Sequence-selective osmium oxidation of DNA: efficient distinction between 5-methylcytosine and cytosine†

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5-Methylcytosine was distinguished from cytosine using the large difference of their osmium oxidation rates, and this reaction was applied to detection of the cytosine methylation status at a specific site of a long sequence using the formation of a bulge structure by hybridization with a guide DNA.

5-Methylcytosine (M) is a common modified pyrimidine base, which frequently appears in genomic DNA, particularly in CpG sequences, and plays a key role in epigenetic events, which strongly affect the control of gene expression and cell differentiation.[1] Analysis of the cytosine methylation status of a gene is very important for understanding the expression mechanism of genetic information. However, to distinguish M from C, *i.e.*, to detect the existence of only one methyl group in a long DNA strand, is not easy. For evaluation of the methylation status of genes, several methods showing a C-positive and M-negative signal have been used,[2] such as a cleavage assay with methylation-insensitive restriction enzymes[3] and bisulfite DNA sequencing.[4] In these cases, primer design and complete modification of DNA are very significant in order to avoid false-positives for M and thus to obtain reliable results. The existence of a more rapid and selective chemical reaction capable of distinguishing between M and C would be promising as a useful method for efficiently analyzing the status of cytosine methylation at a specific site in a gene. The reactions of pyrimidine bases T and C are well known, such as photodimerization,[5] Michael addition,[6] and oxidation,[7] but the number of reports on the chemistry of M is still limited. When we find an effective condition for M-selective reaction, this method would give full play to its ability for sequence-selective epigenotyping.

In this paper, we report M-selective oxidation. M was oxidized efficiently by exposure to a reaction mixture containing an osmium complex, making possible a clear distinction from very weak oxidation of C. This result is applicable to a novel method for the sequence-selective typing of cytosine methylation status using a bulge formation at the target site (Fig. 1).

We initially prepared a ^{32}P-labelled oligodeoxynucleotide, 5′-^{32}P-d(AAAAAAGNGAAAAAA)-3′ (**ODN(N)**, N = M or C), that possessed a single pyrimidine base. **ODN(N)** was added to a mixture (Os-mix) of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), and 100 mM bipyridine in 100 mM Tris–HCl buffer (pH = 7.7), 1 mM EDTA, and 10% acetonitrile,

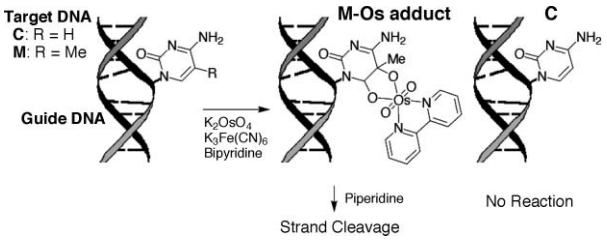

Fig. 1 Schematic illustration of a sequence-selective oxidation of 5-methylcytosine (M) using a single base bulge formation.

and then the reaction mixture was incubated at 0 °C for 5 min. The oxidized strand was cleaved at a damaged pyrimidine base with hot piperidine (90 °C, 20 min), and the products were analyzed as a band for a shortened strand using polyacrylamide gel electrophoresis. The result for the reaction of **ODN(N)** is shown in Fig. 2. The strand cleavage at C of **ODN(C)** was negligible (lane 1), whereas **ODN(M)** was sequence-selectively cleaved at M (lane 2). The MALDI-TOF MS data of **ODN(M)** treated with Os-mix suggested the formation of an adduct in which the osmium complex is attached to **ODN(M)**.[8] This adduct was converted into a product in which M was oxidized to 5-methylcytosine glycol, when treated with sodium sulfite.[9] The osmium oxidation of a nucleoside, 5-methyl-2′-deoxycytidine, was also carried out, and the production of 5-methyl-2′-deoxycytidine *cis*-glycol was confirmed by NMR and mass spectra.[10]

