

# Effects of DNA<sub>3'</sub>pp<sub>5'</sub>G capping on 3' end repair reactions and of an embedded pyrophosphate-linked guanylate on ribonucleotide surveillance

Mathieu Chauleau<sup>†</sup>, Ushati Das<sup>†</sup> and Stewart Shuman<sup>\*</sup>

Molecular Biology Program, Sloan-Kettering Institute, New York, NY 10065, USA

Received December 5, 2014; Revised February 19, 2015; Accepted February 20, 2015

## ABSTRACT

When DNA breakage results in a 3'-PO<sub>4</sub> terminus, the end is considered 'dirty' because it cannot prime repair synthesis by DNA polymerases or sealing by classic DNA ligases. The noncanonical ligase RtcB can guanylate the DNA 3'-PO<sub>4</sub> to form a DNA<sub>3'</sub>pp<sub>5'</sub>G<sub>OH</sub> cap. Here we show that DNA capping precludes end joining by classic ATP-dependent and NAD<sup>+</sup>-dependent DNA ligases, prevents template-independent nucleotide addition by mammalian terminal transferase, blocks exonucleolytic proofreading by *Escherichia coli* DNA polymerase II and inhibits proofreading by *E. coli* DNA polymerase III, while permitting templated DNA synthesis from the cap guanosine 3'-OH primer by *E. coli* DNA polymerase II (B family) and *E. coli* DNA polymerase III (C family). Human DNA polymerase β (X family) extends the cap primer predominantly by a single templated addition step. Cap-primed synthesis by templated polymerases embeds a pyrophosphate-linked ribonucleotide in DNA. We find that the embedded ppG is refractory to surveillance and incision by RNase H2.

## INTRODUCTION

The synthesis and repair of DNA rely on enzymatic reactions at 3' ends, whereby the terminal 3'-OH primes nucleotide addition by DNA polymerases and 3'-OH/5'-PO<sub>4</sub> nick sealing by DNA ligases. Yet, many biological scenarios generate 'dirty' DNA 3'-PO<sub>4</sub> ends that cannot prime repair synthesis or ligation. The textbook view had been that evasion of the dirty end problem requires removal of the 3'-PO<sub>4</sub> (referred to as 'end-healing') and/or resection of 3' nucleotides. We recently described a new pathway of DNA 3'-PO<sub>4</sub> end-processing by the non-canonical RNA ligase *Escherichia coli* RtcB (1,2) in which RtcB reacts with GTP to form a covalent RtcB-(histidyl)-GMP intermedi-

ate and then transfers GMP to a DNA 3'-PO<sub>4</sub> to form a DNA<sub>3'</sub>pp<sub>5'</sub>G 'cap' (3) (Supplementary Figure S1). The implications of DNA 3' capping for DNA repair are potentially profound, given the wide phylogenetic distribution of RtcB enzymes (4–7) and the prevalence of 3'-PO<sub>4</sub> ends *in vivo*, generated either directly by DNA damage or as repair intermediates. To that end, we are addressing the impact of DNA capping on repair reactions at DNA 3' ends.

In an initial study (8), we found that capping protects DNA 3' ends from resection by *E. coli* exonucleases I and III and from end-healing by T4 polynucleotide 3' phosphatase. To our surprise, we found that the cap is an effective primer for DNA synthesis by exemplary members of two different families of repair polymerases. *E. coli* DNA polymerase I (A family) and *Mycobacterium smegmatis* DinB1 (Y family) extend the DNAppG primer to form an alkali-labile DNApp(rG)pDNA product. For these two polymerases, dNTP addition depended on pairing of the cap guanine with an opposing cytosine in the template strand (8). We concluded that the biochemical impact of DNA capping is to prevent resection and healing of a 3'-PO<sub>4</sub> end, while permitting DNA synthesis, at the price of embedding a ribonucleotide and a pyrophosphate linkage in the repaired strand.

How general a property is it of DNA polymerases that they can utilize a capped DNA strand as a primer? To address this issue here, we expanded our analysis to an exemplary B family repair polymerase (*E. coli* DNA polymerase II; Pol II) (9), a C family replicative polymerase (*E. coli* DNA polymerase III; Pol III) (10), a template-dependent family X polymerase (human DNA polymerase beta; Pol β) (11) and a template-independent family X polymerase (calf thymus terminal transferase) (12). We find that Pol II, Pol III and Pol β are adept at templated nucleotide additions to the cap guanylate primer terminus. By contrast, terminal transferase cannot extend a cap primer because it is sensitive to the cap pyrophosphate linkage.

Classic DNA ligases seal nicks with 3'-OH and 5'-PO<sub>4</sub> ends via three nucleotidyl transfer steps (13). In the first step, nucleophilic attack by ligase on the α phosphorus of ATP or NAD<sup>+</sup> results in release of PP<sub>i</sub> or NMN and for-

<sup>\*</sup>To whom correspondence should be addressed. Tel: +1 212 639 7145; Email: s-shuman@ski.mskcc.org

<sup>†</sup>These authors contributed equally to the paper as first authors.

mation of a covalent ligase–adenylate intermediate in which AMP is linked via a P–N bond to N $\zeta$  of a lysine. In the second step, the AMP is transferred to the 5' end of the 5'-PO $_4$  DNA strand to form DNA-adenylate, A $_5$ pp $_5$  DNA. In the third step, ligase catalyzes attack by the 3'-OH of the nick on DNA-adenylate to join the polynucleotides and release AMP. Can DNA ligases seal a nick with a 3' capped DNAppG $_{OH}$  terminus and thereby embed the pyrophosphate linked guanylate? We find here that exemplary ATP-dependent and NAD $^+$ -dependent DNA ligases are unable to seal a DNAppG $_{OH}$  strand.

A key question is whether and how DNA caps might be removed. We reported initially that aprataxin, an enzyme implicated in repair of A $_5$ pp $_5$  DNA ends formed during abortive ligation by classic ligases (14,15), has a vigorous DNA 3' de-capping activity, converting DNAppG to DNA $_3$ p and GMP (8). Here we examine the ability of the proofreading exonucleases associated with DNA polymerases to excise the DNA 3' cap. We find that the cap structure precludes proofreading by *E. coli* DNA polymerase II and strongly inhibits proofreading by *E. coli* DNA polymerase III.

Finally, the ease with which templated DNA polymerases embed pyrophosphate-linked guanylates raises the issue of whether and how these putative 'lesions' might be surveilled and removed after they are embedded in duplex DNA. Recent studies have highlighted a pathway of ribonucleotide excision repair (RER) that locates and removes single ribonucleotides embedded when DNA polymerases utilize an rNTP substrate instead of a cognate dNTP for templated synthesis (16). RER is initiated by ribonuclease H2 (RNase H2), an enzyme that incises a single (dN)p(rN) phosphodiester linkage in duplex DNA to generate a nick with—(dN) $_{OH}$  and p(rN)p(dN)-termini (17). Subsequent RER steps include resection of the ribonucleotide 5' end and repair DNA synthesis from the 3'-OH DNA end (16). Here we show that a DNA-embedded ribonucleotide 5' pyrophosphate (dN)pp(rG) is refractory to incision by RNase H2.

## MATERIALS AND METHODS

### Enzymes

*Escherichia coli* RtcB, *Chlorella virus* DNA ligase and *E. coli* DNA ligase (LigA) were produced in *E. coli* and purified as described (2,20,21). *E. coli* DNA polymerase II and DNA polymerase III core were a generous gift of Dr Kenneth Mariani. *E. coli* RNase H2 and calf thymus terminal transferase were purchased from New England Biolabs (NEB). Human DNA polymerase  $\beta$  was purchased from Novoprotein.

### Substrates

The 5'  $^{32}$ P-labeled pDNAp and pDNA $_{OH}$  strands were prepared by enzymatic phosphorylation of 20-mer  $_{HO}$ CTAGAGCTACAATTGCGACC oligonucleotides, with either 3'-PO $_4$  or 3'-OH termini, by using [ $\gamma$  $^{32}$ P]ATP and the phosphatase-dead mutant T4 Pnkp-D167N. The radiolabeled pDNAp and pDNA $_{OH}$  strands were gel-purified. The 5'  $^{32}$ P-labeled pDNApp(rG) $_{OH}$  and

pDNApp(dG) $_{OH}$  strands were prepared by incubating pDNAp with a 20-fold molar excess of RtcB in the presence of 2 mM MnCl $_2$  and 1 mM GTP or dGTP for 20 min at 37°C, followed by gel-purification. Primer-templates for DNA polymerase assays were prepared by annealing the radiolabeled pDNA $_{OH}$ , pDNAp, and pDNAppG primers to a four-fold molar excess of the unlabeled template DNA strands specified in the figures. The duplex substrates for RNase H2 assays were prepared by reaction of Klenow DNA polymerase and dNTPs with the 5'  $^{32}$ P-labeled primer-templates with p(dG) $_{OH}$ , r(rG) $_{OH}$ , or pp(rG) $_{OH}$  primer termini, after which the resulting filled-in blunt duplexes were recovered by phenol–chloroform extraction and ethanol precipitation.

