Ribonucleotides embedded in yeast genomic DNA are targets of RNase H2 and the nucleotide excision repair system

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Ribonucleotides embedded in yeast genomic DNA are targets of RNase H2 and the nucleotide excision repair system

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This thesis is dedicated to my family, who has always offered me unconditional love and support, not only throughout the course of this study, but also throughout my entire life.

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SUMMARY

Increasing evidence suggests that ribonucleotides may represent one of the most common non-standard nucleotides found in genomic DNA. Therefore, it is important to understand the extent to which ribonucleotides alter genomic integrity and the cellular mechanisms that are responsible for removing them. We developed oligonucleotide-driven gene correction assays in the yeast *Saccharomyces cerevisiae* to show that, if not removed, mispaired and paired ribonucleotides embedded in genomic DNA serve as templates for DNA synthesis and could cause genetic change. We found that RNase H type 2 targets single paired and mispaired ribonucleotides, as well as a stretch of two or three ribonucleotides embedded in DNA, and the nucleotide excision repair system can target single paired ribonucleotides as damage.

CHAPTER 1

INTRODUCTION

1.1 Background

The presence of ribonucleotides in genomic DNA poses a threat to the genomic integrity of cells, frequently resulting in deleterious mutations and even cell death. Numerous studies suggest that ribonucleotides may represent one of the most abundant non-standard nucleotides found in genomic DNA. During DNA replication and repair, DNA polymerases[1-7] and DNA primases[8,9] often incorporate ribonucleotides into DNA. In addition, oxidative damage of DNA can convert a deoxyribonucleotide into a ribonucleotide[10]. Yeast replicative DNA polymerases incorporate ribonucleotides into genomic DNA at frequencies of approximately two per kilobase pair, making ribonucleotides the most abundant form of potential DNA damage in the cell[11]. In comparison to deoxyribonucleotides, ribonucleotides have a reactive 2' hydroxyl on the sugar group that renders the DNA backbone more susceptible to strand cleavage[11]. This can distort the double helix DNA backbone[12], resulting in genome instability[13], defective replication[14] or transcription[13], and mutagenesis[13]. To maintain genomic integrity, yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) cells utilize several DNA repair mechanisms, including the ribonuclease (RNase) H class of enzymes[14], such as RNase H type 2 (RNase H2), and the nucleotide excision repair (NER) system[15]. Defects in RNase H type 2 are associated with the neuroinflammatory disorder, Aicardi Goutiéres Syndrome[16], and defects in the NER system are associated with the rare genetic disorders, xeroderma pigmentosum, trichothiodystrophy, and Cockayne syndrome[17].

Storici et al. (2007) showed that RNA can serve as a template for DNA repair during double-strand break (DSB) repair and directly transfer genetic information to genomic DNA in

yeast[18]. Utilizing the method demonstrated by Storici et al. (2007), we developed oligonucleotide-driven gene correction assays in *S. cerevisiae* to demonstrate that, if not removed, mispaired and paired ribonucleotides embedded in genomic DNA serve as templates for DNA synthesis during DSB repair and could cause a genetic change. *In vitro*, RNase H2 has been shown to specifically target and cleave isolated ribonucleotides embedded in genomic DNA even when mispaired[19,20]. However, until now, a detailed study of the *in vivo* substrate specificity of RNase H2 has been missing. The NER system has been found to remove a variety of bulky DNA lesions that distort the double helix DNA backbone[15]; however, until now, no studies have analyzed whether the NER system also targets ribonucleotides embedded in genomic DNA as damage.

1.2 Specific Aims

The specific aims of this study were to analyze the *in vivo* substrate specificity of RNase H2 and to determine whether the NER system can target ribonucleotides as damage.

1.3 Hypothesis

RNase H2 targets paired and mispaired ribonucleotides embedded in the genomic DNA of *S. cerevisiae* during DSB repair, and the NER system targets ribonucleotides embedded in the genomic DNA of *S. cerevisiae* as damage during DSB repair.

1.4 Literature Review

In 2012, Reijns et al. discovered that RNase H2 is required for the proper embryonic growth and development of mice[16]. In the absence of RNase H2, mice embryos accumulate more than one million ribonucleotides per cell embedded in their genomic DNA, which results in genome instability and a p53-dependent DNA damage response[16]. Wahba et al. (2011) showed that yeast DNA and RNA hybrids often form naturally due to transcriptional errors, but they are typically removed by RNase H1 and RNase H2[21]. Cells that were defective in transcriptional repression, RNA degradation, and RNA export showed increased formation of RNA:DNA hybrids[21]. Lazzaro et al. (2012) demonstrated that the accumulation of ribonucleotide monophosphates into the genome causes replication stress and leads to toxic consequences, particularly in the absence of RNase H1 and RNase H2[22].

