Recognition of threosyl nucleotides by DNA and RNA polymerases

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ABSTRACT

α-L-Threose nucleic acids (TNA) are potentially natural nucleic acids that could have acted as an evolutionary alternative to RNA. We determined whether DNA or RNA polymerases could recognize phosphorylated threosyl nucleosides. We found that for both the Vent (exo⁻) DNA polymerase and HIV reverse transcriptase K_m values were increased and k_{cat} values decreased for the incorporation of tTTP in comparison to their natural counterparts. Our results suggest that TNA may have played a role in the evolution of the DNA–RNA–protein world. Thus, TNA may be a candidate for further studies in evolutionary chemistry and biology.

INTRODUCTION

To understand the chemical etiology of nucleic acids, the Eschenmoser group has mapped the structural neighborhood of RNA and has studied the potential of these polymers to function as evolutionary alternatives to the nucleic acid system.

The potentially natural nucleic acid analogs α-L-threose nucleic acids (TNA) (Fig. 1), a tetrose-based oligonucleotide that can hybridize with TNA, RNA and DNA, can be considered as an RNA alternative (1,2). Being potentially synthesized from natural starting materials, investigation of the functional properties of the TNA system should be interesting. These experiments may be very useful to analyze the potential of using TNA in molecular evolution. Furthermore, these studies are also important to further characterize the structure–activity relationship of the polymerases. Previously, Szostak and co-workers found that certain DNA polymerases could copy limited stretches of a TNA template (3). In our investigation we will focus on the recognition of phosphorylated threosyl nucleosides by DNA and RNA polymerases.

MATERIALS AND METHODS

Chemicals and DNA

DEAE–Sephadex A25 was obtained from Sigma. Highly purified 2′-deoxynucleoside triphosphates used in DNA polymerase reactions and the Mono Q 5/5 and 10/10 columns were purchased from Pharmacia.

Synthesis of α-L-threofuranosyl nucleotides

1-(α-L-Threofuranosyl)-thymine was synthesized as previously described (4). The synthesis of 1-(α-L-threofuranosyl)-uracil was done accordingly. These nucleoside analogs were converted to their respective α-L-threofuranosyl nucleoside triphosphates in a two step reaction. First, synthesis of the α-L-threosyl nucleoside monophosphate and, in a second step, synthesis of the α-L-threofuranosyl nucleoside triphosphate.

α-L-Threosyl nucleoside monophosphates

The synthesis of α-L-threosyl nucleoside monophosphate was done in a two step reaction. First, synthesis of the 2′-O-benzoyl-nucleoside monophosphate and, in a second step, deprotection to form the nucleoside monophosphate.

A cold mixture of dried pyridine (2.5 ml), POCl$_3$ (0.112 mmol, 1.5 equiv.) and triazole (0.225 mmol, 3 equiv.) was stirred under nitrogen in a dry round-bottom flask. After 10 min, the 2′-O-benzoyl nucleoside (0.075 mmol, 1 equiv.) was added at 0°C while stirring. As soon as the starting material had completely disappeared (TLC CH$_2$Cl$_2$:acetone 7:3, 15–20 min), the reaction was quenched by pouring the mixture into a cold solution of 1 M TEAB (pH 7.5) (2.5 ml). The solution was concentrated to dryness. Mass spectrometry showed the obtained reaction product to be the desired 2′-O-benzoyl nucleoside containing a phospho-triazolide group. Hydrolysis of the triazolide group could be accomplished by dissolving the residue in water and adjusting the sample to pH 4 with an aqueous solution of H$_3$PO$_4$. Formation of the desired monophosphate could be followed by TLC (isopropanol/NH$_4$OH/H$_2$O 60:30:10). The R_f value for the monophosphate residue containing the triazole group was 0.8 whereas that of the residue without the triazole moiety was 0.4. After ~6 h the conversion was complete and the solution was concentrated under vacuum.

