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Mismatch repair-independent tandem repeat sequence instability resulting from ribonucleotide incorporation by DNA polymerase ε

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ABSTRACT

During DNA synthesis *in vitro* using dNTP and rNTP concentrations present *in vivo*, yeast replicative DNA polymerases α, δ and ε (Pols α, δ and ε) stably incorporate rNTPs into DNA. rNTPs are also incorporated during replication *in vivo*, and they are repaired in an RNase H2-dependent manner. In strains encoding a mutator allele of Pol ε (*pol2-M644G*), failure to remove rNMPs from DNA due to deletion of the *RNH201* gene encoding the catalytic subunit of RNase H2, results in deletion of 2–5 base pairs in short repetitive sequences. Deletion rates depend on the orientation of the reporter gene relative to a nearby replication origin, suggesting that mutations result from rNMPs incorporated during replication. Here we demonstrate that 2–5 base pair deletion mutagenesis also strongly increases in *rnh201Δ* strains encoding wild type DNA polymerases. As in the *pol2-M644G* strains, the deletions occur at repetitive sequences and are orientation-dependent, suggesting that mismatches involving misaligned strands arise that could be subject to mismatch repair. Unexpectedly however, 2–5 base pair deletion rates resulting from loss of *RNH201* in the *pol2-M644G* strain are unaffected by concomitant loss of *MSH3*, *MSH6*, or both. It could be that the mismatch repair machinery is unable to repair mismatches resulting from unrepaired rNMPs incorporated into DNA by M644G Pol ε, but this possibility is belied by the observation that Msh2–Msh6 can bind to a ribonucleotide-containing mismatch. Alternatively, following incorporation of rNMPs by M644G Pol ε during replication, the conversion of unrepaired rNMPs into mutations may occur outside the context of replication, e.g., during the repair of nicks resulting from rNMPs in DNA. The results make interesting predictions that can be tested.

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1. Introduction

Most DNA polymerases are relatively efficient at excluding ribonucleoside triphosphates (rNTPs) from being incorporated into DNA during DNA synthesis *in vivo* ([1–4] and references therein). Nonetheless, rNTP exclusion by DNA polymerases is not absolute, and the concentrations of rNTPs in cells are much higher than the concentrations of dNTPs [3,5,6]. These facts imply that some rNTPs will be incorporated into DNA *in vivo*, and that they will eventually be removed to maintain the chemical identity of organisms whose genomes are comprised of DNA. In support of these possibilities, we recently reported [7] that rNTPs are incorporated during replication by *Saccharomyces cerevisiae* DNA polymerase ε (Pol ε), a putative leading strand replicase [8,9]. We also reported that these rNMPs are efficiently removed from DNA by RNase H2-dependent repair. However, in yeast strains harboring a mutator allele (*pol2-M644G*) encoding a form of Pol ε with a 10-fold higher than normal propensity to incorporate rNMPs into DNA, deletion of the *RNH201* gene encoding the catalytic subunit of RNase H2 results in replication stress and genome instability [7]. This genome instability primarily involves the deletion of 2–5 base pairs in short repetitive DNA sequences. Interestingly, many of these deletions occurred at locations where the mutation rate is high only when the *URA3* reporter gene is in one of its two possible orientations relative to the nearest origin of replication. This orientation bias, and the fact that the mutagenesis is observed in the *pol2-M644G* strain encoding a mutator derivative of the putative leading strand replicase, raises interesting questions about DNA replication fidelity, but in this case for discrimination against the sugar rather than discrimination against an incorrect or misaligned base.

The first studies to demonstrate that deletion of the gene encoding the catalytic subunit of RNase H2 was mutagenic [10,11] were performed in strains encoding wild type DNA polymerases. The

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mutagenesis observed in those studies was suggested to reflect aberrant processing of rNMPs incorporated by the Pol α–associated RNA primase that initiates Okazaki fragments, and the increase in overall mutation rate was small. In strains harboring the *pol2-M644G* allele, loss of RNase H2 activity was much more mutagenic, suggesting that the mutagenesis depends on unrepaired rNMPs incorporated during leading strand replication by the M644G mutator derivative of Pol ε. However, the same study also confirmed the earlier work [10,11], by demonstrating that deleting *RNH201* in strains with wild type polymerases increased mutation rates by only about 2-fold at each of three different reporter loci in yeast. Thus, existing evidence leaves open the question of whether unrepaired rNMPs incorporated during replication by wild type replicative DNA polymerases, rather than rNMPs incorporated by RNA primase, are in fact mutagenic. Here we address this question by defining specific mutation rates in *rnh201Δ* strains encoding wild type replicases. We find that 2–5 base pair deletion rates in repetitive sequences are elevated an average of 65-fold, and the site specificity of mutagenesis recapitulates that seen in the *pol2-M644G rnh201Δ* strains. These data strongly support the conclusion that rNMPs incorporated during normal DNA replication are highly mutagenic if not repaired by RNase H2.

