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A Unique Unnatural Base Pair Between a C Analogue, Pseudoisocytosine, and an A Analogue, 6-Methoxypurine, in Replication

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Abstract—Pseudoisocytidine, a C-nucleoside analogue of cytosine, has two possible isomers of the H1- and H3-forms. Enzymatic incorporation experiments confirmed the existence of the two isomers in solution, and the 2′-deoxyribonucleoside triphosphate of pseudoisocytosine (PIC) was incorporated into DNA opposite both guanine and 6-methoxypurine (M) by the Klenow fragment of *Escherichia coli* DNA polymerase I. In addition to the PIC•M pairing in replication, M also functioned as an A analogue and T was efficiently incorporated opposite M. Thus, the PIC•M pair is regarded as a base pair between a C analogue and an A analogue, and can mediate the interconversion between the G•C and A•T base pairs. The combination of PIC and M could be used as a G•C↔A•T transition mutagen.

The nucleoside of pseudoisocytidine (PIC) has been developed as a candidate for an antileukemic compound and functions as a cytidine analogue that is subject to neither enzymatic cleavage of the glycosidic bond nor enzymatic deamination.[1–4] Apart from its antibiotic activity, PIC has a unique structural property and has another possible isomer of the H3-form, as well as the C analogue of the H1-form (Fig. 1a). Actually, as the H3-form, PIC has been used to stabilize triple-strand formation with the G•C pair.[5] Although we examined the structure of PIC by NMR, the two isomers were not identified clearly. We have now examined these isomers of PIC by enzymatic incorporation experiments using DNA templates containing guanine and 6-methoxypurine (M) as the pairing partners with the PIC H1- and H3-forms, respectively. This PIC•M pair is interesting, because the nucleoside of M, as well as O^6-methylguanosine,[6,7] may function as an A analogue, and the PIC•M pair is, in other words, a base pair between the C analogue and the A analogue (Fig. 1b). This suggests that the PIC•M pair can mediate the interconversion between the G•C and A•T base pairs (Fig. 1b), and thus we examined the PIC•M pair as a potential mutagen.

The 2′-deoxyribonucleoside of PIC was synthesized according to the literature,[2,5] and was converted to the 2′-deoxyribonucleoside 5′-triphosphate (dPIC-TP). The molar absorption coefficient of dPIC-TP (4.6×10^3 at 264 nm) at pH 7.0 was determined by quantitative analysis of the phosphorus in the compound. The 2′-deoxynucleoside of M was synthesized from 2′-deoxyinosine,[8] and was converted to the amidite for synthesis of DNA templates. Under alkaline conditions with concentrated ammonia at 60 °C for 8 h, the nucleoside of M was transformed to 2′-deoxyinosine and 2′-deoxyadenosine in ~10% and ~53% yields, respectively. Thus, for DNA synthesis, base-labile protection, using phenoxyacetyl for A, *p*-isopropylphenoxyacetyl for G, and acetyl groups for C, was used (Glen Research),[9] and

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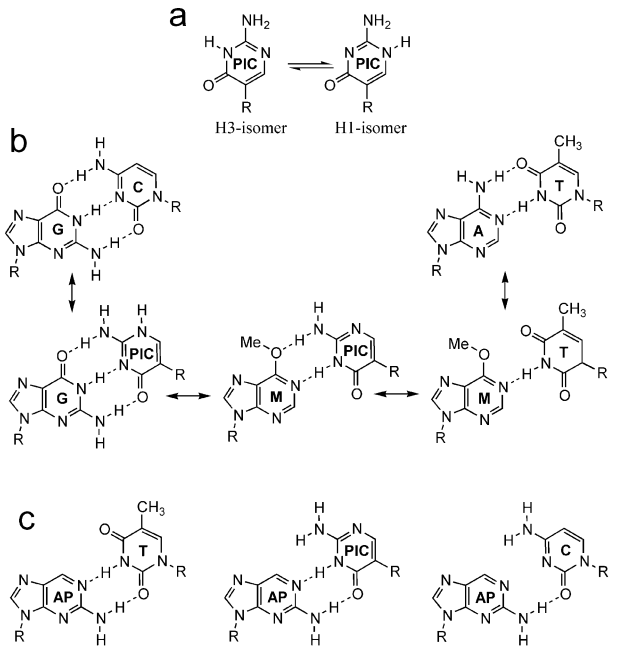

