Enzymatic Primer-Extension with Glycerol-Nucleoside Triphosphates on DNA Templates

Jesse J. Chen1*, Ching-Hsuan Tsai1, Xin Cai1, Allen T. Horhota2, Larry W. McLaughlin2, Jack W. Szostak1*

1 Howard Hughes Medical Institute, and Department of Molecular Biology and Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, Massachusetts, United States of America, 2 Department of Chemistry, Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts, United States of America

Abstract

**Background:** Glycerol nucleic acid (GNA) has an acyclic phosphoglycerol backbone repeat-unit, but forms stable duplexes based on Watson-Crick base-pairing. Because of its structural simplicity, GNA is of particular interest with respect to the possibility of evolving functional polymers by *in vitro* selection. Template-dependent GNA synthesis is essential to any GNA-based selection system.

**Principal Findings:** In this study, we investigated the ability of various DNA polymerases to use glycerol-nucleoside triphosphates (gNTPs) as substrates for GNA synthesis on DNA templates. Therminator DNA polymerase catalyzes quantitative primer-extension by the incorporation of two glyceronucleotides, with much less efficient extension up to five glyceronucleotides. Steady-state kinetic experiments suggested that GNA synthesis by Therminator was affected by both decreased catalytic rates and weakened substrate binding, especially for pyrimidines. In an attempt to improve pyrimidine incorporation by providing additional stacking interactions, we synthesized two new gNTP analogs with 5-propynyl substituted pyrimidine nucleobases. This led to more efficient incorporation of gC, but not gT.

**Conclusions:** We suggest that directed evolution of Therminator might lead to mutants with improved substrate binding and catalytic efficiency.

Introduction

The ribose and 2-deoxyribose components of the backbones of RNA and DNA have long been thought to play a critical role in stabilizing Watson-Crick base pairing and maintaining fidelity in polymerase-mediated information transfer. Having survived billions of years of evolution on Earth, ribose has been considered to be one of the indispensable building blocks of contemporary life [1]. However, recent studies from the Eschenmoser group of nucleic acid analogs with altered sugar-phosphate backbones demonstrate dramatically that D-ribofuranose is not a chemical requirement for the ability of a prebiotically plausible nucleic acid to function as the genetic information carrier [2]. Indeed, a nucleic acid with a simpler backbone structure, such as (3′→2′)-α-L-threose nucleic acid (TNA, Figure 1), might have been an evolutionary progenitor of DNA or RNA [3].

Recently, the Meggers group synthesized S-Glycerol nucleic acid (GNA), a nucleic acid analog with an acyclic, phosphoglycerol repeating unit (Figure 1) [4,5]. Like TNA, GNA is a nucleic acid with a simplified backbone that can form stable duplexes. As a first step toward understanding the evolutionary potential of GNA, we have recently shown that Bst DNA polymerase can catalyze faithful (but limited) DNA synthesis on a GNA template even though the DNA product cannot form a stable duplex with the GNA template [6]. This surprising observation suggests that, in contrast to common belief [7], stable heteroduplex formation is not required for genetic information transfer between two nucleic acid systems—a short base-paired region stabilized by the enzyme appears to be sufficient.

In addition to exploring GNA molecules as a genetic information carrier, we are also interested in probing their ligand binding and catalytic abilities. We envision that an *in vitro* selection strategy, similar to that used in RNA aptamer or artificial ribozyme selection [8], can be used to isolate functional GNA constructs. This selection strategy requires a polymerase that can synthesize GNA in a template-directed fashion. We have previously synthesized the four canonical glycerol-nucleoside triphosphates (gNTPs with A, G, T, or C, Figure 2) and demonstrated that several DNA polymerases can incorporate a single glyceronucleotide onto a DNA primer/template using...
gNTPs as the substrates [9]. Incorporation of a second glyceronucleotide was not observed in that study, which we attributed to the poorly constrained nature of the acyclic glycerophosphate backbone of GNA and/or the unstable nature of a GNA/DNA duplex [9].

