REGULATORY FACTORS AND PATHWAYS INFLUENCING POL II rRNA SYNTHESIS IN *SACCHAROMYCES CEREVISIAE*

A DISSERTATION

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BY

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DENTON, TEXAS

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To the Dean of the Graduate School:

I am submitting herewith a dissertation written by Gunisha Sagar entitled "Regulatory Factors and Pathways Influencing Pol II rRNA Synthesis in *Saccharomyces cerevisiae.*" I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Molecular Biology.

Heather Conrad-Webb, Ph.D., Major Professor

We have read this dissertation and recommend its acceptance:

Department Chair

Accepted:

Dean of the Graduate School

DEDICATION

To my Teachers and Husband

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ABSTRACT

GUNISHA SAGAR

REGULATORY FACTORS AND PATHWAYS INFLUENCING POL II rRNA SYNTHESIS IN *SACCHAROMYCES CEREVISIAE*

DECEMBER 2008

rRNA synthesis in *Saccharomyces cerevisiae* is strictly regulated by the physiological status of the cell and its response to environmental cues. This necessitates activation of a cell signaling pathway and a regulatory response. Under ideal growth conditions, rRNA is synthesized by combined activities of RNA polymerase I and II. However, in response to mitochondrial dysfunction or the absence of Pol I transcription factor UAF, the contribution of Pol II rRNA synthesis increases significantly. To identify factors regulating Pol II rRNA expression in ρ^+ cells (cells with mitochondrial DNA), a Yeast Knock Out library in ρ^+ homozygous diploids was screened. Each of the knock-out strains, lacking one of the non-essential genes was transformed with reporter constructs bearing Pol II rDNA promoter fused to either the *E. coli lacZ* or yeast *URA3* reporter genes and was surveyed for defects in Pol II rRNA synthesis.

From over approximately 2800 deletion strains, five candidate genes (*FKH2, PBS2, RTG2, PHO3* and *OCA4*) were identified that exhibited reduced Pol II promoter function. *FKH2* encodes for a cell cycle specific transcription factor. Fkh2 may

regulate Pol II rDNA expression during the G2 phase of cell cycle. Pbs2p and Hog1p, members of the HOG pathway, also enhanced Pol II driven rRNA synthesis, implicating the involvement of HOG pathway in Pol II rDNA transcription. Rtg2p, a member of retrograde pathway, was found to influence Pol II rDNA transcription suggesting that mitochondrial dysfunction and low levels of nitrogen trigger the polymerase switch. Rtg2p most likely up-regulates Pol II rDNA transcription as a part of a novel chromatin remodeling complex, SLIK (SAGA-like). In addition, *OCA4* along with other members (*OCA1* and *OCA3*) of a novel complex 490 is found to significantly affect Pol II rDNA transcription. Pho3p, a acid phosphatase is most likely indirectly involved in Pol II rDNA transcription.

Our original hypothesis that polymerase II switch may be a backup mechanism for cells undergoing stress is supported by the involvement of Retrograde and HOG pathways in triggering Pol II driven rRNA synthesis in ρ^+ strains of *Saccharomyces cerevisiae.*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The ribosome, a macromolecular machinery of 4 megadaltons, is indispensable for cell survival as it is the hub for the synthesis of all proteins (Preiss and Hentze, 2003). It is a ribonucleoprotein particle composed of two subunits (Venema and Tollervey, 1999). The 60S or large subunit contains three rRNA (Ribosomal RNA) molecules, 25S, 5.8S and 5S rRNA complexed with 46 ribosomal proteins. In contrast, the smaller 40S subunit has a single 18S rRNA and 32 proteins (Verschoor et al., 1998). In addition, ribosome has binding sites for tRNAs, a mRNA channel and a peptidyl transferase center (Liljas, 1999). Surprisingly, rRNA lines these sites. The 16S rRNA promotes favorable interaction of mRNA with tRNA while the 23S and 25S rRNA constitute a major part of the peptidyl transferase activity in prokaryotes and eukaryotes, respectively (Ramakrishnan, 2002).

The 25S, 18S and 5.8S rRNA genes are transcribed as a single primary RNA Pol I transcript within the fibrillar center of the nucleolus, while the 5S rRNA gene is transcribed independently by RNA Pol III (Scheer et al., 1993). The primary Pol I transcript is cleaved within the 3' ETS (External Transcribed Spacer region) by Rnt1p, an exoribonuclease (Venema and Tollervey, 1999). The cleavage by Rnt1p is likely to be co-transcription and results in the production of a 35S rRNA precursor. This 35S pre-rRNA transcript is then subjected to processing and various post transcriptional

modifications to form mature rRNAs (Kressler et al., 1999; Venema and Tollervey, 1999).

Within the dense fibrillar region of nucleolus, modifications and processing of rRNA does not take place on naked pre-rRNA, but occur within pre-ribosomal particles formed as a result of association of ribosomal proteins and association factors which are imported to nucleus through the nuclear pore complexes (Rout et al., 1997; Scheer et al., 1993; Trapman et al., 1975). This results in the formation of a 90S pre-ribosomal RNA particle, comprising of U3 snoRNA, 35S rRNA and processing factors involved in the synthesis of 40S pre-ribosomal subunit. Interestingly, the processing factors involved in the synthesis of 40S pre-ribosomal particle assemble co-transcriptionally onto pre-35S rRNA as soon as the 18S rRNA is transcribed (Dlakic, 2005). The 35S pre-rRNA undergoes various covalent modifications such as the pseudouridylation of uridine nucleotide by H/ACA-box SnoRNPs (small nucleolar ribonucleoprotein particles) and methylation of 2'-hydroxyl residue of ribose by C/D box snoRNPs within the 90S pre-ribosomal RNA particle. Following these modifications and the cleavage of 5' end of 35S pre-rRNA by U3snoRNP, the 32S pre-rRNA is cleaved to form the 20S and 27S pre-rRNA precursors (Venema and Tollervey, 1999). The 20S and 27S precursors are exported out of nucleolus to nucleoplasm as a part of pre-40S and pre-60S particles, respectively (Dlakic, 2005; Kressler et al., 1999). In cytoplasm, the 20S rRNA is further processed to18S rRNA within the pre-ribosomal particle and the 27S rRNA is cleaved by an exosome to form 25S and 5.8S rRNA (Kressler et al., 1999; Venema and Tollervey, 1999).

In cytoplasm ribosomal subunits complete assembly following the incorporation of all r-proteins of 60S subunit along with 5S, 5.8S and 25S rRNA and completion of final stages of maturation of 18S rRNA within the 43S subunit to form a 40S subunit. Thus, most part of ribosomal biogenesis occurs in the nucleolus, but is completed in cytosol (Dlakic, 2005).

The overall rate of ribosomal synthesis depends on the synthesis of rRNA, a structural component of ribosome. Almost two-thirds of the ribosome is composed of rRNA (Rakauskaite and Dinman, 2008). Besides playing a structural role, the 25S rRNA forms a part of a functional peptidyl transferase center (PTC) of the ribosome and plays a role in peptide bond formation during protein synthesis (Rakauskaite and Dinman, 2008). In an actively growing cell, rRNA synthesis accounts for approximately 80% of total transcriptional activity (Planta, 1997; Rudra and Warner, 2004). However, upon depletion of nutrients rRNA synthesis drops rapidly (Li et al., 2006; Moorefield et al., 2000; Shulman et al., 1977). Thus, in response to different environmental cues, rRNA synthesis is regulated at various levels of transcription such as initiation, elongation, degradation, and processing of rRNA by a variety of regulatory factors (Moss, 2004; Warner, 1989). rRNA levels vary with growth rate. In a fast growing culture, rRNA constitutes 85% of total RNA but the level drops as growth rate decreases (Waldron and Lacroute, 1975). As considerable amount of cellular energy is diverted toward ribosome biogenesis, the synthesis of both rRNA and ribosomes is tightly regulated in response to physiological

demands (Moss, 2004; Warner, 1999). It is thus necessary to study the conditions and factors that regulate such a vital process.

In *Saccharomyces cerevisiae,* 150-200 copies of rDNA units comprising of 18S, 25S, 5.8S and 5S genes are arranged head to tail as 9.1 Kb tandem arrays on the long arm of chromosome XII (Nomura, 2001; Petes, 1979). The number of rDNA repeats vary with the genetic background, age of the strain and the environmental conditions (Reeder, 1999; Vu et al., 1999). The nontranscribed spacer regions, NTS1 and NTS2 separate the two consecutive rRNA gene clusters, while the 5S rRNA gene separates the nontranscribed region into NTS1 and NTS2 (Philippsen et al., 1978). Each rDNA transcription unit, composed of 25S, 18S and 5.8S rRNA genes, is transcribed in the same orientation by RNA polymerase I (Pol I) as a single 35S rRNA precursor and a 5S rRNA gene borne within the rDNA repeat is transcribed in the opposite orientation by RNA Pol III (Warner, 1989) (Figure 1).

Pol I Mediated Transcription of rDNA Genes

Pol I transcription of rDNA transcription unit (25S, 18S and 5.8S rRNA genes) is mediated by the binding of Pol I to rDNA Pol I promoter. The Pol I rDNA promoter is located 150 bp (+5 to –155) upstream of RNA Pol I transcriptional start site (Musters et al., 1989; Nomura, 2001).

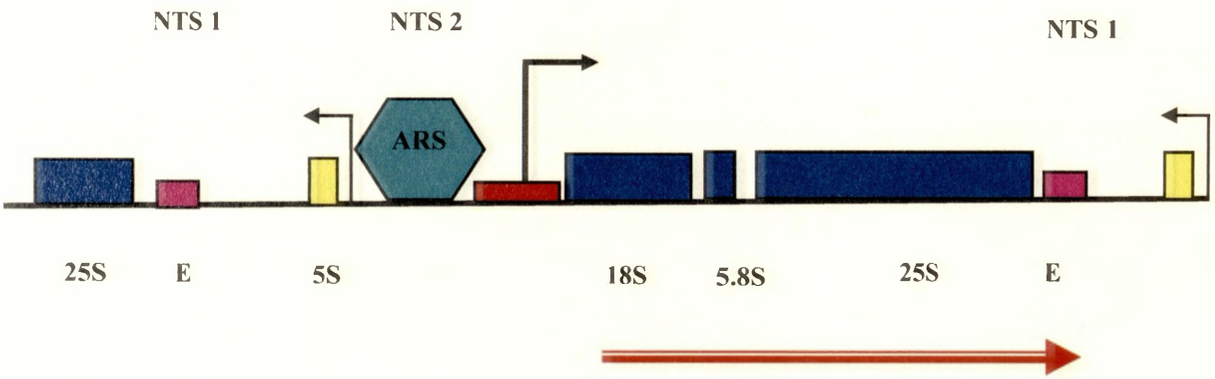

Figure 1. Yeast rDNA Repeat.
The rDNA gene clusters borne on the long arm of chromosome XII are transcribed (red arrow) by binding of RNA Pol I to a Pol I promoter (red box) as a 35S rRNA precursor (red arrow). Pol I transcription is enhanced by an enhancer element located at the end of 25S rRNA gene (pink box) (Johnson and Warner, 1989). The Autonomously Replicating Sequence (ARS) (aqua hexagon). A 5S rRNA gene (yellow) is transcribed in opposite orientation by RNA polymerase I

In a bipartite model, Pol I promoter is composed of an Upstream Control Element or Upstream Promoter Element (UCE/UPE) and Core Element (CE) (Choe et al., 1992; Keys et al., 1994; Kulkens et al., 1991). The upstream control element is required for transcriptional activation, whereas the core element is required for accurate and basal transcription (Keys et al., 1996) (Figure 2).

The primary event in Pol I mediated transcription is the sequence specific recognition of UCE by Pol I transcription factor, UAF (Nomura, 2001). Binding of UAF to rDNA promoter commits the rDNA template to transcription (Planta, 1997). UAF recruits the Core Factor (CF) and TATA binding protein (TBP) to the UCE of Pol I promoter and results in the formation of a preinitiation complex (PIC). CF, a complex of Rrn6p, Rrn7p and Rrn11p, facilitate promoter opening by unfolding chromatin as shown by DNase I footprinting and transcription on immobilized templates (Figure 2) (Aprikian et al., 2001). Under favorable growth conditions, the phosphorylated form of Rrn3p associates with RNA polymerase I and renders Pol I competent to form a transcriptionally active initiation complex (Grummt, 2003).

The association of Rrn3p with Pol I is growth regulated. There are limiting amounts of active Pol I-Rrn3p complexes in stationary phase cell cultures (Milkereit and Tschochner, 1998; Sandmeier et al., 2002). In addition, Target of Rapamycin (TOR) signaling pathway also regulates the recruitment of Rrn3p-Pol I complex to Pol I promoter in vivo (Claypool et al., 2004). *RRN3* gene was found to be a suppressor of *Cbf5-1,* suggesting that Cbf5p, a low affinity centromeric DNA binding protein may also be involved in Pol I rRNA synthesis (Cadwell et al., 1997). Cbf5p was identified as a part of a ribonucleoprotein complex along with Pol I, TBP, Rrn3p, Rrn5p, Rrn7p, Reb1p, Nop1p, Nhp2p and Rrp5p by mass spectrometry (Fath et al., 2000).

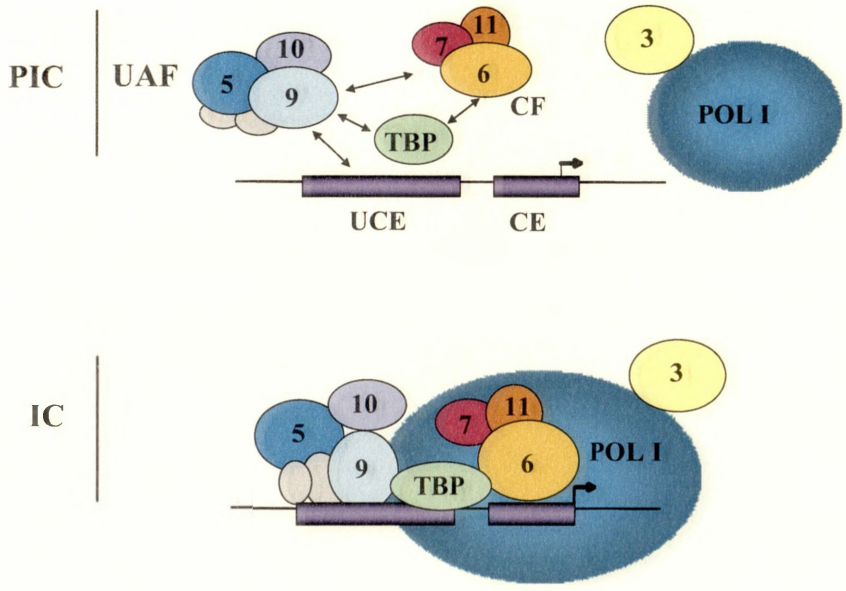

Figure 2. Activation of rDNA Pol I Promoter by RNA Polymerase I.
Upstream Activating Factor (UAF), a complex of (Rrn5p, Rrn9p, Rrn10p, H3 and H4) binds to the Upstream Core Element (UCE). Upon binding, UAF recruits Core Factor (CF), a complex of Rrn6p, Rrn7p and Rrn11p to Core Element (CE). TATA binding Protein (TBP) associates with both CF and UAF at TATA box. Association of UAF-TBP-CF results in the formation of a Preinitiation Complex (PIC). Formation of PIC promotes the recruitment of RNA Pol I and Rrn3p complex and results in the formation of Initiation Complex (IC). Once IC is formed, Pol I mediated transcription is initiated from the TATA box within Pol I promoter (Jodhka, 2004).

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rRNA synthesis (Cadwell et al., 1997). Cbf5p was identified as a part of a ribonucleoprotein complex along with Pol I, TBP, Rrn3p, Rrn5p, Rrn7p, Reb1p, Nop1p, Nhp2p and Rrp5p by mass spectrometry (Fath et al., 2000). After assembly of preinitiation complex (CF and UAF), the RNA polymerase I-Rrn3p complex is recruited to rDNA Pol I promoter and this marks a transition from a pre-initiation to an initiation phase (Yamamoto et al., 1996) (Figure 2). Transcriptionally active RNA Pol I complex then promotes initiation of transcription from the transcription initiation site (+1). Once transcription is initiated, TBP, CF and Pol I-Rrn3p complex dissociates from the template (Aprikian et al., 2001) (Figure 2).

Pol I transcription efficiency is enhanced by the binding of Reb1p to two binding sites within NTS (Warner, 1989). One of the binding sites, located within the enhancer element, terminates transcription; whereas, the other site located 60 bp upstream of the 5' end of Pol I promoter enhances Pol I transcription (Kulkens et al., 1992; Planta, 1997). The deletion of both Reb1p binding sites from the chromosomal rRNA repeats is known to drastically reduce Pol I transcription to 25% (Kulkens et al., 1992).

A looping or a three dimensional matrix-attachment model was proposed for Reb1p mediated enhancer function (Warner, 1989). According to this model, oligomerization of Reb1p to the nucleolar matrix results in looping of rDNA repeats which in turn brings all Pol I rDNA units to one side and intergenic spacers carrying 5S rRNA gene to the other side of the spatial complex or ribomotor. Because of looping, enhancer-terminators of at least two neighboring Pol I promoters are brought in close proximity.

By virtue of close proximity of two promoters, the Pol I and its associated factors from one unit can pass directly to another unit without being released in the nucleoplasm, resulting in increased transcriptional efficiency (Planta, 1997; Warner, 1989).

Conditions Affecting rRNA Synthesis

rDNA transcription is regulated by different growth and environmental conditions. Psoralen crosslinking of proteins with DNA and electron microscopic studies have shown that yeast modulates rDNA transcription by regulating relative levels of active and inactive copies of rRNA genes under different growth conditions (Dammann et al., 1993). In addition, rDNA transcription is also regulated by modulating transcriptional initiation frequency, that is.the rate of loading of active Pol I complexes at Pol I rDNA promoter (Fahy et al., 2005; French et al., 2003). Some of the conditions that determine the overall rate of rDNA transcription include growth rate, defects in the secretory pathway, and heat shock.

Transcription of rRNA genes is coordinated with cellular growth (Warner, 1989, 1999). An exponentially growing culture has a higher rate of rRNA transcription than a stationary phase culture (Ju and Warner, 1994). As well, pre-rRNA synthesis is reduced by 10 fold in a stationary phase culture (Moss, 2004). This differential regulation may be due to either alteration in the proportion of active rRNA genes (Dammann et al., 1993; Reeder, 1999), due to variation in transcription initiation frequency or a combination of both mechanisms (Fahy et al., 2005; French et al., 2003). As cells undergo a transition from exponential to stationary phase, the number of active rRNA genes decrease

(Dammann et al., 1993) through the action of chromatin remodeling deacetylase complex, Rpd3p (Sandmeier et al., 2002). In fact, cells in the stationary phase have reduced rRNA synthesis due to reduced polymerase loading or decreased initiation frequency (Sandmeier et al., 2002). The reduced initiation frequency in stationary cultures is attributed to the lack of active Pol I-Rrn3p complexes (Milkereit and Tschochner, 1998; Yamamoto et al., 1996). The levels of Rrn3p-Pol I complexes at the Pol I promoter is also regulated by TOR pathway in response to nutrient levels (Claypool et al., 2004; Powers and Walter, 1999). Thus, rRNA synthesis is mainly regulated at the level of initiation of transcription from active genes and the proportion of active rDNA copies is not a major determining factor in stationary phase cultures (Fahy et al., 2005).

Regulation of rRNA synthesis by polymerase loading appears to be the primary form of regulation in most conditions. Polymerase initiation frequency in two exponentially growing yeast strains with differing numbers of rRNA genes (140 rRNA genes and 42 rRNA genes) showed equal rate of rDNA transcription due to modulation of initiation frequency i.e rate of initiation per gene (French et al., 2003). All rRNA genes were active in a 42 copy strain, while, not all rRNA genes were active in the 140 copy strain. Despite these differences both the strains exhibited a similar rate of rRNA synthesis primarily due to increased Pol I loading in 42 copy strain (French et al., 2003). Thus regulation of rDNA transcription at the level of initiation is of a greater significance than regulating the proportion of active opened genes in both stationary and exponential cultures (Fahy et al., 2005; French et al., 2003).

Disruption of the secretory pathway results in transcriptional repression of rRNA genes as well as Ribosomal Protein (RP) genes. A direct correlation between a defect in the secretory pathway and rRNA synthesis came from studies with temperature sensitive (Ts) mutants of *SLY1*. In addition to a defect in endoplasmic reticulum (ER) to Golgi complex transport, a temperature sensitive *sly1* mutants have a drastic reduction of rRNA genes and RP genes transcription (Mizuta and Warner, 1994). The coupling of secretory pathway and ribosome synthesis is apparently due to a feedback response from stretch sensors of "Wsc" subfamily located within the cell wall (Li et al., 2000). Defects in the secretory pathway that cause an increase in cell mass without a concomitant increase in plasma membrane also trigger the cell wall integrity pathway to repress ribosome synthesis (Nierras and Warner, 1999).

Heat shock also causes a transient decline in synthesis of 35S pre rRNA (Liu et al., 1996; Warner, 1989). Hybridization studies show that rDNA transcription is inhibited at the level of initiation upon heat shock (Veinot-Drebot et al., 1989). Pol I rDNA transcription is also reduced at high temperature of 42.5°C due to relocation of many nuclear proteins to cytosol causing the nucleolus, the site of rDNA transcription, to disassemble (Liu et al., 1996).

Pol II Driven Transcription of rDNA Genes

The rRNA genes have an endogenous cryptic Pol II promoter that overlaps the Pol I promoter (Conrad-Webb and Butow, 1995) (Figure 3). In respiratory deficient strains of *S. cerevisiae*, this cryptic promoter is used to synthesize functional rRNA (Conrad-Webb

and Butow, 1995). The transcriptional activation of the promoter by RNA polymerase II results in the transcription of 25S, 18S, and 5.8S rDNA genes as a single 35S rRNA precursor and this phenomenon is referred to as Pol I to Pol II switch or polymerase switch (Figure 3). Intriguingly, a similar Pol II rDNA promoter overlapping the Pol I promoter is found in rRNA genes of other eukaryotic organisms such as plants, monkeys and other mammals (Pikkard, 1994; Smale and Tjian, 1985; Sollner-Webb and Mougey, 1991).

In wild-type yeast, Pol II rDNA promoter is not active within the chromosomal rDNA repeats (Conrad-Webb and Butow, 1995), but is suppressed by binding of the Pol I transcriptional factor UAF to the overlapping promoter region (Oakes et al., 1999; Siddiqi et al., 2001; Vu et al., 1999). Repression of Pol II rRNA transcription may be brought about by localizing rDNA chromatin structure to regions of the nucleolus that are inaccessible to Pol II, but accessible to Pol I (Vu et al., 1999). The histone deacetylase, Rpd3p, is important for Pol II mediated rDNA transcription from the chromosomal context in UAF mutants. A large decrease in Pol II transcriptional activity of rRNA genes ($\geq$70%) relative to a control RPD3 *uaf*Δ strain was seen in a double mutant *rpd3*Δ *uaf*Δ by primer extension analysis, suggesting Rpd3p role in maintaining rDNA chromatin conducive for Pol II rDNA transcription (Oakes et al., 2006).

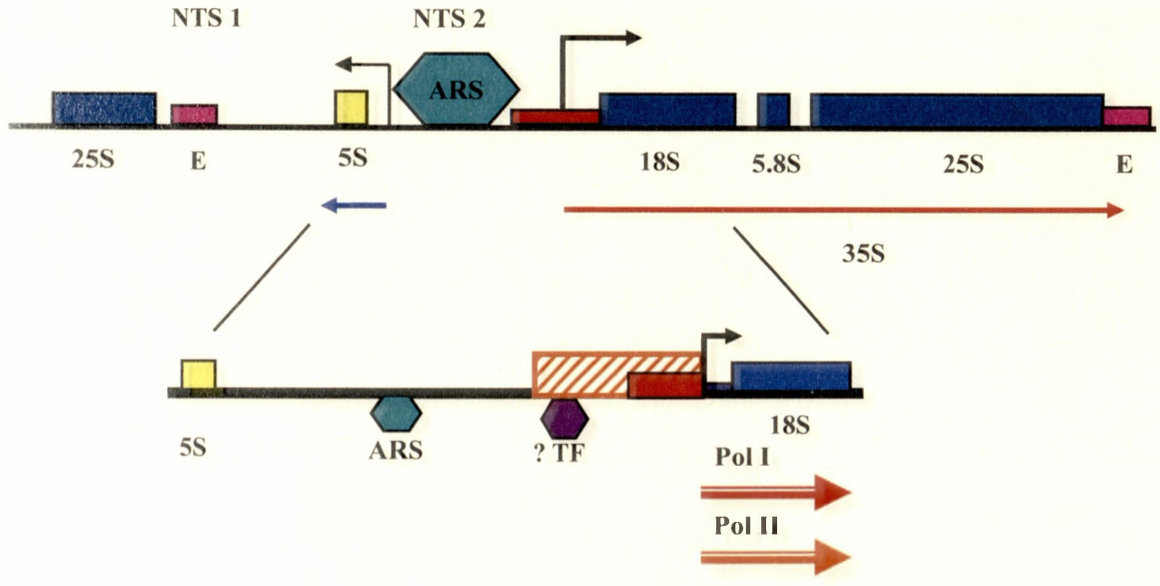


Figure 3. The Polymerase Switch.
Under normal conditions in ρ^+ strains of *S. cerevisiae*, rDNA gene clusters borne on the long arm of chromosome XII are transcribed (red arrow) by binding of RNA Pol I to a Pol I promoter (red box) as a 35S rRNA precursor (red arrow). Pol I transcription is enhanced by an enhancer element located at the end of 25S rRNA gene (pink box) (Johnson and Warner, 1989). The Autonomously Replicating Sequence (ARS) is shown as an aqua hexagon. A 5S rRNA gene is transcribed in opposite orientation by RNA Polymerase III (blue arrow). A loss of UAF or mitochondrial dysfunction triggers Pol II transcription from an activated Pol II rDNA promoter (orange cross-hatched box) and results in the synthesis of 35S rRNA precursor (orange arrow). However, an unidentified transcription factor (lilac hexagon) may also trigger Pol II driven rDNA transcription in ρ^+ strains of *S. cerevisiae*.