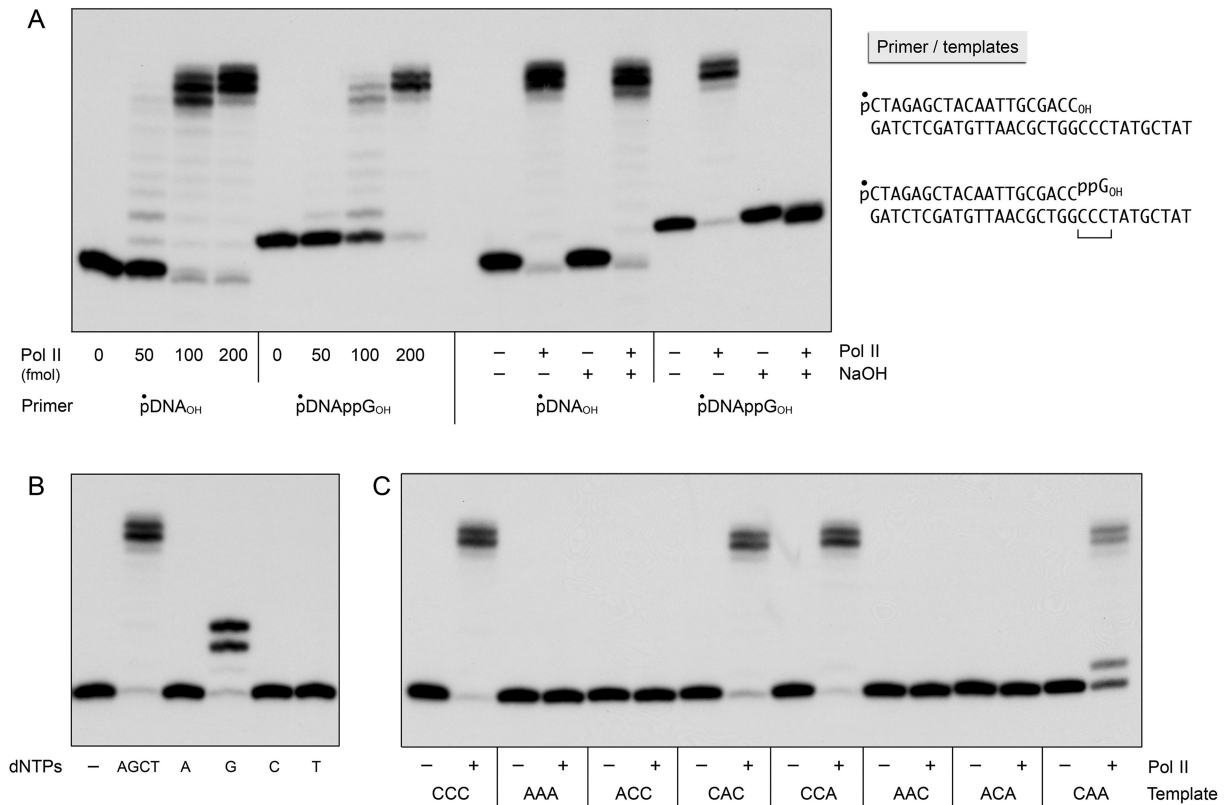
## RESULTS AND DISCUSSION

### DNA $_3$ pp $_5$ G ends are extended by DNA polymerase II

*Escherichia coli* DNA polymerase II (Pol II) is a B family enzyme with polymerase and proofreading activities (9). We compared the ability of Pol II to extend primer-templates formed by annealing 5'  $^{32}$ P-labeled 20-mer pDNA $_{OH}$  or pDNAppG strands to a complementary 31-mer DNA strand to form a 20-bp duplex with an 11-nt single-strand 5' tail (Figure 1A). The sequence of the tail immediately flanking the duplex junction consisted of three consecutive deoxycytidines that could potentially pair with the 3' cap guanylate of the pDNAppG strand. Reaction of the pDNA $_{OH}$  primer-template (200 fmol) with 50, 100 and 200 fmol Pol II in the presence of all four dNTPs resulted in the extension of 19%, 91%, and 93% of the input primer strand (Figure 1A, left panel). At 200 fmol Pol II, the predominant outcomes were the addition of 10 nt (one step short of the end of the template strand) or 11 nt (complete fill-in synthesis to the end of the template strand). Pol II (200 fmol) extended 88% of the input pDNAppG primer-template to yield the same distribution of fill-in products (Figure 1A, left panel). At 100 fmol Pol II, 52% of the input pDNAppG strand was extended.

The kinetic profiles for templated extension of pDNA $_{OH}$  and pDNAppG primer strands (20 nM) by 20 nM Pol II are shown in Supplementary Figure S2A, in which the percent of primer strand elongated by one or more steps of dNMP addition is plotted as a function of time. Both primers were elongated quickly: 73% for pDNA $_{OH}$  and 60% for pDNAppG after 15 s, the earliest time sampled; and in high yield at the endpoint, which was 95% for pDNAp(dG) $_{OH}$  and 92% for pDNApp(rG) $_{OH}$ . There were no significant differences in the product size distributions at any time sampled (not shown). We surmise that the rate of the first Pol II dNMP addition to the DNAppG cap is within a factor of two of a standard DNA $_{OH}$  primer and that there are no durable kinetic obstacles to further dNMP additions. [Analogous kinetic profiles for pDNA $_{OH}$  and pDNAppG primer extension by *E. coli* Klenow Pol I are shown in Supplementary Figure S2B. Pol I elongated 83% of the pDNA $_{OH}$  primer and 33% of the pDNAppG primer after 5 s; the endpoint values were 94% and 89%, respectively.]

Direct proof that the cap guanosine 3'-OH was the primer nucleophile for the first step of dNMP addition by Pol II was obtained by treating the products of fill-in synthesis



**Figure 1.** DNAppG primes DNA synthesis by *Escherichia coli* polymerase II. (A) Cap priming of DNA synthesis embeds the cap ribonucleotide. Left panel: reaction mixtures (10  $\mu$ l) containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5' <sup>32</sup>P-labeled pDNA<sub>OH</sub> or pDNAppG primer-temple (depicted at right with the <sup>32</sup>P-label denoted by •) and 0, 50, 100 or 200 fmol Pol II as specified were incubated at 37°C for 30 min, then quenched with 10  $\mu$ l of 90% formamide, 50 mM EDTA. Right panel: reaction mixtures (10  $\mu$ l) containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5' <sup>32</sup>P-labeled pDNA<sub>OH</sub> or pDNAppG primer-temple and either no enzyme (-) or 200 fmol Pol II (+) were incubated at 37°C for 30 min. The reaction mixtures were adjusted to 50 mM EDTA and then supplemented with either 1.2  $\mu$ l 3 M NaOH (+) or 1.2  $\mu$ l 3 M NaCl (-) and incubated at 22°C overnight, after which the NaOH mixtures were neutralized by adding 1.2  $\mu$ l 3 M HCl. The reaction products were analyzed by urea-PAGE and visualized by autoradiography. (B) dNTP requirement for cap-primed DNA synthesis. Reaction mixtures (10  $\mu$ l) containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 0.2 pmol 5' <sup>32</sup>P-labeled pDNAppG<sub>OH</sub> primer-temple, either no dNTPs (-), 4 mM dNTPs (AGCT) or 1 mM individual dNTPs (A, G, C or T) and no enzyme (-) or 200 fmol Pol II (+) were incubated at 37°C for 30 min. (C) Template requirement for cap-primed DNA synthesis. Reaction mixtures (10  $\mu$ l) containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5' <sup>32</sup>P-labeled pDNAppG<sub>OH</sub> primer-temple substrates with different trinucleotide template sequences flanking the primer 3' end (as specified below the lanes) and either no enzyme (-) or 200 fmol Pol-II (+) were incubated at 37°C for 30 min. The reaction mixtures were supplemented with formamide, EDTA and the products were analyzed by urea-PAGE and visualized by autoradiography.

on the pDNA<sub>OH</sub> and pDNAppG primer-templates with NaOH (0.3 M for 16 h at 22°C), which will cleave any ribonucleoside embedded in a DNA polynucleotide via base-catalyzed attack of the ribose O2' on the (rN)pN phosphodiester. Whereas extension of the pDNA<sub>OH</sub> primer by Pol II yielded a filled-in strand that was resistant to alkali (i.e. an all-DNA strand), the filled-in product of pDNAppG extension was sensitive to alkali, being converted to a shorter pDNAppGp species (Figure 1A, right panel). We conclude that the cap guanylate, and thus the cap pyrophosphate linkage, is embedded in the product of DNA synthesis: pDNApp(rG)pDNA.

