Efforts toward Expansion of the Genetic Alphabet: Replication of DNA with Three Base Pairs

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The faithful pairing of nucleobases, through complementary hydrogen-bond (H-bond) donors and acceptors, forms the foundation of the genetic code. However, there is no reason to assume that the requirements for duplex stability and replication must limit the genetic alphabet to only two base pairs, or for that matter, hydrogen-bonded base pairs.[1] Expansion of this alphabet to contain a third base pair would allow for the encoding of additional information and would make possible a variety of in vitro experiments using nucleic acids with unnatural building blocks. In recent progress toward expanding the genetic alphabet,[2] a variety of unnatural nucleobases have been characterized that form pairs based not on hydrogen bonds but rather on interbase hydrophobic interactions. One of the most promising of these third base-pair candidates is the self-pair formed between two 7-azaindole nucleosides (**7AI**, Figure 1a). However, following incorporation, **7AI** inhibits continued primer extension. Here we show that mammalian polymerase β can efficiently extend primers containing **7AI** and, when combined with the Klenow fragment of *Escherichia coli* polymerase I (KF), can efficiently replicate DNA containing the unnatural self-pair with reasonable fidelity.

Pol β is a mammalian polymerase that functions primarily to gap-fill during base excision repair. Pol β can also substitute for DNA pol I in the synthesis of the short stretches of DNA required to join Okazaki fragments in *E. coli*, which suggests that the polymerase is able to play a role in replicative synthesis.[3] Pol β can perform trans-lesion synthesis on templates containing abasic sites,[4] cis-syn thymine dimers,[5] and cisplatin adducts, all of which are strong replication blocks to most DNA polymerases. Interestingly, during in vitro DNA replication, pol β can replace other stalled polymerases at a modified base and extend the 3′-terminus of the intermediate.[6]

Pol β incorporation kinetics were first examined with fully natural DNA using a 23-nt primer annealed to a 45-nt template (Scheme 1) that contained a native base (dG, dA, dT, or dC) at position **X**. Steady-state kinetics were performed using literature procedures.[2] For the natural bases, pol β incorporated the correct dNTP at 37 °C with efficiencies of 3.4×10^5 M^{-1} min^{-1} for dATP opposite dT; 1.9×10^6 M^{-1} min^{-1} for dTTP opposite dA; 6.6×10^5 M^{-1} min^{-1} for dCTP opposite dG; and 7.0×10^5 M^{-1} min^{-1} for dGTP opposite dC.

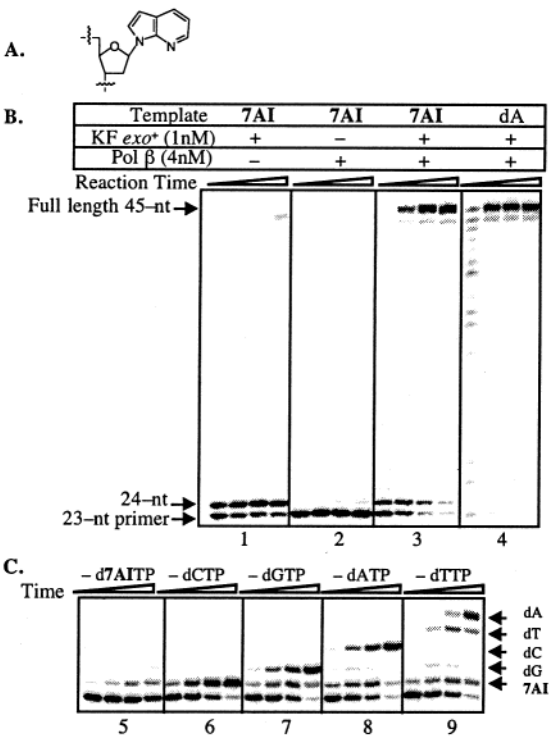

Figure 1. (A) d**7AI** nucleoside. (B) Extension of 23-nt primer (with 45-nt template). Reactions 1, 2, and 3 contain either KF *exo*$^+$, pol β, or both, respectively. Template **X** = **7AI**. The four lanes for each reaction correspond to reaction times of 15, 30, 60, 120 min. Reaction 4 is the same as reaction 3, except template **X** = dA and the reaction times were 3, 10, 30, and 60 min. (C) Fidelity of replication. Reactions 5−9 are identical to reaction 3, except each contains only four of the requisite five nucleoside triphosphates, and the reaction times were 5, 10, 20, and 30 min. The omitted triphosphate is indicated along the top of the gel. The template base that resulted in the termination of primer extension is indicated along right edge of the gel.

