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Unlocking the steric gate of DNA polymerase η leads to increased genomic instability in *Saccharomyces cerevisiae*

Katherine A. Donigan[a,1], Susana M. Cerritelli[b,1], John P. McDonald[a], Alexandra Vaisman[a], Robert J. Crouch[b,*], Roger Woodgate[a,*]

[a] *Laboratory of Genomic Integrity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-3371, USA*
[b] *Program in Genomics of Differentiation, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA*

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ABSTRACT

DNA polymerase η (pol η) is best characterized for its ability to perform accurate and efficient translesion DNA synthesis (TLS) through cyclobutane pyrimidine dimers (CPDs). To ensure accurate bypass the polymerase is not only required to select the correct base, but also discriminate between NTPs and dNTPs. Most DNA polymerases have a conserved "steric gate" residue which functions to prevent incorporation of NMPs during DNA synthesis. Here, we demonstrate that the Phe35 residue of *Saccharomyces cerevisiae* pol η functions as a steric gate to limit the use of ribonucleotides during polymerization both *in vitro* and *in vivo*. Unlike the related pol ι enzyme, wild-type pol η does not readily incorporate NMPs *in vitro*. In contrast, a pol η F35A mutant incorporates NMPs on both damaged and undamaged DNA *in vitro* with a high degree of base selectivity. An *S.cerevisiae* strain expressing pol η F35A (*rad30*-F35A) that is also deficient for nucleotide excision repair (*rad1Δ*) and the TLS polymerase, pol ζ (*rev3Δ*), is extremely sensitive to UV-light. The sensitivity is due, in part, to RNase H2 activity, as an isogenic *rnh201Δ* strain is roughly 50-fold more UV-resistant than its *RNH201*$^{+}$ counterpart. Interestingly the *rad1Δ rev3Δ rad30*-F35A *rnh201Δ* strain exhibits a significant increase in the extent of spontaneous mutagenesis with a spectrum dominated by 1 bp deletions at runs of template Ts. We hypothesize that the increased mutagenesis is due to rA incorporation at these sites and that the short poly rA tract is subsequently repaired in an error-prone manner by a novel repair pathway that is specifically targeted to polyribonucleotide tracks. These data indicate that under certain conditions, pol η can compete with the cell's replicases and gain access to undamaged genomic DNA. Such observations are consistent with a role for pol η in replicating common fragile sites (CFS) in human cells.

1. Introduction

Maintenance of genomic integrity depends on the ability of cells to repair high levels of DNA damage that occur daily. Numerous DNA repair processes have evolved to contend with this damage, but persistent unrepaired lesions encountered by the replication fork can lead to replication stalling and ultimately, to fork collapse and double strand breaks. To circumvent these devastating outcomes, cells can switch to using specialized polymerases that are capable of bypassing a variety of DNA lesions through a process commonly called "Translesion DNA Synthesis" (TLS) [1,2]. After the lesion has been bypassed, the TLS polymerase may synthesize a few additional nucleotides before dissociating to allow the replicative polymerase to continue genome duplication with high fidelity. While TLS allows cells to avoid the immediate consequences of fork stalling, this often comes at the price of mutagenesis. TLS is an error-prone process, as TLS polymerases generally exhibit low fidelity when replicating undamaged DNA [3–5]. The best characterized TLS polymerases belong to the Y-family of DNA polymerases [6]. They lack intrinsic exonuclease activity and are characterized by spacious active sites that accommodate DNA lesions and may facilitate misincorporations on undamaged DNA [7–9].

Abbreviations: pol, polymerase; TLS, translesion DNA synthesis; RER, ribonucleotide excision repair; dNTP, deoxyribonucleoside triphosphate; NTP, ribonucleoside triphosphate; dNMP, deoxyribonucleoside monophosphate; NMP, ribonucleoside monophosphate; RNase H2, ribonuclease H2; CFS, common fragile sites; CPDs, cyclobutane pyrimidine dimers; rA, adenosine mono phosphate.

* Corresponding author. Fax: +1 301 217 5815.
E-mail address: woodgate@nih.gov (R. Woodgate).
[1] Considered joint first authors.

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The Y-family DNA polymerase, pol η plays a critical role in the tolerance of cyclobutane pyrimidine dimers induced by UV radiation [10]. While pol η exhibits extremely low-fidelity on undamaged DNA, it bypasses thymidine dimers with relatively high fidelity by inserting dAMP and extending beyond the lesion [4,11]. No other DNA polymerase has been shown to be as capable of accurate and efficient bypass of these lesions, presumably due to their extremely bulky and distorted structure. Given pol η's important role in lesion bypass and its low fidelity on undamaged DNA, its expression and access to DNA must be tightly controlled. In humans, a deficiency in pol η function leads to a variant form of the cancer-prone syndrome *Xeroderma Pigmentosum*, and up-regulation of pol η in tumors has been linked to chemotherapeutic drug resistance [12,13]. *Saccharomyces cerevisiae* pol η, which is encoded by the *RAD30* gene, has been shown to be solely responsible for bypassing T-T CPDs [14] and deletion of *RAD30* sensitizes cells to UV radiation [15,16].

In addition to its role in TLS after UV damage, pol η has been recently implicated in DNA synthesis through natural replication barriers that create replication stress during S phase genomic duplication [17]. Replication forks stall at chromosomal common fragile sites (CFS), including non-B DNA structures, regions of mononucleotides repeats and low complexity A–T rich sequences [18]. At these sites, pol η replaces the replicative polymerases thereby avoiding genome instability associated with under-replicated DNA regions.

