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RECOGNITION OF A NON-STANDARD BASE PAIR BY THERMOSTABLE DNA POLYMERASES

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Abstract: Examination of several commercially available thermostable DNA polymerases identifies 9°N DNA polymerase as single enzyme that could incorporate two components of an expanded genetic alphabet, 2,4-diaminopyrimidine and xanthosine as deoxynucleoside triphosphate opposite their cognate base in a DNA template. © 1998 Elsevier Science Ltd. All rights reserved.

Over the past decade nucleic acids have become important both for diagnostic and therapeutic applications.[1] In this regard, the SELEX methodology (Systematic Evolution of Ligands by Exponential Enrichment), a combinatorial chemistry technology that uses oligonucleotides as the source of compounds, is a very powerful tool.[1] Recently, this methodology was used to identify oligonucleotides that bind specific targets ('aptamers') and that serve as catalysts ('ribozymes') for various reactions.[2] However, it is obvious that the sequence diversity for a nucleïc acid containing the four standard bases (n^4) is remarkably lower than the sequence diversity for a polypeptide of the same length (n^{20}). In addition, recent reports suggest that oligonucleotides that are built solely from the four standard nucleotides might also lack functional and/or structural diversity.[3]

Work in these laboratories suggested that extra 'letters' of an expanded genetic alphabet following the Watson-Crick base pairing rules might help to overcome these problems. In this context, the non-standard base pair between 2,4-diaminopyrimidine (pyDAD, presenting an hydrogen bond donor-acceptor-donor pattern) and xanthosine (puADA, presenting an hydrogen bond acceptor-donor-acceptor pattern, trivially designated X) appears to be especially interesting (Fig. 1).[4] For example, it has been shown that both non-standard bases have the appropriate stability needed to participate in a biopolymer used to store and express genetic information.[4] Further, in contrast to standard nucleotides both molecules are likely to be charged on the nucleobase under physiological conditions. The pK_a for deprotonation of the free dX at its N-3 atom was determined to be 5.7, the pK_a for protonation of the pyDAD nucleobase was found to be 6.7.[5]

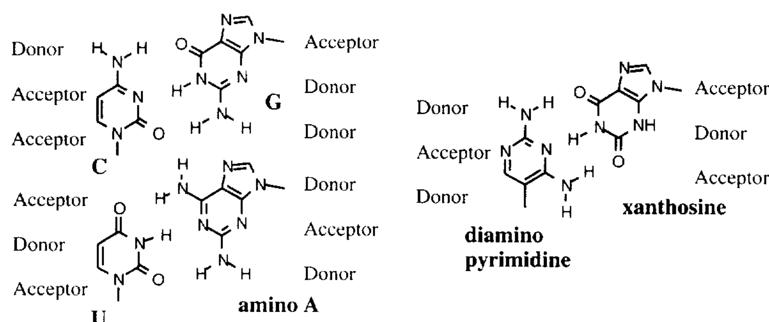

Fig. 1 Two standard and one non-standard base pair that meet the constraints imposed by the Watson-Crick formalism.

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By using the SELEX methodology the impact of these non-standard bases on the catalytic property or binding affinity of an oligonucleotide should be, in principle, easily examined. However, this method requires nucleic acid sequences that can be amplified by a thermostable DNA polymerase. To date, only HIV-1 reverse transcriptase is known to allow incorporation of both dXTP and the d(pyDAD)TP opposite their cognate base in the template.[6] Therefore, we examined commercially available thermostable DNA polymerases for their ability to form the d(pyDAD)-dX base pair.

Synthesis of non-standard nucleic acids substrates was performed as previously described.[6] The primer was synthesized by Microsynth (Windisch, Switzerland), purified by PAGE and finally labeled at the 5'-end with *Redivue* [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (Life Technologies). Standard dNTPs were purchased from Pharmacia. Primer elongation experiments with thermostable DNA polymerases were performed as previously reported.[6,7]

When Vent DNA polymerase (*Thermococcus litoralis*) was challenged by a template containing the d(pyDAD) nucleobase, no elongation of the primer past the non-standard base was observed, both in the absence and presence of dXTP (Fig. 2a, lanes 1-2). Similar results were seen when the experiment was repeated with a template containing dX, again both in the absence and presence of the d(pyDAD) nucleobase (Fig. 2a, lanes 3-4). Experiments with other thermostable DNA polymerases identified other enzymes that fall into the same class, namely DNA polymerases from *Thermus thermophilus* (Tth), *Pyrococcus furiosus* (Pfu), and *Pyrococcus woesii* (Pwo) (data not shown).

