Published in final edited form as:

J Mol Biol. 2009 June 5; 389(2): 264–274. doi:10.1016/j.jmb.2009.04.023.

Kinetic Analysis of Base Pairing Preference for Nucleotide Incorporation Opposite Template Pyrimidines by Human DNA Polymerase ϵ

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SUMMARY

DNA polymerase (pol) ϵ , a member of the mammalian Y-family of DNA polymerases involved in translesion DNA synthesis, has been previously suggested to peculiarly utilize Hoogsteen base pairing for DNA synthesis opposite template purines, unlike pols η and κ which utilize Watson-Crick base pairing. To investigate the possible roles of Hoogsteen, Watson-Crick, and wobble base pairing modes in the selection of nucleotides opposite template pyrimidines by human pol ϵ , we carried out kinetic analyses of incorporation of modified purine nucleoside triphosphates including 7-deazapurines, inosine, 2-aminopurine, 2,6-diaminopurine, and 6-chloropurine, which affect H-bonding in base pair formation opposite template pyrimidines. Carbon substitution at the N7 atom of purine nucleoside triphosphates, which disrupts Hoogsteen base pairing, only slightly inhibited DNA synthesis opposite template pyrimidines by pol ϵ , which was not substantially different from human pols η and κ . Opposite template T, only the relative wobble stabilities (inferred from the potential numbers of H-bonds, steric, and electrostatic interactions but not measured) of base pairs were positively correlated to the relative efficiencies of nucleotide incorporation by pol ϵ but not the relative Watson-Crick or Hoogsteen stabilities, unlike pols η and κ . In contrast, opposite C only the relative Watson-Crick stabilities of base pairs were positively correlated to the relative efficiencies of nucleotide incorporation by pol ϵ , as with pols η and κ . These results suggest that pol ϵ might not indispensably require Hoogsteen base pairing for DNA synthesis opposite pyrimidines but rather might prefer wobble base pairing in the selection of nucleotides opposite T and Watson-Crick base pairing opposite C.

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Supplementary Data Supplementary data associated with this article can be found in the on-line version. Supplementary material contains supplementary Figs. S1-S5 showing the possible base pairing interactions of purine derivatives with pyrimidine bases T and C in Watson-Crick, wobble, and Hoogsteen geometry and also the pre-steady-state kinetic data.

Keywords

DNA polymerase; translesion synthesis; wobble base pairing; Watson-Crick base pairing; Hoogsteen base pairing

Introduction

High fidelity of DNA replication is essential for the maintenance of the genomic integrity and the survival of organisms.¹ Historically this fidelity has been mainly attributed to the simple classical notion that all DNA polymerases utilize Watson-Crick base pairing (A-T or G-C) for the selection of the correct complementary nucleotides opposite normal template bases during DNA synthesis.² Indeed several factors such as Watson-Crick base pair H-bonding, geometry, and base stacking in the polymerase active site have been suggested to be important in high nucleotide selectivity by DNA polymerases, although conclusions about the major contributing factor have been controversial.³⁻⁶

Recently two major exceptions have been found to the Watson-Crick rules in nucleotide selection among four eukaryotic Y-family DNA polymerases, which are believed to be major players in translesion DNA synthesis. REV1 catalyzes the preferential insertion of dCTP opposite normal template bases and the various DNA lesions such as *N*²-alkylG adducts and abasic sites⁷⁻⁹ by utilizing a unique mechanism of protein-template-directed nucleotide incorporation.¹⁰ DNA polymerase ι (pol ι) has also been previously suggested to insert nucleotides opposite template purines by distinctively utilizing Hoogsteen base pairing.¹¹⁻¹³ In contrast, two other Y-family DNA polymerases, pols η and κ , have been shown to require Watson-Crick base pairing for nucleotide insertion.^{14,15}

Pol ι , found in some higher eukaryotes including mammals, has unusual enzymatic properties in incorporating nucleotides opposite unmodified template purines and pyrimidines.¹⁶⁻¹⁸ Pol ι preferentially inserts the correct nucleotides T and G opposite template bases A and C, respectively. However, pol ι preferentially inserts not only C but also T opposite a template purine G and inserts A but G (more efficiently) opposite a template pyrimidine T. It has been suggested that the unusual nucleotide selectivity of pol ι opposite template purines A and G might be related to the utilization of Hoogsteen base pairing (Fig. 1c), which thus may facilitate the replicative bypass of DNA lesions disturbing the Watson-Crick H-bonding face of purine templates, e.g. some *N*²-alkylG adducts.^{11,12,19} However, the base pairing mode used by pol ι in nucleotide incorporation opposite template pyrimidines T and C still remains to be elucidated. Although speculation about wobble base pairing of dGTP with templating T by pol ι has been made,²⁰ it is still unclear if the nucleotide selectivity opposite template pyrimidines T and C by pol ι can also be attributed to the mode of Hoogsteen base pairing of nucleotides with the template pyrimidines T and C in a similar way as with the template purines A and G, or to another mode of base pairing (Fig. 1).

