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Inhibition of Sma I, Ava I, Nae I and Xma I endonuclease activities by the methylation of DNA with Hpa II methylase

Choi, W.S., S.C. Kang, J.S. Seo*, and O.J. Yoo

Department of Biological Science and Engineering Korea Advanced Institute of Science and Technology Seoul 131, Korea

**Department of Biochemistry and Cancer Research Institute College of Medicine, Seoul National University*

제한효소 Sma Ⅰ, Xma Ⅰ, Ava Ⅰ, Nae Ⅰ의 DNA 절단반응에 있어서 Hpa Ⅱ methylation의 억제효과

최우성 · 강선철 · 서정선* · 유욱준

한국과학기술원 생물공학과

*서울대학교 의과대학 생화학교실 및 암연구소

Abstract: The DNA methylated by Hpa II methylase was not cleaved by Sma, I, Ava I and Nae I endonucleases. This experimental data could be interpreted as strong evidences that Sma I, Ava I and Nae I methylases which yet to be isolated would methylate on the inmost cytosine nucleotide within their hexameric recognition sequences. The facts that Sma I, Ava I and Nae I endonucleases can not cleave the DNA methylated by Hpa II methylase are the valuable informations for protecting DNAs upon cleavage reactions by Sma I, Ava I and Nae I endonucleases especially for cDNA insertion experiments into vector DNAs using Sma I, Ava I and Nae I oligonucleotide linkers. In the case of Xma I endonuclease, partially cleaved DNA fragments were observed although the reaction rate was greatly decreased. This result implies that the methylation site of Xma I methylase which yet to be isolated would not be the same as that of Hpa II methylase in Xma I sequence.

Key Words: restriction modification engyme, DNA protein interaction.

Hpa II methylase recognizes the DNA sequences, 5 -CC*GG-3 , and methylates on the internal cytosine nucleotide as indicated by an asterisk (6). Since the Hpa II specific sequence is the central tetrameric sequences of the hexameric enzymes, Sma I (5 -CCCGGG-3'), Xma I (5'-CCCGGG-3'), Ava I (5'-CPyCGPuG-3') and Nae I (5 -GCCGGC-3'), Hpa II methylase can modify the inmost cytosine nucleotides of the hexameric sequences, 5'-CCCGGG-3' and 5'-GCCGGC-3'. In this paper we will describe *in vitro* methylation of DNAs by Hpa II methylase, which can partially or completely protect the DNA from the cleavage reactions of the four related hexameric endonucleases.

MATERIALS AND METHODS

Materials

S-adenosyl-methionine was purchased from Sigma. Restriction endonucleases were purchased from New England BioLabs. Hpa II methylase and Hpa II endonuclease were isolated in our laboratory as described previously (6)(7). Plasmid

pUC9 DNA and M13mp9 DNA were isolated by the procedure of Clewell *et al.* (2). All other chemicals used for this experiments are reagent grade.

Restriction endonuclease reactions

Alu I, Ava I, Hha I, Hpa II, Nae I, Sma I and Xma I endonuclease reaction mixtures contained 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 6mM 2-mercaptoethanol, 0.1 mM EDTA and optimum concentrations of NaCl (KCl for Sma I) with indicated amounts of DNA. In every case, the reaction mixture was incubated at 37°C (25°C for Sma I) for 30 min to 3 hrs with 1 to 10 units of the enzymes.

DNA methylation *in vitro*

For the completion of the Hpa II methylation reaction, prolonged incubation was necessary with an excess amount of enzyme. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 50 μg/m*l* bovine serum albumin, 10 mM 2-mercaptoethanol, 80 μM S-adenosylmethionine, 10 to 20 μg/m*l* of DNA, and 120 units of enzyme in 25 μl. After 3 hrs of incubation at 37°C, 1 μl of 2.5 mM S-adenosylmethionine and 50 units of enzyme were freshly added. And the reaction was continued for 3 additional hours.

Analysis of restriction fragments

To analyze the cleavage product clearly, pUC9 and M13mp9 RF DNAs were treated with Nar I and Bgl II endonucleases, respectively. The resulting linear pUC9 and M13mp9 DNA have one of 5′-CCCGGG-3′ and 5′-GCCGGC-3′, respectively. The analyses of the DNA fragments after digestion with endonucleases were performed on 1% agarose horizontal gels (8 × 9 × 0.3 cm). Electrophoresis was carried out at 60 volts for 3 hrs at room temperature in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). DNA was visualized by staining with 1 μg/m*l* of ethidium bromide for 30 minutes.