The 2′-O-benzoyl nucleoside monophosphate was suspended in ammonia-saturated H$_2$O (1.5 ml) and stirred at room temperature for 2 h. Ammonia and water were evaporated and co-evaporated twice with water. Formation of the deprotected reaction product could be followed by TLC (isopropanol/NH$_4$OH/H$_2$O 60:30:10) ($R_f \approx 0.2$). The resulting white solid was then dissolved in water and adsorbed on silica gel (5 g) packed with isopropanol/NH$_4$OH/H$_2$O (60:10:5)

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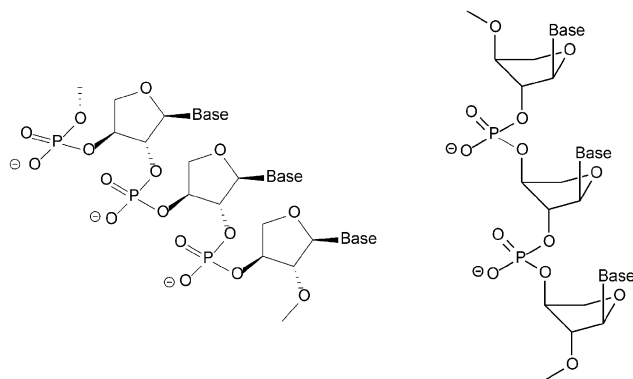
Figure 1. Constitution, configuration and conformation (with linearized backbone) of an α-L-threofuranosyl (3′→2′) oligonucleotide strand (TNA).

using a mixture of isopropanol/NH$_4$OH/H$_2$O (60:30:10) as eluent. The desired fractions were collected, evaporated to dryness and co-evaporated twice with water, producing a colorless syrup. The nucleoside monophosphate was then dissolved in water and this solution was passed through an AgI-X4 column in the free acid form by elution with 10 column volumes of water. Fractions containing the desired monophosphate were collected and the aqueous solution was adjusted to pH 7 with a solution of NaOH followed by evaporation under vacuum. The yield of pure monophosphate, after drying, amounted to ~80%.

1-(α-L-Threosyl)-thymine monophosphate. ^{31}P NMR (T$_{tmp}$) δ (p.p.m.) (D$_2$O) 4.188 (s). ^{13}C NMR (D$_2$O): δ = 93.71 (C-1′), 82.36 (d, C-2′, J = 4.9 Hz), 79.81 (d, C-3′, J = 3.9 Hz), 76.87 (d, C-4′, J = 2.9 Hz). Exact mass calculated for C$_9$H$_{13}$N$_2$O$_8$P$_1$ [M+Na] = 331.03074; found 331.0289.

1-(α-L-Threosyl)-uracil monophosphate. ^{31}P NMR (T$_{ump}$) δ (p.p.m.) (D$_2$O) 3.569 (s). ^{13}C NMR (D$_2$O): δ = 94.43 (C-1′), 82.25 (d, C-2′, J = 4.9 Hz), 79.77 (d, C-3′, J = 4.9 Hz), 77.91 (d, C-4′, J = 3.9 Hz). Exact mass calculated for C$_8$H$_{11}$N$_2$O$_8$P$_1$ [M–H] = 293.01747; found 293.0160.

α-L-Threofuranosyl nucleoside triphosphates

Triphosphate synthesis was carried out according to the method of Hoard and Ott (5).

The resulting triphosphates were purified on a DEAE–Sephadex A25 column in the bicarbonate form. The column was washed with H$_2$O and eluted with a 0–1 mM TEAB gradient. TEAB was removed by repeated evaporation with methanol. Subsequently, the desired product was converted to its sodium salt by addition of a 1 M solution of sodium iodide in acetone. The precipitate was collected by centrifugation, washed with acetone (four 1 ml portions) and dried over phosphorus pentoxide under vacuum and analyzed by NMR. The yield of pure triphosphate, obtained after conversion to the sodium salt and drying, was 65%.

