THE ROLE OF RAD52P ISOFORMS IN DIRECT REPEAT-MEDIATED DELETION EVENTS IN *Saccharomyces cerevisiae*

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ABSTRACT

The yeast *Saccharomyces cerevisiae* is a single-celled facultatively anaerobic fungus. Like all eukaryotic organisms, *S. cerevisiae* contains mitochondria, and mitochondrial DNA (mtDNA), that encodes many of the proteins necessary for oxidative phosphorylation. Similar to nuclear DNA, an accumulation of mutations in mtDNA can be detrimental to cell function. Therefore, mtDNA must be maintained and protected from damage. *RAD52* is a nuclear gene that codes for Rad52p, a specific protein involved in homologous DNA recombination and double-strand break repair. The open reading frame of this gene contains five potential start codons that may drive expression in either the nucleus or the mitochondria. The focus of this study was to determine the role of these different start codons on Rad52p expression in the nucleus and the mitochondria. To do so, we performed a series of fluctuation analyses to measure the rate at which yeast strains with mutated start codons underwent direct repeat-mediated deletion, which is a function of Rad52p. Our data suggest that translation from the first two start codons yields a mitochondrial isoform of the protein, while expression from the last three start codons yields a nuclear isoform.

INTRODUCTION

The mitochondrion has been called the powerhouse of the cell and is responsible for the production of adenosine triphosphate (ATP) during the process of oxidative phosphorylation. ATP is readily converted into useable energy by the cell and therefore a sufficient and stable supply is crucial to cell survival and many homeostatic cellular processes. Several clinically relevant disorders are caused by impaired or nonfunctional mitochondria, including Leber's hereditary optical neuropathy, which causes a loss of vision, Kearns-Sayre Syndrome, which compromises most sensory and vital organ systems, and maternally inherited diabetes with deafness, to name a few (Solano *et al*., 2001). Each of these disorders is caused by mutations in mitochondrial genes.

The mitochondrial genome consists of a circular DNA molecule that in humans contains 16,569 base pairs encoding 37 genes for ribosomal ribonucleic acids (rRNA), transfer RNAs (tRNA), and 13 polypeptides (Solano *et al*., 2001). Mitochondrial DNA is organized in structures known as nucleoids. Similar to bacterial cells in which the nucleoid region is exposed to the cell cytoplasm, the nucleoid in a mitochondrion is exposed to the mitochondrial matrix (Williamson & Fennel, 1979). Direct exposure of the mitochondrial genome to the matrix can lead to potential problems of DNA stability. For example, exposure of mtDNA to reactive oxygen species and other sources of oxidative stress in the matrix can

alter DNA structure, causing breaks in the DNA and other forms of damage (Stuart & Brown, 2006). It therefore seems logical that the mitochondrian has mechanisms in place to repair and correct any damage to its DNA. The processes mediating nuclear DNA repair have been well characterized, but the same cannot be said of mtDNA (Kalifa *et al*., 2009).

One protein that has been implicated in DNA repair is Rad52p, encoded by *RAD52,* a nuclear gene that contains five potential start codons (De Mayolo *et al.,* 2006). Rad52p was first discovered in yeast strains that were sensitive to gamma radiation. These strains lacked functional Rad52p and were unable to repair double-stranded breaks in DNA caused by gamma radiation (Resnick, 1969). It was subsequently determined that Rad52p is a DNA binding protein that plays an important role in homologous recombination and double-strand break repair by acting as part of a DNA repair mechanism involving many different proteins in the Rad52 epistasis group (Mortensen *et al*., 1996; 2002; Symington, 2002). Repair mechanisms of this type have been implicated directly in maintaining the integrity of nuclear DNA (De Mayolo *et al*., 2006). Presumably due to the five alternative start codons, multiple isoforms of Rad52p have been identified through differences in their electrophoretic mobility, and those translated from the last three start codons have been shown to be sufficient for recombination and the repair of gamma ray-induced double-stranded DNA breaks in the nucleus (De Mayolo *et al*., 2006).

