

A single nucleotide incorporation step limits human telomerase repeat addition activity

 Yinnan Chen[†], Joshua D Podlevsky[†], Dhenugen Logeswaran^{id} & Julian J-L Chen^{* id}

Abstract

Human telomerase synthesizes telomeric DNA repeats (GGTTAG)_n onto chromosome ends using a short template from its integral telomerase RNA (hTR). However, telomerase is markedly slow for processive DNA synthesis among DNA polymerases. We report here that the unique template-embedded pause signal restricts the first nucleotide incorporation for each repeat synthesized, imparting a significantly greater K_M . This slow nucleotide incorporation step drastically limits repeat addition processivity and rate under physiological conditions, which is alleviated with augmented concentrations of dGTP or dGDP, and not with dGMP nor other nucleotides. The activity stimulation by dGDP is due to nucleoside diphosphates functioning as substrates for telomerase. Converting the first nucleotide of the repeat synthesized from dG to dA through the telomerase template mutation, hTR-51U, correspondingly shifts telomerase repeat addition activity stimulation to dATP-dependent. In accordance, telomerase without the pause signal synthesizes DNA repeats with extremely high efficiency under low dGTP concentrations and lacks dGTP stimulation. Thus, the first nucleotide incorporation step of the telomerase catalytic cycle is a potential target for therapeutic enhancement of telomerase activity.

Keywords deoxynucleoside diphosphate; DNA polymerase; processivity; reverse transcriptase; ribonucleoprotein

Subject Categories DNA Replication, Repair & Recombination

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Introduction

The ends of linear eukaryotic chromosomes are capped by telomeres, an array of short DNA repeats bound by specific telomeric proteins (Arnoult & Karlseder, 2015). Telomere length is crucial for chromosome integrity and maintained by the unique cellular reverse transcriptase, telomerase, that adds telomeric DNA repeats (GGTTAG)_n processively onto chromosome ends (Wu *et al*, 2017b). Human telomerase utilizes a short 11-nt template from the long non-coding 451-nt telomerase RNA (TR) for DNA repeat synthesis

catalyzed by the telomerase reverse transcriptase (TERT) subunit (Podlevsky & Chen, 2012; Hockemeyer & Collins, 2015). The 5' boundary of the TR template prevents non-template usage to ensure telomeric DNA synthesis fidelity and is physically safeguarded in different species by divergent structural elements: a distal RNA helix and single-stranded nucleotide span in vertebrates (Chen & Greider, 2003a), TERT-binding TR elements in ciliates (Jansson *et al*, 2015; Jiang *et al*, 2015), or a template-adjacent RNA helix in fungi (Tzfati *et al*, 2000; Seto *et al*, 2003; Qi *et al*, 2013). The human TR (hTR) template 5' boundary is further protected by the template-embedded pause signal, the first dT:rA base pair in the DNA product/RNA template hybrid, that restricts DNA synthesis beyond the 5' boundary (Brown *et al*, 2014). Upon completing synthesis of a DNA repeat and reaching the 5' template boundary, telomerase regenerates the RNA template for subsequent repeat synthesis (Wu *et al*, 2017a). There have been identified telomerase mutations which impair specifically repeat addition processivity and not catalytic activity, yet result in stem cell defects that manifest as a spectrum of short telomere syndromes (Robart & Collins, 2010; Alder *et al*, 2011; Gramatges *et al*, 2013; Zaug *et al*, 2013).

Telomerase repeat addition processivity relies on a highly complex and unique catalytic reaction cycle that comprises two distinct phases: (i) synthesis of a single telomeric repeat with six consecutive nucleotide incorporation steps and (ii) regeneration of the template for additional telomeric repeat synthesis (Podlevsky & Chen, 2012). Telomerase catalyzes the nucleotide incorporation reaction with an active site that comprises a triad of invariant aspartic acids universally conserved in DNA polymerases (Lingner *et al*, 1997). The template regeneration phase is unique to telomerase and is accomplished by a "template translocation" mechanism whereby the template dissociates from and then realigns with the newly extended DNA product (Autexier & Lue, 2006). Recent findings indicate that template translocation is a rapid process (Parks & Stone, 2014), although the precise mechanism remains enigmatic. Telomerase-synthesized DNA products are predominantly released from the enzyme between two consecutive cycles of repeat synthesis, which generates the characteristic 6-nt ladder banding pattern of the products (Greider, 1991). However, the specific steps that promote product release and limit processive repeat synthesis during telomerase catalytic cycle remain elusive.

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Several intrinsic enzymatic determinants or reaction conditions that affect specifically repeat addition processivity have been identified. These reaction conditions, which include high dGTP concentration, stimulate telomerase repeat addition activity through an unknown mechanism (Hammond & Cech, 1997; Maine *et al*, 1999; Sun *et al*, 1999; Hardy *et al*, 2001; Wu *et al*, 2017a). Herein, we report that human telomerase exhibits lower kinetics specifically for the incorporation of the first nucleotide, a dG residue, of each telomeric repeat synthesized. This lower incorporation kinetics is mediated by the template-embedded pause signal that arrests nucleotide synthesis at the end of the template and remains active following template translocation, therefore impairing processive repeat synthesis. Elevated dGTP concentrations increase the incorporation efficiency of the first nucleotide for each repeat synthesis and consequently stimulate both processivity and rate of telomerase repeat addition. These results reveal a critical step in the telomerase catalytic cycle that underlies the dGTP stimulation of human telomerase repeat addition.

Results

Human telomerase template-embedded pause signal mediates high K_M for nucleotide incorporation

Telomerase synthesizes DNA at an exceedingly lower rate than most DNA polymerases (Hwang *et al*, 2014), which is presumably due to the unique telomerase catalytic cycle for processive short DNA repeat synthesis. During each of the reiterated cycles, telomerase catalyzes the incorporation of six consecutive deoxynucleotides, dG¹, dG², dT³, dT⁴, dA⁵, and dG⁶, onto the 3' hydroxyl of the DNA primer (Fig 1A), followed by template translocation to regenerate the template for the next cycle of repeat synthesis. With template translocation having been reported to be a rapid process (Parks & Stone, 2014), we investigated whether any of the six-nucleotide incorporations limit overall telomerase repeat addition activity, especially as specific TR template residue mutations have been shown to affect telomerase enzymatic function (Gilley *et al*, 1995; Gilley & Blackburn, 1996; Drosopoulos *et al*, 2005; Brown *et al*, 2014). The kinetics of the individual nucleotide incorporation steps have not been previously assessed due to technical complications associated with analyzing processive telomerase enzymes. To overcome this technical difficulty, we employed template-free (TF) human telomerase (Qi *et al*, 2012) that catalyzes a non-processive DNA synthesis reaction using RNA templates supplied *in trans*, which permits a simplified and defined primer extension assay for determining the nucleotide incorporation efficiency at each individual position across the hTR template. Human TF telomerase was reconstituted by assembling *in vitro*-expressed human TERT protein in rabbit reticulocyte lysate with the two essential hTR fragments, CR4/5 and a template-free pseudoknot fragment lacking the template sequence (Figs 1B and EV1). This TF telomerase was assayed for nucleotide incorporation with a series of DNA/RNA duplex substrates comprising a DNA primer and an RNA oligo serving as the template (Fig EV2). The preassembled DNA/RNA hybrid substrates were specifically designed with permuted telomeric sequences and a short RNA template for measuring the K_M of nucleotide incorporation specifically for dG¹, dT³, dA⁵, and dG⁶, corresponding to positions 1,

3, 5, and 6 in the hTR template (Figs 1A and EV2). The K_M measurements used extremely low concentrations of TF telomerase enzyme and excess nucleotide substrates ranging from 2 to 200 μ M. With either 2 or 200 μ M nucleotide substrate, the product formation over the incubation time remained linear indicating that the initial velocity of the reaction was measured (Appendix Fig S1). Remarkably, the K_M for incorporating dG¹ was approximately 120 μ M, exceptionally higher than the K_M for incorporating dT³, dA⁵, and dG⁶ that ranged from 3 to 31 μ M (Fig 1C). To further investigate whether this was a result of nucleotide identity or the incorporation position within the template sequence, we altered the identity of the nucleotide incorporated at positions 1, 5, and 6 to dA¹, dT¹, dG⁵, and dT⁶ (Fig EV2). Altering the identity of the nucleotide incorporated at these positions did not substantially change the high K_M for nucleotide incorporation at position 1, nor the low K_M at other positions measured (Fig 1C). Interestingly, the dG-to-dT transversion at positions 1 and 6 noticeably increased K_M from 120 to 400 μ M and from 5 to 14 μ M, respectively (Fig 1C). This is consistent with results reported for *Tetrahymena* telomerase, where the K_M for incorporating dT was slightly higher than for dG (Lee & Blackburn, 1993; Collins & Greider, 1995). Overall, these results suggest that the high K_M of the first nucleotide incorporation is position-specific relative to the template sequence and independent of the identity of the nucleotide incorporated.

The incorporation of the first nucleotide, dG¹, perfectly coincides with the DNA synthesis pause site governed by the pause signal embedded in the hTR template sequence (Fig 1C). The template-embedded pause signal dT:rA base pair forms with the incorporation of dT³ and mediates DNA synthesis arrest after the incorporation of the three subsequent nucleotides, dT⁴, dA⁵, and dG⁶ (Brown *et al*, 2014). We hypothesized that the pause signal arresting DNA synthesis through an elevated K_M for nucleotide incorporation at the pause site would be mitigated with increased dGTP concentrations. To test this hypothesis, we designed a DNA/RNA hybrid substrate with a dT:rA pause signal embedded within and a single-stranded RNA template that allows for the incorporation of four nucleotides, corresponding to dA⁵, dG⁶, dG¹, and a non-telomeric dC² (Fig 1D). The pause signal would arrest DNA synthesis at the pause site following the incorporation of ³²P-dA⁵ and dG⁶. In the presence of only ³²P-dATP, a single ³²P-dA⁵ residue was incorporated to the DNA primer, generating a single band (Fig 1D, lane 1). The inclusion of 5 μ M dGTP in the reaction permitted the incorporation of dG⁶, while the subsequent dG¹ incorporation was inhibited by the pause signal (Fig 1D, lane 2). Increasing the dGTP concentration to 20 or 50 μ M increasingly overcame this DNA synthesis arrest and resulted in the dG¹ incorporation, generating a pronounced third band (Fig 1D, lanes 3 and 4). These nucleotide incorporations were template-dependent as the fourth band corresponding to a dC² incorporation was generated only in the presence of dCTP (Fig 1D, lane 5). These results reveal that elevated dGTP concentrations effectively overcome the DNA synthesis arrest at the pause site, which is governed by the pause signal and mediated through the high K_M of the dG¹ incorporation.