Scheme 1

23-nt primer	5′-TAATACGACTCACCTATAGGGAGA-3′
24-nt primer	5′-TAATACGACTCACCTATAGGGAGA**7AI**-3′
25-nt primer	5′-TAATACGACTCACCTATAGGGAGA**7AI**C-3′
26-nt primer	5′-TAATACGACTCACCTATAGGGAGA**7AI**CG-3′
27-nt primer	5′-TAATACGACTCACCTATAGGGAGA**7AI**CGA-3′
45-nt template	3′ATTATGCTGAGTGATATCCCTCT**X**GCTAGGTTACG-GCAGGATCGC-5′

The unnatural d**7AI**TP was then evaluated as a substrate for pol β, using the same 23-nt primer and 45-nt template containing **7AI** at position **X**. Pol β inserted d**7AI**TP, as well as each natural dNTP, opposite **7AI** with a k_{cat}/K_M that was less than 10^3 M^{-1} min^{-1}. These rates are significantly slower than those for native synthesis and more than 10^2 slower than the rate at which KF inserts d**7AI**TP opposite **7AI**. Therefore, in contrast to KF, pol β does not efficiently insert the unnatural triphosphate opposite any base in the template, including **7AI**.

To examine the pol β-mediated extension of the **7AI**:**7AI** self-pair, a 24-nt primer containing **7AI** at the 3′-terminus (Scheme 1) was annealed to the 45-nt template containing **7AI** at position **X**. Remarkably, extension of the **7AI**:**7AI** self-pair by insertion of dCTP opposite dG, proceeded with an efficiency of 3.5×10^5 M^{-1} min^{-1}, which is virtually identical to that measured for the insertion of dCTP opposite dG following a dA:dT base pair. Apparently, pol β is not deleteriously affected by the presence of the unnatural terminus and thus can extend the primer past the unnatural base with a native-like efficiency.

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Interestingly, the activities of KF and pol β are exclusive and complementary with regard to the **7AI** self-pair: KF efficiently synthesizes the self-pair, and pol β efficiently extends it. Given the low processivity of both enzymes, a binary polymerase system should be able to replicate DNA containing **7AI**. To test this notion, we characterized extension of the 23-nt primer oligonucleotide in the presence of each polymerase separately, as well as in the presence of both polymerases together (Figure 1b). In all cases, the concentration of each of the five dNTPs (four natural dNTPs and d**7AI**TP) was 100 μM, and exonuclease proficient KF (KF *exo*$^+$) was used to optimize fidelity. In reactions containing only KF *exo*$^+$, an equilibrium was established between primer and $n + 1$ product due to the presence of both polymerase and exonuclease activities, while no $n + 2$ product was detected. These results are consistent with the kinetic data described above, showing that KF is able to efficiently insert d**7AI**TP opposite **7AI** but is not able to efficiently continue primer extension. In the reaction containing only pol β, no $n + 1$ product (which would result from the insertion of d**7AI**TP opposite **7AI**) was observed, again consistent with the steady-state kinetics. However, in the presence of both enzymes, efficient full-length product synthesis was evident, and the rate for full-length synthesis was not significantly compromised by the presence of the unnatural base. We also examined the fidelity of the binary polymerase system using the 23-nt primer with only four of the required five triphosphates (Figure 1c). It is apparent that when a given triphosphate is absent, synthesis proceeds along the template until the complementary base is reached and then terminates. Thus, when used together, KF *exo*$^+$ and pol β can efficiently, and with reasonable fidelity, replicate DNA containing three base pairs.