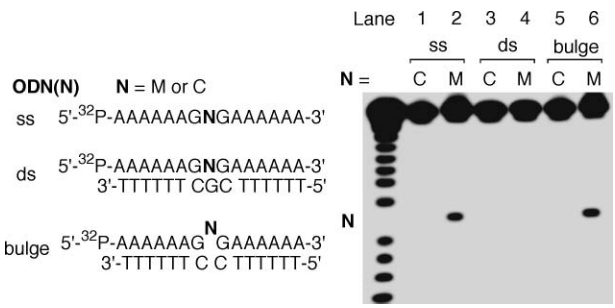

Fig. 2 Sequence of ^{32}P-labeled **ODN(N)** and PAGE analysis of strand cleavage through Os oxidation and hot piperidine treatment. **ODN(N)** was incubated in a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris–HCl buffer (pH = 7.7) and 10% acetonitrile at 0 °C for 5 min, and then treated with hot piperidine (90 °C, 20 min). The products were analyzed using polyacrylamide gel electrophoresis. Lane 1, single strand, N = C; lane 2, single strand, N = M; lane 3, full-matched duplex, N = C; lane 4, full-matched duplex, N = M; lane 5, bulged duplex, N = C; lane 6, bulged duplex, N = M.

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On the other hand, the duplex formation strongly suppressed M oxidation. We examined the reaction for the hybrid of **ODN(M)** and the complementary strand 5′-d(TTTTTTCGCTTTTTT)-3′. The strand cleavage of the duplex was negligible, which is quite different from the effective cleavage of single-stranded **ODN(M)** (lanes 3 and 4). This weak reactivity is probably attributed to inhibition of the attack of an osmium complex on the π-orbital of a C5–C6 double bond by the base stacking of the duplex structure.

The modulation of oxidation efficiency by DNA conformational transition strongly suggests that a bulge structure formation, *i.e.*, partial single strand formation, at a target site would induce a sequence-selective M oxidation (Fig. 1). We hybridized **ODN(N)** with a single nucleotide-shortened strand, 5′-d(TTTTTTCCTTTTTT)-3′ (T_m = 18 °C). By treatment of the bulged DNA duplex with Os-mix at 0 °C for 5 min, efficient strand cleavage was observed at the bulged M site of **ODN(M)**, like the reaction for a single-stranded **ODN(M)** (lanes 5 and 6 in Fig. 2). This result is applicable to sequence-selective oxidation of a DNA sequence containing multiple methylation sites. The p53 DNA fragment containing two target sites was prepared[11] and hybridized with two types of "guide DNAs", which were designed for forming a sequence-specific bulge structure at each target site (Fig. 3a). Treatment of the DNA duplexes with Os-mix resulted in the strand cleavage at a bulged M site of each hybrid (target1 for guide1 and target2 for guide2). In contrast, a cleavage band did not appear at a non-bulged M site, which was protected completely from oxidation.

The oxidation efficiency of M-bulged duplex in Os-mix is quite different from that of the C-bulged duplex. The oxidation of these bulged duplexes observed for the first minute was fitted to a first-order rate equation. The calculated rate constants for M-bulged and C-bulged duplexes were 1.11×10^{-2} and 2.51×10^{-5} s^{-1}, respectively. Electron donation to a C5–C6 double bond by a methyl group strongly contributes to efficient reaction for the M base. It has been reported that the calculated and experimental ionization potentials of M are much lower than those of C.[12] Additionally, we ascertained (through optimizing the reaction conditions) that the addition of bipyridine as a ligand to an osmium ion and potassium hexacyanoferrate(III) as an osmium activator to a reaction mixture is essential for acceleration of the reaction.[13]

M-Selective strand modification will be very effective for the epigenotyping of genes. We prepared a short fragment of p53 gene sequence containing a methylation hot spot that causes a C-to-T transition mutation related to a colon, breast, and hematological cancer (60 nt, 100 fmol),[11] and tested an epigenotyping assay using the protocol described above (Fig. 3b).[14] For this assay, we also prepared a guide DNA fixed on polystyrene beads to facilitate the protocol. After generation of the bulged structure by hybridization of the target DNA with the guide DNA, the mixture was incubated in Os-mix at 0 °C for 5 min, and then washed with buffer and 7 M urea. After the recovered DNA strand was treated with hot piperidine, the DNA amplification was monitored with real-time PCR using a TaqMan probe. As a result, a sigmoid curve showing efficient DNA amplification was observed for a non-methylated sequence (**p53(C)$_{bulge}$**). This is similar to the behavior of the amplification curve for a full-matched duplex (**p53(M)$_{fullmatch}$**). In contrast, a curve with a retarded start and a small curvature was displayed for a methylated sequence in 40 PCR cycles (**p53(M)$_{bulge}$**), and this is completely distinguishable from the control curve. We

