RIBONUCLEOTIDES IN YEAST GENOMIC DNA ARE TARGETS OF RNASE H2 AND NUCLEOTIDE EXCISION REPAIR

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RIBONUCLEOTIDES IN YEAST GENOMIC DNA ARE TARGETS OF RNASE H2 AND NUCLEOTIDE EXCISION REPAIR

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[To the students of the Georgia Institute of Technology]
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SUMMARY

Ribonucleotides can be incorporated into the yeast genome through a variety of mechanisms, including through DNA polymerazation, DNA priming, and oxidative damage. Ribonucleotides contain a reactive 2’ hydroxyl group on the sugar, which can distort the DNA double helix and lead to defective replication and transcription and ultimately mutagenesis. Ribonucleotide excision repair (RER) has been found to remove ribonucleotides through the enzyme RNase H2, though the in vivo substrate specificity is not known. Nucleotide excision repair (NER) removes bulky lesions formed in DNA, however its role in the extraction of ribonucleotides has not yet been determined in eukaryotes. Previously developed oligonucleotide-driven gene correction assays in *Saccharomyces cerevisiae*, or baker’s yeast, have shown that paired and mispaired rNMPs embedded into genomic DNA, if not removed, serve as templates for DNA synthesis and can result in a genetic alteration. We implemented this assay to examine whether RNase H2 and NER can target specific rNMPs in DNA. Our results deliver new evidence that RNase H2 specifically recognizes isolated paired and mispaired rNMPs embedded in yeast genomic DNA and that the NER mechanism can recognize an isolated paired rNMP as damage during DNA double-strand break repair in yeast.
CHAPTER 1
INTRODUCTION

Ribonucleotides are the building blocks of RNA, which are a group of molecules that drive the transcription, translation, and regulation of genes [1]. Ribonuclease H’s (RNase H) remove ribonucleotides that have been mistakenly integrated into the DNA chromosome, which is a process essential to stabilizing the genome due to the presence of a reactive 2’ hydroxyl group on the ribose sugar of a ribonucleotide [2]. There are two types of RNase H enzymes, RNase H1 and H2, the latter being the key player in ribonucleotide excision repair (RER) in eukaryotes [4]. Mutations in any of the three RNase H2 subunits (rnh201, rnh202, and rnh203) cause a serious neurological disorder called Aicardi-Goutières syndrome (AGS) [5]. AGS occurs rarely, however due to its pathogenic resemblance to diseases such as lupus and congenital HIV, it is predicted that understanding the genetics behind AGS could explain the pathogenesis for these other disorders as well [6].

In this study, we will determine the in vivo substrate specificity of RNase H2 in RER and also whether RAD14, a key protein in eukaryotic nucleotide excision repair, which focuses on removing DNA bulky lesions, can remove ribonucleotides as well. Previous studies have shown in vitro substrate specificities of RNase H2 in which the enzyme excises only single ribonucleotides and where RNase H1 excises stretches of ribonucleotides [7]. However, no in vivo studies have been performed to support or refute this data. Also it is not yet known whether eukaryotic nucleotide excision repair can recognize ribonucleotides in addition to DNA bulky lesions [8,12]. We hypothesize that
*in vivo*, both RNase H2 and *RAD14* are able to excise paired and mispaired ribonucleotides from the genome.
CHAPTER 2
LITERATURE REVIEW

Ribonucleotides are integrated in the DNA during replication or repair, but as ribose is a significantly more unstable molecule compared to deoxyribose due to its 2’ reactive hydroxyl group, they increase the rate of short deletions in the DNA by sensitizing the DNA to enzymatic nicking [2, 3]. Ribonucleotides also alter the double helix structure of DNA causing a change in DNA conformation from the B form, with equally available major and minor grooves in the double helix, to the A form, with a deep major groove in the double helix which could potentially affect DNA polymerase ε’s ability to synthesize new DNA from the parental strand [9, 10]. DNA polymerases have been found to incorporate ribonucleotides at a rate of two ribonucleotides/kb, making this occurrence the predominant form of potential DNA damage in the cell [4]. Therefore, without proper extraction of these ribonucleotides, the cell could face major complications during DNA replication and repair. This extraction is carried out by a mechanism called ribonucleotide excision repair (RER) and the main protein involved in eukaryotic RER is RNase H2 [4].