In contrast to UAF mutation, mutations in Pol I or other Pol I transcription factors such as CF and Rrn3p do not independently lead to Pol II transcription of rDNA (Reeder, 1999). In polymerase switch strains, rDNA repeats increase in number from 80 to 400 and this expansion is a cause rather than a consequence of a switch (Nomura, 2001; Reeder, 1999)

Under normal growth conditions in wild-type yeast cells, rDNA genes are almost exclusively transcribed by RNA Pol I. The transcription from Pol II rDNA promoter overlapping Pol I promoter is suppressed by a functional UAF (Vu et al., 1999). However, a loss of UAF or mitochondrial dysfunction triggers chromatin remodeling of the rDNA repeat resulting in excision of rDNA episomes and Pol II rDNA transcription from rDNA episomes (Conrad-Webb and Butow, 1995) (Figure 4).

A Pol I to Pol II switch in rRNA synthesis occurs in other eukaryotes from plants to mammals. Transfection of *Arabidopsis thaliana* protoplasts with Pol I reporter constructs bearing the firefly luciferase gene under the regulation of wild-type rDNA promoter of *Brassica oleracea* resulted in high levels of Pol II dependent luciferase expression (Doelling and Pikaard, 1996). This suggests that foreign *B. oleracea* rRNA gene promoter is recognized preferentially by Pol II in *Arabidopsis* (Doelling and Pikaard, 1996). Likewise, preferential transcription of HSV *TK* gene occurs by Pol II in monkey cells transfected with plasmid bearing herpes simplex virus (HSV) thymidine kinase (TK) reporter gene fused to human rDNA Pol I promoter bearing 5' deletion (Δ5'-83').

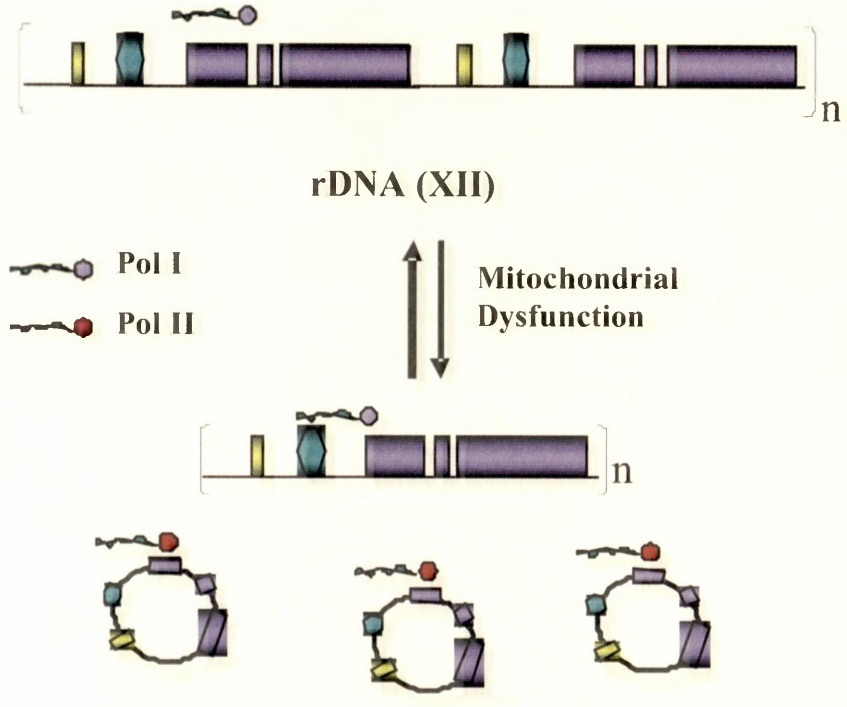


Figure 4. Activation of Pol II rDNA Promoter on Episomes.
Expansion of rDNA repeats on Chrm XII as a result of loss of mitochondrial DNA or loss of UAF favors high rate of homologous recombination between the rDNA repeats leading to the emergence of episomes with a single rDNA transcription unit. The Pol II rDNA promoter overlapping the Pol I promoter is activated on these episomes as a result of binding of RNA Pol II. Transcriptional activation of Pol II promoter leads to transcription of rDNA genes as a single functional 35S rRNA precursor. However, the rDNA transcription unit borne as a linear array on chromosomal is transcribed by Pol I (Conrad - Webb and Butow, 1995).

This deletion in the upstream control sequence of human rDNA Pol I promoter allows transcription initiation by Pol II from an alternate start site within the core region (Smale and Tjian, 1985). The conservation of a Pol I to Pol II polymerase switch in the synthesis of rRNA from such diverse organisms as yeast to humans suggests that an evolutionarily conserved mechanism may play an important role in rRNA synthesis.

Since the Pol I to Pol II switch is conserved during evolution, it may serve as a back up mechanism for rRNA synthesis in yeast to humans. In yeast, Conrad-Webb and Butow (1995) have reported polymerase switch as a rescue mechanism for rRNA synthesis in respiratory deficient mitochondrial (ρ^0) cells. In addition, both ρ^+ and ρ^0 cells lacking Pol I due to a disruption of a *RPA135* gene, survive by Pol II rDNA transcription (Conrad-Webb and Butow, 1995).

Regulatory Factors Influencing Pol II rRNA Synthesis

A Polymerase switch is also reported in ρ^+ cells in response to varied environmental conditions (Ahmed, 2001). Deletion analysis of the NTS2 region of the rDNA promoter showed that the Pol II rRNA synthesis from the episomal context in 161U7ρ^0 cells is due to the activation of Pol II rDNA promoter by at least two regulatory factors (Jodhka, 2004). Deletion of the Reb1p binding site within the Pol II rDNA promoter significantly reduces β-galactosidase activity per plasmid copy number to 41.3%, suggesting that Reb1p is necessary for transcriptional activation of rDNA genes by RNA Pol II in 161U7ρ^0 cells (Jodhka, 2004). However, in ρ^+ cells a functional Reb1p binding site is not necessary for the activation of Pol II promoter suggesting that Reb1p is not involved in

Pol II rDNA transcription in ρ^+ cells (Butler and Kadonaga, 2002). Thus, regulatory mechanisms may vary between ρ^+ and ρ^0 cells. Since a double mutant bearing a deletion upstream of Reb1p binding site and a mutation in Reb1p binding site has drastically reduced β-galactosidase activity per plasmid copy number (15 % in both ρ^+ and ρ^0 cells), another factor or factors might bind to the upstream region of rDNA Pol II promoter and promote recruitment of Pol II transcriptional machinery.

In order to identify regulatory factors influencing Pol II rRNA synthesis in *S. cerevisiae*, a homozygous Yeast Knock-Out collection was screened for single gene deletions that alter Pol II rRNA synthesis. This approach provides major advantages over the classical methods of random mutagenesis and conventional screening as the mutant phenotype represents a complete loss of function of the gene. By virtue of high rates of homologous recombination in yeast, each of the 4757 non-essential genes in a homozygous diploid strain of *S. cerevisiae* (BY4743 ρ^+) is replaced by a KanMX4 cassette (Figure 5).

Introduction of rDNA reporter plasmids into each of the deletion strains allows the rapid assessment of the consequences of each deletion on Pol II rRNA synthesis. Thus, novel regulators or members of the known signaling pathways in yeast can be identified. These factors may be activators or repressors depending upon their affect on Pol II rDNA expression. In addition, known members of stress related signaling pathways can be tested by the introduction of the reporter plasmids in the same manner. Identification of

members of known signaling pathways or known transcription factors can identify environmental triggers for polymerase switch in *S. cerevisiae*.

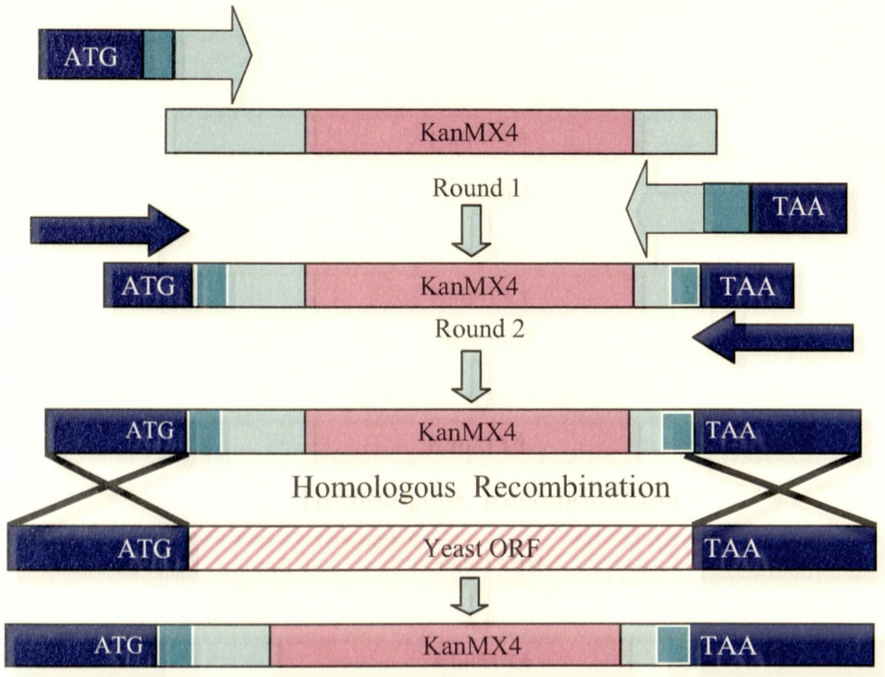


Figure 5. Creation of Yeast Knock-Out Strains by Homologous Recombination.
Each non-essential gene (ORF) is replaced by a KanMX4 cassette (Pink) by homologous recombination. The KanMX4 gene in each mutant is flanked by two distinct 20 nucleotide sequences that serve as a molecular bar code to uniquely identify each deletion mutant (Dark Teal) (Winzeler, 1999).
Modified from www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html

Objectives of the study are as follows:

1. To identify regulatory factors influencing Pol II rRNA synthesis in *Saccharomyces cerevisiae.*
2. To investigate the influence of the components of the selected signaling pathways on Pol II rRNA synthesis in *Saccharomyces cerevisiae.*

Identification of novel signaling pathways or cellular regulatory factors that trigger Pol II rRNA switch will aid in understanding the mechanism of rRNA regulation in *S. cerevisiae.* Also it will provide an insight to different environmental stress conditions that trigger polymerase switch in ρ^+ cells. Identification of transcription factors required for Pol II rRNA synthesis will aid in elucidating the mechanism for the polymerase switch. In addition, a connection between Pol II rRNA synthesis and cell cycle may be established as many factors regulating cell cycle progression are also responsive to different environmental cues and stressors. The coupling of Pol II rRNA synthesis to a specific signaling pathway activated in response to a stressor will further provide a conclusive evidence for it to act as a backup mechanism in ρ^+ cells also. In addition, polymerase switch is evolutionary conserved from yeast to mammals implicating that it is a universal rescue mechanism under stress or conditions impairing Pol I mediated rDNA synthesis.

CHAPTER II

METHODS

Isolation of Plasmid DNA

Large Scale Rapid Isolation of Plasmid DNA

A single bacterial colony containing the plasmid of interest (prDNAURA3 or pFES17) was inoculated into 250 ml of LB–Amp broth (0.5% yeast extract, 1% casein hydrolysate, 0.5% NaCl and 50 μg/ml ampicillin). The cells were grown overnight at 37°C in a controlled environment shaker incubator (New Brunswick Scientific Cooperation, Inc). The cells were pelleted in bottles (500 ml) at 4°C in a Sorvall Superlite-GS3 rotor at 5400 rpm for 15 minutes. The cell pellet was resuspended in 6 ml of alkaline lysis solution I or STE [15 % sucrose, 0.025 M Tris-HCl (pH 8.0), 0.01M EDTA (pH 8.0)] and 2 mg/ml lysozyme. The cells were transferred to a 50 ml centrifuge tube and incubated for 10 minutes on ice. Following addition of 12 ml of freshly prepared alkaline lysis solution II (0.2M NaOH and 1% SDS), the cell contents were thoroughly mixed by inverting and incubated for 10 minutes at room temperature. Following incubation, 7.5 ml sodium acetate (pH 4.6) was added, mixed by inversion and incubated in ice water for 20 minutes. The cell lysate was centrifuged for 15 minutes in a Sorvall Superlite-GS3 rotor at 7300 rpm. The supernatant was separated from the white precipitate of chromosomal DNA, RNA, SDS protein and cell wall complexes.

The supernatant was transferred to a new 50 ml centrifuge tube and 50 µl of 1 mg/ml RNase was added. An equal volume (25:24:1) of phenol: chloroform: isoamylalcohol was added to the supernatant and centrifuged at 10,000 rpm for 10 minutes at 4°C in a SA600 rotor. The aqueous phase containing the plasmid DNA was separated from the organic phase and re-extracted with equal volume of phenol: chloroform: isoamylalcohol (25:24:1). For precipitation of DNA, aqueous phase was incubated with 100 % of cold ethyl alcohol for at least 30 minutes on ice. The DNA pellet was obtained by centrifuging at 4°C at 10,000 rpm for 20 minutes. The DNA pellet was dissolved in 1.7 ml of sterile water. To the resuspended pellet, 0.4 ml of 4M NaCl was added along with 2 ml of 13% PEG 8000 (Sigma) and entire contents were mixed. The solution was incubated on ice for an hour, followed by centrifugation in a microcentrifuge (Marathon MicroA) at 10,000 rpm for 15 minutes. The pellet was washed twice with 5 ml of 70 % ethanol and either air dried or dried in a speedvac SVC 100 (SAVANT). The pellet was resuspended in 100 µl of Tris-EDTA (0.1 M Tris HCl, pH 8.0., 0.01 M EDTA, pH 8.0) or in sterile water.

Small Scale Isolation of Plasmid DNA: Qiagen Plasmid Midi Kit

A single *E. coli* colony bearing either of the two plasmids of interest (prDNA*URA3* or pFES17) was used to inoculate 100 ml of LB-Amp broth (0.5% yeast extract, 1% casein hydrolysate, 0.5% NaCl and 50 µg/ml ampicillin). The cells were grown overnight at 37°C in a controlled environment shaker incubator (New Brunswick). The cells were harvested at 6000 rpm in a Sorvall GS3 rotor.

The bacterial pellet was resuspended in 10 ml of ice cold P1 buffer containing RNase. To the resuspended pellet, 10 ml of buffer P2 was added and cells were incubated for 5 minutes. Following incubation in buffer P2, 10 ml of chilled buffer P3 was added. The slurry was centrifuged at 11800 rpm in a Sorvall SA 600 rotor for 30 minutes at 4°C. The supernatant containing the plasmid DNA was promptly separated and re-centrifuged at 11800 rpm in a Sorvall SA 600 rotor for 15 minutes at 4°C. The clear supernatant was passed through a Qiagen–tip 100 pre-equilibrated with 10 ml of QBT buffer. The Qiagen tip was washed twice with QC buffer to remove all contaminants and excess polysaccharides. The plasmid DNA was eluted with 15 ml of QF buffer. The DNA was precipitated by using a QIAprecipitator midi module and eluted in 1 ml of TE buffer or water (www.qiagen.com).

Quantification of Plasmid DNA

The plasmid DNA was quantified by UV spectrophotometery (Spectronic Genesys 5) and the quality of the preparation was checked by carrying out agarose gel electrophoresis following digestion with appropriate restriction enzymes (Glasel, 1995). The integrity of double stranded plasmid DNA (prDNAURA3 or pFES17) was verified by carrying out single or double digestion with Type II Restriction Enzymes (New England Biolab) followed by gel electrophoresis. The NEB online sequence analysis tool was used to estimate the size of various digestion fragments (Vincze et al., 2003).

Transformation Strategies

Multiwell Transformation Strategy

A single 96 well microtiter plate bearing the strains with homozygous deletion in the non-essential genes (YKO collection provided by *Saccharomyces* Genome Deletion Project Consortium & distributed by Research Genetics) was transferred to YPD petri plates (150×15mm) by using a pinning device or a replicator (Boekel). The strains were grown for 2 days at 30°C. After 2 days of growth, strains were stamped with a pinning device to 96 well brick or deep well dish of 1.2 ml capacity (Phenix M) containing 500 µl of YPD. The brick was air sealed with a gel permeable sealing membrane (Breathe Easy, 6×3.25 inches) to provide aeration and prevent contamination. The cells were mixed in a vortex with a foam adapter for 96 well deep dishes. The bricks were incubated at 30°C in an orbital shaker at 200 rpm for at least overnight at an angle sufficient for growth of all strains. Following growth, cells were collected by centrifugation in a Sorval Swinging Bucket SH300 rotor with an attachment (Sorval) for a deep well dish. After removal of supernatant, cells were vortexed to loosen the pellet. To each well, 100 µl of 105 X transformation mix (pH 5.0-5.6) containing the following components: [0.1459 gm of DTT (Promega), 1.89 ml of 1M lithium acetate (Sigma), 7.56 ml of PEG 4000 (BDH), 47.25 µl of acetic acid, 525 µl of sterile water, 210 µg of plasmid & 525 µl of 10 mg/ml denatured salmon sperm DNA] were added. The cells were mixed by pipeting and the multiwell dish was incubated at 30°C for 30 minutes.

The cells were given a heat shock at 42°C for an hour. Following heat shock at 42°C, cells were spun down. The transformation mix was removed with a multichannel 8 well pipette (RAININ). The cells were resuspended in 100 µl of selective liquid media, vortexed and incubated at 30°C for overnight. The following day the cells were stamped with a pinning device or a replicator (Boekel) onto selective plates to select for transformants (Kushner et al., 2003).

Multiwell transformation was initially undertaken to enhance the transformation process. However, due to either inefficient penetration of heat or due to poor growth of mutant cells in a multiwell dish the transformation efficiency was low. Thus, to enhance the transformation efficiency different modifications in the standard protocol were tried.

Modified Yeast Transformation Protocol

The strains were individually grown overnight in 5 ml YPD liquid media in glass test tubes (Pyrex) at 30°C. The OD_{600} of the overnight cultures were taken. The cells were transferred to 15 ml sterile tubes and centrifuged for 5 minutes in a Sorval Swinging Bucket SH300 rotor at 3000 rpm. The cells were washed in 5 ml sterile water. If OD_{600} was close to 1, cells were re-suspended in 500 µl of 0.1 M Lithium Acetate (LiAc) and the entire amount was transferred to a single well of a deep well dish. However, if OD_{600} exceeded 1.2 then cells were suspended in 500 µl of 0.1M LiAc and only 250 µl was transferred to a single well of a deep well dish. The position of the well was marked on a paper grid with the corresponding deletion strain. The cells were pelleted in a deep well dish at 3000 rpm in a SH3000 rotor for 10 minutes. The entire supernatant was removed

with a multichannel pipette (RAININ). Thirty six milliliters of 100X transformation mix: [24 ml PEG 3500 (50% W/V), 3.6 ml 1M lithium acetate, 5.0 ml of boiled salmon sperm DNA (USB), 3.4 ml of plasmid DNA solution (10μg of each plasmid)] was prepared in a 50 ml tube. Three hundred and sixty microliters of transformation mix was then added to each well with a multichannel pipette. The cells were incubated at 30°C for 30 minutes and heat shocked at 42°C for 40 minutes. The cell pellet was obtained by centrifuging at 3000 rpm in Sorval SH3000 rotor for 5 minutes. The transformation mix was removed with a multichannel pipette (RAININ). The cells were suspended in 250 μl of sterile water and 225 μl of cells were plated on the selective media plates. The plates were incubated for 2 days at 30°C to obtain transformants.

High Efficiency Transformation

The deletion strains were grown overnight in 50 ml of YPD media containing 1 % Yeast extract, 2 % Peptone and 2 % Glucose (YPD) at 30°C to achieve maximum cell density in a 250 ml conical flask or grown in 10 ml of YPD media in a test tube. The cells were harvested at 5000 rpm for 5 minutes in a sterile 50 ml centrifuge tube. After a wash with 25 ml of sterile water the harvested cells were suspended in 1.0 ml of 0.1M LiAc and transferred to a 1.5 ml microfuge tube. The cells were pelleted at top speed in a microfuge for 15 sec and the LiAc was removed with a micropipette. The cells were resuspended in ~400 μl of 0.1 M LiAc. After vortexing, 50 μl of cell suspension was transferred to a labeled 1.5 ml microfuge tube. To 50 μl of cells, the components of a transformation mixture were added in order: 240 μl of 50 % w/v Polyethylene Glycol

(PEG 4000), 36 µl of 1.0 M LiAc, 25 µl of salmon sperm DNA (2 mg/ml) and plasmid DNA (0.1-10 µg) in 50 µl of water. The cells were vortexed until the pellet was completely suspended in the transformation mixture. The cells were incubated at 30°C for 30 minutes and heat shocked at 42°C for 20-25 minutes for uptake of plasmid DNA. Following heat shock, cells were pelleted and the transformation mix was removed. The cells were washed with 500 µl of sterile water and resuspended in 0.2-1.0 ml of sterile water. The cells (0.1-0.2 ml) were plated on the selective plates to screen for transformants (Gietz and Schiestl, 1995).

Screening Transformants

The deletion strains were initially transformed with a single plasmid prDNA*URA3* bearing *URA3* gene under the influence of dual rDNA Pol I and Pol II promoter. The transformants were selected on SC-His media [0.67% yeast nitrogen base, 1% ammonium sulfate, 2% glucose, 0.19% SC-His (Q-BIOgene) and 2% agar]. The transformants were patched along with the positive control ($Ura3^+$) and the negative control ($URA3^-$) on SC-His media. The patched transformants were then replicated from SC-His plate to SC-His, SC-His-Ura [0.67% yeast nitrogen base, 1% ammonium sulfate, 2% glucose, 0.2% SC-His-Ura (US biologicals) and 2% agar] and the commercially prepared FOA (5 Fiuoro-Orotic Acid). FOA selects for uracil auxotrophs. However, due to lack of significant growth differences between the positive and negative controls on FOA plates, the FOA plates were not used for further screening. Tranformants growing slower than the positive control ($URA3^+$) on SC-His-Ura plate were selected.

The prospective candidates with compromised growth were then transformed with the rDNA-*lacZ* reporter construct (pFES17) to rule out false positives. Transformants harboring both plasmids (prDNA*URA3* and pFES17) were selected on SC-His-Leu [0.67% yeast nitrogen base, 1% ammonium sulfate, 2% glucose, 0.17% SC-His-Leu (Q-BIOgene) and 2% agar] plates. The transformants were replica plated to SC-His-Leu, SC-His-Leu-Ura [0.67% yeast nitrogen base, 1% ammonium sulfate, 2% glucose, 0.166 % SC-His-Leu-Ura (Q-BIOgene) and 2% agar] and SC-His-Leu+Xgal [0.67% yeast nitrogen base, 1% ammonium sulfate, 2% glucose, 0.17% SC-His-Leu(Q-BIOgene), 0.06 g X-Gal (5-Bromo-4-Chloro-3-Indonyl-Beta-D Galactoside) in 3 ml of N,N dimethyl formamide and 2% agar] plates. The transformants that were white or light blue in X-gal indicator plates were also assayed for reduced growth in liquid Ura$^-$ media. The same procedure was employed to transform the deletion candidates with only rDNA-*lacZ* bearing reporter construct (pFES17) to assay for β-galactosidase activity.

Growth Assays

The candidate deletion strain or the wild-type positive control (*URA3*$^+$) was grown in 10 ml of SC-His media at 30°C. The concentration of cell was adjusted approximately to the same OD_{600} value with sterile water. This adjustment for OD was necessary to eliminate growth differences between the wild-type positive control & deletion strains due to variation in amount of cells. After adjustment of OD_{600} for all strains, cells were diluted 1:5 in SC-His media. An equal amount of cells (200 or 500 µl) were used to inoculate 250 ml Erlenmeyer Flasks containing 100 ml of SC-His or SC-His-Ura media.

The cells were grown at 30°C in Innova 4300 incubator shaker at 200 rpm. At various time intervals absorbance at 600 nm was determined and a growth curve was plotted for all prospective candidates. The growth profile of both the wild-type and the mutant was compared in the presence and absence of uracil in selective media. Those prospective candidates with a greater reduction in growth in Ura$^-$ media compared to wild-type positive control were used for further studies.

Confirmation of Deletion Strains by PCR

Each gene disruption was confirmed by PCR. By employing the primer design program of *Saccharomyces* Genome Database (http://seq.yeastgenome.org/cgi-bin/web-primer3), primers complementary to sequences approximately 100 or 200 bases upstream and downstream of the non-essential gene were designed. These primers were synthesized by Biosynthesis (http://www.biosyn.com). The primers were reconstituted in 0.2 ml sterile water and their concentration was determined by measuring the absorbance at 260 nm. The 5X solution of the primers were used as stock for all PCR reactions.

Taq Polymerases from various sources were used to confirm various deletions as no one kit enzyme provided optimum conditions for amplification. This could be due to preferences or specificity of different versions of thermostable enzymes for different template DNA, primers or requirement of varied magnesium ion concentration (buffers). This necessitated optimization for annealing temperatures for primers to anneal to their template DNA (Table 1).

