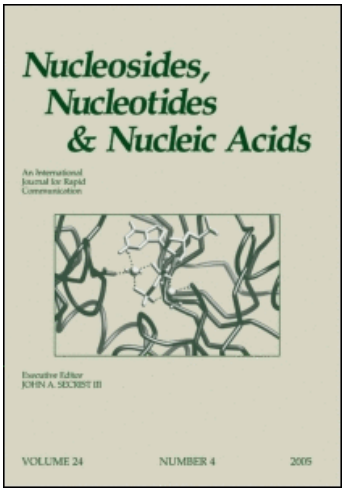

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RECOGNITION OF 2'-DEOXYISOINOSINE TRIPHOSPHATE BY THE KLENOW FRAGMENT OF DNA POLYMERASE I

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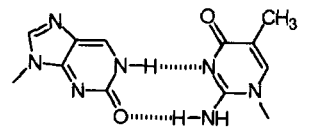
ABSTRACT : The capability of the Klenow fragment deficient in 3'→5' exonuclease activity (KFexo$^-$) to incorporate 2'-deoxyisoinosine (disoI) opposite canonical bases and 5-methyl-2-deoxyisocytidine (d^{5Me}isoC) was examined. The triphosphate of disoI is incorporated opposite d^{5Me}isoC designed as its pairing partner. It is also incorporated opposite T and G according to a pairing scheme involving its minor tautomeric form (the O^{2}H tautomer).

INTRODUCTION

Alternative base pairing schemes hold great promise in basic and applied fields. One trend is to develop additional base pairs to diversify the coding and catalytic capacities of nucleic acids. Along this line, specific copying of pyrimidine and purine analogs[1-3] as well as of non hydrogen-bonding pairs of aromatic substituents of bases[4,5] were shown to occur in replication reactions. A second trend is to substitute a base in one or several of the four natural pairs by an unnatural partner so as to modulate the recognition of canonical bases. Along this line, ambiguous partners able to pair to several canonical bases are being explored in several laboratories[6-10] and specific partners with improved pairing stability with G and T[11] were described.

We have previously evaluated the stability of isoI/^{5Me}isoC base pair[12] by thermal denaturation experiments performed on heptadecanucleotides containing at the central position either isoI/^{5Me}isoC or any combination of isoI with natural bases.[13] The non-canonical base pair was found to be as stable as an A/T pair, which is consistent with the base pairing initially postulated (Figure 1).

As does isoG,[14] isoI can adopt two tautomeric forms in solution, the N^{1}H major one (lactam) and the O^{2}H minor one (lactim).[15] The relative stability of T/isoI and G/isoI pairs can be interpreted by a pairing mode involving either the lactam or the lactim form



isoI ^{5Me}isoC

Figure 1

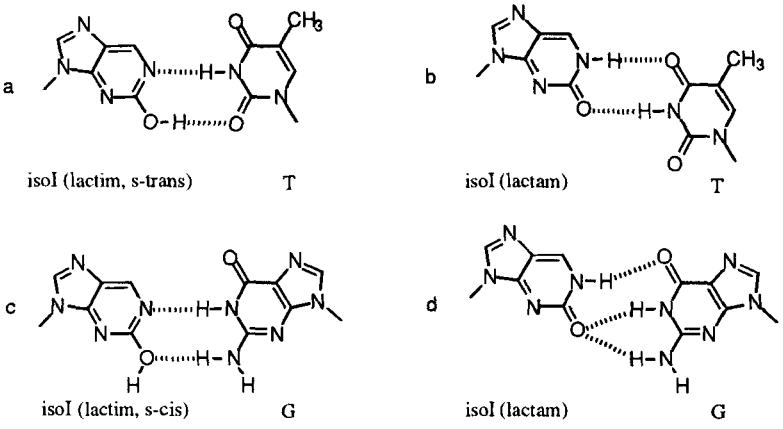


Figure 2: Possible isoI:T and isoI:G base pair configurations.

according to Watson-Crick or wobble pairing mode (Figure 2). This ambiguous base pairing makes isoI a potential mutator tool.

Here we present the results of our investigations on the pairing abilities of isoI and natural bases during replication using the Klenow fragment of DNA polymerase I deficient in 3'→5' exonuclease activity (KFexo$^-$). The ability of KFexo$^-$ to incorporate disoITP opposite ^{5Me}isoC or canonical bases was investigated. Whether canonical triphosphates are incorporated opposite isoI in a DNA template was also tested.