1-(α-L-Threosyl)-thymine triphosphate. ^{31}P NMR (T$_{ttp}$) δ (p.p.m.) (D$_2$O) –8.104 (d, γ-P), –12.212 (d, α-P), –22.214 (t, β-P). Exact mass calculated for C$_9$H$_{15}$N$_2$O$_{14}$P$_3$ [M–H] = 466.96578; found 466.9663.

1-(α-L-Threosyl)-uracil triphosphate. ^{31}P NMR (T$_{utp}$) δ (p.p.m.) (D$_2$O) –7.761 (d, γ-P), –12.014 (d, α-P), –21.791 (t, β-P). Exact mass calculated for C$_8$H$_{13}$N$_2$O$_{14}$P$_3$ [M–H] = 452.95013; found 452.9509.

HPLC

Ion exchange HPLC was performed using a L-6200 A Merck-Hitachi pump with UV monitoring. For analytical purposes, samples were separated on a Mono Q 5/5 column using a TEAB gradient (pH 7.5) with a flow rate of 1.0 ml min^{-1}. The TEAB in the gradient was increased from 0 to 0.5 M over 30 min. For preparative separation, a semi-preparative Mono Q 10/10 column was used with a flow rate of 2 ml min^{-1} and an analogous gradient (0–0.5 M TEAB over 40 min). Reversed phase HPLC on a C$_{18}$ column (self-packed, 12–40 μM, 250 × 9 mm) was performed using solvents A (0.1 M TEAB, 1% CH$_3$CN, H$_2$O) and B (90% CH$_3$CN in 0.1 M TEAB) and a linear gradient of 1–15% solvent B over 30 min at 2 ml min^{-1}.

Spectroscopy

Samples for NMR spectroscopy were prepared in a non-buffered solution of 99.96% D$_2$O. The ^{31}P NMR spectra were recorded with a Varian Unity 500 spectrometer with 85% H$_3$PO$_4$ in H$_2$O as internal standard.

Mass spectrometry

HPLC coupled to mass spectrometry (HPLC/MS) was performed on a capillary chromatograph (CapLC; Waters, Milford, MA). Columns of 150 mm × 0.3 mm (LCPackings, San Francisco, CA) were used. Oligonucleotides (1.25 pmol) were eluted with a triethylammonium:1,1,1,3,3,3-hexafluoro-2-propanol:acetonitrile solvent system adapted from Apffel *et al.* (6). The flow rate was 5 μl min^{-1}. The content of the organic phase in the gradient was increased by 1% min^{-1}. Electrospray spectra in negative ion mode were acquired on a quadrupole/orthogonal acceleration time-of-flight mass spectrometer (Q-Tof-2; Micromass, Manchester, UK) equipped with a standard electrospray source. Scan time was set to 2 s. The combined spectra from a chromatographic peak were deconvoluted using the MaxEnt algorithm of the software (Masslynx 3.4; Micromass).

Oligodeoxyribonucleotides

Oligodeoxyribonucleotides P1, P2 and T3 were purchased from Eurogentec. All other oligodeoxyribonucleotides were synthesized on an Applied Biosystems 392 DNA Synthesizer at 1 μmol scale using phosphoramidites from Glen Research. The concentrations were determined using a Varian Cary 300 Bio UV-spectrophotometer.

The lyophilized oligonucleotides were dissolved in DEPC-treated water and stored at –20°C. The primer oligonucle-otides were 5′-^{32}P-labeled with [γ-^{32}P]ATP (4500 Ci/mmol, 10 mCi/ml) (Pharmacia) using T4 polynucleotide kinase (Life Technologies) following standard procedures. Labeled oligo-nucleotides were further purified using a NAP-5™ column (Pharmacia).

