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Brief report

Replication of ribonucleotide-containing DNA templates by yeast replicative polymerases

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ABSTRACT

The major replicative DNA polymerases of *S. cerevisiae* (Pols α, δ, and ε) incorporate substantial numbers of ribonucleotides into DNA during DNA synthesis. When these ribonucleotides are not removed *in vivo*, they reside in the template strand used for the next round of replication and could potentially reduce replication efficiency and fidelity. To examine if the presence of ribonucleotides in a DNA template impede DNA synthesis, we determined the efficiency with which Pols α, δ, and ε copy DNA templates containing a single ribonucleotide. All three polymerases can replicate past ribonucleotides. Relative to all-DNA templates, bypass of ribo-containing templates is slightly reduced, to extents that depend on the identity of the ribo and the sequence context in which it resides. Bypass efficiencies for Pols δ and ε were increased by increasing the dNTP concentrations to those induced by cellular stress, and in the case of Pol ε, by inactivating the 3′-exonuclease activity. Overall, ribonucleotide bypass efficiencies are comparable to, and usually exceed, those for the common oxidative stress-induced lesion 8-oxo-guanine.

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1. Introduction

The stability of eukaryotic genomes partly reflects the fact that they are comprised of DNA rather than RNA, the latter being more sensitive to strand cleavage due to the presence of a reactive 2′ hydroxyl on the ribose ring. DNA polymerases maintain DNA content by preventing incorporation of ribonucleoside triphosphates (rNTPs) during DNA synthesis [1]. This rNTP exclusion is efficient but not perfect, as revealed by several studies showing that polymerases insert rNTPs during DNA synthesis *in vitro* [1–14] and during repair of double strand breaks in DNA [15,16]. The probability that ribonucleoside monophosphates (rNMPs) will be stably incorporated into DNA *in vivo* is increased by the much higher concentrations of rNTPs as compared to dNTPs in cellular nucleotide pools [2,17,18]. Indeed, during DNA synthesis in reactions containing rNTP and dNTP concentrations measured in yeast cell extracts, the major replicative DNA polymerases of *S. cerevisiae*, DNA polymerases α (Pol α), δ (Pol δ) and ε (Pol ε), stably incorporate rNMPs into DNA in amounts suggesting that rNMPs could be the most common of all non-canonical nucleotides introduced into the eukaryotic genome [18]. However, previous studies suggested that rNMPs in DNA may be removed by RNase H and FEN1 to maintain genome stability [19,20].

Discrimination against stable rNTP incorporation *in vitro* varies in the order Pol δ > Pol ε > Pol α [18]. In a model wherein Pol ε is the major leading strand replicase and Pol δ and Pol α primarily participate in replicating the lagging strand template [21,22], reviewed in [23] and also see [24], these rNTP incorporation propensities suggest that the majority of rNTPs may be incorporated into DNA during leading strand replication. For that reason, we recently investigated the consequences of rNTP incorporation into DNA *in vivo* in yeast strains encoding a mutant form of Pol ε (*pol2-M644G*) with an increased tendency to incorporate rNTPs into DNA [25]. When the *RNH201* gene encoding the catalytic subunit of RNase H2 was deleted, this *pol2-M644G rnh201Δ* strain accumulated rNMPs in genomic DNA, progressed more slowly through S-phase, had elevated dNTP pools and an increased rate of spontaneous mutagenesis that included 2–5 bp deletions in repetitive sequences [25]. These data indicate that rNTPs are incorporated during replication *in vivo* and are normally removed by RNase H2-dependent repair. Failure to remove rNMPs elicits a replicative stress response and destabilization of the nuclear genome. The deleterious effects could result from strand cleavage at rNMPs, and/or possibly from difficulty in replicating templates containing unrepaired rNMPs. Indeed, a previous study revealed that Pols α and δ had difficulty in replicating a DNA template that contained a tract of four rNMPs, but both polymerases were able to bypass given

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a sufficient amount of enzyme and time [26]. Here we complement our earlier studies of Pols α, δ and ε incorporation of rNTPs into DNA from cellular nucleotide pools by quantifying the efficiencies with which these polymerases bypass DNA templates containing a single rNMP. We find that all three polymerases bypass rNMPs, with efficiencies that are slightly reduced and comparable to those for the common oxidative stress-induced lesion 8-oxo-guanine (8-oxo-G) [27,28].

