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**A second function of pseudouridine synthase,
Pus3p, independent of Ψ formation,
impacts *HOT1*-associated recombination,
growth rate, and temperature tolerance
in *Saccharomyces cerevisiae*.**

A Thesis Submitted to the University Honors Program
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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, the protein product of the *DEG1* gene, Pus3p, is characterized as a pseudouridine (Ψ) synthase. Disruption of *DEG1* results in various mutant phenotypes including depressed growth, temperature sensitivity at 37°C, decreased tRNA pseudouridylation, and decreased rates of *HOT1*-associated recombination. In order to investigate the relationship between the various mutant phenotypes, we assayed for the ability of a *deg1* partial-deletion mutant and two novel *deg1* point mutants to restore wild-type *DEG1* functions in a *deg1*-deletion yeast strain. Our results suggest that the effect of *DEG1* disruption on *HOT1*-associated recombination, growth rate, and temperature tolerance results from a function of Pus3p independent of its role as a pseudouridine synthase.

INTRODUCTION

HOT1 is a *cis*-acting recombination hotspot associated with the promoter of the 35S rRNA gene that is situated within the tandem array of rDNA repeats. Although the mechanism by which *HOT1*-associated recombination occurs is unknown, numerous genes have been implicated in regulating *HOT1* activity (Lin and Keil, 1990; Prusty, 1999). Lin and Keil (1990) used UV mutagenesis to generate *HOT1* hypo-recombination and hyper-recombination yeast strains. Five distinct *HOT1* hypo-recombination mutants were classified as *hrm1*, *hrm2*, *hrm3*, *hrm4*, or *hrm5* and evaluated for growth rate, temperature tolerance, and sensitivity to γ -radiation. *Hrm3-1* displays slow growth, temperature sensitivity at 37°C, and a wild-type phenotype for sensitivity to γ -radiation. Hepfer *et al.* (unpublished) have shown that the phenotypes associated with *hrm3-1* result from a point mutation in a previously characterized gene, *DEG1* (DEpressed Growth; Carbone *et al.*, 1990). *DEG1* encodes Pseudouridine Synthase 3 (Pus3p), belonging to the TruA family of pseudouridine (Ψ) synthases, which catalyzes the formation of Ψ_{38} and Ψ_{39} in the anticodon loop of various yeast tRNAs (Lecoite *et al.*, 1997). The *hrm3-1* mutation disrupts Pus3p by substituting phenylalanine for Ser¹⁶⁷ (Hepfer *et al.*, unpublished).

Four distinct families of Ψ synthases, identified through sequence alignment, each contain invariant lysine and aspartic acid residues (Koonin, 1996). It has subsequently been shown in the RluA and TruB families that the invariant lysine residue is dispensable (Spedalieri *et al.*, 2000). Mutagenesis studies have confirmed; however, that mutation of

the invariant aspartic acid residue blocks the formation of Ψ in three of the four groups of *E. coli* Ψ synthases including RluA (Huang *et al.*, 1998a; Vidhyashankar *et al.*, 1999), TruB (Vidhyashankar *et al.*, 1999), and TruA (Huang *et al.*, 1998b). The invariant aspartic acid residue has been shown to be directly involved in the mechanism of Ψ formation, and conversion of the invariant aspartic acid to glutamic acid in *E. coli* Ψ synthase I is sufficient to eliminate Ψ formation (Huang *et al.*, 1998a). Due to the highly conserved nature of this residue, it is assumed that the mechanism of Ψ formation is similar in yeast, and that conversion of this residue to glutamic acid will be sufficient to eliminate Ψ formation in yeast Ψ synthases.

Initially we planned to investigate the importance of Ser¹⁶⁷, Ser³⁹⁷, and the invariant aspartic acid (Asp¹⁵¹) as they pertain to *HOT1*-associated recombination, growth rate, and temperature tolerance in *Saccharomyces cerevisiae*. In addition, we intended to examine the impact of Pus3p truncation resulting from the deletion of a 312 bp *KpnI-KpnI* fragment of *DEG1* created by Adamczyk (2001) or the induction of an amber mutation within *DEG1*, which would effectively remove 11 basic amino acids from the carboxyl terminus of Pus3p. We were successful in creating *deg1* mutant alleles to analyze the importance of Ser¹⁶⁷, Asp¹⁵¹, and the 312 bp *KpnI-KpnI* fragment of *DEG1*. We chose the substitution of Glu for Asp¹⁵¹ (D151E) since the two amino acids both contain carboxylic acid side groups and substitution of the invariant glutamic acid residue for the invariant aspartic acid was sufficient to eliminate Ψ formation in TruA *E. coli* Ψ synthase (Huang *et al.*, 1998b). We chose the substitution of Ala for Ser¹⁶⁷ (S167A) since

the side groups of these two amino acids are similar in size. The S167A substitution is essentially the removal of the hydroxyl group at residue 167 of Pus3p.

Several theories have been postulated regarding the cause of depressed growth in *deg1* yeast strains. Overexpression of an 850 bp portion of *DEG1* has been shown to induce depressed growth in wild-type yeast, suggesting that depressed growth may result from competition between wild-type and mutant Pus3p (Carbone *et al.*, 1991). It has also been proposed that the metabolic effects of decreased tRNA pseudouridylation may slow cellular metabolism by affecting the rate of mRNA decoding on the ribosome or inducing a prokaryotic-like attenuation mechanism in mitochondria (Lecoite *et al.*, 1997). We show here that the effect of *DEG1* disruption on *HOT1*-associated mitotic recombination, growth rate, and temperature tolerance may not be attributable to the metabolic effect of decreased tRNA pseudouridylation; rather these effects may result from a function of Pus3p independent of its role as a pseudouridine synthase.

MATERIALS AND METHODS

Yeast Strains. The genotypes of yeast strains *deg1*(Δ -45-1234) and *deg1*(S167F/*hrm3*-1) are described in Table 1. Both strains contain the *HOT1*::*his4* Δ ::*URA*::*his4*-260 assay on chromosome III and the *lys*- Δ BX::*CAN*::*LYS* assay on chromosome II as described previously (Lin and Keil, 1990). *Deg1*(Δ -45-1234) is a *deg1*-deletion strain in which a 1279 bp *XhoI*-*KpnI* fragment, corresponding to most of the *DEG1* gene, has been removed (Figure 1, Appendix A). *Deg1*(S167F/*hrm3*-1) is a *deg1* strain containing the original *hrm3*-1 mutation (substitution of T for C, 500 bases

downstream of the start codon, Appendix A), which encodes Pus3p^{S167F} (Hepfer *et al*, unpublished).

Mutagenesis. Removal of the 312 bp *KpnI-KpnI* fragment from *DEG1* was described previously (Adamczyk, 2001). Point mutants of *DEG1*, resulting in the Pus3p amino acid conversions Aspartate to Glutamate at residue 151 and Serine to Alanine at residue 167 (D151E and S167A, respectively) were produced by the Megaprimer method of site-directed mutagenesis (Sarkar and Sommer, 1990; Figure 2a) using *Pfu* DNA Polymerase and *Taq* DNA Polymerase, respectively, according to the manufacturer's instructions (Stratagene, La Jolla, CA). YCp*Lac111-DEG1::901C2* is a construct of the shuttle vector YCp*Lac111* and a 1.6 kbp *EcoRI-EcoRI* fragment of *S. cerevisiae* chromosome VI containing all of the *DEG1* gene (tagged with epitope 901) and its promoter sequences (Appendix A; Steudel, 1998). YCp*Lac111-DEG1::901C2*, linearized with *EcoRV* according to the manufacturer's instructions (NEB, Beverly, MA), served as the template for PCR mutagenesis. The mutant allele, *deg1*(D151E), was generated using primers 302 and D151E in the first round of PCR to produce Megaprimer 302-D151E, which was subsequently used with primer 303 in the second round of PCR to amplify *deg1*(D151E). The mutant *deg1*(S167A) was produced using primers 302 and S167A in the first round of PCR to generate Megaprimer 302-S167A, which was subsequently used with primer 301 in the second round of PCR to produce *deg1*(S167A). Megaprimer 303-S397A and Megaprimer 303-Y433amber were produced; however, amplification of the megaprimers in the second round of PCR was unsuccessful. Primer sequences and binding sites are listed in Table 2. Primers D151E, S167A, S397A, Y433amber, and 303

were purchased from Operon Technologies (Alameda, CA); primers 301 and 302 were a gift from Ralph Keil (Milton S. Hershey Medical Center, Hershey, PA). All reactions were run as follows: 5 min at 95°C in the absence of DNA polymerase; and after DNA polymerase was added, 30 cycles of denaturation (95°C for 45 s), annealing (48°C for 45 s), and extension (72°C for 3.2 min), except for the second round of PCR using *Pfu* DNA Polymerase, in which denaturation occurred at 98°C for 45 s. Following each reaction, products were size-separated by electrophoresis at 50V on a 0.8% preparatory agarose gel in 1 x TAE (then purified using the glass bead method of the GENE CLEAN II gel purification system (Bio 101 Inc., Vista, CA) or size exclusion chromatography using QIAquick PCR Purification protocol according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA).