DNA polymerase reactions

End-labeled primers were annealed to their template by combining primer and templates in a molar ratio of 1:2.5 and heating the mixture to 75°C for 10 min, followed by slow

cooling to room temperature over a period of 2.5 h. For the incorporation of one tTTP or tUTP, the primer P1 was annealed to template T2 or T3. A series of 15 µl reactions was performed for two enzymes: Vent (exo⁻) DNA polymerase (New England BioLabs) and HIV reverse transcriptase (Amersham Biosciences). The final mixture contained 50 nM primer–template complex (in their respective reaction buffer supplied with the polymerase), 10 µM, 100 µM or 1 mM triphosphate building blocks and different enzyme concentrations. In the control reaction with the natural nucleotide 10 µM dTTP was used. The mixture was incubated at 37 (HIV reverse transcriptase) or 55°C [Vent (exo⁻) DNA polymerase] and aliquots were quenched after 10, 30 and 120 min.

For the further incorporation of a natural guanosine triphosphate (dGTP) after incorporation of tTTP or tUTP, the primer P1 was annealed to template T3. The final mixture contained 50 nM primer–template complex and 10 µM tTTP or tUTP, 1 µM dGTP and 0.01 U µl⁻¹ Vent (exo⁻) DNA polymerase and 0.5 U µl⁻¹ HIV reverse transcriptase. The mixture was incubated at 37 (HIV reverse transcriptase) or 55°C [Vent (exo⁻) DNA polymerase] and aliquots were taken at 10, 30 and 120 min.

For HPLC/MS experiments the buffer was replaced by a solution containing 20 mM Tris base, 2 mM Mg(OAc)$_2$ and 0.1% Triton X-100 adjusted to pH 8.8 with HCl.

Transcription reaction

Primer P2 was annealed to template T1 by combining primer and template in the molar ratio 1:2 and heating the mixture to 75°C for 10 min, followed by slow cooling to room temperature over a period of 2.5 h. The reaction was initiated by addition of 2 µl of enzyme dilution to a reaction mixture of 18 µl. The incubation mixture consisted of 250 nM hybrid, reaction buffer (supplied with the RNA polymerase), 0.167 µM [γ-³²P]GTP (Amersham Biosciences), 10, 100 or 1000 µM triphosphate building block (tUTP or rUTP) and 5 U µl⁻¹ RNA polymerase. The mixture was incubated at 37°C and the reaction was quenched after 10, 30 and 120 min.

Kinetics experiments

To determine the kinetic parameters of the incorporation of tTTP or dTTP, a steady-state kinetic assay was performed. The reaction mixture was prepared by adding Vent (exo⁻) DNA polymerase or HIV reverse transcriptase to primer P1 annealed to template T3, buffer and tTTP or dTTP. The final mixture (20 µl) contained 0.05 or 0.005 U µl⁻¹ Vent (exo⁻) DNA polymerase and 0.5 or 0.01 U µl⁻¹ HIV reverse transcriptase, the commercially supplied buffer, 250 nM primer–template complex and various concentrations of tTTP or dTTP. A concentration range of 0.1–12.5 µM was used for Vent (exo⁻) DNA polymerase and a concentration range of 1.56–100 µM was used for HIV reverse transcriptase. Reactions were incubated at 55 or 37°C. Reaction times were between 2 and 20 min. The kinetic constants, V_{max} and K_m, were determined from a Michaëlis–Menten plot, using GraphPad Prism version 3.02.

Electrophoresis

All polymerase reactions were quenched by the addition of a double volume of loading buffer (90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 50 mM EDTA).

Primers

| 5' | CAGGAAACAGCTATGAC | 3' | P1 |
| 5' | TAATACGACTCACTATAG | 3' | P2 |

Templates

3'	ATTATGCTGAGTGATATC**AAAAAG** 5'	T1
3'	GTCCTTTGTCGATACTG**AAAAAAA** 5'	T2
3'	GTCCTTTGTCGATACTG**ACTGTGTGTGTG** 5'	T3

Figure 2. Sequences of primers and templates used in the polymerase incorporation experiments.

Samples were heated at 70°C for 5 min prior to analysis by electrophoresis for 2–3 h at 2000 V on a 0.4 mm 20% denaturing gel in the presence of a 100 mM Tris–borate, 2.5 mM EDTA buffer, pH 8.3. Products were visualized by phosphorimaging. The amount of radioactivity in the bands corresponding to the products of enzymatic reactions was determined using the Optiquant image analysis software (Perkin Elmer).