To further explore the connection between the pause signal and the high K_M for dG¹ nucleotide incorporation, we eliminated the pause signal from the DNA/RNA hybrid substrate by mutating the base pair dT:rA to dA:rU and then measured K_M for the dG¹ nucleotide incorporation (Figs 1E and EV3). This transversion

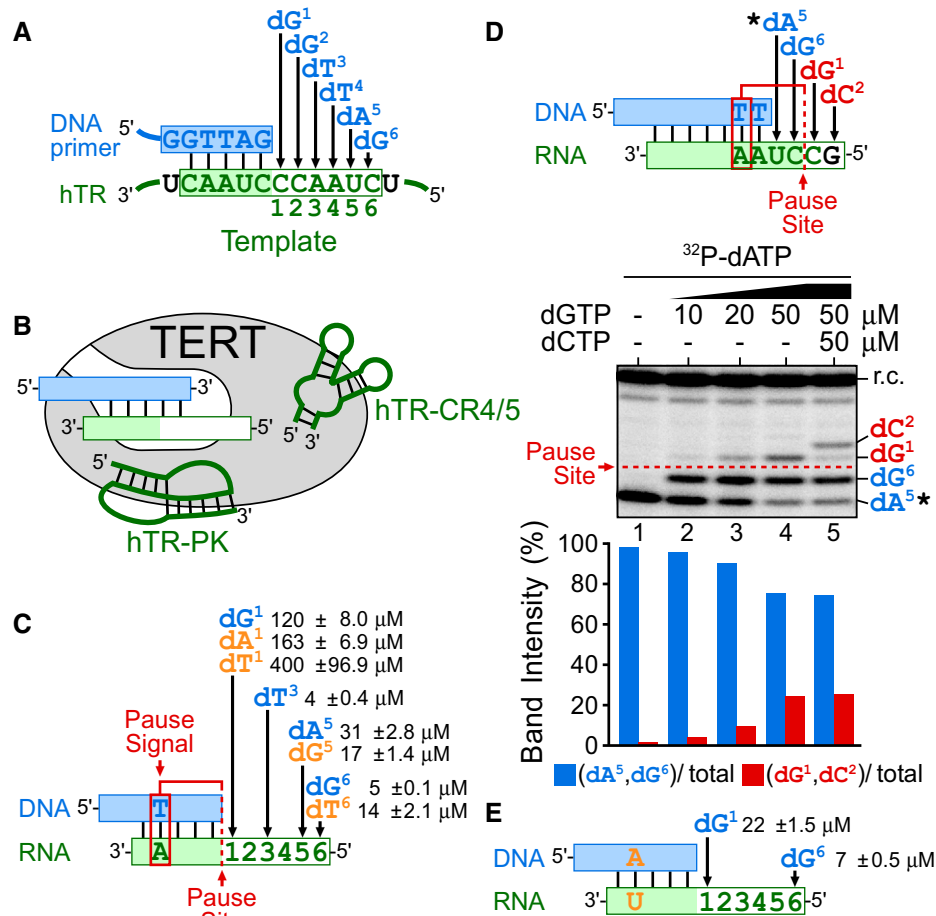


Figure 1. An elevated K_M for the dG¹ nucleotide incorporation by human telomerase.

- A** Schematic of the six-nucleotide incorporations catalyzed by human telomerase. Human telomerase catalyzes incorporations of six deoxynucleotides, dG¹, dG², dT³, dT⁴, dA⁵, and dG⁶, directed by the integral hTR template (green box). The hTR template contains two regions: an alignment sequence (shaded) for base pairing with the DNA primer and the actual templating sequence for specifying DNA polymerization. Numbers below the hTR template region denote the order and position of the six deoxynucleotide incorporations.
- B** Composition of template-free (TF) telomerase. TF telomerase was reconstituted by assembling *in vitro*-expressed human TERT protein with the two essential hTR fragments, CR4/5 and the pseudoknot (PK) that had the template region excised. Pre-annealed DNA primer (blue box) and RNA template (green box) hybrids were used as substrates for the TF telomerase activity assay.
- C** K_M for incorporating nucleotides dG¹, dT³, dA⁵, and dG⁶ by TF telomerase. The dG¹ incorporation is located adjacent to the pause site (red dashed line) specified by the pause signal (red box), a dT:rA base pair in the DNA/RNA hybrid. For measuring the K_M of specific nucleotide incorporations, different DNA/RNA hybrid substrates with permuted sequences were used (Fig EV2). The K_M for incorporating non-telomeric nucleotides (orange) with corresponding template mutations are denoted.
- D** High dGTP concentrations overcome the pause signal-mediated DNA synthesis arrest. Primer extension assays performed using the TF telomerase and a DNA/RNA hybrid substrate in the presence of 0.165 μM ³²P-dATP (asterisk) with a titration of dGTP (10, 20 and 50 μM). The DNA/RNA hybrid substrate allows for the incorporation of four nucleotides: dA⁵, dG⁶, dG¹, dC², as depicted. The pause signal (red box) and the pause site (red dashed line) in the DNA/RNA substrate arrests DNA synthesis after the incorporation of dA⁵ and dG⁶ (blue) reducing the incorporation efficiency for dG¹ (red). A radiolabeled DNA recovery control (r.c.) was added before product purification and precipitation. Nucleotide incorporation beyond the pause site was quantitated by the intensity of dG¹ and dC² products (red) over the total intensity of products.
- E** Removal of pause signal decreases K_M for dG¹ incorporation. The DNA/RNA hybrid substrates with the pause signal dT:rA mutated to dA:rU (orange) were used to determine K_M for nucleotide incorporation at positions dC² and dG⁶ (Fig EV3).

mutation has previously been shown to effectively inactivate the pause signal and permit DNA synthesis beyond the pause site (Brown *et al.*, 2014). As expected, the removal of the pause signal significantly reduced the K_M for dG¹ incorporation from 120 to 22 μM (Figs 1E and EV3). In contrast, the loss of the pause signal in the DNA/RNA duplex did not affect the K_M for incorporating dG⁶ (Fig 1C and E). We thus conclude that the template-embedded pause signal has an inhibitory effect specifically on the dG¹

nucleotide incorporation, mediated through a high K_M exclusively for this incorporation.

dGTP stimulates telomerase repeat addition processivity and rate

It has been previously proposed that the template-embedded pause signal may have dual functions (Brown *et al.*, 2014): arresting DNA

synthesis at the end of the template and inhibiting the first nucleotide incorporation after template translocation (Fig 2A). Processive repeat addition by telomerase necessitates reiterated cycles of nucleotide addition. The inhibitory effect of the pause signal on the first nucleotide dG¹ incorporation would impair initiating synthesis of each repeat, negatively affecting processive telomerase repeat addition. As high dGTP concentrations can effectively overcome the pause signal-mediated inhibition of dG¹ incorporation (Fig 1D), we hypothesized that high dGTP concentrations would facilitate progression into subsequent catalytic cycles and in turn promote telomerase repeat addition activity, generating more high molecular weight (M.W.) DNA products. In fact, dGTP-dependent stimulation of repeat addition activity has been previously reported for human and ciliate telomerases (Hammond & Cech, 1997; Maine *et al*, 1999; Sun *et al*, 1999; Hardy *et al*, 2001). However, the underlying mechanism for dGTP stimulation of telomerase repeat addition remained unclear. To ascertain that this previously reported nucleotide stimulation of human telomerase is specific to dGTP, and not dATP or dTTP, we reconstituted wild-type human telomerase with full-length, template-bearing hTR in human HEK293 cells and assayed the immuno-purified telomerase enzyme for repeat addition activity with a telomeric DNA primer (TTAGGG)₃ (Fig 2B and C). To determine the effects of dGTP and dATP concentrations on telomerase repeat addition, we performed the conventional telomerase primer extension assay in the presence of ³²P-dTTP with increasing concentrations (10, 50, and 200 μM) of dGTP or dATP individually (Fig 2B). Elevated dGTP concentrations at 50 or 200 μM significantly increased the relative ratio of high over low M.W. products by approximately 1.5-fold (Fig 2B, lanes 1, 4, and 5), while varying the concentration of dATP had no significant effect on repeat addition (Fig 2B, lanes 1–3). To examine the effects of dTTP, a similar assay was performed in the presence of ³²P-dATP. Similarly, increased dGTP concentrations increased repeat addition efficiency by approximately 1.5-fold (Fig 2C, lanes 1, 4, and 5), while elevated dTTP did not significantly increase repeat addition efficiency, but seemed to slightly increase the length of the highest M.W. products (Fig 2C, lanes 1–3). The slight increase of the highest M.W. products with elevated dTTP is consistent with a previous report (Drosopoulos & Prasad, 2010). Additionally, increasing nucleotide concentrations from 10 to 100 μM did not appear to alter single repeat synthesis, as nucleotide incorporation efficiency was saturated at 10 μM (Appendix Fig S2). These data clearly demonstrate that human telomerase repeat addition was stimulated specifically by increased concentrations of dGTP, and not dATP or dTTP. The increased ratio of higher over lower M.W. products could arise from an increase in the processivity and/or the rate of repeat addition, two independent attributes of the telomerase enzyme (Drosopoulos *et al*, 2005; Xie *et al*, 2010). To investigate the underlying mechanism for dGTP stimulation of telomerase repeat addition, we employed two specific telomerase activity assays to measure the processivity separately from the rate of repeat addition in the presence of elevated dGTP concentrations.

To specifically measure the processivity of telomerase repeat addition, we designed a telomerase product release assay that quantitates repeat addition processivity based on the distribution of DNA products released from the enzyme (Fig 3A). Enzyme-bound DNA products are still undergoing additional rounds of repeat addition, and the inclusion of these premature intermediates would influence

the measurement of telomerase repeat addition processivity. For this assay, human telomerase was reconstituted in HEK 293FT cells and the immuno-purified enzyme bound to beads was incubated with the telomeric DNA primer (TTAGGG)₃ in the presence of different concentrations of dGTP or dATP. The DNA products released from the immobilized telomerase enzyme were isolated from the supernatant and analyzed to determine the probability of each successive DNA repeat addition. This probability was calculated from the slope of “products left behind” (Lattrick & Cech, 2010) by plotting the intensity of major DNA products with the number of repeats (3–12) added (Fig 3A and B). The slope of the plot corresponds to the repeat addition processivity and was used to compare the relative repeat addition processivity across different assay conditions (Fig 3B). The results of this product release assay found that increasing dGTP concentration from 10 to 100 μM resulted in a twofold increase in processivity, while increasing dATP concentrations to 100 μM had no significant effect on processivity (Fig 3C). Thus, high dGTP concentration effectively stimulates the processivity of human telomerase repeat addition.

We next investigated the effects of elevated dGTP concentrations on the rate of repeat addition. To specifically measure repeat addition rate, we employed a pulse-chase time course assay to track the increasing size of the DNA products undergoing processive repeat addition over time (Fig 3D). For this pulse-chase assay, immuno-purified telomerase was initially incubated exclusively with ³²P-dTTP to radioactively label the DNA primer and this pulsed reaction was chased with cold nucleotides to track the processive enzyme–DNA complexes over time. The repeat addition rate was 0.86 repeat/min measured under the chase condition with 10 μM dGTP and 100 μM of dTTP and dATP. However, when chased with the dGTP concentration increased to 100 μM, the repeat addition rate nearly doubled to 1.45 repeat/min (Fig 3E, left panel). In contrast, when the reaction was chased with either 10 or 100 μM dATP, in the presence of 100 μM dGTP and dTTP, the repeat addition rate remained unchanged at 1.21 or 1.27 repeat/min, respectively (Fig 3E, right panel). Thus, dGTP concentration is a crucial determinant for the rate, in addition to the processivity, of human telomerase repeat addition.