In the present study, we show that certain DNA polymerases can incorporate at least two GNA residues onto a growing primer. Kinetic analysis of Therminator-catalyzed single glyceronucleotide incorporation suggested that pyrimidine glyceronucleotides were weakly bound substrates. In an attempt to improve pyrimidine incorporation, we synthesized two 5-propynyl substituted gNTP analogs, to improve the base-stacking interaction between the incoming gNTP and the terminal nucleotide of the primer (Figure 2). This modification led to improved efficiency of incorporation of gCTP, but not of gTTP.

**Results**

**Polymerase Screens**

Using a primer extension assay, we screened a number of polymerases (including Taq, Bst, DeepVent (exo-), Therminator, Sequenase, Superscript II, and HIV-1 reverse transcriptase) for their ability to catalyze GNA synthesis on a DNA template. In order to enhance our chances of observing primer-extension by more than just one base as previously observed [9], we used long incubations under a variety of conditions. We found that a number of polymerases were able to incorporate more than one GNA monomer at a 1:1 enzyme:primer/template ratio (Figure 3). The Therminator polymerase utilized most of the primer and generated a significant fraction of +2 extended products within 1 h (Figure 3). After 8 h, Deep Vent exo- polymerase had a higher yield of +2 extended primer, but Therminator appeared to synthesize small amounts of +3, +4, and +5 products. We therefore decided to characterize primer-extension and substrate utilization by Therminator in greater detail. Raising the assay temperature from 55°C to 75°C (Therminator’s optimum temperature) resulted in decreased efficiency of GNA incorporation. Therefore, all the subsequent kinetic studies were performed at 55°C. Neither the addition of Mn²⁺ nor substitution of adenosine with diaminopurine-2’-deoxyriboside in the template significantly improved the efficiency of GNA synthesis by this enzyme, using the four canonical gNTPs.

The products of Therminator-catalyzed, single and double glyceronucleotide incorporation were characterized by MALDI-TOF MS. The expected mass difference between a primer extended with one deoxyribonucleotide vs. one glyceronucleotide is 42 Daltons. As shown in Table 1, the observed masses for single glyceronucleotide or deoxyribonucleotide incorporation on Template A are consistent with the calculated values. The identity of the products of two sequential nucleotide incorporations (gTMP+dAMP, gTMP+gAMP, 2 gAMP, or 2 gGMP on Template B) was also confirmed by MALDI-TOF MS (Table 1).

**Kinetic analysis of glycerol-nucleotide addition to a DNA primer**

To obtain insight into the process of GNA synthesis catalyzed by Therminator on DNA templates, we performed steady-state kinetic experiments and measured the $k_{cat}$ and $K_m$ values for the four canonical gNTP substrates (Figure 2). The kinetic parameters for addition of a single glyceronucleotide to an all-DNA primer are summarized in Table 2. Because the kinetic experiments were performed at 55°C instead of Therminator’s optimum temperature of 75°C, decreased $k_{cat}$ values were observed for regular dNTPs ($2–7 \text{ s}^{-1}$ at 55°C, Table 2) compared with those at 75°C ($\approx 15 \text{ s}^{-1}$ calculated from specific activity provided by the
Therminator showed similar \( k_{\text{cat}} \) values for gATP compared to dATP, and for gGTP compared to dGTP, while slower kinetics were observed for gTTP and gCTP (by 5-fold and 3-fold, respectively) compared with TTP and dCTP. All 4 gNTPs showed higher \( K_m \) values than the corresponding dNTPs, with an 20 fold increase for gATP and gGTP and a 200–300 fold increase for gTTP and gCTP. As a result, the decrease of catalytic efficiency \( (k_{\text{cat}}/K_m) \) was much more dramatic for pyrimidine than for purine gNTPs (1000-fold versus only 20-fold). Such discrimination between purine and pyrimidine triphosphate substrates is not observed for threose-nucleoside triphosphates (tNTPs) [10]. One possible explanation for this result is that base-stacking interactions between the incoming nucleoside triphosphate and the primer play a more prominent role in gNTP binding than in dNTP or tNTP binding.