2. Materials and methods

2.1. Materials and reagents

DNA modification and restriction enzymes were from New England Biolabs (Ipswich, MA), oligonucleotides were from Integrated DNA Technologies (Coralville, IA), rNMP containing oligonucleotides were from Dharmacon RNAi Technologies Thermo Scientific (Lafayette, CO), and dNTPs were from Amersham Biosciences (Piscataway, NJ).

2.2. Enzymes and DNA substrates

S. cerevisiae four-subunit wild type (WT) Pol ε and the N-terminal Pol ε_{152} fragment in both exonuclease proficient (Pol ε_{152} exo$^+$) and deficient (Pol ε_{152} exo$^-$) forms, were expressed and purified as previously described [29,30]. The catalytic subunit of *S. cerevisiae* polymerase Pol α was purified as described [31]. *S. cerevisiae* three subunit Pol δ was purified as described [32]. Oligonucleotide primer-templates (Table 1) were prepared as described [18].

2.3. Bypass efficiency assay

For Pol α, reaction mixtures contained 20 mM Tris (pH 8), 200 μg/mL BSA, 2 mM DTT, 10 mM $MgCl_2$, 1.5 fmol of Pol α, 2 pmol (67 nM) primer-template, 12 μM dGTP, 14 μM dCTP, 16 μM dATP, and 30 μM dTTP. These dNTP concentrations are those measured earlier [18] in extracts of the wild type yeast strains. For Pol δ, the reaction mixture contained 20 mM Tris (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 90 mM NaCl, 8 mM Mg acetate, 8–17 fmol Pol δ, 2 pmols (67 nM) primer-template and either cellular dNTP concentrations or 10-fold higher concentrations. For four-subunit Pol ε (1.7 fmol), Pol ε_{152} exo$^+$ (4 fmol), and Pol ε_{152} exo$^-$ (13 fmol), the reaction mixture contained 40 mM Tris (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 100 mM NaCl, 8 mM Mg acetate, 2 pmols (67 nM) primer-template, and either cellular dNTP concentrations or 10-fold higher concentrations. All components except the polymerase were mixed on ice and then incubated at 30 °C for 1 min. The polymerase was added to initiate the reaction and aliquots were removed at 2, 4, 6 and 20 min. An equivalent volume of formamide loading dye (95% deionized formamide, 25 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol) was added to terminate the reaction. This mixture was heated to 95 °C for 3 min and the DNA products were separated by electrophoresis through an 8% denaturing polyacrylamide gel containing 25% formamide. A PhosphorImager and Image Quant software (Molecular Dynamics) were used to visualize and quantify the DNA products. The efficiency of insertion opposite individual template positions and the relative bypass probability were calculated as previously described [33].

3. Results

3.1. Measuring rNMP bypass parameters

The rNMP bypass parameters for Pols α, δ and ε were determined using DNA substrates whose template strands contain a single rG, rC, rA or rU in the same sequence context, corresponding to a dG in the all-DNA control template (Table 1). In the images shown in Fig. 1, this position is marked with a R, and it is designated as position zero in Fig. 2, with flanking template positions numbered as −1 for the preceding incorporation or with a plus for the subsequent incorporations needed for complete bypass. We also used a second template with a rG located at the immediate 5′ position instead (Table 1 substrates 6 and 12), referred to as 5′-rG, to examine bypass efficiency for the same base (rG) in a different sequence context. Reaction mixtures contained primer-templates in sufficient excess over polymerase to generate DNA products where the probability of termination of synthesis at each template position was constant over a 2–6 min time course (with a few exceptions, as noted below). DNA synthesis reaction mixtures contained the physiologically relevant, slightly biased dNTP concentrations (12 μM dGTP, 14 μM dCTP, 16 μM dATP, and 30 μM dTTP) measured in extracts of the yeast strains used in our genetic studies [18,25]. In some cases, 10-fold higher dNTP concentrations were used to approximate the dNTP pools induced by cellular stress [34].