First nucleotide incorporation following template translocation mediates nucleotide-specific stimulation of telomerase repeat addition

The synthesis of a telomeric DNA repeat comprises three dG incorporations: dG¹, dG², and dG⁶ (Fig 4A). We hypothesized that the high dGTP concentration stimulated telomerase repeat addition by overcoming the high K_M for dG¹ incorporation. To determine whether the dG¹ incorporation is specifically responsible for the dGTP stimulation of telomerase repeat addition, we generated three hTR template mutants hTR-51U, hTR-50U, and hTR-46/52U that individually altered the three dG nucleotide incorporations to dA¹, dA², and dA⁶, respectively. We reconstituted these telomerase template mutants in human HEK293 cells and assayed the immuno-purified enzyme by conventional telomerase primer extension assay in the presence of ³²P-dTTP with either 10 or 100 μM of dGTP or dATP. Remarkably, of these three mutants, only the hTR-51U mutant had a pronounced increase of the higher M.W. products with increased dATP (Fig 4B, lanes 4 and 5) and was unaffected by

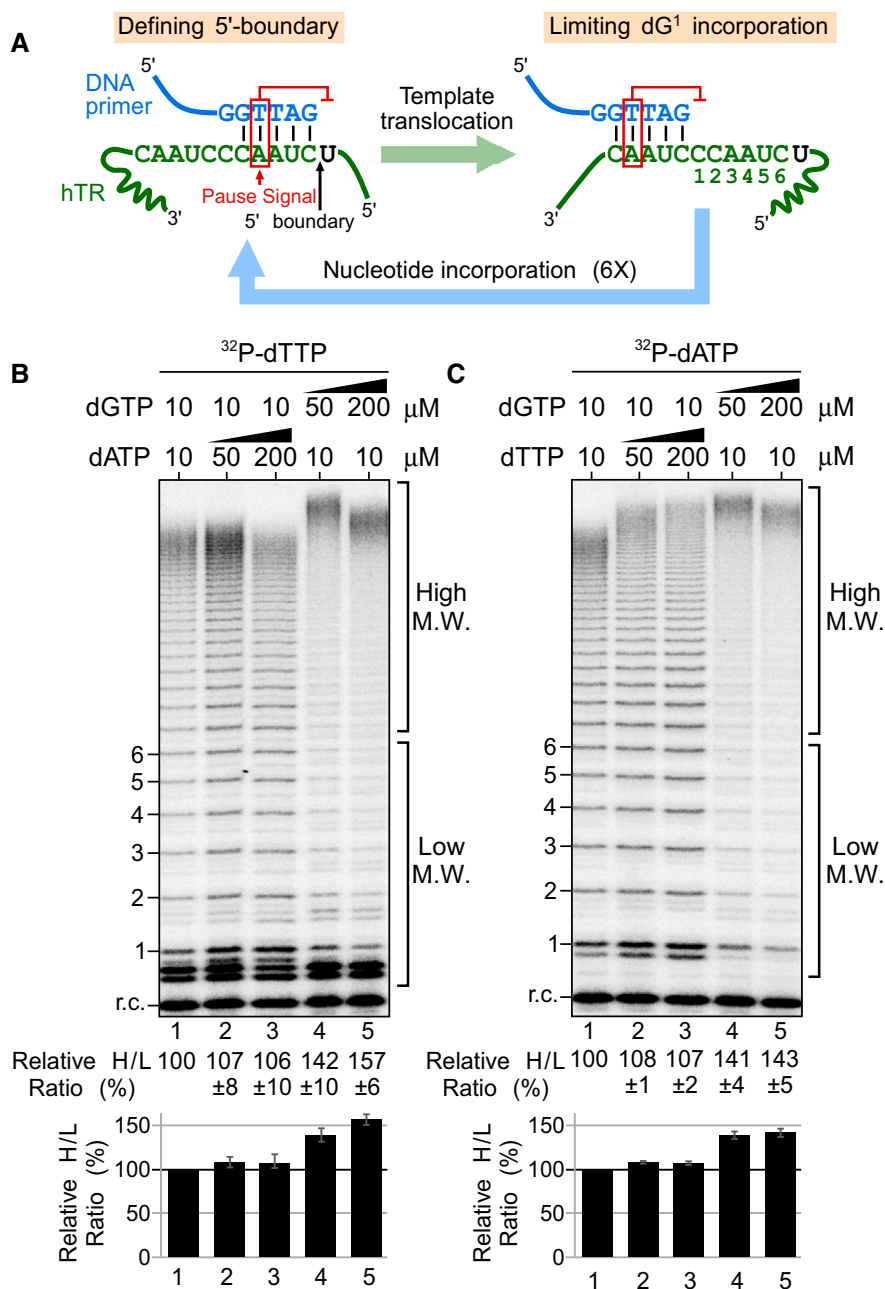


Figure 2. dGTP-dependent repeat addition stimulation of human telomerase.

- A** Schematic of the dual function of the template-embedded pause signal (red box). The pause signal defines the 5' template boundary (black arrow) by inhibiting non-telomeric DNA synthesis beyond the template boundary (Brown *et al.*, 2014). A putative additional function of the pause signal is to limit dG¹ incorporation post-template translocation.
- B, C** Direct primer extension assays were performed with telomerase enzyme reconstituted *in vivo* and immuno-purified. Telomerase was assayed in the presence of either (B) 0.165 μM ³²P-dTTP, 10 μM dTTP and a range of dGTP or dATP concentrations, or (C) 0.165 μM ³²P-dATP, 10 μM dATP and a range of dGTP or dTTP concentrations. A radiolabeled DNA recovery control (r.c.) was added before product purification and precipitation. Numbers to the left of the gel denote the number of repeats added to the telomeric primer. Ratio as a percent for the intensity of high over low M.W. DNA products generated relative to the reaction with the low nucleotide concentrations. A bar graph of the relative ratio of high/low M.W. DNA products are shown below the gel. The error bars represent standard error of the mean determined from three independent replicates.

increased dGTP (Fig 4B, lanes 4 and 6). Similar to wild type, the hTR-50U and hTR-46/52U mutant telomerases had increased repeat addition efficiencies with increased dGTP and were unaffected by

increased dATP (Fig 4B, lanes 6–12). It is interesting to note that the hTR-46/52U mutant telomerase had significantly lower repeat addition efficiency, producing less high M.W. products (Fig 4B, lanes 1

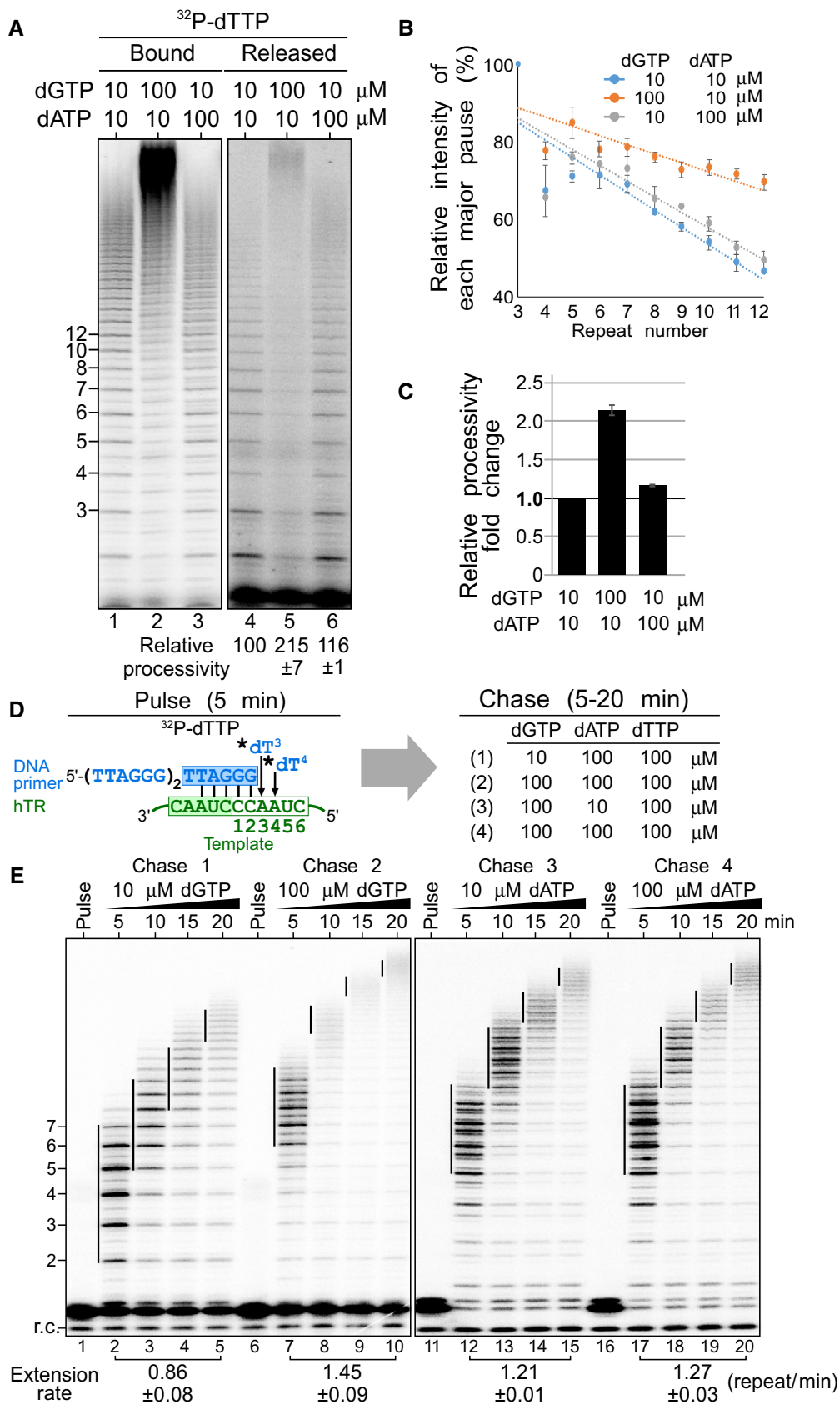


Figure 3.

Figure 3. Effects of dGTP on repeat addition processivity and rate.

- A Stimulation of repeat addition processivity with dGTP. *In vivo*-reconstituted telomerase enzyme was assayed by the product release analysis in the presence of 0.165 μM ^{32}P -dTTP, 10 μM dTTP as well as either 10 or 100 μM of dGTP and dATP. The enzyme-bound and released DNA products were separated and individually analyzed.
- B Quantitation of repeat addition processivity. The intensities of the released DNA products with 3–12 repeats added were quantitated and normalized to the intensity of the product with 3 repeats added. The relative intensities of major bands were plotted against the number of repeats added to determine slopes that correspond to the relative processivity from each reaction (see Materials and Methods section). The error bars represent standard error of the mean determined from two independent replicates.
- C Relative repeat addition processivity under different nucleotide concentrations. A bar graph indicates the fold change of the relative repeat addition processivity under either 10 or 100 μM of dGTP and dATP. The error bars represent standard error of the mean determined from two independent replicates.
- D Schematic of pulse-chase time course analysis. During the pulse reaction, the DNA primer (TTAGGG)₃ was labeled with 0.165 μM ^{32}P -dTTP (asterisk) by the telomerase enzyme. During the chase reaction, the enzyme-bound radiolabeled DNA primer was extended processively with telomeric repeats under 100 μM cold dTTP as well as either 10 or 100 μM of dGTP and dATP for 5, 10, 15, or 20 min.
- E Repeat addition rate measured by the pulse-chase time course analysis under differing nucleotide concentrations. The vertical lines on the gel indicate the major bands of telomere products synthesized during the chase reactions. Repeat addition rates are expressed as repeats per minute (see Materials and Methods) and indicated below the gel. The standard error of the mean was determined from two independent replicates.

and 10). This was likely due to the less stable dA:rU base pairing located at the end of the primer/template duplex, which would negatively impact primer realignment efficiency during template translocation. Noticeably, the hTR-51U and hTR-50U mutant telomerases generated DNA products with altered banding profiles (Fig 4B). This is seemingly due to the substitution of a dA instead of a dG residue at the specified template positions affecting nucleotide incorporation efficiency, as it has been reported that telomerase is sensitive to template sequence alterations (Gilley *et al.*, 1995; Drosopoulos *et al.*, 2005). The template mutant hTR-50U appeared to have a lower incorporation efficiency for dG⁶ residue, resulting in the accumulation of the DNA product after dA⁵ incorporation visible as an additional band (Fig 4B, lanes 7 and 8), which was effectively alleviated by high dGTP (Fig 4B, lane 9). Nonetheless, by changing the nucleotide incorporation from dG¹ to dA¹ with the hTR-51U template mutation, we effectively changed the stimulation of human telomerase repeat addition from dGTP to dATP-dependent. This further supports the hypothesis that the dG¹ nucleotide incorporation is a critical step for human telomerase repeat addition.