RESULTS

Plasmid pUC9 and M13mp9 RF DNAs have 11 and 18 Hpa II sites, respectively. All of these sites were methylated by Hpa II methylase as described in "Methods". The DNAs thus methylated were completely resistant against the cleavage reactions of Hpa II endonuclease (Fig. 1). The results shown in Fig. 1 also indicate that the Hpa II methylase and endonuclease used do not have any considerable amounts of contaminating methylase or nuclease activities, and recognize the same nucleotide sequence with associated specificities as described earlier (6)(7).

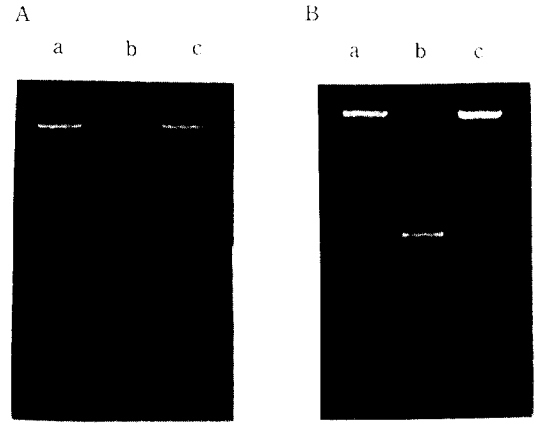

Fig. 1. *DNA methylation by Hpa II methylase. A: Linearized pUC9 DNA methylated by Hpa II methylase was completely resistant to Hpa II endonuclease. The detailed reaction conditions are explained in "Methods". Lane a, methylated DNA by Hpa II methylase. Lane b, unmethylated DNA was digested with 10 units of Hpa II endonuclease for 1.5 hours at 37°C. Lane c, methylated DNA by Hpa II methylase was treated with 10 units of Hpa II endonuclease for 1.5 hours at 37°C. B: The same experiment has been performed as A except that linearized pUC9 DNA was replaced with linearized M13mp9 DNA.*

Sma I, Ava I and Nae I endonucleases can not cleave the DNA methylated by Hpa II methylase

Sma I endonuclease recognize the hexanucleotide sequences 5′-CCC↓GGG-3′, and cleaves at the point indicated by the arrow (3). Since the internal tetrameric sequence, 5′-CCGG-3′, is identical with Hpa II sequence, it was expected that the methylation by Hpa II methylase within the hexameric sequence could inhibit the cleavage reactions by the related hexameric enzymes. The results shown in Fig. 2 indicates that the methylation by Hpa II methylase completely inhibited the cleavage reaction by Sma I

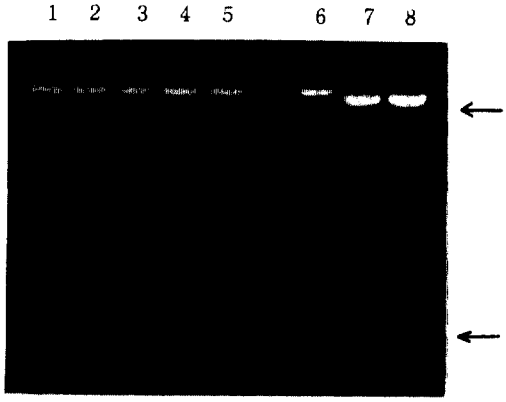

Fig. 2. *Sma I endonuclease activity on the methylated DNA by Hpa II methylase. Sma I endonuclease activity was totally inhibited by the methylation on the inmost cytosine residue in Sma I sequences. The detailed reaction conditions are described in "Methods". Lane 1. methylated pUC9 linear DNA by Hpa II methylase. Lane 2,3,4 and 5, methylated pUC9 linear DNA by Hpa II methylase treated with 10 units of Sma I endonuclease for 30 min, 1 hour, 2 hours and 3 hours, respectively. Lane 6, unmethylated pUC9 linear DNA. Lane 7 and 8, unmethylated pUC9 linear DNA treated with 10 units of Sma I endonuclease for 30 min, 1.5 hours, respectively.*

