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Brief Communication

Ribonucleotide incorporation by yeast DNA polymerase ζ

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ABSTRACT

During replication in yeast, the three B family DNA replicases frequently incorporate ribonucleotides (rNMPs) into DNA, and their presence in the nuclear genome can affect genome stability. This prompted us to examine ribonucleotide incorporation by the fourth B family member, Pol ζ, the enzyme responsible for the majority of damage-induced mutagenesis in eukaryotes. We first show that Pol ζ inserts rNMPs into DNA and can extend primer termini containing 3′-ribonucleotides. We then measure rNMP incorporation by Pol ζ in the presence of its cofactors, RPA, RFC and PCNA and at normal cellular dNTP and rNTP concentrations that exist under unstressed conditions. Under these conditions, Pol ζ stably incorporates one rNMP for every 200–300 dNMPs incorporated, a frequency that is slightly higher than for the high fidelity replicative DNA polymerases. Under damage-induced conditions wherein cellular dNTP concentrations are elevated 5-fold, Pol ζ only incorporates one rNMP per 1300 dNMPs. Functional interaction of Pol ζ with the mutasome assembly factor Rev1 gives comparable rNMP incorporation frequencies. These results suggest that ribonucleotide incorporation into DNA during Pol ζ-mediated mutagenesis *in vivo* may be rare.

1. Introduction

Due to the strict geometry of their active sites and their intrinsic 3′-5′-proofreading activities, replicative DNA polymerases (replicases) rarely misincorporate incorrect deoxynucleotides into DNA. While replicative polymerases also efficiently discriminate against insertion of rNTPs into DNA (reviewed in [1]), rNTPs are present in cellular nucleotide pools at much higher concentrations than dNTPs [2,3], thereby significantly increasing the probability of ribonucleotide incorporation during DNA replication. Moreover, unlike base–base mismatches, newly inserted ribonucleotides (rNMPs) are poorly proofread by DNA polymerases (Pols) δ and ε [4,5]. As a consequence, rNMPs are incorporated during DNA synthesis *in vitro*, as well as during DNA replication *in vivo*, with consequences that can be both beneficial, when used as a signal for mismatch repair, but also deleterious when not properly repaired [6–8].

In studies using physiological rNTP and dNTP concentrations, the frequency of rNMP incorporation by replicative polymerases *in vitro* varies depending on the DNA polymerase, the identity of the ribonucleotide and the local DNA sequence context, as well as on the assay used. In experiments using oligonucleotide DNA substrates, average rNMP incorporation frequencies by Pol δ, Pol ε and Pol α are 1 in 5000; 1 in 1250 and 1 in 625, respectively, with site-to-site variations of more than 10-fold [3]. In an assay that copies a single-stranded plasmid DNA template that more closely resembles condition under which DNA replication occurs, rNMP incorporation frequencies by Pol δ and Pol ε are 1 in 720 and 1 in 640, respectively. These frequencies are not substantially affected by the presence of the accessory factors, i.e. the single-stranded binding protein RPA, the replication clamp PCNA, and its loader RFC [9]. Differences in average ribonucleotide incorporation frequencies between the two assays may reflect sequence context effects and/or potential hotspots for rNMP incorporation.

Pol ζ is a B-family DNA polymerase that has a major role in translesion DNA synthesis (TLS) in eukaryotes and is responsible for the bulk of mutations induced by DNA damage (reviewed in [10]). About half of the spontaneously arising mutations can be ascribed to the participation of Pol ζ during synthesis at stalled DNA replication forks [11]. Pol ζ may also be involved in the repair of DNA double strand breaks [12,13]. Pol ζ-dependent mutagenesis *in vivo* is consistent with its lack of proofreading activity and its ability to efficiently extend mismatched primer termini. Moreover, yeast

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Table 1
dNTP:rNTP discrimination factors for exonuclease-deficient yeast Pols ζ, α and δ.

Nucleotide	Pol ζ	Pol α [b]	Pol δ-exo⁻ [c]
dA: rA	4700	10,000	31,000
dT: rU	1600	8000	20,000
dG: rG	690	3000	4800
dC: rC	470	3800	3000
BPS Error Rate [a]	130×10^{-4}	9.6×10^{-4}	13×10^{-4}

[a] From [14]. BPS, base pair substitution.
[b] From [3].
[c] Determined as describe in [3], but for exonuclease-deficient Pol δ.

Pol ζ generates single base–base mismatches during DNA synthesis *in vitro* at rates that are about ten-fold higher than for naturally proofreading-deficient Pol α or proofreading-deficient variants of Pol δ and Pol ε (Table 1) [14]. This fact indicates that Pol ζ discrimination against insertion of incorrect bases is lower than its B-family siblings. We therefore reasoned that it may also discriminate poorly against rNMPs, and thereby contribute to incorporation in cells, particularly in response to DNA damage when Pol ζ is actively engaged. Indeed, TLS in *Escherichia coli* is accompanied by frequent ribonucleotide incorporation, and if the dNMP/rNMP discrimination capacity of the machinery is reduced, genome stability is negatively affected [15].

Here we have measured the ability of Pol ζ to misincorporate rNMPs into DNA using different types of assay and replication conditions. Surprisingly, our data indicate that Pol ζ discrimination against ribonucleotide incorporation is only slightly lower than for the replicative DNA polymerases. Under TLS conditions, rNMP incorporation is even lower than that occurring during normal DNA replication. Given that Pol ζ likely performs much less DNA synthesis in cells than do the replicases, these results suggest that ribonucleotide incorporation into DNA by Pol ζ may be rare *in vivo*.