Previous research has suggested the presence of an upstream mitochondrial localization sequence near the first and second putative start codons of the *RAD52* gene (Sia *et al*., unpublished data). Therefore, it was hypothesized that translation from start codons further upstream might create a Rad52p isoform that is localized to the mitochondrion instead of the nucleus. It is generally accepted that ribosomes start translation at the first start codon of an mRNA's coding region due to an appropriate recognition sequences around it (Nakamoto, 2009). However, under certain conditions, ribosomes may bypass specific start codons and begin translation at others due to a process called "leaky scanning" (Kozak, 2002). Leaky scanning can be due to factors such as the specific nucleotide sequence surrounding a start codon, the length of the 5′ mRNA leader sequence, as well as the three-dimensional conformation of the mRNA (Hamilton *et al*., 1987; Kovak, 1990; 1991).

In the present study, we monitored changes in the rate of recombination in either the nucleus or the mitochondria of yeast after mutating different Rad52p start codons. Although it is not yet known why this takes place, we present evidence suggesting that a mitochondrial isoform of Rad52p is indeed encoded by sequence information located further upstream in the *RAD52* gene.

MATERIALS AND METHODS

Yeast Strain Start Codon Mutations

Several different strains of yeast were previously created for use in this study (Sia *et al*., unpublished data). The different yeast strains contained point mutations in each of the potential *RAD52* start codons. These strains contained either a missense or a nonsense mutation (Figure 1) that adversely impacted transcription of the *RAD52* gene. A wild type strain of yeast was used as a positive control while a previously created *RAD52* knockout strain was used as a negative control.

Figure 1. Start codon mutations used in the study. The wild type gene is shown at the top. Mutated sequences are aligned below the wild type gene with the specific mutations highlighted in red. All potential start codons (some mutated) are highlighted in yellow. The types of mutations include (in order from the top): a missense mutation in the first start codon, a missense mutation in the second start codon, missense mutations in both the first and second start codons, a missense mutation in the third start codon, a nonsense mutation in the third start codon, and a nonsense mutation in the fifth start codon.

Media Used

Four types of media were used to grow the yeast (see Kalifa & Sia, 2007): Synthetic Defined medium lacking arginine and uracil (SD-Arg-Ura), Yeast-Peptone-Dextrose medium (YPD) (1% Bacto™ yeast extract (Becton-Dickinson, Franklin Lakes, NJ), 2% Bacto™ peptone (Becton-Dickinson), and 2% dextrose), Synthetic Defined medium lacking tryptophan (SD-Trp), and Yeast-Peptone-Glycerol medium (YPG) (1% Bacto™ yeast extract (Becton-Dickinson), 2% Bacto™ peptone (Becton-Dickinson), and 2% glycerol).

Nuclear and Mitochondrial Reporter Constructs

DNA reporter constructs, which monitor the ability of a cell to create proteins that enable them to live on specific selective media, were used to determine if direct repeat-mediated deletion (DRMD) and recombination took place within the yeast strains. The nuclear reporter used in the study measured the ability of the yeast to synthesize tryptophan, an essential amino acid (Figure 2A), while the mitochondrial reporter measured the ability of the yeast to synthesize Subunit II of cytochrome c oxidase, a protein required by the electron transport chain (Figure 2B).

