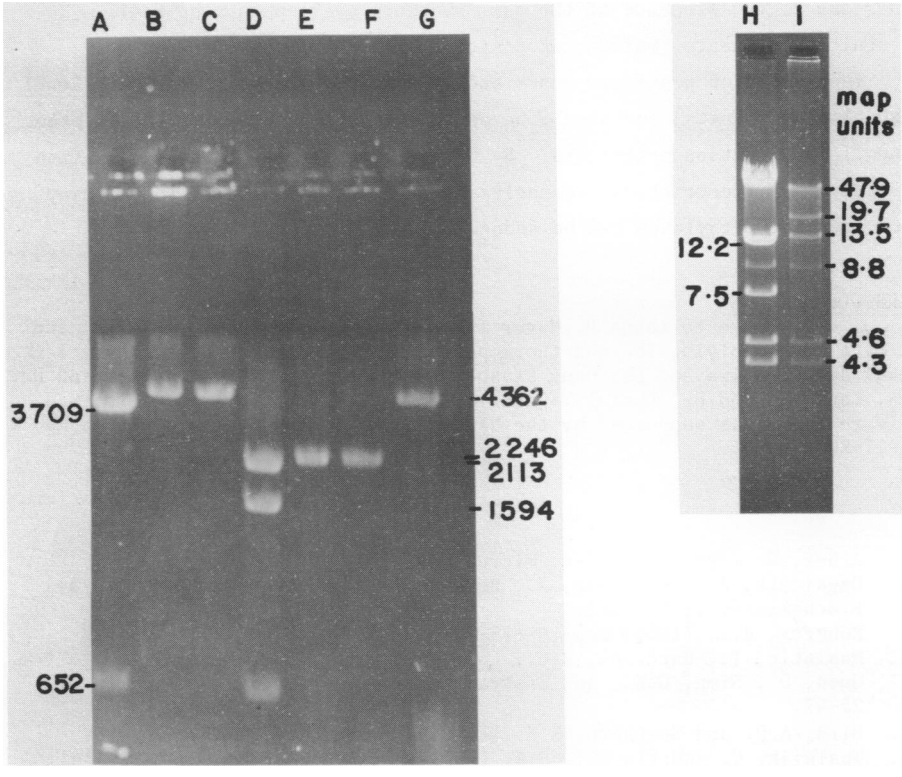

Fig. 4. 1% Agarose TBE gel electrophoresis; (A) one µg pBR322, EcoRI and SalI digestion. (B) one µg pBR322+MTaqI+SAM, then EcoRI digestion. (C) one µg pBR322+MTaqI+SAM, then EcoRI and SalI digestion. (D) one µg pBR322, EcoRI and AccI digestion. (E) one µg pBR322+MTaqI+SAM, then EcoRI and AccI digestion. (F) one µg pBR322+MThI+SAM, then EcoRI and AccI digestion. (G) one µg pBR322, EcoRI digestion. (H) Two µg λdam⁻ DNA+MTaqI (no SAM) then HindIII and XhoI digestion. (I) one µg λdam⁻ DNA+MTaqI+SAM, then HindIII and XhoI digestion. The fragment sizes for λ DNA are given in map units (16). In each case the digestion mix contained 3 units of restriction enzyme in the recommended buffer (New England Biolabs) and digestion was performed for one hour.

(16) MboI cannot (17). Thus the sequence, 5' TCG^{me}ATC 3', which contains an overlapping M.TaqI and MboI sequence, will be cut by Sau3A but not MboI. Some such sequences exist in plhage λ DNA (fig. 3 lanes L and M). TaqI cleavage is similarly blocked by M.Eco dam^{+} methylation (19) which has the specificity G^{me}ATC (19). This phenomenon is shown in Figure 3 lane N. The fragment designated 3 is in fact a partial caused by fusion of fragment 3

and 7 due to the presence of the dam$^+$ sequence overlapping the TaqI recognition sequence, GATCGA, at position 1125.

The isolation procedure described here was also used to purify M'EcoRI (unpublished results) and should be applicable to the purification of other type II modification methylases. By determining the effect of methylation on cleavage by restriction endonucleases, the site of methylation of many site specific methylases can be deduced (18).

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