3.2. rNMP bypass by Pol α

When copying the all-DNA control template (Fig. 1A, left), Pol α is moderately processive (Fig. 2A). However, when copying the corresponding rG-containing template, the termination probability after insertion opposite rG (position 0) is significantly higher (44%

Table 1
Primer-template sequences.

Substrate	NMP	Primer-Template Sequence
		ACTCTTTTGGGACCGCAATG-5′
1	dG	ACGTCGTGACTGAGAAAACCCTGGCGTTACCCA-3′
		ACTCTTTTGGGACCGCAATG-5′
2	3′-rG	ACGTCGT**g**ACTGAGAAAACCCTGGCGTTACCCA-3′
		ACTCTTTTGGGACCGCAATG-5′
3	rC	ACGTCGT**c**ACTGAGAAAACCCTGGCGTTACCCA-3′
		ACTCTTTTGGGACCGCAATG-5′
4	rA	ACGTCGT**a**ACTGAGAAAACCCTGGCGTTACCCA-3′
		ACTCTTTTGGGACCGCAATG-5′
5	rU	ACGTCGT**u**ACTGAGAAAACCCTGGCGTTACCCA-3′
		ACTCTTTTGGGACCGCAATG-5′
6	5′-rG	ACGTCG**g**TACTGAGAAAACCCTGGCGTTACCCA-3′
		GGCCCAACTGGAAACCTCAGCTGGACGTCTTTAAGTGACC-5′
7	dG	CATGATTACGAATTCCAGCTCGGTACCGGGTTGACCTTTGGAGTCGACCTGCAGAAATTCACTGG-3′
		GGCCCAACTGGAAACCTCAGCTGGACGTCTTTAAGTGACC-5′
8	3′-rG	CATGATTACGAATTCCAGCTCG**g**TACCGGGTTGACCTTTGGAGTCGACCTGCAGAAATTCACTGG-3′
		GGCCCAACTGGAAACCTCAGCTGGACGTCTTTAAGTGACC-5′
9	rC	CATGATTACGAATTCCAGCTCG**c**TACCGGGTTGACCTTTGGAGTCGACCTGCAGAAATTCACTGG-3′
		GGCCCAACTGGAAACCTCAGCTGGACGTCTTTAAGTGACC-5′
10	rA	CATGATTACGAATTCCAGCTCG**a**TACCGGGTTGACCTTTGGAGTCGACCTGCAGAAATTCACTGG-3′
		GGCCCAACTGGAAACCTCAGCTGGACGTCTTTAAGTGACC-5′
11	rU	CATGATTACGAATTCCAGCTCG**u**TACCGGGTTGACCTTTGGAGTCGACCTGCAGAAATTCACTGG-3′
		GGCCCAACTGGAAACCTCAGCTGGACGTCTTTAAGTGACC-5′
12	5′-rG	CATGATTACGAATTCCAGCTCG**g**TACCGGGTTGACCTTTGGAGTCGACCTGCAGAAATTCACTGG-3′

Ribonucleotides are boldfaced, underlined and in lowercase.

