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*Sse*8387I, a useful eight base cutter for mammalian genome analysis (influence of methylation on the activity of *Sse*8387I)

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ABSTRACT

To develop restriction enzymes that are useful for genome analysis, we previously performed screening and isolated *Sse*8387I from *Streptomyces* sp. strain 8387. *Sse*8387I is a restriction enzyme that recognizes 5′-CCTGCA/GG-3′ and cleaves DNA at the site shown by the diagonal (*Nucleic Acid Res.*, 18, 5637–5640). The present study evaluated the effects of methylation that is important when *Sse*8387I is used for genome analysis. *Sse*8387I lost cleavage activity after methylation of adenine or methylation of cytosine at any site in the recognition sequence. However, the recognition sequence of *Sse*8387I contains no CG sequence, which is the mammalian methylation sequence. In addition, we evaluated the effects of methylation of CG at sites other than the recognition sequence. The cleavage activity of *Sse*8387I was maintained even when CG sequences were present immediately before or after, or near the recognition sequence, and cytosine was methylated. These results suggest that CG methylation does not affect the cleavage activity of *Sse*8387I. Therefore, *Sse*8387I seems to be very useful for mammalian genome analysis.

INTRODUCTION

Many type II restriction enzymes have been isolated from various microorganisms (1). All microorganisms with type II restriction enzymes produce methylase corresponding to each enzyme to protect their genome DNA from the self-produced restriction enzymes. Methylases produced against each enzyme recognize the same sequence recognized by the corresponding restriction enzymes. They methylate adenine or cytosine in this sequence, preventing DNA cleavage by the restriction enzymes (1). In other words, restriction enzymes cannot cleave DNA methylated by the corresponding methylase. The activity of restriction enzymes is often affected by methylation of sites other than the site methylated by the corresponding methylase (2).

For mammalian genome DNA analysis, eight base cutters such as *Not*I, *Sfi*I and *Fse*I are used (3–5). Their cleavage sites are used for the production of genomic maps. However, these eight base cutters are sensitive to the 5′-^{m}CG-3′ sequence that acts as a methylation signal in vertebrates (2,6). Therefore, eight base cutters that are not affected by CG methylation may be more useful than the above eight base cutters for genome analysis. We previously isolated *Sse*8387I, a type II restriction enzyme that recognizes 5′-CCTGCAGG-3′ (7). *Sse*8387I activity may not be affected by CG methylation because the sequence recognized by this enzyme does not contain CG sequences. For mammalian genome DNA analysis, we evaluated the effects of methylation, including CG methylation near the recognition sequence of *Sse*8387I, on the cleavage activity of this enzyme.

MATERIALS AND METHODS

Materials

*Pst*I methylase (M. *Pst*I) and CpG methylase (M. *Sss*I) were obtained from NEB. λ-DNA, restriction enzymes and T4 polynucleotide kinase were products of Takara Shuzo. All synthetic oligonucleotides were produced by the Takara Custom Service Center. All enzymes were used according to the manufacturer's instructions. [γ-^{32}P]ATP (>5000 Ci/mmol) was from Amersham. All other chemicals were of reagent grade.

Preparation of λ-DNA methylated with *Pst*I methylase

Methylation reaction was done at 37°C, in 100 µl of reaction mixture that contained 50 mM Tris–HCl (pH 7.5), 10 mM Na_2EDTA, 10 mM 2-mercaptoethanol, 80 mM *S*-adenosylmethionine, 20 U M. *Pst*I and 10 µg λ-DNA. The reaction was stopped by extraction of the reaction mixture with phenol, and then methylated λ-DNA was recovered by ethanol precipitation.

Preparation of λ-DNA methylated with CpG methylase

Methylation reaction was done at 37°C, in 100 µl of reaction mixture that contained 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 160 mM *S*-adenosylmethionine, 20 U M. *Sss*I and 10 µg λ-DNA (8,9). The reaction was stopped by extraction of the reaction mixture with phenol, and then methylated λ-DNA was recovered by ethanol precipitation.