We next sought to determine whether the dATP-dependent stimulation of hTR-U51 mutant telomerase affects specifically the processivity and/or the rate of repeat addition. We examined the repeat addition processivity of the hTR-51U mutant with our product release assay (Fig 5A). As expected, the hTR-51U mutant had a 35% increase of telomerase repeat addition processivity in the presence of 100 μM dATP and no effect with 100 μM dGTP (Fig 5A–C). This result indicates that the first nucleotide incorporation in the telomerase catalytic cycle is an important determinant for repeat addition processivity. Following this, we then examined the contribution of the first nucleotide incorporation efficiency on the repeat addition rate. We performed a pulse-chase time course assay with the hTR-51U mutant to measure the repeat addition rate in the presence of either 10 or 100 μM of dATP or dGTP (Fig 5D). When the reactions were chased with 100 μM dATP, the repeat addition rate was dramatically increased to 3.15 repeat/min from 1.27 repeat/min with 10 μM dATP—a near twofold increase (Fig 5E). In contrast, increasing dGTP from 10 to 100 μM did not increase the repeat addition rate of hTR-51U mutant, which remained similar from 2.99 to 2.95 repeat/min (Fig 5E). Our data indicate that the concentration of the nucleotide incorporated as the first residue of a telomeric DNA repeat is crucial for the processivity as well as the rate of human telomerase repeat addition.

Telomerase can incorporate deoxynucleoside diphosphates as substrate

It was previously reported that *Tetrahymena* telomerase accumulates high M.W. DNA products in the presence of elevated dGTP, dGDP, or even dGMP, leading to the hypothesis that a secondary guanosine binding site was responsible for the dGTP-dependent repeat addition stimulation (Hardy *et al.*, 2001). However, our hTR51U telomerase mutant exhibited dATP-dependent stimulation of repeat addition processivity, opposing a secondary guanosine binding site in human telomerase. To investigate the possibility of a more general purine nucleoside binding site for repeat addition stimulation, we examined whether dGMP or dGDP stimulates human telomerase repeat addition. Our result showed that dGMP, at either 10 or 100 μM , failed to stimulate telomerase repeat addition in a reaction containing 10 μM dGTP, 10 μM dTTP, and 100 μM dATP (Fig 6A, lanes 1 and 2). Interestingly, increasing dGDP concentrations from 10 to 100 μM generated a noticeable increase in telomerase repeat addition efficiency (Fig 6A, lanes 3 and 4). To investigate this dGDP-dependent stimulation of repeat addition, we examined whether dGDP functions as a substrate for nucleotide incorporation by telomerase. By replacing dGTP with either dGDP or dGMP in the telomerase reaction, we showed that human telomerase can effectively incorporate dGDP as substrate for telomeric DNA synthesis (Fig 6A, lanes 8–9), yet not dGMP (Fig 6A, lanes 6–7). The purity of the dGMP, dGDP, and dGTP nucleotides was assessed by MALDI-MS analysis to ensure no dGTP contamination (Appendix Fig S3). We further examined telomerase for utilizing dADP as substrate. Similarly, human telomerase can effectively incorporate dADP, and not dAMP (Fig EV4A and B). To eliminate the possibility of γ -phosphate transfer from dATP in the reaction to dGDP, we performed a telomerase reaction lacking any nucleoside triphosphates using a ^{32}P end-labeled DNA primer with exclusively deoxynucleoside diphosphates: dGDP, dADP, and dTDP as substrates (Fig EV4C). This deoxynucleoside diphosphates-only telomerase reaction generated a significant level of repeat addition activity (Fig EV4D, lanes 1–2), which, however, was consistently lower than the activity from the deoxynucleoside triphosphates reaction (Fig EV4D, lanes 3–4). These results suggest that deoxynucleoside diphosphates are sufficient substrates, yet less effective than deoxynucleoside triphosphates for human telomerase DNA synthesis.

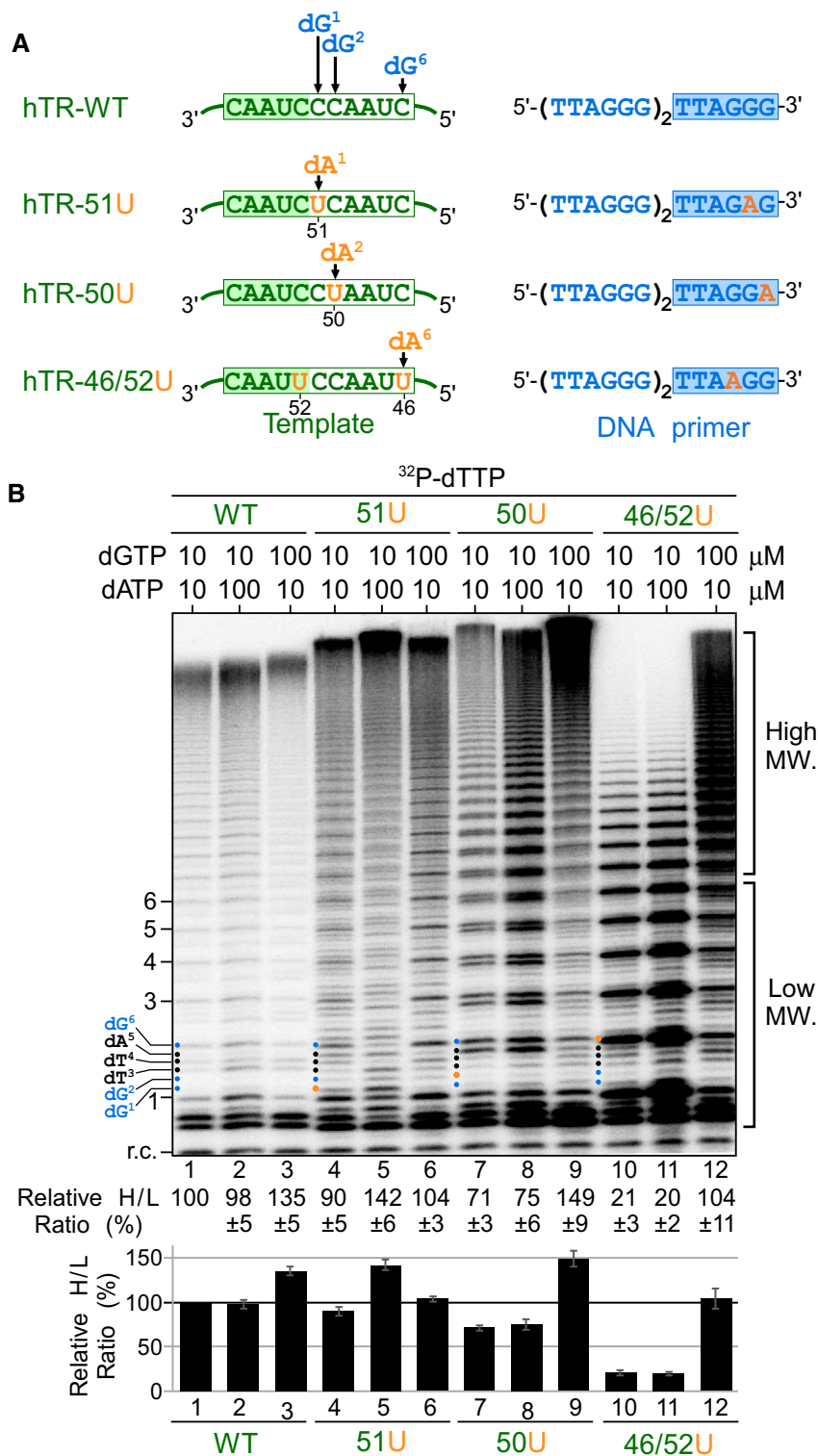


Figure 4. The dG¹ incorporation and telomerase repeat addition activity.

A Sequences of hTR mutant templates and DNA primers used in this assay. The wild-type hTR template specifies for the incorporation of three dG residues, dG¹, dG², and dG⁶. The mutant hTR templates, 51U, 50U, and 46/52U, harbor rC-to-rU template mutations (orange) that specify incorporations of non-telomeric dA¹, dA², and dA⁶ (orange), respectively. The telomerase template mutants were assayed with corresponding DNA primers as depicted.

B Direct primer extension assays of telomerase template mutants. Wild-type and mutant telomerases were reconstituted *in vivo* and the immuno-purified enzymes were assayed in the presence of 0.165 μM ³²P-dTTP, 10 μM dTTP as well as either 10 or 100 μM of dGTP and dATP. Ratio as a percent for the intensity of high over low M.W. DNA products generated relative to the wild-type telomerase reaction with the low nucleotide concentrations. A bar graph of the relative ratio of high/low M.W. DNA products are shown below the gel. Standard error of the mean was determined from three independent replicates.