RESULTS

Incorporation of tTTP and tUTP into a DNA hybrid and extension by DNA polymerases

In a first series of primer extension assays we investigated the ability of two DNA polymerases, Vent (exo⁻) DNA polymerase and HIV reverse transcriptase, to extend a DNA primer, annealed to a DNA template, by threosyl nucleotides. Both enzymes have been shown to efficiently incorporate modified nucleotides into a DNA hybrid (7,8). For these experiments end-labeled primer P1 was annealed to template T2, having a seven adenine base overhang (Fig. 2). Both the Vent (exo⁻) DNA polymerase (Fig. 3) and the HIV reverse transcriptase (Fig. 4) are able to incorporate two tTTP or tUTP nucleotides into the DNA hybrid. For the Vent (exo⁻) DNA polymerase weaker bands could be seen corresponding to a primer extension with three or four threosyl building blocks (Fig. 3). However, incorporation of the second modified nucleotide is more difficult than incorporation of the first one and further chain elongation seems to stop after incorporation of the second threosyl nucleotide. The same incorporation profile was found using an excess of polymerase (data not shown). Under the conditions used for HPLC/MS the primer was extended by two threofuranosyl nucleotides (Fig. 5).

In a second series of primer extension assays, we investigated further elongation with a natural dGTP building block following incorporation of tTTP and tUTP. For these experiments the end-labeled primer P1 was annealed to template T3 (Fig. 2). Both the Vent (exo⁻) DNA polymerase (Fig. 6) and the HIV reverse transcriptase (Fig. 7) are able to further extend the primer with a natural dGTP building block following incorporation of tTTP or tUTP. Quantitative data for the
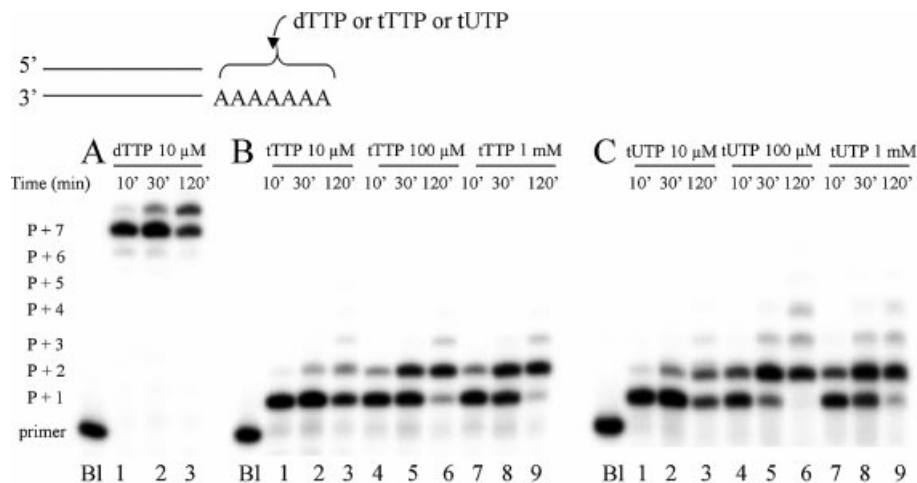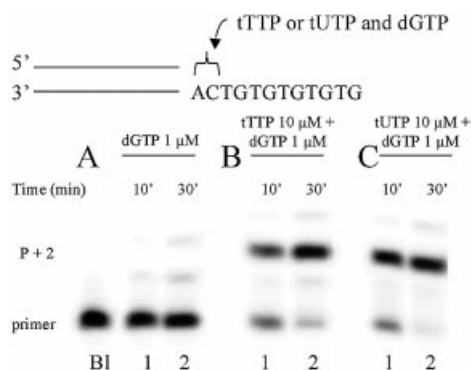
Figure 3. Phosphorimages of the enzymatic incorporation of tTTP and tUTP into a 50 nM hybrid P1/T2 in the presence of 0.05 U µl⁻¹ Vent (exo⁻) DNA polymerase. (**A**) 10 µM dTTP. (**B**) Lanes 1–3, 10 µM tTTP; lanes 4–6, 100 µM tTTP; lanes 7–9, 1 mM tTTP. (**C**) Lanes 1–3, 10 µM tUTP; lanes 4–6, 100 µM tUTP; lanes 7–9, 1 mM tUTP. Bl indicates the blank reaction in the absence of NTPs.
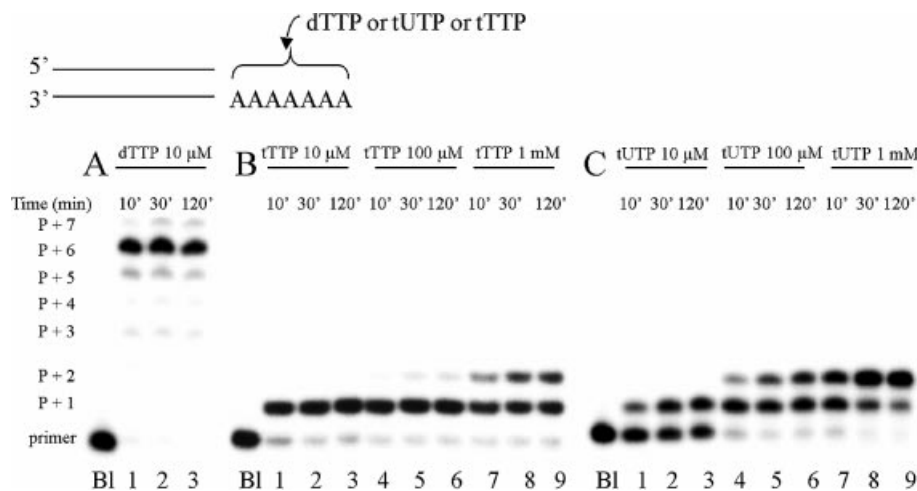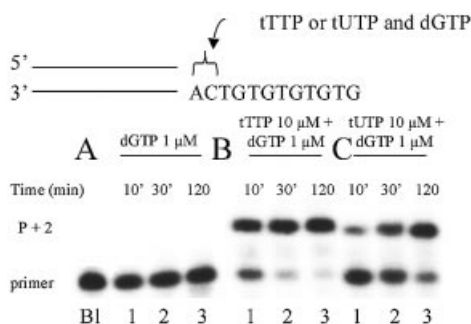