Subcloning and Bacterial Transformation. Following PCR mutagenesis, the resulting *deg1* alleles were cleaved at the terminal restriction enzyme sites *EcoRI/EcoRI* for *deg1*(S167A) and *EcoRI/SacI* for *deg1*(D151E) and *deg1*(Δ 55-366) and cloned into the *EcoRI* site or *EcoRI/SacI* site of YCp*Lac111* using T₄ DNA Ligase. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and used according to the manufacturer's instructions. Ligations were performed overnight at 16°C, with a 4:1 molar ratio of insert to vector, using T₄ DNA Ligase according to the manufacturer's instructions (NEB). Fifty μ l of subcloning efficient DH5 α competent cells (BRL, Life Technologies, Rockville, MD) were transformed using 2.5 μ l of each ligation reaction (>10 ng of each Yc*pLac111-deg1* construct), and screened on LB plates containing

ampicillin (50 µg/ml), X-gal (40 mg/ml), and IPTG (40 mg/ml). Transformants were identified by a blue/white screen, and white colonies were selected as candidate transformants. Plasmids were isolated from each type of transformed cell using the alkaline lysis method and subsequent phenol/chloroform extraction and ethanol precipitation as described by Maniatis *et al.* (1982). Plasmids containing *deg1* mutant alleles were confirmed by restriction enzyme digestion using *NgoMIV* and *AatII* (double digest) or *KpnI* according to the manufacturers instructions (NEB, results not shown). Desirable transformed strains were stored at -70°C in ~20% glycerol.

Yeast Transformation and Phenotypic Screening. Saturated cultures of yeast strain *deg1*(Δ-45-1234) grown in SC-ura were diluted 1:20 in SC-ura, and grown at 30°C for 4 h on a shaker platform (200 rpm). Cells were harvested by centrifugation at 2000g for 5 min, washed once with sterile ddH₂O, and suspended in a final concentration of 10.2 mg/ml lithium acetate, 10 mM Tris HCl, 1mM EDTA, 10% DMSO, and 1 mg/ml boiled salmon sperm DNA prepared according to Maniatis *et al.* (1982). *YCpLac111-deg1* recombinant plasmids were obtained from transformed bacteria using the Qiagen Plasmid Midi kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions, and used to transform the *deg1*-deletion yeast strain, *deg1*(Δ-45-1234) (~950 ng of plasmid/75 µl cell suspension). Six volumes of 50% polyethylene glycol (average MW 3350, in 10.2 mg/ml lithium acetate, 10 mM Tris HCl, and 1mM EDTA) were then added to the cell suspension. Each suspension was incubated at 30°C for 30 min, incubated at 42°C for 20 min, centrifuged at 1500g for 40 s, re-suspended in 300 µl ddH₂O and plated onto SC-ura-

leu plates. Ura⁺ Leu⁺ transformants were screened further by replica plating onto a variety of selective media to verify that each had the expected phenotypes (dropout media was a generous gift from Ralph Keil, Milton S. Hershey Medical Center, Hershey, PA).

Temperature Tests. Ten μ l aliquots of saturated culture and a 10^{-1} dilution of each yeast strain were spotted onto duplicate SC-ura-leu plates and incubated at 30°C or 37°C. Growth was assessed after 48 h.

Recombination Assay. The recombination assays used in this study are described in Figures 4 and 5. An aliquot of cells for each yeast strain was streaked from saturated culture or frozen stock onto SC-ura-leu plates and grown at 30°C in order to isolate three independent colonies. Each colony was placed in a tube containing 1 ml SC-ura-leu then grown to saturation (48 h at 30°C). Each saturated culture was diluted 1:10,000 in ddH₂O, then 10 μ l of this was spotted onto an SC-leu plate to determine the initial cell counts per ml. Twenty μ l of each 1:10,000 dilution was used to inoculate 4 ml of SC-leu that was evenly distributed between four tubes and incubated at 30°C for 3 days. Serial dilutions of three of the four tubes were prepared, and 10 μ l aliquots were spotted onto SC-leu (10^{-4}), SC-his, SC-arg+CAN, SC+5FOA (concentrated, 10^{-1} , 10^{-2} , and 10^{-3}). Colonies were counted after two days and recombination rates were determined using the method of Drake (1970). Statistical significance was evaluated using the median test (Siegal, 1956).

Growth Curve Analysis. Starting from saturated cultures grown in SC-ura-leu, triplicate 1:10,000 dilutions of each yeast strain were prepared in 4 ml of SC-ura-leu and incubated overnight at 30°C. Beginning 9 h after dilution, the absorption at 595 nm was recorded for each tube every hour for 17 h. Absorption values below 0.1 or above 1.0 were not used in subsequent calculations. Doubling time was determined from a semi-logarithmic plot of A_{595} versus time.

RESULTS

Sequence Alignment. In order to identify conserved regions, amino acid sequences of other α synthases were compared to Pus3p using BLAST. Figure 1 shows the alignment of the Pus3p amino acid sequence to other yeast α synthases (Pus1 and Pus2) and homologous α synthases from other species (*Mus musculus* Pus3p, *C. elegans* E02H1.3, and *E. coli* TruA). Within the six examined sequences, Asp¹⁵¹ is conserved throughout; however Ser¹⁶⁷ is shared only with the homologous proteins in *C. elegans* and *Mus musculus*.

Mutagenesis. The Megaprimer method of site-directed mutagenesis (Sarkar and Sommer, 1990; Figure 2a) was used to generate the *deg1* mutant alleles, *deg1*(D151E) and *deg1*(S167A). Megaprimer 303-S397A and Megaprimer 303-Y433amber were produced (Figure 2b); however, amplification of these megaprimers in the second round of PCR was unsuccessful (results not shown).

Temperature Resistance. Various *deg1* alleles were used to transform the *deg1*-deletion yeast strain, *deg1*(Δ -45-1234), and assayed for the ability to restore temperature

resistance at 37°C. Strains *deg1*(pS167A), *deg1*(pD151E), *deg1*(pΔ55-366), and pDEG1, are transformed *deg1*(Δ-45-1234) yeast carrying the Yc*pLac111* plasmid with *deg1* alleles *deg1*(S167A), *deg1*(D151E), *deg1*(Δ55-366), and wild-type *DEG1* respectively (Table 1). Strain *deg1*(Δ-45-1234) was transformed using the empty vector Yc*pLac111* to serve as a negative control. Strain *deg1*(S167F/*hrm3-1*) containing the nuclear *deg1* mutant allele, was also used as a negative control. As expected, substantial growth was observed for both cell densities in all strains at 30°C (Figure 3). Strains *deg1*(Δ-45-1234) and *deg1*(S167F/*hrm3-1*) did not grow at all at 37°C. Strain *deg1*(pΔ55-366) also displayed the temperature sensitive phenotype. Strain *deg1*(pS167A) grew only slightly at 37°C. Both strains *deg1*(pD151E) and pDEG1 were comparably resistant to 37°C. This suggests that Pus3p Ser¹⁶⁷, but not Asp¹⁵¹, is critical to the function of *DEG1* associated with temperature tolerance. Strain *deg1*(pΔ55-366) behaved like the negative controls *deg1*(Δ-45-1234) and *deg1*(S167F/*hrm3-1*), implying that the *DEG1* activity associated with temperature resistance was not functional in these strains.