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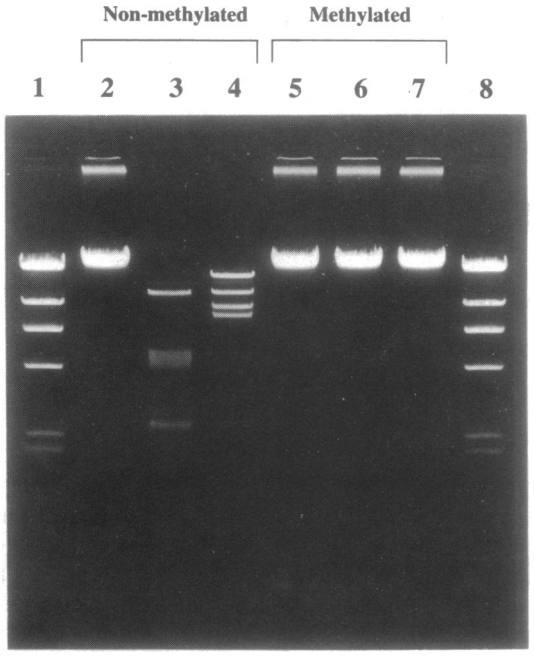


Figure 1. Cleavage of λ-DNA methylated with *Pst*I methylase. Lanes 1 and 8: λ-*Hin*dIII digests as size markers, lane 2: non-methylated λ-DNA was not digested, lane 3: non-methylated λ-DNA digested with *Pst*I, lane 4: non-methylated λ-DNA digested with *Sse*8387I, lane 5: methylated λ-DNA was not digested, lane 6: methylated λ-DNA digested with *Pst*I, lane 7: methylated λ-DNA digested with *Sse*8387I.

Restriction enzyme digestion of λ-DNA

Restriction enzyme digestion of λ-DNA was done in 50 µl of optimum reaction mixture that contained 1 µg λ-DNA, at optimum temperatures for each restriction enzyme. The reaction was stopped by addition of a termination dye (0.25% bromophenol blue, 20% glycerol and 50 mM Na_2EDTA) and heating at 65°C for 5 min. The fragments were separated by electrophoresis at 100 V for 1–2 h in a 0.7% agarose gel containing 0.5 mg/ml ethidium bromide.

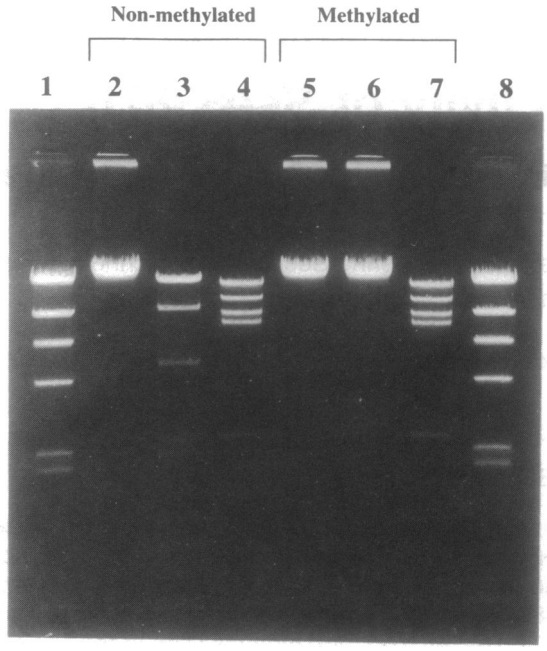
Preparation of end-labeled substrate DNAs

Synthetic oligonucleotides (5′-AACGCCTGCAGGCGAAG-3′) containing no or single 5-methylcytosine at various positions of cytosine (Fig. 3B, position nos 2–6) were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and the labeling reaction was stopped by heating at 95°C for 10 min. End-labeled substrate DNAs were prepared by annealing respective end-labeled synthetic oligonucleotides and complementary synthetic oligonucleotides which contained no or single 5-methylcytosine at the corresponding position with labeled synthetic oligonucleotides.