To further evaluate the binary polymerase system, we quantitated the contribution of three separate steps to the overall fidelity: the relative rates for insertion of the correct versus incorrect triphosphate by KF opposite **7AI** in the template; the preferential removal of mispairs between **7AI** and a natural base by KF exonuclease activity; and the relative rates that the **7AI** self-pair and mispairs are extended by pol β. To determine the fidelity of KF-mediated insertion, steady-state kinetics were performed at 37 °C (the optimal temperature for the binary polymerase system) with the 23-nt primer and 45-nt template containing **7AI** at position **X** (Scheme 1). KF inserts d**7AI**TP opposite **7AI** in the template with an efficiency of 7.8×10^5 M^{-1} min^{-1}. This rate is 20 to 110-fold more efficient than insertion of any natural triphosphate (see Supporting Information). To approximate the proofreading activity, steady-state rate constants for exonuclease activity (k_{exo}) were determined under saturating duplex conditions (see Supporting Information). KF exonuclease activity was less efficient with the correct **7AI** self-pair, relative to mispairs that result from the incorporation of a natural triphosphate opposite **7AI** in the template. Natural bases mispaired opposite **7AI** are excised with a rate that varies between 5.0 and 7.5 min^{-1}, which is 3 to 5-fold more efficient than the rate for **7AI**. A final contribution to fidelity arises from preferential extension of the **7AI** self-pair by the pol β-mediated addition of the next correct nucleoside triphosphate. As mentioned above, the unnatural pair is extended with an efficiency of 3.5×10^5 M^{-1} min^{-1}, while mispairs between **7AI** in the template and a natural base at the primer terminus are extended 7 to 100-fold less efficiently (see Supporting Information). While it is difficult to approximate the overall fidelity, we note that each step that is sensitive to the unnatural base, namely, KF incorporation, KF proofreading, and pol β extension, makes a positive contribution to fidelity. We are in the process of quantitating the fidelity with which the binary polymerase system synthesizes DNA containing the unnatural base pair. The fidelity is obviously not as good as the highly evolved natural systems of replication; however, we are confident that the fidelity is reasonable on the basis of the steady-state kinetics as well as the data presented in Figure 1.

To address the fidelity of continued primer extension by the binary polymerase system, 25-nt, 26-nt, and 27-nt primers were used to examine addition of a second (dGTP opposite dC), third (dATP opposite dT), and fourth (dTTP opposite dA) nucleotide past the unnatural base in the growing primer strand (Scheme 1). The steady-state rate data is reported in Supporting Information. The efficiency and fidelity of continued synthesis by both enzymes compares favorably with that observed during synthesis of DNA containing only natural bases.

Nature has evolved a conserved system of information storage and retrieval that is based on the specific complementary nucleobase H-bonds. However, there is no reason H-bonding should be uniquely capable of supporting the intermolecular interactions required for information storage and replication. We have demonstrated that hydrophobicity, long known to be a dominant force in proteins[7] is also capable of providing the intermolecular interactions required for nucleic acid structure and function, including stability in duplex DNA, enzymatic triphosphate insertion, proofreading, and extension. Nucleoside analogues that are not constrained to have complementary H-bonding patterns greatly increase the number of potential unnatural base pair candidates for expansion of the genetic code. We are currently examining other unnatural nucleobases that are designed around alternate scaffoldings. We hasten to point out that despite the efficient extension of the **7AI**:**7AI** self-pair, pol β is not able to extend primers containing a variety of other unnnatural bases,[8] implying that the ability of a given polymerase to extend a given unnatural base pair will likely need to be evaluated on a case-specific basis. Toward this goal, we are currently examining a wide variety of enzymes, including pol κ, pol η, pol ζ, and pol V, which like pol β have been demonstrated to efficiently catalyze primer extension past a modified base in the template.[9] We are also currently examining the ability of the binary polymerase system to replicate DNA containing multiple unnatural bases in the template. These studies may allow for the development of in vitro replication conditions for additional unnatural base pairs, and will continue to identify the replication activities that are important for our efforts to replicate unnatural DNA in vivo.

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Supporting Information Available: Experimental protocols and data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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