To better understand the importance of base pairing mode for the nucleotide insertion opposite template pyrimidines by human pol ι , we performed primer extension and the steady-state kinetic analyses by utilizing the nucleotides of various modified purines and

investigated the relationship between the relative insertion efficiencies of the modified purine nucleotides opposite template base T (or C) by pol α and the relative base pair stabilities of the modified purines and T (or C) in either Watson-Crick, wobble, or Hoogsteen geometries. We provide evidence that N7 atom-based Hoogsteen H-bonding is not essential for efficient nucleotide insertion opposite template pyrimidines by pol α , based on the results of studies with 7-deazapurine nucleotides. On the contrary, our results suggest that pol α favors insertion of nucleotides with high stabilities in wobble geometry opposite template T but favors nucleotides with high stabilities in Watson-Crick geometry for nucleotide incorporation opposite template C, as demonstrated by correlation analyses between the insertion efficiencies of modified purine nucleotides opposite template T (or C) and the relative base pair stabilities in either geometry. The implications of the distinctive nucleotide selectivity for pol α opposite template pyrimidines are discussed in comparison with pols η and κ .

Results

Incorporation of Single Modified-Purine Nucleotide Opposite T and C by Human Pols α , η , and κ

In order to evaluate the possible qualitative differences in nucleotide selectivity for incorporation opposite normal template T and C by human pols α , η , and κ , each of the various modified purine nucleotides (Fig. 2) was used in primer extension assays with 14-mer/18-mer primer/template duplexes containing T or C (Table 1) at position 15 of the template (Fig. 3). Opposite T, pol α incorporated dGTP, d7DGTP, and dITP much better than dATP, d7DATP, d2ATP, or dDAPTP, whereas pol κ incorporated dATP, d7DATP, d2ATP, and dDAPTP much better than dGTP, d7DGTP, or dITP and pol η also hardly incorporated dGTP, d7DGTP, or dITP (Fig. 3a), indicating that pol α might have an uncommon mode of nucleotide selection opposite template T, in the opposite manner to pols η and κ . Opposite C, pols α , η , and κ all incorporated dGTP, d7DGTP, and dITP better than the other nucleotides (Fig. 3b), indicating that those pols might have a similar mode of nucleotide selection opposite template C.

Effect of 7-Deazapurine Nucleotides on DNA Synthesis by Human Pols α , η , and κ

In order to determine if pol α requires Hoogsteen base pairing in polymerization opposite template pyrimidines, two types of nucleotides (normal or 7-deazapurine) were used in primer extension assays. The substitution of a carbon for a N7 nitrogen of purine nucleotides, which can abolish H-bond formation with a N7 nitrogen, disrupts only Hoogsteen base pairing but not Watson-Crick or wobble base pairing with the pyrimidines (T or C) (Fig. 1). The effects of 7-deazapurine nucleotides were tested with pols α , η , and κ . For qualitative analysis of the effects of 7-deazapurine nucleotides on DNA synthesis across template pyrimidines, increasing concentrations of pols were used in 15 min incubations with 14-mer/18-mer primer/template complexes in the presence of all four nucleotides (containing either normal or 7-deazapurine nucleotides). Pol α readily extended 14-mer primers, across T and C, in proportion to enzyme concentration and yielded mainly 1- to 3-base-extended products regardless of the presence of normal or 7-deazapurine nucleotides (Fig. 4), although the extent of DNA synthesis was slightly reduced in the case of the 7-

deazapurine nucleotides. Similarly, DNA syntheses across T and C by pols η and κ were not inhibited in the presence of 7-deazapurine nucleotides (Fig. 4), indicating that nucleotide insertion across template pyrimidines by these pols ι , η , and κ is relatively unaffected by 7-deaza modification of purine nucleotides.

For the quantitative analysis of the effects of 7-deaza substitution at the purine ring on nucleotide insertion opposite template pyrimidines, steady-state kinetic parameters were determined for single (normal or 7-deazapurine) nucleotide incorporation opposite T and C (Table 2). Opposite T, the catalytic efficiencies (specificity constants, k_{cat}/K_m) for 7-deaza-dATP incorporation by pols ι , η , and κ were only slightly reduced or unchanged (1.8-, 1.0-, and 1.5-fold respectively) compared to those for dATP incorporation. The k_{cat}/K_m for 7-deaza-dGTP incorporation opposite T by pol ι was also slightly reduced (3.2-fold) compared to that for dGTP incorporation. Opposite C, the values of k_{cat}/K_m for 7-deaza-dGTP insertion by pols ι , η , and κ were also only slightly changed (3.3-fold decreased, 2.3-fold increased, and 1.3-fold decreased, respectively) compared to those for dGTP incorporation. Interestingly, the K_m values for nucleotide incorporation by pol ι were decreased about 3-fold (possibly decreasing K_d) but accompanied by a 6- to 10-fold decrease of k_{cat} (possibly decreasing k_{off} for the product) by 7-deaza substitution, not seen with pols η and κ , although the exact interpretation is not feasible in this system.³

