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Determination of Methylation Site of DNA-Methyltransferase *NlaX* by a Hybrid Method

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ABSTRACT

Using a new method based on a combination of bisulfite reaction, the repair enzyme uracil-DNA glycosylase, and synthetic oligodeoxyribonucleotides, the methylation site of DNA-methyltransferase *NlaX* (*M·NlaX*) from *Neisseria lactamica* was established to be the inner cytosine in the double-stranded pentanucleotide recognition sequence 5'-CCNGG-3' (where N = any nucleoside). 5-Methylcytosine (*m*⁵C) type modification by *M·NlaX* was confirmed by the use of oligonucleotide substrates that contain 5-fluoro-2'-deoxycytidine.

INTRODUCTION

Type II DNA-methyltransferases are widely investigated because of their protective role in prokaryotic biology and their common features with the eukaryotic methyltransferases regarding the possible mechanisms of DNA-protein interaction (1,8,21). These enzymes methylate the deoxyadenosine (dA) or deoxycytidine (dC) residues in defined double-stranded DNA sequences such as those recognized by restriction endonucleases. Moreover, the dC residues can be modified at either position 4 or 5 in a heterocyclic base (3).

Neisseria lactamica produces at least three methyltransferases, two of which are associated with the cognate restriction endonucleases *NlaIII* and *NlaIV* that recognize the nucleotide sequences 5'-CATG-3' and 5'-GGNN-CC-3' (N = any nucleoside), respectively (9,11). Cloning of the adenine-specific *NlaIII* methyltransferase-encoding gene had identified a potential *m*⁵C DNA-methyltransferase (designated *M·NlaX*), with no apparent counterpart endonuclease (9). The predicted amino

acid sequence of this "orphan" *M·NlaX* showed approximately 66% sequence identity with those of the methyltransferase *SsoII* (*M·SsoII*) and methyltransferase *ScrFI* polypeptides isolated from *Shigella sonnei* and *Streptococcus cremoris*, respectively (7). Although they are of varying size, all three methyltransferases are predicted to recognize the sequence 5'-CCNGG-3'. Evidently, further biochemical studies of methyltransferase *NlaX* will be facilitated by knowing the exact DNA methylation site. Here we undertook an approach that it is easier to find a non-methylated dC than a methylated one in cytosine-type methyltransferases of which the recognition site contains only two dC(s). To this end, we describe a method (Figure 1) by which sodium bisulfite was used to efficiently convert dC to deoxyuridine (dU), leaving 5-methyl-2'-deoxycytidine and N4-methyl-2'-deoxycytidine residues unchanged (22). The dU residue is subsequently detected by a repair enzyme, uracil-DNA glycosylase (UDG). In this manner, we established the methylation site of *M·NlaX*.

MATERIALS AND METHODS

Enzymes and Chemicals

The recombinant *M·SsoII*, *M·NlaX*, and restriction endonuclease *SsoII* (*R·SsoII*), each containing an amino-terminal His₆ sequence as an affinity tag, were purified using a two-step procedure involving heparin-sepharose and Ni-NTA-agarose chromatography (7). *E. coli* UDG was purchased from New England Biolabs (Beverly, MA, USA), bacteriophage T4 polynucleotide kinase from SibEnzyme (Novosibirsk, Russia), sodium metabisulfite from Merck KGaA (Darmstadt, Germany), hydroquinone from Sigma (St. Louis, MO, USA), and [γ -³²P]ATP from Isotope (Obninsk, Russia). All other chemicals were analytical-grade commercial products.

Oligonucleotide Preparation

The oligonucleotides were synthesized on a model 380B DNA synthesizer (Applied Biosystems, Foster City,

CA, USA) using standard phosphoramidite chemistry. Oligonucleotides containing 5-fluoro-2'-deoxycytidine (f⁵dC) were produced as described previously (24). Labeling of oligonucleotides at their 5'-terminal were carried out using [γ -³²P]ATP and T4 polynucleotide kinase. Oligonucleotides were annealed by mixing an equimolar amount of the radiolabeled strand with the complementary unlabeled strand. Samples were heated at 90°C in a water bath for 5 min and then left to cool slowly to ambient temperature.

