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# THE EFFECTS OF DNA METHYLATION BY Hha I METHYLASE ON THE CLEAVAGE REACTIONS BY Hae II, Aha II AND Ban I ENDONUCLEASES

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Summary: The DNA methylated by Hha I methylase was resistant against cleavage of Hae II or Aha II endonuclease indicating that the methyl group of the C5 position of the inmost cytosine nucleotide interferes with the interaction between the enzyme and the hexameric recognition sequence. Considering that Hae II or Aha II methylase has not been isolated yet, the result explained above is a useful information for protecting a double stranded DNA from being cleaved by Hae II or Aha II endonuclease. In contrast to Hae II or Aha II endonuclease, Ban I endonuclease which also has Hha I sequence as its tetrameric core was able to cleave the same DNA normally. This result suggests that the C5 position of the inmost pyrimidine nucleotide is not an important contact point between Ban I endonuclease and its hexameric recognition sequence. 

Restriction methylases recognize the same specific DNA sequences as do their associated restriction endonucleases (1) (2). Methylation of these sequences protects DNA against cleavage by homologous endonucleases as well as heterologous restriction enzymes with the same recognition specificity. Recently, it has been also shown that DNA modified within the central tetramer of a recognition sequence is protected against cleavage by related hexameric enzymes having that central tetrameric core with a few exceptions (3). However DNA methylation by Hha I methylase and the activities of the related restriction enzymes on the modified DNA have not been reported.

Hha I methylase recognizes the DNA sequence, 5'-GC$\overset{*}{G}$C-3', and methylates on the internal cytosine nucleotide as indicated by an asterisk (4). Since the Hha I specific sequence is the central tetrameric sequences of the hexameric

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enzymes, Hae II (5'-PuGCGCPy-3'), Aha II (5'-GPuCGPyC-3') and Ban I (5'-GGPyPuCC-3'), Hha I methylase can methylate the internal cytosine nucleotides of the four hexameric sequences.

In this paper we will describe the effects of the methylation by Hha I methylase on the internal cytosine nucleotides within the related hexameric restriction sequences. We will also discuss the specific interactions between Ban I endonuclease and its recognition sequences.

## MATERIALS AND METHODS

Materials: Plasmid pUC9 DNA and M13mp9 RF DNA were isolated by the method of Clewell and Helinsky (5) and Zinder et al. (6), respectively. Plasmid pDR 323 was constructed in this laboratory. T4 DNA ligase, restriction endonucleases and methylases were from New England BioLabs. S-Adenosylmethionine was from Sigma. All other chemicals used for this experiment are reagent grade.

DNA methylation in vitro: For the completion of the Hha I methylation reaction, prolonged incubation was necessary with an excess amount of methylase and S-Adenosylmethionine. The 50 µℓ reaction mixture contained 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 3 mM S-Adenosylmethionine, 10-20 µg/ml of DNA and 100 units of the enzyme. After incubation of 4 hours at 37°C, 1 µℓ of 32 mM S-Adenosylmethionine and 20 units of the enzyme were freshly added. The reaction mixture was incubated at 37°C for 3 additional hours. The DNA thus methylated was totally resistant against the cleavage reaction of Hha I endonuclease.

Restriction endonuclease reactions: Endonuclease reactions were performed in 10 µℓ volume containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ , 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 µg of DNA, optimal concentrations of NaCl and indicated amounts of enzyme. Each reaction mixture was incubated 37°C for 30 min to 3 hours and then stopped by incubation at 70°C for 10 min. The reaction mixtures were saved at -20°C for further analyses.

Construction of plasmid pDR 323 DNA: To facilitate the analyses of DNA fragments after restriction endonuclease reactions, it was necessary to construct a plasmid DNA having smaller number, possibly less than four, of recognition sequences of the restriction endonucleases to be used. For this purpose pUC9 DNA was digested with Pvu II endonuclease and the digested DNA fragments were separated by 1% agarose gel electrophoresis (7). From the two pUC9-Pvu II fragments, the larger one (which is shorter than pUC9 DNA by 323 bp) was eluted out from the gel and then treated with T4 DNA ligase. The resulting circular DNA (pDR 323) contains three each of Hae II and Aha II sites and two of Ban I site.