Steady-state Kinetics of Nucleotide Incorporation Opposite T and C

To quantitatively analyze insertion efficiency of modified purines which could affect the Watson-Crick and wobble base pairing with T and C, steady-state kinetic parameters were determined for incorporation of individual purine-modified nucleotides into 14-mer/18-mer duplexes opposite T or C (Tables 3 and 4). Insertion efficiencies of various purine-modified nucleotides opposite template pyrimidines—which can form base pairs in Watson-Crick and wobble geometries with varying H-bonds, steric, and electrostatic interactions—can be useful indirect indicators for base pairing preferences opposite template pyrimidines by DNA polymerases, as successfully applied before in studies with pol η .²¹ The relative stabilities of base pairs were arbitrarily calculated as the potential number of attractive H-bonding donor-acceptor pairs minus the potential number of repulsive donor-donor or acceptor-acceptor pairs in either base pair geometry, as described previously.²¹ Possible attractive and repulsive interactions for Watson-Crick or wobble base pairings of the modified purines with T and C are shown in supplementary Figs. S1-S3. The catalytic efficiencies (k_{cat}/K_m) for nucleotide incorporation by pols ι , η , and κ were compared with the relative stabilities of the corresponding base pairs in either Watson-Crick or wobble geometries (Tables 3, 4 and Fig. 5). With pols η and κ , dATP (forming two Watson-Crick (W-C) H-bonds), dDAPTP (forming three W-C H-bonds), and d2APTP (forming two W-C H-bonds) were incorporated opposite T with much higher k_{cat}/K_m values (7- to 63-fold with pol η ; 7- to 144-fold with pol κ) than d6CPTP, dGTP, or dITP, which can form at most one Watson-Crick H-bond (Table 3). Opposite C, dGTP (forming three W-C H-bonds) and dITP (forming two W-C H-bonds) were incorporated with much higher k_{cat}/K_m values (49- to 135-fold with pol η ; 16- to 164-fold with pol κ) than d2APTP, d6CPTP, dATP, and dDAPTP, which can form at most one W-C H-bond (Table 4). Thus, the relative efficiencies for nucleotide incorporation opposite T and C by pols η and κ were found to show

significant positive correlation with the relative stabilities of the corresponding base pairs in Watson-Crick (W-C) geometry (Fig. 5a-d).

However, with pol α nucleotides such as dGTP, d7DGTP, and dITP, which cannot form any Watson-Crick H bonds opposite T but can form two wobble H-bonds, were incorporated with higher k_{cat}/K_m values (3- to 28-fold) than nucleotides such as dDAPTP, dATP, and d2APTP, which can form two or three W-C H-bonds but form no wobble H-bond (Table 3). In contrast, opposite C the nucleotides such as dGTP and dITP, which can form three or two W-C H-bonds respectively, were incorporated with much higher k_{cat}/K_m values (27- to 114-fold) than d2APTP, d6CTP, dATP, and dDAPTP, which can form at most one W-C H-bond (Table 4). Thus, the relative efficiencies for nucleotide incorporation opposite T by pol α appeared to have a significant positive correlation only with the relative wobble stabilities of base pairs (Fig. 5e and 5g), whereas the relative efficiencies for nucleotide incorporation opposite C appeared to show a significant positive correlation only with the relative Watson-Crick stabilities of base pairs (Fig. 5f and 5h). There was also no significant positive correlation between the relative efficiencies for nucleotide incorporation opposite T and C by pol α and the Hoogsteen stabilities of base pairs (Fig. 5i and 5j), indicating little contribution of Hoogsteen base pairing in base incorporation opposite template pyrimidines.

Discussion

In this work we provide kinetic evidence that pol α favors Watson-Crick base pairing opposite template C but wobble base pairing opposite template T for efficient nucleotide incorporation. In order to characterize the base pairing mode of pol α for nucleotide incorporation opposite template pyrimidines, we adopted a strategy, developed previously, of using the modified purine nucleotides that confer the varying degree of stabilities in Watson-Crick, wobble, or Hoogsteen base pairing with T and C.²¹ No strong inhibitory effect of 7-deaza-dATP or 7-deazadGTP, which lack the N7 nitrogen, was observed on pol α -catalyzed DNA synthesis opposite template pyrimidines, and no significant positive correlation between the catalytic efficiencies of pol α -catalyzed nucleotide insertion opposite template T and C with the relative Hoogsteen stabilities of the corresponding base pairs also suggests that pol α may not require Hoogsteen H-bonding at the N7 position of the purine nucleotide for the efficient incorporation opposite template pyrimidines. A significant positive correlation between the catalytic efficiencies of pol α -catalyzed nucleotide insertion opposite template T seen only with the relative wobble stabilities of the corresponding base pairs suggests that pol α may use wobble base pairing for the efficient base incorporation opposite template T (Fig. 1b). In contrast, the significant positive correlation between the catalytic efficiencies of pol α -catalyzed nucleotide insertion opposite template C, observed only with the relative Watson-Crick stabilities of the corresponding base pairs, suggests that pol α requires Watson-Crick base pairing for efficient base incorporation opposite template C (Fig. 1a, right panel).

