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Mode of action of the *Spiroplasma* CpG methylase M.SssI

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The cytosine DNA methylase from the wall-less prokaryote, *Spiroplasma* strain MQ1 (M.SssI) methylates completely and exclusively CpG-containing sequences, thus showing sequence specificity which is similar to that of mammalian DNA methylases. M.SssI is shown here to methylate duplex DNA processively as judged by kinetic analysis of methylated intermediates. The cytosine DNA methylases, M.HpaII and M.HhaI, from other prokaryotic organisms, appear to methylate in a non-processive manner or with a very low degree of processivity. The *Spiroplasma* enzyme interacts with duplex DNA irrespective to the presence of CpG sequences in the substrate DNA. The enzyme proceeds along a CpG-containing DNA substrate molecule methylating one strand of DNA at a time.

Processive methylation; M.SssI methylase; M.HpaII methylase; M.HhaI methylase

1. INTRODUCTION

While most prokaryotic DNA methylases recognize and methylate unmethylated discrete sites which are composed of 4–6 bases, the mammalian methylase methylates cytosine residues exclusively at CpG-containing sequences. Mammalian methylases methylate hemimethylated DNA with higher efficiency when compared with duplex unmethylated DNA as substrate. In contrast, most prokaryotic methylases methylate unmethylated duplex DNA and hemimethylated DNA with similar efficiency [1]. While a DNA methylase from the wall-less bacteria, *Spiroplasma* strain MQ1 (M.SssI), displays the substrate specificity characteristic of prokaryotic methylases, its sequence specificity is identical to that of the mammalian methylases, methylating exclusively CpG sequences [2,3]. The gene encoding the *Spiroplasma* methylase (*sssIM*) has been cloned and expressed in *E.coli* [3]. Sequencing of the gene and examination of the derived amino acid sequence of the enzyme revealed the conserved domains which are common to all prokaryotic Type II cytosine DNA methylases [3–5], and are also present to some extent in mouse DNA methylase [6].

The catalytic features displayed by M.SssI prompted us to examine its mode of action. Early studies suggested that the mammalian methylase methylates its substrate by a processive mechanism [7]. Studies that addressed questions concerning the mechanism of action of prokaryotic methylases either could not determine processivity, as was the case with MHhaI [8], or suggested that each methylation event involves binding of the enzyme to a specific site on the DNA followed by dissociation of the enzyme from its substrate. This non-processive mechanism had been proposed for the modification enzyme, M.EcoRI, [9] and the *E. coli* DAM methylase [10]. It should be noted that these two enzymes are adenine DNA methylases.

Evidence is presented here to suggest that the M.SssI methylase can interact with duplex DNA regardless of the presence of CpGs, and methylates the DNA processively on one strand at a time. In contrast, two other cytosine DNA methylases (M.HpaII and M.HhaI) act non-processively or with lower processivity than that displayed by M.SssI.

2. MATERIALS AND METHODS

2.1. *DNA substrates and enzymes*

RF I DNA of the bacteriophage ϕX 174 was prepared from lysates of *E. coli* C which were infected with the lysis-defective ϕX 174 *am3* mutant, and purified by isopycnic centrifugation.

CpG-rich- and CpG-deficient DNA fragments were prepared by PCR using a Promega Co. *Taq* polymerase with a MJ Research Inc. thermal controller programmed for 1 min at 94°C, 1 min at 55°C and 1 min at 73°C for 35 cycles. A 450 bp CpG-rich (24 CpG sites) fragment was derived from a plasmid containing the 3′ CpG island of the mouse apoAI gene [11], and a 600 bp DNA fragment completely devoid of CpGs was derived from a plasmid containing the 5′ portion of the *sssIM* gene [3].

M.HpaII, M.HhaI, M.SssI and T4 DNA polymerase were acquired from New England Biolabs. T4 DNA ligase, Klenow polymerase, *Pst*I, *Ava*II, *Hpa*II, *Cfo*I, and *Msp*I were purchased from Boehringer-Mannheim. Methylases used in this study were partially purified commercial products (specific activities: M.SssI, 32 U/μg protein; M.HpaII, 114 U/μg protein; M.HhaI, 139 U/μg protein).

