DNA Repair 11 (2012) 649–656

Contents lists available at SciVerse ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Proofreading of ribonucleotides inserted into DNA by yeast DNA polymerase ε

Jessica S. Williams[a,1], Anders R. Clausen[a,1], Stephanie A. Nick McElhinny[a], Brian E. Watts[a], Erik Johansson[b], Thomas A. Kunkel[a,*]

[a] Laboratory of Molecular Genetics and Laboratory of Structural Biology, National Institute of Environmental Health Sciences, NIH, DHHS, Research Triangle Park, NC 27709, USA
[b] Department of Medical Biochemistry and Biophysics, Umeå University, Umeå SE-901 87, Sweden

ARTICLE INFO

Article history:
Received 12 March 2012
Received in revised form 11 May 2012
Accepted 16 May 2012
Available online 8 June 2012

Keywords:
DNA replication
DNA polymerase ε
Ribonucleotides
Exonuclease
Proofreading

ABSTRACT

We have investigated the ability of the 3′ exonuclease activity of *Saccharomyces cerevisiae* DNA polymerase ε (Pol ε) to proofread newly inserted ribonucleotides (rNMPs). During DNA synthesis *in vitro*, Pol ε proofreads ribonucleotides with apparent efficiencies that vary from none at some locations to more than 90% at others, with rA and rU being more efficiently proofread than rC and rG. Previous studies show that failure to repair ribonucleotides in the genome of *rnh201Δ* strains that lack RNase H2 activity elevates the rate of short deletions in tandem repeat sequences. Here we show that this rate is increased by 2–4-fold in *pol2-4 rnh201Δ* strains that are also defective in Pol ε proofreading. In comparison, defective proofreading in these same strains increases the rate of base substitutions by more than 100-fold. Collectively, the results indicate that although proofreading of an 'incorrect' sugar is less efficient than is proofreading of an incorrect base, Pol ε does proofread newly inserted rNMPs to enhance genome stability.

Published by Elsevier B.V.

1. Introduction

Replicative DNA polymerases almost always insert correct deoxynucleoside triphosphates (dNTPs) into correctly aligned primer-templates. When they occasionally do generate mismatches, these can be excised by the 3′–5′ exonuclease activities associated with many replicases, and rare mismatches that escape this proofreading can be corrected by mismatch repair (MMR). Operating in series, polymerase selectivity and the two error correction processes assure high fidelity DNA replication and stabilize the eukaryotic nuclear genome over many generations.

This understanding of high fidelity DNA replication has emerged from studies of base–base and insertion–deletion mismatches. DNA polymerases also discriminate well against inserting an 'incorrect' sugar, *i.e.*, a ribonucleoside triphosphate (rNTP) (reviewed in [1,2]). However they do so imperfectly, and the probability that a rNTP may be inserted is further increased by the fact that cellular rNTP concentrations are much higher than dNTP concentrations [3,4]. In fact, during DNA synthesis *in vitro* in reactions containing the concentrations of dNTPs and rNTPs measured in extracts prepared from asynchronously growing, log phase *Saccharomyces cerevisiae* cells, all three major yeast family B replicases, DNA polymerases α, δ, and ε (Pols α, δ, and ε), incorporate substantial numbers of rNTPs into DNA [5]. In these experiments, one ribonucleotide (rNMP) was stably incorporated for every 625, 5000 or 1250 deoxyribonucleotides (dNMPs) incorporated by Pols α, δ and ε, respectively. Interestingly, Pols δ and ε incorporate rNMPs despite the fact that they have intrinsic 3′–5′ exonuclease activities that are well known to efficiently proofread single base–base mismatches. This and the fact that urepaired ribonucleotides incorporated into DNA result in replicative stress and genome instability [6], motivated the current investigation of whether rNMPs inserted into DNA by Pol ε can be proofread by its intrinsic 3′ exonuclease.

The possibility that ribonucleotides might be proofread by Pol ε is suggested by previous studies of two family B homologs of Pol ε, T4 DNA polymerase [7] and φ29 DNA polymerase [8]. The intrinsic 3′–5′ exonuclease activity of both polymerases can excise ribonucleotides from 3′-termini in primer-template DNA. Moreover, φ29 Pol extends a primer with a terminal rG less efficiently than it extends a primer with a terminal dG [8], thereby potentially increasing the probability of excision rather than extension. This may be important because studies of single base–base mismatches clearly show that the balance between excision and extension determines proofreading efficiency (reviewed in [9,10]). However, neither the φ29 Pol nor the T4 Pol study measured actual proofreading, *i.e.*, excision of a newly inserted ribonucleotide during an ongoing polymerization reaction. Thus, the efficiency with which a base pair containing an incorrect sugar is proofread during DNA synthesis, if at all, is largely unexplored. It is also currently unknown whether failure to proofread newly incorporated rNMPs has biological consequences. Interest in whether ribonucleotides can be

* Corresponding author. Tel.: +1 919 541 2644; fax: +1 919 541 7880.
E-mail address: kunkel@niehs.nih.gov (T.A. Kunkel).
[1] These authors contributed equally to this work.

1568-7864/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.dnarep.2012.05.004

proofread is increased by the demonstration that the other mechanism for correcting replication errors, DNA mismatch repair, does not prevent the genome instability associated with unrepaired ribonucleotides incorporated during DNA replication by Pol ε in yeast [11].