In *S. cerevisiae,* two DNA repair mechanisms, the mismatch repair system and RNase H2, compete to remove single mispaired ribonucleotides embedded in genomic DNA[13]. Shen et al. (2011) observed that, in the absence of mismatch repair and RNases H, ribonucleotide-driven gene modification increased by a factor of 47 in yeast[13]. Furthermore, Shen et al. discovered that RNase H2 specifically targets mispaired ribonucleotides in *S. cerevisiae*[13]. However, it is still unclear whether the NER system targets ribonucleotides embedded in genomic DNA as damage.

CHAPTER 2

MATERIALS AND METHODS

We utilized four strains of *S. cerevisiae*, including the wild type (WT) (genotype: *alf* Δ*ho MAT*α-*inc* Δ*mata*::*hisG* Δ*hml*::*ADE1* Δ*hmr*::*ADE1 ade1 leu2-3,112 lys5 trp1*::*hisG ura3-52 ade3*::*GAL-HO leu2*::HO_{cs}), *rnh201*-Δ, *rad14*-Δ, and *rnh201*-Δ*rad14*-Δ. Since Rnh201 is the catalytic subunit of RNase H2[19], and Rad14 is a damage recognition protein in the NER system[23], the *rnh201*-Δ mutant cells were deficient in RNase H2 function, *rad14*-Δ mutant cells were deficient in the NER system, and the *rnh201*-Δ*rad14*-Δ double mutant cells were deficient in both DNA repair pathways.

First, we transformed Leucine auxotrophic (Leu^-) *S. cerevisiae* cells to Leucine prototrophic (Leu^+) cells using one DNA-only oligonucleotide and four RNA-containing oligonucleotides. In our WT strain, the *LEU2* gene was disrupted with an HO endonuclease recognition site, thus generating Leu^- cells. Next, we created a double-strand break at the HO site using the galactose-inducible HO endonuclease. We then transformed these cells using oligonucleotides as templates for DNA repair synthesis of the *leu*2 locus, thus removing the HO site, restoring a functional *LEU2* gene and generating Leu^+ cells (Figure 1). The DSB repairing oligonucleotides contained a site with a single, two, or three ribonucleotides, and also contained a silent point mutation (A → G transition) in the vicinity of the ribonucleotide site that, if transferred to chromosomal DNA, resulted in the creation of the *Stu*I restriction enzyme site, which served as marker for DSB repair by the oligonucleotides (Figure 1). Second, we amplified the *leu*2 locus by polymerase chain reaction (PCR) using primers upstream and downstream from the DSB site and external to the sequence of the repairing template oligonucleotides. Third, we digested the resultant PCR products with the *Stu*I restriction enzyme. If the PCR products

were cut by the *Stu*I restriction enzyme, then the oligonucleotides were used as templates for repair of the *leu*2 locus, and the ribonucleotides were not removed by another DNA repair mechanism. Lastly, we calculated the percentages of *Stu*I site cut and performed the Mann-Whitney U Test between the four strains of *S. cerevisiae* and the WT for each oligonucleotide.