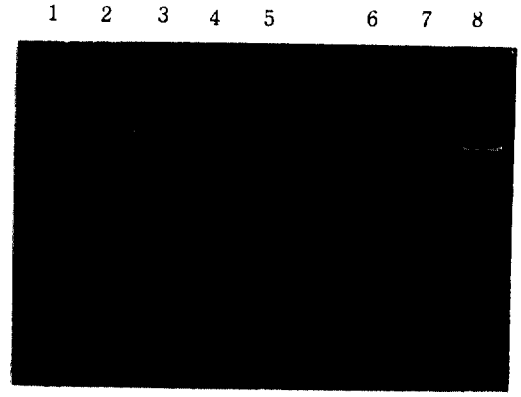

Fig. 3. *Ava I endonuclease activity on the methylated DNA by Hpa II methylase. Ava I endonuclease activity was totally inhibited by the methylation on the inmost cytosine residue in Ava I sequences (note that pUC9 DNA used in this experiment has only one of the two different Ava I sequences which can be methylated by Hpa II methylase). The detailed reaction conditions are described in "Methods". Lane 1, methylated pUC9 linear DNA by Hpa II methylase. Lane 2,3,4, and 5, methylated pUC9 linear DNA by Hpa II methylase treated with 4 units of Ava I endonuclease for 30 min, 1 hour, 2 hours and 3 hours, respectively. Lane 6, unmethylated pUC9 linear DNA. Lane 7 and 8, unmethylated pUC9 linear DNA treated with 4 units of Ava I endonuclease for 30 min, 1.5 hours, respectively.*

endonuclease even after 3 hours of incubation (Fig. 2, lane 5). The position of the two final restriction fragments generated from unmethylated pUC9 linear DNA after treatment with Sma I endonuclease are marked by arrows on the right side of the gel, although the lower band is extremely faint.

The same results have been obtained when Ava I and Nae I endonucleases were used (Fig. 3 and Fig. 4). Ava I and Nae I endonucleases recognize 5′-C↓PyCGPuG-3′ (4) and 5′-GCC↓GGC-3′ (1) respectively, and cleave at the points indicated by the arrows. Among the two kinds of Ava I sequences, 5′-CCCGGG-3′ and 5′-CTCGAG-3′, only the former sequence would be methylated by Hpa II methylase while the later sequence would not be methylated by the enzyme and would be cleaved by Ava I endonuclease. To facilitate the experiment with Ava I endonuclease, linearized pUC9 DNA was used because it has only one Ava I sequence which can be methylated by Hpa II methylase. Linearized pUC9 DNA by Nar I endonuclease generated two fine fragments after the reaction by Ava I endonuclease (Fig. 3). In the case of the experiment with Nae I endonuclease, M13mp9 DNA (linearized by Bgl II endonuclease) which contains one Nae I sequence, was used. Although the enzyme activity of Nae I endonuclease was slow on linearized M13mp9 DNA, the reaction was almost completed after 1.5 hours of incubation of the unmethylated DNA with the enzyme at 37°C (Fig. 4, lane 8). The final two restriction fragments are marked with arrows on the side of the gel. With the DNA methylated by Hpa II methylase, neither Ava I nor Nae I endonuclease could cleave the DNA even after 3 hours of incubation at 37°C (Fig. 3, lane 5 and Fig. 5, lane 5).

Xma I endonuclease was able to cleave the DNA methylated by Hpa II methylase with decreased reaction rate

Xma I endonuclease which is an isoschizomer of Sma I endonuclease recognizes the same hexameric sequence, 5′-C↓CCGGG-3′, but cleaves dif-