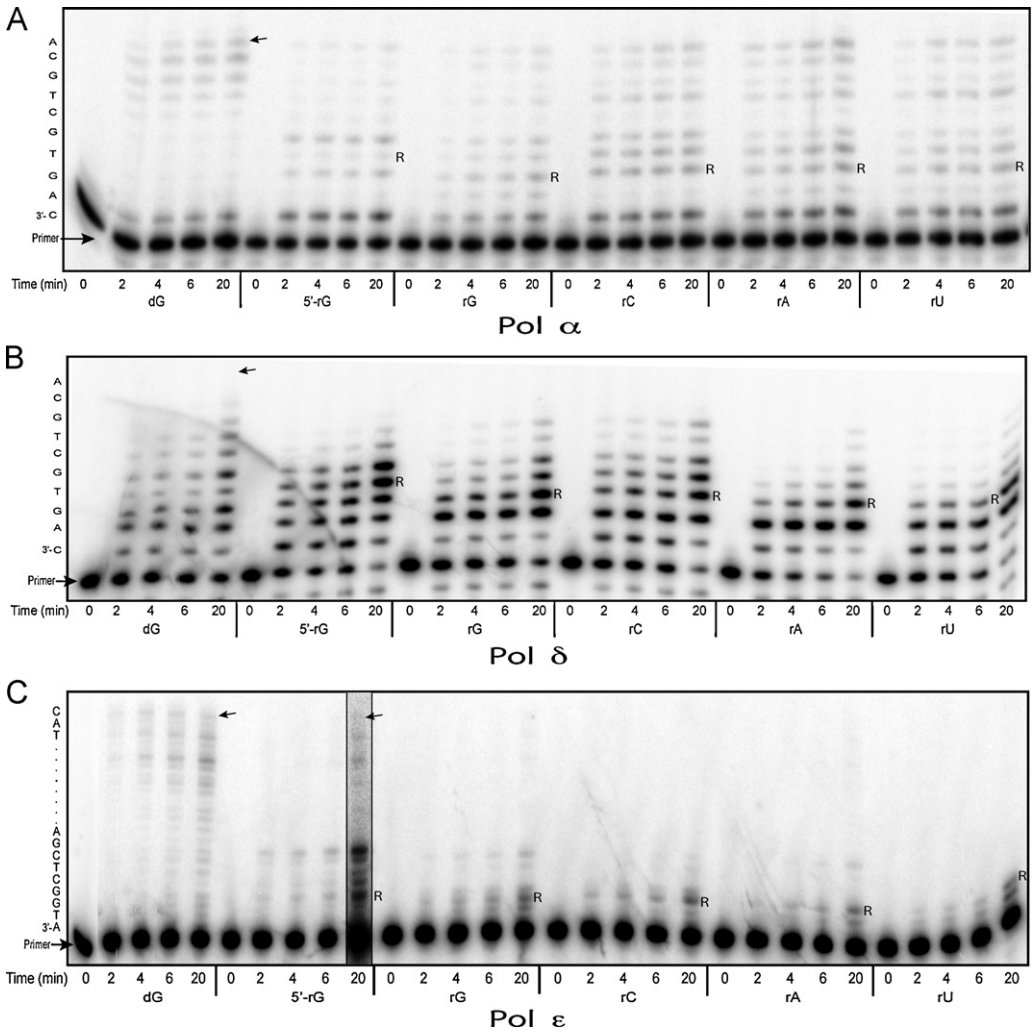

Fig. 1. PAGE phosphorimages of the bypass of a single rNMP by Pols α, δ, and ε. Gel image of DNA products for primer extension reactions with (A) Pol α using substrates 1–6, (B) Pol δ using substrates 1–6, and (C) Pol ε using substrates 7–12. The template sequence is shown to the left of the image and the arrow depicts the location of full-length product. The "R" represents the location of the corresponding rNMP in the template. No enzyme was added to the un-extended primer (designated 0 min). The products of reactions incubated for 20 min were not used for quantification, and are shown only to illustrate that some bypass occurs with all six templates. (C) The boxed lane for the 5′-rG template at 20 min depicts an increase in the grey scale to show the presence of full-length product. For the rA- and rU-containing templates, some products of the 6-min reactions resulted from multiple cycles of extension, so this time point was not used for quantification. Additional control reactions were performed to compare the efficiency of bypass of four different all-DNA control templates, containing dG, dC, dA or dT at the position corresponding to the rNMP. Variations in bypass efficiencies among these controls were 2-fold or (usually) less (data not shown). Thus, results for the control template shown here were used to calculate relative bypass efficiencies for all five rNMP-containing templates (Table 2).