In RER, RNase H2 makes a single-strand nick on the 5’ end of the ribonucleotide, leaving a 3’ OH upstream of the ribonucleotide that DNA polymerase can polymerize to. When this occurs, the strand containing the ribonucleotide is displaced, creating a flap. FEN1 endonuclease arrives and cuts this flap and finally DNA ligase attaches the two ends together [4]. In an in vitro study, RNase H2 has been shown to excise only single-embedded ribonucleotides in the genome, whereas RNase H1 excises ribonucleotides in stretches of four or more [7]. However no studies have shown the substrate specificities of RNase H’s in vivo. Another DNA repair mechanism in the cell is nucleotide excision repair, or NER. NER removes bulky lesions in the DNA that is mostly commonly formed
due to ultraviolet exposure [8]. These bulky lesions primarily consist of cyclobutane pyrimidine dimers (CPD), however NER has been found to potentially recognize any damage that causes major distortion of the double helix [11]. In NER, the DNA is incised on both sides of the lesion and removes a 25-30 nucleotide-long fragment, which is followed by repair synthesis and ligation by DNA polymerase and ligase. NER has recently been discovered to play a role in the removal of ribonucleotides in prokaryotic genomes, but it has not yet been uncovered whether NER proteins in eukaryotes, such as its key protein RAD14, also play a role in eukaryotic ribonucleotide excision repair [12, 13].

When genes encoding RNase develop a mutation that affects RNase H’s function, this can lead to a serious neurological disorder called Aicardi-Goutières syndrome (AGS), which has symptoms resembling a brain viral infection [5]. AGS can cause early progressive encephalopathy, intercerebral calcifications, and elevated levels of white blood cells and interferon-α in the cerebrospinal fluid [4, 5]. Children having this disorder exhibit lack of progression in motor, language, and social proficiency. AGS does not have a cure at this time and method of pathogenicity is still uncertain [14]. AGS is a very rare genetic disorder, but due to its pathogenic similarity to acquired autoimmune diseases such as lupus and congenital HIV, it is projected that illustrating the genetics behind AGS will ultimately elucidate the pathogenesis of the aforementioned disorders [6]. The most well-known disorder that is associated with defective NER is Xeroderma Pigmentosum (XP), an autosomal recessive disease in which the patients afflicted are dangerously sensitive to sunlight that make them highly susceptible to skin cancer, while exhibiting other neurological defects as well [11]. Other disorders associated with NER deficiency include Cockayne syndrome (CS), a severe neurological and developmental disorder, and trichothiodystrophy (TTD), a rare autosomal recessive disorder in which the symptoms can range from brittle hair to considerable developmental defects and high child mortality [15].
In our study, we will determine the \textit{in vivo} substrate specificity of RNase H2 in RER and also whether \textit{RAD14}, the main protein in nucleotide excision repair, can remove ribonucleotides as well. We hypothesize that both RNase H2 and \textit{RAD14} are able to remove paired and mispaired ribonucleotides from the yeast genome.
CHAPTER 3

MATERIALS AND METHODS

Step 1:

*S. cerevisiae* strains used in this study and their genotype are listed in Table 1. First, we transformed Leu' yeast cells, which have a *leu2* disrupted by a HO cut-site, with RNA-containing oligonucleotides by inducing a double-strand break (DSB) at the locus by expression of HO endonuclease under the control of *pGAL*. To restore this site, we introduced paired and mispaired ribonucleotide-embedded oligonucleotides into the genome to function as a template for DSB repair. When the HO cut-site was repaired using these oligonucleotides, a *StuI* restriction enzyme site was created in the *LEU2* gene. This site assists us in determining whether RNase H type 2 and/or *RAD14* play a role in removing ribonucleotides from DNA. The oligonucleotides used for DSB repair include one containing only DNA nucleotides, another containing a mispaired ribonucleotide within the *StuI* site sequence, another containing a paired ribonucleotide near the *StuI* site, another containing a stretch of two ribonucleotides near the *StuI* site, and lastly one containing a stretch of three ribonucleotides near the *StuI* site (Figure 1).

