Effects of DNA₃′pp⁵′G capping on 3′ end repair reactions and of an embedded pyrophosphate-linked guanylate on ribonucleotide surveillance

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ABSTRACT

When DNA breakage results in a 3′-PO₄ 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′-PO₄ to form a DNA₃′pp⁵′G OH cap. Here we show that DNA capping precludes end joining by classic ATP-dependent and NAD⁺-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 template-attachment 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′-PO₄ nick sealing by DNA ligases. Yet, many biological scenarios generate ‘dirty’ DNA 3′-PO₄ 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′-PO₄ (referred to as ‘end-healing’) and/or resection of 3′ nucleotides. We recently described a new pathway of DNA 3′-PO₄ end-processing by the non-canonical RNA ligase Escherichia coli RtcB (1,2) in which RtcB reacts with GTP to form a covalent RtcB-(histidinyl)-GMP intermediate and then transfers GMP to a DNA 3′-PO₄ to form a DNA₃′pp⁵′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′-PO₄ 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 DNAppG(DNA 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′-PO₄ 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′-PO₄ ends via three nucleotidyl transfer steps (13). In the first step, nucleophilic attack by ligase on the α phosphorus of ATP or NAD⁺ results in release of PP₁ or NMN and for-

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mation of a covalent ligase–adenylate intermediate in which AMP is linked via a P–N bond to Nε of a lysine. In the second step, the AMP is transferred to the 5′ end of the 5′-PO₄ DNA strand to form DNA-adenylate, A₅′pp₅′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 DNAppG₅′OH terminus and thereby emit the pyrophosphate linked guanylate? We find here that exemptly ATP-dependent and NAD⁺-dependent DNA ligases are unable to seal a DNAppG₅′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 A₅′pp₅′DNA ends formed during abortive ligation by classic ligases (14,15), has a vigorous DNA 3′ de-capping activity, converting DNAppG to DNA₃′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 template 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)₉O₉H 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 Marians. *E. coli* RNase H2 and calf thymus terminal transferase were purchased from New England Biolabs (NEB). Human DNA polymerase β was purchased from Novoprotein.

### Substrates

The 5′ ³²P-labeled pDNApp and pDNA₅′OH strands were prepared by enzymatic phosphorylation of 20-mer HOCTAGAGCTACAATTGCGACC oligonucleotides, with either 3′-PO₄ or 3′-OH termini, by using [γ³²P]ATP and the phosphatase-dead mutant T₄ Pnkp-D167N. The radiolabeled pDNApp and pDNA₅′OH strands were gel-purified. The 5′ ³²P-labeled DNApp(dG)₅′OH and pDNA₅′OH strands were prepared by incubating pDNA with a 20-fold molar excess of RtcB in the presence of 2 mM MnCl₂ 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 pDNA₅′OH, pDNApp, and pDNA₅′OH 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 with dNTPs with the 5′ ³²P-labeled primer-templates with p(dG)₅′OH, p(rG)₅′OH, or pp(rG)₅′OH primer termini, after which the resulting filled-in blunt duplexes were recovered by phenol–chloroform extraction and ethanol precipitation.

## RESULTS AND DISCUSSION

DNA₅′pp₅′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′ ³²P-labeled 20-mer pDNA₅′OH or pDNA₅′OH 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 deoxyctydines that could potentially pair with the 3′ cap guanylate of the pDNA₅′OH strand. Reaction of the pDNA₅′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 pDNA₅′OH primer-template to yield the same distribution of fill-in products (Figure 1A, left panel). At 100 fmol Pol II, 52% of the input pDNA₅′OH strand was extended.

The kinetic profiles for templated extension of pDNA₅′OH and pDNA₅′OH 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 pDNA₅′OH and 60% for pDNA₅′OH after 15 s, the earliest time sampled; and in high yield at the endpoint, which was 95% for pDNA₅′OH and 92% for pDNA₅′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 DNA₅′OH primer and that there are no durable kinetic obstacles to further dNMP additions. [Analogous kinetic profiles for pDNA₅′OH and pDNA₅′OH primer extension by *E. coli* Klenow Pol I are shown in Supplementary Figure S2B. Pol I elongated 83% of the pDNA₅′OH primer and 33% of the pDNA₅′OH 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 μl) containing 10 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5'-32P-labeled pDNAOH or pDNAppG primer-template (depicted at right with the 32P-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 μl of 90% formamide, 50 mM EDTA. Right panel: reaction mixtures (10 μl) containing 10 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5'-32P-labeled pDNAOH or pDNAppG primer-template 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 μl 3 M NaOH (+) or 1.2 μl 3 M NaCl (−) and incubated at 22°C overnight, after which the NaOH mixtures were neutralized by adding 1.2 μl 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 μl) containing 10 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5'-32P-labeled pDNAppG primer-template (depicted at right with the 32P-label denoted by •) and 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 μl) containing 10 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5'-32P-labeled pDNAppG primer-template 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 pDNAOH 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 pDNAOH 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-template 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 products 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$_3$pp$_5$G ends are not proofread by DNA polymerase II**