Figure 4. Phosphorimages of the enzymatic incorporation of tTTP and tUTP into a 50 nM hybrid P1/T2 in the presence of 0.05 U µl⁻¹ HIV reverse transcriptase. (**A**) 10 µM dTTP. (**B**) Lanes 1–3, 10 µM tTTP; lanes 4–6, 100 µM tTTP; lanes 7–9, 1 mM tTTP. (**C**) Lanes 1–3, 10 µM tUTP; lanes 4–6, 100 µM tUTP; lanes 7–9, 1 mM tUTP. Bl indicates the blank reaction in the absence of NTPs.
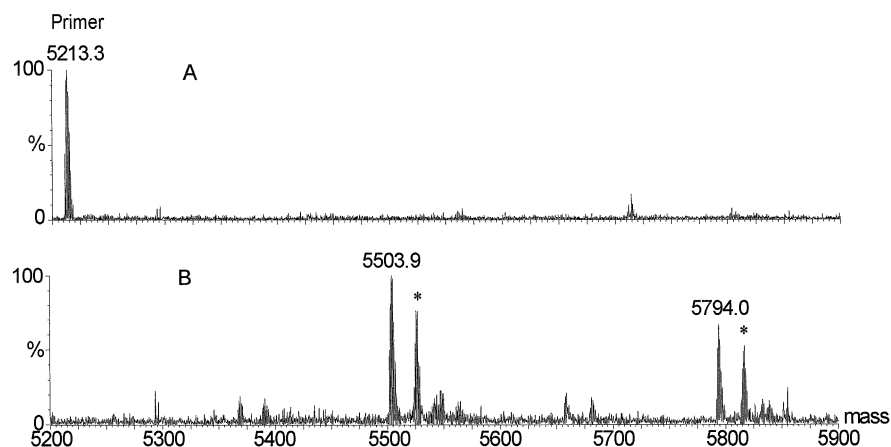
Figure 5. Deconvoluted mass spectra of (**A**) the primer 5′-CAG GAA ACA GCT ATG AC-3′ after HPLC of the hybrid and separation of the two DNA strands and (**B**) the extended primer by one and two threofuranosyl nucleotides. Peaks indicated by an asterisk denote cation adducts (Na⁺ or Mg²⁺).