Restriction enzyme digestion of end-labeled substrate DNAs

Restriction enzyme digestion of end-labeled substrate DNAs was done in 10 µl of optimum reaction mixture containing 0.1 pmol of end-labeled substrate DNAs at 37°C for 4 h. The digestion mixtures were electrophoresed on 12% denaturing polyacrylamide gel.

(A)



(B)

Lambda phage

2545	TGCTGGCGAG	CCTGCAGG	AAGAGGATGG
2809	ACGCAGGGGA	CCTGCAGG	ATTTTATGTA
11824	ACGGAAAAT	CCTGCAGG	CGATTACAA
19822	ACAGTGTCAT	CCTGCAGG	TTGACGTTT
36990	TTGGAGCCAA	CCTGCAGG	TGATGATTAT

Figure 2. (**A**) Cleavage of λ-DNA methylated with CpG methylase. Lanes 1 and 8: λ-*Hin*dIII digests as size markers, lane 2: non-methylated λ-DNA was not digested, lane 3: non-methylated λ-DNA digested with *Mlu*I, lane 4: non-methylated λ-DNA digested with *Sse*8387I, lane 5: methylated λ-DNA was not digested, lane 6: methylated λ-DNA digested with *Mlu*I, lane 7: methylated λ-DNA digested with *Sse*8387I. (**B**) The neighboring sequences of the recognition site with *Sse*8387I in λ-DNA. The methylation sites with CpG methylase were indicated by an open bar.

RESULTS

Effects of 6-methyladenine in the recognition sequence

The six bases in the middle of the recognition sequence of *Sse*8387I are identical to that of *Pst*I. M. *Pst*I is known to methylate adenine in the recognition sequence into *N*-6-methyladenine (1). Therefore, using M. *Pst*I, we evaluated the effects of *N*-6-methyladenine in the recognition sequence on *Sse*8387I activity.

Figure 1 shows cleavage patterns after digestion with *Sse*8387I or *Pst*I using M. *Pst*I-methylated λ-DNA or non-methylated λ-DNA as a substrate. The non-methylated λ-DNA was cleaved by both *Pst*I and *Sse*8387I (lanes 2–4). On the other hand, M. *Pst*I-methylated λ-DNA was not cleaved by either *Pst*I or *Sse*8387I (lanes 5–7).