2.2. *Initial rate measurements*

Activity of M.SssI was assayed by initial rate measurements of incorporation of [*methyl*-^{3}H]*S*-adenosylmethionine (AdoMet, specific

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activity 15 Ci/mmol, Amersham, England) into ϕX 174 RF DNA under the following assay conditions: 50 mM Tris-HCl, pH 8, 50 mM NaCl, 10 mM EDTA, 5 mM dithiotreitol, 1.3 μM AdoMet, 0.1 mg/ml bovine serum albumin, in a 50 μl volume, at 37°C. The reaction was terminated by the addition of 0.2 ml 0.5% sodium lauryl sulfate/0.5 N NaOH and incubated for 2 h at 60°C. The DNA was extracted with chloroform:isoamyl alcohol (24:1), co-precipitated with 10 μg calf thymus DNA as carrier in 10% trichloroacetic acid onto Whatman GF/C filters, washed twice with 5% trichloroacetic acid, dried, and radioactivity counted in a liquid scintillation counter.

2.3. *Partial methylation and digestion of DNA*

ϕX 174 RF DNA was partially methylated in vitro with M.SssI, M.HpaII, or M.HhaI methylase, in a buffer containing: 50 mM Tris-HCl, pH 8, 10 mM EDTA, 5 mM dithiotreitol, 0.16 mM AdoMet, (New England Biolabs), 0.1 mg/ml bovine serum albumin, in 10–60 μl final volumes, at 28°C for time intervals as indicated in the figures. Reactions were terminated by the addition of 5 vols. of preheated (65°C) restriction buffer (10 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 5 mM dithiotreitol) and kept in a heated bath at 65°C for 20 min. This DNA was used without further purification for restriction enzyme digestion. All digestions were carried out for 2 h with an excess of restriction enzyme (5–10 U/μg DNA).

*Pst*I fragments (where indicated) were gel-purified with glass beads (GeneClean, vio 101) and end-labeled for 30 min at 37°C with T4 DNA polymerase using [α^{32}P]dCTP (specific activity 3,000 Ci/mmol, Amersham), along with 0.25 mM dATP, dGTP and dTTP, in a buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiotreitol.

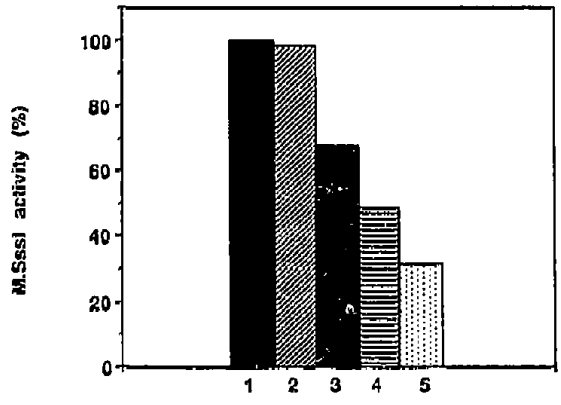

Fig. 1. Activity of M.SssI in the presence of CpG-lacking competitor DNA. The apoAI 450 bp fragment (CpG-rich) prepared as described in Materials and Methods was used as substrate. DNA methylation reactions were assayed by incorporation of [^{3}H]AdoMet into DNA as described in Materials and Methods for 4 min. Using 4 U M.SssI/ml reaction mixture, the reaction was linear with time for at least 10 min. A DNA fragment devoid of CpG sites prepared as described in Materials and Methods was included in the reaction mixture at different concentrations. 1, CpG-rich fragment alone (0.3 μg DNA); 2, CpG-rich fragment (3 μg DNA); 3, CpG-rich fragment (0.3 μg) and CpG-lacking fragment (0.3 μg) (1:1); 4, CpG-rich fragment (0.3 μg) and CpG-lacking fragment (0.9 μg) 1:3); 5, CpG-rich fragment (0.3 μg) and CpG-lacking fragment (3 μg) (1:10). In the control reaction (0.3 μg CpG-rich DNA) the activity obtained (100%) was 3 pmol methyl incorporated per U M.SssI per h. Results represent the average values of duplicates, representative of three different experiments.