DNA 3′ capping by RtcB at a 3′-PO$_4$ 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′-32P-labeled primer templates in which the primer strand 3′ terminal guanosine nucleotide—either p(dG)$_{OH}$, p(rG)$_{OH}$ or pp(rG)$_{OH}$—was opposite a mismatched dA nucleotide in the template strand (Figure 2). Reaction of the mispaired p(dG)$_{OH}$ primer-template with Pol II and Mg$^{2+}$ (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)$_{OH}$ 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)$_{OH}$ primer-template (Figure 2, right panel), signifying that the cap pyrophosphate linkage is not hydrolyzed by the Pol II proofreading exonuclease.

**DNA$_3$pp$_5$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$_{OH}$ 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$_{OH}$ 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 dNTPs, omission of dNTPs resulted in complete resection of the pDNA$_{OH}$ 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$_{OH}$ 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$_{OH}$ 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 μl) containing 20 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 5’-32P-labeled pDNApGOH or pDNAppGOH primer-template (depicted at bottom with the 32P-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 μl) containing 20 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 10 mM DTT, 4% glycerol, 0.2 pmol 5’-32P-labeled pDNApGOH or pDNAppGOH 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 μl) containing 10 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 10 mM DTT, 4% glycerol, 0.2 pmol 5’-32P-labeled pDNApGOH or pDNAppGOH 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 μl 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 ppGOH 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 pDNAppGOH primer was at least 75-fold less than its activity on the pDNApGOH primer.

**DNA₃ pp₅-G ends are extended by human DNA polymerase β**

Pol β, the prototypical family X polymerase (11), is a central agent of the base excision repair pathway. Pol β catalyzes templated primer extension and gap filling reactions; it lacks a 3′ proofreading activity. Reaction of 0.2, 1 and 2 pmol human Pol β with 0.2 pmol 5’-32P-labeled pDNAp(dG)OH primer-template and dNTPs resulted in the extension of 55%, 90%, and 87% of the input primer strand, with 1 and 2 pmol Pol β effecting complete fill-in synthesis, entailing 10 dNMP addition steps (Figure 4). Similarly, 43%, 71%, and 88% of the ribo-terminated pDNAp(rG)OH primer-template was extended by 0.2, 1, and 2 pmol Pol β, again with fill-in synthesis being the majority outcome at 2 pmol Pol β. We found that 29%, 56%, and 74% of the 3′-capped pDNAp(rG)OH primer was extended by 0.2, 1 and 2 pmol Pol β, signifying that the cap pyrophosphate *per se* had little effect on the extent of primer utilization compared to the pDNAp(rG)OH control. The salient finding here was that the predominant outcome of the Pol β reaction with the capped primer was a single step of nucleotide addition, with comparatively scant progression to fill-in synthesis (Figure 4). Thus Pol β was adept at adding a dNMP to a DNAp(pG)OH primer to form a DNAp(rG)p(dN) product, but less proficient at extending this species further. We infer that Pol β 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 β reactions with pDNAp(dG)OH and pDNAp(rG)OH primer-templates (Figure 5). Ninety percent of the control pDNAp(dG)OH 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 β: (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 DNA₃ pp₅-G cap guanine with a template cytosine nucleobase**

We surveyed DNA polymerases I, II, III and β for their ability to extend the pDNAp(rG)OH 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
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.

**DNA₃₃′pp₅′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′ 32P-labeled 21-mer primer strands was prepared in which the 3′ terminal guanosine nucleotide was p(dG)OH, pp(dG)OH, p(rG)OH or pp(rG)OH (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 input 5′ 32P-labeled pDNAp(dG)OH strand was ligated by ChVLig and EcoLigA, respectively, there was no detectable sealing of the 32P-labeled pDNApp(rG)OH 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 DNAppGOH strand to a 5′-PO₄ 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′ 32P-labeled pDNAp(dG)OH or pDNAp(rG)OH strands on the 3′-OH side of the nick. We found that ChVLig and EcoLigA sealed 88% of the input 32P-labeled pDNAp(dG)OH strand, but there was no detectable sealing of the 32P-labeled pDNAp(rG)OH strand by either ligase (not shown). We conclude that the pyrophosphate bridge of the 3′ cap precludes 3′-OH/5′-PO₄ nick sealing by canonical ATP-dependent and NAD⁺-dependent DNA ligases.