Rate of *HOT1*-associated recombination. Wild-type *DEG1* and various mutant *deg1* strains (Table 1) were assayed for *HOT1*-associated recombination (Ura⁻ or His⁺) and non-*HOT1* recombination (Can^R) as described in Figures 4 and 5. Figure 6 displays the relative abundance of recombinants and Table 3 quantifies the rates of recombination for each of the six strains. The rate of non-*HOT1* recombination was not significantly different between any of the strains studied. The rate of *HOT1*-associated recombination (determined by the number of Ura⁻ and His⁺ recombinants) in strains *deg1*(pS167A),

deg1(p Δ 55-366), *deg1*(Δ -45-1234), and *deg1*(S167F/*hrm3-1*) is significantly lower as compared to wild-type strain, pDEG1 ($p \leq 0.01$); however, the recombination rate of strain *deg1*(pD151E) is comparable to wild type. This suggests that Pus3p Asp¹⁵¹ is dispensable to the function of *DEG1* associated with *HOT1* recombination; however, Pus3p Ser¹⁶⁷ and the 312 bp *KpnI-KpnI* fragment of *DEG1* must be present for normal rates of *HOT1*-associated recombination.

Growth Rate. The effect of *deg1* disruption on doubling time was evaluated (Table 3). The most rapid growth rates were observed in yeast strains *deg1*(pD151E) and pDEG1 (doubling times of 135 ± 2 min and 144 ± 5 min, respectively), indicating that Pus3p Asp¹⁵¹ is dispensable to the function of *DEG1* associated with normal growth rate. Doubling times for strains *deg1*(pS167A), c, and *deg1*(S167F/*hrm3-1*) were less than that of the wild-type strain, pDEG1, and within one standard deviation of the negative control strain, *deg1*(Δ -45-1234) (Table 3), indicating that Pus3p Ser¹⁶⁷ and the 312 bp *KpnI-KpnI* fragment of *DEG1* must be present for normal growth rates in *S. cerevisiae*.

DISCUSSION

We have created constructs of YCp*Lac111* containing *deg1*(Δ 55-366), *deg1*(S167A), or *deg1*(D151E) which encode Pus3p(Δ 55-366), Pus3p^{S167A}, and Pus3p^{D151E}, respectively. The constructs were used to transform the *deg1*-deletion yeast strain, *deg1*(Δ -45-1234), and assayed for the ability to restore wild-type growth rates,

temperature tolerance, and *HOT1*-associated recombination rates. Yeast strains used in this study are described in Table 1.

Strain *deg1*(p Δ 55-366) behaved similarly to the control strains, *deg1*(Δ -45-1234) and *deg1*(S167F/*hrm3*-1), indicating that the 312 bp *KpnI-KpnI* fragment of *DEG1* must be present for normal Pus3p functions. The deletion impacts Pus3p by removing nearly a quarter of the protein (amino acids 19 through 122), including a conserved sequence motif of unknown function shared among TruA-like Ψ synthases (Lecoite *et al.*, 1998). The *deg1*(Δ 55-366) mutation does not remove or alter the *DEG1* regions encoding Pus3p Asp¹⁵¹ or Ser¹⁶⁷. Carbone *et al.* (1991) have shown that overexpression of the 850 bp terminal part of the gene induces the slow growth phenotype in wild-type yeast, possibly as a result of competition between truncated Pus3p and wild-type Pus3p. Our results show that expression of the *deg1* mutant allele *deg1*(Δ 55-366), which contains the in-frame 850 bp terminal part of the gene, is not capable of restoring the wild-type growth rate in the *deg1*-deleted yeast strain *deg1*(Δ -45-1234).

In investigating the relationship between the various phenotypes resulting from *DEG1* disruption, we did not want to exclude the possibility that Pus3p may have multiple catalytic regions. Therefore, we chose to convert the aspartic acid residue that is invariant in Ψ synthases to glutamic acid—a mutation subtle enough that it should not alter the overall conformation of the protein, but likely sufficient to disrupt Ψ synthase function (Huang *et al.*, 1998b). Although we did not examine Ψ synthase activity in the mutants produced in this study, it has previously been shown in *Escherichia coli* that the

invariant aspartic acid residue forms a covalent complex with U39 of target tRNAs, and its conversion to glutamic acid prevents Ψ formation, most likely by sterically inhibiting the formation of this complex (Huang *et al.*, 1998a). Our results have indicated that Pus3p Asp¹⁵¹ is not essential for normal rates of *HOT1*-associated mitotic recombination, normal growth rate, or temperature tolerance in *Saccharomyces cerevisiae*.

The ability of the mutant allele *deg1*(D151E) to restore normal growth rates as well as normal rates of *HOT1*-associated recombination and temperature tolerance in *deg1*-deleted yeast, suggests that a putative second function of Pus3p may regulate these activities, although the exact nature of this activity is unknown. It has been shown that RNA Polymerase III mutations that attenuate tRNA metabolism strongly reduce rRNA transcription by RNA Polymerase I (Briand *et al.*, 2000). If such a cross-talk mechanism exists between tRNA metabolism and rRNA transcription, it could account for the decreased rates of *HOT1*-associated recombination observed in several of the *DEGI*-disrupted strains. Sequences of *HOT1* essential for recombination stimulatory activity coincide with the enhancer and transcription initiation site for RNA Polymerase I (Voelkel-Meiman *et al.*, 1987). In addition, deletions within the RNA Polymerase I enhancer region affecting *HOT1* activity often block rRNA transcription (Huang and Keil, 1995). It is possible that the effect of *DEGI* disruption on growth rate, *HOT1*-associated recombination, and temperature tolerance could be brought about by adversely affecting tRNA metabolism, such that RNA Polymerase I activity is decreased; however, transcription assays have failed to show that *DEGI* disruption affects transcription by RNA Polymerase I (Fogel, 2000; Hepfer *et al.*, unpublished).

One of the first isolated *HOT1* hypo-recombination mutants, *hrm3-1*, displays slow growth and temperature sensitivity at 37°C (Lin and Keil, 1990). The *hrm3-1* mutation disrupts Pus3p by substituting phenylalanine for Ser¹⁶⁷ (Hepfer *et al.*, unpublished). Initially, we assumed that the mutant phenotypes seen in Pus3p^{S167F} yeast resulted from an overall distortion of the enzyme, since phenylalanine is a highly hydrophobic amino acid and the region around Ser¹⁶⁷ is highly hydrophilic (Kyte-Doolittle Hydrophilicity Plot, MacVector, Oxford Molecular Group, Beaverton, OR; results not shown). However, by evaluating yeast strains with a *deg1* allele created by inducing a conversion of Ser¹⁶⁷ to alanine, we have shown that this residue is critical for normal rates of *HOT1*-associated mitotic recombination, normal growth rate, and temperature tolerance. The functional significance of Ser¹⁶⁷ is most likely independent of Ψ synthase activity since it lies outside the four functional motifs common to all Ψ synthases (Koonin, 1996) and the six conserved sequence motifs of TruA-like proteins (Lecoite *et al.*, 1998). This could indicate that Pus3p contains two catalytically distinct domains corresponding to the regions around Asp¹⁵¹ and Ser¹⁶⁷. This seems unlikely, however, given the proximity of Asp¹⁵¹ and Ser¹⁶⁷ (separated only by 15 aa). It is possible that Ser¹⁶⁷ may play a role in tRNA recognition specific to Pus3p, which could explain why it is not highly conserved. If this were true, we would not expect pseudouridylation at U38 or U39 of yeast tRNAs by Pus3p^{S167A}, since proper recognition of tRNAs must occur prior to Ψ formation. In contrast, if Pus3p contains catalytically distinct domains corresponding to the regions near Asp¹⁵¹ and Ser¹⁶⁷ we would expect pseudouridylation at U38 or U39 of yeast tRNAs by Pus3p^{S167A}.

Gutgsell *et al.* (2000) have suggested that the primary function of pseudouridine synthases may be to act as RNA chaperones, aiding in the folding of RNAs. To support their hypothesis, Gutgsell *et al.* (2001) have demonstrated that mutants of the RluD rRNA Ψ synthase, RluD^{D139T} and RluD^{D139N}, can rescue normal growth rates but not Ψ formation in RluD-deficient *E. coli*. According to this hypothesis, Ψ formation may simply act as a dissociation signal that is not critical for cell viability. Indeed, the only identified function of Ψ in tRNA involves tRNA species in which Ψ is found in the anticodon (Senger *et al.*, 1997; Tomita *et al.*, 1999; and Charette and Gray, 2000). Pseudouridine synthases are not the first tRNA modifying enzyme suspected of having dual functions. A similar situation has been described for tRNA methylation. *E. coli* tRNA(m⁵U54)methyltransferase has been shown to be essential for viability; however, methylation of U54 is not (Persson *et al.*, 1992). Our results support Gutgsell's hypothesis in that they suggest that Pus3p may have multiple functions, and that the functions that effect *HOT1*-associated recombination, growth rate, and temperature tolerance are independent of Ψ formation.

