J|A|C|S
COMMUNICATIONS

Published on Web 08/08/2002

Site-Specific Discrimination of Cytosine and 5-Methylcytosine in Duplex DNA by Peptide Nucleic Acids

Akimitsu Okamoto, Kazuhito Tanabe, and Isao Saito*

Department of Synthetic Chemistry and Biological Chemistry, Faculty of Engineering, Kyoto University, and SORST, Japan Science and Technology Corporation, Kyoto 606-8501, Japan

Received April 10, 2002

5-Methylcytosine (^{m}C) is involved in the regulation of gene expression and gene silencing.[1] Moreover, ^{m}C is believed to cause about one-third of all transition mutations responsible for human genetic diseases and cancer.[2] Therefore, it has become clinically important to know the methylation status at specific sites in genomic DNA. There are many reports mapping methylation patterns in genomic DNA using the Maxam and Gilbert chemical modification[3] or the polymerase chain reaction (PCR) after sodium bisulfite-mediated conversion of cytosine to uracil.[4] However, these protocols for the detection of cytosine methylation require complicated and tedious procedures. In addition, the resulting data contain too much information on the cytosine methylation of whole DNA sequences for effective analysis of their functions. Therefore, the development of a simple and convenient method for the site-specific discrimination of cytosine methylation is imperative for genomic studies.

Here we report a simple method for the discrimination of cytosine and ^{m}C in duplex DNA using peptide nucleic acids (PNAs).[5] To our knowledge, this is the first direct method for detecting methylated cytosines site-specifically in duplex DNA. We developed the detection method using a complex produced by PNA-assisted DNA displacement in combination with fluorescence resonance energy transfer (FRET). After treatment of the complex with a restriction enzyme, strong fluorescence emission was observed for the complex containing C (C-complex) in the target sequence, whereas the fluorescence intensity for the complex containing ^{m}C (^{m}C-complex) was extremely weak.

The protocol for site-specific discrimination of C and ^{m}C is outlined in Figure 1. Duplex DNA containing C at the target site was incubated with two complementary PNAs to displace the DNA strand, and the DNA target site was displaced as a "P-loop" [6] (complex I in Figure 1). Subsequently, a fluorescent probe oligonucleotide (PO), with fluorescein attached to the 5′ end and a dabsyl group to the 3′ end as a quencher, was added to hybridize with the P-loop to produce a fluorescently labeled duplex, "PD-loop" [7] (complex II in Figure 1). The resulting complex was further treated with a restriction enzyme. The duplex containing C at the target site should produce a strong fluorescence emission when the PO is digested, whereas little or no fluorescence emission should be observed with the duplex containing ^{m}C at the target site, because ^{m}C inhibits digestion by the restriction enzyme.[8]

Initially, we investigated an 80mer DNA duplex containing C (**DNA 1**/**DNA 3**) or ^{m}C (**DNA 2**/**DNA 3**) at the *Hha*I recognition site (5′-G**X**GC-3′, **X** = C or ^{m}C). DNA and PNA oligomers used in this study are summarized in Table 1. According to the protocol described in Figure 1, complex II was prepared with **PO 1** and **PNA 1**, and the fluorescence emission resulting from the enzymatic digestion of complex II was monitored. Figure 2 shows the time

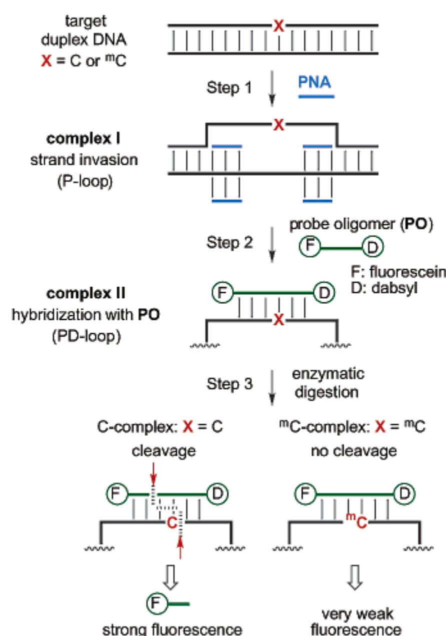

Figure 1. Protocol for site-specific discrimination of cytosine (C) and 5-methylcytosine (^{m}C) in duplex DNA. Step 1: strand invasion of target duplex DNA by PNAs. Step 2: hybridization of displaced DNA strand with probe oligomer (**PO**). Step 3: specific cleavage by restriction enzyme. "F" and "D" of PO denote fluorescein and dabsyl groups, respectively.

course for the fluorescence intensity of complex II treated with *Hha*I. The fluorescence emission for the C-complex increased remarkably with increasing incubation time. On the other hand, fluorescence emission for the ^{m}C-complex was very weak. These results suggest that it is possible to judge the methylation status of cytosine at specific sites in duplex DNA by fluorescence microscopic analysis of an *Hha*I-treated fluorescein-containing complex.