Figure 1. The two isomers of PIC and the PIC•M pair. The H3- and H1-forms of PIC (a). The interconversion between the C•G and T•A pairs through the PIC•M pair (b). Unnatural T•AP, PIC•AP, and C•AP pairs (c). R in the pyrimidine analogues indicates 2′-deoxyribose 5′-triphosphate, and R in the purine analogues indicates ribose in DNA templates.

the deprotection was carried out by a treatment with concentrated ammonia at room temperature for 2 h.

The efficiency of the incorporation of PIC into DNA was assessed by the steady-state kinetics of single-nucleotide insertion experiments.[10,11] The exonuclease-deficient Klenow fragment (Amersham USB) was used with combinations between nucleoside triphosphates and double-stranded templates (35-mer) with a ^{32}P-labeled primer (20-mer), in which various bases in the templates were adjacent to the 3′ end of the primer. As a pairing partner of PIC, we also used 2-aminopurine (AP),[12–15] which would pair with the H3-isomer of PIC through two hydrogen bonds, and compared the insertion efficiency of the PIC•AP pairing with those of the T•AP and C•AP pairings (Fig. 1c). The single-nucleotide insertion products were analyzed by gel electrophoresis after an incubation at 37 °C. The insertion efficiency of each substrate opposite each base in the template is shown in Table 1. The incorporation efficiency (V_{max}/K_M) correlated well with the number of hydrogen bonds in the base pair (Table 1 and Fig. 1). The only exception was the T•M pairing, which has a relatively high incorporation efficiency (1.8×10^5) as compared to other single-hydrogen-bonded base pairs, such as the C•M and C•AP pairs. This might reflect the good shape fitting between the pairing bases.[16,17]

The correlation between the number of hydrogen bonds and the incorporation efficiency of the base pairings indicated the existence of the two PIC isomers, and specific incorporation was observed in each of the PIC•M and PIC•G pairings (Table 1). The triphosphate of PIC was incorporated into DNA opposite M with a relatively high incorporation efficiency ($V_{max}/K_M = 6.0\times10^4$). In addition, PIC was incorporated opposite G with similar efficiency (7.6×10^6) to the C•G pairing (9.9×10^6). The PIC•AP pairing (2.1×10^4) is less effective than the T•AP pairing (2.0×10^5), but more effective than the C•AP pairing (1.0×10^4), indicating the partial existence of the H3-form of PIC. In addition, efficient pairing between T and M was observed. The incorporations of T (1.8×10^5) and PIC (6.0×10^4) opposite M were superior to that of C opposite M (1.8×10^3). Thus, M functions as an A analogue rather than a G analogue, and PIC behaves as the C analogue, but the isomer of PIC enables the PIC•M pairing in replication.

Next, to examine whether the extension is continued after the incorporation of PIC opposite M, we carried out the primer extension using the intrinsic 3′-exonuclease proficient Klenow fragment (Takara). As shown in Fig. 2, the polymerase efficiently continued the extension after the incorporation of either PIC or T opposite M (Fig. 2, lanes 7 and 8). In the absence of dCTP, the full-length product (29-mer) was obtained (Fig. 2, lane 8), indicating that the extension could continue after the incorporation of PIC opposite G at position 28. On the other hand, the extension after the C•A or PIC•A pairing was much less efficient (Fig. 2, lanes 2 and 4) as compared to that of the natural T•A pairing (Fig. 2, lane 3).