Table 1

Optimization of PCR Conditions for Deletion Strain Candidates

Gene	Forward Primer Sequence Reverse Primer Sequence	F.P (Tm)	R.P (Tm)	Annealing Temp (°C)	PCR Kit	Size wild-type product (bp)	Ideal Buffer (Bold)
AZF1	5'TTTCGCTTCTTTGAGAAGTGGAG3' 5'GTGACACTTGTGTAAGTTTGACG3'	70	76	65	FS	3058	Premix C
FKH2	5'TGGTTCCGCATTTCTAAAGGT3' 5'TCAAGGATGCAAACACAGCA3'	60	58	54	T	3203	10X Enzyme Buffer
HOG1	5'TTATACGGGAGGATCTTCGA3' 5'TGATAAACAAACAATACGCCA3'	58	56	50	FS	1923	All premixes **(K)** except J.
MKS1	5'ATGTGCATGCCGTTGTTACA3' 5'TGACGTTTTTGTTGCAGTTG3'	58	56	51	FS	2612	Premix E,C,B & F
OCA4	5'GACACGGGGAAAATCTTTAC3' 5'AAAACGAGGATTATGAGGATG3'	58	58	53	T	1680	10X Enzyme Buffer
PBS2	5'GAGCGATTTCGTGAGCCATA3' 5'TATTGACGTCCACATCGCTT3'	60	58	54	FS	2628	Premix B,C, E, **(F),** H & I
PHO3	5'TATTAGTCGCCGCTTAGGCAC3' 5'ATCGAATTGCACTCCTGCAAC3'	64	62	54	FS	2032	All except **(B)** G & J.
RTG2	5'AGTCACATGACCGCGATAAGCGAT3' 5'TTCACGGGATTTATTCGTGACG3'	72	64	59	FS	2178	Pre Mix **(B)**
SWI5	5'GATCTGCTCTATAAACAATGATTGAGTAC3' 5'TTACCCACATTCTCCACTCTTCCA3'	78	74	65	FS	2652	Premix C
TRP1	5'CACAGGTAGTTCTGGTCC3' 5'TTGTCTCCACACCTCCGC3'	56	58	53	FS	602	All Premixes

F.P: Forward Primer, R.P: Reverse Primer, FS: Fail Safe, T: Takara

In all cases PCR was performed with the wild-type total DNA derived from the control strain (BY4743ρ^+) and DNA from the candidate strain bearing the deletion. The PCR products of wild-type and the deletion strain were compared by carrying out agarose gel electrophoresis with standard 1 Kb ladder (Invitrogen) or 1 Kb^+ Ladder (Invitrogen). Initially, Ready To Go PCR Beads (Amersham Biosciences) were tried for testing all deletion candidates with the wild-type control strain. The PCR bead containing stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of pure ReTaq DNA polymerase and reaction buffer was reconstituted to a final volume of 25 µl with template DNA, 5' primer, 3'primer and water. After reconstitution the concentration of each dNTP was 200 µM in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM $MgCl_2$ (http://www.amersham biosciences.com). For a standard 1X reaction/tube approximately 1 to 5 µl of total DNA (50-100 ng) was used along with 1µl of 5'and 3' primers (0.5µM). The reaction volume was brought to 25 µl with sterile water.

A master mix containing primers and water was prepared. Equal aliquots of the master mix were distributed among the PCR (0.2 ml, thin walled) tubes. Appropriate volume of DNA was added and the tubes were thermocycled in Mastercycler PCR machine (Eppendorf) under standard reaction conditions for 30 cycles: denaturation was carried out at 92.5°C for 1.5 minutes; annealing was carried out for 1.5 minutes at varied annealing temperatures depending upon the template and primer pairs; primer extension was carried out at 72°C for 3 minutes. The final extension was carried out at 72°C for 10 minutes. However, for some deletion strains where the standard program run at one

particular annealing temperature did not result in a PCR product a gradient PCR was also carried out to determine the optimum annealing temperature. If PCR beads did not result in a PCR product, another PCR enzyme TaKaRa Ex Taq™ Hot Start (TaKaRa Biotechnology) was tried. For a single PCR reaction (25µl), 2.5 units of enzyme (0.25µl), 2µl of 10X *Ex Taq* Buffer, 2.0µl of dNTP mixture (2.5 mM of each nucleotide) and 1.0µl of 5' and 3' primer (0.5µM) was used. The volume was brought to 25µl with sterile DNase free water. Template DNA (< 1µg) was added to each (24 µl) aliquot of the master mix. The contents were mixed in a 0.2 ml PCR tube and thermocycled with standard program at an annealing temperature optimal for the primer-template pair. Annealing temperatures had to be optimized for different templates and primer pairs by carrying out a gradient PCR. Fail Safe ™ PCR reaction kit marketed by Epicentre (www.epicentre.com) was finally used for reactions not resulting in a PCR product with above two kits. This kit contains a set of twelve reaction premixes with different concentrations of $MgCl_2$, 4 dNTPs, a buffered salt solution with Fail Safe PCR enhancer (with betaine) and a thermostable fail safe PCR enzyme. For a single 25 µl fail safe reaction, 12.5 µl of each premix (A–L) was mixed with 12.5 µl of master mix, 1.0 µl each of 5' and 3' primer (~ 1 µM), 1.0 µl of DNA ranging from (1-500 ng) and the volume was made up with water. The reactions were set on ice and 12.5 µl of master mix was mixed with each of the premixes. A standard PCR program was used first to find the premix reaction mix that was optimum for each primer and template pair at a particular annealing temperature. For this purpose the wild type DNA from a HB2 strain was used.

Once the optimum reaction condition was identified, the same condition was used with the DNA of the candidate and the wild-type strain (BY4743ρ^+). In some cases where the PCR reaction resulted in a similar sized PCR product for the deletion candidate and the wild-type strain, their PCR product was digested with a unique restriction enzyme that cleaves within the KanMX4 module of the deletion strain but does not cleave the wild-type strain. Thus, the wild-type strain DNA should result in a single band and the deletion candidate should yield two or more bands depending upon number of restriction sites.

Conversion of ρ^+ to ρ^0 Cells

To determine the effect of candidate deletions on Pol II r DNA expression in ρ^0 cells, ρ^0 derivatives of ρ^+ candidates were made. The ρ^0 derivatives of BY4743ρ^+ and candidate ρ^+ deletion strains were made by growing ρ^+ strains for four consecutive passages in YPD (1 % yeast extract, 2 % peptone and 2 % dextrose) media with ethidium bromide at the final concentration of 0.4 mg/ml at 30°C. Following growth in YPD ethidium bromide, 100 µl of the cells were subcultured in 10 ml of YPD media. The cells were serially diluted in YPD liquid media and streaked for singles on YPD agar plates. The colonies were then replicated to YPG (1 % yeast extract, 2 % peptone and 2% glycerol) and YPD plates. The ρ^0 colonies showing no growth on YPG plates but depicting growth on YPD plates were picked for further verification. These colonies were checked for the complete absence of mitochondrial DNA by probing their DNA with a mitochondrial DNA-specific probe by either colony or dot blots hybridization.

β-Galactosidase Assay

The Pol II rDNA promoter expression in candidates was analyzed by determining β-galactosidase activity/reporter gene copy in whole cell extracts. For the quantitative estimation of β-galactosidase activity, the wild-type and various candidates were transformed with only one reporter plasmid, pFES17. The cells were grown in selective SC-Leu-raffinose (0.67 % yeast nitrogen base, 1 % ammonium sulfate, 2 % glucose & 0.2 % of Sc-Leu)(drop out synthetic mix-leucine w/o nitrogen base, US Biological) to an OD_{600} between 0.9-1.0. The cells were harvested as three 7.5 ml aliquots, pelleted and resuspended in 300 µl of cold breaking buffer (0.1M Tris-HCl, pH 8.0, 0.001 M dithiothreitol, and 20 % glycerol). These cell pellets were frozen at -80°C and used as early as possible to make crude extracts. In addition, the remaining culture was collected for DNA isolation for the determination of plasmid copy number as three 15 ml aliquots as cell pellets.

To assay for the β-galactosidase activity, crude cell extract was made for each candidate and the positive control (BY4743 ρ^+ + pFES17). To prepare a crude extract the cell pellets were thawed on ice and transferred to a labeled microfuge tube. The cells were vortexed for 6 minutes with glass beads (0.5 mm) in the presence of 12.5 µl of proteinase inhibitor, PMSF (phenylmethyl sulfonyl fluoride, 40 mM in 100% Isopropanol). An additional 250 µl of cold breaking buffer was added, vortexed for a minute and transferred to a new labeled microfuge tube. The extract was centrifuged at 10000 rpm for 15 min to obtain a clear crude extract. The quantitative determination of β-

galactosidase activity in the clear crude extract was assayed by measuring the production of ONP (o-nitrophenyl) at 420 nm from the substrate ONPG (o-nitrophenyl-β-D-galactosidase). Each extract was assayed a minimum of two times with each assay conducted in triplicate. To ensure accuracy, multiple extracts from each sample were assayed independently. The total β-galactosidase activity was normalized to total protein content of the cell, determined by carrying out Bradford Assay (Bradford, 1976). Each extract was assayed in triplicates for total protein. β-galactosidase activity was calculated by using the formulae: $OD^{420} \times 1.7 / 0.0045 \times$ protein (mg) × extract volume (ml) × time (min) and was expressed in nmoles/ minute/ mg protein (Miller, 1972).

β-galactosidase activity was normalized for copy number. This normalization is important because the 2μ origin borne on the plasmid pFES17 results in multiple plasmid copies per cell. Since the goal is to compare differential *lacZ* expression in the wild-type and deletion candidates, it is mandatory that the differences in *lacZ* expression among the wild-type and deletion candidates are due to the effect of a particular deletion in the candidate strain and not due to differential levels of plasmid DNA per cell (Conrad-Webb and Butow, 1995).

Copy Number Determination

Isolation of Total Yeast DNA

The desired strain bearing the plasmid (pFES17) was grown in 10 ml of YPD (1 % yeast extract, 2 % peptone and 2 % dextrose) for overnight at 30°C in Innova 4300 shaker incubator (New Bruswick Scientific). The cells were harvested by centrifuging at 4300

rpm in a Swinging Bucket rotor (SH3000) for 5 minutes. The cells were washed with 5 ml of sterile water and transferred to a 1.5 ml microfuge tubes. The cells were centrifuged for a minute in a microfuge (Spectrafuge 16M, Labnet). The pellet was re-suspended in 200 µl of breakage buffer (2 % triton X-100, 1 % SDS, 0.1 M NaCl, 0.01 M Tris HCl and 0.001M EDTA, 200 µl (1:1 phenol: chloroform), 0.3 gm glass beads (0.5 mm., Biospec products). The cells were mixed by vortexing for 6 minutes. The cells were centrifuged for 5 minutes in a microfuge to get a clear lysate. The supernatant or clear lysate was transferred to a new microfuge tube. The total DNA in the supernatant was precipitated by adding 1 ml of ice cold 100 % absolute ethyl alcohol. The DNA pellet was suspended in 400 µl of TE & RNase mixture [0.1M Tris HCl, pH 8.0., 0.01 M EDTA, pH 8.0 and 20 µl of RNase (30 µg /ml)] and incubated for at-least 30 minutes at 37°C. After incubation, 10 µl of 4M ammonium acetate was added along with 1 ml of ethanol. The DNA pellet was obtained after centrifuging in a microfuge (Spectrafuge 16M, Labnet) for 15 minutes. The pellet was washed twice with 500 µl of 70 % ethanol to remove excess salts. Following 70 % wash the pellet was re-suspended in 50-100 µl of sterile water. The total DNA was quantified by UV spectrophotometry and checked for the absence of RNA by agarose gel electrophoresis.

Amplification of TRP1 Fragment by PCR

The genomic DNA from either HB2 strain or (BY4743ρ^+ +*Lac*Z44) was isolated (as mentioned above). The DNA was dialyzed in a mini dialysis unit/ Slide-A-Lyzer (PIERCE) with a molecular weight cut off of 3500 to obtain a salt free DNA for synthesis

of probe. The mini dialysis unit was covered with a cap aseptically and placed in a floatation device in a beaker filled with TE buffer (0.01M Tris & 0.01M EDTA, pH 8.0). Dialysis was allowed to go on for at least 15 minutes at room temperature. An aliquot of DNA was run in a 0.7% agarose gel to check for the absence of RNA. After gel electrophoresis, 5µl of dialyzed DNA was used as a template for PCR with *TRP1* primers at annealing temperature of 53°C (optimized by doing a gradient PCR) with Takara Enzyme (Takara Bio Inc) following manufactures recommendations. The band isolated *TRP1* product (~ 4 µl) was used as a template for subsequent multiple PCR reactions with Takara reagents for amplification of *TRP1* DNA.

Purification of TRP1 Fragment

Zymoclean Gel DNA kit (Zymoresearch) was used to band isolate PCR amplified *TRP1* fragment from 1 % agarose gel. The DNA band from the agarose gel was excised with a sterile razor blade and trimmed to obtain a minimum gel volume. Three volume of ADB (Agarose Dissolving Buffer) was added to each volume of agarose gel. The agarose gel was heated with buffer at 55°C for 5-10 minutes until the gel was completely dissolved. The melted agarose solution was loaded onto a Zymo-Spin column of capacity 600 µl and the column was placed in a 2 ml collection tube. The column was centrifuged at full speed (≥10,000g) for 5-10 seconds and the flow through in the collection tube was discarded. The column was washed twice; once with 200 µl of wash buffer for 5-10 seconds and one more time with 200 µl of wash buffer for 30 seconds. DNA was eluted

from the column by the addition of either 8 µl of TE buffer (0.01M Tris-HCl, 0.001M EDTA with optimum pH 8.0) or sterile water.

Quantification of TRP1 Fragment

To determine DNA concentration purified *TRP1* fragment (~70-100 ngs) was electrophoresed along with 5 µl of low DNA mass ladder (Invitrogen) in a 0.7 % agarose gel made in 1X TBE buffer (89 mM Tris Base, 89 mM boric acid (USB), 2mM EDTA, pH 8.0) and 0.5ug/ml ethidium bromide (BIORAD). The low DNA mass ladder generates fragments ranging from 100 bp-2000 bp. The *TRP1* fragment was quantitated in relation to low DNA mass ladder (Invitrogen) by using either the Quantity One program of FX Molecular Imager (Bio-Rad) or Alpha Innotech Gel Doc system.

Labeling DNA: (TRP1, pGEM-3Zf (-) and Mitochondrial DNA)

The NEBlot® kit (New England Biolabs) or Sequenase ™ Random Primer Labeling Kit was used for the preparation of labeled probes. The DNA was labeled by using random priming reaction. Approximately 30 ngs of *TRP1* band isolated DNA or pUC19 DNA was labeled with ^{32}P dCTP in a standard 50 µl reaction. The *TRP1* or pUC19 DNA equivalent to 30 ngs was made up to a volume of 38 µl with water and denatured for 5 minutes in a heating block at 95-100°C. The denatured DNA was placed in ice for 5 minutes. The various components of kit were then added in order: 10 X labeling buffer (5 µl), dNTP mixture (2 µl of each dNTP), α ^{32}P dCTP (3000 ci/mM; 50 µCi), and 5 units of DNA Polymerase I-Klenow Fragment (1µl). The labeling reaction was allowed to proceed for at least an hour at 37°C. The labeled probe was purified by passing the probe

through a sephadex® G-50 column. This was necessary to remove the unicorporated nucleotides from the probe as they would result in a high background on the filter following hybridization. For the preparation of a sephadex® G-50 column a 3 ml syringe was packed with sephadex beads (Sigma) pre-equilibrated in TE buffer pH 8.0) under gravity. The column was placed in a 15 ml falcon tube and was packed by centrifugation for 5 minutes. Following centrifugation, the column was placed in a labeled falcon tube with a labeled microfuge tube. The probe was then added to the center of the column and centrifuged for 5 minutes (Sambrook et al., 1989). The eluted probe was denatured for 5 minutes at 95-100°C and cooled on ice for 5 minutes. The only difference between the NEBlot® kit and the Sequenase ™ Random Primer Labeling Kit is that in the later the template DNA and 10 X primer mix (5µl) is made up to 38 µl with water. To ensure probe excess multiple labeling reactions for pGEM-3Zf (-) and *TRP1* were prepared.

Dot Blot Hybridization

β-galactosidase activity was normalized for copy number by carrying out dot blot hybridization assay. This assay gives an estimate of the relative number of plasmid copies to genomic DNA. The total cellular DNA (plasmid as well as genomic) was isolated (Hoffman & Winston, 1987). Approximately 7 µg of DNA was diluted to a final volume of 234 µl in sterile water. 166 µl of 20X SSC (3M NaCl, and 0.3 M sodium citrate pH 7.0) was added. The DNA solution was denatured at 80°C for 10 minutes. Following denaturation, DNA was neutralized with 135 µl of 1M Tris-HCl, pH 4.0 and 10 µl of 1 % bromophenol blue. Increasing folds (1.0X, 2.0X, 3.0X and 4X) of DNA for each sample

was blotted to duplicate filters (Amersham Pharmacia Biotech) by using the manifold Schleicher and Schuell DNA blot apparatus. The DNA was immobilized to the filter by UV crosslinking at 1200 microjoules using a stratalinker. The nylon membranes were pre-hybridized for overnight at 42°C in a hybridization roller bottle (Bellco Glass Inco, Auto Blot) in appropriate amounts of Hybrisol® buffer (Chemicon International). The duplicate filters were hybridized with either of the two different α^{32}p dCTP labeled probes (pGEM-3Zf (-) or *TRP1* fragment) prepared by using Random Priming kit (New England BioLab). To detect for the multicopy pFES17 plasmid, one of the membranes was hybridized to approximately 120-150 ng of [α^{32}p] labeled pGEM-3Zf (-) plasmid probe at 42°C for overnight. Approximately 80-100 ng of labeled *TRP1* PCR fragment was used as a single copy probe to detect genomic DNA. The other membrane was hybridized with labeled *TRP1* PCR fragment at 42°C for overnight. The membranes were washed twice at room temperature in 2XSSC and 0.1% SDS for 20 minutes and washed twice in 0.1X SSC with 0.1 % SDS for 30 minutes at 42°C to remove non specific binding. The filters were exposed to Kodak FX Molecular Phosphor Imager screens for appropriate time to get maximum counts without saturation. The relative counts were calculated by using Quantity One software of FX Molecular Imager (Bio-Rad). The ratio of slopes of pGEM-3Zf (-) and *TRP1* were obtained from X-Y scatter plots between concentration of DNA and densitometric counts would give the relative number of reporter gene copies/genomic DNA for each sample. This value was used to estimate β-galactosidase activity/copy for each candidate after normalizing to one with wild-type.

This was done to have a single reference while comparing copy number of different assays done at different time.

Colony Hybridization

Colony hybridization was carried out to check for the complete absence of mitochondrial DNA in Rho^0 derivatives of the candidate strains with and without plasmid pFES17or to create a Rho^0 derivative of the wild-type strain ($BY4743\rho^+$) that served as a control for β-galactosidase enzyme assays. This method was employed to rule out the possibility of occurrence of other petite mutants (Rho^-), bearing a part of the mitochondrial genome amongst the Rho^0 population that could be induced by insufficient ethidium bromide treatment. It also facilitates labeling of entire nylon filter bearing the DNA from many strains with $\alpha^{32}p$ labeled total mitochondrial DNA. The labeling was done by using random priming kit distributed by New England Biolab. A sterile whatman filter was placed on a YPD media plate and a Hybond N filter (Amersham Pharmacia Biotech) was placed on the top of the whatman. Both positive control DNA ($BY4743\rho^+$) and the negative control DNA (BY4743 ρ°) was inoculated as a patch in a grid pattern on the filter. The filters were incubated at 30°C for 2 days. Duplicate grids were created on control plates. Nylon filter with colonies facing up was transferred to a whatman filter saturated with 20 ml solution containing: 0.05M EDTA and 2.5% beta-mercaptoethanol (Sigma). The filters were kept undisturbed for 15 minutes in a closed petri plate. Following incubation, the nylon membrane was transferred to a whatman saturated with approximately 3 mg of mureinase (US Biological) in citrate buffer pH 5.8 (1.2 M

sorbitol, 0.02 M potassium phosphate, and 0.02 M citric acid. 0.001 mM EDTA) for 3 hrs at 37°C in an airtight box. After 3 hrs of incubation, the nylon filter was transferred to a whatman paper saturated with 0.5 M NaOH for 2 minutes and this was done twice to ensure denaturation. The nylon filter was transferred twice to a whatman paper saturated with neutralization buffer (1 M Tris, pH 8.0 and 1.5 M NaCl) for 2 minutes and UV crosslinked at 1200 microjoules using a stratalinker. The nylon filters were then prehybridized in hybrisol and hybridized at 42°C with 5 µl of ^{32}P labeled total mitochondrial DNA probe. The probe was labeled by using Random Primer NEBlot® Kit.

Statistical Analysis of β-Galactosidase Activity/Copy Number

For each experiment at least two independent cell extracts for the control and the test samples were assayed for β-galactosidase activity per copy number. For reproducibility of results each extract was assayed at least two times in triplicates. For both control and test samples average mean and standard error of mean was calculated and compared. An independent student's *t*-test was performed by using SPSS software to find out if there were significant differences between the sample and control group for each experiment. An independent *t*-test was employed because the wild-type and mutants were two different populations and the sample size was less than 30. A two tailed independent student's *t*-test at 0.05 level of significance was employed to reject the null hypothesis: that there are no differences between the wild-type and the mutants and accept the alternate hypothesis: that there are significant difference between two groups. A two

tailed test was employed as the mutant could have higher as well as lower β-galactosidase activity/copy number as compared to wild-type. Since the standard error of mean of the wild-type and mutant population differed considerably, an unequal variance was assumed while determining *t* test.

In some cases outliners in the wild-type population were removed from the experimental data. To determine an outliner, a proportion score was determined for each wild-type entry by dividing the value with the wild-type population mean. The wild -type entries having a proportion value differing remarkably from rest of the wild-type population were removed. An independent student's *t*-test at $p < 0.05$ was conducted for all wild-types excluding the wild-type outliners and mutants.

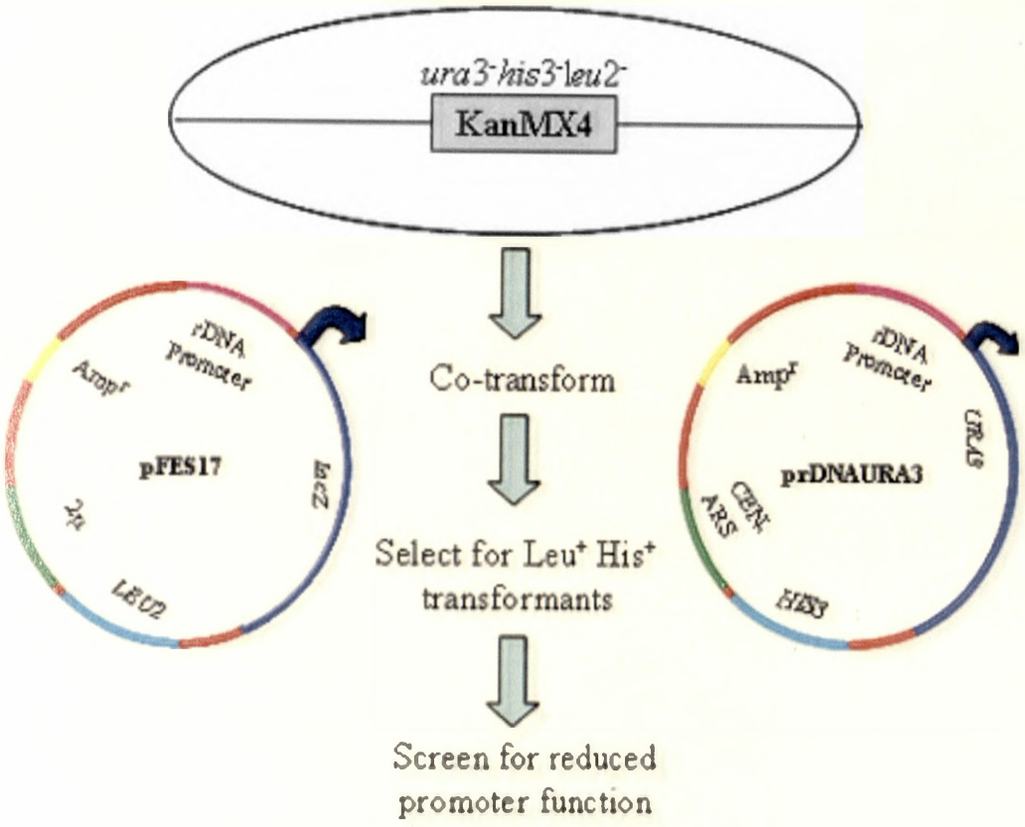
The lower and upper limits of 95% confidence interval difference values were subtracted and added respectively to the wild-type population mean to obtain a new confidence interval. This confidence interval was extended by the average standard deviation of the entire wild-type population, obtained after removing the values corresponding to the outliners. If the mean value of the mutant was within this extended confidence interval then it was not considered statistically significant. However, if the mutants mean value was outside the extended confidence interval it was considered significant.

CHAPTER III

RESULTS

A Screen for Regulatory Factors Influencing Pol II rRNA Synthesis

To identify regulatory factors reducing Pol II rRNA synthesis in *Saccharomyces cerevisiae,* the homozygous Yeast Knock-Out (YKO) collection was screened for single gene deletions that reduced Pol II rRNA synthesis. Two reporter plasmids, prDNA*URA3* or pFES17 were employed to assess Pol II rDNA promoter function (Figure 6). The reporter plasmid prDNA*URA3* (7.8Kb) bears the yeast *URA3* gene under the influence of dual Pol I and Pol II rDNA promoters. The *URA3* gene encodes for orotidine 5'phosphatide decarboxylase, one of the key enzymes of the uracil biosynthetic pathway (Lacroute, 1968). Since the prDNA*URA3* plasmid bears yeast (*CEN-ARS*) sequence, it is maintained as a single copy plasmid. In contrast, the other reporter plasmid pFES17 (9.5Kb) bears *E.coli lacZ* gene under the control of dual Pol I and Pol II rDNA promoters. As a high copy shuttle plasmid, it contains 2μ replication origin from the yeast 2μ plasmid and replicating sequences (Ori) from *E.coli* (Figure 6). Strains defective in Pol II rRNA synthesis will have little or no β-galactosidase activity, while wild-type strains will have robust activity and will be blue on X-Gal indicator plates. In the screen, both reporter plasmids were transformed into homozygous diploid deletion strains to reduce the incidence of false positive candidates.



	X-Gal Media	Ura⁻ Media
Wild-Type	Blue	Wild-Type Growth
Candidates	Light Blue	Slow Growth

Figure 6. Screen for Regulatory Factors for Reduced Promoter Function.
Deletion strains were co-transformed with two reporter plasmids pFES17 and prDNA*URA3*. The plasmid pFES17 bears *E.coli lacZ* reporter gene under the influence of the dual Pol I-Pol II rDNA promoter, 2μ replication origin (2μ plasmid), *LEU2* and ampicillin resistance gene (Amp^r) as selectable markers in yeast and *E. coli*, respectively. The plasmid prDNA*URA3* bears the *URA3* gene under the influence of the dual Pol I – Pol II rDNA promoter, *CEN–ARS* (Centromere and Autonomous Replicating Sequence), *HIS3* gene and ampicillin resistance gene (Amp^r) as selectable markers in yeast and *E. coli*, respectively.