RESULTS AND DISCUSSION

The synthesis of 2'-deoxyisoinosine 5'-triphosphate (disoITP) was performed *via* the corresponding 5'-monophosphate. Phosphorylation of 3'-*O*-acetyl-2'-deoxyisoinosine using 2-cyanoethylphosphate and DCC[16] in pyridine resulted in a low yield of isolated compound. Therefore 3'-*O*-acetyl-2'-deoxyisoinosine was treated with 2-cyanoethyl-*N*,*N*-

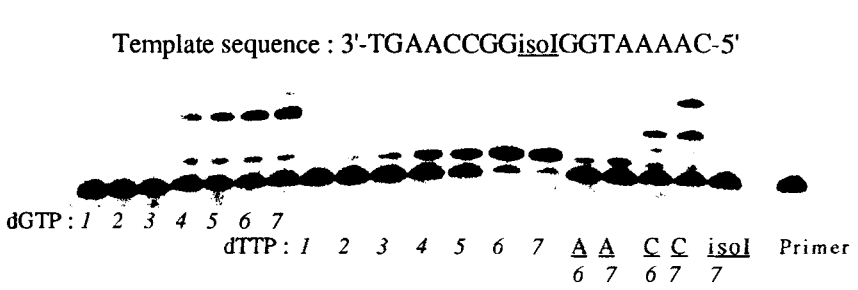


Figure 3 : Primer extension catalyzed by KFexo⁻. Deoxytriphosphates present are indicated below with variable concentrations :*1* : 100 nM, *2* : 500 nM, *3* : 1 μM, *4* : 5 μM, *5* : 10 μM, *6* : 100 μM, *7* : 1 mM. A, C, isoI indicate dATP, dCTP and disoITP.

diisopropylchlorophosphoramidite in the presence of DIEA in dry dichloromethane. After 1 h, 1*H*-tetrazole was added to the reaction mixture, followed 20 min later by aqueous iodine solution. The resulting phosphodiester[17] was isolated in 53% yield after purification by chromatography on C18 column. Treatment with a 1% solution of sodium methylate in methanol afforded disoIMP in 81% yield.[18] The triphosphate was obtained by condensation of disoIMP as morpholidate[19] (prepared according to reported procedures[20]) with pyrophosphate in DMF. The triphosphate was purified by anion-exchange chromatography on DEAE-cellulose, and isolated as sodium salt on cation-exchange resin (Dowex 50X8, Na^+ form). The pure triphosphate was characterized by NMR and mass spectrometry.[21]

The DNA templates and primer were synthesized on a Expedite Millipore synthesizer and purified by reverse phase HPLC. The primer (5'-ACTTGGCC-3') was labeled at the 5'-end with Redivue [γ-^{32}P]ATP using T4 polynucleotide kinase, then annealed with different templates (17-mers) containing either isoI, ^{5Me}isoC or natural bases at the 9th position. Primer elongation experiments catalyzed by KFexo⁻ were performed at 23 °C using isoI or natural bases as incoming triphosphates.[22]

Firstly, we investigated the possible misincorporation of natural nucleotides opposite isoI in a DNA template (Figure 3). When KFexo⁻ was incubated in the presence of dGTP, a +3 product was formed with little pausing at position +1 (opposite isoI), however the elongation was partial (c.a. 30%) even at high concentration (1 mM). In the presence of dTTP, a +1 product was detected at 1 μM with a complete elongation of the primer at 100 μM. When high concentrations of dATP or dCTP were used, traces of elongated products were detected. No incorporation of isoI face to itself was observed. When higher concentrations of triphosphates and KFexo⁻ were used, the same tendency (T>G>A,C) was observed with higher efficiency of incorporation (data not shown).

Template sequence :
3'-TGAACCGGisoIGGTAAAAC-5'

Figure 4 : Primer extension catalyzed by KFexo⁻.
Deoxytriphosphates present (50μM) are indicated below, lanes 1: dTTP, 2: dTTP +dCTP,
3: dGTP+dCTP, 4: dGTP+ dCTP+dATP,
5: dTTP+dATP+ dCTP.

In an other seriès of experiments,[23] we examined primer elongation using the same template in the presence of more than one dNTP. Using a mixture of dTTP and dCTP (Figure 4, lane 2), KFexo⁻ elongated the primer past the non-canonical base with a first pause at the position +3 (elongation stopped one base before T in the absence of dATP), the +8 product corresponds to further elongation after misincorporation opposite next T until 16-mer. When dTTP, dCTP and dATP were added (lane 5), similar result was obtained: KFexo⁻ was able to elongate the primer without truncated products except a +1 product, suggesting that the probable isoI/A mismatch was not further elongated by KFexo⁻ neither was the isoI/C mismatch (lanes 3 and 4).

