Communication

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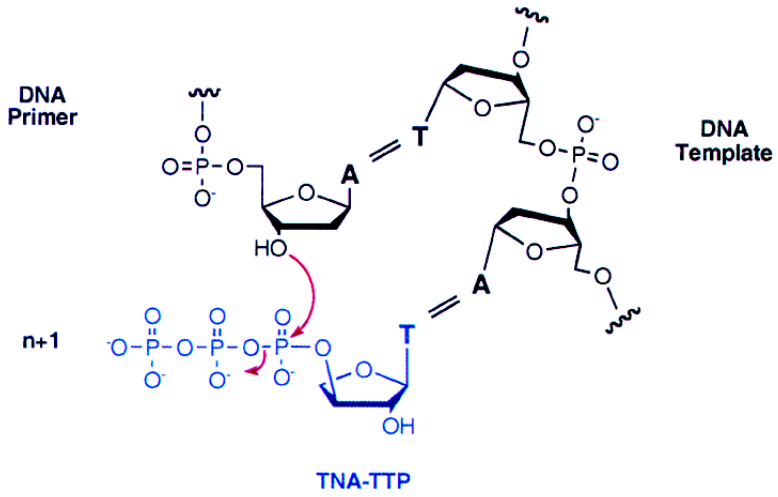

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TNA Synthesis by DNA Polymerases

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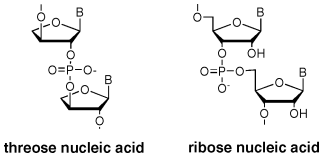
The systematic evaluation of structures related to RNA[1] led to the recent discovery that (3′,2′)-α-L-threose nucleic acid (TNA) oligonucleotides are capable of Watson−Crick base-pairing with complementary DNA, RNA, and TNA oligonucleotides.[2] These observations, coupled with the chemical simplicity of threose relative to ribose, make TNA very interesting as a potential progenitor of RNA.[3] For this reason, we would like to compare the functional properties of TNA and RNA by attempting to evolve TNA aptamers and catalysts by in vitro selection.[4,5] This will require polymerases capable of copying a library of random DNA sequences into a library of TNA sequences for selection, and then copying the surviving TNA sequences back into DNA for amplification by PCR. We have recently identified several DNA polymerases capable of faithfully copying a short TNA template into DNA.[6] We now wish to report that some DNA polymerases are also capable of TNA synthesis on a DNA template when given the appropriate TNA triphosphate. These enzymes are candidates for directed evolution into more efficient DNA-dependent TNA polymerases.



We used a primer extension assay to compare the ability of a set of DNA polymerases to synthesize TNA by extension of a DNA primer annealed to a DNA template, in the presence of chemically synthesized α-L-threofuranosyl thymidine-3′-triphosphate **1** (tTTP, Figure 1A). A synthetic DNA primer was annealed to either of two DNA templates in which the three template bases following the primer were adenine (A) or diaminopurine (D) (Figure 1b).

We screened several different types of DNA polymerases including the Klenow fragment of DNA pol I (exo-), the thermophilic DNA polymerases Taq, Bst pol I, and Deep Vent (exo-), the bacteriophage T7 (exo-), and its commercial version Sequenase, the viral reverse transcriptases HIV RT, MMLV, and its mutated commercial version Superscript II, as well as the repair polymerases Pol β, DinB, and Dbh (data not shown). All enzymes tested except DinB and Dbh were able to extend all of the DNA primer by at least one tT nucleotide. The DNA polymerases Bst pol I, Deep Vent (exo-), and HIV RT were capable of catalyzing multiple tT additions to the DNA primer (Figure 2). Deep Vent (exo-) was by far the most effective at TNA synthesis (lanes 5−7). The HIV reverse transcriptase is unusual in catalyzing the rapid incorporation of two successive tT residues, but showing virtually no subsequent incorporation even with longer reaction times and additional polymerase. Only for Deep Vent (exo-) did the substitution of adenosine by diaminopurine in the template significantly enhance TNA synthesis, leading to quantitative incorporation of three tT residues. This substitution has previously been shown to increase