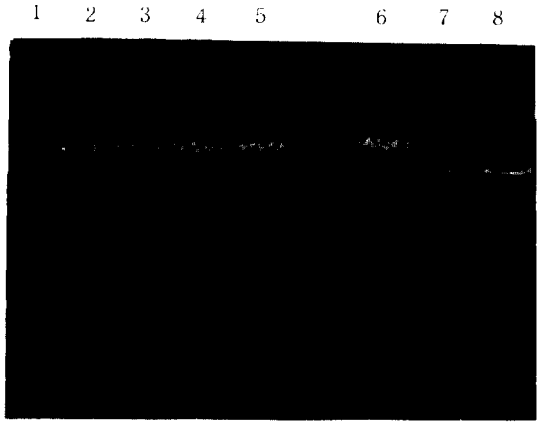

Fig. 4. *Nae I endonuclease activity on the methylated DNA by Hpa II methylase. Nae I endonuclease was totally inhibited by the methylation on the inmost cytosine residue in Nae I sequences. The detailed reaction conditions are described in "Methods". Lane 1, methylated M13mp9 RF linear DNA by Hpa II methylase. Lane 2,3,4, and 5, methylated M13mp9 RF linear DNA by Hpa II methylase treated with 5 units of Nae I endonuclease for 30 min, 1 hour, 2 hours and 3 hours, respectively. Lane 6, unmethylated M13mp9 RF linear DNA by Hha I methylase. Lane 7 and 8, unmethylated M13mp9 RF linear DNA by Hha I methylase treated with 5 units of Nae I endonuclease for 30 min, 2 hours, respectively.*

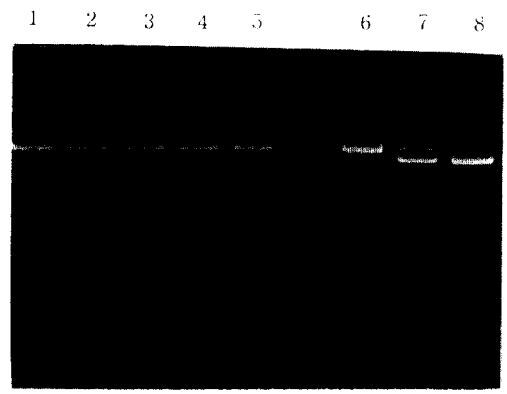

Fig. 5. *Xma I endonuclease activity on the methylated DNA by Hpa II methylase. Xma I endonuclease activity was partially inhibited by the methylation on the inmost cytosine residue in Xma I sequence. Only a fraction of the DNA was cleaved even after 3 hours of incubation (lane 5) indicating that the reaction rate was decreased. The detailed reaction conditions are described in "Methods". Lane 1, methylated pUC9 linear DNA by Hpa II methylase. Lane 2,3,4 and 5, methylated pUC9 linear DNA by Hpa II methylase treated with 1 units of Xma I endonuclease for 30 min, 1 hour, 2 hours and 3 hours, respectively. Lane 6, unmethylated pUC9 linear DNA. Lane 7 and 8, unmethylated pUC9 linear DNA treated with 1 unit of Xma I endonuclease for 30 min and 1.5 hours, respectively.*

ferently. The arrow indicates the cleavage site. The methylation by Hpa II methylase on the inmost cytosine residue within Xma I site greatly protected the sequence against the cleavage reaction of Xma I endoncuelase (Fig. 5). As we can see from Fig. 5 lane 5, only a small fraction of the methylated DNA was cleaved by Xma I endonuclease even after 3 hours of incubation at 37°C. When compared with the reaction rate with the unmethylated DNA (Fig. 5, lane 8), it is clear that the reaction rate with the methylated DNA was greatly decreased.

DISCUSSIONS

DNAs methylated on the inmost cytosine residue by Hpa II methylase within the central tetramer were protected against cleavage reactions by the related hexameric enzymes (Xma I, Ava I, Sma I and Nae I endonucleases) having that central tetrameric core. When treated with Sma I, Ava I or Nae I endonucleases, the DNA methylated by Hpa II methylase was totally resistant against the cleavage reactions (Fig. 2,3, and 4). These results could be the strong evidences that the specific methylation sites of Sma I, Ava I and Nae I methylases which yet to be isolated are the same as the case of Hpa II methylase. However, it cannot be ruled out that a methylation near or within the recognition sequence may have the same effect as the specific methylation upon cleavage reaction of the cognate endonuclease.

The results that Sma I and Nae I endonucleases (note that one of the two Ava I sequences can not be methylated by Hpa II methylase) cannot cleave the DNAs methylated by Hpa II methylase is valuable informations for protecting DNAs upon cleavage reactions by those restriction enzymes. One of the example is as follows. The cDNAs ligated with Sma I or Nae I linker DNAs should be cut with Sma I or Nae I endonuclease before being joined into vector DNAs. In this case Hpa II methylase which yet to be isolated can be used instead of Sma I or Nae I methylase for protecting the cDNAs from the

cleavage reactions.