## RESULTS

pDR 323 DNA which has been constructed as described in 'methods' contains three each of Hae II and Aha II sites, two of Ban I sites and 19 of Hha I sites. All of the Hha I sites were methylated by Hha I methylase and protected against the cleavage reactions by Hha I endonuclease (Fig. 1 lane

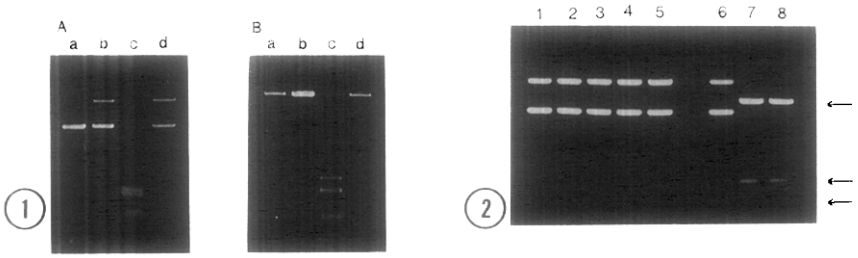


Fig. 1. Completion of DNA methylation by Hha I methylase. The DNA methylated by Hha I methylase was treated with Hha I endonuclease and the reaction products were analyzed by 1% agarose gel electrophoresis. A: The detailed reaction conditions using pDR 323 DNA are explained in MATERIALS AND METHODS. Lane a, unmethylated DNA; Lane b, methylated DNA by Hha I methylase; Lane c, unmethylated DNA digested with 16 units of Hha I endonuclease for 90 min at 37°C; Lane d, methylated DNA by Hha I methylase treated with 16 units of Hha I endonuclease for 90 min at 37°C. B: The same experiment has been performed as A except that pDR 323 DNA was replaced with linearized M13 mp9 DNA.

Fig. 2. Hae II endonuclease activity on the DNA methylated by Hha I methylase. The detailed reaction conditions using pDR 323 DNA and 6 units of Hae II endonuclease are explained in MATERIALS AND METHODS. The reaction products were analyzed by 1% agarose gel electrophoresis. Lanes 1 to 5, methylated DNA treated with Hae II endonuclease for 0, 0.5, 1.0, 2.0 and 3.0 hours at 37°C, respectively. Lanes 6 to 8, unmethylated DNA treated with 6 units of Hae II endonuclease for 0, 0.5 and 1.5 hours, respectively. The positions of the three final cleavage products are indicated by the arrows.

d). These results shown in Fig. 1 also indicate that the Hha I methylase and endonuclease used do not have any considerable amounts of contaminating methylase or nucleases activities, and recognize the same nucleotide sequence with associated specificities as described earlier (8).

Hae II endonuclease activity was completely inhibited by the methylation of DNA with Hha I methylase: Hae II endonuclease recognizes the hexameric sequence 5'-PuGCGC$\overset{\downarrow}{\text{P}}$y-3', and cleaves at the point indicated by the the arrow (5). Since the internal tetrameric sequence, 5'-GCGC-3', is identical with Hha I sequence, it is expected that the methylation by Hha I methylase within the hexameric sequence could inhibit the cleavage reaction by Hae II endonuclease. The result shown in Fig. 2 indicates that the methylation by Hha I methylase completely inhibited the cleavage reaction by Hae II endonuclease even after 3 hours of incubation with 6 units of the enzyme (Fig. 2, lane 5) at 37°C. The resulting three DNA fragments after completion of Hae

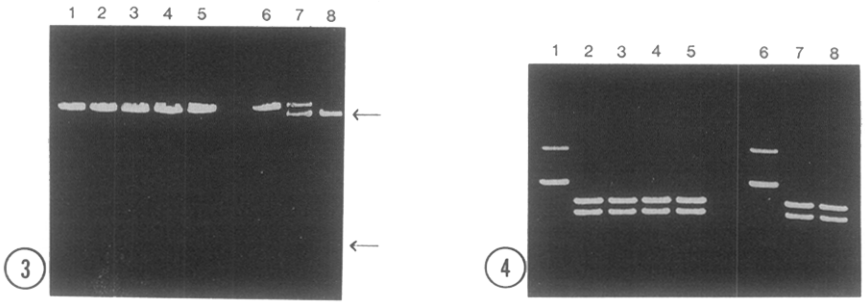


Fig. 3. Aha II endonuclease activity on the DNA methylated by Hha I methylase. The detailed reaction conditions using M13 mp9 linear DNA and 3 units of Aha II endonuclease are explained in MATERIALS AND METHODS. The reaction products were analyzed by 1% agarose gel electrophoresis. Lanes 1 to 5, methylated DNA treated with Aha II endonuclease for 0, 0.5, 1.0, 2.0 and 3.0 hours at 37°C, respectively; Lanes 6 to 8, unmethylated DNA treated with Aha II endonuclease for 0, 0.5 and 1.5 hours at 37°C, respectively. Only lane 8 shows the completion of the reaction. The positions of the final cleavage products are indicated by the arrows.