3. RESULTS AND DISCUSSION

As a first step towards the elucidation of the mode of action of M.SssI we have explored the possibility that the interaction of M.SssI with its substrate DNA is directed by the presence of CpG sites in the DNA. To this end, we have examined the effect of CpG-lacking and CpG-rich DNA on the activity of the methylase by kinetic experiments. M.SssI methylase activity was assayed by incubating the enzyme with a 450 bp fragment containing 24 CpG sites available for methylation under conditions where the reaction was linear for at least 10 min at 37°C. To examine the effect of competitor DNA on the initial rate of the reaction, incubations were carried out for 4 min at 37°C. A 600 bp fragment completely devoid of CpG residues was added to the methylation reaction to examine whether a CpG-lacking DNA would compete with the CpG-rich substrate for the binding of methylase molecules. As can be seen in Fig. 1 a, 10-fold excess of CpG-containing DNA had no effect on methylase activity (columns 1 and 2). In contrast, when a DNA fragment totally devoid of CpG sites was added to the reaction in an equal amount to the CpG-rich substrate DNA, the initial rate of methylase activity decreased by about 30% (Fig. 1, column 3). When a 3- and 10-fold excess of the CpG-lacking fragment was included, DNA methylation was reduced by 50 and 70%, respectively (Fig. 1, columns 4 and 5). The significant decrease in enzyme activity in the presence of CpG-lacking DNA suggested that the methylase was sequestered by the excess inert DNA, indicating that M.SssI can interact with DNA devoid of CpG residues.

Interestingly, the inhibitory effect of CpG-lacking competitor DNA on the initial rate was not directly proportional to its relative concentration. This observation may suggest that the methyl-transfer reaction is the rate-limiting event in the reaction. On the other hand, the observed competition with CpG-lacking DNA may be consistent with the possibility that the methylase acts by a processive mechanism, which is characteristic of many methyl transfer events following a single binding event.

To test the hypothesis that M.SssI acts processively, we have analyzed the kinetics of the methylation reaction driven by the M.SssI methylase on ϕX 174 RF DNA as substrate. The accumulation of completely methylated product molecules and partially methylated intermediates of the methylation reaction was monitored. Samples were withdrawn from the reaction at various time intervals, the reaction was terminated, DNA was digested with *Hpa*II, and digests electrophoresed and visualized by ethidium bromide staining. (*Hpa*II recognizes the sequence, CCGG, and is sensitive to methylation at the inner cytosine residue of this sequence.) Since *Hpa*II sites (a subset of the CpGs present in ϕX 174 RF DNA) are subject to methylation by M.SssI (Fig. 2D), the banding pattern observed was assumed to reflect the pattern of methylation by M.SssI.