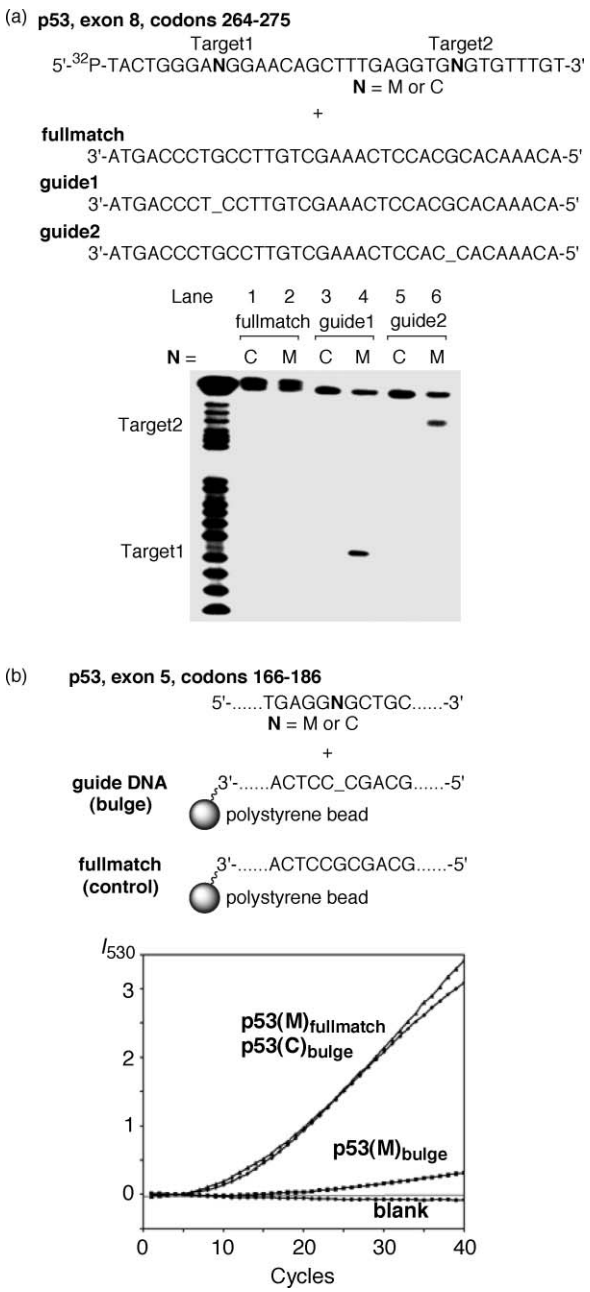

Fig. 3 (a) Sequences for a sequence-specific oxidation study and a polyacrylamide gel electrophoresis analysis of the reaction. The same condition as described in Fig. 1 was used for the reactions. (b) Real-time PCR amplification. DNA samples were incubated in a solution of 10 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris–HCl buffer (pH = 7.7) and 10% acetonitrile at 0 °C for 5 min. The target DNA was removed from supports by washing with 7 M urea, and then treated with hot piperidine (90 °C, 20 min). The process of PCR amplification of the treated DNA samples was monitored by the fluorescence of the TaqMan probe (λ_{exc} = 470 nm, λ_{em} = 530 nm). PCR amplifications were performed, using 2 μL of 1 μM TaqMan probe and 5 units of HotStarTaq DNA polymerase, over 40 cycles at 94 °C for 0.5 min, to 50 °C for 0.5 min, to 72 °C for 1 min.

easily and efficiently achieved the detection of the existence of only one methyl group in a long DNA strand using the combination of sequence-specific osmium oxidation and PCR amplification.

In conclusion, we have described M-selective oxidation and its application to epigenotyping. M was efficiently and easily modified through osmium oxidation. M-Selective oxidation is applicable to detection of the cytosine methylation status at a specific site of a long sequence using the formation of a bulge structure by hybridization with a guide DNA. This is a conceptually new M-positive epigenotyping method, and will serve as an alternative method for the epigenotyping of a very small amount of gene sample.

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8 [(**ODN(M)** + OsO_4(Bpy)–H)$^-$], calcd 5067.54, found 5067.92; [(**ODN(M)**+OsO_3(Bpy)–H)$^-$], calcd 5051.54, found 5051.36; [(**ODN(M)** + OsO_2(Bpy)–H)$^-$], calcd 5035.54, found 5035.15.

9 [(**ODN(M)** + 2OH–H)$^-$], calcd 4691.14, found 4691.96.

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13 Refer to the the ESI.

14 A color version of Fig. 3b is available the ESI.