Digestion of the complex II with a different restriction enzyme was also examined. *Hap*II restriction enzyme cleaves the 5′-CCGG-3′ sequence, and the cleavage is suppressed by the presence of ^{m}C at the recognition site.[9] According to the protocol described above, we examined the discrimination of cytosine and ^{m}C using the PD-loop complex system on 80mer duplex DNA (**DNA 4**/**DNA 6** and **DNA 5**/**DNA 6**) containing 5′-C**X**GG-3′ (**X** = C for **DNA 4**, or ^{m}C for **DNA 5**). As a result of *Hap*II digestion of complex II containing **PO 2** and **PNA 1**, strong fluorescence derived from the cleavage of PO was observed for 5′-CCGG-3′, whereas the fluorescence was very weak for 5′-C^{m}CGG-3′, as was observed for *Hha*I digestion.

We next tested the site-specific detection of ^{m}C in a 72mer duplex DNA fragment (**DNA 7**/**DNA 9** for the C-complex, and **DNA 8**/**DNA 9** for the ^{m}C-complex), which corresponds to codons 270−

* Address correspondence to this author. E-mail: saito@sbchem.kyoto-u.ac.jp.

10.1021/ja0264955 CCC: $22.00 © 2002 American Chemical Society

Table 1. DNA and PNA Oligomers Used in This Study

Target Duplex DNAs[a]	
5′-(22mer)GG*TTTTTTTTTT*GTCA**GXGC**ATGG*TTTTTTTTTT*AG(22mer)-3′	
DNA 1: **X** = C; **DNA 2**: **X** = mC	
DNA 3: complementary strand of **DNA 1** and **DNA 2**	
5′-(22mer)GG*TTTTTTTTTT*GTAC**CXGG**GATG*TTTTTTTTTT*AG(22mer)-3′	
DNA 4: X = C; **DNA 5: X** = mC	
DNA 6: complementary strand of **DNA 4** and **DNA 5**	
5′-(18mer)TT*CTCTTCCTCT*GT**GXGC**CG*GTCTCTCCCA*GG(22mer)-3′	
DNA 7: X = C; **DNA 8: X** = mC	
DNA 9: complementary strand of **DNA 7** and **DNA 8**	
Probe Oligonucleotides (POs)[a,b]	
PO 1	5′-FAAACCAT**GCGC**TGACAAAD-3′
PO 2	5′-FAAACATC**CCGG**GTACAAAD-3′
PO 3	5′-FGACCG**GCGC**ACAGAD-3′
Peptide Nucleic Acids (PNAs)[c]	
PNA 1	H-TTTTTTTTTT-NH_2
PNA 2	H−CTCTTCCTCT-NH_2
PNA 3	H-GTCTCTCCCA-NH_2

[a] Recognition sites of restriction enzymes *Hha*I and *Hap*II are shown in bold, and PNA-binding sites are in italic. [b] "F" and "D" denote fluorescein and dabsyl, respectively. [c] In **PNA 1−3**, "H" denotes a free amine end, and "NH_2" denotes a carboxamide end.

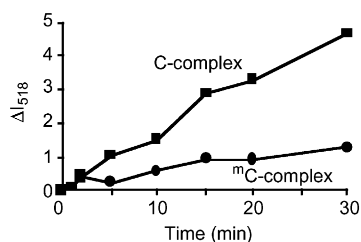

Figure 2. Change in fluorescence intensity (ΔI) of the complex II treated with *Hha*I. Fluorescence was monitored at 518 nm. Fluorescence intensities are designated (■) for C-complex (**DNA 1**/**DNA 3**/**PNA 1**/**PO 1**) and (●) for mC-complex (**DNA 2**/**DNA 3**/**PNA 1**/**PO 1**). Conditions used were 90 nM target duplex DNA, 18 μM PNA, 900 nM PO, 10 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 10 mM DTT, 50 mM sodium chloride, 10 units of enzyme, and an incubation time of 60 min at 37 °C.

294 of exon 8 in the human p53 gene.[10] The target 5′-GCGC-3′ site on the duplex DNA is one of the sequences where cytosine methylation has been observed. By treating complex II containing **PO 3**, **PNA 2**, and **PNA 3** with the restriction enzyme *Hha*I, the fluorescence emission of the C-complex was enhanced with increasing incubation time, whereas the fluorescence intensity for the mC-complex was considerably weaker than that for the C-complex. Fluorescence microscopic analysis of these *Hha*I-treated mixtures was carried out in agarose gel to avoid diffusion of the fluorescent products.[11] Figure 3 shows the fluorescence image of the gel piece. In the gel containing the C-complex, strong green fluorescence was observed (Figure 3a), whereas only faint fluorescence was detectable for gel containing the mC-complex (Figure 3b). Thus, the fluorescence experiment indicates that site-specific detection of mC in a target DNA sequence is feasible by detection of the fluorescence produced from cleavage of the PO by an appropriate restriction enzyme.