No read-through past a templating d(pyDAD) nucleobase was also observed with Deep Vent DNA polymerase (*Pyrococcus sp.*), both in the absence and presence of dXTP (Fig. 2b, lanes 1-2). In contrast, Deep Vent DNA polymerase was able to elongate a primer past dX in the template, both in the absence and presence of d(pyDAD)TP (Fig. 2b, lanes 3-4). However, a quantitative Phosphor Imager analysis[8] revealed that the amount of products formed past the non-standard base in the template in the absence of d(pyDAD)TP was identical to the amount of products formed in its presence. This suggests that primer elongation past dX in the template is exclusively due to misincorporation of dG and/or dT opposite dX. Experiments with the DNA polymerase from *Thermus aquaticus* (Taq) yielded identical results with respect to the incorporation of both dXTP and d(pyDAD)TP opposite the cognate base in the DNA template (data not shown).

More positive results were seen with HotTub DNA polymerase (*Thermus 'ubiquitos'*). When this thermostable DNA polymerase was challenged by a template containing the d(pyDAD) nucleobase in the absence of dXTP, DNA synthesis stopped at the positions primer+4 and primer+5 and no further elongation of the primer past the non-standard base was observed (Fig. 2c, lane 1). However, in the presence of dXTP read-through past the d(pyDAD) nucleobase in the template and synthesis of full-length product occured (Fig. 2c, lane 2). This implies that HotTub DNA polymerase could form the dX-d(pyDAD) to some extent. In contrast, our results strongly suggest that HotTub DNA polymerase could not incorporate d(pyDAD)TP opposite dX in the DNA template (Fig. 2c, lanes 3-4). Similar to Deep Vent DNA polymerase the Phosphor Imager analysis[8] did not reveal any difference between the amount of products formed past dX in the template in the absence and presence of d(pyDAD)TP.

Surprising results were obtained with 9°N DNA polymerase (*Thermococcus sp.*). Similar to HotTub DNA polymerase 9°N DNA polymerase was able to specifically incorporate dXTP opposite the d(pyDAD) nucleobase in the template and to synthesize full-length product (Fig. 2d, lanes 1-2). However, primer elongation also mostly

stopped one base before the position of the non-standard base (primer+4). When incubating 9°N DNA polymerase in the absence of d(pyDAD)TP with a template containing dX large amounts of truncated products (position primer+4 and primer+5) were detected (Fig. 2d, lane 3). In addition, longer products were also formed due to misincorporation of standard nucleotides opposite dX in the template. However, when repeating this experiment in the presence of d(pyDAD)TP pausing at positions primer+4 and primer+5 was significantly reduced and large amounts of products past dX in the DNA template were observed (Fig. 2d, lane 4). The quantitative analysis yielded that at least 45% of the products formed past dX in the template derived from specific incorporation of d(pyDAD)TP opposite dX.

