Site-specific Transition of Cytosine to Uracil via Reversible DNA Photoligation

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We report that deamination coupled with 5-carboxyvinyldeoxyuridine-mediated photobranching causes the heat-induced transition of cytosine to uracil with high efficiency without any side reaction.

The development of a technique for artificial site-specific mutation will be a useful genetic tool for preparing non-native proteins. The mutation of nucleic acid bases is also caused by intrinsic factors such as sunlight and ultraviolet light. In living cells, transition of C is also caused by the formation of pyrimidine dimer in the 5'-CT-3', 5'-TC-3' and 5'-CC-3' sequence by UV irradiation coupled with enzymatic repair. The deamination of cytosine in cyclobutane pyrimidine dimers was assigned as a key step in these transitions based on the reduced half-life of deamination to a matter of hours when the 5,6 bond is saturated compared to 30,000 years' half-life for the deamination of normal monomeric cytosine. While many kinetic and biological studies on such deamination have already been reported, a method for C to U transition based on this deamination process with site-specificity has not been reported. We have recently reported a template-directed DNA photoligation mediated by 5-vinyldeoxyuridine (VU) via [2+2] cycloaddition between the U base and terminal C. In fact, the rapid deamination of the photoligated dimer in an enzymatic digestion process has been also observed in this study. We have also reported the photochemical synthesis of branched oligodeoxynucleotides (ODNs) via 5-carboxyvinyldeoxyuridine (CVU). We therefore report here the artificial site-specific transition from cytosine to uracil mediated by reversible DNA photoligation. The efficient, selective transition of cytosine to uracil was achieved by photochernical branched ODN formation at the target cytosine and the successive photosplitting of the branched ODN after heat treatment.

CVU-containing ODN was synthesized according to standard phosphoramidite chemistry on a DNA synthesizer using phosphoramidite of methyl ester of CVU coupled with the post-modification procedure after ODN synthesis (Scheme 1). Figure 1 shows a schematic chart for the method for site-selective transition from cytosine to uracil and the detection system of uracil as a cleavage band in PAGE analysis. The photoradiation of cytosine-containing ODN at 366 nm while addressing the CVU-containing hairpin template ODN introduced the branch structure at the target cytosine via [2+2] cycloaddition between the CVU base and the target cytosine. The deamination of the target cytosine was promoted site-specifically with heat treatment to produce a uracil analogue in ligated ODN. After heat treatment, uracil-containing ODN was produced by photosplitting at 302-nm irradiation. The uracil base produced in ODN was removed with uracil DNA glycosylase (UDG), and the resulting abasic site was converted into strand breaks with piperidine treatment, which was detected by PAGE analysis (Fig. 1). When 32P-5'-end-labeled 11mer 5'-
The rate constant for deamination at 37 °C was similar to that at 1.8 orders of magnitude greater than that of 2.66 for deamination. This acceleration in the rate constant for deamination at 37 °C was not conducted (Fig. 2, lane 6). These results clearly indicate that the cleaved band seen in lane 5 was caused by the transformation from cytosine to uracil at the target site via the deamination of the C<>U photoadduct by heating at 90 °C and photosplitting at 302 nm. This method for the transition of cytosine to uracil proceeded effectively at every step. Uracil formation was also directly confirmed by the HPLC analysis of enzymatic digestion of isolated ligated 17mer after heat treatment.

The temperature dependency of this deamination process of C<>U-containing ODN 3 was examined in duplex and C<>C dimer-containing ODN in duplex and C<>C dimer-containing ODN in duplex. The effect of pH for deamination was also examined at 37 °C. However no difference was observed in its rate constant for deamination at pH 5.0, pH 7.0 and pH 9.0. This indicates that the protonation of N3 of the cytosine analogue and attack by water or hydroxyl at C4 of the cytosine analogue in this addition elimination pathway of deamination might also play an important role in the kinetics of deamination. These results indicate that the cytosine residue in the C<>CVU photoadduct was easily transformed to uracil residue at higher temperatures.7