To monitor Pol II rRNA expression with prDNA*URA3* plasmid, the controls and transformants were replicated to selective media with and without uracil. The wild-type BY4743 ρ^+ strain containing the prDNA*URA3* reporter plasmid served as a positive control for Pol II rDNA promoter function, while the wild-type BY4743 ρ^+ strain containing the parent vector of prDNA-*URA3,* pRS423, served as a control for the Ura minus phenotype representing the absence of Pol II rDNA promoter function. This vector has the *HIS3* gene as a selectable marker, but lacks the *URA3* reporter gene. Transformants growing slower than the wild-type control in media devoid of uracil were chosen for further study.

Table 2

Transformation Controls

Strains	Phenotype	Genotype	X-Gal Media	Ura$^-$ Media
BY4743ρ^+	WT	*his3$^-$leu2$^-$ura3$^-$*	NA	-
BY4743ρ^+ prDNA*URA3*	Ura3$^+$	*HIS3$^+$leu2$^-$URA3$^+$*	NA	+
BY4743ρ^+ pRS423	Ura3$^-$	*HIS3$^+$leu2$^-$ura3$^-$lacZ*	NA	-
BY4743ρ^+ pFES17	Leu2$^+$lacZ$^+$	*ura3$^-$his3$^-$LEU2$^+$lacZ*	Blue	-
BY4743ρ^+ pFES17 prDNA*URA3*	Leu2$^+$Ura3$^+$lacZ$^+$	*HIS3$^+$LEU2$^+$URA3$^+$lacZ*	Blue	+

NA: Not Applicable

To rule out false positives, candidate deletions growing slower than the control (BY4743 ρ^+ prDNA*URA3*) in Ura$^-$ media were transformed with the reporter plasmid pFES17. Transformants and the wild-type control, BY4743 ρ^+ pFES17, were transferred to X-Gal indicator plates to assess Pol II rDNA promoter function. The wild-type strain containing both reporter genes grew well on Ura$^-$ media and was blue on X-Gal indicator plates. In contrast, the candidate deletions with reduced Pol II rDNA promoter function grew slower than the wild-type on Ura$^-$ media and were white or light blue on X-Gal indicator plates. Initially, the multi-well transformation strategy was used to hasten the process, as ninety-six strains could be transformed at one time. However, frequently no transformants were obtained for specific deletion strains with multiwell transformation protocol, despite improvements to the protocol. To remedy the low transformation rate the multi-well transformation method was replaced by the High Efficiency Transformation method (Gietz et al., 1992). In the latter method, each strain was individually grown and transformed, thereby resulting in greater numbers of transformed strains.

Overall, 2661 strains were successfully transformed out of 3161 strains attempted (Table 24; Appendix G). Approximately 500 strains were difficult to transform because of growth constraints presumably due to the specific deletion. As expected, several transformants (31/3161) grew slower in Ura$^-$ media, but were blue on X-Gal indicator plates. Since they were blue on X-Gal indicator plates, they were categorized as false positives. On the other hand, seven out of 2661 transformants were Ura$^+$, but white on X-

Gal indicator plates. Since these transformants were Ura^+, they were also considered false positives. Both categories of false positives were not selected for further study. Out of 2661 transformants, four candidates fulfilling the selection criteria of reduced growth on selective Ura^- media and being either white or light blue on the X-Gal indicator plates were chosen for further study. Since Pol II rRNA synthesis may be triggered in response to varied stresses, the members of other stress response pathways such as General Stress Response (GSR), Heat Shock (HS) and retrograde response were also transformed with the reporter plasmids used for the initial screen for regulatory factors (Tables 2 and 3). Many of the deletion strains in selected pathways showed reduced growth on Ura^- media, but were blue on X-Gal indicator plates. However, *RTG2*, a member of the retrograde regulatory pathway had reduced growth on Ura^- media and was light blue on an X-Gal indicator plate (Table 4).

Overall, five candidate genes were selected for further study (Table 4). As expected, these candidates may control Pol II rRNA synthesis by acting as transcription or regulatory factors of signaling pathways.

FKH2 is a transcription factor of the forkhead family that regulates cell cycle. *OCA4* is a cytoplasmic protein required for brome mosaic virus replication in *S. cerevisiae*. *PHO3* is a constitutively expressed acid phosphatase. *PBS2* is a MAPKK (Mitogen Activated Protein Kinase Kinase) and a member of High Osmolarity Glycerol pathway that is activated by osmotic stress.

Finally, *RTG2* is a member of retrograde regulation that is involved in transmission of signals from mitochondria to nucleus in response to mitochondrial dysfunction (Table 4).

Table 3

Signaling Pathways Potentially Triggering Pol II rRNA Synthesis

Standard Name	Function	Pathway	Growth on Ura⁻ media	Growth on X-Gal media
MSN2	TF activated upon GSR	GSR	Reduced	Blue
MSN4	TF activated upon GSR	GSR	Enhanced	Light Blue
HSP12	Regulates *MSN* 2 and 4	NA	Enhanced	Blue
RTG1	Transcription factor	Retrograde	Reduced	Blue
RTG2	Transcription factor	Retrograde	Reduced	Light Blue
TPK2	PKA	Ras-cAMP	Wild-Type	Blue
SMK1	MAPKK	Spore wall assembly	Reduced	Blue
BCK1	MAPKKKK	Cell wall integrity	Reduced	Blue
KSS1	MAPK	Pheromone & Filamentous growth	NA	Blue

GSR: General Stress Response, MAPK: Mitogen Activated Protein Kinase, MAPKK: Mitogen Activated Protein Kinase Kinase, MAPKKK: Mitogen Activated Protein Kinase Kinase Kinase, PKA: 3', 5'cyclic AMP dependent protein kinase, TF: Transcription Factor.

Table 4

Candidate Strains Affecting Pol II rRNA Synthesis

Standard Name	Systematic Name	Function	Growth on Ura⁻ Media	Growth on X-Gal Media
FKH2	YNL068C	Transcription Factor	Reduced	Light Blue
OCA4	YCR095C	Cytoplasmic protein required for Brome Mosaic Virus replication in *S. cerevisiae*	Reduced	Light Blue
PHO3	YBR092C	Constitutively expressed acid phosphatase	Reduced	Light Blue
PBS2	YJL128C	MAPKK of HOG pathway	Reduced	Light Blue
RTG2	YJL252C	Retrograde regulation	Reduced	Light Blue

HOG: High Osmolarity Glycerol Pathway, MAPKK: Mitogen Activated Protein Kinase Kinase.

Since several promising candidates were identified, efforts were focused on these candidates. Screening of the remaining approximately 1500 deletion strains is ongoing. To confirm slow growth on Ura$^-$ media, the deletion candidates were tested by conducting liquid growth assays in the presence and absence of uracil. The wild-type and candidate strains were grown overnight at 30°C in selective media containing uracil.

Equal number of cells was transferred to selective media with and without uracil and growth was monitored. In the exponential phase, the deletion strains show a greater reduction in growth in the absence of uracil as compared to the wild-type suggesting that the slow growth was due to the reduced Pol II promoter function (Figures 7-9). The deletion strain *pbs2*-Δ continued growing slower than the Ura3$^+$ wild-type control in Ura$^-$ media even after 25 hours.

The growth of other candidates (*fkh2*-Δ, *oca4*-Δ, *pho3*-Δ and *rtg2*-Δ) approached the wild-type control (Ura3$^+$) during the stationary phase in Ura$^-$ media (Figures 7-9). This may be due to reduced Ura3p synthesis from the Pol II rDNA promoter that is inadequate for growth during the exponential phase, but sufficient to support growth during stationary phase.

To quantitate reduced Pol II rDNA promoter activity, the deletion candidates containing the *lacZ* reporter plasmid, pFES17 were assayed for β-galactosidase activity per copy number in whole cell extracts. To ensure accuracy, the enzyme activity was determined for at least two of the cell extracts assayed in multiple independent trials.

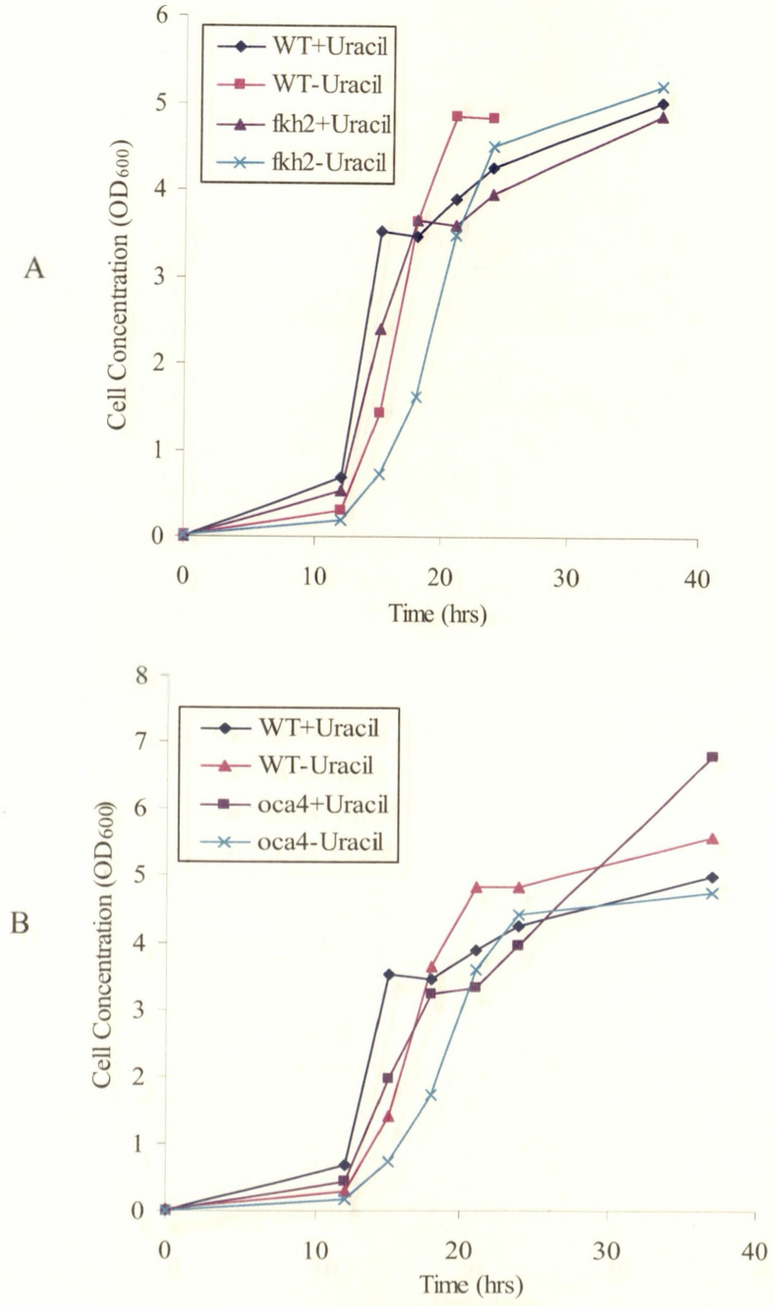


Figure 7. Growth Curves of Wild-Type, *fkh2*-Δ and *oca4*-Δ Strains.
The wild-type and mutants with reporter plasmid prDNA*URA3* were grown in SC-His media. At time T_0, equal numbers of cells were transferred to SC-His media with and without uracil. Growth was monitored at OD_{600} over time at 30°C. The mutants (*fkh2*-Δ and *oca4*-Δ) grew slower than wild-type in Ura^- media (Representative Experiment).

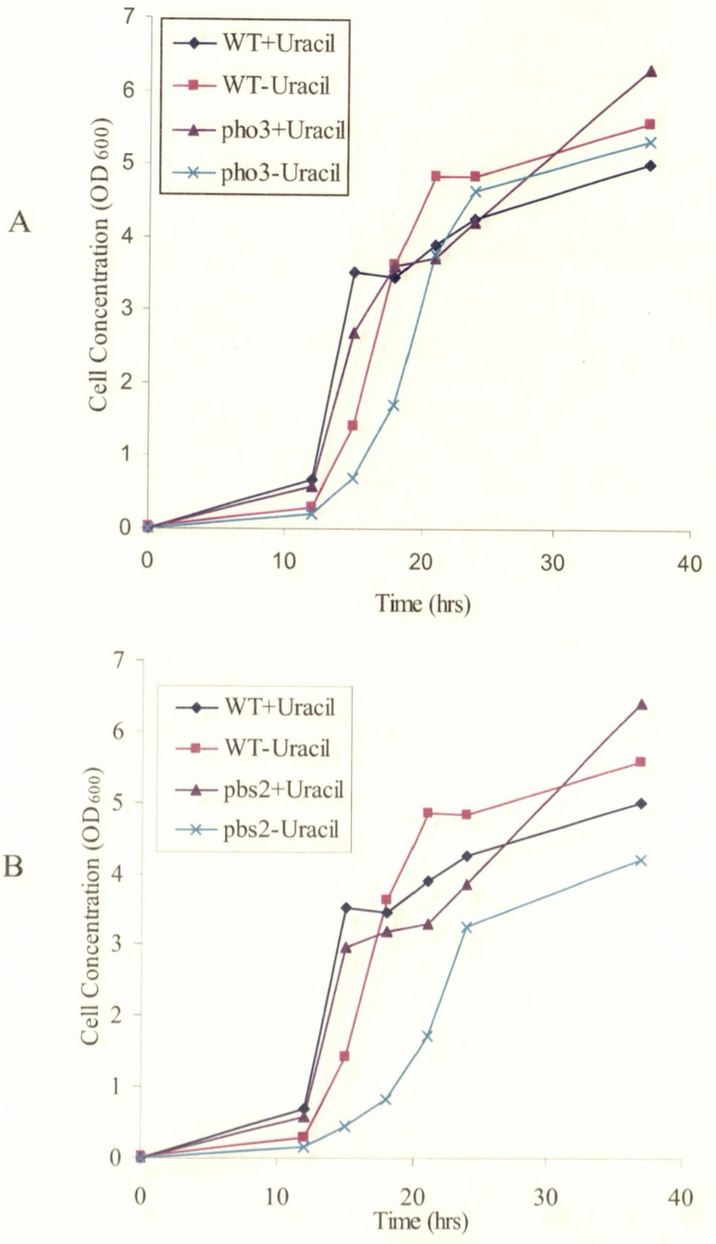


Figure 8. Growth Curves of Wild-Type, *pho3*-Δ and *pbs2*-Δ Strains.
The wild-type and mutants with reporter plasmid prDNA*URA3* were grown in SC-His media. At time T_0, equal numbers of cells were transferred to SC-His media with and without uracil. Growth was monitored at OD_{600} over time at 30°C. The mutants (*pho3*-Δ and *pbs2*-Δ) grew slower than wild-type in Ura⁻ media (Representative Experiment).

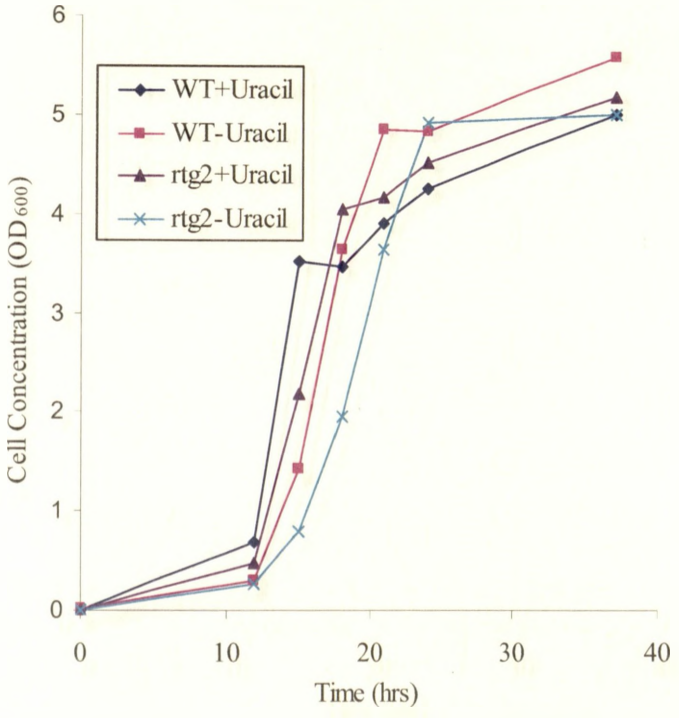


Figure 9. Growth Curves of Wild-Type and *rtg2*-Δ Strain.
The wild-type and mutants with reporter plasmid prDNA*URA3* were grown in SC-His media. At time T_0, equal numbers of cells were transferred to SC-His media with and without uracil. Growth was monitored at OD_{600} over time at 30°C. The mutant *rtg2*-Δ grew slower than wild-type in Ura$^-$ media (Representative Experiment).

As predicted from the screen, all four candidates had reduced β-galactosidase activity per copy number. The deletion of the *FKH2* gene in *fkh2*-Δ strain had the most drastic reduction in β-galactosidase activity per copy number to 16.7% of wild-type activity (77.2 units ± 3.7) [$t(45.54) = 29.47$, $p < 0.05$] (Table 5). In addition, *oca4*-Δ, *pho3*-Δ and *pbs2*-Δ had approximately 60% of wild-type enzymatic activity and these reductions in average enzymatic activities were also statistically significant with *t*-test as $t(56.49) = 12.04$, $t(57.96) = 11.99$ and $t(42.08) = 7.14$ respectively at $p<0.05$ (Table 5; Figure 10).

To confirm the effect of candidate deletions on rDNA Pol II expression, the β-galactosidase activity per copy number was determined for above-mentioned strains (*fkh2*-Δ, *oca4*-Δ, *pho3*-Δ and *pbs2*-Δ) in a second independent experiment. As in the first experiment, *fkh2*-Δ had a significantly lower average enzymatic activity per copy number (90.5 units ± 4.72) [$t(33.60) = 25.33$, $p < 0.05$], representing 28.3% of wild-type (Table 6; Figure 11). In addition, *pho3*-Δ showed a significant reduction in average enzymatic activity (166 ± 4.12) corresponding to ~ 60% of wild-type activity [$t(57.96) = 11.00$, $p < 0.05$]. Although smaller reductions were seen with *oca4*-Δ mutant (86% of wild-type) and *pbs2*-Δ (79.2% of wild-type) as opposed to ~60% in experiment I, the average enzymatic activities remained statistically significant with *t*-test for *oca4*-Δ as [$t(42.08) = 7.14$, $p< 0.05$] and for *pbs2*-Δ as [$t(25.99) = 3.78$, $p < 0.05$].

Table 5

Average β-Galactosidase Activity/Copy Number of the Deletion Candidates (Trial 1)

	Cell Extract 1				Cell Extract 2								
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Average	Average ±SEM	%WT
BY4743ρ^+	415.0	448.5	428.2	443.2	425.2	458.8	423.7	432.2	591.7	596.5	466.3		100
BY4743ρ^+	420.1	423.6	290.3	291.2	440.8	445.2	396.8	389.9	537.0	572.5	420.8		
BY4743ρ^+	443.9	462.6	374.8	400.8	487.0	466.7	440.0	453.8	596.7	630.7	479.2		
BY4743ρ^+	477.0	477.0	428.3	440.7	466.0	473.7	432.8	475.4	615.5	628.7	493.1	463.6 ±12.6	
Δ*fkh2*ρ^+	77.3	77.9	68.5	72.7	77.7	76.8	78.1	76.8	108.6	109.7	83.0		
Δ*fkh2*ρ^+	69.9	47.7	58.0	59.3	73.2	68.4	68.8	68.4	103.0	103.5	72.3	*77.2 ±3.7	16.7
Δ*oca4*ρ^+	217.2	246.9	210.3	207.3	234.2	237.1	260.6	248.0	342.1	328.6	257.2		
Δ*oca4*ρ^+	293.8	325.4	256.1	310.0	242.4	237.7	231.4	245.1	338.5	336.6	280.4	*267.5 ±10.3	57.7
Δ*pho3*ρ^+	281.8	303.1	264.2	264.2	246.9	251.3	260.2	254.0	363.9	344.6	283.6		
Δ*pho3*ρ^+	272.3	344.8	259.2	248.3	246.0	233.3	240.3	239.2	320.8	322.3	272.7	*278 ±9.0	60
Δ*pbs2*ρ^+	324.1	329.2	281.1	253.1	246.4	246.4	247.5	271.6	394.4	397.4	293.8		
Δ*pbs2*ρ^+	424.4	417.7	266.6	270.6	265.4	282.9	282.9	294.5	434.3	443.1	324.8	*318.7 ±15.9	68.7

Mutants with values significantly different from (BY4743ρ^+, wild-type) with $p < 0.05$ by Independent Student's *t* test (two-tailed). SEM: Standard Error of Mean.

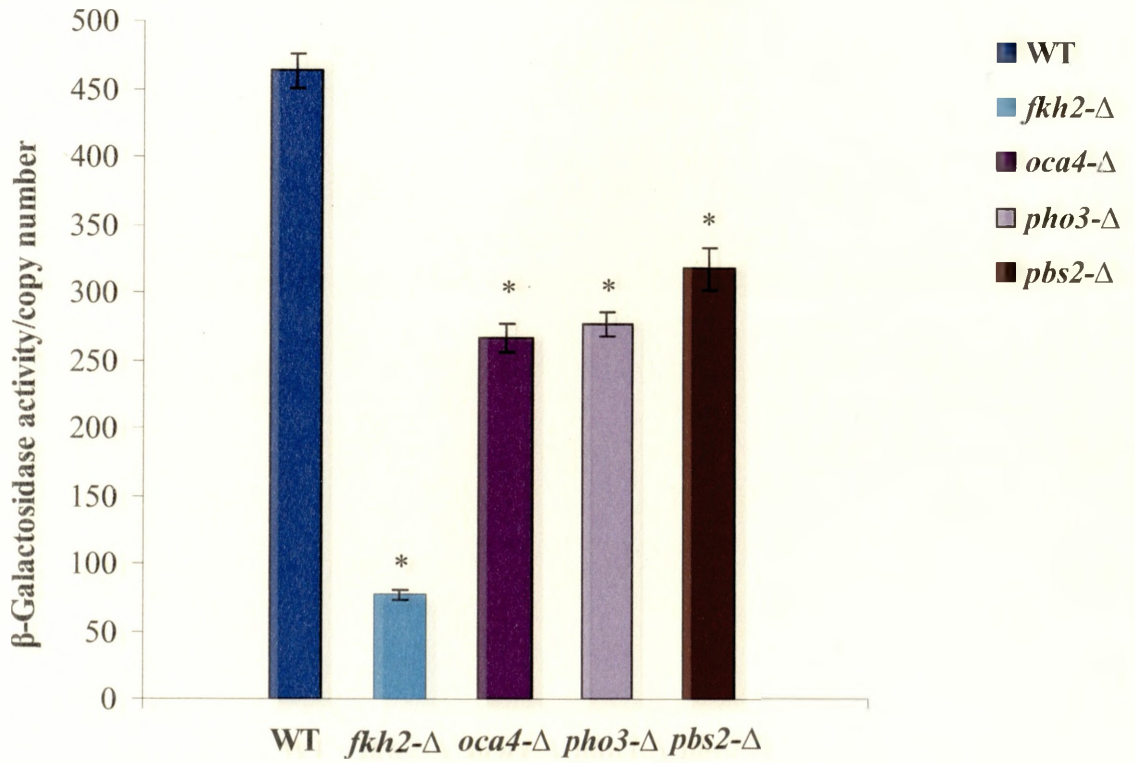


Figure 10. Average β-Galactosidase Activity/Copy Number of Candidates (Trial 1). The wild-type and the deletion candidates were grown in SC-Leu-raffinose media. Multiple extracts of each strain were assayed for β-galactosidase activity/copy number. The results are plotted as average β-galactosidase activity per copy number ± SEM (Standard Error of Mean). An asterisks indicates that the values are significantly different from the wild-type control ($p<0.05$) as determined by Independent Student's *t*-test (two tailed) assuming unequal variances.