Currently we are sequencing *deg1*(D151E), *deg1*(S167A), and *deg1*(Δ 55-366) to verify the identity of the *deg1* mutants alleles, and we are investigating Ψ formation in the yeast strains described in this paper. In addition, it is possible that the substitution of glutamic acid for aspartic acid at residue 151 was not sufficient to eliminate pseudouridine synthase activity in Pus3p. Since we have shown that transformation of *deg1*(Δ -45-1234) yeast with *deg1*(D151E) restores wild-type growth rates, temperature tolerance,

and *HOT1*-associated recombination rates, it would be advisable to determine if a less-conservative substitution, for instance Pus3p^{D151A}, is capable of restoring normal phenotypes. In addition, other researchers in the Hepfer laboratory are exploring the impact of Pus1p and Pus2p deletion in *S. cerevisiae*. Such experiments will help determine whether the effect of *DEG1*-disruption on *HOT1*-associated recombination, growth rate, and temperature tolerance is due to errors in tRNA metabolism or to an as-of-yet unknown function of Pus3p, independent of tRNA metabolism, and dependent upon Pus3p Ser¹⁶⁷.

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TABLE 1. Yeast strains and constructs used in this study

Strain or Construct	Description
Strains	
HRM552	MATa <i>ade2-1 ade5 ura3-52 can1 trp1-HIII leu2-3.112 his4-260::URA3::his4Δ::HOT1 lys2-ΔBX::CAN1::LYS2 deg1(Δ136-1414)::TRP1</i>
HRM607	MATa <i>ade2-1 ade5,7 ura3-52 can1 trp1-HIII LEU his4-260::URA3::his4Δ::HOT1 lys2-ΔBX::CAN1::LYS2 deg1(S167F)</i>
DEG1a	HRM552 carrying YCp <i>Lac111-deg1(S167A)</i>
DEG1b	HRM552 carrying YCp <i>Lac111-deg1(D151E)</i>
DEG1c	HRM552 carrying YCp <i>Lac111-deg1(Δ235-546)</i>
DEG1d	HRM552 carrying YCp <i>Lac111-DEG1</i>
DEG1e	HRM552 carrying YCp <i>Lac111</i>
Constructs	
YCp <i>Lac111-DEG1</i>	YCp <i>Lac111</i> plasmid carrying the epitope-tagged, <i>DEG1::901</i> , and promoter sequences oriented with the start codon near the vector multicloning site.
YCp <i>Lac111-deg1(S167A)</i>	YCp <i>Lac111</i> plasmid carrying the epitope-tagged <i>deg1</i> mutant allele, <i>deg1(S167A)::901</i> , and promoter sequences oriented with the start codon near the vector multicloning site.
YCp <i>Lac111-deg1(D151E)</i>	YCp <i>Lac111</i> plasmid carrying the epitope-tagged <i>deg1</i> mutant allele, <i>deg1(D151E)::901</i> , and promoter sequences oriented with the start codon near the vector multicloning site.
YCp <i>Lac111-deg1(Δ235-546)</i>	YCp <i>Lac111</i> plasmid carrying the epitope-tagged <i>deg1</i> mutant allele, <i>deg1(Δ235-546)::901</i> , and promoter sequences oriented with the start codon near the vector multicloning site.

TABLE 2. Primers used in this study

Name	Binding Site ^a	Sequence ^b
301	1403-1419 (coding strand)	<u>GATGAAAAGAATTCTAGTCTTCAAG</u>
302	-180--164 (noncoding strand)	<u>TTATTGCTGCTGAATTCTTAATATTATC</u>
303	1409-1419 (coding strand)	CCCGGGTACCGAGCTCGAA <u>ATAT</u> AGTC
S167A	490-509 (coding strand)	GTAAATTGG <u>C</u> ACGGACTTC
D151E	442-460 (coding strand)	CTCCCTT <u>TCT</u> CTGTTCTGCC
S397A	1178-1216 (noncoding strand)	CAAACA <u>ACTTCG</u> CAAAGAC
Y433amber	1292-1305 (noncoding strand)	GTAAATGCTAAATAG <u>T</u> CCAAG

^a Numbers specify the distance from the start codon; Appendix A

^b Underlined bases indicate mismatches; restriction enzyme sites are italicized (*EcoRI* = *GAATTC*; *SacI* = *GAGCTC*)

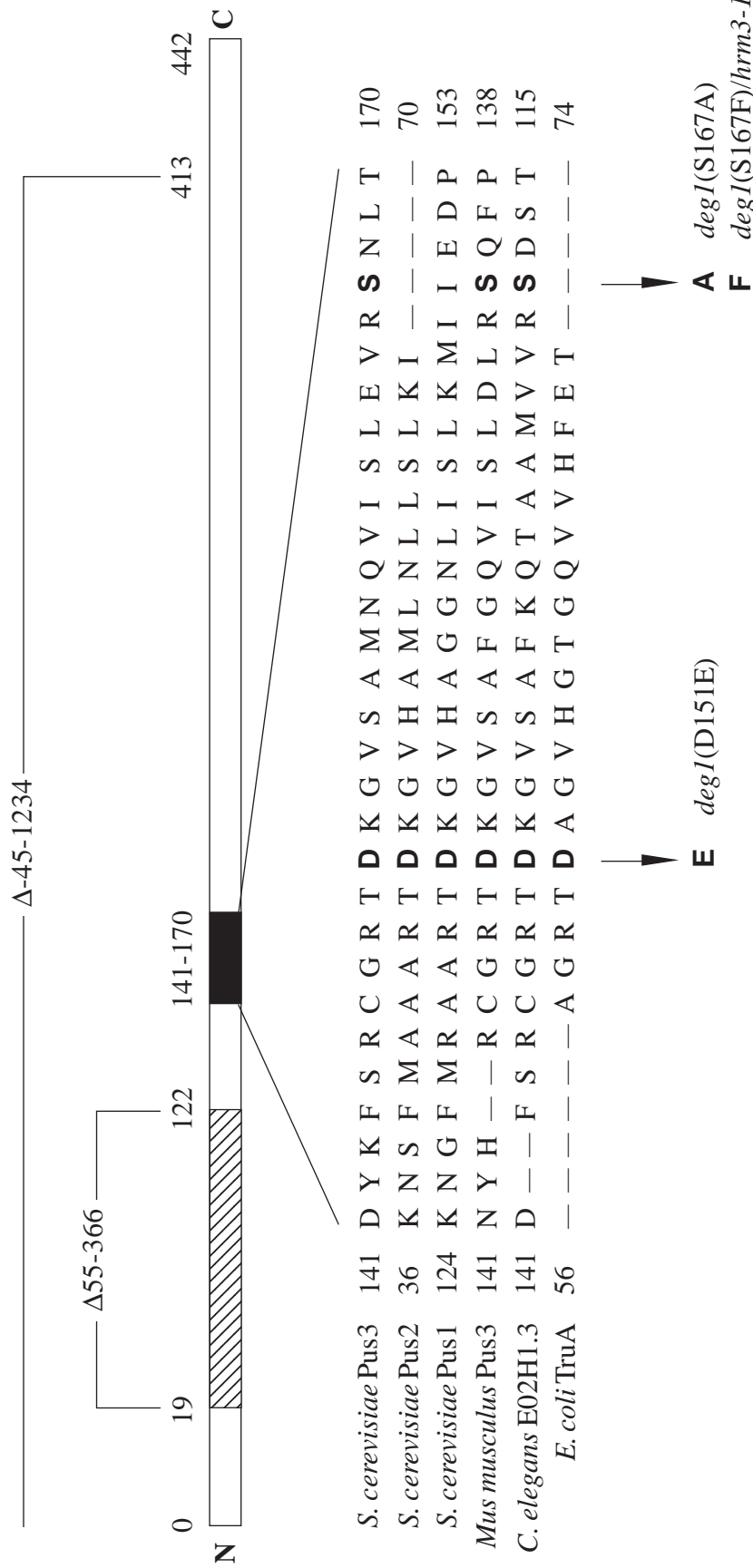


FIGURE 1. Aligned amino acid sequences of the *Saccharomyces cerevisiae* *DEG1* gene product, Pus3p, with other pseudouridine synthases. Bold residues denote the invariant aspartic acid residue (D) and the serine residue (S) that were targeted for mutagenesis in this study. The cross-hatched region indicates the location of the 312 bp *KpnI-KpnI* deletion which removes 104 amino acid residues near the N terminus of Pus3p. The *deg1* deletion (Δ 45-1234) removes most of the coding region of the gene as shown. Positions of point mutations analyzed in this study are indicated. Numbers represent the distance from the start codon of the gene or the distance from the N terminus of the protein.