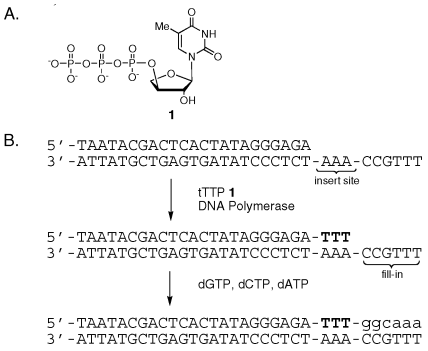


Figure 1. (A) Structure of tTTP substrate. (B) Sequence of primer−template complex. TNA product of DNA template is shown in bold with continued DNA extension from TNA adduct given in lower case.

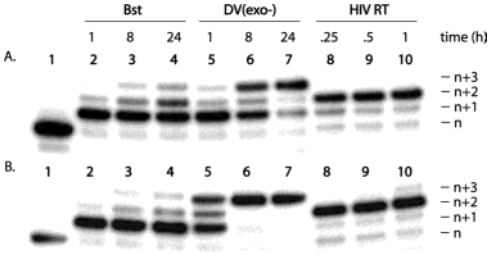


Figure 2. tTTP primer extension. Products of tTTP **1** extension on a 5′-^{32}P-labeled 23-mer primer annealed to a DNA template containing either adenosine (A) or diaminopurine (B) at the insertion site. Primer extension experiments were performed with 250 nM primer/template and 0.5 μL DNA polymerase. Reaction progress over time was analyzed by denaturing polyacrylamide gel electrophoresis for experiments using Bst (lanes 2−4), DV^{exo-} (lanes 5−7), and HIV RT (lanes 8−10).[9]

the stability of DNA/TNA heteroduplexes and the efficiency of TNA-directed nonenzymatic ligation.[7]

To understand the various factors that influence the rate of TNA synthesis, we have examined the catalytic efficiency of three aspects of the TNA synthesis reaction: (1) extension of a DNA primer by one TNA nucleotide; (2) subsequent addition of a second and third TNA nucleotide; and (3) extension of a primer ending in either one or three TNA nucleotides by DNA synthesis. Using the steady-state method,[8] values for K_m, V_{max}, and catalytic efficiency (V_{max}/K_m) were determined for dT and tT **1** insertion opposite a single A in the DNA template. This method provides an accurate means for comparing enzyme selectivity for threose- versus deoxyribose-nucleoside triphosphates.

As expected, the enzyme with the least kinetic discrimination between tTTP **1** and dTTP was the thermophilic polymerase Deep Vent (exo-) (Table 1), with a kinetic selectivity of ∼0.01. HIV reverse transcriptase was more selective (∼0.0013), and Bst pol I was the most selective (>0.00018) for DNA. Comparison of K_m and V_{max} values suggests that all three enzymes show a similar decrease in tTTP binding (1−300-fold, corresponding to a loss of

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Table 1. Steady-State Kinetic Parameters for Polymerase-Mediated Single-Nucleotide Extension of TNA **1** on a DNA Template[a]

```
5′-d-TAATACGACTCACTATAGGGAGA
3′-d-ATTATGCTGAGTGATATCCCTCTACCGTTT
```

enzyme	NTP	K_m (μM)	V_{max} (%/min)	V_{max}/K_m (%/min M)
Bst	dTTP	0.31 ± 0.01	0.4 ± 0.01	1.3×10^6
	tTTP	95 ± 26	0.022 ± 0.01	231
$DV^{(exo-)}$	dTTP	0.52 ± 0.14	0.26 ± 0.2	5.0×10^5
	tTTP	42 ± 21	0.21 ± 0.15	5.0×10^3
HIV RT	dTTP	0.22 ± 0.07	0.71 ± 0.05	3.2×10^6
	tTTP	20 ± 4.3	0.081 ± 0.03	4.1×10^3

[a] Assay conditions: Reactions were initiated by adding 10 μL of 2x dTTP or tTTP **1** to an equal volume of the reaction mixture containing 250 nM primer−template complex, 1−2.5 nM enzyme, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 2 mM $MgSO_4$, 100 μM DTT, 25 μg/μL BSA, incubating for 1−20 min at either 42 or 55 °C, and quenching with the addition of 10 μL loading buffer (1x TBE/8 M urea/dye). A 10 μL aliquot was then analyzed by 20% denaturing polyacrylamide gel electrophoresis. Resulting bands were quantified using a PhosphorImager (Molecular Dynamics).