As compared with currently available methods for evaluating the methylation status of DNA,[3,4] a major advantage of our method

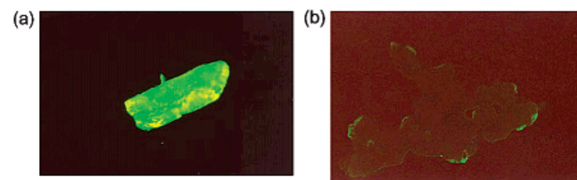

Figure 3. Microscopic observation of the gel slice containing the *Hha*I-digested complex to detect cytosine methylation. (a) Fluorescence micrograph of the C-complex produced from **DNA 4**/**DNA 6**. (b) mC-complex produced from **DNA 5**/**DNA 6**.

is that cytosine methylation can be detected optically without time-consuming procedures such as duplex denaturation and electrophoresis, although the general utility of our methods is limited by the sequence restrictions imposed on the target sequences and the well-described limitations for duplex invasion by PNAs.[7,12]

In summary, we have developed a simple method for site-specific detection of mC in duplex DNA using PNA. Strong fluorescence was detected by the enzymatic digestion of the C-complex, whereas the digestion of the mC-complex produced only a weak fluorescence. Site-specific discrimination of cytosine methylation using the PD-loop complex is extremely simple compared with other mC detection methods. This method provides a useful tool for analyzing DNA methylation.

Supporting Information Available: Experimental procedures and spectral data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Jones, P. A.; Takai, D. *Science* **2001**, *293*, 1068−1070. (b) Martienssen, R. A.; Colot, V. *Science* **2001**, *293*, 1070−1074. (c) Cogoni, C. *Annu. Rev. Microbiol.* **2001**, *55*, 381−406. (d) Paszkowski, J.; Whitham, S. *Curr. Opin. Plant Biol.* **2001**, *4*, 123−129 and references therein.
(2) Spruck, C. H., III; Rideout, W. M., III; Jones, P. A. In *DNA Methylation: Molecular Biology and Biological Significance*; Jost, J. P., Saluz H. P., Eds.; Birkhäuser Verlag: Basel, Switzerland, 1993; pp 487−509. (b) Esteller, M.; Garcia-Foncillas, J.; Andion, E.; Goodman, S. N.; Hidalgo, O. F.; Vanaclocha, V.; Baylin, S. B.; Herman, J. G. *N. Engl. J. Med.* **2000**, *343*, 1350−1354. (c) Robertson, K. D. *Oncogene* **2001**, *20*, 3139−3155.
(3) Church, G. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1991−1995.
(4) Frommer, M.; McDonald, L. E.; Millar, D. S.; Collis, C. M.; Watt, F.; Grigg, G. W.; Molloy, P. L.; Paul, C. L. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 1827−1831. (b) Herman, H. G.; Graff, J. R.; Myöhänen, S.; Nelkin, B. D.; Baylin, S. B. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9821−9826.
(5) For a recent review, see: Nielsen, P. E. *Acc. Chem. Res.* **1999**, *32*, 624−630.
(6) Smulevitch, S. V.; Simmons, C. G.; Norton, J. C.; Corey, D. R. *Nat. Biotechnol.* **1996**, *14*, 566−568. (b) Bukanov, N. O.; Demidov, V. V.; Nielsen, P. E.; Frank-Kamenetskii, M. D. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5516−5520.
(7) For a recent report on the PD-loop, see: Kuhn, H.; Demidov, V. V.; Coull, J. M.; Fiandaca, M. J.; Gildea, B. D.; Frank-Kamenetskii, M. D. *J. Am. Chem. Soc.* **2002**, *124*, 1097−1103.
(8) Ehrlich, M.; Wang, R. Y.-H. *Science* **1981**, *212*, 1350−1357. (b) Korch, C.; Hagblom, P. *Eur. J. Biochem.* **1986**, *161*, 519−524.
(9) Butkus, V.; Petrauskiene, L.; Maneliene, Z.; Klimasauskas, S.; Laucys, V.; Janulaitis, A. *Nucleic Acids Res.* **1987**, *15*, 7091−7102.
(10) Tornaletti, S.; Pfeifer, G. P. *Oncogene* **1995**, *10*, 1493−1499.
(11) Hashimoto, S.; Wang, B.; Hecht, S. M. *J. Am. Chem. Soc.* **2001**, *123*, 7437−7438.
(12) Kurakin, A.; Larsen, H. J.; Nielsen, P. E. *Chem. Biol.* **1998**, *5*, 81−89. (b) Kuhn, H.; Demidov, V. V.; Nielsen, P. E.; Frank-Kamenetskii, M. D. *J. Mol. Biol.* **1999**, *286*, 1337−1345.

JA0264955