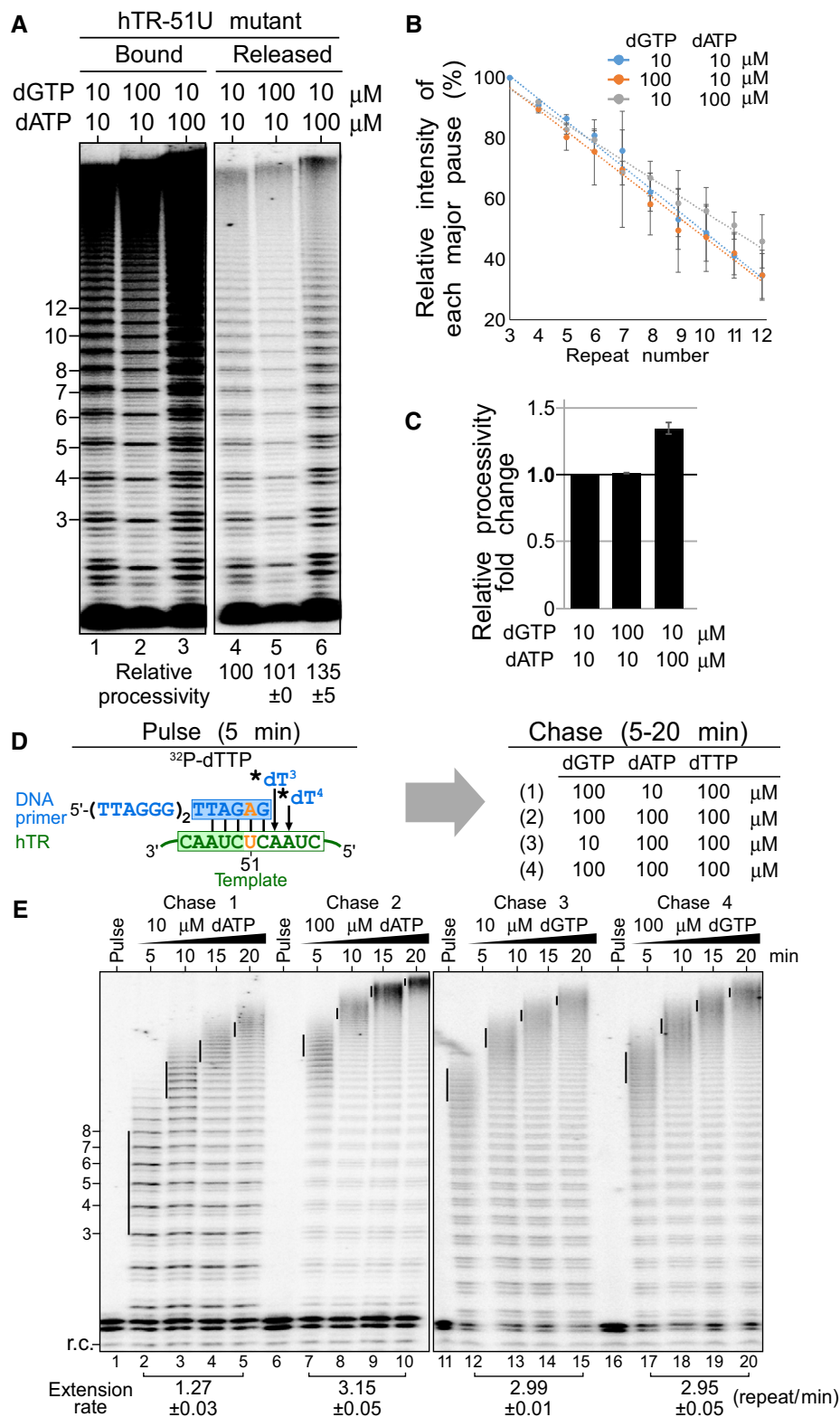


Figure 5.

We sought to assess the pervasiveness of DNA polymerases for utilizing deoxynucleoside diphosphates for DNA synthesis. In addition to telomerase examined in this study, it has been previously

reported that the HIV RT and a bacteriophage DNA polymerase are capable of using deoxynucleoside diphosphates as substrate (Yang *et al*, 2002; Garforth *et al*, 2008). We examined three RTs: TF

Figure 5. Effects of dATP on repeat addition processivity and rate for the telomerase mutant hTR-51U.

- A dATP-dependent stimulation of repeat addition processivity with the hTR-51U mutant. *In vivo*-reconstituted telomerase enzyme was assayed by the product release analysis in the presence of 0.165 μM ^{32}P -dTTP, 10 μM dTTP as well as either 10 or 100 μM of dGTP and dATP. The enzyme-bound and released DNA products were separated and individually analyzed.
- B Quantitation of repeat addition processivity. The intensities of the released DNA products with 3–12 repeats added were quantitated and normalized to the intensity of the product with 3 repeats added. The relative intensities of major bands were plotted against the number of repeats added to determine the slopes that correspond to the relative processivity from each reaction. The error bars represent standard error of the mean determined from two independent replicates.
- C Relative repeat addition processivity under different nucleotide concentrations. A bar graph indicates the fold change of the relative repeat addition processivity under either 10 or 100 μM of dGTP and dATP. The error bars represent standard error of the mean determined from two independent replicates.
- D Schematic of the pulse-chase time course analysis for the telomerase mutant hTR-51U. During the pulse reaction, the DNA primer (TTAGGG)₂TTAGAG was labeled with 0.165 μM ^{32}P -dTTP (asterisk) by the telomerase hTR-51U enzyme. During the chase reaction, the enzyme-bound radiolabeled DNA primer was extended processively with telomeric repeats under 100 μM cold dTTP as well as either 10 or 100 μM dGTP and dATP for 5, 10, 15, or 20 min.
- E Repeat addition rate measured by the pulse-chase time course analysis under differing nucleotide concentrations. A radiolabeled DNA recovery control (r.c.) was added before product purification and precipitation. The vertical lines on the gel indicate the major bands of telomere products synthesized during the chase reactions. Repeat addition rates are expressed as repeats per minute and indicated below the gel. The standard error of the mean was determined from two independent replicates.

telomerase, AMV, and TGIRT III group II intron RTs, and three DNA-dependent DNA polymerases: Taq, T4, and the Klenow fragment, for incorporating dGDP into DNA products using corresponding DNA/RNA or DNA/DNA duplex substrates and labeling ^{32}P -dATP (Figs 6B and C, and EV5). Except for the T4 DNA polymerase (Fig EV5, lane 7), all other examined enzymes showed significant incorporation of dGDP and not dGMP as substrate (Fig 6B, lane 4 and 8; Fig 6C, lanes 3; and 7; Fig EV5, lane 3). All enzymes analyzed showed template-directed nucleotide incorporations lacking non-specific terminal transferase activities. Therefore, the utilization of deoxynucleoside diphosphate as substrate for DNA synthesis is ubiquitous among DNA polymerases. Moreover, the dGDP-dependent stimulation of human telomerase repeat addition is likely from active incorporation of dGDP as substrate rather than secondary-site binding.

Removal of the pause signal eliminates dGTP-specific stimulation of human telomerase repeat addition

With our TF telomerase system, we showed that the template-embedded pause signal is responsible for the high K_M of the dG¹ incorporation (Fig 1E), a crucial determinant for repeat addition processivity and rate (Fig 3). To assess whether the pause signal limits repeat addition in a processive telomerase reaction, we generated a human telomerase template mutant, termed Δ pause, that lacks the pause signal by mutating the four rA residues (nt positions 48, 49, 54, and 55) in the template to rU residues (Fig 7A). Our previous study indicated that each of the rA residues in the TR template must be altered to completely eliminate the pause signal (Brown *et al.*, 2014). We reconstituted wild-type and the Δ pause mutant human telomerase in HEK293 cells and assayed the immuno-purified enzyme with corresponding DNA primers and a titration of dGTP from 10 to 50 μM (Fig 7B). In sharp contrast to the wild-type telomerase that had the expected dGTP-dependent stimulation of repeat addition activity (Fig 7B, lanes 1–3), the Δ pause mutant had strikingly high repeat addition activity with only 10 μM dGTP, which remained unchanged with 100 μM dGTP (Fig 7B, lanes 4–6). Interestingly, the Δ pause template mutant had an altered major banding pattern compared with the wild-type enzyme, which was presumably due to a shift of the limiting nucleotide incorporation from dG¹ to the three dA residues for the mutant enzyme (Fig 7B, lane 4). We then analyzed the Δ pause mutant for repeat

addition processivity and rate separately (Fig 8). The Δ pause mutant had an approximately onefold greater processivity at either 10 or 100 μM dGTP compared to the wild-type enzyme at 10 μM dGTP (Fig 8A–C). The repeat addition rate of the Δ pause mutant was found to be 1.70 repeat/min at 10 μM dGTP and 1.65 repeat/min with 100 μM dGTP (Fig 8D and E), which is significantly higher than the 0.7 and 1.3 repeat/min of the wild-type enzyme at 10 and 100 μM dGTP, respectively (Fig 3D and E). These results strongly support the pause signal is responsible for the intrinsically low processivity and rate, as well as the dGTP-specific stimulation of human telomerase repeat addition.

Discussion

Telomerase is an RNA-dependent DNA polymerase specialized in adding telomeric DNA repeats onto chromosome ends (Podlevsky & Chen, 2016). Apart from other DNA polymerases, telomerase employs a highly orchestrated, yet poorly understood, catalytic cycle to regenerate the RNA template for processive synthesis of multiple telomeric DNA repeats (Wu *et al.*, 2017b). The total number of DNA repeats added by a given telomerase enzyme in a single turnover is determined by the two tangibly separate attributes of the telomerase enzyme: the processivity and the rate of repeat addition (Podlevsky & Chen, 2012; Schmidt & Cech, 2015). The processivity of telomerase repeat addition corresponds to the probability of continuous DNA repeat synthesis over complete product release following each catalytic cycle of six-nucleotide incorporations. Distinct from processivity, the rate of repeat addition corresponds to the number of telomeric repeat synthesized per unit time. The repeat addition rate is contributed by: (i) the rate of individual nucleotide additions to the primer and (ii) the rate of template regeneration by template translocation. In this work, we discovered a previously unknown role of the telomerase template-embedded pause signal for inhibiting the dG¹ incorporation step, the first nucleotide of each telomeric DNA repeat. This slow dG¹ incorporation is a decisive step that affects the rate of the telomerase reaction. Failure to incorporate the dG¹ residue prevents processive additional cycles of repeat synthesis, which prompts complete DNA product release and terminates the reaction (Fig 9).

As depicted in our working model of the telomerase catalytic cycle (Fig 9), each repeat synthesis comprises the addition of six

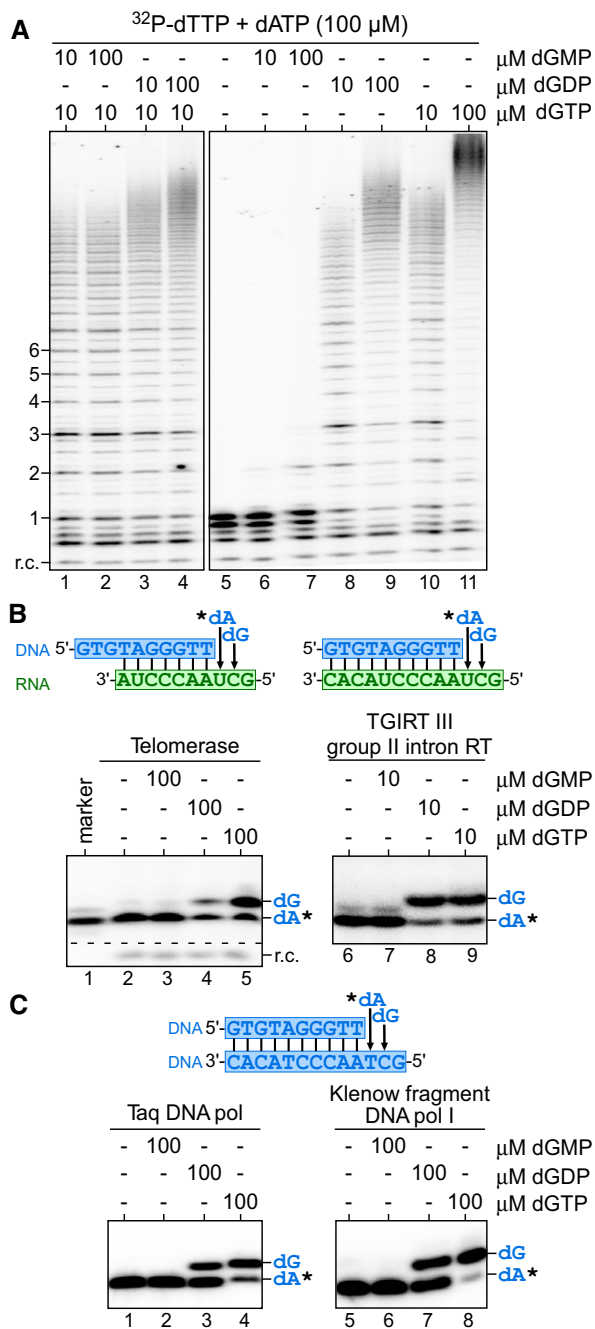


Figure 6. Telomerase utilizes deoxynucleoside diphosphates as substrate.