Four mutant strains of *Saccharomyces cerevisiae* were used: wild-type, *rnh201*, *rad14*, and *rnh201 rad14*. The wild-type is used as the control, the *rnh201* mutant cannot form RNase H2 proteins, the *rad14* mutant cannot form *RAD14* proteins, and the double mutant is not able to form either RNase H2 or *RAD14*, which is used to determine whether there is a synergistic effect of deleting both proteins on the removal of ribonucleotides.
Steps 2 and 3:

Once the oligonucleotides have been transformed into the cells, we amplified the \textit{LEU2} gene by using PCR (polymerase chain reaction). We first digest the PCR product with \textit{Bam}H1 restriction enzyme, which will allow us to confirm that the oligonucleotides we introduced were used as template for double strand break repair at the HO site since the \textit{Bam}H1 site is positioned directly at the break site. Then, we digested the PCR product with the \textit{Stu}I restriction enzyme. The \textit{Stu}I site is only generated if the oligonucleotides we introduced are present in the genome unharmed. However, if the ribonucleotide(s) is removed from the sequence, meaning they were removed by a protein other than RNase H2 or \textit{RAD14}, then the site would not be present in the \textit{LEU2} gene. Based on the percentage of \textit{Stu}I sites cut, we are able to determine the role that RNase H2 and/or \textit{RAD14} play in ribonucleotide excision repair.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tr>
<td>\textit{WT}</td>
<td>FRO-767,768 (\textit{alf \Delta ho \Delta hml::ADE1 MATa-inc \Delta hmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL::HO leu2::HOcs \Delta mata::hisG})</td>
</tr>
<tr>
<td>\textit{rnh201}</td>
<td>FRO-984,985 (FRO-767,768 \textit{\Delta rnh201::kanMX4})</td>
</tr>
<tr>
<td>\textit{rad14}</td>
<td>YS-388,389 (FRO-767,768 \textit{\Delta rad14::kanMX4})</td>
</tr>
<tr>
<td>\textit{rnh201 rad14}</td>
<td>YS-390,391 (FRO-767,768 \textit{\Delta rnh201::hygMX4 \Delta rad14::kanMX4})</td>
</tr>
</tbody>
</table>

\textbf{Table 1.} \textit{Saccharomyces cerevisiae} strains used in this study with genotypes.
Figure 1. Different oligonucleotide substrates used as a template for DNA double strand break repair. Repair occurred at the HO site, transforming the Leu⁻ cells back to Leu⁺. LEU2.R1m signifies a single mispaired RNA-containing oligonucleotide, LEU2.D1mR1p signifies a single-paired RNA-containing oligonucleotide, LEU2.D1mR2p signifies a two-paired RNA-containing oligonucleotide, and LEU2.D1mR3p signifies a three-paired RNA-containing oligonucleotide. The green line delineates the StuI restriction enzyme site that is created due to the transformation.
CHAPTER 4

RESULTS

The \textit{StuI} site is present in the genome only if the DNA has not been targeted by any proteins for excising ribonucleotides. The \textit{p}-values are determined by comparing each strain to the wild-type. All strains that were repaired using the DNA oligonucleotide exhibit high percentages of \textit{StuI} sites, similar to what is seen in the wild-type (Table 2 and Figure 2).

In the single mispaired RNA-containing oligonucleotide (LEU2.R1m), \textit{rnh201} exhibits a significantly higher number of \textit{StuI} sites compared to that of the wild-type, \textit{rad14} does not exhibit a significant change in frequency, and the double mutant (\textit{rnh201 rad14}) is found to have a statistical difference as well.