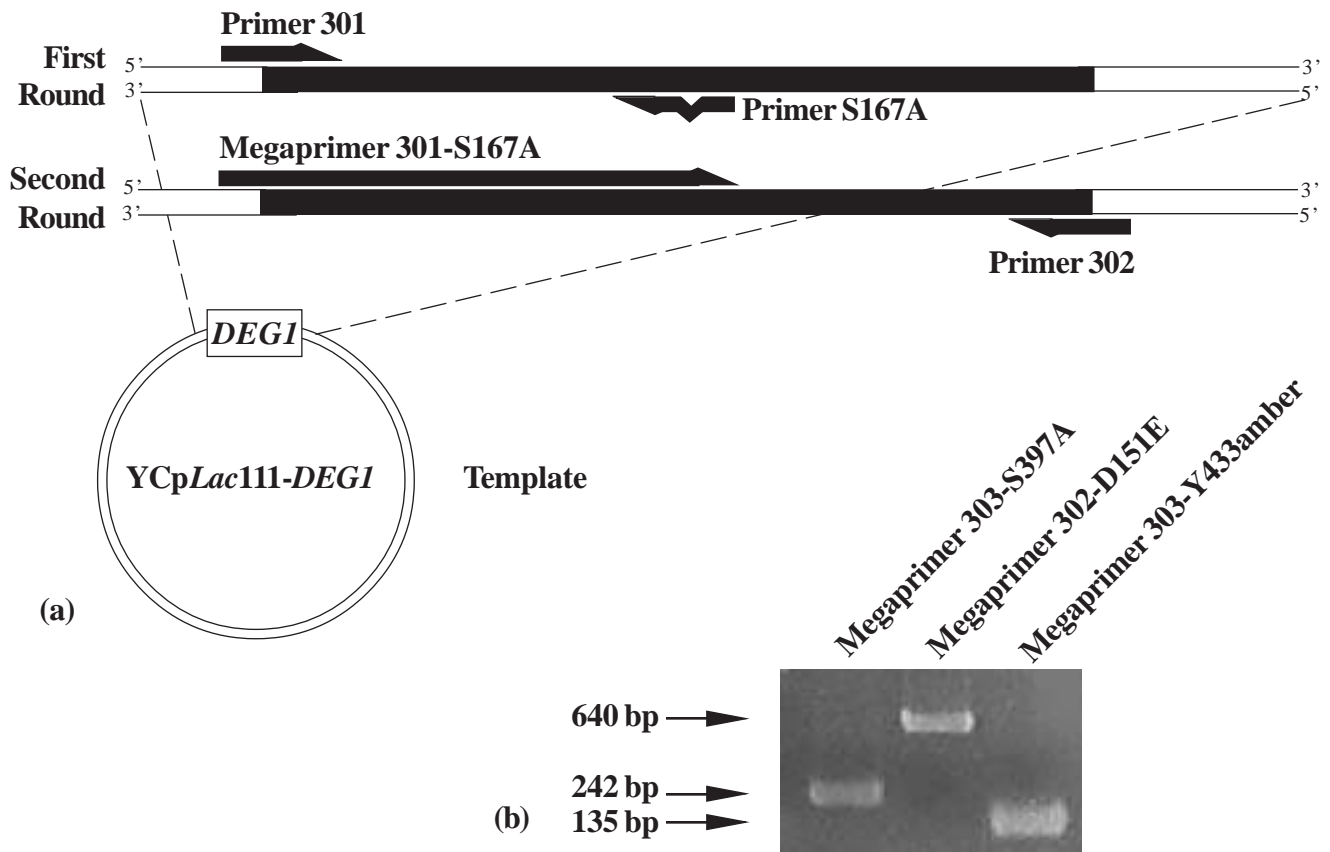


FIGURE 2. (a) The production of *degI*(S167A) by the Megaprimer method of site-directed mutagenesis (Sarkar and Sommer, 1990). The cross-hatched region represents the *DEGI* open reading frame. The point mutation was introduced within Primer S167A during the first round of PCR where Primer 301 and Primer S167A produce Megaprimer 301-S167A. The *degI*(S167A) mutant allele was amplified in the second round of PCR using Megaprimer 301-S167A and Primer 302. Primer sequences and binding sites are described in Table 2. (b) Following the first round of PCR, a 2 μ l aliquot of each reaction was size separated on an 0.8% agarose gel in 1 x TAE to verify that the Megaprimers were amplified.

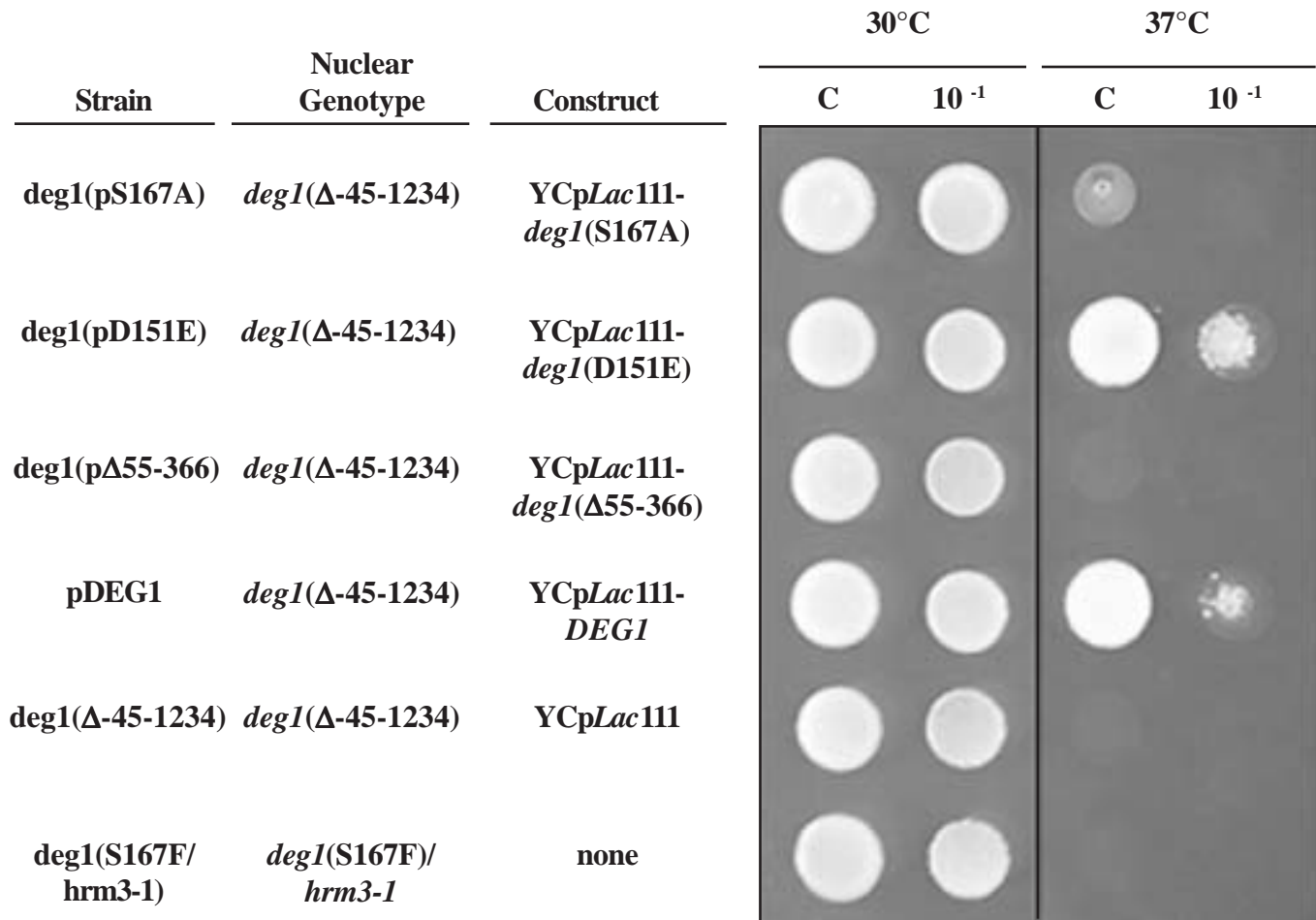


FIGURE 3. Comparison of temperature tolerance in wild-type *DEG1* and various *deg1* strains. YCp*Lac111*-based constructs containing various disruptions of *DEG1*, wild-type *DEG1* or the empty YCp*Lac111* vector were used to transform *deg1*(Δ-45-1234) yeast. Saturated cultures (C) of each strain and a 10⁻¹ dilution were spotted onto duplicate SC-ura-leu plates and incubated at 30°C or 37°C for 48 hours.