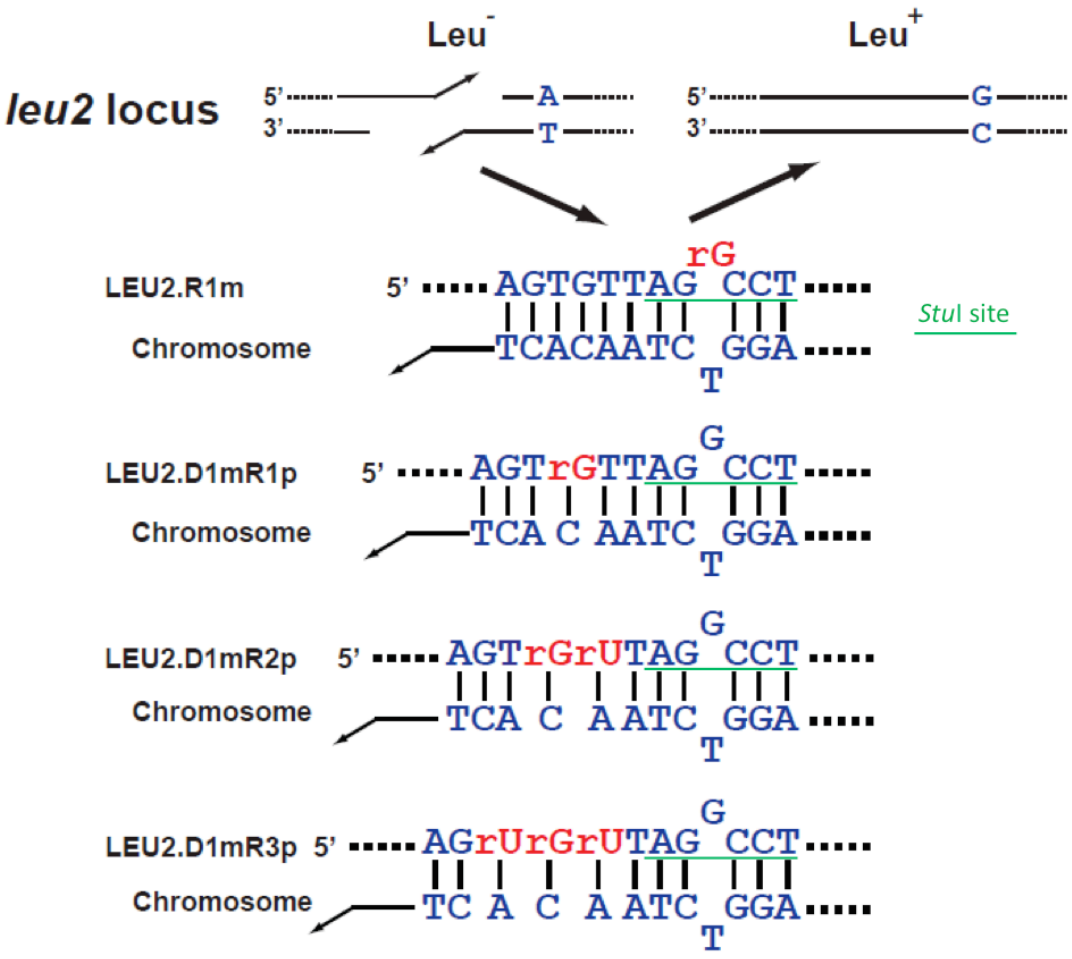

Figure 1. Scheme of chromosomal DSB repair by RNA-containing oligonucleotides. Schematic representation of the DSB repair at the *leu*2 locus with the four RNA-containing oligonucleotides in which rG either forms a single mispair with genomic DNA (LEU2.R1m), a single pair with the genomic DNA (LEU2.D1mR1p), a pair in a stretch of rGrU with the genomic DNA (LEU2.D1mR2p), or a pair in a stretch of rUrGrU with the genomic DNA (LEU2.D1mR3p).

CHAPTER 3

RESULTS

We found statistically significant differences between the percentage of *Stu*I cut Leu^+ transformants of the WT and the *rnh201*-Δ mutant strain as well as the *rnh201*-Δ*rad14*-Δ double mutant strain for the LEU2.R1m oligonucleotide. We also discovered statistically significant differences between the percentage of *Stu*I cut Leu^+ transformants of the WT and the *rnh201*-Δ mutant strain, the *rad14*-Δ mutant strain, and the *rnh201*-Δ*rad14*-Δ double mutant strain for the LEU2.D1mR1p oligonucleotide. In addition, we determined statistically significant differences between the percentage of *Stu*I cut Leu^+ transformants of the WT and *rnh201*-Δ mutant strain as well as the *rnh201*-Δ*rad14*-Δ double mutant strain for the LEU2.D1mR2p oligonucleotide. Furthermore, we found statistically significant differences between the percentage of *Stu*I cut Leu^+ transformants of the WT and *rnh201*-Δ mutant strain as well as the *rnh201*-Δ*rad14*-Δ double mutant strain for the LEU2.D1mR3p oligonucleotide.