No extension of the pDNAppG primer-temple by Pol II was seen in the absence of exogenous dNTPs (Figure 1B). By performing the reactions in the presence of single dNTPs, we found that dGTP was the sole effective substrate for nucleotide addition by Pol II (Figure 1B), indicating that the first steps of synthesis were templated by the cytosines in the single-strand tail. The predominant prod-

ucts seen when Pol II acted on the pDNAppG primer in the presence of only dGTP comprised a mixture of primers extended by two or three nucleotide steps (Figure 1B), which suggests that the  $n + 2$  product pDNApp(rG)GG can slip back on the template CCC sequence to allow incorporation of a third dGMP nucleotide when the 'correct' dNTP is unavailable for addition at the next template position.

The role of the template sequence in cap primer utilization by Pol II was addressed in the experiment shown in Figure 1C, in which the identities of the three template strand nucleobases immediately flanking the primer terminus were varied as cytosine or adenine and the primer-templates were reacted with polymerase plus all four dNTPs. Changing CCC to AAA abolished dNMP addition to the pDNAppG strand, signifying that the cap guanine must pair with a template cytosine in order to prime synthesis. By surveying all C/A combinations within the trinucleotide, we found that a template cytosine immediately opposite the cap guanosine was necessary and sufficient for effective priming of fill-in

synthesis (CAC, CCA and CAA template trinucleotides did prime), whereas any template containing adenine opposite the cap guanosine was inactive (i.e. ACC, AAC and ACA did not prime) (Figure 1C). We surmise that Pol II does not allow the longer pyrophosphate linkage of the cap to reach across the non-complementary template A so that the cap guanosine primer can pair productively with the second cytosine within the ACC template trinucleotide.

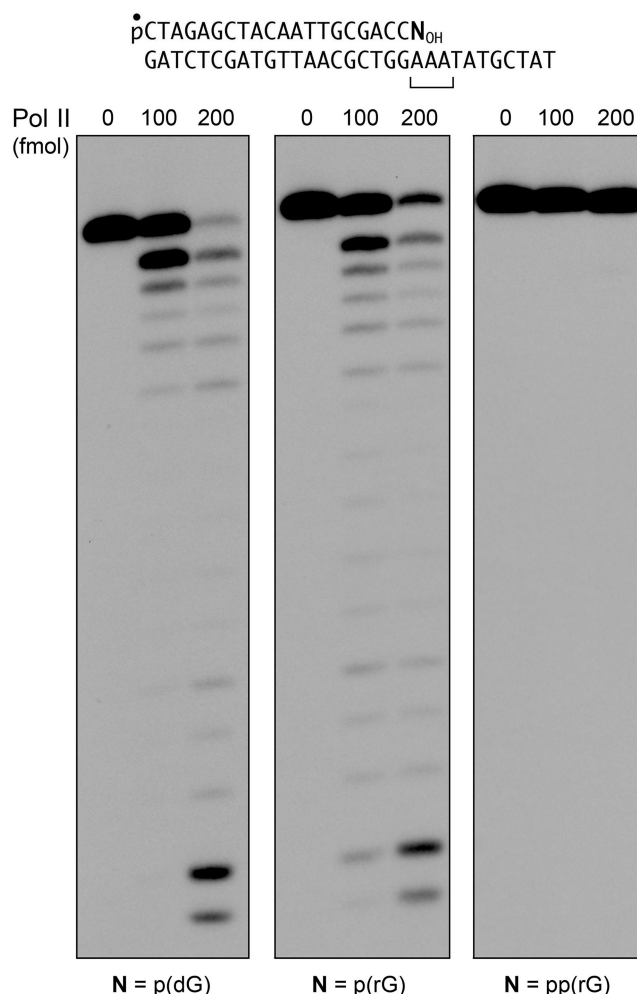
### DNA<sub>3</sub>pp<sub>5</sub>G ends are not proofread by DNA polymerase II

DNA 3' capping by RtcB at a 3'-PO<sub>4</sub> end in gapped duplex DNA or nicked duplex DNA will most often result in addition of a pyrophosphate-linked guanosine opposite a dA, dG or dT nucleotide to which it does not form a canonical base pair. In that event, the mismatched cap might be a substrate for proofreading by a DNA repair polymerase. To test this idea in the case of *E. coli* Pol II, we prepared a series of 5' <sup>32</sup>P-labeled primer templates in which the primer strand 3' terminal guanosine nucleotide—either p(dG)<sub>OH</sub>, p(rG)<sub>OH</sub> or pp(rG)<sub>OH</sub>—was opposite a mismatched dA nucleotide in the template strand (Figure 2). Reaction of the mispaired p(dG)<sub>OH</sub> primer-template with Pol II and Mg<sup>2+</sup> (in the absence of dNTPs) triggered resection of the mismatched end by the 3'-to-5' proofreading exonuclease inherent to Pol II. Forty percent of the input primer ends were shortened by exposure to 100 fmol Pol II, most by a single nucleotide step; 200 fmol Pol II resected 86% of the input primer by as many as 14–15 nt (Figure 2, left panel). Pol II displayed similar proofreading activity on a mispaired p(rG)<sub>OH</sub> primer-template, 39% and 71% of the ribo-terminated primer strand was shortened during a reaction with 100 and 200 fmol Pol II (Figure 2, middle panel). The salient finding was that the Pol II was inert with the mismatched pp(rG)<sub>OH</sub> primer-template (Figure 2, right panel), signifying that the cap pyrophosphate linkage is not hydrolyzed by the Pol II proofreading exonuclease.

### DNA<sub>3</sub>pp<sub>5</sub>G ends are extended, but inefficiently proofread, by DNA polymerase III core

*Escherichia coli* DNA polymerase III (Pol III) is the exemplary bacterial replicative polymerase. The core module of Pol III comprises a heterotrimer of three subunits: a 130 kDa polymerase encoded by *dnaE*; a 27 kDa proofreading exonuclease encoded by *dnaQ*, and a 9 kDa non-catalytic subunit encoded by *holE* (10,18,19). The DnaE protein is a C family polymerase (10). Here we tested the efficacy of 3'-capped DNA as a primer for *E. coli* Pol III core. A control reaction of the pDNA<sub>OH</sub> primer-template with 0.2, 2 and 4 pmol of Pol III core in the presence of all four dNTPs resulted in the extension of 88%, 96%, and 97% of the input primer strand (Figure 3A). The 3' cap was an effective primer, with 0.2, 2 and 4 pmol of Pol III core catalyzing the extension of 72%, 87%, and 89% of the input pDNA<sub>pp</sub>G strand (Figure 3A).

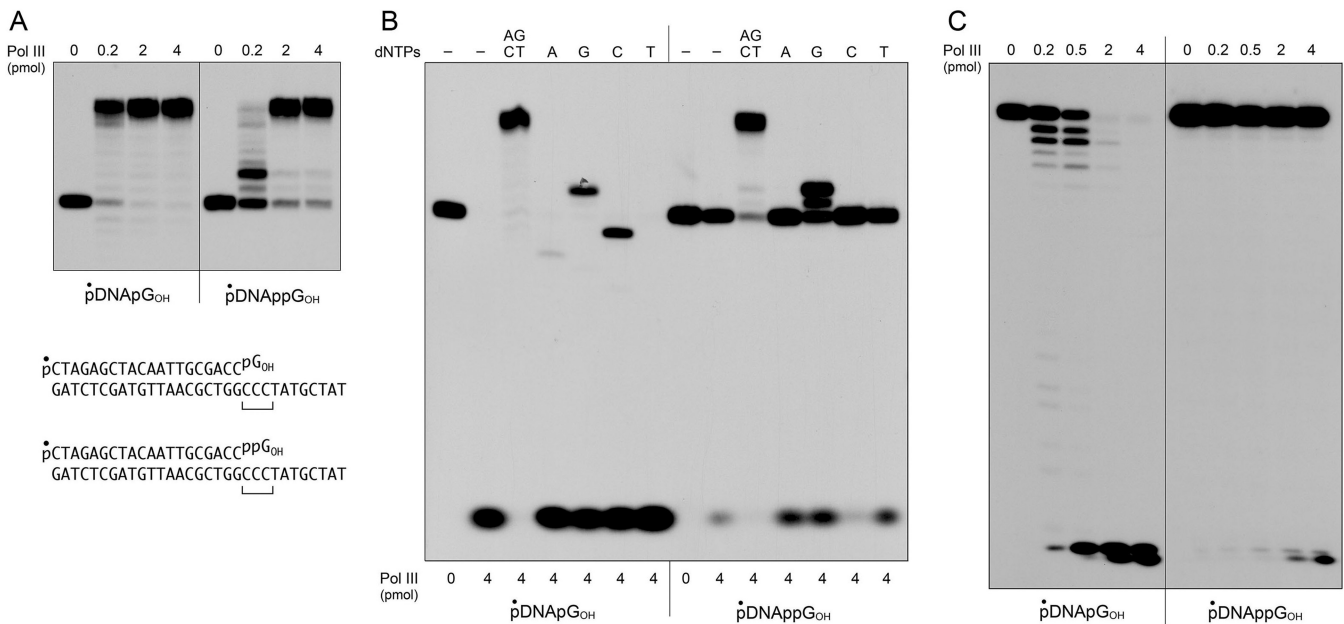
The proofreading function of the Pol III core complex was tested by reacting it with the primer-template DNA in the presence of magnesium in the absence of dNTPs or in the presence of a single dNTP. Whereas Pol III core activity was exclusively fill-in synthesis in the presence of all four