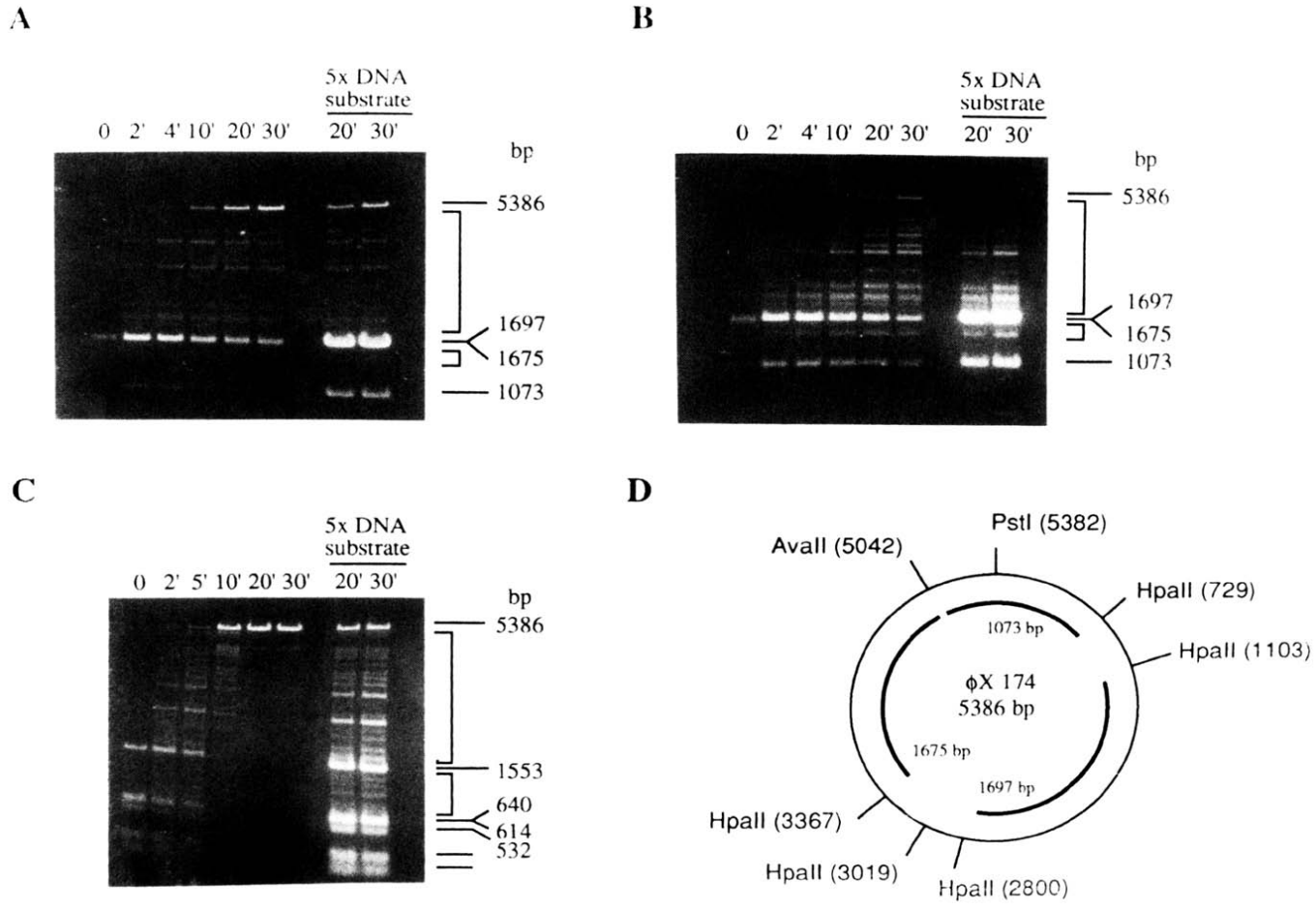

Fig. 2. Processivity of the prokaryotic methylases M.SssI, M.HpaII and M.HhaI. ϕX 174 RF DNA was methylated in vitro as described in Materials and Methods with (a) M.SssI (4 U/μg DNA), (B) M.HpaII, (0.4 U/μg DNA), (C) M.HhaI (1 U/μg DNA). The different enzyme concentrations were used in an attempt to adjust reaction rates. 10 min after the onset of the reaction an aliquot was withdrawn and a 5-fold excess of DNA substrate was added. Reactions were terminated as described in Materials and Methods at the time intervals (in min) indicated in the figure. Zero lanes represent ϕX DNA digested with the corresponding restriction enzymes. The DNA was digested to completion with *Hpa*II for A and B, and with *Cfo*I for C. All samples were then digested by *Ava*II to avoid the complicated banding pattern produced by the presence of closed circular DNA. The DNA digests were electrophoresed on a 0.8% agarose gel in 40 mM Tris-acetate, pH 8.2, 2 mM EDTA (TAE buffer), and visualized by ethidium bromide staining. Bands indicated by size were *Hpa*II fragments originating from the unmethylated ϕX 174. The 5,386 bp band correspond to the linear, fully methylated product. Bracketed bands correspond to intermediates of the methylation of reaction. (D) Relevant restriction map of the ϕX 174 genome. Positions of the *Hpa*II, *Pst*I, and *Ava*II restriction sites on the ϕX 174 DNA sequence [16] are indicated in parentheses. The three major bands of the *Hpa*II/*Ava*II digestion are drawn in thick lines. Restriction of ϕX 174 DNA with *Cfo*I/*Ava*II produces 19 fragments and were not depicted for simplicity.