Here we investigate proofreading of ribonucleotides that are incorporated by *S. cerevisiae* Pol ε, which has been inferred to be the primary leading strand replicase [12]. This initial focus on Pol ε is based on the fact that Pol ε incorporates rNMPs during DNA synthesis *in vitro* [5] and *in vivo* [6], and failure to remove these rNMPs due to a defect in RNase H2-dependent repair increases the rate of 2–5 base pair deletions in tandem repeat DNA sequences [6]. Our biochemical and genetic results support the conclusion that during replication by Pol ε, exonucleolytic proofreading can remove newly inserted ribonucleotides and thereby enhance genome stability. We further show that editing an incorrect sugar in DNA is substantially less efficient than editing single base–base mismatches.

2. Materials and methods

2.1. Biochemistry

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

2.2. Polymerases and DNA substrates

Wild type (WT) and exonuclease-deficient *S. cerevisiae* Pol ε were expressed and purified as previously described [13,14]. Oligonucleotide primer-templates (Fig. 1A) were prepared as described [5].

2.3. Extending a ribo-terminated primer terminus

Reaction mixtures contained 50 mM Tris (pH 7.5), 0.1 mg/ml BSA, 2 mM DTT, 8 mM $MgCl_2$, 100 nM primer-template containing either a 3′-deoxy-terminated primer or a 3′-ribo-terminated primer (both labeled with 5′-γ-^{32}P), and the concentrations of dNTPs (16 μM dATP, 30 μM dTTP, 12 μM dGTP, 14 μM dCTP) previously measured in extracts of asynchronous, logarithmically growing budding yeast cells [5]. Reactions were initiated by adding either exonuclease-proficient or exonuclease-deficient Pol ε (10 nM). For this assay, we used the catalytically active 152-kDa N-terminal fragments of the Pol ε catalytic subunit, enzymes that we previously demonstrated [14] are reasonable surrogates for the intact, four subunit Pol ε holoenzymes used for the proofreading assays described below. All components were mixed on ice and incubated for 30 min at 30 °C. Aliquots of reaction products were incubated with either water or 0.3 M KOH for 2 h at 55 °C. An equal amount of formamide loading dye (95% deionized formamide, 25 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol) was added, the mixture was heated to 95 °C for 3 min, and the products were separated by electrophoresis through an 8% denaturing polyacrylamide gel. A phosphoimager and Image quant software (GE Healthcare) were used to visualize and quantify the products.

2.4. Stable incorporation of rNMPs into DNA

Stable incorporation of rNMPs into DNA was assessed as previously described [5]. Reaction mixtures (20 μl) contained 2.0 pmol (100 nM) of a 70-mer template annealed to a 5′-[γ-^{32}P]-labeled 40-mer DNA primer, and 10 nM Pol ε. The dNTP concentrations were as mentioned above, and the rNTP concentrations were rATP, 3000 μM; rCTP, 500 μM; rGTP, 700 μM; and rUTP, 1700 μM, as previously measured in extracts of asynchronous, logarithmically growing budding yeast cells [5].

2.5. Yeast strain construction

S. cerevisiae strains used are isogenic derivatives of strain Δ|(-2)|-7B-YUNI300 (*MATa CAN1 his7-2 leu2-Δ::kanMX ura3-Δ trp1-289 ade2-1 lys2-ΔGG2899-2900* [15]). Relevant strain genotypes are listed in Supplemental Table S1. The *pol2-M644G*, *rnh201Δ* and *pol2-M644G rnh201Δ* strains were described previously [6,12]. The *pol2-4* strain was constructed using the integration-excision method. A wild type strain was transformed with BamHI-linearized pJB1 plasmid (containing a fragment of the *POL2* gene with the D290A and E292A mutations [16]. Following verification of correct integration at the *POL2* locus, *URA3* was excised and *pol2* mutations were confirmed by DNA sequencing. The *pol2-4 rnh201Δ* mutant was generated by deletion–replacement *of RNH201 via* transformation with a PCR product containing the hygromycin-resistance cassette (HYG-R) amplified from pAG32 [17] and flanked by 60 nucleotides of sequence homologous to the intergenic regions upstream and downstream of the *RNH201* ORF. Transformants that arose from homologous recombination were verified by PCR analysis. The *URA3* reporter gene was introduced into the *pol2-4* and *pol2-4 rnh201Δ* strains in either orientation 1 or orientation 2 at position *AGP1* [15] by transformation of a PCR product containing *URA3* and its endogenous promoter flanked by sequence targeting the reporter to *AGP1*.

2.6. Strain growth and phenotypic analysis

Strains were grown in rich medium (YPDA: 1% yeast extract, 2% bacto-peptone, 250 mg/l adenine, 2% dextrose, 2% agar for plates). Spot assays were performed by plating 10-fold serial dilutions of exponentially growing cells onto YPDA in the absence or presence of the indicated concentrations of hydroxyurea (HU; Sigma H8627). Plates were incubated at 30 °C and photographed after 3 days of growth.

2.7. Measurement of spontaneous mutation rates and sequence analysis

Spontaneous mutation rates were measured by fluctuation analysis as described previously [18]. For each 5-FOA-resistant mutant that was sequenced, an independent colony was patched to YPDA and then replica plated to medium containing 5-fluoro-orotic acid (5-FOA). Genomic DNA from a single 5-FOA resistant colony from each patched colony was isolated and the *ura3* gene was PCR-amplified and sequenced. Rates of individual mutation classes were calculated by multiplying the fraction of that mutation type by the total mutation rate for each strain.

3. Results

3.1. rNTP insertion and extension in vitro by wild type and exonuclease-deficient Pol ε

In our initial study of ribonucleotide incorporation by yeast replicases [5], we reported that in reactions containing only one correctly paired dNTP or rNTP present at its cellular concentration, rNTP insertion by exonuclease-proficient Pol ε at a specific template position was lower than dNTP insertion by a factor of 500–6700-fold, depending on the identity of the base. When the