### Table 1. MALDI-TOF MS analysis of single and double nucleotide incorporation by Therminator DNA polymerase.a.

| XY | NTP substrate | Nucleotide incorporated | Calc| Obsd |
|----|---------------|-------------------------|-----|------|
| Template A | CA | none | none | 6126.1 | 6126.2 |
| AG | TTP | T | 6430.3 | 6431.5 |
| TG | dATP | dA | 6439.3 | 6439.5 |
| CA | dGTP | dG | 6455.3 | 6455.3 |
| GA | dCTP | dC | 6415.3 | 6415.3 |
| AG | gTTP | gT | 6388.3 | 6388.3 |
| AG | gU\text{prop}TP | gU\text{prop} | 6412.3 | 6411.9 |
| TG | gA TP | aA | 6397.3 | 6396.8 |
| CA | gGTP | gG | 6413.3 | 6413.4 |
| GA | gCTP | gC | 6373.3 | 6373.3 |
| GA | gC\text{prop}TP | gC\text{prop} | 6411.3 | 6410.8 |
| Template B | AT | gTTP+dATP | gT+gA | 6701.5 | 6700.0 |
| AT | gTTP+gATP | gT+gA | 6659.5 | 6658.9 |
| TT | gATP | gA | 6685.6 | 6667.7 |
| CC | gCTP | gC | 6415.3 | 6415.3 |

aSequences of the primer and the template:
- Primer: 5'\text{\textendash}TAA TAC GAC TCA CTA TAGGG-3'.
- Template: 3'\text{\textendash}ATT ATG CTG AGT GAT ATC CC XY ACA TCT ATC-5'.

### Table 2. Kinetic analysis of single-nucleotide incorporation by Therminator DNA polymerase.a.

| XYb | NTP | \( K_m \) (\( \mu \text{M} \)) | \( k_{\text{cat}} \) (s\( ^{-1} \)) | \( k_{\text{cat}}/K_m \) (s\( ^{-1} \text{M}^{-1} \)) |
|-----|-----|-----------------|----------------|----------------|
| TG  | dATP | 0.59±0.11 | 3.7±0.5 | 6.2×10^6 |
| CA  | dGTP | 0.47±0.27 | 2.0±0.3 | 4.3×10^6 |
| AG  | TTP  | 0.67±0.14 | 4.2±0.4 | 6.4×10^6 |
| DG  | TTP  | 0.35±0.10 | 6.8±0.6 | 1.9×10^7 |
| GA  | dCTP | 0.32±0.17 | 3.2±0.8 | 1.0×10^7 |
| TG  | gATP | 14.0±6.7 | 4.7±0.8 | 3.4×10^4 |
| CA  | gGTP | 10.6±4.7 | 2.3±0.1 | 2.1×10^5 |
| AG  | gTTP | 129.4±77.3 | 0.8±0.3 | 5.8×10^3 |
| DG  | gTTP | 54.3±8.3 | 1.6±0.4 | 3.0×10^4 |
| AG  | gU\text{prop}TP | 139.5±14.5 | 1.5±0.3 | 1.1×10^4 |
| DG  | gU\text{prop}TP | 41.4±4.7 | 1.7±0.2 | 4.1×10^4 |
| GA  | gCTP | 89.5±38.6 | 1.3±0.6 | 1.4×10^4 |
| GA  | gC\text{prop}TP | 3.3±0.4 | 1.9±0.2 | 5.8×10^5 |

aSequences of the primer and the template:
- Primer: 5'\text{\textendash}TAA TAC GAC TCA CTA TAGGG-3'.
- Template: 3'\text{\textendash}ATT ATG CTG AGT GAT ATC CC XY ACA TCT ATC-5'.
- \( ^b \)D denotes 2,6-diaminopurine-2'-deoxyribonucleotide.