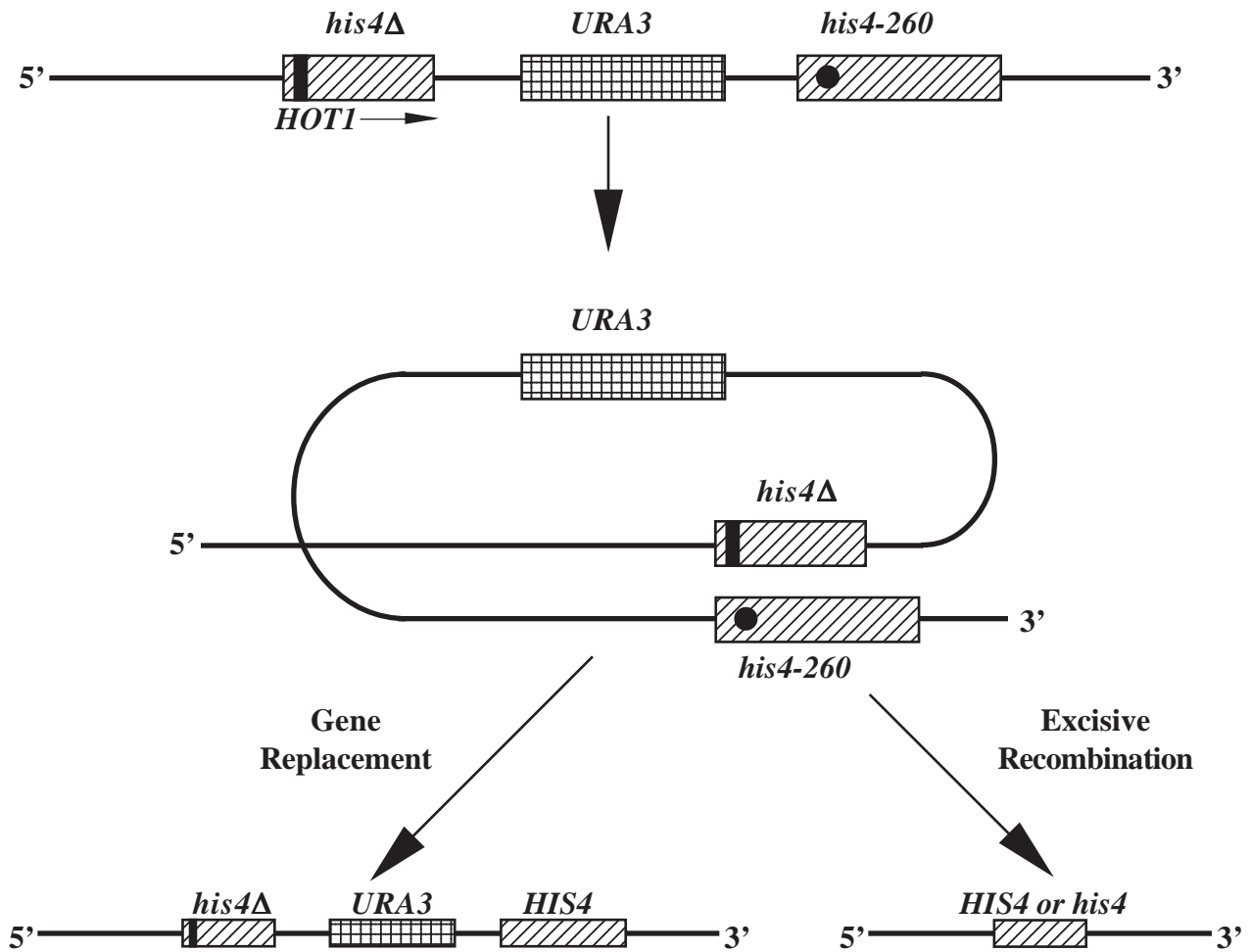


FIGURE 4. Assay for *HOT1::his4Δ::URA3::his4-260* recombination present on chromosome III. The *URA3* gene is flanked by a truncated *his4Δ* gene and a *his4-260* point mutant (indicated by the dark circle) with *HOT1* situated within the *his4Δ* gene (indicated by the dark bar). Excisive recombination removes the *URA3* gene, resulting in Ura⁻ His⁺ or Ura⁻ His⁻ recombinants. Gene replacement allows restoration of the *HIS4* gene without *URA3* excision, resulting in Ura⁺ His⁺ recombinants.