How are unrepaired rNMPs in DNA converted into deletions? In the field of cancer research, the deletion of repeat units from repetitive DNA sequences is called microsatellite instability (MSI), and MSI is an established hallmark of tumors with defects in repair of errors arising by strand misalignments that occur during DNA replication (reviewed in [12–14]). The 2–5 base pair deletions in repetitive sequences observed in the *pol2-M644G rnh201Δ* strains [7] can be considered a specialized form of this type of misalignment mutagenesis (see further discussion below). Theoretically, the deletions could result from rNMP incorporation during replication, followed by strand slippage during the next round of replication as a DNA polymerase attempts to bypass the rNMP that remains in the template strand because RNase H2 is absent. In this model, a mismatch containing a 2–5 base loop in the template strand should arise that is stabilized by the correct base pairs possible at repetitive sequences [15,16]. Such deletion mismatches are predicted to be subject to MMR. Perhaps especially important is MMR initiated by Msh2–Msh3 (MutSβ), which has evolved to repair loop mismatches in this size range (reviewed in [17–19]). Here we test this prediction by comparing the rates of 2–5 base pair deletions in *pol2-M644G rnh201Δ* strains that are proficient in MMR to strains that are deficient in MMR repair. The results show that loss of MMR has little effect on the 2–5 base pair deletion rate. This suggests that either MMR does not repair deletion mismatches promoted by rNMPs incorporated into DNA by M644G Pol ε, or that the deletion mismatches are formed during a DNA transaction that is not subject to MMR, e.g., during processing of rNMPs remaining in DNA when RNase H2 is defective.

2. Materials and methods

2.1. Construction of yeast strains

S. cerevisiae strains were isogenic derivatives of strain Δ|(-2)|-7B-YUNI300 (*MAT*a *CAN1 his7-2 leu2-*Δ::*kan*MX *ura3-*Δ *trp1-289 ade2-1 lys2-*Δ*GG2899-2900*) [20]. The *rnh201Δ, pol2-M644G* and *pol2-M644G rnh201Δ* strains have been described previously [7]. The *msh3Δ* variants were constructed by deleting and replacing *MSH3* via transformation with a PCR product containing the nourseothricin resistance cassette (NAT-R) amplified from pAG25 [21] and flanked by 50 nucleotides of sequence homologous to the intergenic regions upstream and downstream of the *MSH3* open reading frame. Transformants that arose by homologous recombination containing the replacement of the *MSH3* open reading frame with NAT-R were isolated on YPDA plates containing 100 μg/ml NAT and verified by PCR analysis and DNA sequencing of genomic DNA. To construct *msh6Δ* and *msh3Δ msh6Δ*, strains, diploid yeast strains were first made heterozygous for *MSH6* (Supplementary Table 1, strains d031 and d032). *MSH6* was removed from diploid cells (ySNM1001 and ySNM1005 [9]) via transformation with a PCR product containing the *TRP1* gene flanked by sequences homologous to 200 nucleotides of intergenic regions upstream and downstream of the *MSH6* open reading frame. Transformants were isolated on complete synthetic media plates lacking tryptophan and verified by PCR analysis to have replaced *MSH6* with *TRP1* via homologous recombination. Dissection of meiotic tetrads confirmed that only one copy of *MSH6* had been deleted. Mating type α (mat α) haploids resulting from these dissections (strains h031 and h032) were mated on YPDA with *pol2-M644G* mating type a (mat a) haploid yeast (above; ySNM70 and ySNM77 [8]). Diploids (nonmaters; strains d231.5 and d232.5) were sporulated and underwent meiotic tetrad dissection. Small samples of *TRP1*$^+$ spore colonies generated (lacking *MSH6*) were taken and the remainder of each colony was replica plated to media containing 5-fluoro-orotic acid (5-FOA). Genomic DNA was isolated from samples corresponding to colonies with elevated mutation rates and the *pol2-M644G* mutation was confirmed by sequencing. Mat α isolates of the *pol2-M644G msh6::TRP1* haploid strains generated (strains h231 and h232) were mated with mat a *pol2-M644G msh3::NAT rnh201::HYG* haploids (above; ABC2001 and ABC2003). Transformants bearing all three markers were selected. The resulting diploids (strains d281 and d282) were homozygous for *pol2-M644G* and heterozygous for *msh6Δ*, *msh3Δ*, and *rnh201Δ*. These were sporulated and meiotic tetrads were dissected, resulting in *pol2-M644G* haploid cells with all combinations of the presence or absence of *MSH6*, *MSH3*, and *RNH201*, spore colonies of which (strains h271, h272, h281, and h282) were used to determine mutation rates and to generate mutation spectra.

2.2. Measurement of spontaneous mutation rates and sequence analysis

Spontaneous mutation rates were measured by fluctuation analysis as described previously [22]. For each *ura3* mutant that was sequenced, an independent colony was patched to YPDA and then replica plated to media 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.

2.3. MutSα binding to a ribo-containing mismatch.

Enzyme mobility shift assays were performed as described, using yeast MutSα purified as previously described [23]. Oligonucleotide heteroduplexes were created by annealing radiolabeled dTCGTTTTACAACGTCGTGAA**T**GAGAAAACCCTGGCGTTACC with unlabeled dGGTAACGCCAGGGTTTTCTC**G**TTCACGACGTTGTAAAACGA or dGGTAACGCCAGGGTTTTCTC**rG**TTCACGACGTTGTAAAACGA (Dharmacon). Bold and underlined bases define the residues creating the rG · dT and dG · dT mispairs. Binding reaction mixtures (20 μl) containing 1 nM heteroduplex and 250, 50 or 10 nM of MutSα were incubated at 25 °C for 10 min. Bound substrate was separated from unbound substrate using a nondenaturing 5% polyacrylamide TBE gel (BioRad) run at at 100 V with 0.5X TBE buffer at 4 °C. Following electrophoresis, gels were dried and exposed to a phosphor screen.