Synthesis of 5-propynyl substituted glycerol-nucleoside triphosphates

In order to test the hypothesis that poor enzymatic incorporation of pyrimidine gNTPs reflects poor base-stacking, we synthesized the C-5-propynyl substituted pyrimidine nucleoside triphosphates (Figure 2). This co-planar nucelobase modification has been proposed to increase base stacking and hydrophobic interactions between base pairs [11]. In the case of GNA synthesis, we thought that a 5-propynyl group in pyrimidine gNTPs might improve binding to the primer/template complex in the active site of polymerase. The synthetic schemes for the preparation of 5-propynyl substituted gNTPs (gU\text{prop}TP and gC\text{prop}TP, or 1u and 1c) are shown in Figures 4 and 5. Two key intermediates, the 5-
propynyl pyrimidine glycerol-nucleosides (4u and 5c), were prepared from 5-iodo-substituted precursors (3u and 3c) and propyne by Sonogashira coupling (Figure 4 and 5) [12,13]. A dibutylaminomethylidene group was used to protect the exocyclic amine of 3c instead of an acetyl or benzoyl group in order to avoid a potential cyclization side-reaction involving the amide and the 5-propynyl group [14]. The presence of a propynyl group in 4u and 5c was confirmed by the characteristic chemical shift (4–5 ppm) of the methyl carbon in 13C NMR [15] together with 1H-NMR and ESI-MS analysis.

gUpropTP and gCpropTP (1u and 1c) were synthesized from the corresponding nucleosides (5u and 6c) using the one-pot, salicylchlorophosphorin approach developed by Ludwig and Eckstein [16]. The final purified products 1u and 1c were characterized by 1H- and 31P-NMR and by ESI-MS. In addition, 1u and 1c have similar UV absorption profiles to those reported for 5-propynyl-deoxyribonucleosides [15].

### Kinetics of DNA primer-extension using 5-propynyl gNTPs

We measured $K_m$ and $k_{cat}$ for Therminator-catalyzed DNA primer-extension, using 5-propynyl gUTP and gCTP as substrates. The results suggest that a stronger base stacking interaction does increase the catalytic efficiency of gNTP incorporation, although the effect is more prominent for gCpropTP than for gUpropTP (Figure 2). Compared to gCTP, gCpropTP had a much lower $K_m$ (3.3 μM vs. 89.5 μM) and a slightly faster $k_{cat}$ (1.9 s$^{-1}$ vs. 1.3 s$^{-1}$) (Table 2). As a result, the catalytic efficiency ($k_{cat}/K_m$) of Therminator with gCpropTP was 40 fold higher than that with gCTP. In fact, gCpropTP had the lowest $K_m$ and highest $k_{cat}/K_m$ of all 6 gNTPs. In contrast, the effect of 5-propynyl substitution on gUTP was modest, and gUpropTP showed a similar $K_m$ value to gTTP with a ~2 fold increase in $k_{cat}$ (Table 2). When diaminopurine was used in the template, both gTTP and gUpropTP had a significantly lower $K_m$ (34.5 μM and 41.4 μM, respectively) than those measured with the dA-containing template (129.4 μM and 139.5 μM, respectively) with slightly increased $k_{cat}$ (Table 2). These results suggest that both H-bonding and base stacking contribute to binding of gNTPs to the primer/template complex and that the synergy of these two interactions determines the overall catalytic efficiency.

### Kinetic analysis of dNMP incorporation on a GNA-terminated primer

We synthesized a primer containing a single GNA monomer (gT) at the 3’-terminus in order to study the effect of the positioning of the nucleophile (2’-OH of GNA vs. 3’-OH of DNA) on the catalytic efficiency of Therminator polymerase. However, kinetic parameters for a 3’-gT-terminated primer were more difficult to obtain than with an all-DNA primer. Under steady-state conditions with a low enzyme:primer/template ratio, we were not able to observe significant primer-extension even at saturating gNTP concentrations. At a 1:1 enzyme:primer/template ratio (1 μM), ~15% extension of the 3’-gT-terminated primer was observed after 24 h with gATP and gGTP, but not with gTTP and gCTP. This observation suggests that, in the active site of Therminator, the conformation of the terminal GNA nucleotide, especially its 2’-OH group, is not optimal (or is poorly constrained) for nucleophilic attack on the incoming triphosphate.
The catalytic efficiency is further reduced by the low affinity of Therminator for gNTP substrates, especially in the case of gTTP and gCTP.