**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⁺-dependent clades of nick-sealing DNA ligases (20,21). We reacted ChVLig and EcoLigA with singly nicked DNAs prepared by annealing 21-mer 5′ 32P-labeled pDNAp(dG)OH or pDNAp(rG)OH strands and an unlabeled 10-mer pDNAOH 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′ 32P-labeled pDNAp(dG)OH strand were ligated by ChVLig and EcoLigA, respectively, there was no detectable sealing of the 32P-labeled pDNAp(rG)OH 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 DNAppGOH strand to a 5′-PO₄ 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′ 32P-labeled pDNAp(dG)OH or pDNAp(rG)OH strands on the 3′-OH side of the nick. We found that ChVLig and EcoLigA sealed 88% of the input 32P-labeled pDNAp(dG)OH strand, but there was no detectable sealing of the 32P-labeled pDNAp(rG)OH strand by either ligase (not shown). We conclude that the pyrophosphate bridge of the 3′ cap precludes 3′-OH/5′-PO₄ nick sealing by canonical ATP-dependent and NAD⁺-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 β. Reaction mixtures (80 μl) contained 10 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 1.6 pmol (20 nM) 5′ P-labeled pDNAOH or pDNAppGOH primer-templates (as illustrated) and 16 pmol (200 nM) Pol β. The reactions were initiated by adding Pol β to mixtures pre-warmed to 37°C. Aliquots (10 μl) were withdrawn after incubation at 37°C for 0.25, 0.5, 1, 2, 5, 10 and 20 min and quenched immediately with 10 μl 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 (≥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 ±SEM.

plex DNA and catalyze metal-dependent hydrolysis of the 5′-flanking (dN)p(rN) phosphodiester to yield a nicked duplex with (dN)OH 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′ 32P-labeled primer templates with p(dG)OH, p(rG)OH or pp(rG)OH 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 32P-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 DNApGcap guanine with a template cytosine nucleobase. Fidelity was tested by reacting DNA polymerases with series of 5′32P-labeled DNNpG primer-template substrates (shown at bottom) with different template homo-trinucleotides NNN flanking the primer 3′ end (as specified below the lanes). (Top left) Reaction mixtures (10 µl) containing 10 mM Tris–HCl, pH 7.9, 50 mM NaCl, 10 mM MgCl2, 1 mM DTT, 4 mM dNTPs, 0.2 pmol 32P-labeled primer-template and 5 U of Klenow Pol I were incubated at 37°C for 20 min. Alternatively, reaction mixtures (10 µl) containing 10 mM Tris–HCl, pH 8.0, 10 mM MgCl2, 10 mM DTT, 4% glycerol, 4 mM dNTPs, 0.2 pmol 32P-labeled primer-template and either 0.2 pmol Pol II (bottom left), 4 pmol Pol III (top right) or 2 pmol Pol β (bottom right) were incubated at 37°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 pN3′pp5′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, insofar 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 pNOH 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 pNOH 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 μl) containing 20 mM Tris-acetate, pH 7.9, 50 mM KOAc, 10 mM Mg(OAc)₂, 0.25 mM CoCl₂, 100 μM dideoxy-TTP, 0.2 pmol 5′-32P-labeled primers with different 3′ terminal nucleotides—either p(dG)OH, pp(dG)OH, p(rG)OH or pp(rG)OH—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 32P-label denoted by •.

Figure 8. The cap pyrophosphate prevents nick sealing by DNA ligases. Left panel: Chlorella virus DNA ligase reaction mixtures (10 μl) containing 50 mM Tris–HCl, pH 7.5, 5 mM DTT, 10 mM MgCl₂, 1 mM ATP, 0.4 pmol 5′-32P-labeled pDNA(p(dG)OH or pDNA(p(rG)OH nicked duplex (shown below with the 32P-label denoted by •) and either no enzyme (−) or 1 pmol ChVLig (+) were incubated at 37°C for 30 min. Right panel: Escherichia coli DNA ligase reaction mixtures (10 μl) containing 50 mM Tris–HCl, pH 7.5, 5 mM DTT, 5 mM MgCl₂, 1 mM NAD⁺, 10 mM (NH₄)₂SO₄, 0.4 pmol pDNA(p(dG)OH or pDNA(p(rG)OH nicked duplex, and either no enzyme (−) or 1 pmol EcoLigA (+) 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.

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 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 ChVLig in complex with 5′-PO₄ nicked DNA (PDB ID: 2Q2U) revealed a tight interface of their adenylyltransferase domains with the three terminal nucleotides (NpNpNpOH) of the 3′-OH strand, including multiple direct atomic contacts to the two phosphodiester bonds (20, 21). To our inspection, it is unlikely that a terminal pyrophosphate linkage could be accommodated at the ligase interface with the nick without
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 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* DinB all perform complete fill-in synthesis on a DNAppG primer template suggest that these polymerases tolerate pyrophosphates in lieu of phosphodiesters at upstream inter-nucleotide linkages in the primer strand. Yet, Pol β appears to be sensitive to a pyrophosphate in lieu of the penultimate linkages in the primer strand. Yet, Pol β 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 NpNOH phosphate in TdT that is absent in Pol β. 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-β-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 β, the corresponding amino acid is a glycine (Gly189). We speculate that this difference might open up more space around the terminal phosphodiester in Pol β so that it can accept the cap pyrophosphate, whereas TdT is constrained from doing so.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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