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Ribonucleotide incorporation, proofreading and bypass by human DNA polymerase δ

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ABSTRACT

In both budding and fission yeast, a large number of ribonucleotides are incorporated into DNA during replication by the major replicative polymerases (Pols α, δ and ε). They are subsequently removed by RNase H2-dependent repair, which if defective leads to replication stress and genome instability. To extend these studies to humans, where an RNase H2 defect results in an autoimmune disease, here we compare the ability of human and yeast Pol δ to incorporate, proofread, and bypass ribonucleotides during DNA synthesis. In reactions containing nucleotide concentrations estimated to be present in mammalian cells, human Pol δ stably incorporates one rNTP for approximately 2000 dNTPs, a ratio similar to that for yeast Pol δ. This result predicts that human Pol δ may introduce more than a million ribonucleotides into the nuclear genome per replication cycle, an amount recently reported to be present in the genome of RNase H2-defective mouse cells. Consistent with such abundant stable incorporation, we show that the 3′-exonuclease activity of yeast and human Pol δ largely fails to edit ribonucleotides during polymerization. We also show that, like yeast Pol δ, human Pol δ pauses as it bypasses ribonucleotides in DNA templates, with four consecutive ribonucleotides in a DNA template being more problematic than single ribonucleotides. In conjunction with recent studies in yeast and mice, this ribonucleotide incorporation may be relevant to impaired development and disease when RNase H2 is defective in mammals. As one tool to investigate ribonucleotide incorporation by Pol δ in human cells, we show that human Pol δ containing a Leu606Met substitution in the polymerase active site incorporates 7-fold more ribonucleotides into DNA than does wild type Pol δ.

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1. Introduction

The presence of a ribonucleotide in DNA is potentially problematic because the 2′-oxygen on the ribose renders the DNA backbone susceptible to cleavage and potentially changes the conformation of the sugar pucker. Thus shortly after the discovery of DNA polymerases [1], it became of interest to examine how effectively DNA polymerases prevent ribonucleotide incorporation during DNA synthesis. Numerous studies since then (reviewed in [2,3]) have revealed that discrimination against ribonucleotide incorporation can be high, but varies widely among DNA polymerases and is not absolute. The probability of ribonucleotide incorporation is increased by the fact that in both budding yeast [4] and in mammalian cells [5], ribonucleoside triphosphate (rNTP) concentrations are higher than deoxyribonucleoside triphosphates (dNTPs) concentrations. These facts led us to examine the frequency of stable ribonucleotide incorporation into DNA by the three DNA polymerases that replicate the budding yeast nuclear genome, Pols α, δ, and ε. In reactions containing physiological concentrations of the rNTPs and dNTPs, these replicases incorporate a surprisingly large number of ribonucleotides into DNA [4]. The biological relevance of these ribonucleotides was revealed by deleting the budding yeast *RNH201* gene that encodes the catalytic subunit of RNase H2, the enzyme that initiates removal of ribonucleotides from DNA (see [6,7] and references therein). Budding yeast *rnh201Δ* strains accumulate large numbers of ribonucleotides in their genome and they have several phenotypes characteristic of replicative stress, including genome instability [6–9]. Large numbers of ribonucleotides are also incorporated by Pol ε into DNA in fission yeast, and these are also removed in a RNaseH2-dependent manner [10]. Moreover, Pol ε from budding yeast can proofread incorporated ribonucleotides, albeit not as efficiently as a misincorporated base [11].

The phenotypes of RNase H2-defective yeast may be relevant to the fact that defects in RNase H2 in humans result in Aicardi–Goutières syndrome, a recessive neuroinflammatory condition with similarities to autoimmune diseases [12]. It is therefore

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of interest to know whether the causes and consequences of ribonucleotide incorporation during DNA replication in yeast extend to higher eukaryotes. Of particular relevance here is a recent study demonstrating that more than a million ribonucleotides are present in the genome of RNase H2-defective mouse embryo cells [13]. These could result from failure to completely remove RNA primers from Okazaki fragments and/or from rNMPs incorporated by DNA polymerases during replication. As an initial step toward understanding the origins of ribonucleotides in the mammalian nuclear genome, and their possible relevance to human biology, we describe the ability to incorporate and proofread ribonucleotides during DNA synthesis *in vitro* by human Pol δ, which has been inferred to be the primary lagging strand replicase [14] and which has high fidelity and can efficiently proofread base-base mismatches [15]. We also examine Pol δ bypass of single and multiple consecutive ribonucleotides in DNA templates. We observe pausing during bypass that may contribute to the replicative stress observed in RNase H2-defective cells. Finally, having previously showed that a Leu612Met variant of yeast Pol δ that has lower than normal fidelity [14] also incorporates increased numbers of ribonucleotides into DNA, here we demonstrate that a similar variant in human Pol δ incorporates large amounts of ribonucleotides into DNA. Overall, the results suggest that the biochemical properties of yeast Pol δ regarding ribonucleotide processing are conserved in human Pol δ, which has several implications that are discussed below.

2. Materials and methods

2.1. Materials and reagents

Oligonucleotides were purchased from Oligos Etc. Inc. (Wilsonville, OR). dNTPs and rNTPs were purchased from GE-Healthcare. Yeast Pol δ was purified as described previously [16]. Yeast PCNA was purified as described previously [17]. Proofreading deficient human Pol δ D402A variants (Exo−) were constructed as previously described [18]. Proofreading proficient (Exo+) and proofreading deficient human Pol δ, Pol δ Leu606Met, and PCNA were purified as described previously [19].