A Direct primer extension assays were performed with telomerase enzyme reconstituted *in vivo* and immuno-purified. Telomerase was assayed in the presence of 0.165 μM ^{32}P -dTTP, 10 μM dTTP, 100 μM dATP as well as combinations of either 10 or 100 μM of dGMP, dGDP, or dGTP.

B Utilization of dGDP by RTs. Specific DNA/RNA hybrid substrates depicted harbor a template sequence for the incorporation of a single dA and a dG residue.

C Utilization of dGDP by DNA-dependent DNA polymerases. The DNA/DNA hybrid substrate depicted harbors a template sequence to allow for incorporation of a single dA and a dG residue. Taq DNA polymerase (pol) and the Klenow fragment of DNA polymerases I were assayed in the presence of 0.165 μM ^{32}P -dATP (asterisk) and either dGMP, dGDP, or dGTP. The identity of nucleotides added are denoted to the right of the gel.

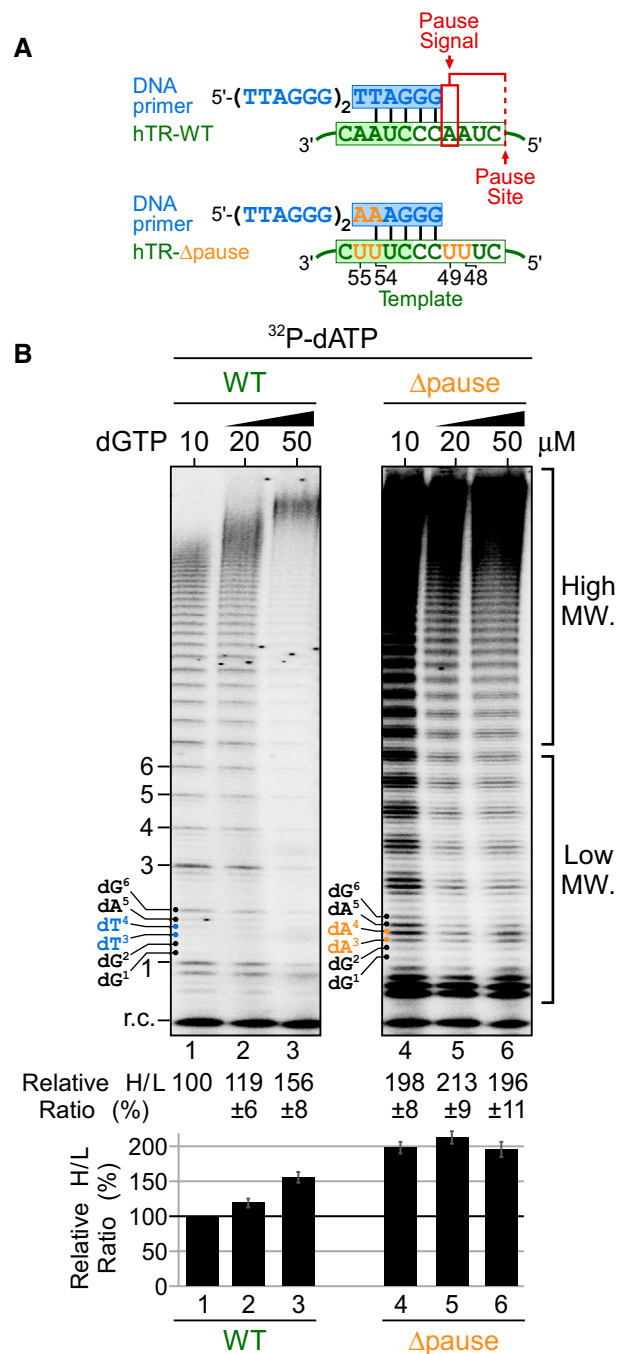


Figure 7. Effects of pause signal removal on telomerase repeat addition.

A Schematic of wild-type and hTR Δ pause template mutant telomerase with corresponding DNA primers. The hTR Δ pause template mutant harbors rA-to-rU mutations (orange) at four residues: 48, 49, 54, and 55 to eliminate the template-embedded pause signal (red box).

B Direct primer extension assays for the telomerase Δ pause template mutant. Wild-type and mutant telomerases were reconstituted *in vivo* and the immuno-purified enzymes were assayed in the presence of 0.165 μM ^{32}P -dATP, 10 μM dATP as well as either 10, 20, or 50 μM of dGTP. Relative repeat addition activity was presented as the ratio of the intensity of high over low M.W. DNA products generated in each reaction normalized to the wild-type enzyme reaction with 10 μM dGTP. A bar graph of the relative ratio of high/low M.W. DNA products are shown below the gel. Standard error of the mean was determined from three independent replicates.

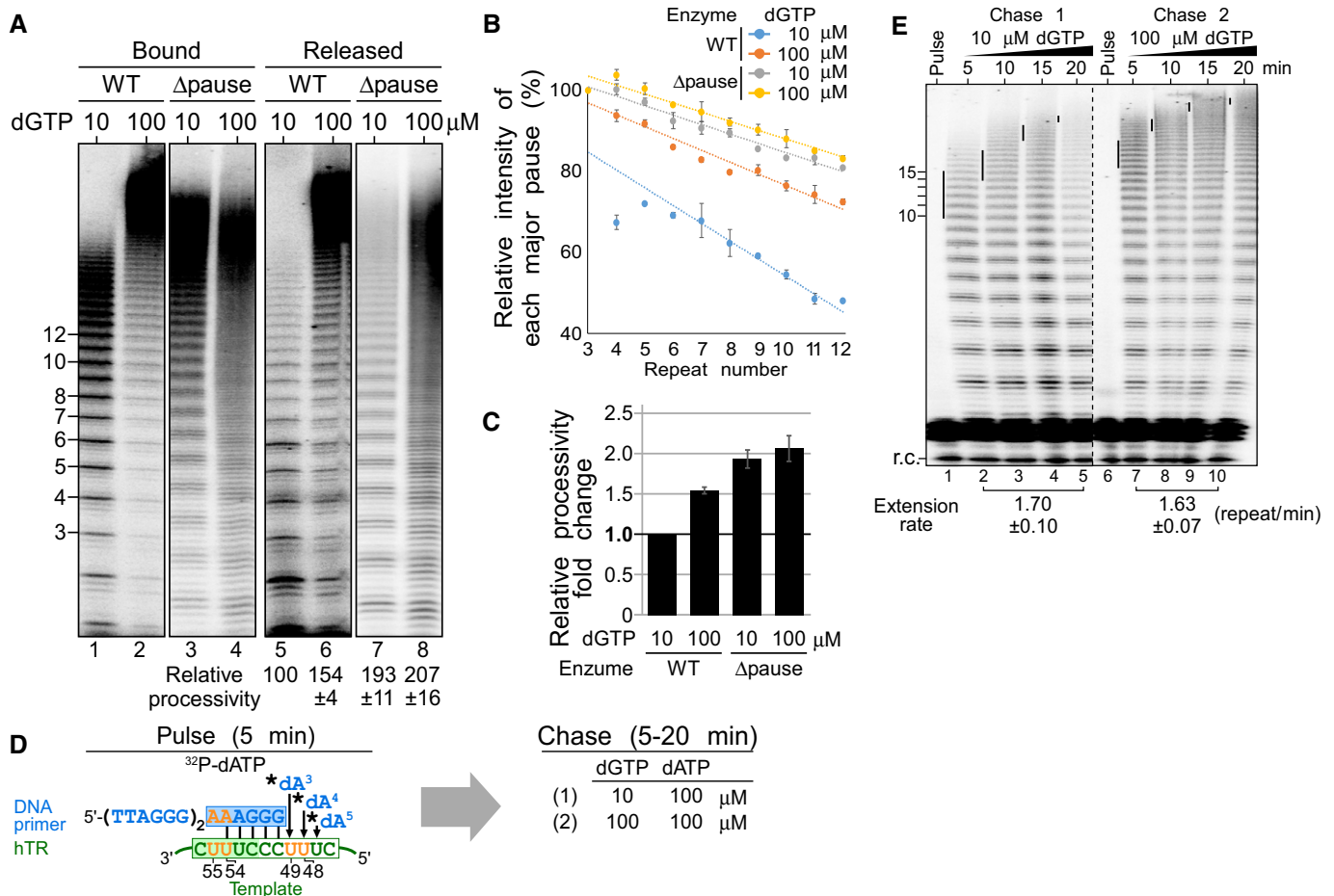


Figure 8. Effects of dGTP on repeat addition processivity and rate of the hTR- Δ pause mutant telomerase.

A Lack of stimulation of repeat addition processivity with dGTP for the telomerase mutant hTR- Δ pause. *In vivo*-reconstituted telomerase enzyme was assayed by the product release analysis in the presence of 0.165 μ M 32 P-dATP, 10 μ M dATP as well as either 10 or 100 μ M of dGTP. The enzyme-bound and released DNA products were separated and individually analyzed.

B Quantitation of repeat addition processivity. The intensities of the released DNA products with 3–12 repeats added were quantitated and normalized to the intensity of the product with 3 repeats added. The relative intensities of major bands were plotted against the number of repeats added to determine the slopes that correspond to the relative processivity from each reaction. The error bars represent standard error of the mean determined from two independent replicates.

C Relative repeat addition processivity under different nucleotide concentrations. A bar graph indicates the fold change of the relative repeat addition processivity under either 10 or 100 μ M of dGTP for wild-type and the hTR- Δ pause mutant. The error bars represent standard error of the mean determined from two independent replicates.

D Schematic of the pulse-chase time course analysis for the telomerase mutant hTR-51U. In the pulse reaction, the DNA primer (TTAGGG)₂AAAGGG was labeled with 0.165 μ M 32 P-dATP (asterisk) by the telomerase hTR- Δ pause enzyme. During the chase reaction, the enzyme-bound radiolabeled DNA primer was extended processively with telomeric repeats under 100 μ M cold dATP as well as either 10 or 100 μ M dGTP for 5, 10, 15, or 20 min.