Table 6

Average β-Galactosidase Activity/Copy Number of the Deletion Candidates (Trial 2)

	Cell Extract 1					Cell Extract 2									
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Average	Average ±SEM	% WT
BY4743ρ^+	305.0	302.8	312.8	318.3	300.5	374.3	346.4	296.5	384.5	268.6	380.2	319.9	325.8		
BY4743ρ^+	267.2	283.3	306.4	313.6	294.0	360.8	328.9	303.9	383.5	250.4	356.2	313.0	313.4	319.6 ±7.71	100
Δ*fkh2*ρ^+	82.5	90.9	91.8	130.7	82.1	102.0	89.6	72.3	95.3	67.3	99.7	81.2	90.5	*90.5 ±4.72	28.3
Δ*oca4*ρ^+	276.1	269.6	268.4	262.7	251.5	321.9	285.9	248.4	316.4	221.4	319.9	250.8	274.4	*274.4 ±9.12	86
Δ*pho3*ρ^+	153.9	148.3	163.4	159.0	161.9	168.1	154.1	143.3	152.8	137.5	165.0	139.2	153.9		
Δ*pho3*ρ^+	167.6	166.9	167.2	170.1	157.3	203.0	199.4	171.6	190.9	157.7	222.3	164.3	178.2	*166 ±4.12	52
Δ*pbs2*ρ^+	259.6	252.9	268.2	266.8	262.5	312.5	304.5	280.3	296.6	257.6	303.0	271.9	278.0		
Δ*pbs2*ρ^+	233.2	231.2	241.7	258.0	237.6	236.2	234.4	206.5	219.2	197.4	248.3	185.3	227.4	*252.7 ±6.75	79.2

* Mutants with values significantly different from (By4743ρ^+, wild-type) with $p < 0.05$ by Independent Student's *t*-test (two-tailed). SEM: Standard Error of Mean

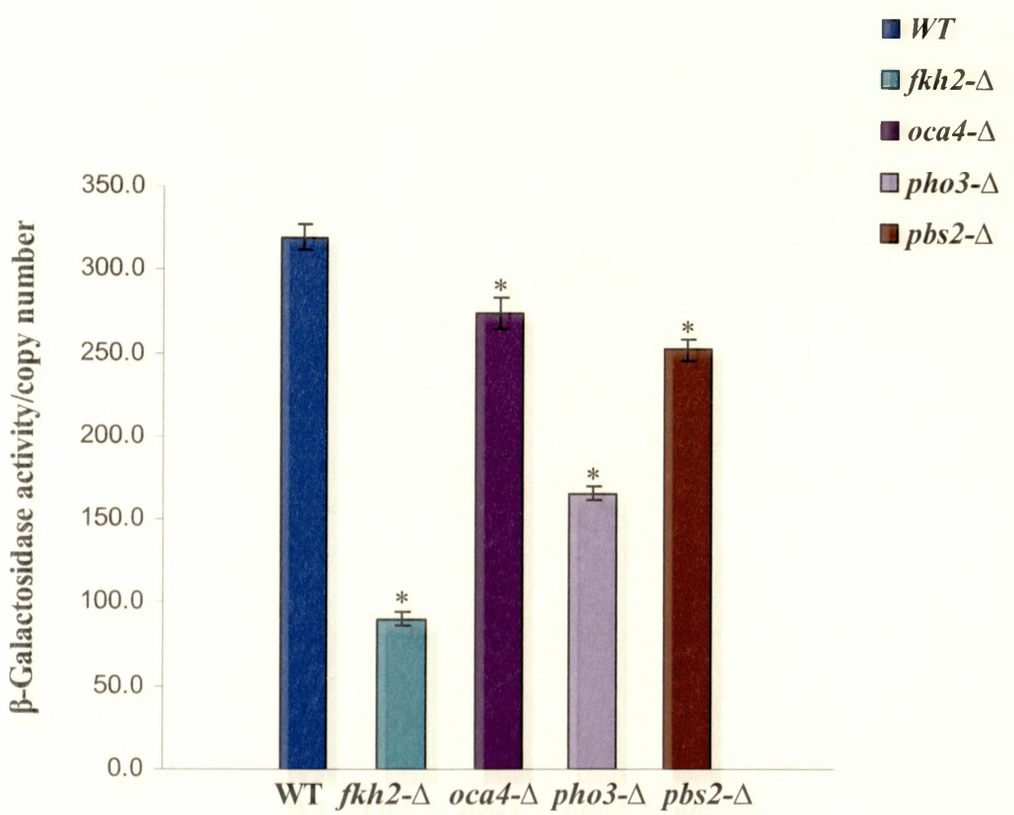


Figure 11. Average β-Galactosidase Activity/Copy Number of Candidates (Trial 2). The wild-type and the deletion candidates were grown in SC-Leu-raffinose media. Multiple extracts of each strain was assayed for β-galactosidase activity/copy number. The results are plotted as Average β-galactosidase activity/copy number ± SEM. An asterisks indicates that the values are significantly different from that of the wild-type control ($p<0.05$) as determined by Independent Student's *t* test (two tailed) assuming unequal variances.

Overall, in both experiments all candidates showed significant reduction in β-galactosidase activity/copy number. The β-galactosidase activity was dramatically lower in *fkh2*-Δ strain (16.7%-28.3%) compared to wild-type. Although, *oca4*-Δ and *pbs2*-Δ showed only a slight reduction (57.7% and 86%) from the wild-type activity respectively, this reduction was still statistically significant at $p < 0.05$ (Table 7). The *pho3*-Δ strain had a modest, but significant reduction (60% and 52%) compared to wild-type activity. In summary, *fkh2*-Δ had the largest reduction, *pho3*-Δ strain showed moderate reduction, while *oca4*-Δ and *pbs2*-Δ showed significant but slight reductions (Table 7).

Table 7

Average β-Galactosidase Activity/Copy Number (Trial 1 & 2)

	Trial 1		Trial 2		Average	
Strain	β-Gal/ Copy # ± SEM	%WT	β-Gal/ Copy # ± SEM	%WT	β-Gal/ Copy # ± SEM	%WT
BY4743ρ^+	463.6 ± 12.6	100	319.6 ± 7.71	100	392	100
Δ*fkh2*ρ^+	77.2 ± 3.7	16.7	90.5 ± 4.72	28.3	84	21.4
Δ*oca4*ρ^+	267.5 ± 10.3	57.7	274.4 ± 9.12	86	271	69.2
Δ*pho3*ρ^+	278 ± 9.0	60	166 ± 4.12	52	222	56.7
Δ*pbs2*ρ^+	318.7 ± 15.9	68.7	252.7 ± 6.75	79.2	285.7	72.8

The Role of *RTG2* in rDNA Transcription

Rtg2p is a regulatory protein required for the activation of RTG pathway. RTG pathway transmits signals from mitochondria to nucleus in response to low glutamate levels or mitochondrial dysfunction (Liu and Butow, 2006). Rtg2p positively regulates retrograde pathway by reversibly binding to the negative regulator (Mks1p). This interaction prevents Mks1p from interacting with the transcription factors, Rtg1p and Rtg3p, resulting in nuclear translocation of Rtg1-3p complex, and activation of *RTG* responsive genes (Liu and Butow, 2006).

To confirm the role played by *RTG2* in Pol II rRNA synthesis, β-galactosidase activity per copy number was determined in crude cell extracts of *rtg2*-Δ strain and compared to wild-type (Table 8). The *rtg2*-Δ strain showed a dramatic reduction in the enzymatic activity to 33.20% of wild-type [$t(21.28)=29.3$, $p<.05$] (Figure 12). Since *RTG2,* a member of RTG regulatory pathway, was shown to markedly affect Pol II rDNA expression, an independent experiment was conducted to examine the role of another member of the pathway, Mks1p. Mks1p regulates RTG pathway in a dual manner. Its binding to Rtg2p activates RTG pathway leading to nuclear translocation of transcription factors Rtg1p and Rtg3p. When bound to Bmh1p and Bmh2p proteins, the pathway is turned off. This inactivation prevents nuclear translocation of transcription factors Rtg1p and Rtg3p as well as their binding to promoter element (R Box) of the retrograde responsive target genes, leading to lack of retrograde response.

Table 8

Average β-Galactosidase Activity/Copy Number (RTG2)

	Cell Extract 1					Cell Extract 2				Average	Average	%WT
Strains	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 1	Assay 2	Assay 3	Assay 4			
BY4743ρ^+	475.4	493.0	458.7	467.2	501.2	452.8	424.8	440.2	433.2	460.7		
BY4743ρ^+	546.6	563.1	477.3	528.1	540.5	510.7	515.5	544.4	560.5	531.9	496.2± 10.57	100.0
$\Delta rtg2\rho^+$	169.4	181.7	159.4	166.6	149.8	185.1	159.1	155.5	157.3	164.9	*164.9 ±3.99	33.2

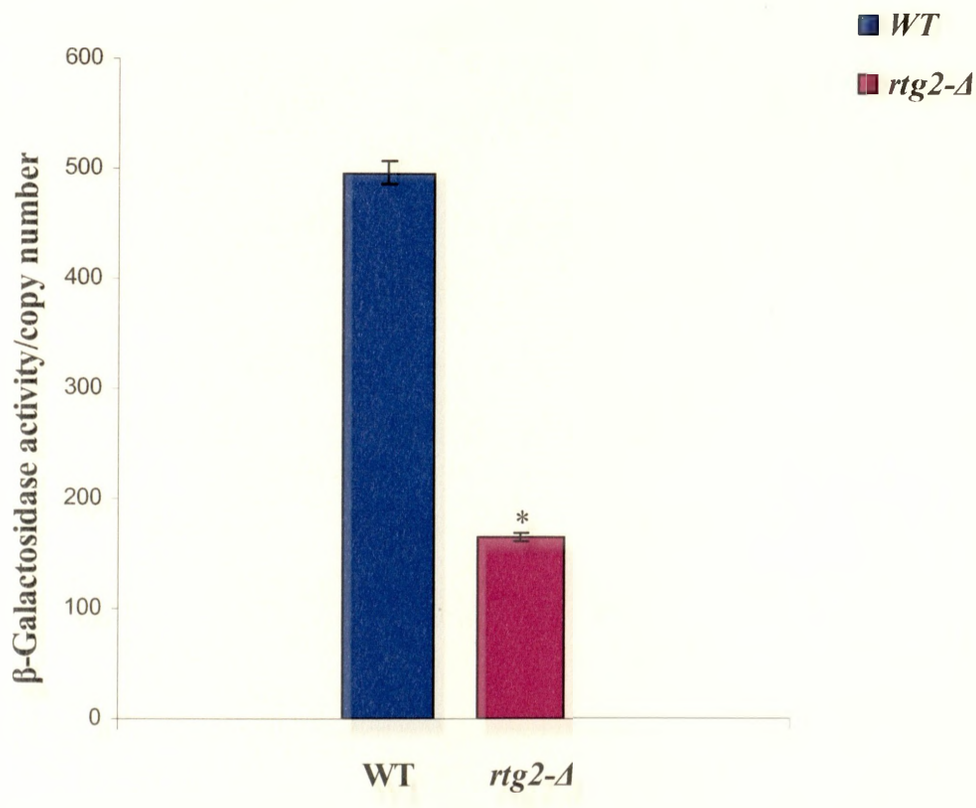


Figure 12. RTG2 is Required for Maximal Pol II rRNA Synthesis.
The wild-type and the deletion candidates were grown in SC-Leu-raffinose media. Multiple extracts of each strain was assayed for β-galactosidase activity/copy number. The results are plotted as Average β-galactosidase activity/copy number ± SEM. An asterisks indicates the values that are significantly different from the wild-type control ($p<0.05$) as determined by Independent Student's *t* test (two tailed) assuming unequal variance

In order to examine the role of the *MKS1* in Pol II rDNA expression, β-galactosidase enzymatic assays were conducted and activity in *mks1*-Δ strain was compared to the wild-type. A several folds increase in constitutive expression of RTG responsive genes such as *CIT2* and *DLD3* in both *mks1*ρ^+-Δ and *mks1*ρ^0-Δ strains suggests that the *mks1*-Δ mutant should also result in an increased average β-galactosidase activity per copy number (Sekito et al., 2002).

In all three experiments the deletion strain *mks1*-Δ had activity greater than 90% of the wild-type. In the first trial experiment, the average β-galactosidase activity per copy number was 391.7 units corresponding to 92% wild-type and was statistically significant [$t(50.58) = 4.8, p<0.05$] (Table 19; Appendix B). In subsequent trials (II &III), (Tables 20-22; Appendix C-E) the β-galactosidase activity per copy number in *mks1*-Δ strain was greater than 100% of wild-type (493.2 and 416) and was not statistically significant from wild type [$t(32) =-0.613, p < 0.05$] and [$t(76) = -14.660, p <0.05$] (Figure 13). As the activity in all three independent cell extracts was greater than 60% of wild-type activity, it suggests that *MKS1* does not play a direct role in Pol II rRNA synthesis.

Table 9

Role of MKS1 in Pol II rRNA Synthesis

	Trial 1		Trial 2		Trial 3		Average	
Strain	β-Gal/ Copy#	% WT	β-Gal/ Copy#	% WT	β-Gal/ Copy#	% WT	β-Gal/ Copy#	% WT
BY4743ρ^+	425.1	100	482.1	100	304	100	403.7	100
Δ*mks1*ρ^+	391.7	92	493.2	102.3	416	137	433.63	107.4

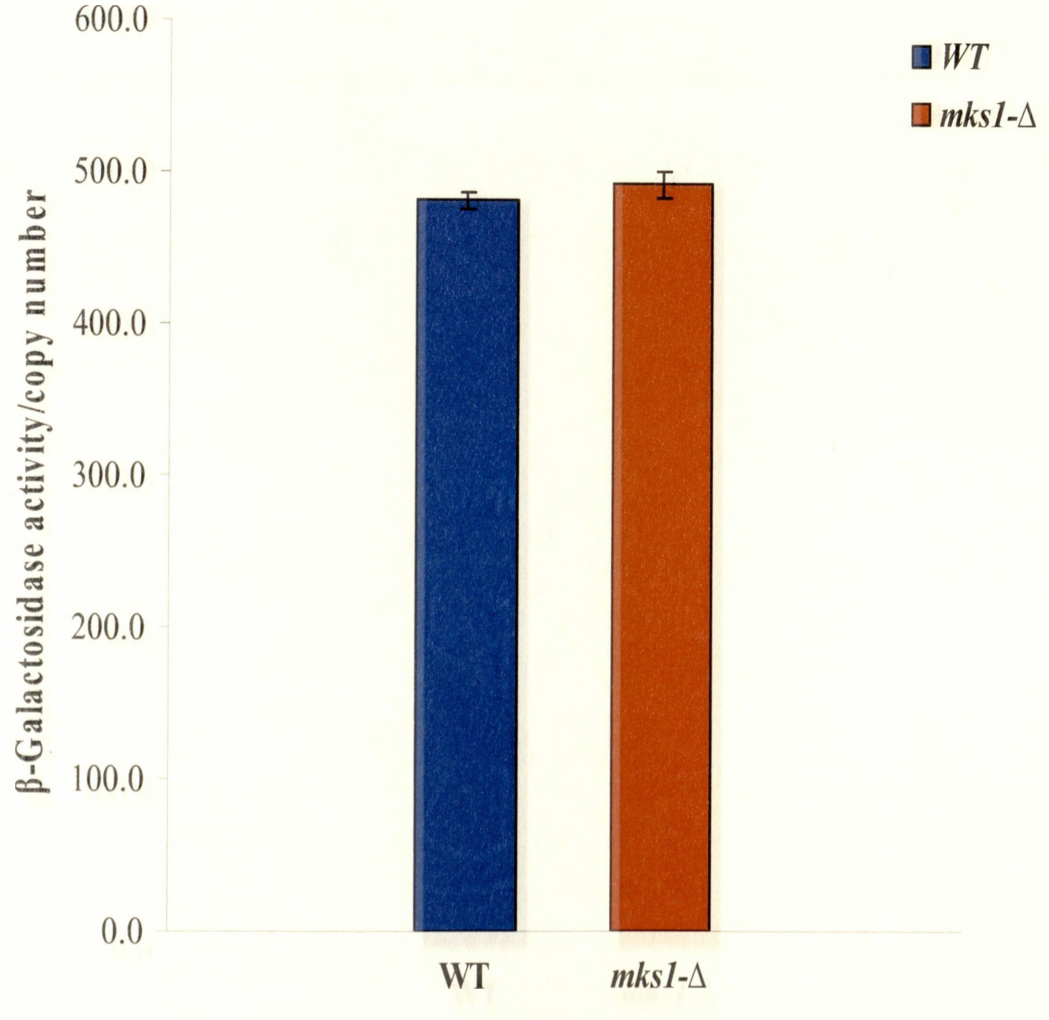


Figure 13. Role of MKS1 in Pol II rRNA Synthesis (Trial 2).
The wild-type and the deletion candidates were grown in SC-Leu-raffinose media. Multiple extracts of each strain were assayed for β-galactosidase activity/copy number. Average β-galactosidase activity/copy number ± SEM is shown.

The Role of HOG Pathway in Pol II rDNA Transcription

Once *PBS2*, a member of High Osmolarity Glycerol (HOG) pathway was identified as a factor required for Pol II driven rDNA transcription (Table 10; Figure 14) the role of other members of the same pathway in rDNA transcription was examined. Hog1p is a MAPK that is activated by MAPKK (Pbs2p) in response to osmotic stress. Activated Hog1p translocates to the nucleus and activates osmotic responsive genes. Activated Hog1p binds to the promoter of osmoresponsive genes and increase their expression. If the HOG pathway directly influences Pol II rDNA promoter expression, then a *HOG1* deletion should also result in reduced Pol II rDNA promoter function. To confirm if the HOG pathway triggers Pol II rRNA synthesis, rRNA expression was examined in *hog1*-Δ strain. Beta-galactosidase activity/copy number was determined in crude cell extracts of *hog1*-Δ strain and compared to wild-type (Table 10).

Aś anticipated, the *hog1*-Δ strain showed a dramatic reduction in β-galactosidase activity/ copy number to 19.0% wild-type [$t(20.03) = 36.07$, $p<0.05$] (Table 10; Figure 14). The activity in other two candidate strains, *fkh2*-Δ (15.7%) [$t(17.37) = 39.33$] and *pho3*-Δ (70% wild-type) [$t(23.65) = 12.15$, $p<0.05$] was also in agreement with previous two experiments (Table 10). In contrast to prior two experiments, the *pbs2*-Δ strain had higher enzymatic activity than wild-type (140%).

Table 10

Role of HOG Pathway in Pol II rRNA Synthesis

	Cell Extract 1					Cell Extract 2				Average	Average ±SEM	%WT
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 1	Assay 2	Assay 3	Assay 4			
BY4743ρ^+	475.4	493.0	458.7	467.2	501.2	452.8	424.8	440.2	433.2	460.7		
BY4743ρ^+	546.6	563.1	477.3	528.1	540.5	510.7	515.5	544.4	560.5	531.9	496.2±10.57	100.0
Δ*fkh2*ρ^+	78.6	80.6	70.8	80.6	80.6	78.7	80.4	77.4	74.8	78.1	*78±1.11	15.7
Δ*pho3*ρ^+	364.0	367.1	312.9	344.9	355.4	355.5	349.8	364.1	352.7	351.8	*351.8±5.43	70.0
Δ*pbs2*ρ^+	769.6	791.3	584.5	689.9	635.7	769.1	634.6	698.5	683.1	695.2	*695.2±23.53	140.0
Δ*hog1*ρ^+	107.1	102.9	111.4	85.8	88	103.4	82.6	93.9	96.6	96.9	*96.9±3.28	19.0

* Mutants with values significantly different from (BY4743ρ^+, wild-type) with $p < 0.05$ by Independent Student's *t*- test (two-tail).

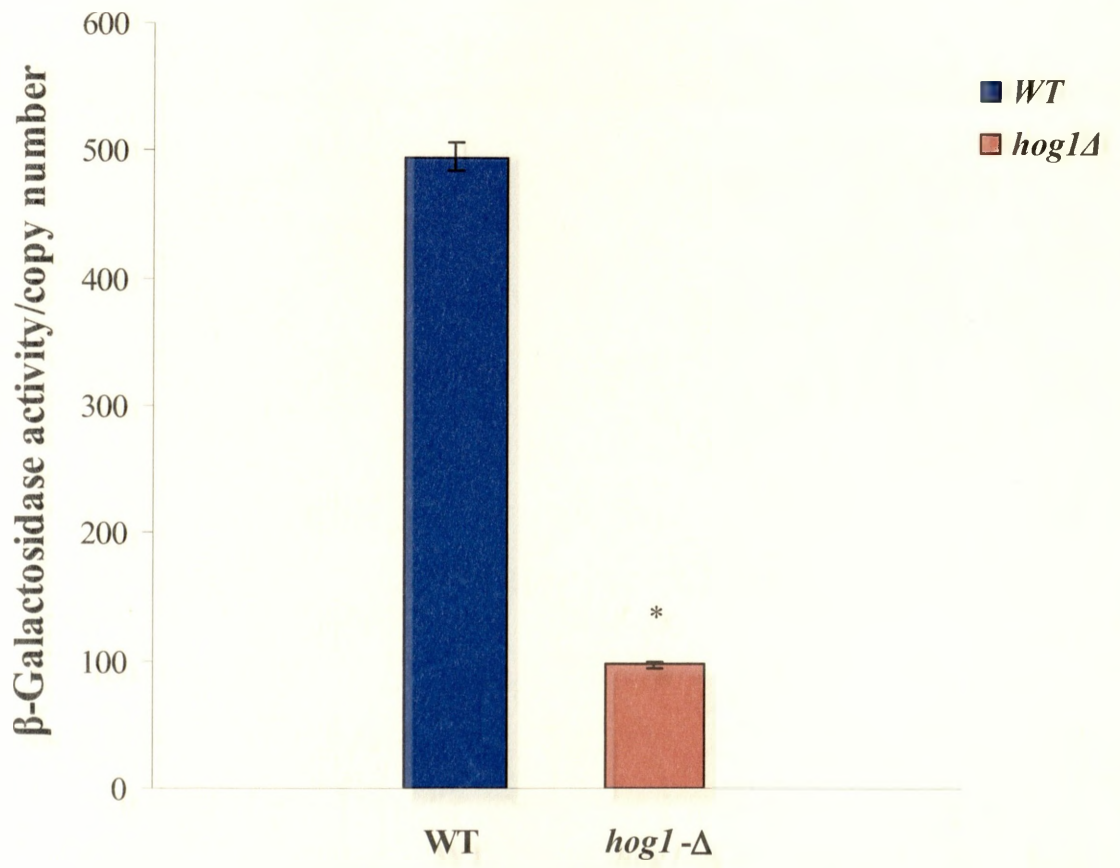


Figure 14. Role of Members of HOG Pathway in Pol II rRNA Synthesis.
The wild-type and the deletion candidates were grown in SC-Leu-raffinose media. Multiple extracts of each strain were assayed for β-galactosidase activity/copy number. Average β-galactosidase activity/copy number ± SEM is shown

However, in the subsequent experiments, the *pbs2*-Δ strain shows reduced β-galactosidase activity/copy number due to variation in copy number. The reduced Pol II rDNA function in the deletion mutants of two consecutive members of the HOG pathway indicates that HOG pathway regulates Pol II rDNA promoter expression. As expected, the activity in other two candidate strains, *fkh2*-Δ (15.7% of wild-type) [$t(17.37) = 39.33$] and *pho3*-Δ (70% of wild-type) [$t(23.65) = 12.15$, $p<0.05$] was in agreement with previous two experiments (Tables 7 and 10).

The Role of Other Members of Complex 490 in Pol II rDNA Expression

S. cerevisiae serves as a model organism to study replication, gene expression and encapsidation of brome mosaic virus, because of the inherent ability of the virus to replicate in yeast. Oca4p was identified as one of the host genes required for replication of brome mosaic virus in *S. cerevisiae* (Chen et al., 2001). It has also been shown to stimulate Pol II rDNA transcription in previous experiments. Besides *OCA4*, the deletion of *OCA1* or *SIW14* also reduced BMV directed reporter gene expression. The *OCA1* or *SIW14* gene encodes a protein phosphatase (Kushner et al., 2003). Recently, Oca4p is documented to be a part of complex 490. This complex also contains *Oca1p* and *Oca3p* (Gavin et al., 2006). Once *OCA4* was confirmed as a candidate affecting Pol II rDNA expression, disruptions of *OCA1* and *OCA3*, members of the complex 490 were tested along with the wild-type controls for reporter function. The average enzyme activities of *OCA4, OCA3* and *OCA1*ρ^+ deletion strains were lower than the wild-type (Table 11; Figure 15).

Table 11

Average β-Galactosidase Activity/Copy Number in Oca-Δ Mutants

	Cell Extract 1							Cell Extract 2					
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Average
BY4743ρ^+	500.6	500.6	501.1	453.6	567.3	550.1	529.0	469.5	479.1	443.1	499.5	419.8	492.8
Δ*oca4*ρ^+	262.9	241.4	253.8	236.2	244.1	247.5	242.8	228.8	267.9	242.4	236.2	234.4	*244.8
Δ*oca3*ρ^+	172.5	141.1	145.3	145.5	152.8	156.5	133.8	158.6	171.0	151.2	166.9	143.6	*153.2
Δ*ca1*ρ^+	326.4	276.7	286.1	271.8	293.4	280.6	256.0	277.4	292.1	259.8	277.4	235.4	*277.7

Strains	β-galactosidase activity/copy number	SEM	%WT
BY4743ρ^+	492.8	12.4	100.0
*Δ*oca4*ρ^+	244.8	3.4	49.7
*Δ*oca3*ρ^+	153.2	3.5	31.1
*Δ*ca1*ρ^+	277.8	6.4	56.4

* Mutants with values significantly different from (BY4743ρ^+, wild-type) with $p < 0.05$ by Independent Student's *t* test (two-tailed).

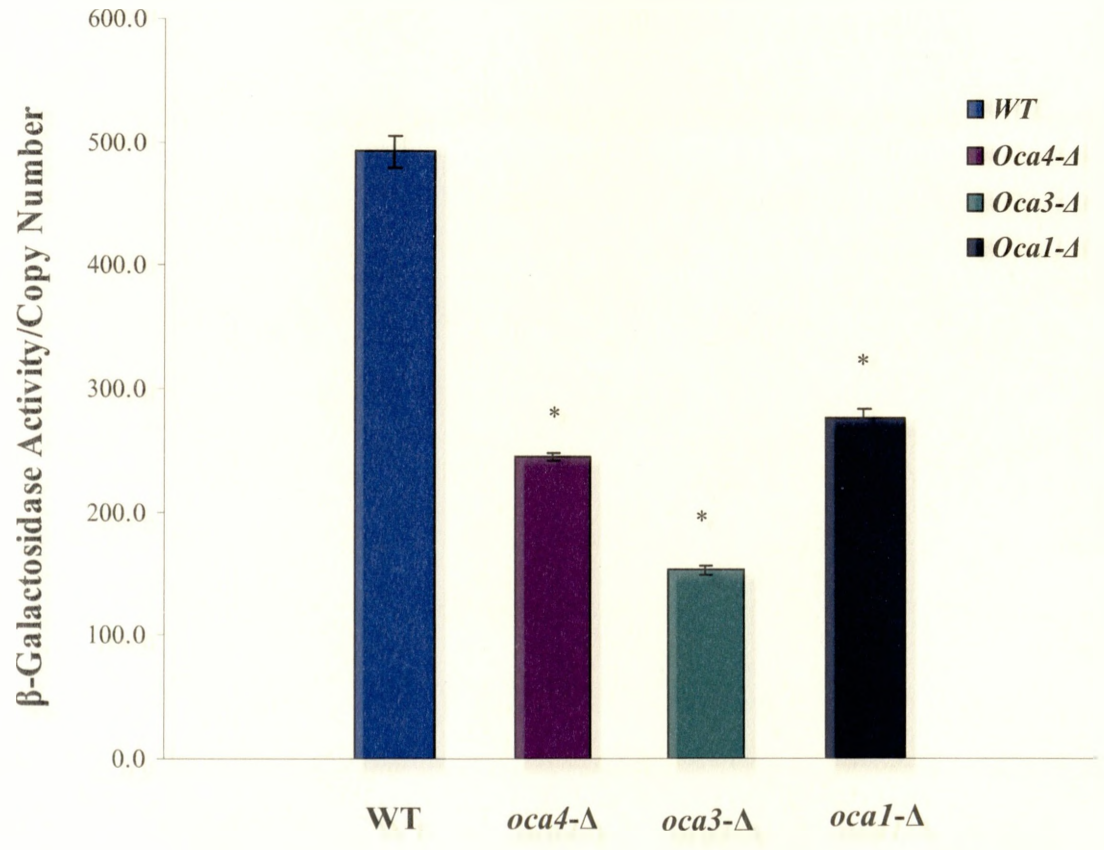


Figure 15. Role of OCAs in Pol II rRNA Synthesis.
The wild-type and the deletion candidates were grown in SC-Leu-raffinose media. Multiple extracts of each strain was assayed for β-galactosidase activity/copy number. The results are plotted as Average β-galactosidase activity/copy number ± SEM. An asterisks indicates the values that are significantly different from the wild-type control ($p<0.05$) as determined by Independent Student's *t* test (two tailed) assuming unequal variances.