In contrast to Sma I, Ava I and Nae I endonucleases, Xma I endonuclease could cleave the DNA modified by Hpa II methylase although the reaction rate was considerably decreased (Fig. 5). This result indicates that the methylated cytosine residue inhibits the proper interaction between Xma I endonuclease and its recognition sequence. This result also implies that the specific methylation site of Xma I methylase, which has not been isolated yet, would not be the same as the case of Hpa II methylase. As we published the date previously (5) that Hind III and Sst I endonucleases produced the specifically nicked circular form of DNA when treated with covalently closed form of DNA, Xma I endonuclease exhibited the same property (data not shown). The specifically nicked DNA may be useful for a number of purposes. The nicking reaction could be used in sequence analysis of DNA. A specifically nicked restriction fragment can be separated into three single strands of different lengths unless the nick is introduced precisely in the middle of the strand. This separation is easier to achieve than the separation of single strands of equal length. Therefore 5′ (or 3′) labeling of specifically nicked fragment, followed by strand separation, provides three starting points for sequence analysis, two from the ends and one from the interior of the fragment.

Another possibility to use the nicking reaction may be the introduction of specific gaps in one DNA strand if the reaction is combined with an appropriate exonuclease. These gaps could be used for specific labeling of one DNA strand, for binding of single-strand specific proteins to defined regions of a DNA and for site-specific mutagenesis.

적 요

Hpa Ⅱ methylase로 처리된 DNA는 Sma Ⅰ, Ava Ⅰ 또는 Nae Ⅰ endonuclease에 의해 절단되지 않았다. 이 사실은 아직 발견되지 않은 Sma Ⅰ, Ava Ⅰ, Nae Ⅰ methylase들이 각각의 specific sequence내에서 가장 중앙의 cytosine을 methylation시킬 것이라는 가정을 강력히 뒷받침 해주고 있다. 또한 이 결과는 cDNA cloning 실험에서 linker DNA를 사용할 때에 응용될 수 있는데, 즉 cDNA를 protection하기 위해서 Sma Ⅰ, Ava Ⅰ 또는 Nae Ⅰ methylase를 발견할 필요가 없이 대신 Hpa Ⅱ methylase를 사용할 수 있게 된 것이다. Xma Ⅰ endonuclease는 위의 경우와 달리 Hpa Ⅱ methylase로 처리된 DNA라도 그 반응속도는 느려졌지만 절단할 수 있었다. 이는 Xma Ⅰ methylase가 발견된다면 methylation site는 Xma Ⅰ sequence내의 가장 중앙에 있는 cytosine이 아닌 다른 곳일 것임을 알려주고 있다.

REFERENCES

1. Christoph, K., Peter, S.N. and Werner W., 1985. Recognition sequences of restriction endonucleases and methylases-a review. Gene., **33:** 1-102.
2. Clewell, D. and Helinski, D.R., 1972. Effect of growth conditions on the formation of the relaxation complex of supercoiled Col El deoxyribonucleic acid and protein in *Escherichia coli.* J. Bacteriol., **110:** 1135-1146.
3. Endow, S.A. and Roberts, R.J., 1977. Two restriction-like enzymes from *Xanthomonas malvacearum.* J. Mol. Biol., **112:** 521-529.
4. Hughes, S.G. and Murray, K., 1980. The nucleotide sequences recognized by endonucleases Ava I and Ava II from *Anabaena variabilis.* Biochem. J., **185:** 65-75.
5. Kang, S.C., Yoon, H. and Yoo, O.J., 1986. DNA protection by methylation with Alu I methylase against cleavages of Hind III, Sst I, Pvu II and Sac I endonucleases. Korean Biochem. J., **19:** 41-46.
6. Mann, M.B. and Smith, H.O., 1977. Specificity of Hpa II and Hae III DNA methylases. Nucl. Acids Res., **4:** 4211-4221.
7. Yoo, O.J. and Agarwal, K.L., 1980. Isolation and characterization of two proteins possessing Hpa II methylase activity. J. Biol. Chem., **255:** 6445-6449.

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