To avoid having covalently closed circular DNA on the gel, which complicates the banding pattern, the DNA was digested with *Ava*II which recognizes a unique site in the ϕX 174 DNA molecule. The results shown in Fig. 2A indicate that the amount of fully methylated molecules (5.4 kb band) increased with time while the relative level of intermediates (bracketed bands) remained virtually constant throughout the entire experiment. Furthermore, at a very early stage of the reaction (4 min), when most of the DNA was completely unmethylated (see 1,697 and 1,675 bp bands), fully methylated molecules were already observed. This data suggested that the enzyme methylates the ϕX DNA molecules processively. To further support this conclusion we challenged the enzyme with a 5-fold excess of substrate, which was added 10 min after the onset of the reaction. As can be seen in Fig. 2A, the excess substrate did not change the relative amounts of intermediates or the rate of formation of completely methylated product molecules. Comparing the progress of the reaction from 10 to 30 min with the original substrate concentration to that obtained with excess substrate, a similar increase in completely methylated products was observed, while the intensity of the bands representing intermediates of the methylation reaction remained at the same levels. The excess substrate was primarily unmethylated as juged by the intensity of the 1697, 1675 and 1073 bp bands. In a processive reaction the presence of intermediates,

at any given time, reflects substrate molecules currently engaged in the methylation reaction. The fact that the intensity of the intermediate bands remained constant, even with excess substrate, strongly supports the suggested processive mode of action of the M.SssI methylase.

Parallel experiments were carried out with two other prokaryotic cytosine DNA methylases, M.HpaII and M.HhaI, to establish their modes of action. ϕX 174 RF DNA has been partially methylated with M.HpaII or with M.HhaI and digested with *Hpa*II or *Cfo*I (an isoschizomer of *Hha*I) respectively. The resulting digestion products were analyzed as described above for M.SssI-methylates DNA. Unlike the digestion pattern observed with M.SssI-methylated DNA, the relative amounts and sizes of intermediate fragments in the *Hpa*II and *Cfo*I digests were constantly changing with time (Fig. 2B and C). Using the arguments discussed above for the M.SssI methylase, the kinetics observed with M.HpaII and M.HhaI suggested that both M.HpaII and M.HhaI methylate non-processively or by a much lower degree of processivity than M.SssI. This conclusion is further supported by the results obtained upon the addition of a 5-fold excess of DNA substrate. In contrast to M.SssI, where the addition of excess substrate had no effect on the reaction kinetics (Fig. 2A), the M.HpaII and M.HhaI reactions were strongly affected. When a 5-fold excess of substrate DNA was added to the reaction the amounts of short intermediates continued to increase at the expense of longer intermediates and fully methylated DNA products (Fig. 2B and C). These results suggest that both M.HpaII and M.HhaI disengage their substrate after binding and methylating one or only a few methylation sites. The enzyme is then available for another binding event taking place on the same or another substrate molecule.

Having established that M.SssI acts upon its substrate duplex DNA in a processive manner, it was of interest to examine whether the enzyme methylates both strands of the DNA simultaneously, or alternatively, methylates each strand individually. In the first case, only fully methylated sites should be observed, while the alternative mechanism would result in hemimethylated sites as well. To distinguish between these two possible mechanisms, ϕX 174 RF DNA was partially methylated in vitro by M.SssI and digested with *Pst*I and *Hpa*II. Digestion with *Pst*I linearizes the ϕX 174 RF DNA and treatment with *Hpa*II digests only at *Pha*II sites which are unmethylated on both strands, since *Hpa*II does not cut at hemimethylated sites [12]. The resulting DNA fragments were separated by gel electrophoresis and full-length linear molecules were extracted from the gel. To assay the methylation status of the *Hpa*II sites, the molecules were end-labelled with T4 DNA polymerase, heat denatured, and reannealed to a 20-fold excess of non-labelled, non-methylated *Pst*I linearized ϕX RF DNA (Fig. 3, lane 3). The labelled reannealed DNA was