Table 2. Steady-State Kinetic Parameters for Deep $Vent^{exo-}$-Mediated Single-Nucleotide Primer Extension[a]

```
5′-d-TAATACGACTCACTATAGGGAGAX
3′-d-ATTATGCTGAGTGATATCCCTCTYCGTTT
```

X	Y (DNA)	NTP	K_m (μM)	V_{max} (%/min)	V_{max}/K_m (%/min M)
-	A	tTTP	42 ± 21	0.21 ± 0.15	5000
tT	AA	tTTP	43 ± 0.8	$5.9 \pm 5 \times 10^{-4}$	14[b]
tTT	AAA	tTTP	183 ± 60	$3.5 \pm 3 \times 10^{-4}$	1.9[c]
tT	A	dGTP	23.1 ± 4.0	0.13 ± 0.003	5600
dT	A	dGTP	1.1 ± 0.5	0.13 ± 0.06	1.2×10^5
tTTT	AAA	dGTP	36 ± 3.2	$8.3 \pm 1.7 \times 10^{-3}$	230
dTTT	AAA	dGTP	1.1 ± 0.6	0.07 ± 0.04	6.4×10^4

[a] See Table 1 for experimental details. [b] 1 h of incubation. [c] 6 h of incubation.

2.8−3.5 kcal/mol of binding energy). Remarkably, Deep Vent (exo-) catalyzes tT and dT incorporation at identical rates, while HIV RT and Bst pol I show additional decreases in catalytic rate.

The efficiency of DNA-directed TNA synthesis by Deep Vent (exo-) allowed us to purify primers ending in one, two, or three tT residues. We first used the primer ending in one tT residue to measure the kinetics of incorporation of a second tT residue (Table 2, line 2). The K_m for tTTP in this reaction was identical to that observed in the incorporation of the first tT residue; however, the rate of incorporation was decreased by a factor of ~355. This observation is consistent with the fact that the attacking hydroxyl in the first step is the normal 3′-OH of deoxyribose, but in the second step it is the 2′-OH of threose. We then measured the kinetics of incorporation of a third tT residue, making use of a primer ending in two tT residues (Table 2, line 3). Surprisingly, at this position, the K_m for tTTP decreased by a factor of ~4.5, while the rate of incorporation was the same as that for the previous position. This may reflect either increasing distortion of the primer/template/enzyme complex, resulting in some disturbance to substrate (tTTP) binding, or possibly a change in the rate-limiting step.

We then measured the kinetics of DNA synthesis by Deep Vent (exo-) following one or three TNA residues on the primer. We measured the incorporation of a single dGTP opposite C in the template (Table 2). The catalytic efficiency is reduced approximately 21-fold when the primer contains one terminal tT residue, relative to dGTP insertion on a completely DNA primer, and an additional 24-fold when three tT residues are present. These observations probably reflect a progressive loss of contacts between the polymerase and the primer strand as the primer is extended with additional TNA residues. This conclusion is consistent with crystallographic data showing a 0.6−1.8 Å displacement of the phosphodiester backbone at the site of a single tT insertion in a standard B-form DNA helix,[10] and with crystal structures of primer/template/polymerase complexes.[11,12] Results very similar to those reported here have also been obtained in the Herdewijn laboratory.[13]

In summary, we have identified Deep Vent (exo-) as a polymerase that is capable of significant TNA synthesis. We suggest that directed evolution of this enzyme might lead to the isolation of a DNA-dependent TNA polymerase capable of extensive TNA synthesis. Such an enzyme, along with a TNA-directed DNA polymerase, would enable in vitro evolution experiments with TNA molecules.

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