In this experiment the average β galactosidase activity/copy number in *Oca4*-Δ strain was 49.7% of the wild type and this was in accordance with a previous experiment (Table 7). The average β galactosidase activity/copy number in *Oca4*-Δ, *Oca3*-Δ, and *Oca1*-Δ strains were 49.7%, 31.1 % and 56.4% of the wild-type and were found to be statistically significant at $p<0.05$ with *t*-test as [$t(12.5)=19.23$], [$t(12.7)=26.23$] and [$t(16.5)=15.3$ respectively] (Table 11; Figure 15). This suggests that the components of OCA complex play a role in regulating rRNA synthesis.

Potential Binding Sites for Transcription Factors in Pol II rDNA Promoter

The entire Pol I and II rDNA promoter region (-1210 to + 44) was searched for potential binding sites of known transcription factors as from screening the YKO library few transcription factors were identified. To find consensus sequences for transcription factors the Yeast Search for Transcriptional Regulators and Consensus Tracking (YEASTRACT; www.yeastract.com) and *Saccharomyces* Genome Database (SGD) (www.yeastgenome.org) were used.

YEASTRACT database contains 284 specific DNA-binding sites for 108 characterized transcription factors. The YEASTRACTs transcription factor binding tool provides a graphical as well as a tabular output for all known yeast transcription factor-binding sites against a query sequence. It also provides information for over 30,990 regulatory associations between transcription factors and target genes (Monteiro, 2008; Teixeira et al., 2006). The SGD provides detailed information for all annotated genes and their products.It gives the sequence for each gene, the known binding sites for regulatory factors, and the interacting partners. The SGD was used to find consensus sequences for

known transcription factors. The rDNA promoter sequence was carefully scanned for these sequences. Most of the transcription factors were shown to bind identical consensus sequences in both databases. Occasionally, the databases showed variation in adjacent bases surrounding the core region. The promoter had multiple potential binding sites for several transcription factors such as Adr1p (4 sites), Ash1p (2 sites), Fkh1/2p (4 sites), Gcr1 (6 sites), Mot 3p (4 sites) and Rtg1/3p (2 sites). Adr1p is a zinc finger transcription factor that activates glucose repressed genes under diauxic transition. Gcr1p serves as a trans-activator for glycolytic genes.

Rtg1p and Rtg3p are transcriptional factors that mediate retrograde response. These sites are scattered along different regions of the rDNA Pol I and II promoter (Figure 16).

Since a disruption of *FKH2* resulted in a dramatic reduction in Pol II promoter expression, the presence of Fkh2p binding sites within the promoter was of great interest. Both Fkh1p and Fkh2p were found to bind to identical consensus sequence. There are three potential Fkh1/2p binding sites within the rDNA promoter region. Two of the sites are located upstream of the critical region at positions -657 and -889 respectively. The other site is positioned further upstream (-1154). Since *fkh2*-Δ strain had the most drastic consequence on Pol II rDNA promoter expression, the promoter region was examined for binding sites for other known factors regulating cell cycle such as Mcm1p, Swi5p and Azf1p. A single binding site was found for both Azf1p and Swi5p.

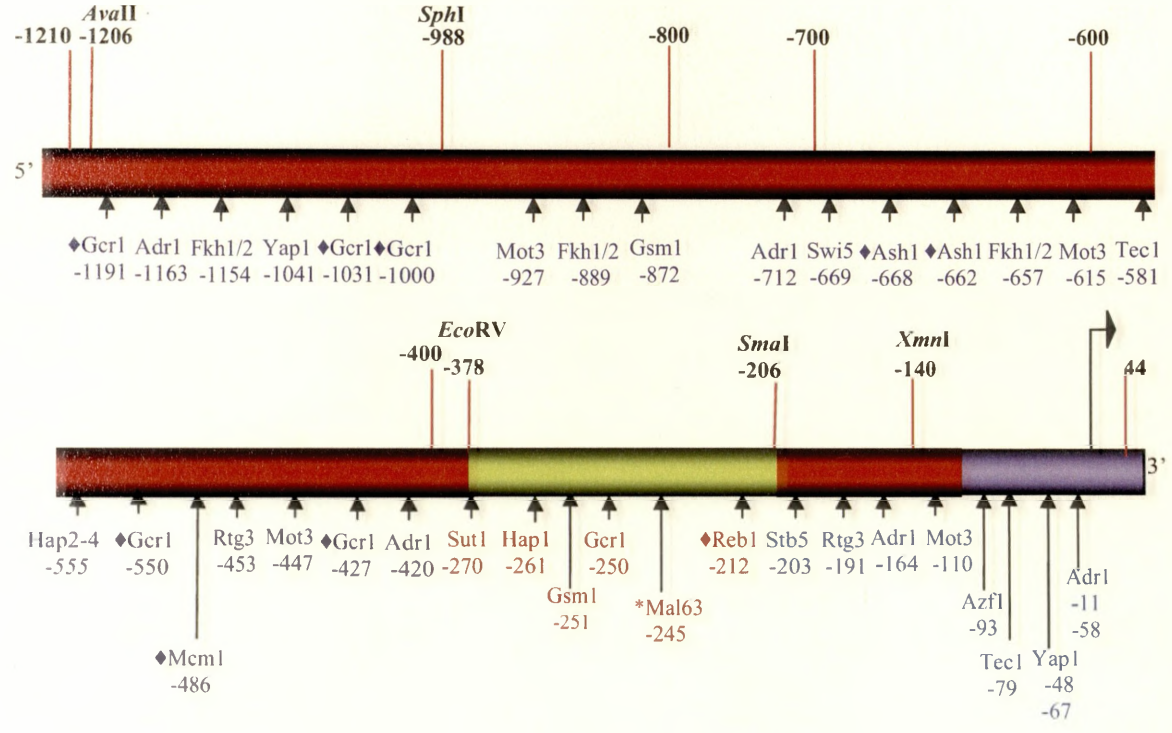


Figure 16. Schematic Representation of TFs on rDNA Pol I and II Hybrid Promoter.

The Pol II rDNA promoter (Red) overlaps the Pol I promoter (Violet). The region from *Eco*RV-*Sma*I site (-378-206) represents the critical region (CR) (yellow). This region is important for Pol II driven rDNA expression. The TFs binding to CR are shown in Red, those binding to the upstream region are shown in purple and those binding to the downstream region are shown in blue. TFs encoded by essential genes are indicated by a diamond.

The Azf1p binding site was found downstream of the critical region (-93) while, Swi5p binding site was located further upstream (-669). There was one binding site for Mcm1p within the rDNA promoter region, located further upstream (-486-476). However, Mcm1p is essential for cell survival and hence its disruption could not be tested. In addition, promoter region was searched for binding sites for other transcription factors responsive to various stresses (osmotic, oxidative and loss of mitochondrial DNA) and metabolites. These transcriptional factors were members of known pathways in *S. cerevisiae* such as RTG, HOG, oxidative stress and energy metabolism.

There are two binding sites for the Rtg3p within the rDNA promoter region. One of the binding site borders the critical region (-191) and the other is positioned upstream of critical region (-453). Rtg3p is a transcriptional activator of RTG pathway. It triggers retrograde response by binding to R element within the promoter of retrograde responsive genes. No binding site for Hog1p or *HOG1* targets was found within the promoter region. Hog1p, a MAPK of the HOG pathway is also known to bind to the promoter of osmoresponsive genes upon activation. The activation of HOG pathway enables cell to combat osmotic stress by increasing synthesis of enzymes involved in glycerol synthesis.

Promoter scanning revealed many transcriptional factors besides Fkh1/2p and Rtg3p. Some of these factors were found to bind upstream of the critical region (-378-1206). These transcriptional factors belong to varied functional categories: cell cycle regulation (Mcm1p, Azf1p and Swi5p), oxidative stress (Mot3p and Yap1p), pseudohyphal growth (Ash1p, Tec1p) and energy metabolism (Gcr1p, Gsm1p, Adr1p, and Hap2/4p).

Many factors such as Sut1p, Hap1p, Gsm1p, Gcr1p, Mal63p and Reb1p were found to bind within the critical region (-378-206) (Tables 12-14). Sut1p is required for induction of hypoxic genes under anaerobic conditions. It is surprising that most of these factors (Hap1p, Gsm1p, Gcr1p and Mal63p) are involved in energy metabolism (Table 12). In 161U7 ρ^0 cells, the deletion of the critical region (-378-206) has shown to reduce the β-galactosidase activity/copy number to 3.5% of wild-type suggesting its importance for Pol II driven rRNA synthesis (Jodhka, 2004). In addition, many factors such as Stb5p, Rtg3p, Adr1p, Mot3p, Azf1p, Tec1p, Yap1p, and Adr1p were found to bind downstream of the critical region (-378-206). Some of these transcription factors bind within the overlapping Pol I-Pol II region (Mot3p, Azf1p, Tec1p, Yap1p, and Adr1p). Yap1p is involved in combating oxidative stress. Azf1p is involved in induction of *CLN3* genes in response to glucose. Some of these downstream factors might also regulate Pol II driven rDNA expression in ρ^+ cells. This is supported from a study in a ρ^0 mutant where deletion of downstream sequences along with the critical region (-141-378) has shown to reduce β-galactosidase activity/copy number to 1.9% of wild-type, suggesting the importance of downstream sequences in Pol II driven rRNA synthesis (Jodhka, 2004). In contrast, sequences upstream of the critical region were not required for Pol II rDNA transcription in ρ^0 cells but may be important for Pol II rDNA transcription in ρ^+ cells. Thus, identification of binding sites of known transcription factors within the rDNA promoter in all three regions would not only clarify the role of Fkh2p in Pol II rRNA synthesis but might also confirm the role of HOG or RTG pathway in rDNA expression in ρ^+ cells.

Table 12

Transcription Factors Binding to rDNA Promoter (Energy Metabolism)

Factor	Consensus Sequence	Position in rDNA Promoter	Database	Function/Pathway
♦Gcr1	CTTCC (1) GAAGG GTAGG(4)	-1191-1187(U) -1031-1027(U) -1000-996(U) -550-546(U) -250-246(C) -427-422(U)	YT	Trans Activator for glycolytic genes, DNA binding protein that binds to the transcriptional activator Gcr2p.
Adr1	TTGGAG (2) GGAGG (4)	-1163-1159(U) -714-709(U) -712-708(U) -164-159(D) -58-54(D) -11-7(D)	YT YG	TF (Zinc Finger) act as an activator of glucose repressed genes under diauxic transition. Adr1 also interacts with Gcn5p via transactivating domain.
Mot3	CAGGCA AAGGCA (2) TAGGTA	-927-922(U) -115-110(D) -447-442(U) -610-615(U)	YT YT YT YT	TF (Zinc Finger) acts as a repressor and an activator, associates with Rox1p and causes repression of subset of hypoxic genes under aerobic condition.
Gsm1	CGGCACCGGATGCGG CCGCTTCCGCTTCCG	-872-858(U) -251-237(C)	YT	TF (Putative Zinc Finger), proposed to be involved in energy metabolism.
Hap2/4	TTATTGGT	-555-549(U)	YT	Heme activated transcription activator, a global regulator of respiratory gene expression.
Mal63	CGCTTCCGC	-245-237(C)	YT	MAL-Activator protein. A transcription factor required for maltose fermentation.

YT: Yeastract, YG: Yeast Genome, TF: Transcription Factor, (C) Critical Region, (U)Upstream Region, (D) Downstream Region. ♦ TFs encoded by essential genes

Table 13

Transcription Factors Binding rDNA Promoter (Stress)

Factor	Consensus Sequence	Position in rDNA Promoter	Database	Function/ Pathway
Rtg1/3	GTCAC(2) GGCAC	-453-449(U) -197-193(D) -204-201(D)	YT YT YT	TF (bHLH), Retrograde regulation.
Sut1	CGCG	-270-267(C)	YG	Interacts with Cyc8p and converts the repressor Cyc8p-Tup1p complex to an activator for induction of hypoxic genes under anaerobic conditions.
Hap1	CCGTATTTTCCG	-261-250(C)	YT	TF (Zinc Finger), responsive to levels of heme and oxygen.
Yap1	TTTGTCA (1) TTAGTCA (1) TTTGTAA (1)	-1041-1034 (U) -48-42 (D) -67-61 (D)	YT,YG YT YT	AP-1-like TF (Basic Leucine Zipper) activates anti-oxidant genes in response to oxidative stress.
Stb5	CGGGG (1)	-207-203(C)	YT	Zinc Finger TF, activator of drug resistance genes, binds Sin Three Binding protein. Activated by oxidative and xenobiotic stress.

YT: Yeastract, YG: Yeast Genome, TF: Transcription Factor, (C) Critical Region, (U)Upstream Region, (D) Downstream Region. ♦ TFs encoded by essential genes, (bHLH) Basic Helix Loop Helix.

Table 14

Transcription Factors Binding rDNA Promoter (Cell Cycle and Pseudohyphal Growth)

Factor	Consensus Sequence	Position in rDNA Promoter	Database	Function/ Pathway
Fkh1/2	TATTTAT TATTTGTT/TATTTGT TGTTTAT	-1154-1148(U) -889-882(U) -657-651(U)	YT YT YT	Fork Head Family TF, regulates transcription of cell cycle genes required for G2/M phase transition.
Swi5	GGCTGA	-669-665(U)	YG	TF activates genes expressed at M/G1 boundary and G1 phase of Cell Cycle.
♦Mcm1	TCCCTTTAGGG	-486-476(U)	YT	Transcription Factor, a part of an activator as well as a repressor complex, regulates transcription of cell cycle genes.
Azf1	AAAAGAAA	-93-89(D)	YT	Asparagine rich Zinc Finger (TF), induction of *CLN3* genes in response to glucose.
Ash1	CTGAT TTGAT	-668-664(U) -662-658(U)	YT YG	GATA like TF (Zinc Finger) Inhibitor of mating type switching in daughter cells and promotes pseudohyphal growth. Ash1p is also a part of Rpd3 HDAC complex.
Tec1	GTAAGA (2)	-576-581(U) -79-74(D)	YT	TF required for Ty1 expression and pseudohyphal growth.
♦Reb1	TTACCCG	-212-206(C)	YT	Pol I termination and Pol II transcription in ρ^0 cells.

YT: Yeastract, YG: Yeast Genome, TF: Transcription Factor, (C) Critical Region, (U)Upstream Region, (D) Downstream Region. ♦ TFs encoded by essential genes.

In order to establish the role these binding factors play in rRNA synthesis, it is prudent to study various disruptions of transcription factors and compare Pol II driven *lacZ* expression in these disruptions with the expression in the wild-type strain. The knock-out strains are available for most of the transcription factors that bind within these regions, except for few that are encoded by essential genes (diamond) and for some difficult to construct deletion strains (asterisk) (Figure 16).

The reporter plasmid, pFES17, was transformed into each of the available YKO deletion strains, bearing deletion for a particular transcription factor. The effect of the deletion of the factor on Pol II driven *LacZ* expression was scored on X-Gal indicator plates over a period of two days. The transformants that were either light blue or white were picked for liquid β-galactosidase assays to determine enzymatic activity/copy number. The deletion candidates that satisfied this selection were: *sut1*-Δ (white), *stb5*Δ (white, light blue), *mot3*-Δ (white), *swi5*-Δ (white and light blue), *azf1*-Δ (white), *adr1*-Δ (white and light blue), *yap1*-Δ (light blue), *hap2*-Δ (light blue) and *hap4*-Δ (white, light blue).

To investigate the role of the above-mentioned factors in Pol II rDNA expression, liquid β-galactosidase enzymatic assays were carried out and β-galactosidase activity /copy number was calculated for each strain and compared to the wild- type in multiple independent assays (Table 23; Appendix F). Yap1p was not selected for enzymatic assay as its binding site was downstream or far upstream from the transcription initiation site. The *stb5*-Δ shows a significant decrease in Pol II promoter expression. The *stb5*-Δ strain

had an average β-galactosidase activity/copy number of 239.5 units corresponding to 57.31% of wild-type activity [$t(42.9) = 33.39$, $p<0.05$] (Table 15; Figure 17).

Table 15

Average β-Galactosidase Activity/Copy Number in Promoter Binding Mutants (Trial 1)

	Average		
Strain	β-Gal /copy number	SEM	%WT
BY4743ρ^+	418.0	4.4	100.00
**mot3*-Δ	511.5	5.9	122.37
**stb5*-Δ	239.5	2.9	57.31
gsm1-Δ	411.3	9.1	98.40
sut1-Δ	417.5	10.5	99.88

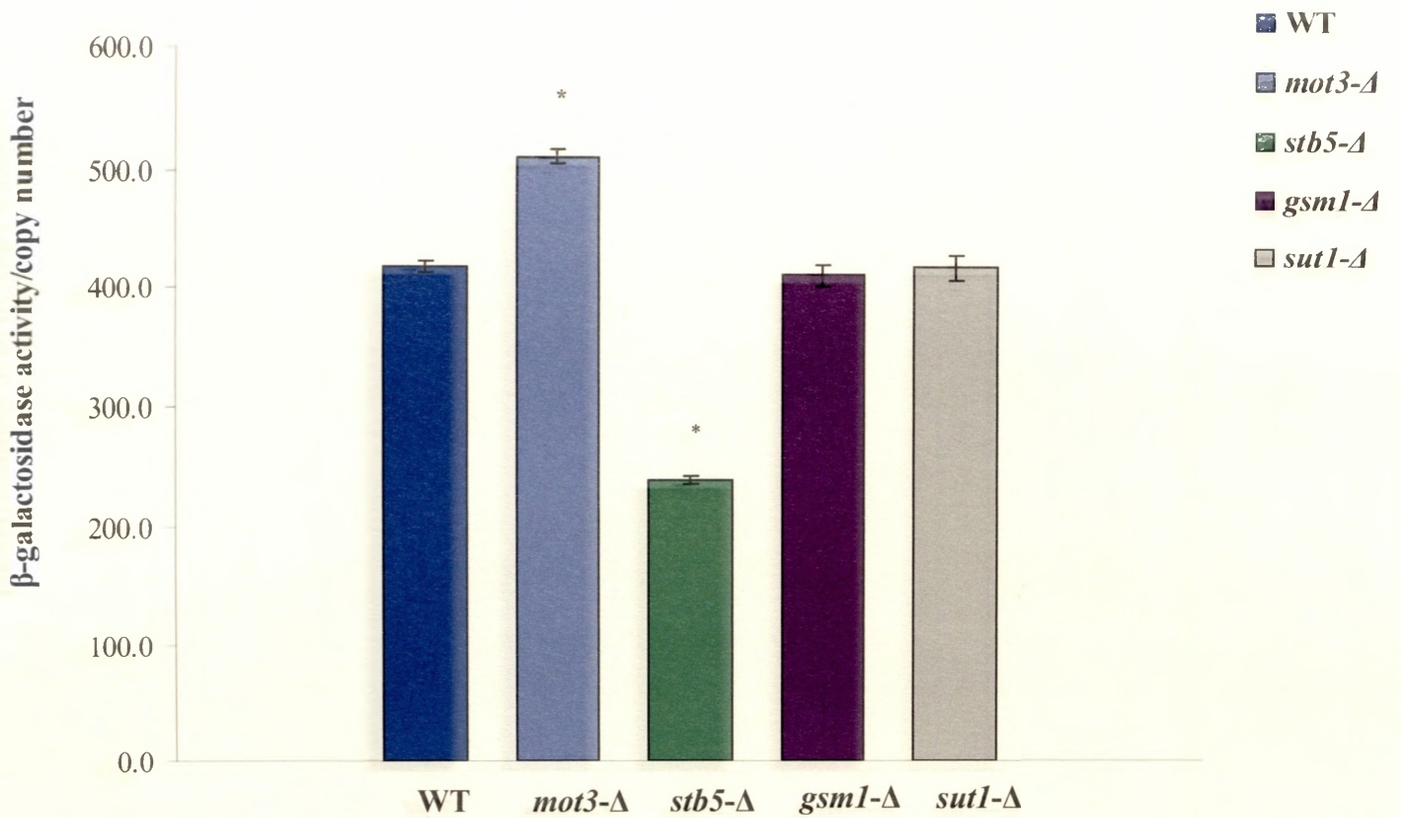


Figure 17. Average β-galactosidase Activity/Copy Number in Potential Promoter Binding Mutants (Trial 1).
The wild-type and the deletion candidates were grown in SC-Leu-raffinose media. Multiple extracts of each strain was assayed for β-galactosidase activity/copy number. The results are plotted as Average β-galactosidase activity/copy number ± SEM. An asterisks indicates the values that are significantly different from the wild-type control ($p<0.05$) as determined by Independent Student's *t* test (two tailed) assuming unequal variances.

Stb5p has a single binding site (-207-203) within the critical region (Table 13; Figure 16) and is known to respond to oxidative and xenobiotic stresses by activating genes of pentose phosphate pathway leading to increased production of NADPH. It was recently found to activate *SNQ2* gene leading to the synthesis of an ABC transporter, conferring pleotropic drug resistance (Larochelle et al., 2006). In contrary, *mot3*-Δ strain had a significant increase in β-galactosidase enzymatic activity/copy number (511.5 units), corresponding to 122.3% of wild type [$t(25.2) = -12.4$, $p<0.05$)]. This suggests that *MOT3* acts as a repressor of Pol II rRNA expression (Table 15; Figure 17). Previous studies have shown that Mot3p protein can serve as a repressor as well as an activator of genes, depending upon the indispensability of the genes under anaerobic condition. *MOT3* along with *ROX1* I cause repression of anaerobic genes (*DAN1/ANB1)* in the presence of oxygen. It is also known to play an important role in modeling cell wall in response to different oxygen levels (Sertil et al., 2007). However, the average β-galactosidase enzymatic activity/copy number was almost similar to wild-type levels (411.3 and 417.5 units) in *gsm1*-Δ and *sut1*- Δ mutant strain, suggesting that *GSM1* and *SUT1* are not involved in Pol II rDNA expression (Table 15; Figure 17).

To confirm the role of *STB5*, *MOT3*, *SUT1* and *GSM1* in Pol II rRNA synthesis their corresponding disruption strains were assayed again in a second trial (Tables 16-18). In addition, transcription factor disruption strains: *adr1*-Δ, *ash1*-Δ, *hap2*-Δ, *hap4*-Δ, *swi5*-Δ and *azf1*-Δ were also tested. In this trial the *mot3*-Δ had higher average β-galactosidase enzymatic activity/copy number than wild type (548 units) and was found to be statistically significant by Independent Student *t*-test [$t(43) = 6.62$, $p<0.05$)] (Table 18).

The *stb5*-Δ mutant showed lower activity than wild type (67%). In this trial, the activity of *stb5*-Δ mutant was higher (67%) than in the previous experiment (57.3%) and was found to be statistically significant ($t(42.5) = 23.7$, $p<0.05$). Also *sut1*-Δ and *gsm1*-Δ mutants had higher activity than the previous experiment. The average β-galctosidase activity/copy number of *sut1*-Δ strain was 195.5% of wild type (981 units) and that of *gsm1*-Δ was 178.4 % of wild type (895.4 units). These activities were also found to be statistically significant at $P<0.05$. The *t*-tests for *sut1*-Δ and *gsm1*-Δ deletion strains were $t(21.0) = 29.5$ and $t(22.3) = 28.47$ respectively at $p<0.05$ by Student's Independent *t* test. These inconsistencies will need to be addressed to determine the role of Gsm1p and Sut1p in Pol II rDNA expression. The *hap2*-Δ, *hap4*-Δ and *swi5*-Δ mutants had drastically high activities than the wild type. Hap2p and Hap4p are members of HAP transcription factor complex that regulate expression of respiratory genes. The average β-galctosidase activity/copy numbers of *hap2*-Δ and *hap4*-Δ strains were 375 and 285 % of wild-type suggesting that these transcription factors repress Pol II rRNA synthesis in ρ^+ strains under these conditions (Table 18). The *t*-tests for *hap2*-Δ and *hap4*-Δ strains were $t(18.9) = 49$ and $t(18.8) = 30.5$ respectively at $p<0.05$. The average β-galctosidase activity/copy number of *SWI5* deletion strain was 1241 units corresponding to 247.3% of wild type (Table 18; Figure 18). The *t*-test for *swi5*-Δ strain was [$t(16.4) = 18.5$) at $p<0.05$. In contrast, *azf1*-Δ showed considerably lower activity of (162.3) units than the wild type (501.7), corresponding to (32.3%) [$t(54.8) = 60.77$, $p<0.05$). The other deletion strains, *adr1*-Δ and *ash1*-Δ had almost 80% of wild- type activity [$t(37.9) = 14.5$ and $t(27.7) = 11.7$] respectively at $p<0.05$ (Table 18; Figure 18).