To quantitatively assess the effect of a 3′-terminal gT residue on the catalytic efficiency of continued polymerization, we determined the kinetic parameters for the incorporation of a single deoxyribonucleotide by Therminator, using a 3′-gT primer. Compared with the all-DNA primer (Table 2), the 3′-gT primer caused 2–4 fold decrease in $k_{cat}$ (Table 3). In addition, an increase in $K_m$ was also observed, which again was more prominent for pyrimidines (∼80 fold) than for purines (4–8 fold) (Table 3). These results suggest that the presence of gT at the 3′-terminus of the primer may interfere with essential conformational changes of the enzyme during its catalytic cycle, resulting in both slower $k_{cat}$ and increased $K_m$ values.

Discussion

Previous studies have shown that certain polymerases are capable of template-directed primer-extension by using as substrates nucleotide analogs with either a shortened (TNA) or an acyclic (flexible nucleic acid, or FNA, Figure 1) sugar moiety [10,17,18]. The active sites of some polymerases are clearly flexible enough to accommodate major sugar modifications [19,20]. However, compared with TNA or FNA, GNA contains both an acyclic and a shortened repeating unit (Figure 1), and it is therefore not surprising that the enzymatic polymerization of gNTPs is quite difficult. In this study, we screened a series of polymerases and identified Therminator as the most efficient GNA polymerase. Not surprisingly, Therminator was also the most efficient polymerase identified in previous efforts to synthesize TNA or FNA on DNA templates [10,18].

Our kinetic analysis of primer-extension with gNTP substrates has provided some insight into the particular problems that prevent efficient Therminator-catalyzed GNA synthesis. Analysis of the addition of the first glycerol-nucleotide to a DNA primer revealed that all six gNTPs examined (the four canonical nucleobases plus the two propynyl-pyrimidines) had $K_m$ values that were similar to those of the corresponding natural dNTPs. Decreased catalytic efficiency was mainly due to higher $K_m$ values, and thus is most likely due to weaker gNTP binding. The same observation was made previously for TNA synthesis catalyzed by Therminator [10]. These results suggest that altered sugar structure on an incoming nucleotide may interfere with essential conformational changes of the enzyme during its catalytic cycle, resulting in both slower $k_{cat}$ and increased $K_m$ values.

Table 3. Kinetic analysis of single deoxyribonucleotide incorporation by Therminator using a GNA-terminated primer.

| XY | NTP | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (s⁻¹ M⁻¹) |
|----|-----|-----------|-----------------|--------------------------|
| TG | dATP | 4.7±2.0   | 0.7±0.2         | 1.4×10⁴                  |
| CA | dGTP | 2.1±1.1   | 1.0±0.1         | 4.7×10⁴                  |
| AG | TTP  | 50.7±38.5 | 0.9±0.2         | 1.8×10⁴                  |
| GA | dCTP | 21.4±7.6  | 1.7±0.6         | 7.8×10⁴                  |

*Sequences of the primer and the template (the lower case denotes the GNA sequence): Primer: 5′-TAA TAC GAC TCA CTA TAG GG T. Template: 3′-ATT ATG CTG AGT ATC CC A XY CAT CTA TC-5′.
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Materials and Methods

Materials

Reagents and solvents were purchased from Sigma-Aldrich. DNA oligonucleotides were purchased from IDT and were purified

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by denaturing polyacrylamide gel electrophoresis. Dibutyrylformamide dimethylacetal [14], R-3-O-(4,4'-dimethoxytrityl)-glycidol (2) [27], and gNTPs (A, G, T, C) [9] were prepared according to published procedures. Taq DNA polymerase was purchased from Roche (1 U is defined as the amount of enzyme that incorporates 10 nmol of total dNTPs into acid soluble material in 30 min at 75 °C). Therminator, Deep Vent (exo-), and Bst polymerases were purchased from New England Biolabs (1 U is defined as the amount of enzyme that incorporates 10 nmol of total dNTPs into acid precipitable DNA within 30 min at 75 °C for Therminator and Deep Vent (exo-) or at 65 °C for Bst). Sequenase was purchased from USB (1 U is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of nucleotide into acid insoluble form in 30 s at 37 °C). Superscript II reverse transcriptase was purchased from Invitrogen (1 U is defined as the amount of enzyme that incorporates 1 nmol of deoxyribonucleotide into acid-precipitable material in 10 min at 37 °C using poly(A)/oligo(T)12-18 as template/primer). HIV-1 reverse transcriptase was purchased from Worthington (1 U is defined as the amount of enzyme that generates 1 μmol of phosphate per minute from poly(A)oligo(T)12-18 as template/primer).