**Figure 2.** The cap pyrophosphate prevents proofreading by *Escherichia coli* polymerase II. Reaction mixtures (10  $\mu$ l) containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 0.2 pmol 5' <sup>32</sup>P-labeled pDNA<sub>pp</sub>(dG)<sub>OH</sub>, pDNA<sub>pp</sub>(rG)<sub>OH</sub> or pDNA<sub>pp</sub>(rG)<sub>OH</sub> primer-template with 'AAA' trinucleotide template sequence flanking the primer 3' end and 0, 100 or 200 fmol Pol II were incubated at 37°C for 30 min, then quenched with formamide, EDTA. The products were analyzed by urea-PAGE and visualized by autoradiography.

dNTPs, omission of dNTPs resulted in complete resection of the pDNA<sub>pp</sub>G<sub>OH</sub> primer strand (Figure 3B). Resection was also the predominant outcome when only one dNTP was provided. For example, 31% of the primers were extended by two templated dGMP additions in the presence of dGTP while 65% of the labeled primers were resected (Figure 3B). The key finding was that the pDNA<sub>pp</sub>G<sub>OH</sub> primer was a comparatively feeble substrate for proofreading by Pol III core, whether in the absence of dNTPs (when 15% of the primers were resected) or in the presence of single dNTPs (when 6–20% of the primers were resected).

Titration of Pol III core in the absence of dNTPs indicated that 60% of the pDNA<sub>pp</sub>G<sub>OH</sub> primer strand was resected at 0.2 pmol of enzyme, predominantly via trimming by one or two proofreading steps (Figure 3C). Increasing levels of Pol III progressively consumed the primer strand and resulted in complete resection to form a radiolabeled



**Figure 3.** Actions of *Escherichia coli* DNA polymerase III at a DNAppG end. (A) Cap priming of DNA synthesis. Reaction mixtures (10  $\mu$ l) containing 20 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5' <sup>32</sup>P-labeled pDNApG<sub>OH</sub> or pDNAppG<sub>OH</sub> primer-template (depicted at bottom with the <sup>32</sup>P-label denoted by ●) and 0, 0.2, 2 or 4 pmol *E. coli* Pol III core complex as specified were incubated at 37°C for 20 min. (B) Effect of dNTP omission on polymerase-exonuclease dynamics. Reaction mixtures (10  $\mu$ l) containing 20 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 0.2 pmol 5' <sup>32</sup>P-labeled pDNApG<sub>OH</sub> or pDNAppG<sub>OH</sub> primer-template, either no dNTPs (-), 4 mM dNTPs (AGCT) or 1 mM individual dNTPs (A, G, C or T) and no enzyme (-) or 4 pmol Pol III (+) were incubated at 37°C for 20 min. (C) Exonuclease titration. Reaction mixtures (10  $\mu$ l) containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 0.2 pmol 5' <sup>32</sup>P-labeled pDNApG<sub>OH</sub> or pDNAppG<sub>OH</sub> primer-template substrate and 0, 0.2, 0.5, 2 or 4 pmol Pol III were incubated at 37°C for 20 min. The reactions were quenched with 10  $\mu$ l of 90% formamide, 50 mM EDTA. The products were analyzed by urea-PAGE and visualized by autoradiography.

end-product that migrated as a dinucleotide (Figure 3C). The ppG<sub>OH</sub> cap protected the primer strand, i.e. 0.2, 0.5, 2, and 4 pmol of Pol III core resected 1.6%, 4.3%, 8.8%, and 15.8% of the input primer to completion, with no apparent accumulation of species trimmed by one or two nucleotides (Figure 3C). From these data, we estimate that the specific activity of the Pol III core exonuclease on the pDNAppG<sub>OH</sub> primer was at least 75-fold less than its activity on the pDNApG<sub>OH</sub> primer.

### DNA<sub>3</sub>pp<sub>5</sub>G ends are extended by human DNA polymerase $\beta$

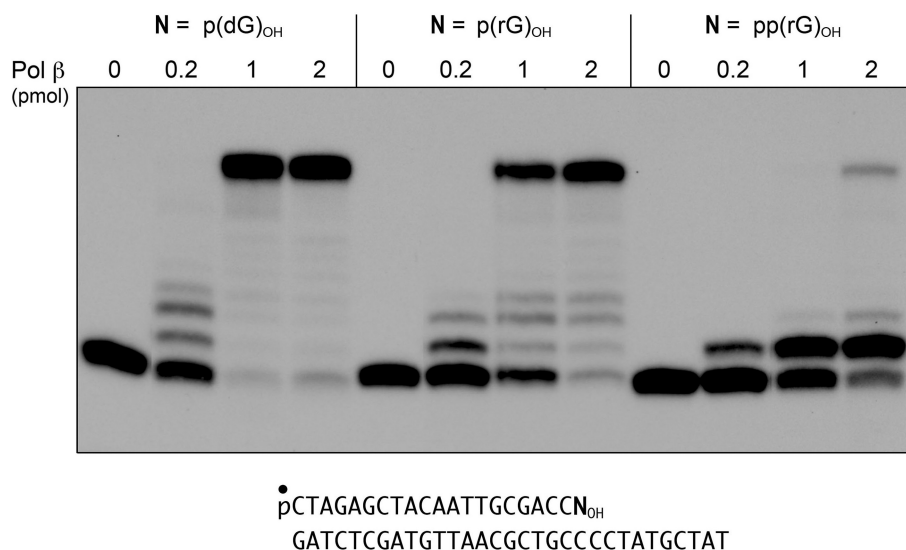
Pol  $\beta$ , the prototypal family X polymerase (11), is a central agent of the base excision repair pathway. Pol  $\beta$  catalyzes templated primer extension and gap filling reactions; it lacks a 3' proofreading activity. Reaction of 0.2, 1 and 2 pmol human Pol  $\beta$  with 0.2 pmol 5' <sup>32</sup>P-labeled pDNAp(dG)<sub>OH</sub> primer-template and dNTPs resulted in the extension of 55%, 90%, and 87% of the input primer strand, with 1 and 2 pmol Pol  $\beta$  effecting complete fill-in synthesis, entailing 10 dNMP addition steps (Figure 4). Similarly, 43%, 71%, and 88% of the ribo-terminated pDNAp(rG)<sub>OH</sub> primer-template was extended by 0.2, 1, and 2 pmol Pol  $\beta$ , again with fill-in synthesis being the majority outcome at 2 pmol Pol  $\beta$ . We found that 29%, 56%, and 74% of the 3'-capped pDNApp(rG)<sub>OH</sub> primer was extended by 0.2, 1 and 2 pmol Pol  $\beta$ , signifying that the cap pyrophosphate *per se* had little effect on the extent of primer utilization compared to the pDNAp(rG)<sub>OH</sub> control. The salient finding here was

that the predominant outcome of the Pol  $\beta$  reaction with the capped primer was a single step of nucleotide addition, with comparatively scant progression to fill-in synthesis (Figure 4). Thus Pol  $\beta$  was adept at adding a dNMP to a DNApp(rG)<sub>OH</sub> primer to form a pDNApp(rG)p(dN) product, but less proficient at extending this species further. We infer that Pol  $\beta$  is inhibited by a pyrophosphate linkage at the *penultimate* backbone position of the primer strand.

This inference was underscored by tracking the time course of the Pol  $\beta$  reactions with pDNAp(dG)<sub>OH</sub> and pDNApp(rG)<sub>OH</sub> primer-templates (Figure 5). Ninety percent of the control pDNAp(dG)<sub>OH</sub> primer was elongated by one or more steps within 15 s; a complete fill-in synthesis product was evident by 30 s and accumulated progressively at 1 and 2 min. The ppG cap exerted several effects on Pol  $\beta$ : (i) the first dNMP addition step was slowed (by at least a factor of five) compared to the control primer; (ii) the  $n + 1$  species accumulated as the predominant product at early times and persisted for up to 20 min; (iii) the fill-in synthesis product appeared at 5 min and increased at 10 and 20 min, concomitant with a decline in the  $n + 1$  species.

### DNA polymerases maintain high fidelity for pairing of the DNA<sub>3</sub>pp<sub>5</sub>G cap guanine with a template cytosine nucleobase

We surveyed DNA polymerases I, II, III and  $\beta$  for their ability to extend the pDNApp(rG)<sub>OH</sub> primer when annealed to a series of template strands that place the cap guanine opposite a C, T, A or G template nucleobase. The consistent finding was that each polymerase extended the cap primer



**Figure 4.** DNAppG primer extension by mammalian polymerase  $\beta$ . Reaction mixtures (10  $\mu$ l) containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5' <sup>32</sup>P-labeled pDNApp(dG)<sub>OH</sub>, pDNApp(rG)<sub>OH</sub> or pDNApp(rG)<sub>OH</sub> primer-templates and 0, 0.2, 1 or 2 pmol of DNA polymerase  $\beta$  as indicated were incubated at 37°C for 20 min, then quenched with formamide, EDTA. The products were analyzed by urea-PAGE and visualized by autoradiography.

when it was across from a C, but catalyzed no detectable nucleotide addition when the cap guanine was opposite T, A or G (Figure 6). Thus, whereas these four DNA polymerases tolerate a 5' pyrophosphate linkage at the primer guanosine terminus, they require a canonical G:C base pair for productive synthesis.