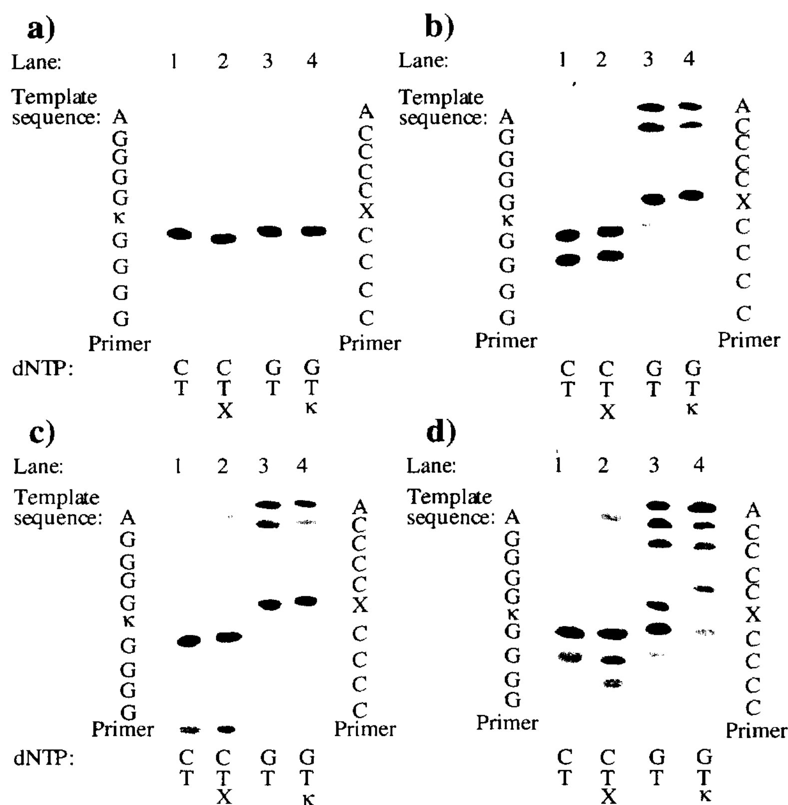

Fig. 2 PAGE analysis of primer extension reaction by (a) Vent, (b) Deep Vent, (c) HotTub, and (d) 9°N DNA polymerase. Deoxynucleoside triphosphates present in solution are indicated below (50 µM each). Incubations were performed at 75°C for 15 min with 0.15 pmol labeled primer/template-complex containing either the d(pyDAD) nucleobase (lanes 1-2) or dX (lanes 3-4) and 0.2 units enzyme in a final volume of 25 µl. X stands for 2'-deoxyxanthosine and κ for the d(pyDAD) nucleobase, respectively.

Results collected in this study with thermostable DNA polymerases confirm and expand data collected recently with mesophilic DNA polymerases.[6] Again, it is demonstrated that both non-standard nucleotides, d(pyDAD) and dX, are not accepted with the same ease than standard nucleotides. However, a structural explanation for the discrimination of thermostable DNA polymerases against both non-standard bases is difficult to assign at this

stage and certainly awaits further studies. To date, only Taq DNA polymerase has been successfully crystallized, but with an incomplete set of substrates.[9]

With 9°N DNA polymerase a single thermostable DNA polymerase has been identified that could incorporate both d(pyDAD)TP and dXTP opposite their cognate non-standard base. In this context, it is tempting to speculate that site-directed mutagenesis of this DNA polymerase will yield a technological powerful tool in the near future for routinely synthesizing oligonucleotides containing both non-standard bases. Interestingly, 9°N DNA polymerase[10] could be aligned by a highly conserved sequence motif[11] to other DNA polymerase sequences where studies already identified residues critical for the non-standard base pair formation.[12]

Results presented in this study shed also light on the evolution of DNA polymerases. To date, it still remains unclear whether all polymerases are derived from an common ancestor and therefore follow the same catalytic mechanism.[13] Experiments with the d(pyDAD)-dX base pair and variant forms suggested that all polymerases must be described individually with respect to their recognition of nucleic acid substrates.[6] This hypothesis is further supported by this study as Vent DNA polymerase which shares over 70% sequence identity with 9°N DNA polymerase, [10] behaves completely different with respect to the recognition of the non-standard base pair.

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8. Radioactivity was quantified using a Phosphor Imager (Molecular Dynamics), with 5 h exposures and the ImageQuant program from Molecular Dynamics. To determine the lower limit of specific formation of the non-standard base pair, the amount of products past the non-standard base in the template was quantified, divided by the total amount of radioactivity in the lane and expressed as a percentage. To correct for non-specific misincorporation of standard nucleobases opposite the non-standard nucleotides the amount of misincorporation of natural dNTPs, determined in a control experiment, was subtracted. This corrected percentage was then reported to the percentage of read-through past the non-standard bases when the cognate non-standard nucleoside triphosphate was present in solution to yield the lower limit of specific formation. Ideally, if no misincorporation of standard nucleosides opposite the non-standard bases occur the percentage specific non-standard base pair formation would be 100%.
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