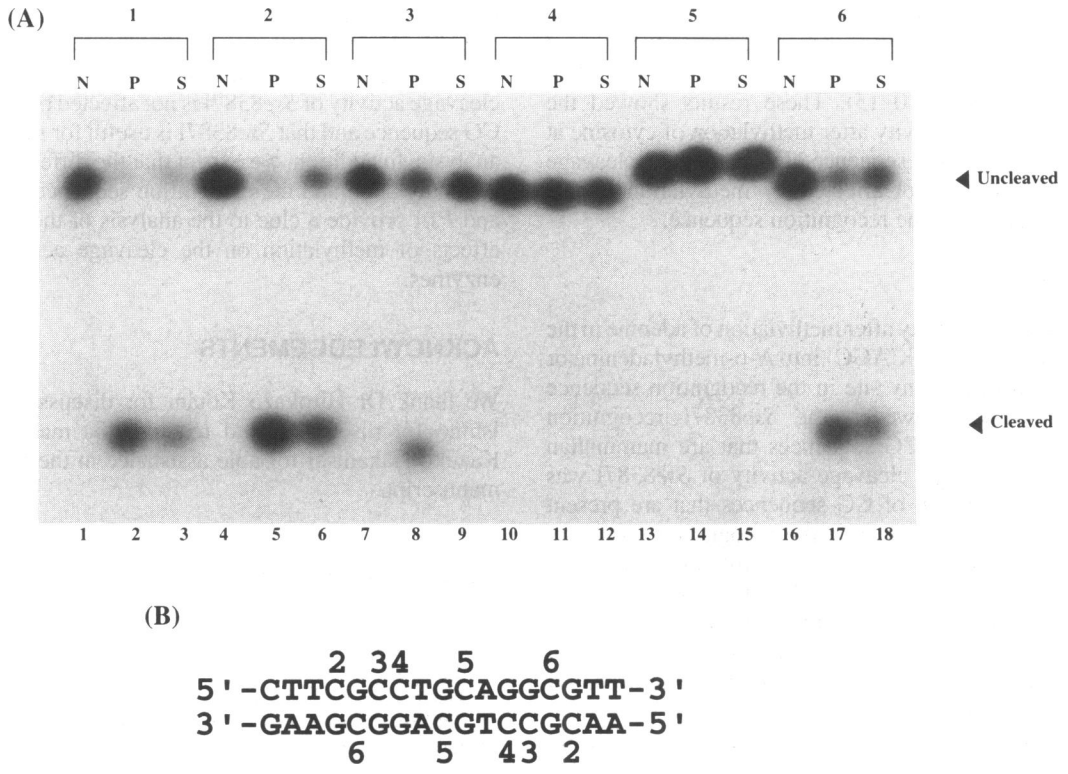


Figure 3. (**A**) Cleavage of synthetic DNAs containing 5-methylcytosine. The block numbers 1–6 in the upper row indicate the position of 5-methylcytosine in synthetic DNAs. Block no. 1: synthetic DNA contained no 5-methylcytosine, block nos 2–6: synthetic DNAs contained 5-methylcytosine at the position nos 2–6 of the sequence listed in (B) respectively. N, P and S in the middle row indicate the digestion of respective synthetic DNAs with various restriction enzymes. N: no digestion, P: *Pst*I digestion, S: *Sse*8387I digestion. (**B**) Sequence of synthetic DNAs and position of 5-methylcytosine. Synthetic oligonucleotide for substrates consist of the sequence listed here. The nos 2–6 above and below the sequence indicate the position of 5-methylcytosine in synthetic DNAs.

Thus, the λ-DNA treated with M. *Pst*I was not cleaved by *Pst*I, confirming λ-DNA methylation by M. *Pst*I. In addition, this M. *Pst*I methylated λ-DNA was not cleaved by *Sse*8387I, showing that *N*-6-methyladenine in the recognition sequence of *Sse*8387I inhibits the cleavage activity of *Sse*8387I.

Effects of 5-methylcytosine

In mammalian DNA, cytosine is methylated in considerable part of CG sequences (6). Therefore, restriction enzymes that are not affected by CG methylation may be more useful for genome analysis. Since the recognition sequence recognized by *Sse*8387I does not contain any CG sequence, *Sse*8387I activity may not affected by CG methylation. Therefore, we evaluated the effects of CG methylation at sites other than the recognition sequence on the cleavage activity of *Sse*8387I. λ-DNA was methylated with CpG methylase, and DNA with artificially methylated CG sequences was prepared (8,9). This λ-DNA was used as a substrate.

Figure 2A shows cleavage patterns after restriction enzyme digestion using λ-DNA methylated with CpG methylase or non-methylated λ-DNA as a substrate. Figure 2B indicates sequences near the *Sse*8387I recognition sequence in λ-DNA (10). As shown in Figure 2A, the non-methylated λ-DNA was cleaved with *M1u*I while the methylated λ-DNA was not cleaved with *M1u*I that is affected by CG methylation. This confirmed complete methylation of λ-DNA by CpG methylase. On the other hand, *Sse*8387I cleaved λ-DNA irrespective of CG methylation. As shown in Figure 2B, CG sequences (indicated by boxes) are present near the *Sse*8387I recognition sequence in λ-DNA. These results suggest that the cleavage activity of *Sse*8387I is not affected by methylation of CG sequences near the recognition sequence.