#### DNA<sub>3</sub>pp<sub>5</sub>G ends are not extended by calf thymus terminal transferase

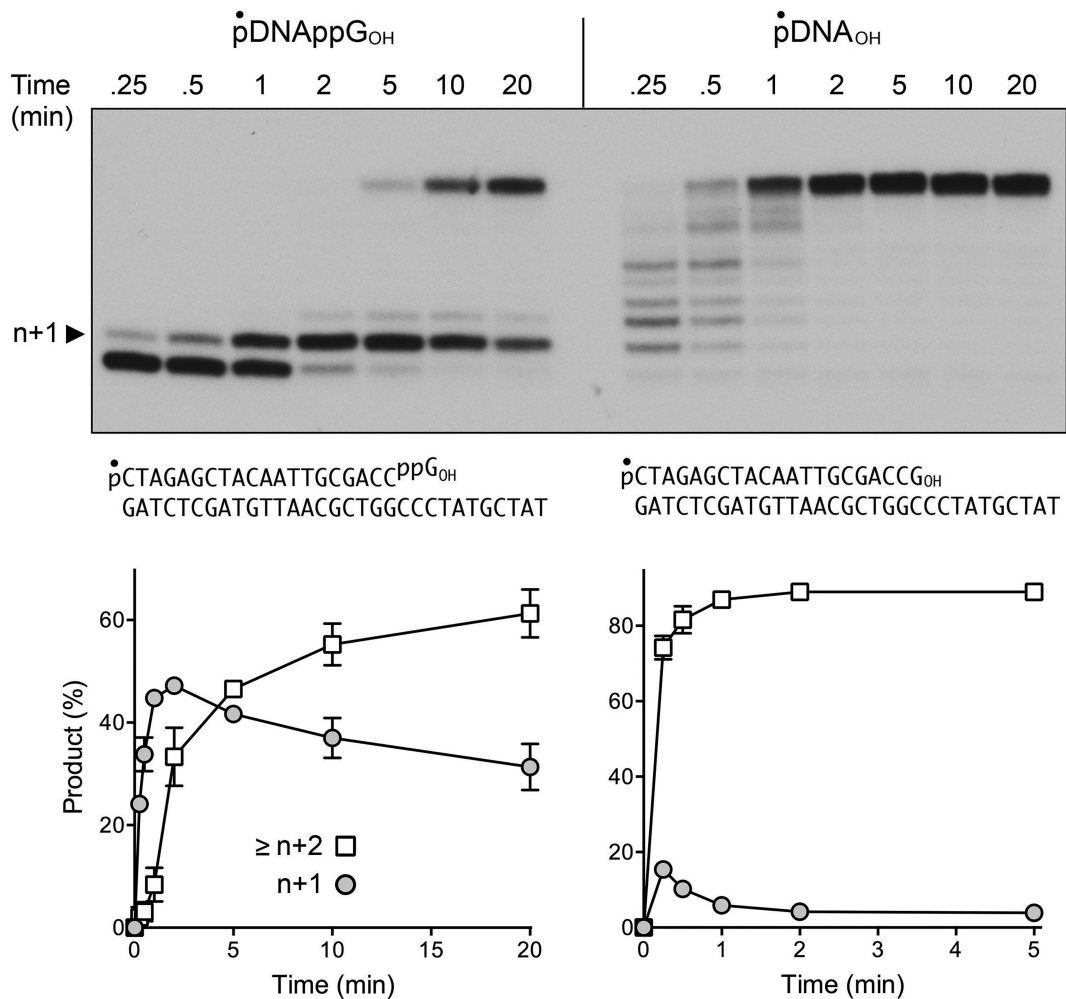
Mammalian terminal transferase (TdT) is an X family DNA polymerase that catalyzes non-templated nucleotide additions to 3'-OH ends of single-stranded DNA primers. The biological function of TdT is to add random segments of junctional DNA during V(D)J recombination of immunoglobulin genes. Calf thymus TdT is well-studied, biochemically and structurally (12), and is a versatile reagent for tagging and labeling polynucleotide 3' ends. Here we tested the effects of 3' capping on single-strand primer extension by calf thymus TdT. A series of 5' <sup>32</sup>P-labeled 21-mer primer strands was prepared in which the 3' terminal guanosine nucleotide was p(dG)<sub>OH</sub>, pp(dG)<sub>OH</sub>, p(rG)<sub>OH</sub> or pp(rG)<sub>OH</sub> (Figure 7). We reacted these primers with 1, 2, 5 or 10 U of TdT in the presence of cobalt (its preferred metal cofactor) and the chain-terminating substrate ddTTP, thereby limiting polymerization to a single step of ddTMP incorporation. Whereas 94% and 98% of the p(dG)<sub>OH</sub> primer strand was extended during a reaction with 1 and 2 U TdT (Figure 7), only 1% of the pp(dG)<sub>OH</sub> primer was extended by 10 U TdT. Thus, the pyrophosphate internucleotide linkage reduced TdT polymerase-specific activity by at least three orders of magnitude. An interesting finding was that a single 3'-terminal ribose sugar diminished TdT activity several-fold; to wit, 17%, 50%, and 95% of the p(rG)<sub>OH</sub> primer strand was extended during a reaction with 1, 2, and 5 U TdT (Figure 7). There was no detectable extension of the pp(rG)<sub>OH</sub> primer by up to 10 U TdT.

#### DNA capping confers resistance to end joining by DNA ligases

*Chlorella* virus DNA ligase (ChVLig) and *E. coli* DNA ligase (EcoLigA) are structurally and biochemically well-characterized exemplars of the ATP-dependent and NAD<sup>+</sup>-dependent clades of nick-sealing DNA ligases (20,21). We reacted ChVLig and EcoLigA with singly nicked DNAs prepared by annealing 21-mer 5' <sup>32</sup>P-labeled pDNApp(dG)<sub>OH</sub> or pDNApp(rG)<sub>OH</sub> strands and an unlabeled 10-mer pDNA<sub>OH</sub> strand to a complementary 31-mer DNA strand to form otherwise identical substrates with either a phosphodiester or pyrophosphate linkage preceding the nick 3'-OH nucleoside (Figure 8). Whereas 83% and 81% of the input 5' <sup>32</sup>P-labeled pDNApp(dG)<sub>OH</sub> strand were ligated by ChVLig and EcoLigA, respectively, there was no detectable sealing of the <sup>32</sup>P-labeled pDNApp(rG)<sub>OH</sub> strand (Figure 8). Because it is well established that ChVLig and EcoLigA are adept at sealing nicks containing a 3'-OH ribonucleotide (21), we considered it unlikely that the general inability to ligate a DNAppG<sub>OH</sub> strand to a 5'-PO<sub>4</sub> strand had to do with the cap being a ribonucleotide. However, to consolidate this point, we prepared otherwise identical singly nicked duplexes with 21-mer 5' <sup>32</sup>P-labeled pDNApp(dG)<sub>OH</sub> or pDNApp(dG)<sub>OH</sub> strands on the 3'-OH side of the nick. We found that ChVLig and EcoLigA sealed 88% of the input <sup>32</sup>P-labeled pDNApp(dG)<sub>OH</sub> strand, but there was no detectable sealing of the <sup>32</sup>P-labeled pDNApp(dG)<sub>OH</sub> strand by either ligase (not shown). We conclude that the pyrophosphate bridge of the 3' cap precludes 3'-OH/5'-PO<sub>4</sub> nick sealing by canonical ATP-dependent and NAD<sup>+</sup>-dependent DNA ligases.