Fig. 4. Ban I endonuclease activity on the DNA methylated by Hha I methylase. The detailed reaction conditions using pDR 323 DNA and 3 units of Ban I endonuclease are explained in MATERIALS AND METHODS. The reaction products were analyzed by 1% agarose gel electrophoresis. Lanes 1 to 5, methylated DNA treated with Ban I endonuclease for 0, 0.5, 1.0, 2.0 and 3.0 hours at 37°C, respectively; Lanes 6 to 8, unmethylted DNA treated with Ban I endonuclease for 0, 0.5 and 1.5 hours at 37°C, respectively.

II endonuclease reactions with unmethylated pDR 323 DNA are shown in Fig. 2, lane 7 and 8.

Aha II endonuclease activity was completely inhibited by the methylation of DNA with Hha I methylase: Aha II endonuclease recognizes the hexameric sequence 5'-GPuCGPyC-3', and cleaves at the point indicated by the arrow (9). Among the four kinds of tetrameric core sequences of this enzyme, only 5'-GCGC-3' can be methylated by Hha I methylase.

To examine the effects of DNA methylation by Hha I methylase upon cleavage reactions by Aha II endonuclease, M13mp9 RF linear DNA was used rather than pDR 323 DNA to obtain simpler reaction products. Unlike pDR 323 DNA, M13mp9 RF DNA has only one Aha II sequence having Hha I sequence (5'-GCGC-3') at the tetrameric core (7). Fig. 3 shows that the methylation by Hha I methylase completely inhibited the cleavage reaction by Aha II endonuclease even after 3

hours (lane 5) of incubation with 3 units of the enzyme at 37°C. The unmethylated M13mp9 RF linear DNA was also treated with Aha II endonuclease as a control. The final two DNA fragments are shown in Fig. 3, lane 8.

Ban I endonuclease can cleave the DNA methylated by Hha I methylase: Ban I endonuclease recognizes the hexanucleotide sequence 5'-GG↓PyPuCC-3', and cleaves at the point indicated by the arrow (9). The two Ban I sequences of pDR 323 DNA are 5'-GGCGCC-3' and 5'-GGCACC-3' (7). Therefore only the former sequence can be methylated by Hha I methylase. However, both methylated and unmethylated Ban I sequences were completely cleaved by Ban I endonuclease (Fig. 4).

## DISCUSSION

The DNA methylated by Hha I methylase was not cleaved by Hae II or Aha II endonuclease but cleaved normally by Ban I endonuclease. The fact that Hae II or Aha II endonuclease can not cleave the DNA methylated by Hha I endonuclease can be explained by one of the following two reasons. 1) Within the hexameric Hae II or Aha II sequence, the specific methylation site of Hae II or Aha II methylase which is yet to be isolated is identical with the site of Hha I methylase. 2) DNA methylation within the hexameric Hae II or Aha II sequence by Hha I methylase can inhibit Hae II or Aha II endonuclease reaction even though the methylation site is different from the site of the cognate restriction methylase. However, it is clear that Hha I methylase can be used for protecting a double stranded DNA from being cleaved by Hae II or Aha II endonuclease. One of the examples is as follows. A double stranded DNA ligated with Hae II or Aha II oligonucleotide linker DNA should be cut with Hae II or Aha II endonuclease, respectively, before being joined into vector DNAs. In this case Hha I methylase can be used instead of Hae II or Aha II methylase which is yet to be isolated.

In contrast to Hae II or Aha II endonuclease, Ban I endonuclease can recognize and cleave its hexameric sequence even after the methylation by Hha I methylase. Considering the fact that the thymidine nucleotide of 5'-GGTACC-

3' or 5'-GGTGCC-3' has already methyl group on C5 position of the pyrimidine ring and Ban I endonuclease has four specific sequences, 5'-GGCGCC-3', 5'-GGCACC-3', 5'-GGTGCC-3' and 5'-GGTACC-3'it is expected that the methylation on the inmost cytosine nucleotide of 5'-GGCGCC-3' by Hha I methylase would not interfere with the specific interaction between Ban I endonuclease and its recognition sequence.

## REFERENCES

1. Arber, W. (1974) Progr. Nucl. Acid Res. Mol. Biol. 14, 1-37.
2. Robert, R. J. (1978) Gene 4, 183-193.
3. Joan, E. B. and Roberts, R. J. (1982) Nucl. Acids Res. 10, 913-934.
4. Mann, M. B. and Smith, H. O. (1979) Proc. of the Conference on Transmethylation, Eds. Usdin E Borchardt RT and Greveling CR Elsevier/North Holland NY 483-492.
5. Clewell, D. B. and Helinsky, D. R. (1972) J. Bacteriol. 110, 1135-1146.
6. Zinder, N. D. and Boeke, J. D. (1982) Gene 19, 1-10.
7. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103-119.
8. Smith, H. O. (1979) Science 205, 455-462.
9. Roberts, R. J. (1984) Nucl.Acids Res. 12, r167-r204.