Our approach of using modified bases in the template DNA and the incoming nucleotide is useful in analyzing the base pairing and selection properties of DNA polymerases for the nucleotide incorporation opposite template bases, in that it has been successfully applied in several studies on the base pairing and selection by various DNA polymerases, e.g. yeast pol

η and human pols α , γ , and ι previously.^{11,21-24} The arbitrary approximation of the base pair stabilities by counting potential H-bonding, steric, and electrostatic interactions in either base pair geometry was adopted in this study, which is based on the assumption that the H-bonding, steric, and electrostatic interactions largely affect the stabilities of the nascent base pair (in either base pair geometry) in the confined polymerase active site.²¹ On that assumption, the relative base pair stabilities can be inferred by using the equation (i.e. the relative base pair stabilities equal the potential number of attractive H-bonding donor-acceptor pairs minus the potential number of repulsive donor-donor or acceptor-acceptor pairs in either base pair geometry). However, the real base pair stability during polymerization should be more complex, beyond our assumptions, and we cannot ignore the other factors contributing to base pairing stability during polymerization, e.g. base stacking (with the primer terminus base) and base (of innate base pair)-amino acid (of polymerase) interactions in the polymerase active site, although these forces were not tested in this study. A possible role of base stacking for catalysis opposite normal template bases has been suggested with yeast pol η by using pyrene nucleotides.²⁵ Interactions of the primer terminus and incoming nucleotides with DNA polymerase has also been suggested to play an important role in both fidelity and catalysis with *Escherichia coli* DNA polymerases I (Klenow fragment).²⁶ However, those factors might not be critical variables in our study design, in that all nucleotide analogues used in this study contain minor groove hydrogen bond acceptors (N3 atom of purine) for polymerase interaction and may inherently retain similar base stacking properties (purine rings). The arbitrary values for base pair stability may denote the relative stabilities of base pair in rank order, even though not being quantitative (and it cannot be assumed that our data are normally distributed in population). Thus, we applied a non-parametric Spearman's rank correlation test to analyze the relationships between the insertion efficiencies of modified bases and the relative stabilities of the corresponding base pairs in Watson-Crick, wobble, and Hoogsteen geometries. We found a positive correlation between the insertion efficiencies of modified bases opposite template bases T or C by pols η and κ and the relative Watson-Crick stabilities of the corresponding base pairs, indicating that pols η and κ favor nucleotides with high Watson-Crick stabilities for the efficient insertion opposite template pyrimidines. Our interpretation of these results corresponds with previous reports that pols η and κ require Watson-Crick base pairing for efficient catalysis.^{14,15,21}

To compare the nucleotide selectivity for incorporation by polymerases we used the steady-state kinetic parameter, k_{cat}/K_m (the specificity constant). Although k_{cat}/K_m has been widely utilized as a general indicator of catalytic efficiency, it should be noted that k_{cat}/K_m has some limitations in describing enzymatic reactions. Especially with processive DNA polymerases, the k_{cat}/K_m might underestimate the catalytic efficiency in single nucleotide insertion kinetics because k_{cat} can reflect the rate of slower DNA dissociation rather than the fast polymerization rate in some cases, e.g. normal base incorporation. However, such a steady-state artifact might be small for distributive Y-family DNA polymerases used in this study. We attempted to measure the pre-steady-state kinetics of dGTP and dATP incorporation opposite template T by pol ι to determine if the step of product DNA release is rate limiting. The burst was marginally detectable and only seen in the case of the most efficient dGTP incorporation (but totally absent in the canonical dATP incorporation)

(Supplementary Fig. S5), indicating that the DNA dissociation step might not really be rate-limiting in polymerization cycles with the nucleotide analogues we used and thus it might not confound the $k_{\text{cat}}/K_{\text{m}}$ derived from steady-state kinetic analysis. In these circumstances, the $k_{\text{cat}}/K_{\text{m}}$ may be a reliable indicator of catalytic efficiency in single nucleotide incorporation by pol ν in our experimental system.