compared to 6.6% with the control template (Fig. 2A)). The termination probability also increased from 4.0% to 29% after extending the deoxyribo-primer terminus paired with the template rG (position +1). The efficiencies of subsequent incorporations required to completely bypass the rG are lower compared to the control template. As a consequence, complete bypass of the rG occurred with 51% efficiency of the corresponding dG in the all-DNA template (Table 2). The rG in the second sequence context (5′-rG) was bypassed with slightly higher efficiency (70%), and again with a significantly increased termination after incorporation opposite the rG and the following position (Fig. 2A). Similar extension reactions using templates containing rC, rA and rU demonstrated that Pol α also efficiently bypasses each of the four different rNMPs (Table 2). In each case, Pol α had difficulty in continuing synthesis after insertion opposite the rNMP and the next template position (Fig. 2A). When a second set of primer-templates was examined (Table 1, substrates 7–12), similar effects of rNMPs on synthesis by Pol α were observed (data not shown).

3.3. rNMP bypass by Pol δ

Quantifying yeast Pol δ bypass parameters under single hit conditions was not feasible with the primer-templates 7–12 (Table 1) because the GG dinucleotide sequence at the 0/+1 position is a strong "natural" pause site for Pol δ even when no rNMP is present. However, using primer-templates 1–6 (Table 1), which have a different template sequence, synthesis was processive enough (Fig. 1B) to quantify termination probabilities at the position of the rNMP and the two immediately surrounding positions (Fig. 2B). With these substrates, relative bypass efficiencies (Table 2) ranged from 62% for rC to 7.4% for rA and rU. These bypass efficiencies reflect a 2- to 3-fold increase in termination probabilities following insertion opposite the rNMP and the flanking positions (Fig. 2B), most especially with the rA and rU-containing templates. A 1.7-fold difference in relative bypass efficiency observed between the two different rG-containing templates indicates sequence context dependence (Table 2).