Figure 6. Phosphorimages of the enzymatic incorporation of tTTP, tUTP and dGTP into a 50 nM hybrid P1/T3 in the presence of 0.01 U μl⁻¹ Vent (exo⁻) DNA polymerase. (**A**) 1 μM dGTP; (**B**) 10 μM tTTP + 1 μM dGTP; (**C**) 10 μM tUTP + 1 μM dGTP. Bl indicates the blank reaction in the absence of NTPs. Full elongation of the primer was achieved after 30 min.

Figure 7. Phosphorimages of the enzymatic incorporation of tTTP, tUTP and dGTP into a 50 nM hybrid P1/T3 in the presence of 0.5 U μl⁻¹ HIV reverse transcriptase. (**A**) 1 μM dGTP; (**B**) 10 μM tTTP + 1 μM dGTP; (**C**) 10 μM tUTP + 1 μM dGTP. Bl indicates the blank reaction in the absence of NTPs.

incorporation of tTTP and tUTP into DNA hybrids P1/T2 and P1/T3 are summarized in Table 1.

The RT experiment was also carried out in the absence of a template. However, in these conditions, we were unable to detect a polymerization reaction of the triphosphates.

Generally, the Vent (exo⁻) DNA polymerase is more efficient than the HIV reverse transcriptase for incorporation of threosyl nucleotides, when a DNA template was used.

Steady-state kinetic analysis of tTTP insertion into a DNA hybrid

To obtain an idea of the efficiency of incorporation by Vent (exo⁻) DNA polymerase and HIV reverse transcriptase of

tTTP in comparison to dTTP, the kinetic parameters K_m and k_{cat} were determined. This experiment was performed according to the standing start assay described by Boosalis *et al.* (9). The target site was placed at the first position downstream of the 5′-end label of the primer. Quantification of radioactive spots, representing polymerized product and remaining primers, allowed calculation of the initial velocity of the reaction. Table 2 lists the kinetic parameters (average of three experiments) of Vent (exo⁻) DNA polymerase and HIV reverse transcriptase for dTTP and tTTP. The kinetic parameters for incorporation of one α-L-threofuranosyl thymine residue revealed, for both the Vent (exo⁻) DNA polymerase and the HIV reverse transcriptase, an increased K_m value in comparison to the K_m for the natural counterpart. Moreover, a decrease in k_{cat} was found for both enzymes in comparison to their natural counterparts. These results indicate that although incorporation of the modified nucleotide is possible, it is less efficient than that of the natural counterpart. The difference in k_{cat} value is, however, not that high (a factor 5–10), and the main difference between threosyl nucleotides and natural nucleotides is observed in the chain elongation reaction.

RNA polymerase reactions

To examine whether RNA polymerases were able to accept tUTP as substrate, a template of the 'overlapping' type was used, incorporating a double-stranded T7 RNA polymerase promotor followed by a single-stranded sequence (10) designed to produce hexanucleotide transcripts (hybrid P2T1). The use of [γ-³²P]GTP as substrate allows incorporation of a radioactive label at the beginning of the transcript and visualization of all products formed under our experimental conditions (results not shown).

Using tUTP as substrate no transcription products were observed under the applied experimental conditions.