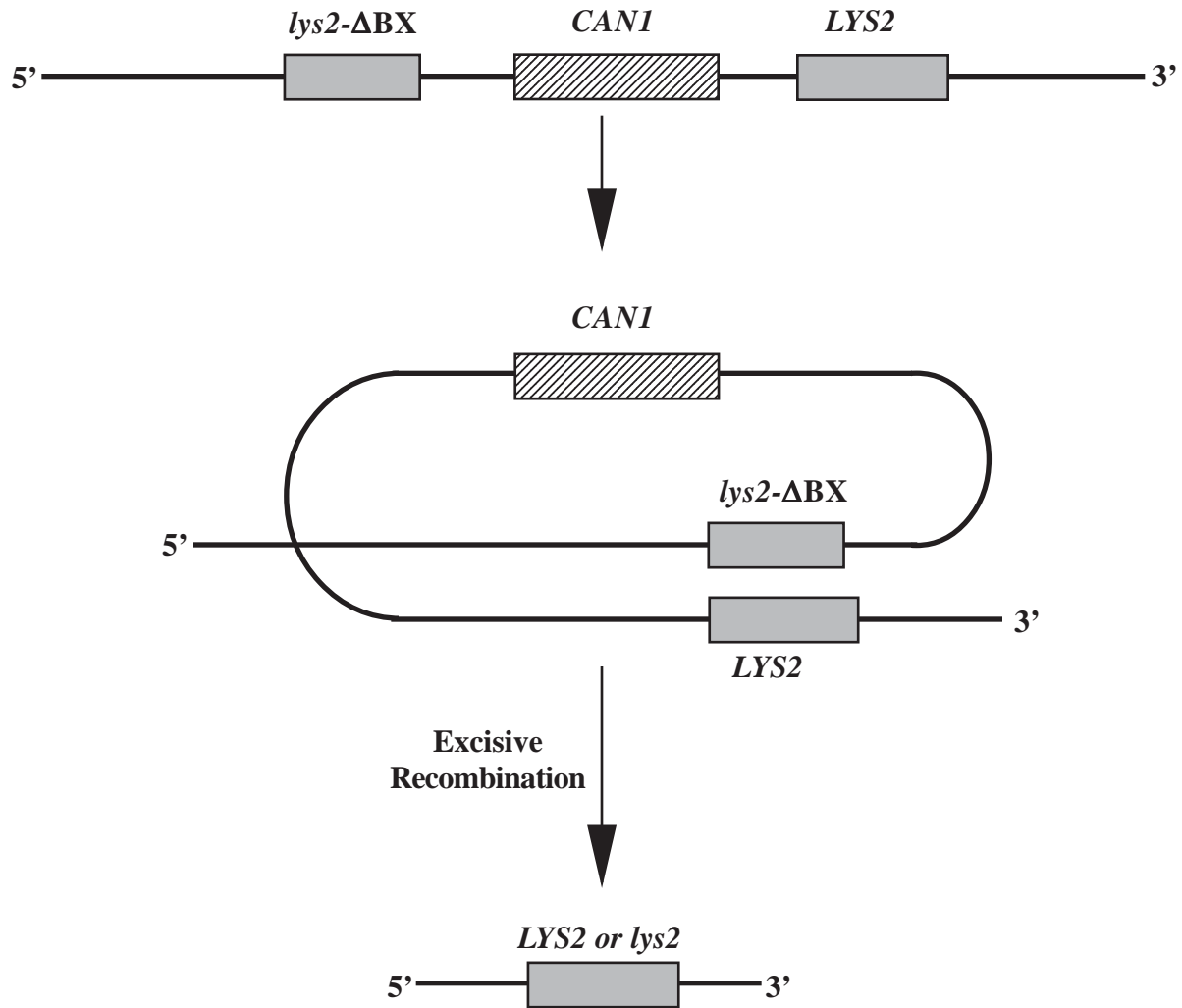


FIGURE 5. Assay for non-*HOT1* *lys2-ΔBX::CAN1::LYS2* recombination present on chromosome II. The *CAN1* gene is flanked by the *lys2-ΔBX* and *LYS2* genes. Excisive recombination eliminates the *CAN1* gene resulting in Can-resistant recombinants.

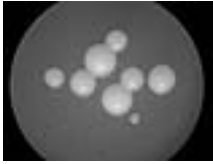
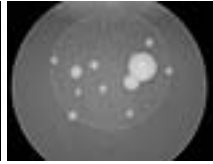
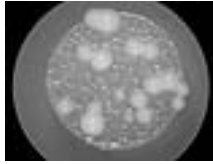















Strain	Nuclear Genotype	Construct	<i>HOT1</i>		Non- <i>HOT1</i>
			Ura ⁻ (10 ⁻²)	His ⁺ (10 ⁻¹)	Can ^R (10 ⁻¹)
deg1(pS167A)	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111-deg1</i> (S167A)			
deg1(pD151E)	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111-deg1</i> (D151E)			
deg1(pΔ55-366)	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111-deg1</i> (Δ55-366)			
pDEG1	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111-DEG1</i>			
deg1(Δ-45-1234)	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111</i>			
deg1(S167F)/ <i>hrm3-1</i>	<i>deg1</i> (S167F)/ <i>hrm3-1</i>	none			

FIGURE 6. Comparison of recombination rates in wild-type *DEG1* and various *deg1* strains. YCp*Lac111*-based constructs containing various disruptions of *DEG1*, wild-type *DEG1*, or the empty vector were used to transform *deg1*(Δ-45-1234) yeast and compared to *deg1*(S167F)/*hrm3-1* yeast with regard to *HOT1*-associated recombination (Ura⁻ or His⁺) and non-*HOT1* recombination (CAN^R).

TABLE 3. The effect of *DEG1* disruption on *HOT1*-associated recombination

Strain	Nuclear Genotype	Construct	Rate (x 10 ⁵) ^a		
			<i>HOT1</i>		<i>Non-HOT1</i>
			Ura ⁻	His ⁺	Can ^R
deg1(pS167A)	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111-deg1</i> (S167A)	16.8 *	2.7 *	7.8
deg1(pD151E)	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111-deg1</i> (D151E)	55.1	19.1	2.2
deg1(pΔ55-366)	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111-deg1</i> (Δ55-366)	23.5 *	4.3 *	3.8
pDEG1	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111-DEG1</i>	86.5	35.5	2.6
deg1(Δ-45-1234)	<i>deg1</i> (Δ-45-1234)	YCp <i>Lac111</i>	12.7 *	3.7 *	6.0
deg1(S167F/ hrm3-1)	<i>deg1</i> (S167F)/ <i>hrm3-1</i>	none	15.2 *	2.4 *	2.5

* Significantly different as compared to YCp*Lac111-DEG1* in *deg1*(Δ-45-1234) yeast at P ≤ 0.01

^a Values represent nine replicates for each genotype in SC medium

TABLE 4. The effect of *DEG1* disruption on growth rate

Strain	Nuclear Genotype	Construct	Doubling time (min) ^a
deg1(pS167A)	<i>deg1</i> (Δ -45-1234)	YCp <i>Lac111-deg1</i> (S167A)	156 \pm 2
deg1(pD151E)	<i>deg1</i> (Δ -45-1234)	YCp <i>Lac111-deg1</i> (D151E)	135 \pm 2
deg1(pΔ55-366)	<i>deg1</i> (Δ -45-1234)	YCp <i>Lac111-deg1</i> (Δ 55-366)	158 \pm 10
pDEG1	<i>deg1</i> (Δ -45-1234)	YCp <i>Lac111-DEG1</i>	144 \pm 5
deg1(Δ-45-1234)	<i>deg1</i> (Δ -45-1234)	YCp <i>Lac111</i>	167 \pm 9
deg1(S167F/hrm3-1)	<i>deg1</i> (S167F)/ <i>hrm3-1</i>	none	185 \pm 13

^aSaturated cultures of each strain were diluted 1:10,000 in SC-ura-leu and grown overnight (~24 h) before growth was observed. Doubling time was determined from a semi-logarithmic plot of A_{595} versus time, using 6-11 data points for each plot. Doubling time represents the average of three replicates for each genotype plus or minus one standard deviation.

APPENDIX A

The 1.6 kbp *EcoRI-EcoRI* fragment of *S. cerevisiae* chromosome VI containing all of the *DEG1* gene (tagged with 901) and promoter sequences (Steudel, 1998). *YCpLac111* vector sequences are not shown. Translated sequence corresponds to Pus3p and epitope 901.

-171	-161	-151	-141	-131
GAATTCTTAA	TATTATCTTA	CTACACCTCA	AATCAATGGG	CTCAGCTCAT
CTTAAGAATT	ATAATAGAAT	GATGTGGAGT	TTAGTTACCC	GAGTCGAGTA
-121	-111	-101	-91	-81
CTTGAAAAGC	TCACTTTGTT	TTTCAGTGAA	AAATTTTTGA	AAGCGAAAAG
GAACTTTTTCG	AGTGAAACAA	AAAGTCACTT	TTTAAAAACT	TTCGCTTTTC
-71	-61	-51	-41	-31
CCGGCTCAAA	ATAAAGAATA	ATATAGTATT	CTCGAGGTGC	CCACATGCAA
GGCCGAGTTT	TATTTCTTAT	TATATCATAA	GAGCTCCACG	GGTGTACGTT
-21	-11	-1	1	10
TCTTTACTGC	CCTACTATAA	CCTCCCTTGA	ATGAGTAATT	TCATTAGAAG
AGAAATGACG	GGATGATATT	GGAGGGAACT	TACTCATTAA	AGTAATCTTC
			M S N	F I R R
			Pus3p	
30	40	50	60	70
GCTAGTTGGG	AAAATGAAAG	CGATTTCAAC	AGGTACCAAT	GCTATTGTTA
CGATCAACCC	TTTTACTTTC	GCTAAAGTTG	TCCATGGTTA	CGATAACAAT
L V G	K M K	A I S T	G T N	A I V
		Pus3p		
80	90	100	110	120
GTAAGAAGGA	CTCCATTTAC	GCAAAGTGGT	CCAAAGAGCA	GCTAATACGA
CATTCTTCCT	GAGGTAAATG	CGTTTGACCA	GGTTTCTCGT	CGATTATGCT
S K K D	S I Y	A N W	S K E Q	L I R
		Pus3p		
130	140	150	160	170
AGGATCACTG	AACTAGAAAA	CGCAAATAAG	CCACATTCTG	AAAAATTCCA
TCCTAGTGAC	TTGATCTTTT	GCGTTTATTC	GGTGTAAAGAC	TTTTTAAGGT
R I T	E L E N	A N K	P H S	E K F Q
		Pus3p		
180	190	200	210	220
GCACATCGAG	GATAACAAAA	AGCGCAAAAT	TTCGCAAGAA	GAGGTTACGA
CGTGTAGCTC	CTATTGTTTT	TCGCGTTTTA	AAGCGTTCTT	CTCCAATGCT
H I E	D N K	K R K I	S Q E	E V T
		Pus3p		
230	240	250	260	270
GAAGCAAGGC	GAAAAAGGCT	CCGAAGAAGT	TTGACTTTTC	TAAACATAAT
CTTCGTTCCG	CTTTTTCCGA	GGCTTCTTCA	AACTGAAAAG	ATTTGTATTA
R S K A	K K A	P K K	F D F S	K H N
		Pus3p		
280	290	300	310	320
ACCAGTTCA	TCGCCTTGAG	ATTTGCCTAT	TTGGGATGGA	ATTACAATGG
TGGTCCAAGT	AGCGGAACTC	TAAACGGATA	AACCCTACCT	TAATGTTACC
T R F	I A L R	F A Y	L G W	N Y N G
		Pus3p		
330	340	350	360	370
CTTAGCTGTT	CAGAAGGAAT	ACACACCGTT	GCCTACAGTA	GAGGGTACCA
GAATCGACAA	GTCTTCCTTA	TGTGTGGCAA	CGGATGTCAT	CTCCCATGGT
L A V	Q K E	Y T P L	P T V	E G T
		Pus3p		