Protection of Synthetic DNA Substrate against *R·SsoII* Digestion

³²P-labeled substrate (3.5 pmol) after annealing by thermal denaturation and renaturation were incubated with 10 U *M·NlaX* or *M·SsoII* in 10 μ L buffer containing 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 7 mM 2-mercaptoethanol, and 0.1 mM S-adenosyl-L-methionine (AdoMet) for 2 h at 37°C. After the reaction, LiClO₄ aqueous solution was added to a final concentration of 1.8 M. DNA was precipitated by acetone, dried, diluted in 50 mM Tris-HCl buffer, pH 7.6, containing 50 mM NaCl, 10 mM MgCl₂, 7 mM 2-mercaptoethanol, and treated with 10 U *R·SsoII* for 1 h at 37°C. The reaction mixtures were diluted in loading buffer A [80% (v/v) aqueous formamide solution containing 1 M EDTA, 0.1% (w/v) xylene cyanol, and 0.1% (w/v) bromophenol blue] and analyzed in a 7 M urea-20% polyacrylamide gel (10), followed by autoradiography.

Bisulfite Conversion Reaction

Aliquots (100 μ L) of a 5 M bisulfite solution (2.5 M sodium metabisulfite, 125 mM hydroquinone, pH 5.0) were added to ³²P-labeled methylated oligonucleotide (10–100 nM). The reaction mixture was incubated for 4 h at 50°C in the dark. The sample was purified using a desalting column (NAP-5; Amersham Biosciences, Piscataway, NJ, USA), eluted in 1 mL distilled water and evaporated to 60 μ L. Freshly prepared NaOH (final concentration, 0.3 M) was added, and the sample was incubated at 37°C for 15 min. The solution was neutralized by the addition of

NH₄OAc, pH 7.0, to 3 M, and the DNA was ethanol-precipitated and dried. The uracil removal was performed in 10 μ L 20 mM Tris-HCl buffer (pH 7.5), 60 mM NaCl, 10 mM EDTA, 0.5 mg/mL BSA. Two units of activity of UDG from *E. coli* were added, and the reaction mixture was incubated at 37°C for 1 h. This solution was dried, and 40 μ L 10% aqueous piperidine were added. After incubation at 90°C for 30 min, piperidine was removed by evaporation on a SpeedVac® (Savant, Holbrook, NY, USA). Traces of piperidine were removed by re-evaporation with water. The DNA fragments were diluted in loading buffer A, and hydrolysis products were analyzed by electrophoresis in a 7 M urea-20% polyacrylamide gel, followed by autoradiography.

Cross-Linking Assay

The cross-linking reactions of methyltransferase (approximately 4 μ g) with 2 pmol ³²P-labeled (in modified strand)

duplex were performed in 20 μ L buffer containing 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 7 mM 2-mercaptoethanol, and 0.1 mM AdoMet. The reactions were incubated at 37°C for 1 h, followed by electrophoresis in a 0.1% SDS-12% polyacrylamide gel (10). Before electrophoresis, samples were diluted and heated for 10 min at 95°C in denaturing loading buffer B [0.0625 M Tris-HCl, pH 6.8, 7 M urea, 10% (v/v) glycerol, 2% SDS, and 0.05% β -mercaptoethanol]. The formation of the oligonucleotide-protein conjugate was detected by autoradiography.

RESULTS AND DISCUSSION

In a previous study, *M-NlaX*-methylated DNA was found to be protected against cleavage by a non-cognate R-*SsoII* (7), which has the recognition sequence of 5'-CCNGG-3' (14). It was assumed that *M-NlaX* recognizes a target sequence very similar if not identi-

Figure 1. Outline of the method for determination of non-methylated dC residue. The steps involved in discriminating alternative site of cytosine methylation (meC) are described in the text. p* signifies end-radiolabeled phosphate in a synthetic oligonucleotide.

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cal to that of *M·SsoII*. To confirm this, two 14-membered DNA duplexes were assembled and labeled after the respective synthesis of the oligonucleotides. Duplex I, of sequence:

5'-AGAGCCAGGTTGGC-3'
3'-TCTCGGTTCCAACCG-5'

contains an A/T base pair (italicized) in the central position of the CCNGG sequence. Duplex II contains a G/C bp, of sequence:

5'-AGAGCCGGGTTGGC-3'
3'-TCTCGGCCCAACCG-5'.