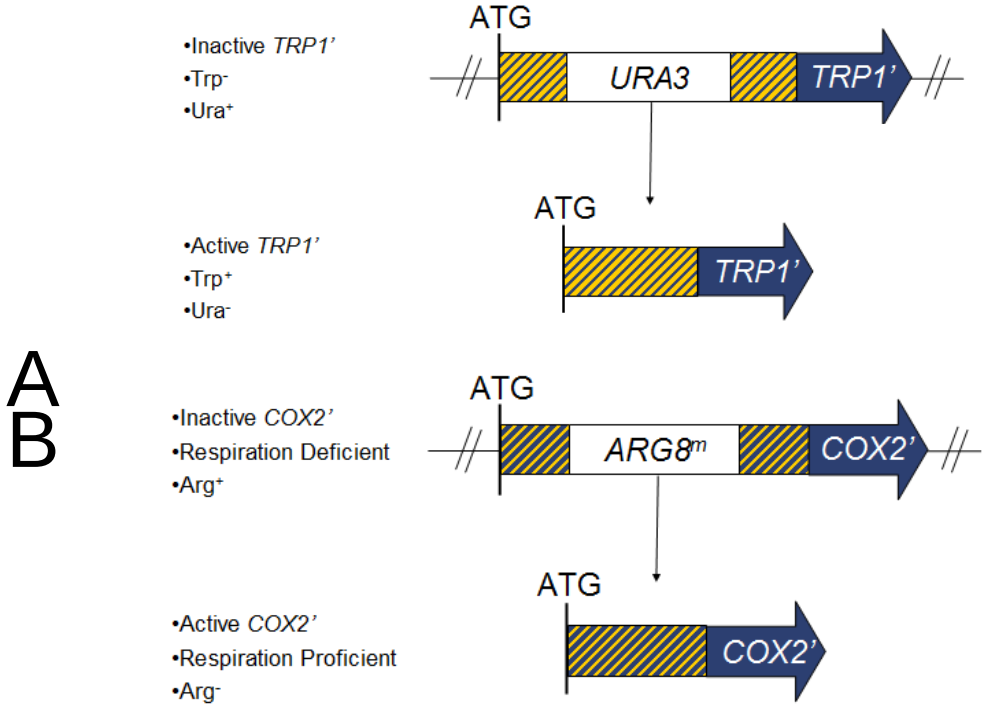

Figure 2. DNA reporter constructs used in the study. A. Diagram of the DNA construct used to detect a direct repeat-mediated deletion (DRMD) event in the nucleus. The *TRP1'* gene is interrupted by the *URA3* gene. When recombination occurs, the cells' ability to synthesize tryptophan is restored and can be detected by plating on selective media (Kalifa *et al*., 2009). B. Diagram of the DNA construct used to detect a DRMD event in the mitochondria. The *COX2'* gene is interrupted by the *ARG8^{m}* gene. When recombination occurs, the cells' ability to synthesize Subunit II of cytochrome c oxidase protein is restored, enabling the cells to undergo cellular respiration. This can be [illegible] media (Phadnis *et al*., 2005).

Measurement of Direct Repeat-Mediated Deletion Events

The ability of yeast to undergo DRMD and homologous recombination was measured using fluctuation analyses (Figure 3). Yeast cells were patched on SD-Arg-Ura and grown for 2 days. Yeast cells that took up the reporter constructs were able to grow on this medium since the constructs contained the arginine and uracil producing genes (Figure 2). Surviving yeast were taken from this medium and streaked to produce single colonies on YPD medium, which was then allowed to grow for three days. Single colonies were then picked, placed in 100µL of water and diluted out as shown in Figure 3. Yeast suspensions from these tubes were spread across the surface of three different media: SD-Trp to measure nuclear recombination, YPG to measure mitochondrial recombination, and YPD which was used as a control and as a basis for calculating the rate of recombination per cell division using the method of the median (Lea & Coulson, 1949).