E Repeat addition rate measured by the pulse-chase time course analysis under differing nucleotide concentrations. A radiolabeled DNA recovery control (r.c.) was added before product purification and precipitation. The vertical lines on the gel indicate the major bands of telomere products synthesized during the chase reactions. Repeat addition rates are expressed as repeats per minute and indicated below the gel. The standard error of the mean was determined from two independent replicates.

nucleotides to the 3' hydroxyl of the DNA primer and then arrested at the 5' end of the hTR template by the physical boundary element (Chen & Greider, 2003a) and the unique template-embedded pause signal (Brown *et al.*, 2014). Following each repeat synthesis, telomerase regenerates the template through a template translocation step, which has recently been shown to be a rapid process (Parks & Stone, 2014) with a rate about 100-fold faster than the overall repeat addition rate measured (Lattrick & Cech, 2010). Thus, the template translocation step is unlikely a major determinant for the rate of

repeat addition (Fig 9). Moreover, the high repeat addition activity and the lack of product release after the dG⁶ incorporation with the Δ pause mutant suggest that template translocation is efficient (Fig 7B, lane 4). Recently, two models have been proposed for the mechanism of template translocation: single-stranded DNA retention by DNA–protein interactions (Wu *et al.*, 2017a) and DNA hairpin formation (Yang & Lee, 2015). Each of these models is possible for template translocation, yet additional work is necessary to establish the definitive mechanism. The data presented herein center on

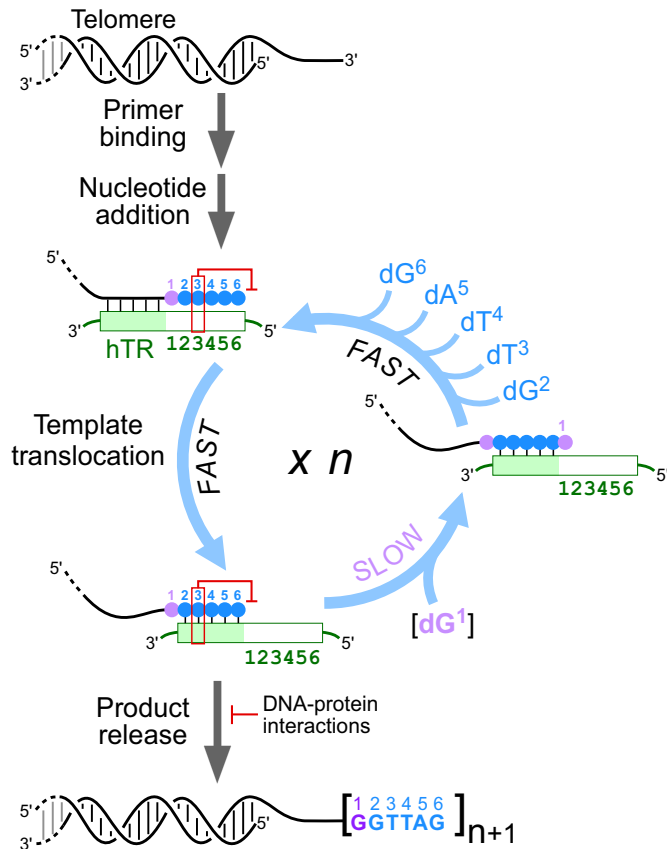


Figure 9. A working model of the telomerase catalytic cycle.

Following telomerase binding to the 3'-end of the telomere, nucleotide addition of six residues (violet and blue circles) proceeds to the end of the template and is arrested by the pause signal (red box) at the pause site. A fast template translocation process regenerates the template by realigning the template relative to the 3'-end of the DNA primer. Progression to the next catalytic cycle is impeded by the still active pause signal from the DNA/RNA hybrid, causing a slow dG¹ residue incorporation (violet) at the pause site. Failure to incorporate the dG¹ residue promotes product release, which is counteracted by DNA-protein interactions through the TERT anchor sites and telomerase accessory POT1-TPP1 protein complex. Successful dG¹ residue incorporation is followed by the rapid incorporation of five additional residues, dG², dT³, dT⁴, dA⁵, and dG⁶ (blue), completing a telomerase catalytic cycle. The number of repeats added corresponds to the number of catalytic cycles completed before complete dissociation of the telomeric DNA from the telomerase enzyme. Increasing the dGTP concentration increases the rate of the slow dG¹ residue incorporation, which increases telomerase repeat addition processivity and rate.

DNA synthesis post-template translocation and neither support nor negate these proposed models. However, mutations across the template that alter the telomeric DNA sequence do not affect the functionality of the pause signal (Brown *et al*, 2014), which would imply that the putative DNA hairpin is largely unaffected by the DNA sequence or is not critical for pause signal-mediated arrest of DNA synthesis. A myriad of intrinsic and extrinsic factors contribute synergistically to template translocation efficiency and repeat addition processivity. These intrinsic factors include the template length that affects template/primer realignment (Chen & Greider, 2003b), TERT anchor sites that bind DNA to prevent complete DNA

dissociation (Jacobs *et al*, 2006; Wyatt *et al*, 2007; Finger & Bryan, 2008; Zaug *et al*, 2008; Akiyama *et al*, 2015), or TERT motifs that enable stable retention of the realigned DNA/RNA hybrid in the active site (Huard *et al*, 2003; Lue *et al*, 2003; Xie *et al*, 2010; Qi *et al*, 2012; Wu *et al*, 2017b). Moreover, telomerase accessory proteins, POT1 (Protection Of Telomeres 1) and TPP1 (TIN2 and POT1-interacting protein 1), have been found to increase processivity by delaying product release through DNA-protein interactions (Lingner *et al*, 1997; Latrick & Cech, 2010). These DNA-protein interactions promote progression into the next cycle of repeat synthesis and augments telomerase repeat addition processivity (Fig 9). In addition to the intrinsic factors, extrinsic factor such as ionic strength, temperature, nucleotide, and primer concentrations (Sun *et al*, 1999). The sheer number of distinct factors that affect telomerase repeat addition processivity underlies the excessive complexity of template translocation.

After a successful template translocation event, the template is regenerated and ready for further nucleotide addition (Fig 9). Nevertheless, the pause signal remains effective following template translocation and inhibits the incorporation of the dG¹ residue through an excessively high K_M for this incorporation (Fig 1C). Our K_M measurements for nucleotide incorporation at specific positions across template were only feasible by using the non-processive TF telomerase (Qi *et al*, 2012) and specifically designed DNA/RNA hybrid substrates with permuted sequences (Fig EV2); these measurements were otherwise challenging and complex with a processive telomerase enzyme. By measuring individual nucleotide incorporation efficiencies with the TF telomerase, we found that removing the pause signal in the DNA/RNA hybrid effectively lowers the high K_M of the dG¹ incorporation to a value comparable to other positions across the template, suggesting that the pause signal is responsible for this inhibitory effect (Figs 1E and EV3). For the processive wild-type telomerase enzyme, the removal of the pause signal from the hTR template dramatically increases repeat addition activity even in the presence of low dGTP concentration at 10 μ M (Fig 7B). This suggests that the dG¹ incorporation is no longer the limiting step for processive repeat addition. The template mutations introduced in the Δ pause mutants modified the sequence of the DNA products, which may affect DNA-protein interactions with TERT anchor sites or accessory proteins. While altering DNA-enzyme binding affinity has the potential to augment repeat addition processivity, it is unlikely that this would simultaneously enhance the rate of repeat addition (Fig 8). Therefore, it is more feasible that the increased rate of repeat addition with the Δ pause mutant was caused by the lower K_M of the dG¹ incorporation. Under saturating dGTP concentrations, the Δ pause mutant and wild-type telomerases had equally high repeat addition activity (Drosopoulos *et al*, 2005), supporting our hypothesis that the dramatically high repeat addition activity of the Δ pause mutant at low dGTP was mainly due to the alleviated dG¹ incorporation. Moreover, the loss of the pause signal noticeably altered the banding pattern of DNA products, which was presumably from new limiting nucleotide incorporations at other template positions and premature product releases.

Upon recognition of the pause signal in the DNA/RNA hybrid, telomerase stalls DNA synthesis precisely at the pause site by the elevated K_M for nucleotide incorporation (Fig 1C). While the underlying mechanism for pause signal recognition requires further

investigation, we speculate that pause signal recognition induces a subtle conformational change in the DNA/RNA hybrid binding site and/or the catalytic site within the TERT protein that manifests as the elevated K_M for nucleotide incorporation. Supporting this hypothesis, previous structural and biochemical studies suggest that telomerase undergoes conformational changes at distinct steps of the catalytic cycle (Mitchell *et al*, 2010; Tomlinson *et al*, 2015; Parks *et al*, 2017). However, determining the exact mechanism for pause signal-mediated arrest of DNA synthesis would require high-resolution structures of the telomerase enzyme complexed with various DNA/RNA hybrids and even the incoming nucleotide bound within the active site.

Beyond compensating for the high K_M of the dG¹ incorporation, high dGTP concentrations have no exotic stimulatory effects on human telomerase. Our results demonstrate that altering the dG¹ incorporation to dA¹ effectively shifted the stimulation of repeat addition processivity and rate from dGTP- to dATP-dependent for human telomerase (Figs 4 and 5). However, the ciliate *Tetrahymena thermophila* telomerase has been reported to have repeat addition processivity stimulated by high concentrations of dGTP, dGDP or even dGMP (Hardy *et al*, 2001). In contrast, our assays with human telomerase failed to show dGMP-dependent stimulation of repeat addition activity (Fig 6A). Thus, the effects of elevated dGTP concentrations on ciliate telomerases appear esoteric with an underlying mechanism distinct from human telomerase. It was interesting to observe that, in addition to dGTP, elevated dGDP also stimulates telomerase repeat addition (Fig 6A, lane 3). Rather than an allosteric effect from a secondary guanosine binding site, dGDP was directly utilized as substrate and effectively increased overall nucleotide concentrations (Fig 6A, lanes 8–9). Furthermore, telomerase assayed with solely deoxynucleoside diphosphates generated a significant amount of DNA products (Fig EV4D, lane 2), eliminating the possibility of phosphate transfer from deoxynucleoside triphosphates to diphosphates. Nonetheless, deoxynucleoside diphosphates are a poorer substrate to telomerase with fivefold to 10-fold lower concentrations in cells than deoxynucleoside triphosphates (Bradshaw & Samuels, 2005). Thus, we do not expect telomerase to utilize deoxynucleoside diphosphates as substrate under cellular conditions. In addition to telomerase, all other RTs and most DNA-dependent DNA polymerases examined in this study are capable to incorporate dGDP into DNA products (Figs 6B and C, and EV5). This, however, is not entirely unexpected as deoxynucleoside triphosphates and diphosphates both permit the same DNA polymerase catalysis with the 3' hydroxyl of the DNA primer attacking the α -phosphate of the incoming deoxynucleotide and the release of inorganic pyrophosphate or monophosphate, respectively (Kornberg, 1969). Interestingly, the Klenow fragment of *Escherichia coli* DNA polymerase that showed dGDP utilization activity in our assay (Fig 6C) has been previously reported inert with deoxynucleoside diphosphates (Kornberg, 1957; Garforth *et al*, 2008). This discrepancy is likely due to the lower nucleotide concentration used in previous studies.

Under estimated intracellular nucleotide concentrations (Bradshaw & Samuels, 2005), telomerase would be efficient for the synthesis of a single repeat, yet limited for the processive synthesis of multiple repeats through multiple catalytic cycles. Conventional telomerase assays have been performed under exceptionally high

concentrations of dATP and dTTP in the range of 100–1,000 μ M, with the radiolabeled dGTP in the very low μ M range (Greider, 1991; Huard *et al*, 2003; Latrick & Cech, 2010; Wu *et al*, 2017a). These skewed assay conditions lead to significant DNA synthesis arrest at the end of the catalytic cycle and generated the characteristic six-nucleotide ladder banding pattern, a hallmark of telomerase activity. Reducing individual nucleotide concentrations below 5 μ M results in intermediate DNA repeat product accumulation due to the stalling of nucleotide addition before reaching the end of the template (Appendix Fig S2). The incorporation of dG¹ is presumably slow under the low nucleotide concentrations *in vivo*, and telomerase would predominately release DNA products terminating with dG⁶ prior to the dG¹ incorporation. The analysis of chromosome terminal sequences found a sharp increase of the specific dG⁶ terminal sequence from about 25% in telomerase-null cells to about 40% in telomerase-positive cells (Sfeir *et al*, 2005), which is consistent with our hypothesis that telomerase generates DNA products with this terminal sequence.