The nucleotide preference of pol ν opposite template T appears to contrast sharply with those of pols η and κ , indicating that the base pairing mode opposite template T by pol ν is different from those used by pols η and κ . Indeed pol ν appeared to disfavor nucleotides with high Watson-Crick stabilities opposite template T, in a reverse manner to pols η and κ . Pol ν also appears to have no substantial preference for nucleotides with high potential for stable Hoogsteen base pairing opposite template T, i.e. no positive correlation between the nucleotide insertion efficiencies opposite template T by pol ν and the relative Hoogsteen stabilities of the corresponding base pairs (Fig. 5i). In contrast, pol ν favors nucleotides with high wobble stabilities opposite template T, suggesting that pol ν may employ wobble base pairing in base discrimination opposite T, in good agreement with a previous report that pol ν prefers to position G opposite T and thus might accommodate well the wobble base pair.²⁰ However, these results contrast strongly with the current view that pol ν discriminates in nucleotide incorporation opposite template purines by Hoogsteen base pairing,^{11,12} suggesting that pol ν has an asymmetric active site to accommodate the nascent base pairs in different geometry depending on the nature of template base. We cannot exclude other factors such as DNA sequence context and metal ions on the base pairing preferences by pol ν . The misinsertion ratios for G opposite T varies considerably (from 3 to 11) in several reports using different sequence contexts.^{16-18,27} Two recent reports indicate that the fidelity of pol ν can be increased up to 5-fold opposite template base T25 or decreased about 5- and 20-fold opposite template bases G and *N*²-ethylG,²⁶ respectively, in the presence of Mn^{2+} instead of Mg^{2+} .

Pol ν appears to employ a Watson-Crick base pairing mode for base incorporation only opposite template base C but not opposite the other bases, i.e. G, A, and T. The complex base selection properties of pol ν might be related to the distinctive conformation of the pol ν active site, which might affect the fitness of the various nascent base pair therein and thus change the catalytic ability to insert the nucleotide. A recent structural study emphasizes the importance of conformational fitness between the pol ν active site and the nascent base pair to form a productive ternary complex poised for catalysis: the incoming pyrimidine nucleotide forces the template purines G and A to rotate from *anti* into *syn* conformation in the rigid active site of pol ν to form productive Hoogsteen base pairing,²⁸ but this phenomenon may not occur in cases with the incoming purine nucleotide opposite the template pyrimidine. Adaptation of template purines to a *syn* conformation to fit into the polymerase active site has also been shown in cases of modified template purines, including the ternary polymerase·DNA·dNTP complexes of a benzo[*a*]pyrene diol epoxide *N*²-G adduct with a Y-family *Sulfolobus solfataricus* DNA polymerase Dpo4,²⁹ 7,8-, dihydro-8-oxo-G with pol β ,³⁰ or 1, *N*⁶-etheno-A31 or *N*²-ethylG32 with pol ν which may facilitate better accommodation and catalysis in the confined active site of a polymerase.

Although initial reports suggested that nucleotide incorporation opposite pyrimidines by pol ι is less efficient (up to several hundred-fold) than opposite purines, a recent report shows that the nucleotide insertion opposite template pyrimidine T has similar efficiency as opposite template purine A in the presence of a physiological concentration of Mg^{2+} (0.25 mM, instead of 5 mM) or Mn^{2+} (75 μ M).³³ Therefore, polymerization opposite template pyrimidines by pol ι might also be as catalytically competent as that opposite template purines under physiological conditions. How could a distinct mode of base pairing opposite template pyrimidines contribute to the fidelity of DNA synthesis by pol ι ? The unusual wobble mode of base selection opposite template T by pol ι might facilitate the incorrect incorporation of G and non-canonical bases (e.g. hypoxanthine) that are highly stable in the wobble geometry opposite template T, but which is not the case opposite template C due to the Watson-Crick base selection. Misincorporation of G or hypoxanthine opposite template T would then generate primarily TA to CG transition mutations, in that human DNA polymerases such as pols α , η and κ preferentially insert C opposite template deoxyinosine.³⁴

In conclusion, our results indicate that human pol ι , one of the Y-family DNA polymerases, may select nucleotides by wobble base pairing for efficient incorporation opposite template T but by Watson-Crick base pairing for incorporation opposite template C. This property of pol ι might promote the erroneous incorporation of nucleotides such as dGTP and the non-canonical dITP, which are highly stable in the wobble geometry opposite normal template base T but not opposite template base C.

Materials and Methods

Materials

Unlabeled dNTPs and T4 polynucleotide kinase were purchased from New England Biolabs (Ipswich, MA). Modified dNTPs were purchased from TriLink Biotechnologies (San Diego, CA). [γ -³²P]ATP (specific activity 3,000 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Bio-spin columns were purchased from Bio-Rad (Hercules, CA). Oligonucleotides were purchased from Midland Certified Reagents (Midland, TX). Recombinant human pol η ,³⁵ pol ι ,¹⁹ and pol κ ³⁶ were expressed in baculovirus-infected insect cell systems and purified to electrophoretic homogeneity as described previously.