Table 16

Average β-Galactosidase Activity/Copy Number in Promoter Binding Mutants (Trial 2; Cell Extract I)

Strain	Cell Extract 1											Average ± SEM
By4743 ρ^+	544.8	495.5	493.4	507.0	538.5	557.4	517.5	479.7	562.7	532.2	546.9	525.1
By4743ρ^+	494.5	491.5	505.5	484.0	515.5	519.5	534.5	474.5	489.5	504.5	506.5	501.8±5.4
Δ *mot3*ρ^+	525.4	539.1	540.6	529.0	575.4	575.4	563.8	510.1	526.1	571.0	594.2	550.0±8.1
Δ *stb5*ρ^+	340.1	332.6	338.7	361.0	371.6	379.4	378.3	303.0	324.8	349.3	343.2	347.4±8.1
Δ *gsm1*ρ^+	869.5	864.8	841.2	952.2	935.7	980.6	975.8	886.0	801.0	931.0	895.5	903.0±17.2
Δ *sut1*ρ^+	882.7	949.2	914.4	1040.9	955.5	1066.2	1063.1	996.6	809.9	1015.6	984.0	970.7±23.9
Δ *adr1*ρ^+	369.0	372.4	357.6	412.6	405.2	426.0	413.9	359.0	332.9	390.5	376.4	383.2±8.6
Δ *ash1*ρ^+	399.8	395.7	390.6	431.2	410.9	465.7	453.5	379.4	348.0	418.0	398.8	408.3±10.0
Δ *swi5*ρ^+	1268.3	1360.3	1353.8	1399.8	1235.5	1531.2	1485.2	1235.3	1215.8	1353.8	1314.3	1341.2±30
Δ *hap2*ρ^+	1826.0	1835.0	1852.8	2022.5	2018.0	2116.2	2080.5	1830.4	1678.7	1960.0	1964.4	1925.9±40
Δ *hap4*ρ^+	1241.8	1164.4	1170.1	1494.2	1560.2	1543.0	1583.1	1397.7	1282.0	1528.6	1508.6	1406.7±49
Δ *azf1*ρ^+	157.5	144.8	145.4	163.8	171.6	167.0	168.3	174.6	165.6	156.0	143.6	159.8±3.3

Table 17

Average β-Galactosidase Activity/Copy Number in Promoter Binding Mutants (Trial 2; Cell Extract 2)

Strain	Cell Extract 2								Average ± SEM
By4743 ρ^+	512.4	525.7	450.4	467.1	484.8	487.8	487.8	501.5	489.7
By4743ρ^+	477.5	458.5	468.5	472.4	494.0	515.6	458.6	507.8	481.6±5.6
Δ *mot3*ρ^+	565.9	571.3	529.2	541.4	528.1	553.5	531.4	535.8	544.6± 6.0
Δ *stb5*ρ^+	316.2	318.4	309.5	310.5	317.2	322.6	331.0	345.3	321.3±4.1
Δ *gsm1*ρ^+	959.0	915.5	869.8	876.2	831.1	782.8	929.4	898.8	882.8±19.9
Δ *sut1*ρ^+	1062.7	1007.5	938.9	921.3	1029.0	1007.0	1033.4	960.9	995.1±17.5
Δ *adr1*ρ^+	421.9	405.6	392.1	375.2	410.7	396.0	427.6	408.5	404.7± 5.9
Δ *ash1*ρ^+			365.9	351.5	390.4	389.0	393.3	386.6	379± 6.8
Δ *swi5*ρ^+			1031.3	973.3	1099.6	1082.6	1089.4	1068.9	1057.5±19.4
Δ *hap2*ρ^+	1901.5	1860.7	1780.7	1687.5	1886.2	1752.6	1884.4	1833.4	1823.4± 26.9
Δ *hap4*ρ^+	1527.7	1583.5	1440.7	1443.9	1411.3	1383.0	1481.7	1461.7	1466.7± 22.7
Δ *azf1*ρ^+	175.5	185.1	164.5	123.9	167.4	175.2	167.9	167.6	165.9± 6.4

Table 18

Average β-Galactosidase Activity/Copy Number in Promoter Binding Mutants (Trial 2; Cell Extracts 1&2)

	Average		
Strain	β-Gal/ copy number	SEM	%WT
BY4743ρ^+	501.7	4.51	100
mot3ρ^+	548.0	5.24	109.2
stb5ρ^+	336.0	5.31	66.97
gsm1ρ^+	895.0	13.1	178.4
sut1ρ^+	981.0	15.5	195.5
adr1ρ^+	392.0	6.04	78.13
ash1ρ^+	394.0	7.6	79.32
swi5ρ^+	1241.0	39.7	247.3
hap2ρ^+	1883.0	27.7	375.3
hap4ρ^+	1432.0	30.08	285.4
azf1ρ^+	162.3	3.3	32.35

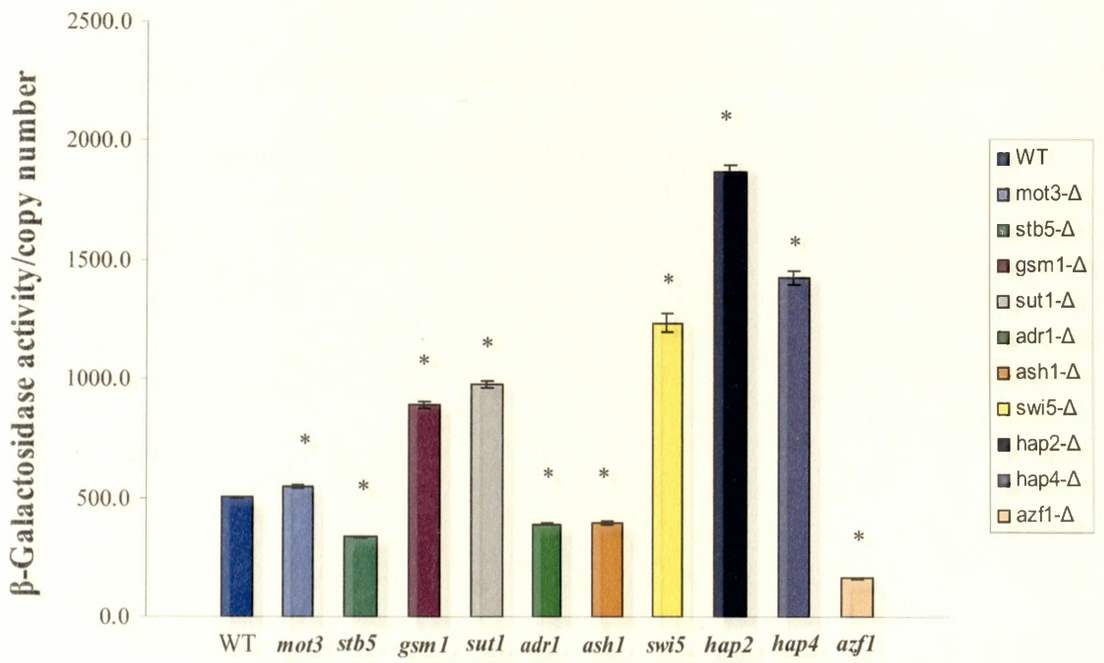


Figure 18. Role of Various Transcription Factors Binding within the rDNA Promoter in rRNA Synthesis.

The wild-type and the deletion candidates were grown in SC-Leu-raffinose media. Multiple extracts of each strain was assayed for β-galactosidase activity/copy number. The results are plotted as average β-galactosidase activity/copy number ± SEM. An asterisk indicates the values that are significantly different from the wild-type control ($p<0.05$) as determined by Independent Student's *t* test (two tailed) assuming unequal variances.

CHAPTER IV

DISCUSSION

To identify regulatory factors influencing Pol II rRNA synthesis in *S. cerevisiae*, the homozygous Yeast Knock Out (YKO) library was screened for non-essential factors that caused reduced Pol II rDNA promoter function. This was achieved by co-transforming deletion strains with two reporter plasmids: pFES17and prDNAURA3 and selecting transformants with reduced Pol II driven expression of the *lacZ* and *URA3* gene. After successfully transforming approximately 2661 of the 4757 strains in the collection, five candidate genes were identified: *FKH2*, *OCA4*, *PHO3*, *PBS2* and *RTG2*.

As expected, these candidates ranged in cellular functions from transcription factors to members of signaling pathways. Fkh2p is a transcription factor belonging to Forkhead Family (Reynolds, 2003). Fkh2p regulates transcription of cell cycle genes such as *CLB2* and *SWI5* (Koranda et al., 2000). The products of these genes are required for cells to progress from G2 to M phase (Koranda et al., 2000). Oca4p and Pho3p are putative phosphatases. Oca4p interacts with Oca1p, Oca3p and Oca5p in the Complex 490 (Gavin et al., 2006), while Pho3p is a constitutively expressed acid phosphatase that is involved in uptake of thiamin and phosphate metabolism (Nosaka, 1990).

Two of the candidates were members of major signaling pathways in *S. cerevisiae*. Pbs2p, a MAPKK, is a member of High Osmolarity Glycerol Signaling pathway which is activated under high osmotic conditions. A second candidate, Rtg2p, is a member of the

retrograde pathway. The retrograde pathway transmits signals from mitochondria to nucleus in response to mitochondrial dysfunction and is also activated by low nitrogen levels (Liu and Butow, 2006). These candidates suggest that at least two stress pathways, HOG and Retrograde, activate Pol II rRNA synthesis. It is possible that other candidate genes remain to be identified.

The YKO collection bears deletions of most of the non-essential genes of *S. cerevisiae;* however, some genes are not included for various reasons. For example, the homozygous diploid mutant of *RPD3* is not included in the collection, because it is defective in sporulation and recombination (www.yeastgenome.org). In contrast, the disruption mutant of *YLR162W* was difficult to create because it lies within a rDNA duplication (Shcherbakova, 2001). In addition, approximately 500 YKO strains were difficult to transform. This could be due to either insufficient aeration in a plastic deep well dish or due to non-uniform mixing of cells with transformation mixture during multiwell transformation. Even culturing individually, some strains were resistant to transformation. This resistance to transformation could be due to a slow growth defect caused by the specific deletion. Since only candidate strains with strong Ura^- phenotype and greatly reduced β-galactosidase activity were chosen, some potential candidates may have been excluded. Finally, strains bearing deletions resulting in increased activity of *lacZ* or *URA3* genes were expected, but they were not observed. Such strains would have to have a dramatic phenotype such as dark blue colonies on X-Gal plate and increased growth in Ura^- media to be noticeable.

Activation of HOG Pathway

The HOG pathway responds to increased extracellular salt concentration (0.5-1.5M) using two independent osmosensors Sko1p and Sln1p (Van Wuytswinkel, 2000) (Figure 19). Upon activation each osmosensor triggers its respective branch of the HOG pathway converges at a common MAPK, Pbs2p. Pbs2p phosphorylates Hog1p and promotes its translocation to nucleus. In nucleus, Hog1p activates its downstream targets including nuclear transcription factors. These factors recruit RNA Polymerase II to promoters of osmoresponsive genes (Saito and Tatebayashi, 2004).

At NaCl levels above 0.3M NaCl, the Sho1 branch is activated (Maeda et al., 1995; O'Rourke and Herskowitz, 2004). The signal from Sho1p is transduced to MAPKK, Ste11, by Ste20p and Ste50p. Ste11p then activates a common MAPKK, Pbs2p and Pbs2p activates Hog1p, a MAPK, by phosphorylation. In response to moderate (0.0625-0.125M NaCl) and extreme (1.5M NaCl) osmolarity, Sln1p serves as an osmosensor and activates Sln1p branch of HOG pathway. However, under iso-osmotic condition Sln1 acts as a negative regulator of HOG pathway by phosphorylating Ssk1 and rendering it inactive (Maeda et al., 1995; O'Rourke and Herskowitz, 2004). Thus, both branches can independently activate Hog1p under varied salt concentrations to ensure protection against osmostress. Sln1 is activated under greater range of conditions (moderate to extreme salt concentrations), whereas Sho1 branch is activated under moderate and high salt condition only.

Hog1 targets orchestrate the cell's response to osmotic stress. These targets include several transcription factors such as Sko1p, Hot1p, Msn 2p, Msn4p, Smp1p and Rck2p

that trigger osmotic response. In addition, Hog1p arrests the cells cycle by regulating cyclin levels (Escote et al., 2004). Activation of Sko1p activates expression of osmoresponsive genes such as *MSN2* and *PTP3* (Proft, 2005) (Figure 19). *MSN2* encodes for a general stress response protein, while *PTP3* encodes for a protein tyrosine phosphatase that down-regulates Hog1p after adaptation (Mattison and Ota, 2000). Hot1p activates expression of genes involved in glycerol synthesis (*GPP1* and *GPD2*) that serve to increase internal osmotic pressure (Rep et al., 1999). Smp1p serves as an activator for *STL1,* a glycerol/H^+ symporter located in plasma membrane as well as *CWP1*, a cell wall mannoprotein (De Nadal et al., 2003). In addition, Hog1p is recruited by specific transcription factors such as Hot1p and Msn1p to promoters of osmoresponsive genes. Hog1p binding facilitates recruitment of Rpd3p, a histone deacetylase, leading to recruitment of Pol II and induction of osmoresponsive genes (De Nadal et al., 2004).

The Role of HOG Pathway in Pol II rRNA Synthesis

Pol II rRNA synthesis is triggered due to the activation of HOG pathway as the *pbs2-*Δ mutant had significant reduction in average β- galactosidase activity per copy (68.7% and 79.2% of wild type) (Table 5 and 6). This suggests that *Pbs2,* a MAPKK of HOG pathway is involved in regulating Pol II rRNA synthesis. To verify the involvement HOG pathway, a disruption of the downstream target of Pbs2p, *hog1*Δ mutant was examined for its effects on Pol II rRNA synthesis.

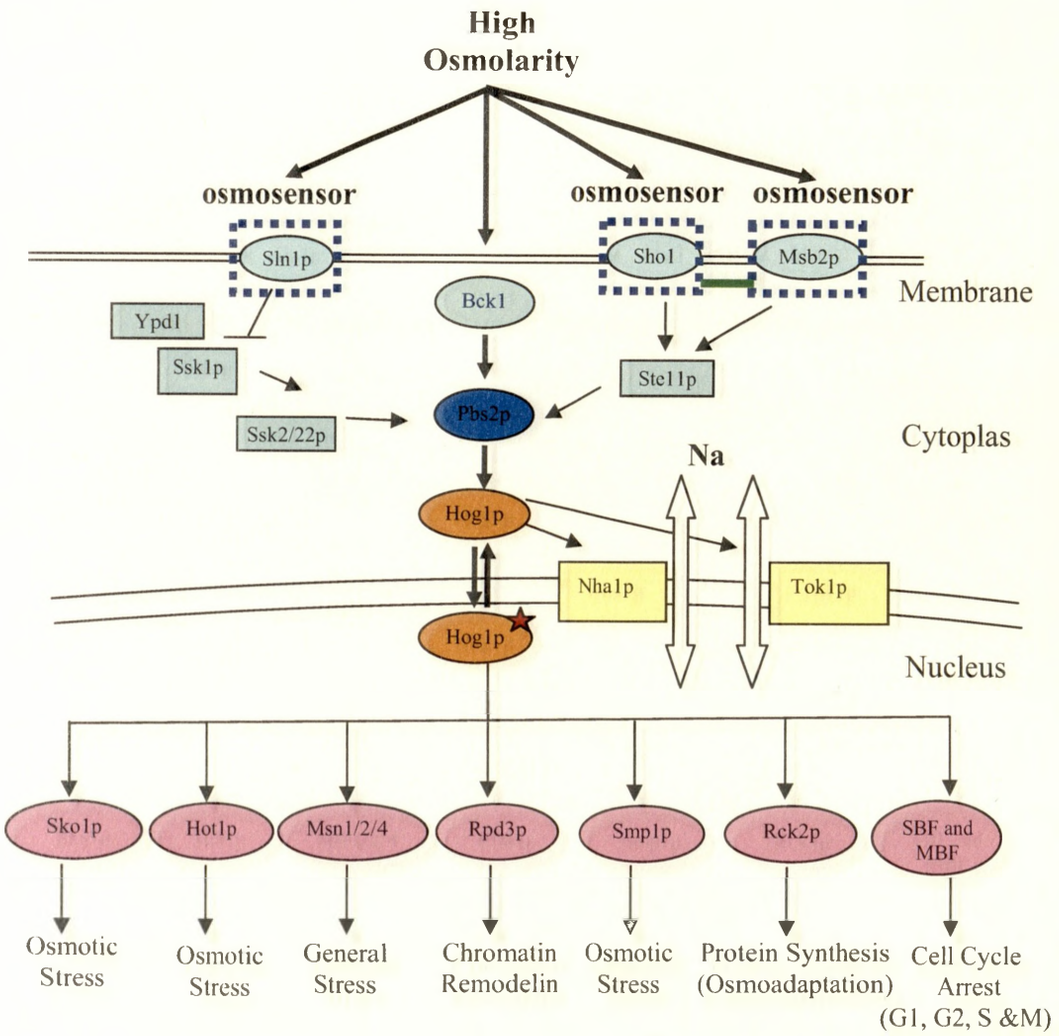


Figure 19. Targets of Hog1p Activated Upon Osmotic Stress.
Three membrane bound osmosensors (Sho1p, Sln1p, Msb2p) sense the differences in osmolarity between the interior and exterior and transduce the signal through the components of MAPK cascade. The sensors are activated by high salt concentration and they independently activate the common MAPKK, Pbs2p (blue oval). Activation of Pbs2p leads to phosphorylation of Hog1p (orange oval with red star) and its translocation to nucleus leading to activation of downstream targets. Hog1p phosphorylates the Na^{+}-K^{+} antiporter (Nha1) and potassium channel (Tok1) leading to efflux of Na^{+}. It is then translocated to nucleus where it activates various nuclear targets: Sko1p, Hot1p, Msn2/4p, Rpd3p, Smp1p, Rck2p and cell cycle regulators. The efflux of Na^{+} allows transcription factors or activators to associate with DNA. These activators then enhance Pol II transcription of osmoresponsive genes Modified from (De Nadal et al., 2002; Westfall et al., 2004).

As expected, the *hog1*-Δ mutant had 19% of wild-type activity suggesting that Hog1p plays a role in Pol II rRNA synthesis (Table 10). Thus, both *PBS2* and *HOG1*, the members of HOG pathway play a role in regulating Pol II rRNA synthesis. Strengthening this assertion, it has shown that high salt conditions (1.0 and 1.5 M NaCl) activate Pol II rDNA transcription in *161U7*ρ^+ haploid strain (Varughese and Conrad-Webb, 2008). The haploid strains were grown at increasing salt concentrations: 0 M, 0.3 M, 0.75 M, 1.0 M and 1.5 M and their β-galactosidase activity per copy number was compared with the same strain grown in the absence of salt. An increase in β-galactosidase activity per copy number was seen with increasing salt concentration. At 1.0 and 1.5 M salt concentrations, the β-galactosidase activity per copy number was two fold the activity of the strain with no salt (Varughese and Conrad-Webb, 2008). In addition, both haploid and diploid cells show induction on X-Gal indicator plates suggesting that high salt conditions (1.0 and 1.5 M) enhance Pol II rRNA synthesis through modest activation of HOG pathway.

The phosphorylated form of Hog1p has many downstream targets that could potentially activate Pol II rRNA synthesis (Figure 19). As some of these potential Hog1p targets (Hot1p, Msn2/4p and Sko1p) are known to interact with DNA directly, the rDNA Pol I-II promoter region was searched for their potential binding sites by using Yeast Search For Transcriptional Regulators And Consensus Tracking (YEASTRACT) (Monteiro, 2008) and *Sacharomyces* Genome Database (SGD) (Cherry, 1998). There were no binding sites for any of these downstream targets within the rDNA promoter.

However, within the rDNA promoter, there were four binding sites for Mot3p, a downstream target of Sko1p, at positions, -110, -447, -615 and -927 (Figure 16). Sko1p causes induction of *MOT3* gene under osmotic stress (Proft, 2005). Mot3p, a transcription factor inhibits induction of hypoxic gene *ANB1* and anaerobic genes *DAN/TIR*. However, the repressor action of Mot3p is antagonized by Rpd3p during anaerobic growth conditions leading to activation of anaerobic genes (Proft, 2005; Sertil et al., 2007). In addition, it is suggested that the Sko1 directed osmotic induction of Mot3p repressor might further repress hypoxic genes during osmotic stress thereby allowing cells to divert energy towards induction of osmoresponsive genes (Proft, 2005). Mot3p is also involved in the activation of *CWP2* gene encoding for an aerobic cell wall mannoprotein. Induction of *CWP2* might bring favorable osmoadaptive changes in cell wall composition (Abramova et al., 2001).

To examine the role of Mot3p in Pol II rDNA synthesis β- galactosidase activity per copy number was compared in wild-type and *mot3*-Δ deletion mutant. The *mot3*-Δ had 122.3% and 109.2% of wild-type activity suggesting that *MOT3* is not influencing Pol II rRNA synthesis under basal conditions (Figures 17 & 18).

However, further studies need to be done to confirm its role at Pol II rDNA promoter. It would be prudent to compare the activity of wild-type and *mot3*-Δ in the presence and absence of osmotic shock.

Basal Expression of Pol II rDNA Genes by Hog1p

Since Hog1p is necessary for both basal and modest osmotic induction in Pol II rRNA synthesis, the mode of activation of Pol II driven rRNA transcription must account for

expression in the absence and presence of osmotic stress. Hog1p is found to circulate between the cytosol and nucleus independent of its phosphorylation state in the absence of osmotic stress (Reiser, 1999) (Figure 20). This low level of nuclear Hog1p could account for the basal level of *lacZ* reporter gene expression as seen in the wild-type haploid and diploid cells without salt (Sagar and Conrad-Webb, 2007; Varughese and Conrad-Webb, 2008).

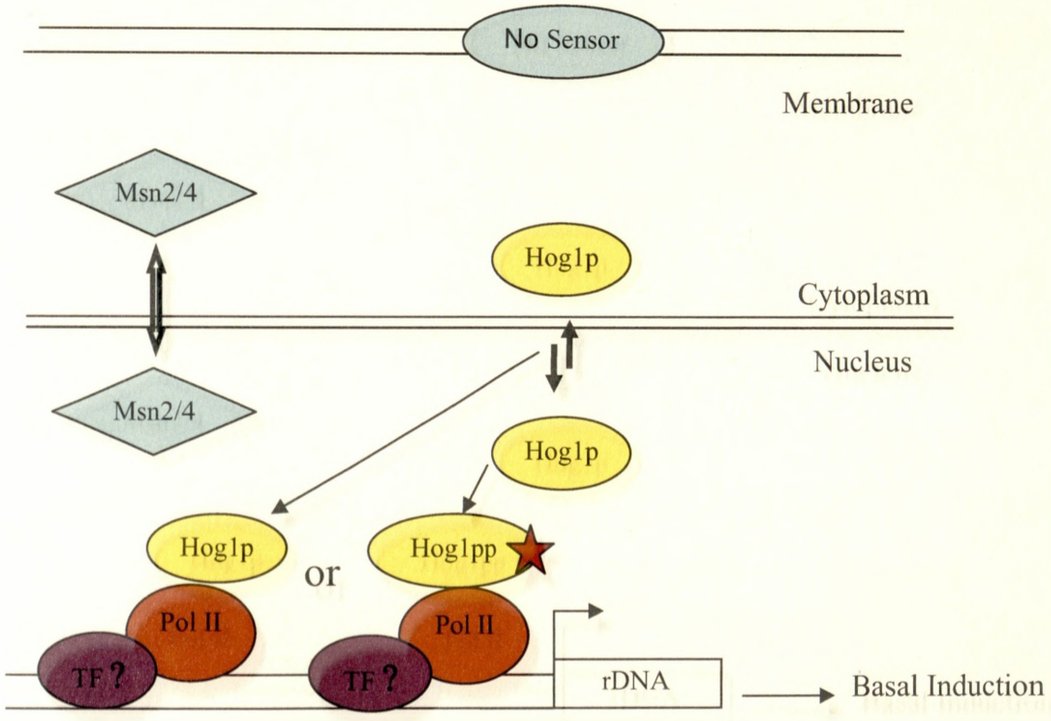


Figure 20. Basal Expression of rDNA Genes Under Non Stress Condition.
Basal activation of rDNA genes could occur in the absence of salt by the Hog1p that circulates between cytoplasm and nucleus. An un-identified nuclear transcription factor (purple oval) may recruit Hog1p (yellow oval) or Hog1pp (yellow oval with star) to DNA. Msn2p (blue diamond) serves as a general transcription factor that also circulates between nucleus and cytoplasm (Modified from Reiser, 1999).

Alternatively, Hog1p may be phosphorylated in response to other signals such as heat, cold, citric acid and arsenic (Lawrence, 2004; Panadero, 2006; Sotelo, 2006; Winkler et al., 2002) (Figure 21). Heat stress activates Hog1p via Sln1 branch of the HOG pathway leading to induction of cold responsive genes (Panadero, 2006).

The members of HOG pathway: Ssk1p, Pbs2p and Hog1p are also involved in conferring resistance to arsenic, implicating activation of HOG pathway via Ssk1 branch in response to metal stress (Sotelo, 2006). These alternative inducers may also result in basal expression of rDNA genes by promoting binding of an unknown transcription factor. Induction of rDNA genes could also occur due to hyperactivation of Hog1p in the absence of salt caused by cross-talk between Cell Wall Integrity and HOG Pathway. This cross-talk is favored by inactivation of protein tyrosine phosphatases (Ptp2p and Ptp3p) that anchor Hog1p and Slt2p in nucleus and cytosol, respectively (Martin, 2005). Inactivation of these protein phosphatases, leads to the activation of Hog1p by Mkk1/2p (MAPKK) of cell wall integrity pathway. Thus, Hog1p can be activated by triggers of cell wall integrity pathway in the absence of protein tyrosine phosphatases or their inactivation, resulting in the induction of osmo-responsive genes (Figure 22).

Regardless of the mode of Hog1p activation, both Hog1p and Rpd3p are required for maximal Pol II rRNA synthesis under non-inducing conditions (Robinson et al., 2008; Sagar and Conrad-Webb, 2007). Amy Robinson (2008) has shown that Rpd3p is required for maximal Pol II rDNA reporter gene expression in the absence of osmotic stress. The *rpd3*-Δ haploid mutant was found to have 7% and 9% of wild-type β-galactosidase activity per copy number.