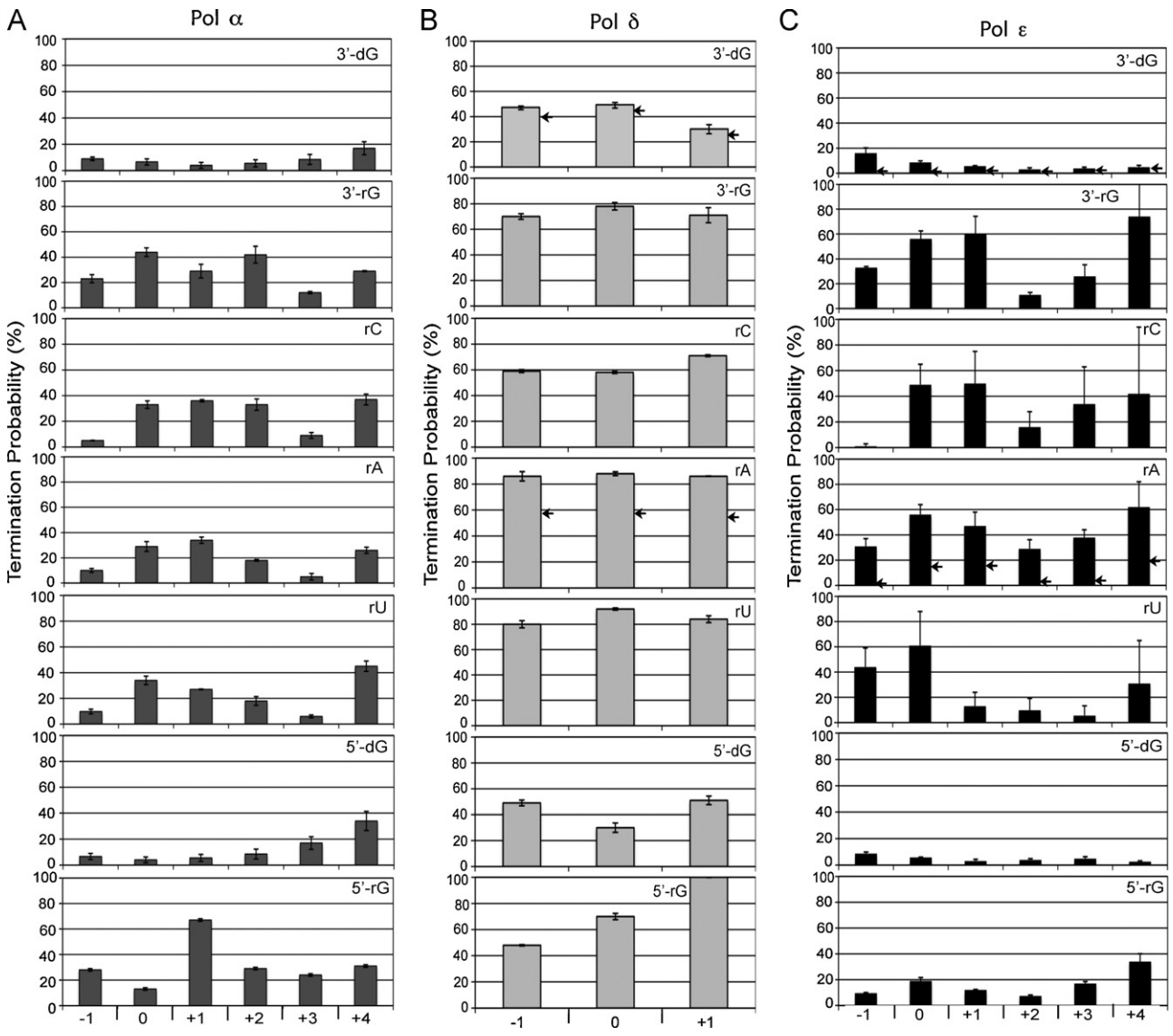

Fig. 2. Polymerase termination probability during rNMP bypass. Gel images of reaction products shown in Fig. 1 were quantified as described in Methods. Bar graph of termination probability (vertical axis, 0–100%) at each incorporation (horizontal axis) for (A) Pol α, (B) Pol δ, and (C) Pol ε. Position "0″ corresponds to the location of the rNMP in the template, −1 indicates the preceding incorporation, and +1 through +4 indicate sequential incorporations after insertion at "0″. Error bars represent the standard deviations. The arrows indicate the values for reactions with 10-fold cellular dNTP concentration.

Table 2
Relative bypass efficiencies.

DNA polymerase	Relative bypass efficiency[a] (%) for					
	rG	5′-rG	rC	rA	rU	8-oxo-G
Pol α	51	70	75	75	70	–
Pol δ	25	43	63	7.4	7.4	15[b], 25[c]
Pol δ high[d]	–	–	–	56	–	–
Pol ε	39	85	53	40	32	ND[e]
Pol ε high[d]	–	–	–	89	–	19[f], 25[f]
Pol ε exo⁻	–	–	–	70	–	–

[a] Bypass probability with rNMP substrate divided by bypass probability with DNA substrate.
[b] 8-oxo-G in first sequence context 3′-CCG$_o$ATTGGGCCAT [27].
[c] 8-oxo-G in second sequence context 3′-TTG$_o$GGCCATGG [27].
[d] 10-Fold higher cellular dNTP concentration.
[e] ND means levels not detected with an estimated S-phase cellular dNTP concentration [28].
[f] Bypass reactions performed with 5- to 8-fold S-phase cellular dNTP concentration [28].