#### Embedded ppG is refractory to incision by RNase H2

RNase H2 enzymes have the distinctive property of being able to recognize a single ribonucleotide embedded in du-



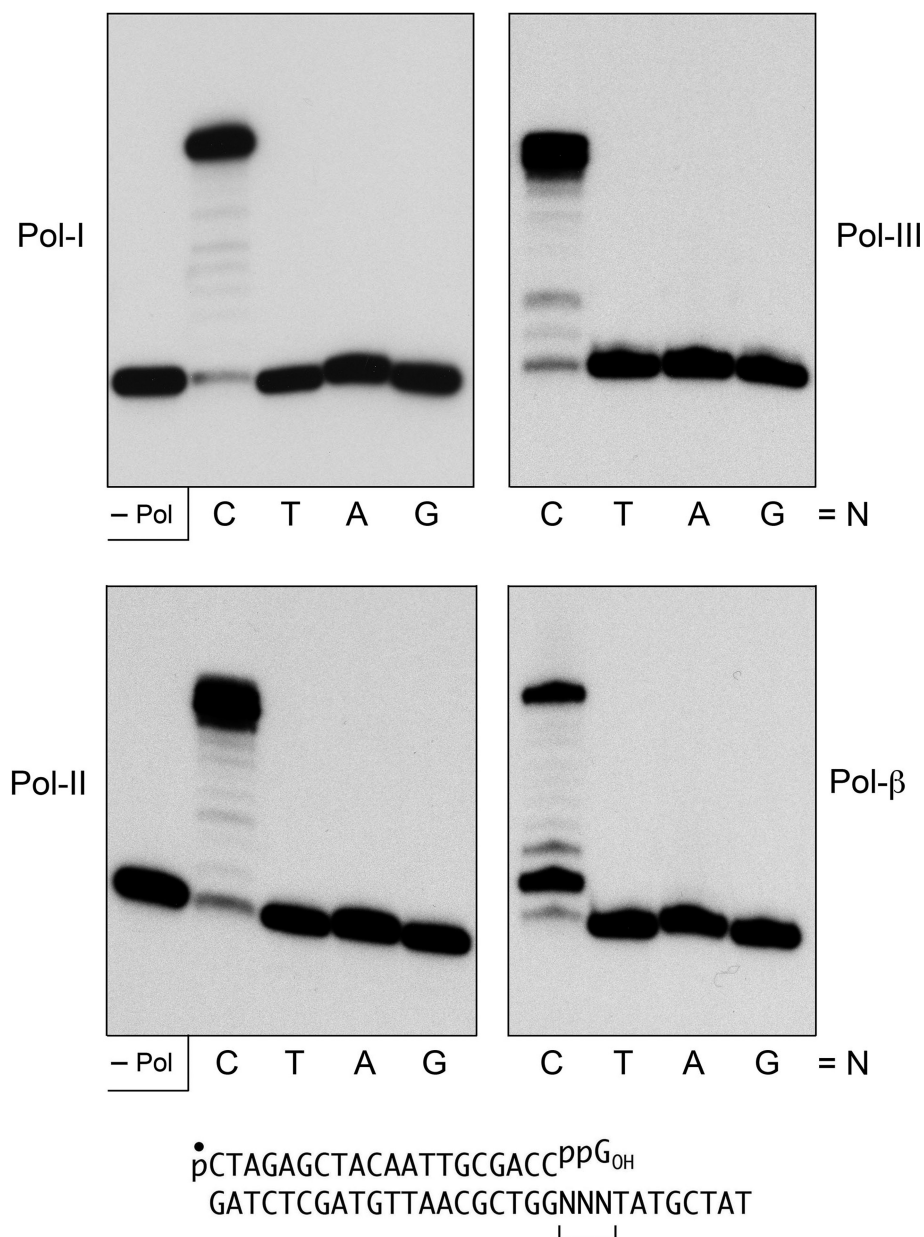
**Figure 5.** Kinetic profile of DNAppG primer extension by Pol  $\beta$ . Reaction mixtures (80  $\mu$ l) contained 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 1.6 pmol (20 nM) 5' P-labeled pDNA<sub>OH</sub> or pDNAppG<sub>OH</sub> primer-templates (as illustrated) and 16 pmol (200 nM) Pol  $\beta$ . The reactions were initiated by adding Pol  $\beta$  to mixtures pre-warmed to 37°C. Aliquots (10  $\mu$ l) were withdrawn after incubation at 37°C for 0.25, 0.5, 1, 2, 5, 10 and 20 min and quenched immediately with 10  $\mu$ l of 90% formamide, 40 mM EDTA. The products were analyzed by urea-PAGE and visualized by autoradiography (top panel). The percent of primer strand extended by one nucleotide ( $n+1$ ) or more than one nucleotide ( $\geq n+2$ ) was quantified by scanning the gels. The product distributions are plotted as a function of reaction time. Each datum in the graph is the average of three experiments  $\pm$ SEM.

plex DNA and catalyze metal-dependent hydrolysis of the 5'-flanking (dN)p(rN) phosphodiester to yield a nicked duplex with (dN)<sub>OH</sub> and p(rN)-termini (17). Thereby, RNase H2 provides a surveillance mechanism that initiates a pathway of RER (16). To probe how a pyrophosphate-linked ribonucleoside, embedded by DNA polymerization from a 3' cap guanosine primer, affects this surveillance mechanism, we employed Klenow DNA polymerase I to fill-in 5' <sup>32</sup>P-labeled primer templates with p(dG)<sub>OH</sub>, p(rG)<sub>OH</sub> or pp(rG)<sub>OH</sub> primer termini. The resulting end-labeled duplexes were isolated and then reacted with *E. coli* RNase H2. RNase H2 quantitatively incised the strand with the embedded p(rG) nucleotide to yield a single <sup>32</sup>P-labeled product indicative of hydrolysis of the (dN)p(rG) phosphodiester (Figure 9). As expected, RNase H2 was unreactive with the all-DNA duplex control substrate (Figure 9). The instructive finding was that RNase H2 failed to incise when an embedded rG was flanked by a 5' pyrophosphate linkage (Figure 9). Thus, a pyrophosphate cap, once embedded by

polymerase, cannot be removed by the RNase H2-initiated pathway of excision repair.

### Conclusions, implications and speculations

We conclude from the results presented here and previously (8) that DNA capping precludes nick sealing by DNA ligases and prevents or inhibits 3' resection by free-standing repair exonucleases and by DNA polymerase-associated proofreading exonucleases, while permitting templated DNA synthesis from the cap guanosine 3'-OH primer by exemplars of five families of DNA polymerases (A, B, C, X and Y). The price of cap-primed DNA repair synthesis is the embedding of a ribonucleotide and an unconventional pyrophosphate linkage in the repaired DNA strand. The pyrophosphate makes the embedded riboguanylate refractory to surveillance and incision by RNase H2, thereby thwarting a key pathway of RER (16).

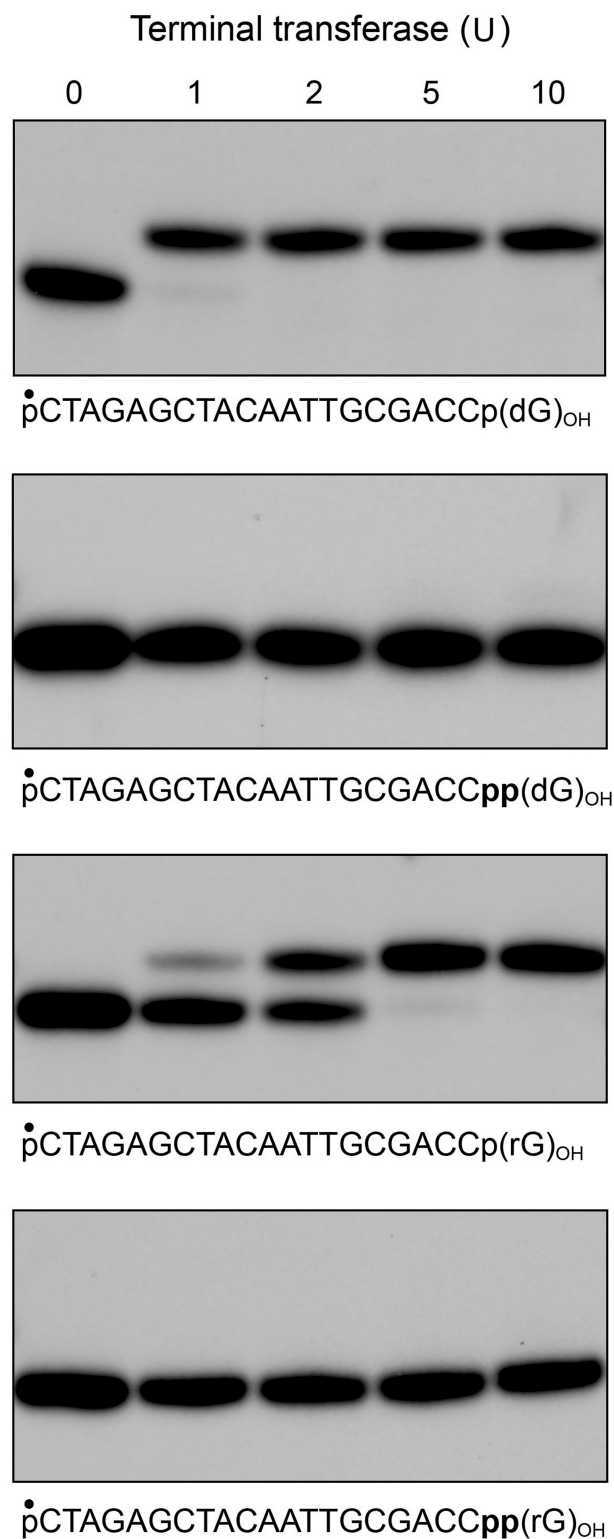


**Figure 6.** DNA polymerases display high fidelity for pairing of the DNAppG cap guanine with a template cytosine nucleobase. Fidelity was tested by reacting DNA polymerases with series of  $5'$ - $^{32}\text{P}$ -labeled pDNAppG<sub>OH</sub> primer-templates (shown at *bottom*) with different template homo-trinucleotides NNN flanking the primer  $3'$  end (as specified below the lanes). (*Top left*) Reaction mixtures ( $10\ \mu\text{l}$ ) containing 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 4 mM dNTPs, 0.2 pmol  $^{32}\text{P}$ -labeled primer-template and 5 U of Klenow Pol I were incubated at  $37^\circ\text{C}$  for 20 min. Alternatively, reaction mixtures ( $10\ \mu\text{l}$ ) containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol  $^{32}\text{P}$ -labeled primer-template and either 0.2 pmol Pol II (*bottom left*), 4 pmol Pol III (*top right*) or 2 pmol Pol  $\beta$  (*bottom right*) were incubated at  $37^\circ\text{C}$  for 20 min. The products were analyzed by urea-PAGE and visualized by autoradiography.