2.2. Ribonucleotide incorporation assay

Stable incorporation of rNMPs by yeast and human Pol δ was analyzed using a substrate made by annealing a 40-mer ^{32}P-labeled primer strand (5′-CCAGTGAATTTCTGCAGGTCGACTCCAAA GGTCAACCCGG) to a 70-mer template strand (5′-ATGACCATGATT ACGAATTCCAGCTCGGTACCGGGTT GACCTTTGGAGTCGACCTGCA-GAAATTCACTGG). Reaction mixtures contained 100 nM DNA substrate, and the reaction buffer contained 20 mM Tris (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 90 mM NaCl, 8 mM Mg-acetate, and 400 nM human or yeast PCNA was added to the reaction mixture. Nucleotide substrates were added at cellular concentrations (Table 1) and contained all eight nucleotides. Reactions were initiated by adding 40 nM human or yeast Pol δ. Incubation was at 37 °C for human Pol δ and at 30 °C for yeast Pol δ. Reactions were terminated after 30 min by adding an equal volume of formamide loading dye, and the reaction products were separated in a denaturing 8% polyacrylamide gel. Full-length reaction products identified by exposing the gel to x-ray film were excised and purified as described [4]. Equivalent amounts of recovered products (as determined by scintillation counting) were treated with either 0.3 M KCl or 0.3 M KOH for 2 h at 55 °C. Following addition of an equal volume of formamide loading dye, equivalent amounts of pre- and postexcision samples were analyzed by electrophoresis in a denaturing 8% polyacrylamide gel. Products were detected and quantified using a PhosphorImager and ImageQuaNT software (Molecular Dynamics).

Table 1
Nucleotide concentrations (μM) in budding yeast [4] and human [5].

Base	Yeast		Human	
	dNTP	rNTP	dNTP	rNTP
A	16	3000	24	3200
C	14	500	29	280
G	12	700	5.2	470
T/U	14	1700	37	570

2.3. Bypass efficiency assay

Reaction mixtures contained 100 nM DNA substrate and the reaction buffer contained 20 mM Tris (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 90 mM NaCl, 8 mM Mg-acetate and 400 nM human or yeast PCNA. The dGTP, dCTP, dATP and dTTP concentrations used are shown in Table 1. All components except the polymerase were mixed on ice and then incubated at 30 °C for 2 min. 40 nM of the polymerase was added to initiate the reaction and aliquots were removed at 3, 6, and 9 min. Following addition of an equal volume of formamide loading dye, samples were analyzed by electrophoresis in a denaturing 12% polyacrylamide gel. Products were detected and quantified using a PhosphorImager and ImageQuaNT software (Molecular Dynamics).

3. Results and discussion

3.1. Abundant ribonucleotide incorporation by human Pol δ

Human Pol δ is a heterotetramer comprised of four subunits: the catalytic subunit (p125) and three accessory subunits (p68, p50 and p12). The frequency with which four subunit human Pol δ stably incorporates ribonucleotides into DNA was examined in DNA synthesis reaction mixtures containing human PCNA and the rNTP and dNTP concentrations estimated to be present in mammalian cells (Table 1 from [5]). Yeast Pol δ is a heterotrimer comprised of three subunits: the catalytic subunit (pol3), and two accessory subunits (pol31 and pol32). A parallel reaction was performed using three subunit yeast Pol δ and yeast PCNA, in this case using the rNTP and dNTP concentrations in yeast (Table 1 from [4]). After extending a 5′-^{32}P-end-labeled 40-mer primer hybridized to a 70-mer template (Fig. 1A), full-length products were gel-purified and treated with 0.3 M KCl (control) or with 0.3 M KOH to hydrolyze the DNA backbone at locations where rNMPs are present. The DNA products were separated by electrophoresis in a denaturing polyacrylamide gel and quantified by phosphorimaging. The results (Fig. 1B) show that 1.2 ± 0.1% of the reaction products generated by human Pol δ (lane 2) were sensitive to alkaline hydrolysis. When divided by the 24 positions quantified here (nucleotides 41 through 65, Fig. 1A), the average value is one incorporated ribonucleotide per 2000 deoxyribonucleotides. This ratio is remarkably similar to that for yeast Pol δ (lane 3, 1.3 ± 0.2% total, 1:1800). We conclude that the ability of Pol δ to incorporate ribonucleotides into DNA during synthesis in the presence of physiological nucleotide pools is conserved between budding yeast and humans. In a model wherein human Pol δ performs about 90% of lagging strand replication of the six billion nucleotide nuclear genome, the results in Fig. 1B predict the incorporation of 3,000,000 ribonucleotides by Pol δ into the nascent lagging strand per round of DNA replication. Given the simplicity of the analysis *in vitro* compared to the complexity of replication *in vivo*, it is amazing that Reijns et al. [13] recently reported the presence of >1,000,000 single or di-ribonucleotides per cell in mouse embryos deficient in RNase H2, the enzyme that initiates removal of ribonucleotides incorporated into DNA during replication in yeast [6]. While the study in mice did