The labeled DNA duplexes I and II were methylated by *M·NlaX* or *M·SsoII*. Incubation of both duplexes I and II with *M·NlaX* in the presence of cofactor AdoMet resulted in full protection of these substrates against hydrolysis by *R·SsoII* (Figure 2). Thus, as in the case of *M·SsoII* (Figure 2), both sequences: 5'-CCA/TGG-3' and 5'-CCG/CGG-3' are recognized by *M·NlaX*.

Which of the two dC residues in the CCNGG recognition sequence does *M·NlaX* methylate? Several approaches have been used for the definition of methylated residue. These include degradation of methylated DNA to small oligonucleotides followed by the identification of methylated fragments and their sequence (2,13), labeling of

substrate DNA with tritium (within methyl groups), cleavage by nuclease and detection of the tritium label within different oligonucleotide fragments (5,15), and the use of MALDI-TOF mass spectrometry (20).

Here we employed an alternative approach that consists of detection of non-methylated dC residue in the recognition site (Figure 1). It relies on the ability of sodium bisulfite to convert dC residues to dU efficiently in ssDNA, under conditions whereby 5-methyl-2'-deoxycytidine and N4-methyl-2'-deoxycytidine residues remain essentially non-reactive (22). The bisulfite reaction together with PCR is usually used in the genomic sequencing technique for the evaluation of the methylation status of CdG islands in DNA and for the mapping of all methylated dC residues in a known nucleotide sequence (4,6). The mechanisms of bisulfite reactions include (i) the addition of bisulfite to the 5-6 double bond of cytosine, (ii) hydrolytic deamination of the resulting cytosine-bisulfite derivative to give an uracil-bisulfite derivative, and (iii) the removal of the sulfonate group by a subsequent alkali treatment to give uracil (18,19,23). The dU residue is easy to detect using repair enzyme UDG. UDG

Figure 2. Methylation assays. Protection of methylated duplexes against *R·SsoII* digestion is seen in lanes 2 and 3. Lanes 1, undigested control of the DNA duplexes. (A) ^{32}P -label in the upper strand of duplex I; (B) ^{32}P -label in the lower strand of duplex I; (C) ^{32}P -label in the upper strand of duplex II; (D) ^{32}P -label in the lower strand of duplex II. Lanes 2 and 3, treatment of duplexes I and II by *M·NlaX* and *M·SsoII*, respectively, plus *R·SsoII*. Lanes 4, non-methylated duplex I and II plus *R·SsoII*. BPB, position of bromophenol blue.

removes uracil with formation of apyrimidinic site in DNA that efficiently hydrolyzes in alkali conditions.

An essential feature of this new method is the design of substrates that do not have dC residues in the 5'-se-

quence flanking the methyltransferase recognition site. To facilitate the separation of the substrate strands in subsequent steps of the experiment, one strand of the DNA duplex should be several nucleotides longer than the other. For this application, DNA duplex III of the following sequence was synthesized as a substrate:

5'-AGAGCCAGGTTGGC-3'
3'-AGTCTCGGTCCAACCGAG-5'.

Accordingly, DNA duplex III containing ^{32}P -label at the 5'-ends was twice incubated with *M·NlaX* to obtain a maximum amount of methylated molecules and to avoid the formation of possibly hemimethylated products. The reaction mixture was treated by *R·SsoII* to remove non-methylated duplex III (step 1). The strands of DNA duplex were separated by electrophoresis in a polyacrylamide gel under denaturing conditions (step 2). The methylated oligonucleotides were isolated from the gel, treated by sodium bisulfite, desalted, and incubated with alkali (step 3). The dU-containing oligonucleotide thus obtained was incubated with UDG and cleaved by 10% aqueous piperidine solution at the place of apyrimidinic site (step 4). The reaction mixture was separated by gel electrophoresis under the denaturing conditions (step 5). To determine the length of the resultant DNA fragment, the 14-membered strand of duplex III was sequenced by the Maxam-Gilbert technique (12) and used as a marker. The accuracy of our method was confirmed by performing the same experiment using *M·SsoII* as a methylating agent. *M·SsoII* is known to methylate the C5 atom of the inner dC residue of its recognition site (14). As a result, oligonucleotide fragments of the same length were found in either case using *M·SsoII* or *M·NlaX* (Figure 3). The fragments corresponded to cleavage of the duplex III 14-membered strand at the position of the first dC residue in the 5'-CCNGG-3' recognition sequence. Taking into account that only two positions for methylation are present in this recognition site, one can conclude that the inner dC is methylated by both enzymes.