In contrast to the slow dG¹ addition, the remaining five nucleotide incorporations appear to be efficient without accumulation of any significant intermediate products (Fig 3A, lane 4). However, in our telomerase product release assay, an intermediate DNA product with only three residues, dG¹, dG², and dT³, added was observed in the enzyme-bound fraction (Fig 3A, lane 1), and not in the released product fraction (Fig 3A, lane 4). This intermediate DNA product likely resulted from a slightly slower incorporation of dT⁴. In support of the slower rate of the dT⁴ incorporation, increasing dTTP concentration to above 10 μ M indeed increases repeat addition rate and generates products with a greater number of repeats added (Fig 2C, lanes 2 and 3), which is consistent with a previous report (Drosopoulos & Prasad, 2010). Together, these results support that the incorporation efficiencies of the remaining five residues, dG², dT³, dT⁴, dA⁵, and dG⁶, minimally contribute to the overall repeat addition rate. The dG¹ incorporation efficiency is the key determinant for both the processivity and the rate of telomerase repeat addition.

Elucidating the mechanism by which telomerase undergoes processive synthesis of hundreds of telomeric DNA repeats has remained challenging. The unique telomerase catalytic cycle relies on a highly orchestrated arrangement of TERT, TR, and accessory proteins to facilitate DNA product retention and effective template regeneration for processive repeat addition. Our findings herein redefine the telomerase catalytic cycle as three critical and distinct steps: (i) rapid template translocation followed by (ii) slow dG¹ incorporation and (iii) efficient addition of the five remaining residues dG², dT³, dT⁴, dA⁵, and dG⁶ (Fig 9). DNA synthesis arrest at the slow dG¹ incorporation step promotes product release and limits processive repeat synthesis. The intrinsic low processivity of human telomerase is beneficial as it affords repeat addition regulation through DNA–protein interactions with TERT anchor sites and telomerase accessory proteins to control product release (Fig 9). Moreover, our findings suggest that telomerase products are released mainly from unsuccessful dG¹ incorporation, instead of failed template translocation. Thus, the slow dG¹ incorporation step and the inhibitory effect of the pause signal limit the processivity and rate of telomerase repeat addition, representing prime targets for therapeutic regulation of telomerase function.

Materials and Methods

In vitro reconstitution of TF human telomerase

Human TERT protein was expressed in rabbit reticulocyte lysate (RRL) from the pNFLAG-hTERT plasmid DNA using the TnT T7 Quick Coupled transcription/translation kit (Promega) following manufacturer's instructions (Xie *et al*, 2010). The hTR pseudoknot (residues 64–184) and CR4/5 (residues 239–328) fragments were *in vitro*-transcribed, gel-purified, and assembled together with the TERT protein in RRL for 30 min at 30°C at a final concentration of 1.0 μM (Qi *et al*, 2012; Brown *et al*, 2014).

In vivo reconstitution of wild-type human telomerase

HEK 293FT cells were grown in DMEM medium (Corning) supplemented with 10% FBS (Atlanta Biological), 1× Penicillin–Streptomycin–Amphotericin B mix (Lonza) and 5% CO₂ at 37°C to 80–90% confluency. Cells in a 6-well plate were transfected with 0.4 μg of pcDNA-NFLAG-hTERT, 1.6 μg of pBS-U1-hTR wild type or template mutants, and 6 μl of FuGENE HD transfection reagent (Promega) following manufacturer's instruction. Cells were harvested 48 h post-transfection, homogenized in HEPES lysis buffer (20 mM HEPES-KOH, pH 7.9, 400 mM NaCl, 0.2 mM EGTA, 2 mM MgCl₂, 10% glycerol, 5 mM β-mercaptoethanol, and 1× complete protease inhibitor cocktail (Roche), 1 mM PMSF), incubated on ice for 30 min and the lysate clarified by centrifugation. Two hundred microliters of cell lysate was combined with 30 μl Anti-FLAG® M2 Beads (Sigma, Cat#A2220) pre-washed with 1× TBS buffer (50 mM Tris–HCl, pH 7.4 and 150 mM NaCl) and incubated at 4°C with gentle rotation for 1 h. The beads were washed three times with 100 μl of 1× TBS buffer and once with 50 μl 1× telomerase reaction buffer (50 mM Tris–HCl, pH 7.5, 3 mM MgCl₂, 50 mM KCl, 2 mM DTT, and 1 mM spermidine), followed by activity assay.

K_M measurement using TF telomerase

One microliter of RRL reconstituted TF telomerase enzyme was assayed in a 10 μl reaction containing 1× telomerase reaction buffer, 40 μM pre-annealed DNA/RNA duplex, specified dNTPs, and 0.165 μM of the denoted α-³²P-dNTP. For measuring the *K_M* values, the activity assays were performed with nucleotide concentrations varying from 0 to 200 μM, or up to 1 mM for high *K_M* measurement. Reactions were incubated at 30°C for 60 min and terminated by phenol/chloroform extraction, followed by ethanol precipitation. The DNA products were resolved on a 15% (wt/vol) polyacrylamide/8M urea denaturing gel, dried, exposed to a phosphorstorage screen, and imaged on a Bio-Rad FX-Pro phosphorimager. The intensities of specific products were normalized to the total product intensity and plotted against the nucleotide concentrations with the Michaelis–Menten equation, $Y = V_{\max} * X / (K_M + X)$, used to fit the nonlinear curve to determine the *K_M* (Prism 5, GraphPad Software).

Activity assay for dNDP and dNMP incorporation

One microliter of RRL reconstituted telomerase enzyme, 1 unit of AMV RT (Promega), 0.5 units of Taq DNA pol III (NEB), 1 unit of T4 DNA pol (Fermentas), or 0.5 units of Klenow fragment of DNA

pol I (Invitrogen) were assayed in 10 μl reactions containing 1× telomerase reaction buffer, 40 μM of denoted pre-annealed DNA/RNA or DNA/DNA hybrid substrates, 100 μM dGTP, dGDP, or dGMP, and 0.165 μM α-³²P-dATP. The assay with TGIRT III group II intron RT (InGex) contained 50 units of enzyme, 1× reaction buffer (20 mM Tris–HCl, pH 7.5, 5 mM MgCl₂, 0.45 M NaCl, 5 mM DTT) and 10 μM dGTP, dGDP, or dGMP, and 0.165 μM α-³²P-dATP. Reactions were incubated at 30°C for 60 min and terminated by phenol/chloroform extraction, followed by ethanol precipitation. The size marker was prepared in a 10 μl reaction containing 1× reaction buffer (100 mM sodium cacodylate, pH 6.8, 1 mM CoCl₂, and 0.1 mM DTT), 1 μM oligonucleotide as indicated, 10 units of terminal deoxynucleotidyl transferase (TdT, Affymetrix), and 0.165 μM α-³²P-dATP (3,000 Ci/mmol, 10 mCi/ml; PerkinElmer). The reaction was incubated at 30°C for 5 s and terminated by addition of 10 μl 2× formamide loading buffer [10 mM Tris–HCl, pH 8.0, 80% (vol/vol) formamide, 2 mM EDTA, 0.08% bromophenol blue, and 0.08% xylene cyanol]. The DNA products were resolved on a 15% (wt/vol) polyacrylamide/8 M urea denaturing gel, dried, exposed to a phosphorstorage screen and imaged on a Bio-Rad FX-Pro phosphor-imager.

Telomerase direct primer extension assay

Twenty microliters of immuno-purified *in vivo*-reconstituted telomerase enzyme on beads was assayed in a 10 μl reaction containing 1× telomerase reaction buffer, 1 μM DNA primer, specified dNTPs, and 0.165 μM of the denoted α-³²P-dNTP. Reactions were incubated at 30°C for 60 min and terminated by phenol/chloroform extraction, followed by ethanol precipitation. The DNA products were resolved on a 10% (wt/vol) polyacrylamide/8 M urea denaturing gel, dried, exposed to a phosphorstorage screen, and imaged on a Bio-Rad FX-Pro phosphorimager. Repeat addition efficiency was estimated as the ratio of the high M.W. DNA products (> 6 repeats added) over the low M.W. DNA products (1–6 repeats added). The cutoff at 6 repeats was arbitrarily chosen to divide the gel into approximately two even sections. The relative high/low ratios of reactions with varying nucleotide concentrations were determined through normalization to the ratio from the low nucleotide concentration reaction.

Telomerase product release assay for repeat addition processivity determination

Twenty microliters of immuno-purified *in vivo*-reconstituted telomerase enzyme on beads was assayed in a 10 μl reaction containing 1× telomerase reaction buffer, 1 μM DNA primer, a range of dNTPs, and 0.165 μM of the denoted α-³²P-dNTP. Reactions were incubated at 30°C for 60 min, and the DNA products in the supernatant were separated from the DNA products bound to the telomerase enzyme immobilized on the beads. Following ethanol precipitation, the DNA products were resolved on a 10% (wt/vol) polyacrylamide/8 M urea denaturing gel, dried, exposed to a phosphorstorage screen, and imaged on a Bio-Rad FX-Pro phosphorimager. Repeat addition processivity was calculated using the equation: $\text{Processivity} = \frac{-\ln 2}{2.303k}$ (Latrick & Cech, 2010). The slope, *k*, was determined by plotting the intensity of each major band, normalized to the intensity of the first band, over the repeat number.

Pulse-chase time course assay

Twenty microliters of immuno-purified *in vivo*-reconstituted telomerase enzyme on beads was initially pulsed with 0.165 μM α - ^{32}P -dTTP for 5 min and then chased with 100 μM dTTP and denoted concentrations of dATP and dGTP. Aliquots of the reactions were terminated by phenol/chloroform extraction at denoted time points, followed by ethanol precipitation. The DNA products were resolved on a 10 (wt/vol) polyacrylamide/8 M urea denaturing gel, dried, exposed to a phosphorstorage screen, and imaged on a Bio-Rad FX-Pro phosphorimager. To determine the rate of repeat addition, the longest DNA products with the highest intensity above initial pulse product bands were used to deduce a “modal band” to calculate the extension rate as previously described (Drosopoulos *et al*, 2005). The modal bands were determined from the intensity traces generated by the ImageJ (NIH) program, with the number of repeats comprising the modal band plotted against the time point of the chase reaction, and the slope of the best-fit trend line determined the rate of repeat addition for the reaction (Appendix Fig S4).

Expanded View for this article is available online.

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Author contributions

JJ-LC conceived the general ideas for this work and designed the experiments; YC performed the human telomerase analysis; DL performed MS analysis; YC, JDP, and DL and JJ-LC analyzed the data. JJ-LC and JDP wrote the manuscript with input from YC and DL.

Conflict of interest

The authors declare that they have no conflict of interest.

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