Table 1. Steady-state kinetic parameters for insertion of single nucleotides into a template-primer duplex by the exonuclease-deficient Klenow fragment

dN′TP

primer 20-mer 5′-^{32}P-ACTCACTATAGGGAGGAAGA

template 35-mer 3′-TATTATGCTGAGTGATATCCCTCCTTCTNTCTCGW **(W=A or T)**

Nucleoside triphosphate (N′)	Template base (N)	Number of H-bonds	K_M (μM)	V_{max} (% min^{-1})	Efficiency (V_{max}/K_M)
C	G	3	0.9 (0.5)	8.9 (2.7)	9.9×10^6
PIC	G	3	1.7 (0.7)	13 (5)	7.6×10^6
T	A	2	1.6 (0.6)	1.7 (0.3)	1.1×10^6
T	AP	2	290 (34)	57 (10)	2.0×10^5
PIC	M	2	480 (180)	29 (10)	6.0×10^4
PIC	AP	2	800 (130)	17 (2)	2.1×10^4
T	M	1	290 (95)	52 (19)	1.8×10^5
C	AP	1	700 (170)	7.1 (1.5)	1.0×10^4
C	M	1	830 (250)	1.5 (0.7)	1.8×10^3

Parenthetic values are standard deviations.

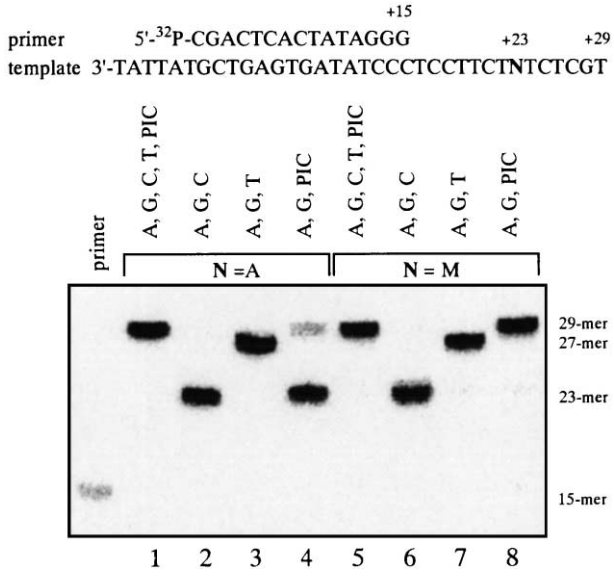

Figure 2. Autoradiograms of denaturing polyacrylamide gels showing primer extensions by the Klenow fragment (exo^+). The extension was carried out at 17 °C for 15 min using 50 nM polymerase.

Similarly, the primer extension was paused after the insertion of C opposite M (Fig. 2, lane 6). These results show the efficient processibility involving the formation of PIC•M, PIC•G, and T•M base pairs, in agreement with the results of the single-nucleotide insertion experiments.

Here, we have demonstrated the base pair formation between the C analogue and the A analogue. This unique property results from the two H1- and H3-isomers of PIC. The natural A•T and G•C pairs could cross-talk with each other through the PIC•M pair (Fig. 1b). Thus, the combination of PIC and M could be used as a potential G•C↔A•T transition mutagen. The nucleotide of 2-aminopurine (AP) is known as this type of mutagen, and can pair with both T and C.[12,18–20] As shown in Table 1, the incorporation experiments show that AP functions as an A analogue, rather than a G analogue, and the incorporation of C opposite AP ($V_{max}/K_M = 1.0\times10^4$) is less efficient than that of PIC opposite M (6.0×10^4). Thus, as compared to AP, the combination of PIC and M would be advantageous for changing the GC contents of genomes and nucleic acid libraries for in vivo and in vitro mutagenesis. Furthermore, the GC contents of the libraries might be controlled by adjusting the ratio between the triphosphates of PIC and M.

References and Notes

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