2. Materials and methods

2.1. Enzymes

RPA, RFC and PCNA were expressed and purified from *E. coli* [16,17]. The two- and four-subunit forms of *Saccharomyces cerevisiae* Pol ζ were purified as described [18]. Rev1 and Rev1 mutants were produced in *S. cerevisiae* and purified from 50 to 100 g of yeast cells, using a modification of protocols described for Pol ζ [18]. Briefly, proteins from the cell lysate were precipitated with 0.3 g/ml ammonium sulfate and purified by glutathione affinity chromatography, followed by GST-tag removal with prescision protease. After additional heparin-sepharose purification, Rev1, and its mutant forms, were dialyzed overnight against storage buffer: 30 mM Hepes (pH 7.4), 200 mM NaCl, 16% glycerol, 0.05% ampholytes, 1 mM DTT. All buffers except for the storage buffer were supplemented with 1 mM EDTA.

2.2. Measuring discrimination against insertion of an rNTP

The assay was performed as described earlier [3]. Briefly, insertion of dA/rA and dG/rG were analyzed using a substrate made by annealing a ^{32}P-labeled primer strand (5′-CTGCAGCTGATGCGC) to a template strand (5′-GTACCCGGGGATCCGTAC(T/C)GCGCATCAGCTGCAG) that either contained a T or a C at the templating position for the incoming nucleotide. Insertion of dC/rC and dT/rU were analyzed using a substrate made by annealing a 5′-^{32}P-labeled primer strand (5′-CTGCAGCTGATGCGA) to a template strand (5′-GTACCCGGGGATCCGTAC(G/A)TCGCATCAGCTGCAG) that contained a G or an A at the templating position for the incoming nucleotide. Assays contained a single dNTP or rNTP at its measured cellular concentration in yeast under normal (unstressed) growth conditions. Reactions were initiated by adding polymerase, and incubation was at 30 °C. In these experiments, we used the two-subunit (Rev3–Rev7) form of yeast Pol ζ. Polymerase concentrations varied from 0.2 to 10 nM and incubation times varied from 1 to 20 min, in all cases resulting in extension of less than 20% of the initial primer. Reactions were analyzed on a denaturing 15% polyacrylamide gel. Products were detected and quantified using a PhosphorImager and ImageQuaNT software.

2.3. Analysis of rNMP incorporation in primed plasmid DNA

rNMP incorporation during DNA synthesis was performed by a protocol described previously with modifications [9]. The 3 kb single-stranded pSKII plasmid DNA was annealed to primer 682 (5′-TATCGATAAGCTTGATATCGAATTCCT) and used as DNA template for rNMP incorporation assays. Where indicated, the 682 primer was 5′-labeled with [γ-^{32}P]ATP. [α-^{32}P] dATP was added to the reactions as radioactive tracer if non-labeled primer was used. Standard reactions contained 40 mM Tris–HCl, pH 7.8, 0.2 mg/ml BSA, 3% glycerol, 80 mM NaCl, 4 nM primed pSKII DNA, 400 nM RPA, 3 nM RFC, 30 nM PCNA, 10 nM of wild type Rev1 or the indicated mutant form of Rev1, and Pol ζ as indicated in legends to figures. All pSKII replication assays contained the 4-subunit form of Pol ζ. Reactions with only dNTPs contain 8 mM Mg-acetate and 50 μM ATP, to ensure efficient PCNA loading by RFC. Reactions with dNTPs and rNTPs contain 13 mM Mg-acetate. Normal levels of rNTPs and dNTPs were: 16 μM dATP, 14 μM dCTP, 12 μM dGTP, 30 μM dTTP, 3000 μM ATP, 500 μM CTP, 700 μM GTP, and 1700 μM UTP [3]. Under TLS conditions, the dNTP concentrations were increased five-fold [2]. Reactions were assembled on ice and preheated at 30 °C for 30 s. The assay was started by adding Pol ζ alone, or an equimolar mixture of Pol ζ and Rev1 (or a mutant form of Rev1) that had been preincubated on ice for 10 min. Reactions were incubated at 30 °C for 15–120 min, stopped with 0.5% SDS and 20 mM EDTA and heated at 55 °C for 10 min. Where indicated, the reaction was treated with 0.3 M NaOH for 2 h at 65 °C in order to hydrolyze the replicated DNA at rNMP positions, and precipitated with ethanol as described [9]. The samples were analyzed by electrophoresis on a 1–3% alkaline denaturating agarose gels at 1.1 V/cm, at 4 °C for 20 h. Under these conditions, denaturation of dsDNA was complete, but hydrolysis at ribonucleotide positions was negligible. Dried gels were quantified using a Typhoon phosphoimager and ImageJ software. Radioactivity distributions as a function of DNA length were determined. In order to convert the radioactivity distribution into product length distribution, the radioactivity distribution was divided by the length distribution in kb. From this normalized distribution, the median length of alkali-stable DNA was calculated. From this, we calculated the incorporation frequency = $(L^-/L^+ - 1)/L^-$, where L^- and L^+ are median product lengths before and after NaOH treatment, respectively. Alternatively, acid-precipitable radioactivity from incorporated [^{32}P]-dAMP was detected by scintillation counting [19].

2.4. Extension of ribonucleotide-containing primer termini

Standard assays were as described above, except that the SKII 682 primer was 5′-Cy3-labelled 5′-TATCGATAAGCTTGATATCGAATTCCTX-3′ with X either dG or rG, or 5′-TATCGATAAGCTTGATATCGAATTCCX-3′ with X either dT or rU. Reactions were incubated at 30 °C for the indicated times and analyzed on a 14% polyacrylamide-7M urea gel. Quantification was performed using fluorescence imaging on a Typhoon system.