In the single paired RNA-containing oligonucleotide (LEU2.D1mR1p), \textit{rnh201} and \textit{rad14} exhibit statistically higher percentages of \textit{StuI} sites when compared to that of the wild-type, and the double mutant exhibits the same significance as well. In the two-paired RNA-containing oligonucleotide (LEU2.D1mR2p), \textit{rnh201} and the double mutant were found to have a statistically higher percentage of \textit{StuI} sites as compared to that of the wild-type, whereas the \textit{rad14} mutant did not show a significant difference. As for the three-paired RNA-containing oligonucleotide (LEU2.D1mR3p), again \textit{rnh201} and the double mutant showed a significantly higher percentage of \textit{StuI} sites as compared to that of the wild-type and the \textit{rad14} mutant did not exhibit a statistical difference (Table 2 and Figure 2).
<table>
<thead>
<tr>
<th></th>
<th>Percentage of <em>Stu</em>I Cut Leu⁺ Transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td><strong>LEU2.D1m</strong></td>
<td>73% (67-88)</td>
</tr>
<tr>
<td>(n=4)</td>
<td>NA</td>
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<tr>
<td><strong>LEU2.R1m</strong></td>
<td>48% (40-58)</td>
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<tr>
<td>(n=6)</td>
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</tr>
<tr>
<td><strong>LEU2.D1mR1p</strong></td>
<td>35% (20-40)</td>
</tr>
<tr>
<td>(n=6)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>LEU2.D1mR2p</strong></td>
<td>35% (25-45)</td>
</tr>
<tr>
<td>(n=4)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>LEU2.D1mR3p</strong></td>
<td>25% (8-33)</td>
</tr>
<tr>
<td>(n=6*)</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Table 2.** Correction of *Stu*I site in Leu⁺ cells. Median percentages of *Stu*I cut Leu⁺ transformants from 4-6 independent transformations are shown with ranges in parentheses or as bars. For each independent transformation, 10 to 20 Leu⁺ transformants were randomly selected for analysis. LEU2.D1m is the DNA-only control. Transformation with no oligo gave no Leu⁺ transformants. Mann-Whitney U test was implemented for statistical analysis. Listed P-values are from tests against the WT. P-values of less than 0.05 are in red.
Figure 2. Correction of StuI site in Leu\(^+\) cells in bar graph form. Median percentages of StuI cut Leu\(^+\) transformants from 4-6 independent transformations are shown as bars. For each independent transformation, 10 to 20 Leu\(^+\) transformants were randomly selected for analysis. LEU2.D1m is the DNA-only control. Transformation with no oligo gave no Leu\(^+\) transformants. P-values determined from tests against the WT. Mann-Whitney U test was implemented for statistical analysis. P-values less than 0.05 are asterisked.
CHAPTER 4
DISCUSSION

The DNA-only oligonucleotide successfully exhibits high percentages for number of StuI sites cut because the proteins should not be targeting the strand as no ribonucleotides are present (Figure 2 and Table 2). Our results have confirmed that RNase H2 targets a single paired and mispaired ribonucleotide in vivo. We also found that RNase H2 targets RNA in a stretch of 2 and 3 ribonucleotides in vivo (Figure 2 and Table 2).

As for the involvement of NER enzymes in excising ribonucleotides, results show that RAD14 targets a single paired ribonucleotide, but does not recognize a mispaired ribonucleotide or stretches of 2-3 ribonucleotides in vivo. Therefore, we have concluded that RER and NER work in coordination in the cell to remove single-paired ribonucleotides in eukaryotes. Our future directions include knocking out RNase H1, the less-involved protein in eukaryotic RER, and observing how this affects the excision of single-paired, two-paired, and three-paired ribonucleotides from the yeast Saccharomyces cerevisiae genome.

This study helps illustrate the mechanism of RER and NER by demonstrating the substrate specificity of the RNase H enzymes as well as RAD14 of NER in a yeast eukaryotic cell. This study will eventually assist the scientific community in finding potential treatments to diseases such as AGS and XP in the future, while additionally having implications towards the study of other similar autoimmune diseases as well.
REFERENCES