Given the crucial role of pol η in TLS and replication through CFS, it is important to determine not only its fidelity in base selection, but also in dNTP/NTP discrimination, the ability of the enzyme to select the correct dNTP from a nucleotide pool dominated by NTPs [19,20]. Incorporation of NMPs into DNA can lead to strand breaks and ultimately, genomic instability. Mechanisms for NMP removal from genomic DNA, termed ribonucleotide excision repair (RER), have been described in both prokaryotes and eukaryotes [21,22]. In eukaryotes, RER is initiated by RNase H2 nicking of the DNA backbone at the site of NMP incorporation, followed by strand displacement DNA synthesis [23]. RNase H2 knockout and hypomorphic mouse embryos exhibit extremely high levels of ribonucleotides in genomic DNA, which leads to early embryonic lethality [24,25]. An alternative, minor pathway of ribonucleotide excision repair initiated by Topoisomerase I has been described for yeast [26,27]. In cells lacking RNase H2, Top1-catalyzed nicking at single NMPs embedded within DNA results in 2–5 base pair deletions within short tandem repeats and leads to replication stress and genome instability [26].

Many DNA polymerases have an aromatic residue in their active site that is positioned to clash sterically with the NTP's 2′ hydroxyl group [28] . The effectiveness of the steric gate varies among polymerases. We have previously identified critical steric gate residues in *Escherichia coli* pol V (Y11) and human pol ι (Y39) [29–31]. Wild-type pol V and pol ι will readily incorporate NMPs opposite undamaged DNA, and elimination of the steric gate in both enzymes markedly increases their capacity to incorporate NMPs.

In this study, we sought to determine the basis for ribonucleotide discrimination by the TLS polymerase pol η and the cellular consequences of pol η-mediated ribonucleotide incorporation in *S. cerevisiae*. We found that wild-type pol η has a minimal ability to incorporate ribonucleotides on damaged or undamaged DNA, but an F35A steric gate mutant readily incorporates the correct ribonucleotide opposite both undamaged and damaged DNA. We evaluated the biological consequences of an F35A substitution in pol η by assaying UV-sensitivity and spontaneous mutagenesis in the *CAN1* gene in a yeast strain deficient for nucleotide excision repair (NER) and the TLS polymerase pol ζ, in the presence or absence of RNase H2. We found that pol η F35A cells are extremely sensitive to UV radiation and that disruption of RER due to deletion of the *rnh201* gene improves UV-survival considerably, but at the same time significantly increases the overall level of spontaneous mutagenesis. In this background, we observed a unique one base pair deletion mutation signature in a stretch of T nucleotides in the *CAN1* gene, which we hypothesize results from error-prone processing of multiple ribonucleotides incorporated by pol η F35A during regular genome duplication.

2. Materials and methods

2.1. Materials

Ultrapure NTPs were purchased from GE Healthcare (Piscataway, NJ) and dNTPs were purchased from Roche (Indianapolis, IN). All oligonucleotides were synthesized by Lofstrand Laboratories (Gaithersburg, MD) and gel-purified prior to use.

2.2. DNA substrate preparation

Primer oligonucleotides were 5′ radiolabeled with ^{32}P by Lofstrand Laboratories. Annealing reactions included 100 nM of radiolabeled primer and 200 nM of unlabelled template in 50 mM Tris–HCl pH 8.0, 5 mM NaCl, 50 μg/ml bovine serum albumin and 1.42 mM 2-mercaptoethanol. Reactions were heated at 95 °C for 5 min, followed by slowly cooling to room temperature over several hours. The DNA substrates used in this study are shown in Table 1.

2.3. Proteins

Wild-type *S. cerevisiae* pol η was purchased from Enzymax (Lexington, KY) and provided at a concentration of 50 ng/μl. The gene for the full-length pol η F35A variant was codon optimized for optimal expression in *E. coli* and synthesized by Genscript (Piscataway, NJ). The full-length *rad30-F35A* gene was subsequently sub-cloned as a *Sal*I-*Hin*dIII fragment into a proprietary *E. coli* expression vector (Enzymax). The pol η F35A protein was expressed and purified by scientists at Enzymax as a custom service and was provided as aliquots ranging in concentration from 30 to 500 ng/μl. Based upon Coomassie blue staining, the preparation of pol η F35A protein is estimated to be greater than 95% pure.

2.4. Primer extension reactions

Standard reaction mixtures (10 μl) contained 70 nm pol η, 10 nm of radiolabeled DNA substrate, 100 μM nucleotide(s) in 1× reaction buffer (2 mM $MgCl_2$, 50 mM NaCl, 40 mm Tris–HCl pH 8.0, 10 mM dithiothreitol, 250 μg/ml bovine serum albumin, 2.5% glycerol). All DNA substrates were confirmed to be >95% annealed by incubating with wild-type pol η and 100 μM of all four dNTPs. The reactions were incubated at 30 °C for 10 min and quenched by the addition of 10 μl of 95% formamide, 10 mM EDTA solution. The samples were heated to 100 °C for 5 min and resolved on an 18% polyacrylamide, 8 M urea gel. Reaction products were visualized and quantified using a Fuji FLA-5100 Phosphorimager and ImageGauge software.

2.5. Electrophoretic mobility shift assays

The DNA binding constant ($K_{D(DNA)}$) for pol η F35A was determined by a gel electrophoretic mobility shift assay as described with modifications [29]. Serial dilutions of the F35A enzyme ranging from 0.1 nM to 1 μM were incubated with radiolabeled DNA substrate (0.2 nM) in binding buffer (10 mM Tris-HCl pH 7.6, 2 mM $MgCl_2$, 50 mM NaCl, 10% glycerol, 0.1% IGEPAL) for 20 min at 30 °C. Samples were loaded onto a 6% Native TAE PAGE gel that had been