Reaction conditions for polymerization assays

Standard DNA polymerase reactions were performed in 50 mM Tris-HCl (pH 7.5) buffer containing 5 mM dithiothreitol (DTT), 100 μ g ml⁻¹ bovine serum albumin (w/v), and 10% glycerol (v/v) with 100 nM primer-template at 37 °C. Primers (14-mer) were 5' end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase and annealed with templates (18-C (or T)-mer). All reactions were initiated by the addition of dNTP and $MgCl_2$ (5 mM final concentration) to preincubated enzyme/DNA mixtures.

Primer extension assays and gel electrophoresis

A ³²P-labeled primer, annealed to a template, was extended in the presence of single or all four dNTPs. Unless indicated, each reaction was initiated by adding 4 μ l of dNTP- Mg^{2+}

solution (final concentrations of 100 μ M of each dNTP and 5 mM MgCl_2) to a preincubated E•DNA complex (final concentrations) of 50 mM Tris-HCl (pH 7.5), 100 nM DNA duplex, 5 mM DTT, 100 μ g bovine serum albumin (BSA) ml^{-1} , and 10% glycerol (v/v)) at 37 °C, yielding a total reaction volume of 8 μ l. After the 10 min, reactions were quenched with a solution of 20 mM EDTA (pH 8.0) in 95% formamide (v/v). Aliquots were separated by electrophoresis on a denaturing gel containing 8.0 M urea and 16% acrylamide (w/v) (from a 19:1 acrylamide:bisacrylamide solution, AccuGel, National Diagnostics, Atlanta, GA) with 80 mM Tris-borate buffer (pH 7.8) containing 1 mM EDTA. Gels were exposed to a phosphorimager screen (Imaging Screen K, Bio-Rad, Hercules, CA). The bands (representing extension of the primer) were visualized with a phosphorimaging system (Bio-Rad, Molecular Imager® FX, Hercules, CA) using the manufacturer's Quantity One Software, Version 4.3.0.

Steady-state reactions

A ^{32}P -labeled primer, annealed to a template, was extended in the presence of increasing concentrations of a single dNTP. The molar ratio of primer/template to enzyme was at least 10:1. Enzyme concentrations and reaction times were chosen so that maximal product formation would be 20% of the substrate concentration. The primer-template was extended with dNTP in the presence of 0.1-5 nM enzyme for 5 or 10 min. All reactions (8 μ l) were done at ten dNTP concentrations and quenched with 9 volumes of a solution of 20 mM EDTA in 95% formamide (v/v). Products were resolved using a 16% polyacrylamide (w/v) electrophoresis gel containing 8 M urea and quantitated by phosphorimaging analysis using a Bio-Rad Molecular Imager FX instrument and Quantity One software. Graphs of product formation vs dNTP concentration were fit using nonlinear regression (hyperbolic fits) in GraphPad Prism Version 3.0 (San Diego, CA) for the determination of k_{cat} and K_{m} values.

Data analysis

Correlation analyses between the relative stabilities of base pairs in either Watson-Crick, wobble, or Hoogsteen geometries and the relative efficiencies for incorporation of the modified purine nucleotides against pyrimidines were performed using a non-parametric Spearman's rank correlation test.³⁷ A one-tailed p value < 0.05 was considered statistical significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank K. C. Angel for technical assistance. This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (No. R01-2007-000-11710-0) (to J. -Y. C.) and United States Public Health Service (USPHS) Grants R01 ES010375 and P30 ES000267 (to F. P. G.).

Abbreviations used

G	guanine
A	adenine
C	cytosine
T	thymine
I	inosine
dNTP	deoxynucleoside triphosphate
d7DATP	7-deazaadenine deoxyribose triphosphate
d7DGTP	7-deazaguanine deoxyribose triphosphate
dDAPTP	2,6-diaminopurine deoxyribose triphosphate
d2APTP	2-aminopurine deoxyribose triphosphate
d6CPTP	6-chloropurine deoxyribose triphosphate
dITP	deoxyinosine triphosphate
pol	DNA polymerase

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Figure 1. Hydrogen bonding in Watson-Crick, wobble, and Hoogsteen base pairs with pyrimidines

(a) T:A and C:G Watson-Crick base pairs. (b) T:G wobble base pair. (c) T:A, T:G, and protonated C⁺:G Hoogsteen base pairs.

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Figure 2. Deoxyribonucleotides of purine derivatives used in this work

dATP, adenine deoxyribose triphosphate; dGTP, guanine deoxyribose triphosphate; d7DATP, 7-deazaadenine deoxyribose triphosphate; d7DGTP, 7-deazaguanine deoxyribose triphosphate; dDAPTP, 2,6-diaminopurine deoxyribose triphosphate; d2APTP, 2-aminopurine deoxyribose triphosphate; d6CPTP, 6-chloropurine deoxyribose triphosphate; dITP, deoxyinosine triphosphate. dR and PPP indicate deoxyribose and triphosphate, respectively.