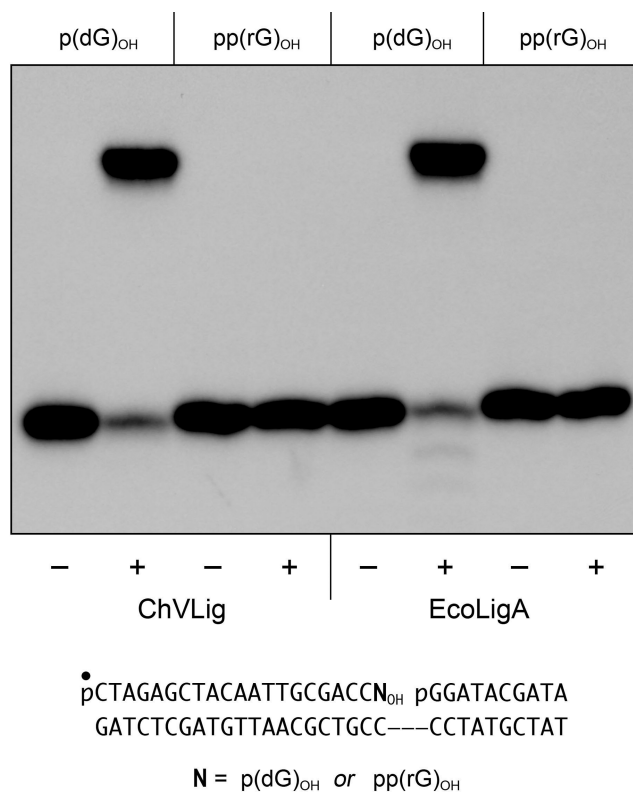
Our findings that the pyrophosphate linkage is resistant to hydrolysis by diverse metal-dependent nucleic acid phosphodiesterases suggest that these enzymes do not easily accommodate a scissile pN<sub>3</sub>pp<sub>5</sub>N dinucleotide in a productive conformation in their respective active sites. It is conceivable that the extra phosphate interferes sterically or electrostatically with nucleic acid binding, coordination of the divalent cation cofactor; and/or metal-catalysis of leaving group expulsion. Indeed, the pyrophosphate linkage engenders 'confusion' for a nucleic acid phosphodiesterase, inso-

far as there are two potential hydrolytic pathways: (i) attack by activated water on the  $5'$  phosphate of the pNppN bridge to form pNp and pN products; or (ii) attack on the  $3'$  phosphate to form pN<sub>OH</sub> and ppN products. In the case of the  $3'$  exonucleases, it is also conceivable that they might reach across the terminal pyrophosphate of a pNpNppN strand and hydrolyze the penultimate phosphodiester to form pN<sub>OH</sub> and pNppN products. Our initial findings indicate that Exo III and Pol II do not execute any of these reactions. It remains to be determined which pathway op-





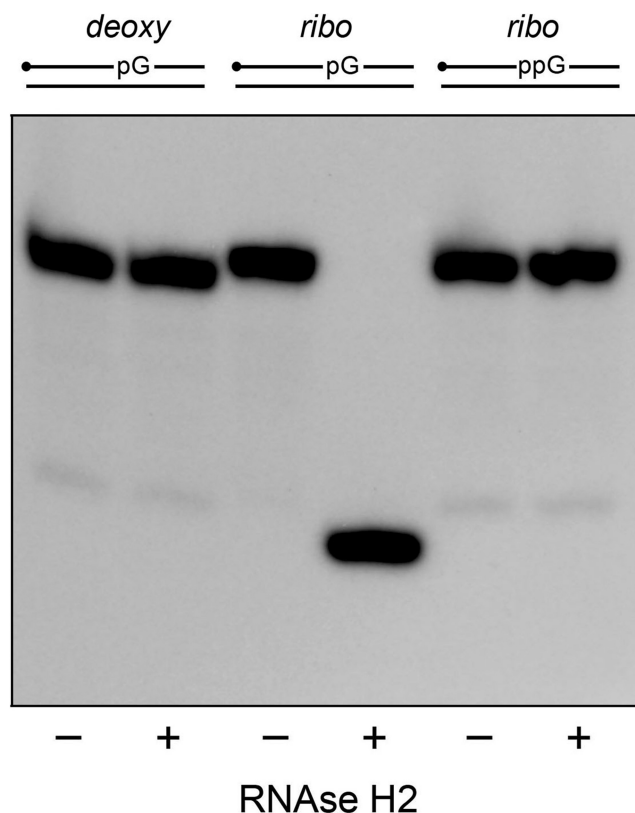
**Figure 7.** The cap pyrophosphate prevents primer extension by terminal transferase. Reaction mixtures (10  $\mu$ l) containing 20 mM Tris-acetate, pH 7.9, 50 mM KOAc, 10 mM Mg(OAc)<sub>2</sub>, 0.25 mM CoCl<sub>2</sub>, 100  $\mu$ M dideoxy-TTP, 0.2 pmol 5' <sup>32</sup>P-labeled primers with different 3' terminal nucleotides—either p(dG)<sub>OH</sub>, pp(dG)<sub>OH</sub>, p(rG)<sub>OH</sub> or pp(rG)<sub>OH</sub>—and 0, 1, 2, 5 or 10 units calf thymus terminal transferase were incubated at 37°C for 20 min. The reactions were quenched with formamide, EDTA. The products were analyzed by urea-PAGE and visualized by autoradiography. The primers are shown below each panel with the <sup>32</sup>P-label denoted by •.



**Figure 8.** The cap pyrophosphate prevents nick sealing by DNA ligases. Left panel: *Chlorella* virus DNA ligase reaction mixtures (10  $\mu$ l) containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 10 mM MgCl<sub>2</sub>, 1 mM ATP, 0.4 pmol 5' <sup>32</sup>P-labeled pDNAp(dG)<sub>OH</sub> or pDNAp(rG)<sub>OH</sub> nicked duplex (shown below with the <sup>32</sup>P-label denoted by •) and either no enzyme (–) or 1 pmol ChV Lig (+) were incubated at 37°C for 30 min. Right panel: *Escherichia coli* DNA ligase reaction mixtures (10  $\mu$ l) containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 5 mM MgCl<sub>2</sub>, 1 mM NAD<sup>+</sup>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.4 pmol pDNAp(dG)<sub>OH</sub> or pDNAp(rG)<sub>OH</sub> nicked duplex, and either no enzyme (–) or 1 pmol Eco LigA (+) were incubated at 37°C for 30 min. The reactions were quenched with formamide, EDTA. The products were analyzed by urea-PAGE and visualized by autoradiography.

tions account for the weak resection activity of the Pol III holoenzyme at a DNAppG end.

The cap pyrophosphate linkage exerts quite disparate effects on enzymes that perform chemistry at the 3'-OH terminus. DNA ligases and DNA polymerases catalyze analogous chemical reactions leading to the templated synthesis of a 3'-5' phosphodiester via the attack of a DNA 3'-OH on a high-energy 5'-phosphoanhydride: either AppDNA for ligases or a dNTP for polymerases. A pyrophosphate in lieu of a phosphodiester at the terminal 3'-OH nucleoside precludes sealing by DNA ligases, even though the phosphodiester is not involved directly in ligation chemistry. The crystal structures of EcoLigA in complex with the nicked DNA-adenylate intermediate (PDB ID: 2WO) and ChV Lig in complex with 5'-PO<sub>4</sub> nicked DNA (PDB ID: 2Q2U) revealed a tight interface of their adenylyltransferase domains with the three terminal nucleotides (NpNpN<sub>OH</sub>) of the 3'-OH strand, including multiple direct atomic contacts to the two phosphodiesters (20,21). To our inspection, it is unlikely that a terminal pyrophosphate linkage could be accommodated at the ligase interface with the nick without



**Figure 9.** A pyrophosphate linkage prevents ribonucleotide surveillance by RNase H2. Reaction mixtures (10  $\mu$ l) containing 20 mM Tris-HCl, pH 8.8, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM KCl, 2 mM  $\text{MgSO}_4$ , 0.1% Triton X-100, 0.2 pmol  $5'$   $^{32}\text{P}$ -labeled duplex DNA with an internal p(dG), p(rG) or pp(rG) linkage as specified, and either no enzyme (-) or 5 U *Escherichia coli* RNase H2 (+) were incubated at 37°C for 30 min. The reactions were quenched with formamide, EDTA. The products were analyzed by urea-PAGE and visualized by autoradiography.

adversely affecting the position and orientation of the 3'-OH with respect to the AppDNA or pDNA strands.