Synthesis of S-1-(5-iodouracil-1-yl)-3-O-(4,4' -dimethoxytrityl)-2,3-propanediol (3u)

5-Iodouracil (3.6 g, 15 mmol) and sodium hydrate (125 mg in 60% mineral oil, 3.0 mmol) were suspended in 25 mL of anhydrous dimethylformamide and the reaction mixture was stirred at room temperature for 30 min. R-3-O-(4,4'-dimethoxytrityl)-glycidol (2) (15 mmol) in 25 mL of anhydrous dimethylformamide was added to the reaction mixture dropwise over 5 min. The reaction mixture was then heated to 100 °C and stirred for 1.5 h. The solvent was then evaporated in vacuo and the resulting pellic was extracted with 50 mL of CH2Cl2 twice. The organic layer was combined, washed twice with saturated sodium bicarbonate (100 mLx2) and once with brine (100 mL), dried over Na2SO4, and evaporated in vacuo. Compound 3u was further purified by silica gel chromatography (0.5% MeOH/CH2Cl2) to afford 3u as an amorphous yellow solid (2.7 g, 4.4 mmol, 29%). 1H NMR (400 MHz, CDCl3) δ: 7.72 (1H, s), 7.41 (1H, d, J = 2.7), 7.39 (1H, s), 7.26-7.32 (6H, m), 6.84 (4H, d, J = 6.8), 4.04-4.07 (2H, m), 3.78 (6H, s), 3.65 (1H, dd, J = 8.4, 14.8), 3.18 (2H, m); 13C NMR (100.5 MHz, CDCl3) δ: 160.6, 158.8, 151.1, 150.5, 144.5, 135.6, 130.1, 128.2, 128.0, 127.2, 113.4, 86.7, 69.2, 67.3, 64.5, 55.4, 52.0; LRMS calcd for [M+Na]+ (C28H27IN2NaO6): 549.2; obsd, 549.0.

Synthesis of S-1-(5-propynyl-uracil-1-yl)-2-O-acetyl-3-O-(4,4'-dimethoxytrityl)-2,3-propanediol (5u)

Compound 4u (0.2 g, 0.38 mmol) was mixed with 4 mL of 10% acetic anhydride in anhydrous pyridine. The reaction mixture was stirred at room temperature for 15 min and was evaporated in vacuo. The crude product was dissolved in 50 mL CH2Cl2, washed twice with saturated sodium bicarbonate (50 mLx2) and once with brine (50 mL), dried over Na2SO4, and evaporated in vacuo. The pellet was then treated with 5 mL of 80% acetic acid in H2O at room temperature for 30 min. The mixture was then evaporated, and the crude 5u was purified by silica gel chromatography (2.5% MeOH/CH2Cl2) to afford 5u as white foam (72 mg, 0.27 mmol, 72%). 1H NMR (400 MHz, CDCl3) δ: 9.61 (1H, br), 7.42 (1H, s), 5.10 (1H, m), 4.17 (1H, dd, J = 4.4, 14.4), 3.88 (1H, dd, J = 6.4, 14.4), 3.73 (2H, m), 2.11 (3H, s). 13C NMR (100.5 MHz, CDCl3) δ: 170.5, 162.6, 150.7, 146.8, 101.1, 91.4, 71.9, 69.8, 60.9, 49.6, 21.1, 4.8; LRMS calcd for [M+Na]+ (C12H14N2NaO5): 289.0800, obsd, 289.0.