Figure 3. Incorporation of purine-modified nucleotides opposite T and C by human pols τ , η and κ

Each of the pols τ (8 nM), η (0.5 nM), or κ (3 nM) was incubated with 3'-³²P-labeled 14-mer/18-T(or C)-mer primer/template duplex DNA (100 nM) in the presence of a single purine-modified deoxynucleotide, 10 μ M (for pol η) or 100 μ M (for pols τ and κ) of dGTP, d7DGTP, dATP, d7DATP, dITP, d2APTP, dDAPTP, or d6CPTP for 10 min at 37°C. (a) template T; (b) template C.

Figure 4. Effect of 7-deazapurine nucleotides on extension of ^{32}P -labeled primers opposite template T and C by human pols τ , η , and κ

The corresponding primer was annealed with each of the two different templates (Table 1) containing an unmodified T or C placed at the 15th position from the 3'-end. Reactions were done for 10 min with a constant concentration of DNA substrate (100 nM primer/template) and increasing concentrations of polymerase as indicated. ^{32}P -labeled primer was extended in the presence of all four dNTPs (100 μM each; C, T, G, A), containing normal purine nucleotides (dGTP and dATP) or 7-deazapurine nucleotides (d7DGTP and d7DATP). The reaction products were analyzed by 16% (w/v) denaturing polyacrylamide gel electrophoresis with subsequent phosphorimaging analysis. (a) 14-mer/18-T-mer primer/template DNA; (b) 14-mer/18-C-mer primer/template DNA.

Figure 5. Comparison between the relative stability of base pairs and the relative efficiency of base incorporation opposite T and C by human pols τ , η , and κ

The ranks of relative efficiencies (Tables 2, 3, and 4) for incorporation of purine derivative nucleotides opposite template T (or C) by pols τ ((e)-(j)), η ((a) and (b)), and κ ((c) and (d)) were plotted against the ranks of relative stabilities of either Watson-Crick ((a)-(f)), wobble ((g) and (h)), or Hoogsteen ((i) and (j)) base pairs between T (or C) and purine derivatives in a graph. For

wobble base pairs, the first term of the relative wobble stabilities opposite template T (Table 3) for the geometry shown in Supplementary Fig. S2(a) and the second term of the relative wobble stabilities opposite template C (Table 4) for the geometry shown in Supplementary Fig. S3(b), which can confer higher stability of base pairs, are used for the plot. For Hoogsteen base pairs, the values of the relative Hoogsteen stabilities derived from the Hoogsteen geometry with T or protonated C^+ (shown in Supplementary Fig. S4) are used for the plot. Spearman's rank correlation test³⁷ was performed using GraphPad Prism software (San Diego, CA) and the calculated correlation coefficients (r_s) and one-tailed p values are shown for each comparison. (a), (c),

(e), (g), and (i) template T; (b), (d), (f), (h), and (j) template C.

Table 1

Oligodeoxynucleotides used in this study

14-mer	5' GGGGAAGGATTCC
18-C-mer	3' CCCCTTCCTAAGGCACT
18-T-mer	3' CCCCTTCCTAAGGTACT

Table 2

Steady-state kinetic parameters for incorporation of normal and 7-deazapurine nucleotides opposite T and C by human pol I

Pol	Template	dNTP	$K_m(\mu M)$	$k_{cat}(s^{-1})$	$k_{cat}/K_m (mM^{-1} s^{-1})$	Relative efficiency	Fold difference in efficiency	Hoogsteen H-bonds
I	18-T	A	630 ± 80	0.010 ± 0.001	0.016	0.062	-	2
		7DA	200 ± 20	0.0017 ± 0.0001	0.009	0.035	1.8-fold lower	1
		G	550 ± 130	0.14 ± 0.01	0.26	1	-	1
		7DG	160 ± 20	0.013 ± 0.001	0.081	0.31	3.2-fold lower	0
		G	750 ± 60	0.18 ± 0.01	0.24	1	-	2
η	18-C	7DG	300 ± 50	0.022 ± 0.001	0.073	0.3	3.3-fold lower	1
		A	1.7 ± 0.1	0.12 ± 0.01	67	1	-	2
		7DA	2.7 ± 0.4	0.18 ± 0.01	67	1	~1.0-fold	1
		G	2.3 ± 0.3	0.12 ± 0.01	77	1	-	2
		7DG	1.1 ± 0.1	0.20 ± 0.01	180	2.3	2.3-fold higher	1
κ	18-T	A	36 ± 4	0.072 ± 0.003	2.0	1	-	2
		7DA	43 ± 10	0.058 ± 0.005	1.3	0.68	1.5-fold lower	1
		G	64 ± 3	0.089 ± 0.001	1.4	1	-	2
		7DG	56 ± 5	0.065 ± 0.002	1.1	0.79	1.3-fold lower	1

Table 3

Steady-state kinetic parameters for incorporation of purine nucleotide derivatives opposite T by human pol I, η , and κ