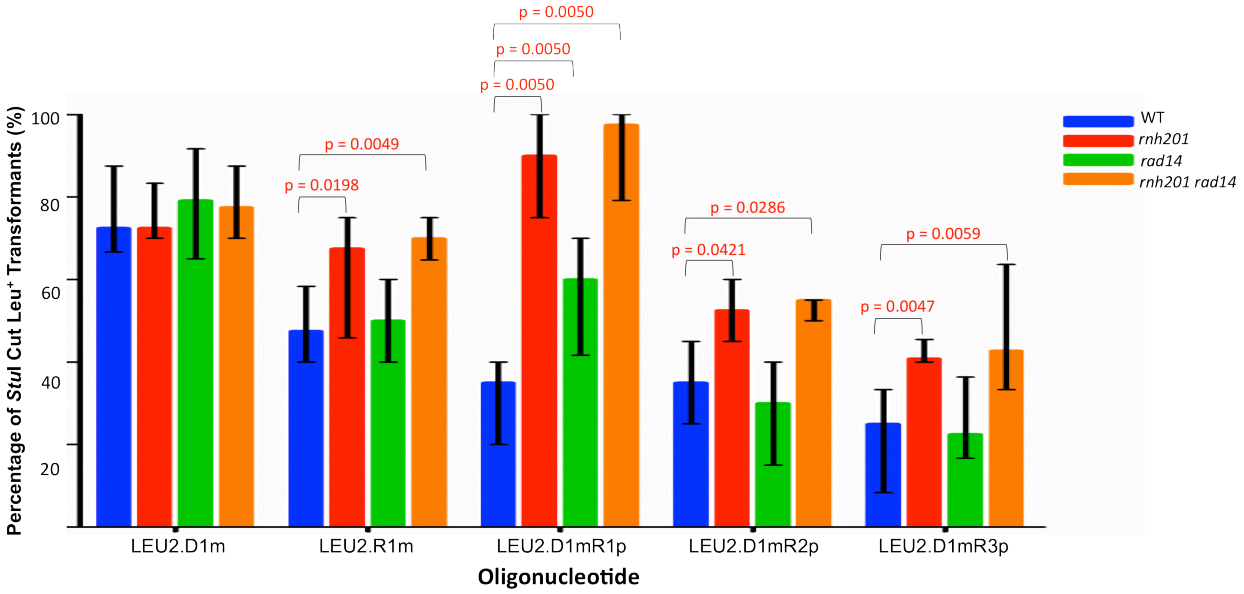

Figure 2. Percentage of *Stu*I cut Leu$^+$ transformants. P-values were calculated by performing the Mann-Whitney U Test against the WT. P-values shown in red were statistically significant according to a 95% confidence interval.

	Percentage of *StuI* Cut Leu$^+$ Transformants			
	WT	*rnh201*-Δ	*rad14*-Δ	*rnh201*-Δ*rad14*-Δ
LEU2.D1m	73%(67-88) NA	73%(70-83) p = 1.0000	79%(65-92) p = 0.7715	78%(70-88) p = 0.5566
LEU2.R1m	48%(40-58) NA	68%(46-75) p = 0.0198	50%(40-60) p = 0.7384	70%(65-75) p = 0.0049
LEU2.D1mR1p	35%(20-40) NA	90%(75-100) p = 0.0050	60%(42-70) p = 0.0050	98%(79-100) p = 0.0050
LEU2.D1mR2p	35%(25-45) NA	53%(45-60) p = 0.0421	30%(15-40) p = 0.4651	55%(50-55) p = 0.0286
LEU2.D1mR3p	25%(8-33) NA	41%(40-45) p = 0.0047	23%(17-36) p = 0.6825	43%(33-64) p = 0.0059

Table 1. Percentage of *Stu*I cut Leu$^+$ transformants. Median percentages of *Stu*I cut Leu$^+$ clones from 80–120 independent clones deriving from 2-4 independent transformations are shown with the ranges. P-values were calculated by performing the Mann-Whitney U Test against the WT. P-values shown in red were statistically significant according to a 95% confidence interval. LEU2.D1m was the DNA-only control. Transformation with no oligonucleotide yielded no Leu$^+$ colonies.

CHAPTER 4

DISCUSSION

Since the results indicated a statistically significant difference between the percentage of *Stu*I cut Leu^+ transformants of the WT and the *rnh201*-Δ mutant for each RNA-containing oligonucleotide, we conclude that RNase H2 targets single mispaired ribonucleotides (rG/T), single paired ribonucleotides (rG/C), as well as a stretch of two or three ribonucleotides (rGrU/CA and rUrGrU/ACA) embedded in the genomic DNA of *S. cerevisiae* during DSB repair. In addition, since the results indicated a statistically significant difference between the WT and the *rad14*-Δ mutant for the LEU2.D1mR1p oligonucleotide, we conclude that the NER system targets single paired ribonucleotides embedded in the genomic DNA of *S. cerevisiae* (rG/C) during DSB repair. In summary, the results of this study support the hypothesis that RNase H2 targets paired and mispaired ribonucleotides embedded in the genomic DNA of *S. cerevisiae* during DSB repair, and the NER system targets isolated ribonucleotides embedded in the genomic DNA of *S. cerevisiae* as damage during DSB repair. This study is the first to show that RNase H2 targets rG/C pairs *in vivo*. This study is also the first to demonstrate that the NER system can recognize ribonucleotide-induced distortion of the DNA double helix backbone as damage. Since distortions of the DNA double helix backbone differ depending on the sequence context, it would be interesting to study the substrate specificity of the NER system for ribonucleotides.

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