Synthesis of S-1-(5-propynyl-uracil-1-yl)-2-O-acetyl-3-O-(4,4'-dimethoxytrityl)-2,3-propanediol (5u)

Compound 5u (53 mg, 200 μmol) was rendered anhydrous by evaporation with anhydrous pyridine (1 mLx3) and was dissolved in 0.8 mL of pyridine/dioxane (1:3). To this mixture, 220 μL of 1 M 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in dioxane was added and the reaction mixture was stirred at room temperature for 10 min. A solution (0.6 mL) of 0.5 M tetra-butylammonium pyrophosphate in dimethylformamide was then added and the reaction mixture was stirred for another 10 min. A solution (7.5 mL) of 1% iodine in pyridine/H2O (98:2) was then added to the above mixture. After 15 min, 5% Na2SO4 aqueous solution was added dropwise until the reaction mixture turned from dark brown to light yellow. The final mixture was evaporated in vacuo and treated with concentrated ammonium solution (10 mL) overnight at room temperature. The mixture was then evaporated in vacuo and the crude product 1u was purified by preparative RP-HPLC using a Varian Microsorb 100-8 C18 column (250×21.4 mm) with a linear gradient from 0 to 20% acetonitrile in 5 mM triethylammonium acetate (pH 7.0) over 20 min with a flow rate of 15.0 mL/min. The fraction containing 1u (retention time 4.5–5.5 min) was combined and lyophilized to yield 84.4 μmol of 1u (42% yield) based on the UV absorption Rf1u: 11.3 mm-1 cm-1. 1H NMR (400 MHz, D2O) δ: 7.84.
Silica gel chromatography (2% methanol/CH₂Cl₂) to yield silica gel chromatography (1%–3% methanol/CH₂Cl₂) as light yellow solid (2.3 g, 3.8 mmol, 38%). 1H NMR (100.5 MHz, CDCl₃) (6H, m), 6.83 (4H, d, J = 6.8), 5.50 (1H, br), 4.14–4.25 (2H, m), 3.69 (2H, s), 3.50 (2H, t, J = 7.6, 14.4), 2.97 (1H, dd, J = 10, 6.4); 13C NMR (100.5 MHz, CDCl₃) δ: 158.3, 157.5, 150.1, 144.9, 136.0, 135.8, 130.0, 128.0, 127.99, 126.9, 113.3, 103.0, 89.2, 86.5, 76.2, 76.1, 70.1, 64.4, 55.3, 54.8, 52.5, 46.1, 31.1, 29.0, 20.2, 19.9, 13.8, 4.6; LRMS calcd for [M+H]+ (C₁₆H₁₄N₃O₃) : 665.3703, obsd, 665.3.

Synthesis of S-1-(5-propynyl-4-N-dibutylaminomethylidenecytosine-1-yl)-2-O-acetyl-2,3-propanediol (6c)

The procedure to synthesize 6c is similar to that of 5u. The yield of 6c from 0.2 g of 5c (0.3 mmol) was 0.11 g (0.24 mmol, 80%). 1H NMR (400 MHz, CDCl₃) δ: 8.83 (1H, s), 7.50 (1H, s), 5.03 (1H, m), 4.21 (1H, dd, J = 4, 14.4), 4.07 (1H, dd, J = 5.2, 14.4), 3.63 (1H, dd, J = 4, 12.4), 3.53 (1H, dd, J = 6.4, 12.4), 3.47 (2H, s), 2.97 (1H, dd, J = 10, 9.6, 2.97 (1H, dd, J = 9.6, 6.8), 1.93 (3H, s); 13C NMR (100.5 MHz, CDCl₃) δ: 171.5, 170.2, 159.1, 157.0, 148.8, 101.5, 89.8, 72.7, 72.3, 60.8, 59.5, 54.3, 46.5, 27.5, 26.6, 21.1, 20.4, 20.0, 4.6; LRMS calcd for [M+H+] (C₁₉H₁₅N₃O₃): 405.2496, obsd, 405.4.

Synthesis of S-1-(5-propynylcytosine-1-yl)-3-O-triphospho-2,3-propanediol (1c)

The procedure to synthesize 1c is similar to that of 1u. The crude product of 1c after concentrated ammonia deprotection was first purified by anion-exchange liquid chromatography using DEAE A25 resin with a gradient of 0–0.5 M triethylammonium bicarbonate. The fraction containing 1c was combined, evaporated in vacuo, and desalted using the preparative RP-HPLC as described for 1u (retention time 8.5 min). The final yield of 1c was 22.3 mmol (11%) based on the UV absorption (ε₂₅₅ nm: 7.7 M⁻¹ cm⁻¹). 1H NMR (400 MHz, D₂O) δ: 7.85 (1H, s), 4.10 (2H, m), 4.01 (1H, m), 3.77 (1H, dd, J = 10, 14.4), 3.52 (1H, dd, J = 7.6, 14.4), 2.02 (3H, s); 31P NMR (161.8 MHz, D₂O) δ: -5.34 (d, J = 21.5), -9.75 (d, J = 19.1), -21.5 (t, J = 21.1); LRMS calcd for [M+H]+ (C₂₁H₃₂N₄O₇P₃) : 461.9874, obsd, 462.0.