3.4. rNMP bypass by Pol ε

We previously reported [18,25] that, in reactions containing equimolar dNTPs (10 μM), yeast Pol ε can bypass a rG in a DNA template, but with 40% relative bypass efficiency and with difficulty in continuing synthesis following insertion opposite the rG and for each of the four additional template positions. When we measured rG bypass using the same primer-template but now in reactions containing dNTP concentrations measured in yeast, similar results were obtained (Fig. 1C), i.e., 39% relative bypass efficiency (Table 2) and increased termination at positions −1 through +4 (Fig. 2C). A comparison of bypass parameters for the rC-, rA- and rU-containing templates reveals a similar pattern of reduced relative bypass efficiencies and increased termination for several incorporations. Similar to Pol δ, Pol ε bypassed the 5′-rG more efficiently (85%) than the first sequence context examined (39%). rNMP bypass by Pol ε was generally more efficient (Table 2), as best exemplified by a 5-fold difference in relative bypass of rA (7.4% for Pol δ, 40% for Pol ε).

3.5. Effect of "stress-induced" dNTP concentrations and 3′-exonuclease on bypass

The above results show that Pol δ and Pol ε copy rNMP-containing templates less efficiently due to difficulty in extending primer termini paired with template rNMPs and when the next incorporation would involve template rNMP at the −2 position. Since Pols δ and ε have difficulty extending these non-canonical substrates, we tested if their extension, and therefore the bypass probability, would increase if the dNTP concentrations were increased to drive polymerization forward. We increased the concentrations of all four dNTPs by 10-fold, which resulted in a 8-fold increase in the efficiency with which Pol δ bypassed rAMP (from 7.4% to 56%, Table 2). This increase results from a reduction in termination following insertion opposite the −1 position, the rAMP and the next nucleotide (values are indicated by the arrows in Fig. 2B). In a similar manner, increasing the dNTP concentrations resulted in a 2-fold increase in the efficiency with which Pol ε bypassed rAMP (from 40% to 89%, Table 2), through a reduction in termination following insertion opposite −1 through +4 (arrows in Fig. 2C). We also tested if primer extension and relative bypass probability would increase if 3′ exonuclease activity was inactivated to eliminate potential idling at rNMP-containing termini. As an initial test, we measured the relative rAMP bypass efficiency of wild type (exonuclease-proficient) Pol ε and its exonuclease-deficient derivative. For this purpose, we used the 152 kDa forms of the Pol ε catalytic subunit (Pol ε_{152} exo$^+$ and exo$^-$) that were previously demonstrated to be reasonable surrogates for the 4-subunit holoenzyme [30]. The relative rAMP bypass efficiency of Pol ε_{152} exo$^+$ was 35%, comparable to the 40% bypass observed with the holoenzyme. The relative rAMP bypass efficiency of Pol ε_{152} exo$^-$ was 2-fold higher (Table 2).

4. Discussion

All three DNA polymerases that replicate the yeast nuclear genome stably incorporate rNMPs into DNA during synthesis, and rNMP incorporation probability varies over a 100-fold range depending on the polymerase, the sequence context and the identity of the rNMP [18]. The present study shows that, in addition to introducing rNMPs into the genome, these same three polymerases can also bypass rNMPs in DNA templates, with efficiencies that vary depending on the polymerase and the identity and location of the rNMP. Polymerase dependence is apparent by comparing the relative bypass efficiencies for each of the five rNMP (columns in Table 2). Variations range from 7.4% to 85%, with Pol α being generally most efficient and Pol δ the least efficient. Bypass efficiency also depends on the identity of the rNMP (rows in Table 2), with a 1.5-fold variation seen with Pol α (51–75%), an approximate 3-fold variation seen with Pol ε (32–85%) and a 8-fold variation seen with Pol δ (7.4–63%).