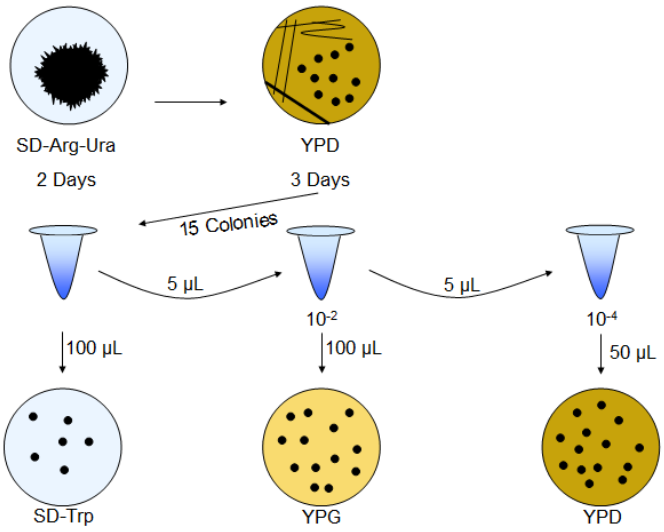

Figure 3. Schematic diagram illustrating the fluctuation test. Cells are initially plated on an SD-Arg-Ura plate for 2 days to isolate yeast that contain DNA reporter constructs. Surviving colonies are then streaked onto a YPD plate and grown for 3 days. Colonies are picked, diluted, and plated onto three separate plate types. The SD-Trp plate tests for nuclear recombination since it lacks tryptophan and any yeast that can grow on it must be able to produce their own tryptophan through a recombination event. The YPG plate tests for mitochondrial recombination because the glycerol in the media is not fermentable; any yeast that can grow on it must have undergone a recombination event to allow cellular respiration. Finally, the YPD plate is used to determine total cell count.

RESULTS AND DISCUSSION

The rate of DRMD for the nuclear reporter construct was tested in each of the different ATG start codon mutation strains, along with a wild type and knockout strain. Two few samples for each strain were obtained to allow a statistical analysis. Nevertheless, the fold changes in rate of recombination per cell division appeared to be similar to wild type in most of the tested strains (Table 1), supporting the idea that with respect to nuclear expression, translation from the last three start codons is efficient (De Mayolo, 2006). Thus, if one start codon is impaired, another may be utilized to make a functioning nuclear version of Rad52p. However, the fold change in strains with a mutation in the fifth start codon were similar to that resulting when *RAD52* gene was completely deleted suggesting that it is required for efficient production of both nuclear and mitochondrial isoforms (Table 1). This can be seen most clearly in Figure 4, which compares the number of DRMD per 10^7 cells between strains.

Yeast Strain	Sample Size	Average Nuclear DRMD Rate	Fold Change from Wild Type	Average Mitochondrial DRMD Rate	Fold Change from Wild Type
Wild Type	5	$11x10^{-7}$	–	$2351x10^{-7}$	–
ATG 1	1	$19x10^{-7}$	1.73 ↑	$593x10^{-7}$	3.96 ↓
ATG 2	2	$11x10^{-7}$	1.00 –	$2801x10^{-7}$	1.19 ↑
ATG 1,2	3	$8x10^{-7}$	1.38 ↓	$1212x10^{-7}$	1.94 ↓
ATG 3	2	$14x10^{-7}$	1.27 ↑	$3151x10^{-7}$	1.34 ↑
ATG 5	3	$4x10^{-7}$	2.75 ↓	$1293x10^{-7}$	1.82 ↓
*RAD52*Δ	3	$3x10^{-7}$	3.67 ↓	$627x10^{-7}$	3.75 ↓

Table 1. The fold change in direct repeat-mediated deletion (DRMD) rate for each type of mutated codon. The yeast strain indicates which codon or group of codons was mutated and the strains are presented in the same order as in Figure 1. The three columns to the left summarize results for the nuclear constructs, while the three columns to the right summarize results for the mitochondrial constructs. An upward arrow indicates an increase, while a downward arrow indicates a decrease, in fold change, relative to the wild type.

The same strains were used to test the DRMD rate for the mitochondrial reporter construct. In this case, there seemed to be no major fold changes in DRMD rate in strains with mutations in the last three start codons (Table 1, Figure 4B). However, strains with a mutation in the first start codon showed greatly reduced DRMD rates (Table 1, Figure 4B), suggesting that the first start codon is essential for creating an isoform of Rad52p that is localized to the mitochondrion. A mutation in ATG 2 is ineffective, implying that ATG 1 is the preferred start codon for the mitochondrial isoform (Table 1, Figure 4B). These findings support previous suggestions that a mitochondrial localization sequence is located upstream from the first two putative start codons in the *RAD52* gene (Sia *et al.*, unpublished data).