Enzyme Screen

A DNA primer (5'-TAA TAC GAC TCA CTA TAG GG-3') was 5'-labeled with [32P] and annealed to a DNA template (3'-ATT ATG CTG AGT GAT A TC CCA TAT CAG-5'), the underlined denotes the region base-paired with the primer). The primer extension experiments of GNA synthesis on the DNA template were preformed with 50 nM primer/template, four gNTPs (A, G, T, and C, 100 µM each), 1 U of Thermostable pyrophosphatase, and polymerase (Taq, 2.5 U; Therminator, 1 U; Deep Vent (exo)-, 1 U; Bst, 4 U; Sequenase, 6.5 U; Superscript II, 100 U; HIV-1 reverse transcriptase, 5 U) in a final volume of 10 µL containing appropriate buffers supplied by the manufacturers. The reaction mixtures were incubated at 55°C for thermophilic enzymes or 37°C for mesophilic enzymes. At 1, 8, and 24 h, an aliquot of 2 µL was removed from the reaction mixture and was analyzed by 20% denaturing polyacrylamide gel electrophoresis.

MALDI-TOF Mass Spectrometry

The single-nucleotide extension reaction mixture (50 µL) contained 20 µM pre-anneled primer/template, 250 µM appropriate dNTP or gNTP, 1 U of thermostable pyrophosphatase, and...
1 U of Thermopanca DNA polymerase. The reactions mixtures were incubated at 55°C for 5 minutes for dNTPs or 8 h for gNTPs. The reaction mixtures were then precipitated by adding 200 μL of ethanol, 50 μL of 2 M ammonium acetate, and 0.5 μL of 3 mg/mL glycogen solution. The samples were redissolved in 0.2 M triethylammonium acetate buffer (pH 7.0) and were absorbed on C18 Zip Tips (Millipore). Samples were eluted with 1.5 μL of a matrix solution containing a 2:1 mixture of 52.5 mg/mL 3-hydroxypropionol acid in 50% acetonitrile and 0.1 M ammonium citrate in water. Eluents were directly spotted onto stainless steel MALDI-TOF plate and were analyzed in positive mode on a Voyager MALDI-TOF mass spectrometer (Applied Biosystems) with an average of 200 scans.

Steady-State Kinetics
Kinetic measurements were performed as previously described using 5'-[32P] labeled primer and the appropriate template for each dNTP or gNTP [10,28]. The single nucleotide extension reactions were initiated by mixing 10 μL of 2 × dNTP or gNTP solution with 10 μL of a solution containing the remaining reaction components. The final reaction mixture of 20 μL contained 1 μM primer/template, 0.05–10 μM dNTP or 0.5–500 μM gNTP, 0.5 U of thermostable pyrophosphatase, and 8.2 nM Thermopanca in 1 × Thermopol buffer (20 mM Tris-Cl, 10 mM KCl, 10 mM (NH4)2SO4, 20 mM MgSO4, 0.1% Triton X-100, 100 μM DTT, pH 8.3). The reaction mixtures were incubated at 55°C. At each time point, an aliquot of 4 μL was removed from the mixture and was quenched with mixing with 4 μL of solution containing 8 M urea, 100 mM EDTA, and 0.05% xylene cyanol and bromophenol blue. Single nucleotide extension was analyzed by 20% denaturing polyacrylamide gel electrophoresis and quantified using a PhosphorImager (Molecular Dynamics) as described before [10].

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Author Contributions
Conceived and designed the experiments: JJC CHT LWM JWS. Performed the experiments: JJC CHT XC. Analyzed the data: JJC CHT XC JWS. Contributed reagents/materials/analysis tools: JJC XC ATH LWM. Wrote the paper: JJC CHT JWS.

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