Thereafter, we investigated the misincorporation of disoITP opposite standard bases in a DNA template. Incorporation of the correct base was performed for comparison. When KFexo⁻ was incubated in the presence of disoITP with a labeled primer/template containing T at position +1 (Figure 5b), elongation was observed at 5 μM; at a higher concentration, a +2 product arised from misincorporation of isoI opposite next G as compared to the misincorporation of A opposite G (Figure 5a). No elongation of the primer was observed in the case of template containing A, C, G or isoI at position +1, even at high concentrations of disoITP (1 mM) (data not shown). The misincorporation of disoGTP opposite T in a template by various polymerases has also been reported.[24]

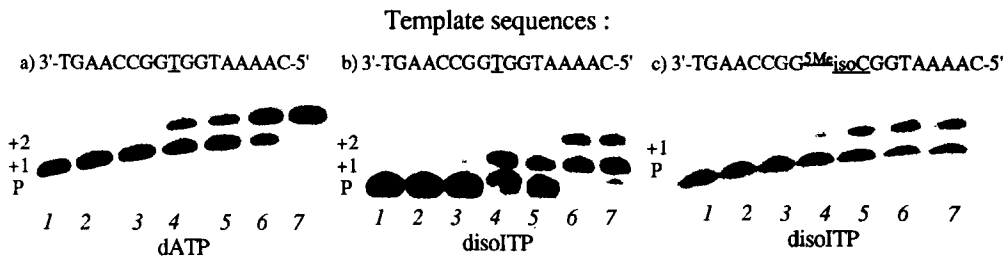


Figure 5 : Primer elongation catalyzed by KFexo⁻. Deoxytriphosphate concentrations are: *1* = 100 nM, *2* = 500 nM, *3* = 1 μM, *4* = 5 μM, *5* = 10 μM, *6* = 100 μM, *7* = 1 mM.

Finally, we examined the incorporation of disoITP opposite ^{5Me}isoC in a DNA template (Figure 5c). As expected, disoITP was incorporated opposite ^{5Me}isoC in a range of concentration comparable to the concentration of disoITP face to T, the incorporation of a non standard base remaining lower than that of canonical base.

CONCLUSION

These results show that the Klenow fragment of DNA pol I accepts isoI as an incoming triphosphate as well as a base in a template. According to the postulated pairing scheme, disoITP is incorporated opposite ^{5Me}isoC in a template. Misincorporation of disoITP opposite T is also observed but with a lower efficiency than the correct base (dATP). The Klenow fragment of DNA pol I is also able to misincorporate dTTP opposite isoI in a template with an efficiency of the same order than the misincorporation of disoITP face to T. The isoI/T base pair can be explained by a Watson-Crick pairing scheme implying the minor tautomeric form of isoI as well as a wobble pairing mode involving the lactam form of isoI. Both of these pairing modes imply two hydrogen bonds. Whereas the G/U mispair adopts a wobble geometry, the isoG/U has the apparent tendency to adopt Watson-Crick geometry.[25] Analogously, isoI might adopt the lactim form for pairing with T. Misincorporations of dGTP opposite isoI in a template, as well as dATP and dCTP with a lower efficiency, are also observed. When standard triphosphates are used together, a template containing the non natural base isoI can be copied by KFexo⁻ affording a full-length reaction product without substantial stop in the DNA synthesis.

These results show that isoI could be used in DNA replication experiments. Misincorporations of dTTP as well as dGTP opposite isoI in a template suggest that isoI

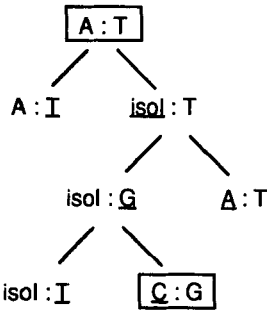


Figure 6: Proposed mutagenic pathways of the base analog isoI through semi-conservative replication. The underlined base is the one incorporated as triphosphate.

might have a mutagenic effect and could induce A→C transitions (Figure 6). Recognition of isoI/^{5Me}isoC base pair by other polymerases still remains to be explored.