A nonsense mutation at ATG 3 returns the DRMD rate close to wild type levels in the nucleus, but the reason for this is not known (Table 1, Figure 4A). It was expected that a nonsense mutation in ATG 5, which is required for production of both nuclear and mitochondrial isoforms, would have similar DRMD rates to that of a complete deletion of *RAD52*. However, this was not supported by the mitochondrial data (Table 1, Figure 4B), and the reason for this has yet to be determined. Future experiments will be needed to explore these findings further and to determine if they are related to the functioning of Rad52p isoforms.

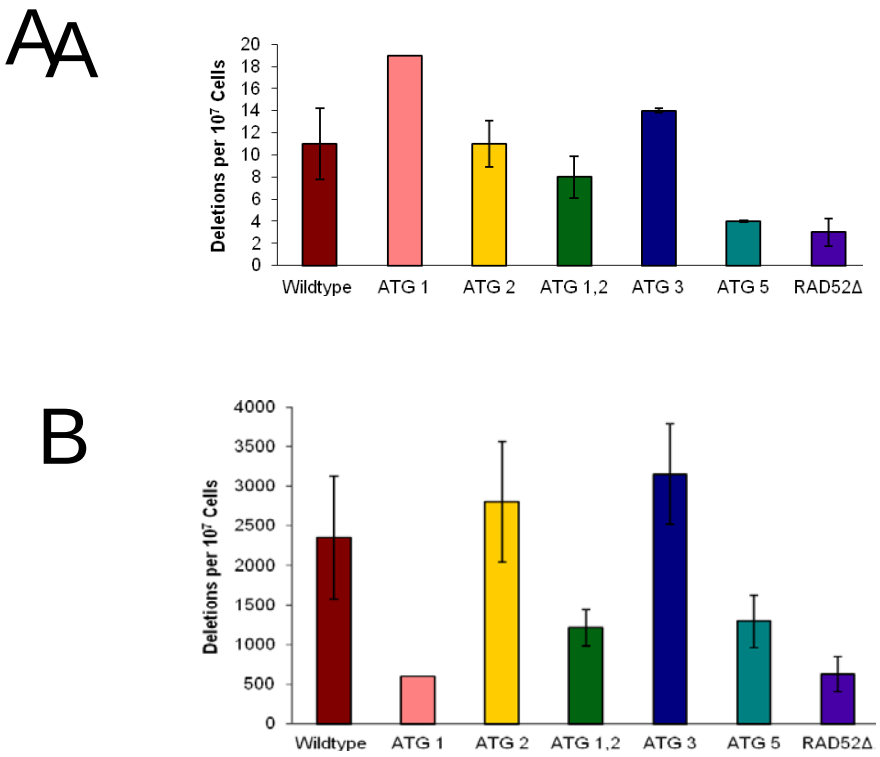

Figure 4. Comparison of average (± SD) DRMD rates. A. Rates measured for the nucleus. Mutations in ATG 3 resulted in DRMD rates similar to wild type since the cell was able to use the ATG 4 or ATG 5 start codons instead. Changes further upstream show no apparent negative impact. B. Rates measured for

the mitochondria. Mutations in ATG 1 resulted in a pronounced decrease in DRMD rates relative to wild type. It is most likely that ATG1 is a preferred start codon for translation of a mitochondrial isoform.

CONCLUSION

The results of this study suggest the presence of a mitochondrial isoform of Rad52p in *S. cerevisiae*. The fact that mutations within the first two start codons had an impact on DRMD rates in the mitochondria, but not in the nucleus, raises the possibility that there are two distinct working forms of this protein. The findings presented here indicate that changing the last three start codons impacts nuclear, but not mitochondrial, function and further support previous studies showing that these start codons are sufficient for nuclear expression of Rad52p. However, additional studies, including fluctuation analysis, will be needed to substantiate these results.

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