Polymerase	dNTP	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)	Relative efficiency	WC stability ^a	Wobble stability ^a
I	DAP	140 $\pm$ 20	0.0013 $\pm$ 0.00001	0.009	0.036	3	-2/-2
	A	630 $\pm$ 80	0.010 $\pm$ 0.001	0.016	0.062	2	-2/-1
	2AP	290 $\pm$ 70	0.0047 $\pm$ 0.0003	0.016	0.064	2	-1/-2
	6CP	250 $\pm$ 20	0.0028 $\pm$ 0.0001	0.011	0.044	1	-1/-1
	G	550 $\pm$ 130	0.14 $\pm$ 0.01	0.26	1	-1	2/0
H	I	1000 $\pm$ 100	0.049 $\pm$ 0.0001	0.049	0.19	-2	2/1
	DAP	13 $\pm$ 2	0.33 $\pm$ 0.01	25	0.38	3	-2/-2
	A	1.7 $\pm$ 0.1	0.12 $\pm$ 0.01	67	1	2	-2/-1
	2AP	40 $\pm$ 6	0.38 $\pm$ 0.02	9.7	0.15	2	-1/-2
	6CP	160 $\pm$ 20	0.35 $\pm$ 0.01	2.2	0.032	1	-1/-1
K	G	170 $\pm$ 40	0.24 $\pm$ 0.01	1.4	0.021	-1	2/0
	I	280 $\pm$ 50	0.31 $\pm$ 0.02	1.1	0.016	-2	2/1
	DAP	60 $\pm$ 10	0.16 $\pm$ 0.01	2.6	1.3	3	-2/-2
	A	36 $\pm$ 4	0.072 $\pm$ 0.003	2.0	1	2	-2/-1
	2AP	96 $\pm$ 17	0.078 $\pm$ 0.004	0.81	0.41	2	-1/-2
G	6CP	340 $\pm$ 10	0.037 $\pm$ 0.001	0.11	0.055	1	-1/-1
	G	1900 $\pm$ 400	0.034 $\pm$ 0.004	0.018	0.009	-1	2/0
	I	1400 $\pm$ 300	0.027 $\pm$ 0.002	0.019	0.010	-2	2/1

^a Calculated as the number of donor-acceptor pairs minus the number of donor-donor or acceptor-acceptor pairs in Watson-crick or wobble geometry. Two values for wobble stabilities are from two types of wobble base pair geometries as shown in supplementary Fig. S2.

Table 4
Steady-state kinetic parameters for incorporation of purine nucleotide derivatives opposite C by human pol *I*, η , and κ

Polymerase	dNTP	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)	Relative efficiency	WC stability ^a	Wobble stability ^a
<i>I</i>	G	750 $\pm$ 60	0.18 $\pm$ 0.01	0.24	1	3	0/0
	I	65 $\pm$ 21	0.0056 $\pm$ 0.0004	0.083	0.35	2	0/-1
	2AP	100 $\pm$ 30	0.00024 $\pm$ 0.00001	0.0025	0.010	0	-1/2
	6CP	160 $\pm$ 50	0.00049 $\pm$ 0.00001	0.0031	0.013	-1	-2/1
	DAP	380 $\pm$ 140	0.00081 $\pm$ 0.00001	0.0021	0.0088	-1	0/2
η	A	1200 $\pm$ 300	0.0038 $\pm$ 0.0003	0.0032	0.013	-2	0/1
	G	2.3 $\pm$ 0.3	0.12 $\pm$ 0.01	77	1	3	0/0
	I	2.5 $\pm$ 0.4	0.13 $\pm$ 0.01	52	0.68	2	0/-1
	2AP	190 $\pm$ 20	0.21 $\pm$ 0.01	1.1	0.014	0	-1/2
	6CP	150 $\pm$ 20	0.12 $\pm$ 0.01	0.8	0.010	-1	-2/1
κ	DAP	580 $\pm$ 130	0.33 $\pm$ 0.03	0.57	0.0074	-1	0/2
	A	74 $\pm$ 22	0.084 $\pm$ 0.010	1.1	0.014	-2	0/1
	G	64 $\pm$ 3	0.089 $\pm$ 0.001	1.4	1	3	0/0
	I	59 $\pm$ 7	0.079 $\pm$ 0.002	1.3	0.93	2	0/-1
	2AP	150 $\pm$ 30	0.012 $\pm$ 0.001	0.080	0.057	0	-1/2
	6CP	190 $\pm$ 20	0.0019 $\pm$ 0.0001	0.010	0.0071	-1	-2/1
	A	5100 $\pm$ 1100	0.016 $\pm$ 0.002	0.0031	0.002	-1	0/2
	DAP	360 $\pm$ 90	0.0031 $\pm$ 0.0002	0.0086	0.0061	-2	0/1

^aCalculated as the number of donor-acceptor pairs minus the number of donor-donor or acceptor-acceptor pairs in Watson-crick or wobble geometry. Two values for wobble stabilities are from two types of wobble base pair geometries as shown in supplementary Fig. S3.