380	390	400	410	420
TTTTGGAGGC	CATGAATAAG	TGTAAACTTG	TTCCCTCGAT	GGTTTTACAA
AAAACCTCCG	GTA CTTATTC	ACATTTGAAC	AAGGGAGCTA	CCAAAATGTT
I L E A	M N K	C K L	V P S M	V L Q
Pus3p				
430	440	450	460	470
GACTATAAAT	TTAGCAGATG	TGGCAGAACA	GACAAGGGAG	TTAGCGCCAT
CTGATATTTA	AATCGTCTAC	ACCGTCTTGT	CTGTTCCCTC	AATCGCGGTA
D Y K	F S R C	G R T	D K G	V S A M
Pus3p				
480	490	500	510	520
GAACCAAGTT	ATATCGTTAG	AAGTCCGTTT	CAATTTAACA	GATGAGGAAC
CTTGGTTCAA	TATAGCAATC	TTCAGGCAAG	GTTAAATTGT	CTACTCCTTG
N Q V	I S L	E V R S	N L T	D E E
Pus3p				
530	540	550	560	570
AGCGGGATCC	GACCAACGAC	AGCAGGGAAA	TACCCTATGT	TCACGTTTTA
TCGCCCTAGG	CTGGTTGCTG	TCGTCCCTTT	ATGGGATACA	AGTGCAAAAT
Q R D P	T N D	S R E	I P Y V	H V L
Pus3p				
580	590	600	610	620
AATCAATTAC	TACCCGACGA	TATTCGTATA	TCAGCTGTCT	GCCTCAGACC
TTAGTTAATG	ATGGGCTGCT	ATAAGCATAT	AGTCGACAGA	CGGAGTCTGG
N Q L	L P D D	I R I	S A V	C L R P
Pus3p				
630	640	650	660	670
GCCACCTAAC	TTTGACGCAA	GATTCAGCTG	TGTTCCACGG	CACTATAAGT
CGGTGGATTG	AAACTGCGTT	CTAAGTCGAC	ACAAGTGGCC	GTGATATTCA
P P N	F D A	R F S C	V H R	H Y K
Pus3p				
680	690	700	710	720
ACATCTTTAA	TGGAAAAAAC	CTTAATATTG	AAAAAATGTC	TAAAGCCGCA
TGTAGAAATT	ACCTTTTTTG	GAATTATAAC	TTTTTTACAG	ATTTCCGGCGT
Y I F N	G K N	L N I	E K M S	K A A
Pus3p				
730	740	750	760	770
TCATATTTTG	TTGGAGAGAG	AGACTTCAGG	AATTTTTGTA	AGCTTGATGG
AGTATAAAAC	AACCTCTCTC	TCTGAAGTCC	TTAAAAACAT	TCGAACTACC
S Y F	V G E R	D F R	N F C	K L D G
Pus3p				
780	790	800	810	820
CTCAAAACAA	ATTACCAATT	TTAAACGAAC	AATAATAAGC	TCAAAAATTC
GAGTTTTGTT	TAATGGTTAA	AATTTGCTTG	TTATTATTTCG	AGTTTTTAAG
S K Q	I T N	F K R T	I I S	S K I
Pus3p				
830	840	850	860	870
TTCCCCTTTC	TGAAACTTTC	TATTGTTTTG	ATCTCGTTGG	TTCAGCATTC
AAGGGGAAAG	ACTTTGAAAG	ATAACAAAAC	TAGAGCAACC	AAGTCGTAAG
L P L S	E T F	Y C F	D L V G	S A F
Pus3p				
880	890	900	910	920
CTATGGCACC	AAGTTCGTTG	CATGATGGCC	ATTCTTTTCT	TAGTTGGTCA
GATACCGTGG	TTCAAGCAAC	GTA C TACCGG	TAAGAAAAGA	ATCAACCAGT
L W H	Q V R C	M M A	I L F	L V G Q
Pus3p				

930	940	950	960	970
ATCACTTGAA	GTGCCGAAA	TTGTCTTGCG	TCTGACTGAT	ATTGAGAAAA
TAGTGAACTT	CACGGCCTTT	AACAGAACGC	AGACTGACTA	TAACTCTTTT
S L E	V P E	I V L R	L T D	I E K
Pus3p				
980	990	1000	1010	1020
CCCCTCAAAG	GCCTGTTTAC	GAAATGGCAA	ATGATATAACC	ATTATTGTTA
GGGGAGTTTC	CGGACAAATG	CTTTACCGTT	TACTATATGG	TAATAACAAT
T P Q R	P V Y	E M A	N D I P	L L L
Pus3p				
1030	1040	1050	1060	1070
TATGATTGTA	AATTTCTCTGA	AATGGATTGG	CAAGAACCTA	CTGTAGATGA
ATACTAACAT	TTAAAGGACT	TTACCTAACC	GTTCTTGGAT	GACATCTACT
Y D C	K F P E	M D W	Q E P	T V D D
Pus3p				
1080	1090	1100	1110	1120
CTATAAGGCA	ATAAAATTCA	CGACAGCTAC	TGAAGCATTG	ACGTTACACT
GATATTCCGT	TATTTTAAGT	GCTGTCGATG	ACTTCGTAAC	TGCAATGTGA
Y K A	I K F	T T A T	E A L	T L H
Pus3p				
1130	1140	1150	1160	1170
ACGAACTTAA	GGCCGCAGTA	TGCAATATTT	TTAAAGATGT	TTTGCCCACA
TGCTTGAATT	CCGGCGTCAT	ACGTTATAAA	AATTTCTACA	AAACGGGTGT
Y E L K	A A V	C N I	F K D V	L P T
Pus3p				
1180	1190	1200	1210	1220
GCAAATACAA	ACAACCTTCTC	AAAGACAATT	ATTAATTTGG	GCGATGGAAG
CGTTTATGTT	TGTTGAAGAG	TTTCTGTAA	TAATTAAACC	CGCTACCTTC
A N T	N N F S	K T I	I N L	G D G R
Pus3p				
1230	1240	1250	1260	1270
AGGTAAAGTA	GTCGGTACCT	ATGTGAAACT	AGAGGATAGA	AGCGTTATGG
TCCATTTTCAT	CAGCCATGGA	TACACTTTGA	TCTCCTATCT	TCGCAATACC
G K V	V G T	Y V K L	E D R	S V M
Pus3p				
1280	1290	1300	1310	1320
AACCTGTCTGA	AGTTGTTAAT	GCTAAATACT	CCAAGAAAAA	GAACAACAAA
TTGGACAGCT	TCAACAATTA	CGATTTATGA	GGTTCTTTTT	CTTGTTGTTT
E P V E	V V N	A K Y	S K K K	N N K
Pus3p				
1330	1340	1350	1360	1370
AATAAGAATC	AGCCATATCA	CATTTGTAGA	GGTTTTACTT	GCTTTAAAAA
TTATTCTTAG	TCGGTATAGT	GTAACATCT	CCAAAATGAA	CGAAATTTTT
N K N	Q P Y H	I C R	G F T	C F K K
Pus3p				
Epitope 901				
1380	1390	1400	1410	1419
ATAAAATAAT	ATATAAACCT	GTATAATATA	ACCTTGAAGA	CTAGAATTC
TATTTTATTA	TATATTTGGA	CATATTATAT	TGGAACCTCT	GATCTTAAG