To evaluate the effects of 5-methylcytosine, DNA with a sequence shown in Figure 3B not containing 5-methylcytosine and DNAs containing 5-methylcytosine at positions 2–6 were synthesized. As a substrate, 5′-end of the strand (5′-AACGC-CTGCAGCGAAG-3′) on the lower part in Figure 3B was labeled with ^{32}P.

Figure 3A shows 12% denaturing polyacrylamide gel electrophoresis of the synthetic DNAs after digestion with restriction enzymes. The synthetic DNA not containing 5-methylcytosine was cleaved with *Pst*I and *Sse*8387I (Fig. 3, lanes 1–3). On the other hand, as shown in Figure 3B, the synthetic DNAs were so designed as to contain CG sequences, which are mammalian methylation sequences, before and after the *Sse*8387I recognition sequence. Both synthetic DNA containing 5-methylcytosine at the site of cytosine in the CG sequence on the 5′-end side of the recognition sequence (Fig. 3B, position no. 2) and DNA containing 5-methylcytosine at the site of cytosine in the CG sequence on the 3′-end side (Fig. 3B, position no. 6) were cleaved with both *Pst*I and *Sse*8387I (Fig. 3A, lanes 4–6 and 16–18). Of the synthetic DNAs containing 5-methylcytosine in the recognition sequence, DNA containing 5-methylcytosine at position 3

(Fig. 3B) was cleaved with *Pst*I but not with *Sse*8387I (Fig. 3A, lanes 7–9). The synthetic DNAs containing 5-methylcytosine at position 4 or 5 (Fig. 3B) were not cleaved with either *Pst*I or *Sse*8387I (Fig. 3A, lanes 10–15). These results showed the following. *Sse*8387I lost activity after methylation of cytosine at any site in the recognition sequence. However, the cleavage activity of *Sse*8387I was not affected by methylation of CG sequences before and after the recognition sequence.

DISCUSSION

*Sse*8387I lost cleavage activity after methylation of adenine in the recognition sequence (CCTGCAGC) into *N*-6-methyladenine or methylation of cytosine at any site in the recognition sequence into 5-methylcytosine. However, the *Sse*8387I recognition sequence does not contain CG sequences that are mammalian methylation sequences. The cleavage activity of *Sse*8387I was not affected by methylation of CG sequences that are present immediately before or after or near the recognition sequence. Therefore, *Sse*8387I seems to be very useful for mammalian genome analysis. On the other hand, in plant genome DNA, CNG sequences are methylated (6). In the *Sse*8387I recognition sequence, C at two sites (Fig. 3B, position nos 4 and 5) is identical to the CNG methylation sequence. This suggests that *Sse*8387I sometimes can not be used for plant genome analysis.

In this study, the effects of methylation was evaluated mainly in terms of mammalian genome analysis. Further studies are needed on 5-hydroxylmethylcytosine contained in DNA in the *Escherichia coli* T even phage (6,11) or 4-methylcytosine due to some methylases (1,6).

Since the six bases in the middle of the *Sse*8387I recognition sequence are identical to the *Pst*I recognition sequence, the effects of methylation on *Sse*8387I were evaluated in comparison with *Pst*I. Only the effects of 5-methylcytosine at position no. 3 (Fig. 3B) differed between the two enzymes. This position is contained in the recognition sequence of *Sse*8387I but not in that of *Pst*I. Thus, both enzymes were affected by methylation in the recognition sequence. However, neither enzyme was affected by methylation at sites other than the recognition sequence. These findings may suggest that methylation at sites other than the recognition sequence does not affect the activity of restriction enzymes. From this viewpoint, and that the recognition sequence of *Sse*8387I does not contain CG sequence, it is probable that the cleavage activity of *Sse*8387I is not affected by methylation of the CG sequence and that *Sse*8387I is useful for mammalian genome analysis. In addition, we expect that the differences in the effects of methylation and the recognition sequence between *Sse*8387I and *Pst*I provide a clue to the analysis of the mechanism of the effects of methylation on the cleavage activity of restriction enzymes.

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