The temperature dependency of this deamination process of C<>CVU photoadduct-containing ODN 3 was examined in duplex in order to understand the deamination kinetics. The deamination reaction was conducted at 37 °C and 90 °C, and the efficiency was estimated by quantifying the intensity of the cleaved band after UDG treatment as described above. The rate constant for deamination was 2.75 x 10⁸ at 90 °C, pH 7.0, which was two orders of magnitude greater than that of 2.66 x 10⁸ at 37 °C, pH 7.0 for deamination.13 This acceleration in the rate constant for deamination is likely attributed not only to a gain in its activation energy for deamination but also to the increased accessibility of the cytosine analogue in denatured DNA to protons and hydroxyl ions. The rate constant for deamination at 37 °C was similar to that at 1.8 x 10⁸ observed in the CCCC target sequence containing d(TGTGAAAAAA)-3’ (ODN 1) and 21mer 5’-d(UCGGTGTTTTACAACGACA)-3’ (ODN 2) were irradiated at 366 nm for 2 h (Fig. 1), the expected ligated 32mer ODN 3 was produced at a 98% yield as determined by the densitometric assay of PAGE (Fig. 2, lane 2). After heating at 90 °C for 2 h (Fig. 2, lane 3), quantitative photosplitting was achieved by 302-nm irradiation for 1 h (Fig. 2, lane 4). A cleavage band was observed by piperidine treatment after enzymatic base excision using UDG after photosplitting (Fig. 2, lane 5).

The mobility of this band was equal to the band that was produced after strand cleavage at the abasic sites by the UDG treatment of authentic deoxyuridine-containing ODN 4 (Fig. 2, lane 8). This cleaved band was not observed when the heating of photoligated ODN 3 at 90 °C was not conducted (Fig. 2, lane 6). These results indicated that the cleaved band seen in lane 5 was caused by the transformation from cytosine to uracil at the target site via the deamination of the C<>CVU photoadduct by heating at 90 °C and photosplitting at 302 nm. This method for the transition of cytosine to uracil proceeded effectively at every step. Uracil formation was also directly confirmed by the HPLC analysis of enzymatic digestion of isolated ligated 17mer after heat treatment.

We further demonstrate that deamination coupled with C<>U-mediated photobranching can be carried out when ODN containing two consecutive cytosines is used as a target sequence. When 32P-5’-end-labeled 20mer 5’-d(TGGCTACGAGCCAACAACAA) (ODN 5) and 27mer 5’-d(UGCGTGTITTCAACCGAGTCTGTA GCCA) (ODN 6) were irradiated at 366 nm for 3 h, the expected ligated 47mer ODN 7 was produced in 80% yield as determined by densitometric assay of PAGE (Fig. 3, lane 2). After heating at 90 °C for 2 h (Fig. 3, lane 3), quantitative photosplitting was achieved by 302-nm irradiation for 1 h (Fig. 3, lane 4). A cleavage band was observed by piperidine treatment after enzymatic base excision using UDG after photosplitting (Fig. 3, lane 5). The mobility of this band was equal to the band that was produced after the strand cleavage at the abasic sites produced by UDG treatment of authentic deoxyuridine-containing ODN 8 (Fig. 3, lane 8). This cleaved band was not observed when heating of photoligated ODN 7 at 90 °C was not carried out (Fig. 3, lane 6) or when piperidine treatment was carried out without heating.
out without UDG treatment after photodigestion (Fig. 3, lane 9). These results clearly indicated that the cleaved band seen in lane 5 was caused by the transformation from cytosine to uracil at the target site via deamination of C<>U photoadduct by heating at 90 °C and photodigestion at 302 nm. It was also found that an efficient transformation from cytosine to uracil occurred at the target cytosine at eleventh position with site-selectivity. These deamination at C at eleventh position and C at twelfth position were 88% and 12% yield, respectively.

The heat-induced deamination of the cytosine residue of a cyclobutane dimer followed by splitting of the photolyase1,2,3 or photoreactivation4,5,6,7 has thus far been known as a method for converting cytosine to uracil. However, these methods are not site-specific. Our deamination method using reversible DNA photodigestion has high sequence selectivity and efficiency at the target cytosine compared with other methods for converting cytosine to uracil using enzymatic or chemical procedures.7,8,9

In summary, we have demonstrated that deamination coupled with CVU-mediated photodigestion causes the heat-induced transition of cytosine to uracil with high efficiency without any side reaction. The present site-specific C to U transition can be widely used for the site-specific mutation of DNA. This method may also find application in mutation not only for DNA but also for messenger RNA.

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Notes and references

5 T. Lindahl and B. Nyberg, Biochemistry, 1974, 13, 3405.
15 Incorporation of CVU into oligonucleotides was confirmed by enzymatic digestion and MALDI–TOF–MS analysis. MALDI–TOF–MS: calecd. for ODN 2 [C<sub>20</sub>H<sub>32</sub>N<sub>4</sub>O<sub>13</sub>P<sub>2</sub>] [(M–H)] 6452.25; found 6452.60.
17 The rate constant (k) for deamination of C<>CVU photoadduct in the ligation site can be calculated from equation (1) where x is the fraction of cytosine converted into uracil which is determined from the intensities of the cleaved bands and t is time of incubation.

\[ k = -\ln(1-x)/t \]