To confirm this result, DNA duplexes IV and V were synthesized in which the inner dC in one of the strands of the recognition sequence was replaced

Figure 3. Size determination of an oligonucleotide fragment in a DNA sequencing gel. Lanes 2–5 are Maxam-Gilbert sequencing reactions (12,16) that serve as marker size. Lane 1 (control), initial non-methylated 14-membered strand of duplex III after treatment by piperidine; lanes 6 and 8 (controls), initial non-methylated 14-membered strand of the same duplex after treatment by sodium bisulfite, UDG, and piperidine; lanes 7 and 9, methylated 14-membered strand of duplex III by *M·NlaX* and *M·SsoII*, respectively, and treatment by sodium bisulfite, UDG, and piperidine solution.

Figure 4. Crosslinking of DNA duplex and methylases. Panel A: duplex IV; Panel B: duplex V. Lanes 1, untreated DNA duplexes IV or V. Lanes 2 and 3, analysis of reaction mixtures after incubation of fl⁵dU-containing DNA duplexes with *M·SsoII* and *M·NlaX*, respectively.

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with a fl⁵dC residue. Because of the amino acid sequence homology of M·NlaX and M·SsoII, we suspected that M·NlaX would methylate the C5 position of the inner cytosine.

Duplex IV:

5'-AGAG**C**FAGGTTGGC-3'

3'-AGTCTCGGTCCAACCGAG-5'

Duplex V:

5'-AGAGCCAGGTTGGC-3'

3'-AGTCTCGGT**F**CAACCGAG-5'
(where **F** = fl⁵dC).

The model of catalysis by m⁵C-methyltransferases suggests that methyl transfer to C5 is essential for making a covalent complex between the enzyme and a substrate. The replacement of methylated deoxycytidine by fl⁵dC results in the formation of an irreversible protein-DNA complex (17). The covalent complex thus formed can be detected easily by 0.1% SDS-PAGE (10).

The incubation of M·NlaX and

M·SsoII with duplexes IV or V in the presence of AdoMet resulted in the formation of the covalent enzyme-substrate complex shown in Figure 4. Thus, this experiment not only served as a positive control demonstrating that the inner dC is methylated by M·NlaX but also permitted the identification of the site cytosine methylation. Hence, these data confirmed the notion that M·NlaX is an m⁵C-methyltransferase.

A lower yield of the cross-linked product in the case of M·NlaX as compared to M·SsoII might be attributed to a lower M·NlaX activity, perhaps as a result of a distinct difference of the two enzymes at the N-terminus. M·NlaX lacks an N-terminal, approximately 70-amino acid residue of M·SsoII, which is needed for its regulatory function (7). M·NlaX can be considered as a natural deleted derivative of M·SsoII. A comprehensive kinetic and thermo-

dynamic study of M·NlaX and M·SsoII interaction with DNA will be performed in the future.

CONCLUSIONS

First, the methylation and position specificity of M·NlaX was established experimentally instead of relying on prediction. The results indicate that, in the double-stranded 5'-CCNGG-3' pentanucleotide sequence, M·NlaX methylates the inner dC residue at the fifth position of the heterocycle. Second, a simple approach of bisulfite reaction in combination with repair enzyme UDG was developed, a technique that could be applied to identifying non-methylated dC residue in recognition sequences that contain two dC residues (e.g., 5'-CCGG-3', 5'-CGCG-3', 5'-GGCC-3', 5'-CCNGG-3', 5'-GGNCC-3', etc.).

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**E.A. Kubareva, J. Walter¹,
A.S. Karyagina², O.V.
Vorob'eva, P.C.K. Lau³,
and T. Trautner¹**

*Moscow State University
Moscow, Russia*

¹*Max-Planck-Institut für
Molekulare Genetic
Berlin, Germany*

²*Institute of Agricultural
Biotechnology*

Moscow, Russia and

³*National Research Council
Canada
Montreal, Quebec, Canada*

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