Three types of sequence context effects are also apparent. One involves approximately 8-fold differences in relative bypass efficiency depending on the identity of the rNMP when flanked by the same neighbors (Table 2). The second is a 2-fold difference in relative bypass efficiency for the same rNMP (rG) when flanked by different neighbors (Table 2). The third context effect includes differences in dNTP insertion probability as bypass proceeds from −1 through +4 (Fig. 2). All three polymerases share a reduced dNTP insertion probability opposite the rNMP (Fig. 2, position 0). Thus, a 2′-oxygen on the sugar of the templating nucleotide of the nascent base pair reduces catalytic efficiency. Similarly, with all three polymerases and all rNMP-containing templates, dNTP insertion is also problematic when the template strand rNMP is paired with the primer-terminal base (Fig. 2, position +1). This reduced efficiency is not surprising, because the primer-terminal base pair of replicative DNA polymerases forms one surface of the nascent base pair binding pocket, whose geometry is critical for efficient insertion [35–37]. Reduced insertion is also observed at the −1 position (several examples depicted in Fig. 2), where the rNMP is in the single stranded DNA immediately adjacent to the nascent base pair binding pocket and will be the next template nucleotide to be copied. The fact that a 2′-oxygen on the sugar of this nucleotide reduces insertion is generally consistent with the fact that certain amino acids in DNA polymerases interact with this nucleotide when it is uncopied and as it is moved into position for catalysis [38–41]. Finally, dNTP insertion is reduced when the template-strand rNMP is embedded in the duplex template-primer at increasing distances upstream of the active site as bypass proceeds. Effects on the insertion efficiency of Pol α and Pol ε are seen for up to four base pairs (Fig. 2A/C). Theoretically, pausing at any of the rNMP or any of the four subsequent positions could slow replication folk progression. A better understanding of the effects of rNMPs in DNA on synthesis should be facilitated by crystal structures of DNA polymerases bound to primer-templates containing rNMPs at various locations as bypass proceeds, as recently accomplished for bypass of a cyclobutane pyrimidine dimer by Pol η [41].

We previously reported that a *pol2-M644G rnh201Δ* double mutant strain accumulates rNMPs in genomic DNA, progresses more slowly through S-phase, has elevated dNTP pools and has an elevated rate of 2–5 bp deletions in repetitive sequences [25]. These data indicate that unrepaired rNTPs incorporated by *pol2-M644G* during replication *in vivo* elicit replicative stress responses and destabilize the nuclear genome. These phenotypes correlate with the difficulty Pols α, δ and ε have in bypassing rNMPs in DNA templates. Nonetheless, the single *rnh201Δ* mutant strain is not sensitive to HU, it grows relatively normal and its dNTP pools are only slightly elevated [25]. These data indicate that unrepaired rNMPs in the nuclear genome by the wild type yeast DNA replicases are tolerated relatively well. To place this tolerance in perspective, we compared the relative bypass efficiencies for rNMPs to published values for 8-oxo-G [27,28], a common lesion generated by oxidative stress and considered to be strongly mutagenic but not particularly cytotoxic. The comparisons (Table 2) reveal that Pol δ and Pol ε can bypass rNMPs at least as efficiently as 8-oxo-G.

The majority of the rNMP bypass efficiency values in Table 2 were determined at dNTP concentrations representative of unstressed cells. These may be minimal estimates, because *rnh201Δ* strains do have slightly elevated dNTP pools [25], and the values in Table 2 for Pol δ and Pol ε demonstrate that relative bypass efficiencies are increased in the presence of "stress-induced" dNTP concentrations. The increased bypass by Pol ε observed at high dNTP concentrations is also consistent with the fact that an exonuclease-deficient derivative of Pol ε copies the rA-containing template-primer 2-fold more efficiently than the exonuclease-proficient Pol ε (Table 2). Collectively, these results suggest that the proofreading exonuclease activity of Pol ε excises dNMPs inserted during rNMP bypass, and that this excision can be prevented either by inactivating the exonuclease or by promoting extension at the expense of excision.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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