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17. Rf (iPrOH/NH_4OH/H_2O : 7/1/2) : 0.30; Rt (0-20% acetonitrile in 10 mM TEAA over 20 min.) : 12.1 min.; ^1H NMR (D_2O) : 2.13 (s, 3H, $COCH_3$); 2.75 (m, 4H, CH_2CN, H2' and H2"); 3.16 (q, 2H, OCH_2); 3.97 (m, 2H, H5' and H5"); 4.10 (m, 1H, H4'); 5.49 (m, 1H, H3'); 6.40 (dd, J = 6.1 Hz, J = 8.2 Hz, 1H, H1'); 8.46 (s, 1H, H8); 8.51 (s, 1H, H6); ^{13}C NMR (D_2O) : 19.8 (OCH_2); 21.0 ($COCH_3$); 37.1 (C2'); 61.1 (d, J = 4.7 Hz, C5'); 66.0 ($\underline{C}H_2CN$); 75.8 (C3'); 83.9 (C1'); 84.3 (d, J = 8.5 Hz, C4'); 119.8 (CN); 124.7 (C5); 139.3 (C6); 147.1 (C8); 158.4 (C2); 159.6 (C4); 174.0 (CO); ^{31}P NMR (D_2O) : 0.23.
18. Rf (iPrOH/NH_4OH/H_2O : 7/1/2) : 0.15; Rt (0-20% acetonitrile in 10 mM TEAA over 20 min.) : 6.7 min.; ^1H NMR (D_2O) : 2.51 and 2.73 (2m, 2x1H, H2' and H2"); 3.99 (m, 2H, H5' and H5"); 4.22 (m, 1H, H4'); 4.69 (m, 1H, H3'); 6.36 (t, J = 6,9 Hz, 1H, H1'); 8.44 (s, 1H, H8); 8.52 (s, 1H, H6); ^{13}C NMR (D_2O) : 39.8 (C2'); 65.1 (d, J = 4.1 Hz, C5'); 72.2 (C3'); 84.0 (C1'); 87.0 (d, J = 8.6 Hz, C4'); 124.9 (C5); 139.3 (C6); 147.7 (C8); 158.6 (C2); 159.7 (C4); ^{31}P NMR (D_2O) : 2.1.
19. Rf (iPrOH/NH_4OH/H_2O : 7/1/2) : 0.45; Rt (0-20% de A dans B en 20 min.) : 9.8 min.; ^1H NMR (D_2O) : 2.56 and 2.77 (2m, 2x1H, H2' and H2"); 3.18 (dd, J = 9.4 Hz, J = 5.0 Hz, 4H, NCH_2); 3.56 (t, J = 4.3 Hz, 4H, OCH_2); 3.98 (m, 2H, H5' and H5"); 4.21 (m, 1H, H4'); 4.71 (m, 1H, H3'); 6.36 (t, J = 6.5 Hz, 1H, H1'); 8.47 (s, 1H, H8); 8.52 (s, 1H, H6); ^{13}C NMR (D_2O) : 39.2 (C2'); 45.1 (CH_2N); 64.8 (CH_2O); 67.4 (d, J = 7.3 Hz, C5'); 71.5 (C3'); 83.9 (C1'); 86.5 (d, J = 4.3 Hz, C4'); 124.7 (C5); 139.2 (C6); 147.4 (C8); 158.3 (C2); 159.4 (C4); ^{31}P NMR (D_2O) : 8.1.
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^{31}P NMR (D_2O) : -21.04 (t, J = 16 Hz); -10.29 (d); -5.30 (d); MS (FAB-) : 491 (M-H)-.

22. Primer (50 pmol) was labeled at the 5'-end by T4 polynucleotide kinase (10 U) in the presence of Redivue [γ-^{32}P]ATP in a total volume of 50 µL. After incubation at 37°C for 30 min., the mixture was heated at 70°C for 15 min. The primer (10 pmol) and the complementary template (20 pmol) in 50 µL buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT) were heated at 80°C for 10 min., then cooled to room temperature over a period of 1 hour. The primer elongation reaction was started by mixing two solutions of equal volume (5 µL). One solution (5 µL) contained the template annealed to the labeled primer (0.5 pmol as the primer, 100 nM) and the polymerase (0.25 U of KFexo$^-$, Biolabs). The second (5 µL) contained the triphosphates (in the range of 100 nM to 1 mM). The mixture was incubated at 23 °C for 20 min.. Reactions were stopped by addition of 2.5 µL of stop buffer, heated at 85 °C for 2 min. then chilled on ice prior to loading on polyacrylamide gel (20%, 7M urea).

23. As described in 22, excepted that the hybrid concentration was 165 nM (with primer/template ratio of 1/1), and the polymerase was 0.16 U/µL.

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