DISCUSSION

Results from insertion studies investigating incorporation of α-L-threofuranosyl uracil or α-L-threofuranosyl thymine into hybrids P1/T2 and P1/T3 (Table 1) revealed that both the Vent (exo⁻) DNA polymerase and HIV reverse transcriptase are able to elongate a DNA primer with one threofuranosyl building block analog opposite its natural counterpart in the template. Both enzymes were also able to further extend the primer with a natural dGTP building block following incorporation of tTTP or tUTP. Incorporation of a second modified nucleotide, however, is much more difficult than incorporation of the first one. Vent (exo⁻) DNA polymerase is more efficient than HIV reverse transcriptase in the

Table 1. Quantitative data for the enzymatic incorporation of tTTP and tUTP and further elongation in the presence of hybrids P1/T2 and P1/T3 by means of Vent (exo⁻) DNA polymerase and HIV reverse transcriptase

	Hybrid P1/T2 tTTP Incorporation of a second tTTP	tUTP Incorporation of a second tUTP	Hybrid P1/T3 tTTP Incorporation of tTTP	Further elongation with dGTP	tUTP Incorporation of tUTP	Further elongation with dGTP
Vent (exo⁻) DNA polymerase	69%	84%	92%	88%	97%	83%
HIV reverse transcriptase	38%	58%	93%	81%	92%	65%

Table 2. Kinetic parameters of Vent (exo⁻) DNA polymerase and HIV reverse transcriptase from a steady-state kinetic analysis of dTTP and tTTP in a DNA hybrid

Enzyme	Substrate	K_m (µM)	k_{cat} (min⁻¹)
Vent (exo⁻) DNA polymerase	dTTP	0.53 ± 0.13	9.46 ± 0.66
	tTTP	1.16 ± 0.31	1.74 ± 0.14
HIV reverse transcriptase	dTTP	2.63 ± 0.43	0.65 ± 0.02
	tTTP	7.3 ± 3.3	0.068 ± 0.008

incorporation of threosyl nucleotides. When we compare these results with previously published data about the incorporation of 1,5-anhydrohexitol nucleotides by Vent (exo⁻) DNA polymerase and HIV reverse transcriptase, we see that both enzymes were also capable of incorporating the anhydro-hexitol analogs (7,8). The efficiency of incorporation of anhydrohexitol nucleotides is higher than that of incorporation of threosyl nucleotides. The 1,5-anhydrohexitol sugar moiety is a rigid six-membered sugar that was developed as a mimic of a natural furanose nucleoside in its C3′-*endo* conformation (11,12). The threofuranosyl nucleoside on the other hand is not a structural mimic of a natural furanose nucleoside but rather a potential functional analog derived from a sugar that contains only four carbons. It seems that polymerases incorporate the structural hexitol nucleoside analog rather than the functional threose analog.

For both the Vent (exo⁻) DNA polymerase and HIV reverse transcriptase K_m values are increased and k_{cat} values are decreased for the incorporation of tTTP in comparison to their natural counterparts. Incorporation of the modified nucleotide is possible, but less efficient than incorporation of the natural counterpart. During the review process an article dealing with TNA synthesis by DNA polymerases was published (13), and results were very similar to those described here. In this article, however, several other DNA polymerases were tested in a similar manner.

Previous studies reported that the kinetics of incorporation of one 1,5-anhydrohexitol nucleotide or one natural deoxy-nucleotide by Vent (exo⁻) DNA polymerase are very similar (7). Moreover, T7 RNA polymerase cannot recognize TNA building blocks. Hexitol building blocks on the other hand have been proven to act as substrates for T7 RNA polymerase (14). This means that all polymerases tested (one DNA polymerase, one RNA polymerase and HIV reverse transcriptase) accept hTTP more easily than tTTP, but we can conclude that of the enzymes tested, DNA polymerase is the most efficient at incorporating threosyl nucleotides into DNA. Therefore, it seems appropriate to use Vent (exo⁻) DNA polymerase for *in vitro* selection of mutated enzymes (to increase incorporation efficiency).

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