By contrast, DNA polymerases readily utilize a DNAppG<sub>OH</sub> primer strand for templated nucleotide addition. We infer that the active sites of DNA polymerases can accommodate the pyrophosphate linkage without grossly perturbing the position and orientation of the 3'-OH nucleophile with respect to the catalytic metal and the incoming dNTP. Moreover, the findings that *E. coli* Pol I, Pol II and Pol III and *M. smegmatis* DinB1 all perform complete fill-in synthesis on a DNAppG primer template suggest that these polymerases tolerate pyrophosphates in lieu of phosphodiester at upstream inter-nucleotide linkages in the primer strand. Yet, Pol  $\beta$  appears to be sensitive to a pyrophosphate in lieu of the penultimate primer strand phosphodiester, because the  $n + 1$  product predominates when Pol  $\beta$  reacts with the DNAppG primer-template. [As a key agent of base excision repair, Pol  $\beta$  specializes in filling single-nucleotide gaps (11).] The crystal structure of Pol  $\beta$  in a closed ternary complex with gapped primer-template and incoming dNTP (e.g. PDB ID: 4KLF) reveals close packing of the second and third inter-nucleotide phosphates at the NpNpNpN<sub>OH</sub> primer terminus against the polymerase, with hydrogen bonds to

these phosphates from the Gly105, Gly107, Ser109 and Ala110 main-chain amides (22). Perturbation of these close contacts by a NpNppNpN<sub>OH</sub> pyrophosphate linkage might explain the  $n + 1$  product accumulation.

The template-independent DNA polymerase TdT is unable to add even a single nucleotide to a primer 3'-OH terminus with a 5'-flanking pyrophosphate linkage. Why is TdT activity abolished by the pyrophosphate, whereas the structurally homologous X family enzyme Pol  $\beta$  is adept at single nucleotide addition to a DNAppG primer-template? We speculate that the difference might be attributable to an atomic interaction with the NpN<sub>OH</sub> phosphate in TdT that is absent in Pol  $\beta$ . In the crystal structure of TdT in a pre-catalytic complex with primer DNA and dTTP (PDB ID: 4I27), His342 sits at the tip of an inter- $\beta$ -strand loop, where it donates a hydrogen bond to the terminal phosphodiester of the primer strand (12). Mutating His342 to alanine is extremely detrimental to TdT polymerase activity (12). In Pol  $\beta$ , the corresponding amino acid is a glycine (Gly189). We speculate that this difference might open up more space around the terminal phosphodiester in Pol  $\beta$  so that it can accept the cap pyrophosphate, whereas TdT is constrained from doing so.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## FUNDING

National Institutes of Health (NIH) [GM46330]. Funding for open access charge: NIH [GM46330].

*Conflict of interest statement.* None declared.

## REFERENCES

- Tanaka, N., Chakravarty, A.K., Maughan, B. and Shuman, S. (2011) Novel mechanism of RNA repair by RtcB via sequential 2',3'-cyclic phosphodiesterase and 3'-phosphate/5'-hydroxyl ligation reactions. *J. Biol. Chem.* **286**, 43134–43143.
- Chakravarty, A.K., Subbotin, R., Chait, B.T. and Shuman, S. (2012) RNA ligase RtcB splices 3'-phosphate and 5'-OH ends via covalent RtcB-(histidiny)-GMP and polynucleotide-(3')pp(5')G intermediates. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 6072–6077.
- Das, U., Chakravarty, A.K., Remus, B.S. and Shuman, S. (2013) Rewriting the rules for end joining via enzymatic splicing of DNA 3'-PO<sub>4</sub> and 5'-OH ends. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 20437–20442.
- Tanaka, N. and Shuman, S. (2011) RtcB is the RNA ligase component of an *Escherichia coli* RNA repair operon. *J. Biol. Chem.* **286**, 7727–7731.
- Tanaka, N., Meineke, B. and Shuman, S. (2011) RtcB, a novel RNA ligase, can catalyze tRNA splicing and *HAC1* mRNA splicing *in vivo*. *J. Biol. Chem.* **286**, 30253–30257.
- Englert, M., Sheppard, K., Aslanian, A., Yates, J.R. and Söll, D. (2011) Archaeal 3'-phosphate RNA splicing ligase characterization identified the missing component in tRNA maturation. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 1290–1295.
- Popow, J., Englert, M., Weitzer, S., Schleiffer, A., Mierzwa, B., Mechtler, K., Trowitzsch, S., Will, C.L., Lührmann, R., Söll, D. *et al.* (2011) HSPC117 is the essential subunit of a human tRNA splicing ligase complex. *Science* **331**, 760–764.
- Das, U., Chaulau, M., Ordonez, H. and Shuman, S. (2014) Impact of DNA<sub>3</sub>pp5'-G capping on repair reactions at DNA 3' ends. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 11317–11322.
- Wang, F. and Yang, W. (2009) Structural insight into translesion synthesis by DNA pol II. *Mol. Cell* **139**, 1279–1289.

10. Lamers, M.H., Georgescu, R.E., Lee, S.G., O'Donnell, M. and Kuriyan, J. (2006) Crystal structure of the catalytic  $\alpha$  subunit of *E. coli* replicative DNA polymerase III. *Cell* **126**, 881–892.
11. Beard, W.A. and Wilson, S.H. (2014) Structure and mechanism of DNA polymerase  $\beta$ . *Biochemistry* **53**, 2768–2780.
12. Gouge, J., Rosario, S., Romain, F., Beguin, P. and Delarue, M. (2013) Structures of intermediates along the catalytic cycle of terminal deoxynucleotidyltransferase: dynamical aspects of the two-metal ion mechanism. *J. Mol. Biol.* **425**, 4334–4352.
13. Shuman, S. (2009) DNA ligases: progress and prospects. *J. Biol. Chem.* **294**, 17365–17369.
14. Ahel, I., Rass, U., El-Khamisy, S.F., Katyal, S., Clements, P.M., McKinnon, P.J., Caldecott, K.W. and West, S.C. (2006) The neurodegenerative disease protein aprataxin resolves abortive DNA ligation intermediates. *Nature* **443**, 713–716.
15. Tumbale, P., Williams, J.S., Schellenberg, M.J., Kunkel, T.A. and Williams, R.S. (2014) Aprataxin resolves adenylated RNA-DNA junctions to maintain genome integrity. *Nature* **506**, 111–115.
16. Sparks, J.L., Chon, H., Ceritelli, S.M., Kunkel, T.A., Johansson, E., Crouch, R.J. and Burgers, P.M. (2012) RNase H2-initiated ribonucleotide excision repair. *Mol. Cell* **47**, 980–986.
17. Rychlik, M.P., Chon, H., Ceritelli, S.M., Klimek, P., Crouch, R.J. and Nowotny, M. (2010) Crystal structure of RNase H2 in complex with nucleic acid reveal the mechanism of RNA-DNA junction recognition and cleavage. *Mol. Cell* **40**, 658–670.
18. Hamdan, S., Carr, P.D., Brown, S.E., Ollis, D.L. and Dixon, N.E. (2002) Structural basis for proofreading during replication of the *Escherichia coli* chromosome. *Structure* **10**, 535–546.
19. Ozawa, K., Horan, N.P., Robinson, A., Yagi, H., Hill, F.R., Jergic, S., Xu, Z.Q., Loscha, K.V., Li, N., Tehei, M. *et al.* (2013) Proofreading exonuclease on a tether: the complex between the *E. coli* DNA polymerase III subunits  $\alpha$ ,  $\epsilon$ ,  $\theta$  and  $\beta$  reveals a highly flexible arrangement of the proofreading domain. *Nucleic Acids Res.* **41**, 5354–5367.
20. Nair, P.A., Nandakumar, J., Smith, P., Odell, M., Lima, C.D. and Shuman, S. (2007) Structural basis for nick recognition by a minimal pluripotent DNA ligase. *Nat. Struct. Mol. Biol.* **14**, 770–778.
21. Nandakumar, J., Nair, P.A. and Shuman, S. (2007) Last stop on the road to repair: structure of *E. coli* DNA ligase bound to nicked DNA-adenylate. *Mol. Cell* **26**, 257–271.
22. Freudenthal, B.B., Beard, W.A., Shock, D.D. and Wilson, S.H. (2013) Observing a DNA polymerase choose right from wrong. *Cell* **154**, 157–168.