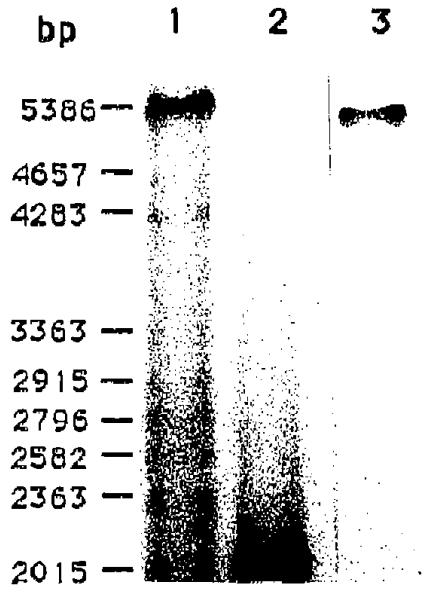

Fig. 3. M.SssI methylates one DNA strand at a time. ϕX 174 RF DNA was partially methylated with M.SssI (0.5 U/μg DNA for 1 h) as described in Materials and Methods. This DNA was digested with *Hpa*II/*Pst*I, electrophoresed on a 0.7% agarose gel in TAE buffer, and the band representing the full-length linear molecule (5,386 bp) was extracted, purified (GeneClean, bio 101), and end-labelled with T4 DNA polymerase (New England Biolabs) using [α^{32}P]dCTP (specific activity 3,000 Ci/mmol, Amersham). This full-length fragment was denatured at 94°C for 2.5 min, and reannealed to a 20-fold excess of non-labelled, non-methylated ϕX 174 RF DNA digested with *Pst*I, in 0.1 M NaCl, 1 mM EDTA at 60°C for 20 min (lane 3). The resulting hybrids were digested with *Hpa*II (lane 1) or *Msp*I (lane 2), and electrophoresed on a 0.7% agarose gel in TEA buffer. The gel was then dried and autoradiographed. The bands correspond to fragments obtained by partial digestion of the labeled (at *Pst*I sites) ϕX 174 DNA molecules which were originally protected from *Hpa*II digestion by methylation on one strand of DNA.

then re-digested with *Hpa*II (Fig. 3, lane 1), or its isoschizomer *Msp*I (Fig. 3, lane 2) electrophoresed and autoradiographed. As can be seen in Fig. 3, lane 1, the complete set of *Hpa*II/*Pst*I intermediates was observed when the reannealed DNA was digested with *Hpa*II. This observation clearly indicates that the full-length, *Hpa*II-resistant molecules were to some extent hemimethylated. It can therefore be concluded that M.SssI methylates on one strand of DNA at a time, whereas methylation of the complementary strand requires a second binding event of the enzyme to the DNA. Such a mode of action has not been shown before for any of the studied prokaryotic methylases, however, similar features were suggested for the rat liver methylase [7].

M.SssI, like all other prokeryotic methylases, methylates unmethylated duplex DNA and hemimethylated DNA at the same rate [3]. In contrast, mammalian DNA methylases show a two-order of magnitude higher activity with hemimethylated DNA than with unmethylated double-stranded DNA substrates [14]. A recent study with the murine DNA methylase showed that this preferential substrate specificity resides in the N-termi-

nal region of the enzyme. Removal of the N-terminal portion of the enzyme releases an inhibitory effect on the de novo activity of the enzyme. As a result, the C-terminal part of the enzyme shows the substrate specificity characteristic of all prokaryotic methylases [15].

Cytosine DNA methylases, of 300–400 amino acids in length, share structural features and contain extensive areas of amino acid homology. In addition, these enzymes contain a non-homologous 'variable region' which is believed to define the sequence specificity of the enzyme [4,5,13]. The murine DNA methylase, although over 1,800 amino acids in length, also displays limited homology to the prokaryotic enzymes in its C-terminal part [6]. We are currently investigating how the various catalytic traits observed in different methylases are reflected in the structural elements of the protein.

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