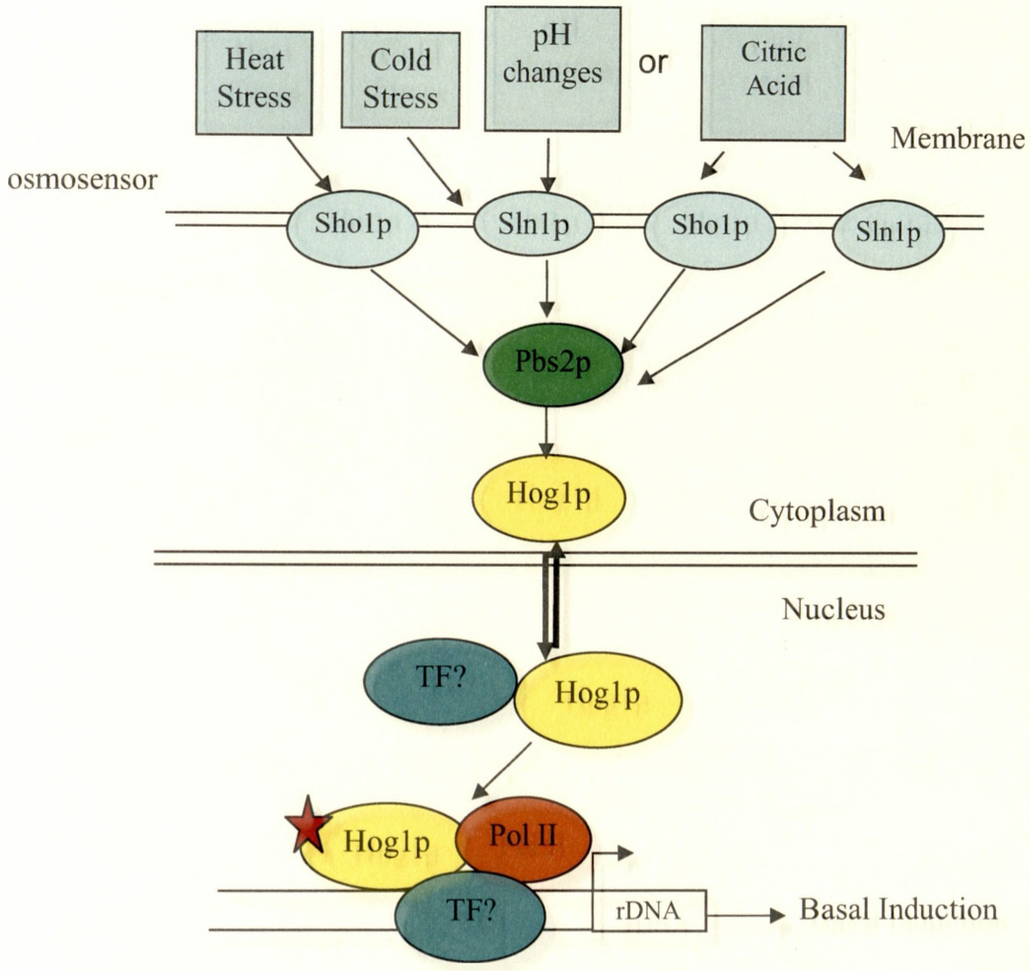


Figure 21. Basal Expression of rDNA Genes Under Stress Conditions Other Than Salt.

Diverse Stresses such as heat, cold, pH changes and citric acid can activate different osmosensors (Sho1p or Sln1p) leading to the activation of MAPKK, Pbs2p. Pbs2p activates Hog1p. The activated Hog1p (yellow oval with star) might be recruited to rDNA promoter by an unknown transcription factor (teal oval) that binds to the rDNA. The binding of TF-Hog1p recruits Pol II to rDNA promoter accounting for basal expression of rDNA genes.

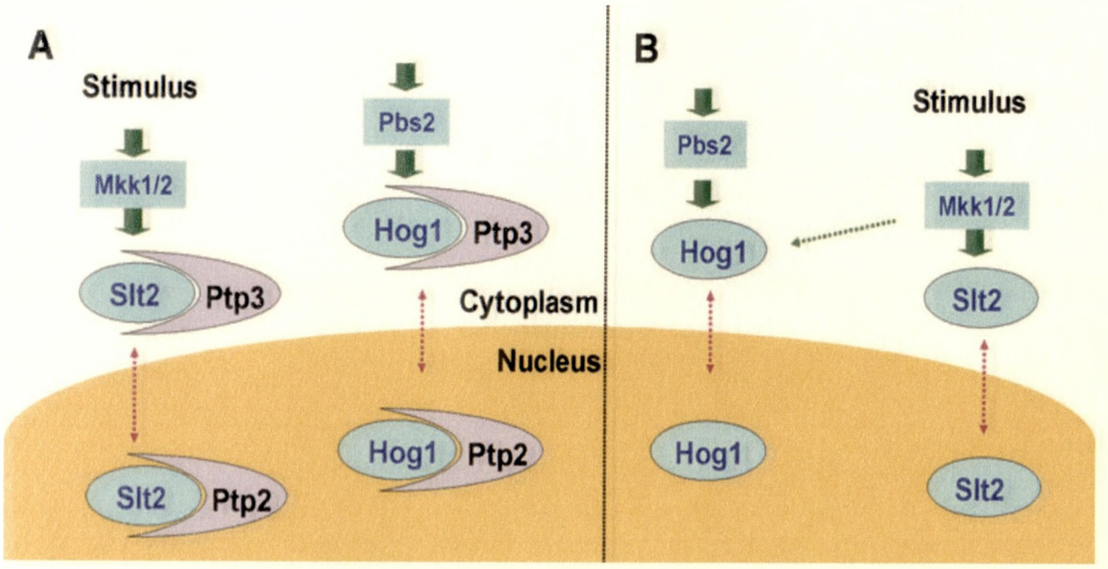


Figure 22. Basal Induction of rDNA Genes Due to a Cross Talk between Cell Wall Integrity Pathway and HOG Pathway.
A. Under normal conditions, a cross-talk between cell wall integrity and HOG pathway is prevented by sequestration of Slt2p, a MAPK of cell wall integrity and Hog1p by protein tyrosine phosphatases (Ptp3) in cytosol and (Ptp2) in nucleus. B. Inactivation of protein phosphatases can lead to activation of rDNA genes by the triggers of cell wall integrity pathway such as Mkk1/2p (Adapted from Martin et al., 2005).

Hog1p may recruit Rpd3p to the rDNA promoter after binding to an unknown transcription factor (TF) or an activator, since Hog1p is known to recruit Rpd3p during osmotic stress (Figure 23) (De Nadal et al., 2004). This recruitment of Hog1p- Rpd3p-TF can promote assembly of Pol II initiation complex along with the Mediator leading to Pol II rDNA expression. One potential target sequence in the critical region of the Pol II rDNA promoter is the M3a motif (Kurdistani et al., 2002).

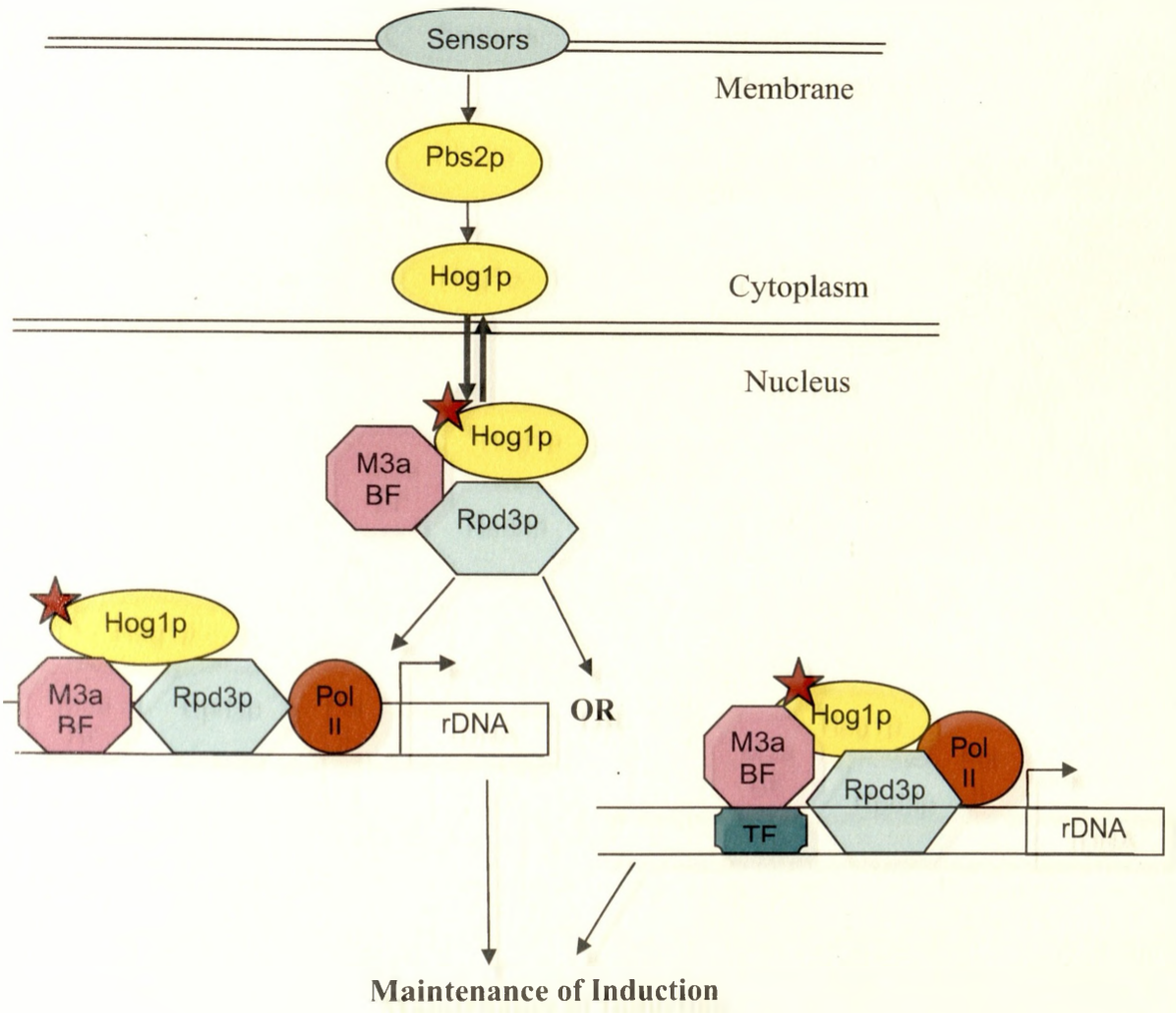


Figure 23. Induced Expression of rDNA Genes (Long Term Osmotic Stress).
Hog1p is phosphorylated upon osmotic stress. The phosphorylated form of Hog1p (yellow oval with star) binds to Rpd3p and M3aBF. The complex (M3aBF-Hog1pp-Rpd3p) binds to rDNA promoter as a single M3a motif sequence is present within the rDNA promoter region. Alternatively, M3aBF may interact with another activator or TF as shown as (Teal box) and then recruit Rpd3p-Hog1p complex to rDNA promoter. The assembly of either M3aBF-Hog1p-Rpd3p or M3aBF-TF-Hog1p-Rpd3p complex can then recruit Pol II and basal transcriptional machinery.

This binding site is also found in Rpd3p target genes usually occurring in the promoter of genes involved in RNA metabolism and global protein synthesis (Tavezoie, 1999). The rDNA promoter has a single M3a binding site (TGAAAAGTTT) for an unknown factor within the critical region (-344-354). M3a may regulate Pol II rDNA expression by itself or interact with another binding partner and recruit Rpd3p to the promoter too (Kurdistani et al., 2002). In addition, M3a binding factor could serve as an activator for recruitment of other transcription factors such as: Sut1, Hap1, Gsm1, Gcr1, Mal63 or Reb1p that may bind within the critical region.

In summary, HOG pathway activates basal as well as induced expression of Pol II rDNA transcription. In the absence of salt, Hog1p may be activated by alternative stresses, cross talk with the cell wall integrity pathway or inactivation of regulating phosphatases. O'Rourke and Herskowitz (2004) have shown that a cluster of 5 genes depend on Hog1p for their basal or modest expression. One of the genes (*YKL161c*) encodes for a MAPK, another gene *RHR2* encodes for a glycerol-3-phosphatase which is involved in production of glycerol and three genes *CWP1*, *SED1* and *PIR3* are involved in the synthesis of cell wall proteins. Pol II rRNA synthesis may fall into this category of genes that are transcribed basally without salt but activated by the classic HOG activation pathway during osmotic stress.

PHO3 As a Potential Regulator of Pol II rDNA Transcription

PHO3 encodes for a constitutively expressed acid phosphatase of 57 Kd (Rogers et al., 1982). It is a soluble thiamin-binding protein that mediates hydrolysis of thiamin phosphate to thiamin in the periplasmic space and promotes its cellular uptake. The

absence of Pho3p results in reduced levels of cellular thiamin. Although always enzymatically active, the levels of Pho3p vary under diverse conditions. Pho3p is present at high levels in cells grown in the presence of inorganic phosphate while Pho3p levels are reduced in the presence of low levels of inorganic phosphate (Tait-Kamradt et al., 1986). The absence of Pho3p alters the cellular levels of phosphates and may indirectly activate Pol II rRNA synthesis in response to phosphate levels.

Role of Components of OCA Complex in Pol II rRNA Synthesis

OCA4 is required for cell cycle arrest in response to oxidative damage of DNA. In addition, Oca4p was identified to be a part of complex 490 in a genome wide study involving Tandem Affinity Purification (TAP) of protein complexes followed by mass spectrometry (Gavin et al., 2006). Oca4p interacts with Oca5p, Oca1p and Oca3p/Siw14p within the complex 490 (Gavin et al., 2006). Both Oca1p and Oca3p share sequence homology with Y-STYX phosphatase, suggesting that all three might have a common role in yeast (Wishart, 1998). To confirm the role of complex 490 in Pol II rRNA synthesis the β-galactosidase activity per copy number in the disruption strains of *OCA1, OCA3* and *OCA4* were compared with the wild-type. In all three deletion strains, the activity was significantly lower than the wild-type confirming the hypothesis that all three proteins (Oca1p, Oca3p and Oca4p) regulate rDNA transcription as a complex. The Oca complex's phosphatase like proteins have structural and functional similarity to dual specificity MAPKs phosphatases such as protein tyrosine phosphatases, Ptp2p and Ptp3p (Wishart, 1998). Thus, Oca proteins might be regulating the phosphatase activity of MAP kinases in response to different stress conditions.

Role of Cell Cycle Transcription Factors in Regulating Pol II rRNA Synthesis

In *Saccharomyces cerevisiae* (>10%) of genes (~800) are transcribed with cell cycle periodicity demonstrating that their transcription is strictly cell cycle regulated (Spellman and Futcher, 1998; Zhu, 2000). Fkh1p and Fkh2p, members of forkhead family of transcription factors regulate transcription of cell cycle genes governing progression from G2/M phase of cell cycle (Koranda et al., 2000). The forkhead family of transcription factors is conserved from drosophila to humans (Kaufmann and Knochel, 1996). Yeast Fkh1p and Fkh2p are 47% identical and 82% similar along the length of Fkh1p (Hollenhorst, 2001). In addition, both factors bind to an identical consensus sequence and display redundancy at specific promoters of *SIC1* gene cluster and *CLB2* cluster (Zhu, 2000).

Despite their structural similarities both Fkh1p and Fkh2p perform distinct functions (Hollenhorst, 2000). The functional difference is not attributed to differences in their DNA binding domains but regulated by their interacting partners (Hollenhorst, 2000). Fkh1p binds to Ndd1 and regulates expression of *SWI5, HAT1, SIR1* and *BUD3* genes (Hollenhorst, 2001). Fkh2p interacts with Mcm1p and Ndd1 (Kumar, 2000; Pic-Taylor et al., 2004) and regulates expression of the *SWI5*, *CLB2, ACE2* and *YJLO51w* genes (Simon, 2001; Zhu, 2000). Fkh1p and Fkh2p alter transcription of *SIC1* gene cluster by regulating expression of *SWI5* and *ACE2*-the controllers of the *SIC1* cluster whose products are required for cells to exit mitosis (Zhu, 2000).

During the G1 phase of cell cycle Fkh2p associates with an essential protein Mcm1p to form a repressor complex (Mcm1p-Fkh2p) at *CLB2* and *SW15* gene clusters (Pic et al.,

2000). Both *CLB2* and *SWI5* gene promoters have Mcm1p binding sites to which Fkh2p binds preferentially as a component of Swi Five Factor (SFF) (Jorgensen and Tyers, 2000). However, Fkh1p binds less efficiently than Fkh2p to *CLB2* and *SWI5* promoters in vivo due to a lack of cooperative association of Fkh1p with Mcm1p. Moreover, the predominance of Mcm1p binding sites within these promoters favors Fkh2p binding (Hollenhorst, 2001). In contrast, Fkh1p primarily binds to promoters devoid of Mcm1p binding sites such as *BUD3* in vivo (Hollenhorst, 2001). The SFF repressor complex remains inactive until S phase (Wittenberg and Reed, 2005) (Figure 24).

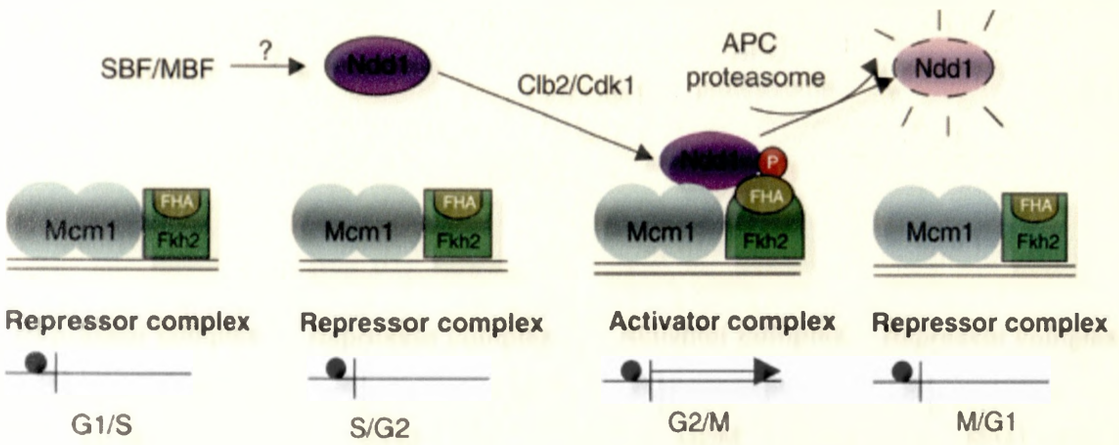


Figure 24. Regulation of Cell Cycle by Transcription Factors.
At G1/S boundary a Mcm1p-Fkh2p complex act as a repressor of *CLB2* cluster genes. During the S/G2 boundary, Ndd1p (purple oval) is synthesized by SBF/MBF complex. Ndd1p is phosphorylated by a Clb2p/Cdk1p complex during G2 phase. The activated (phosphorylated Ndd1p) phosphorylates Fork Head Associated (FHA) domain of Fkh2p. The phosphorylation brings about a conformational change in Fkh2p and converts it to an activator from a repressor. The activated ternary complex (Mcm1p-Fkh2p-Ndd1p) recruits Pol II basal machinery and activates transcription of cell cycle genes at G2/M boundary. In mitosis Ndd1p is degraded by APC proteasome following ubiquitination. Degradation of Ndd1p restores Mcm1p-Fkh2p complex to its repressive state (Wittenberg and Reed, 2005).

At the G1/S boundary of cell cycle, SCB binding factor, SBF (Swi4/ Swi6) and MCB binding factor, MBF (Mbp1/Swi6) facilitate the synthesis of Ndd1p, a coactivator of Fkh2p(Wittenberg and Reed, 2005) (Figure 24). During G2, Ndd1p, is phosphorylated in cell cycle dependent manner by Clb2/Cdk (Figure 23)(Darieva, 2003; Ubersax, 2003). The phosphorylated active form of Ndd1p binds to the Fork Head Associated domain of Fkh2p and converts Fkh2p to an activator (Jorgensen and Tyers, 2000; Simon, 2001) (Figure 24). The assembly of a ternary complex of Mcm1p-Fkh2p-Ndd1p to the promoters of cell cycle genes such as *SWI5* and *ACE2* promotes recruitment of Pol II machinery, leading to their activation in a cell cycle dependent manner (Jorgensen and Tyers, 2000). During mitosis Ndd1p is ubiquitylated and degraded by APC proteasome complex. Degradation of Ndd1p by the APC proteasome terminates transcription of *CLB2* gene cluster and restores the Fkh2p-Mcm1p complex to a repressive state (Loy, 1999) (Figure 24).

Role of Fkh2p: Transcriptional Activator of Pol II rRNA Synthesis

Disruption of *FKH2*, the gene for a cell cycle transcription factor, resulted in the most dramatic effect on Pol II rRNA synthesis reducing expression to 16.7% to 21.4% of wild-type (Table 7). Interesting, *fkh1*- Δ mutant phenotype was similar to the wild-type suggesting that a unique function of Fkh2p may be essential for triggering Pol II synthesis of rRNA. A search for Fkh2p potential binding sites in the rDNA promoter revealed three potential Fkh1/2p binding sites. Two of these sites are located upstream of

the critical region (-657 and -889) whereas, the third site is located further upstream (-1154).

It is possible that any of these sites are used depending on the cell cycle stage and the availability of a particular interacting factor. Simon *et al* (2001) have shown an association of Fkh1p and Fkh2p with genes expressed in G1 and S phase independent of Mcm1p. In addition, a single factor may bind to different consensus sequences at different promoters of cell cycle genes. For example there is variation in the Mcm1p consensus binding motif amongst its G2/M and M/G1 targets (Simon, 2001). There is a single Mcm1p binding site (-486) positioned upstream of the critical region (Figure 15). However since *MCM1* is an essential gene, it could not be tested.

The rDNA promoter also contains additional sites for three cell cycle transcription factors: Ash1p, Azf1p and Swi5p. There are two Ash1p binding sites (-662, -668) in close proximity to Fkh1/2p binding site (-657); however, disruption of *ASH1* modestly reduced Pol II rDNA promoter function (79.32%) of wild-type. Intriguingly, Ash1p is a component of large co-repressor complex of the hisone deacetylase, Rpd3L (Carrozza, 2005). Ash1p and Ume6p serve as integral DNA-binding gene-specific subunits of Rpd3L complex that can directly recruit the complex to specific promoters without the involvement of other co-activators or co- repressors (Carrozza, 2005).

A model can be proposed for Fkh2p mediated activation of rDNA genes during the G2 phase (Figure 25). During the G2 phase, Mcm1p-Fkh2p repressor complex bound to the rDNA promoter at one of its binding sites located at positions -657, -889 and -1154 within the rDNA Pol I-II promoter. The complex is activated by Ndd1p by

phosphorylation. The activated form of Fkh2p associates with Rpd3L and recruits Rpd3L complex to one of the Ash1p binding sites (-662 and -668) within the rDNA promoter to activate Pol II rDNA transcription. The activated Fkh2p-Rpd3L complex then recruits Pol II machinery to rDNA promoter to facilitate Pol II driven rDNA expression during the G2 phase (Figure 25).

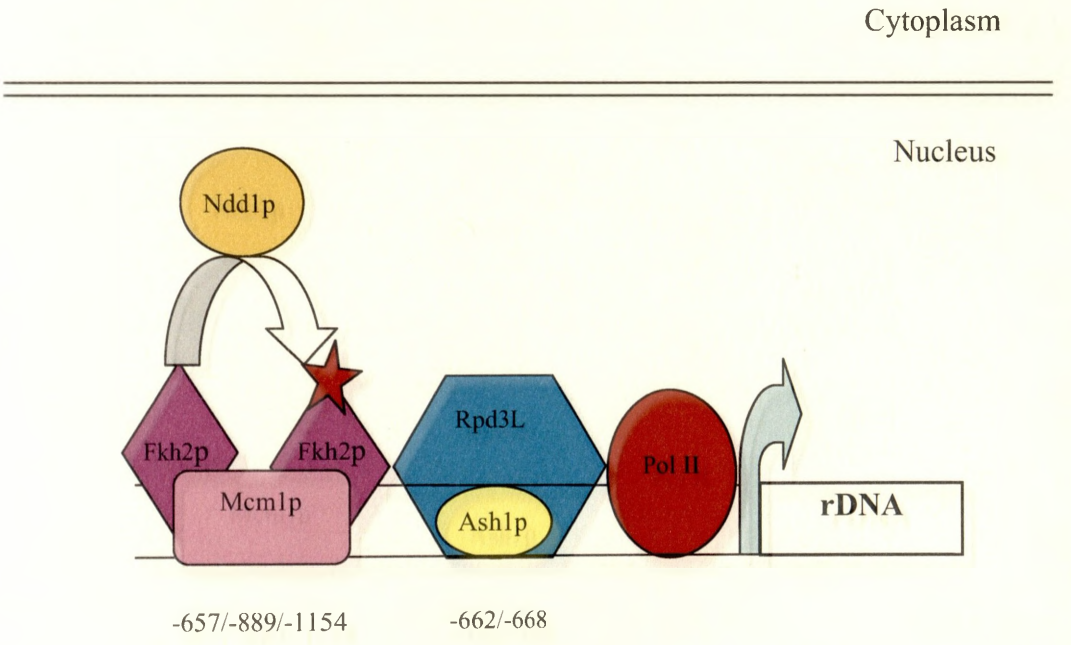


Figure 25. Role of Fkh2p in Pol II rDNA Expression.
During the G2 phase of cell cycle, Ndd1p (dirty yellow oval) activates Fkh2p bound as Fkh2p-Mcm1p complex to one of its three binding sites in rDNA promoter by phosphorylation (purple diamond with red star). Upon activation Fkh2p associates with Rpd3L and recruits it to one of the Ash1p binding sites (-662 and/or -668). The recruitment of the Fkh2p-Rpd3Lcomplex to the promoter leads to activation of rDNA genes during the G2 phase of cell cycle as Fkh2p is activated by Ndd1p during the G2 phase only.

Alternatively, Fkh2p might promote *SWI5* gene transcription during the G2 phase and enhance synthesis of Swi5p. However, data with *Swi5*-Δ strain was ambiguous and must be repeated. Thus, the precise role of Swi5p in Pol II rDNA transcription could not be determined from the study. In addition, Fkh2p might also activate another downstream factor such as Ash1p and promote its binding to Pol II rDNA promoter to trigger rDNA transcription. This is supported by the presence of Ash1p binding sites (-662 and -668) close to Fkh2p binding sites (-657, -889 and -1154) within the rDNA promoter.

Disruption of the *AZF1* gene, a cell cycle transcription factor, resulted in a dramatic reduction of Pol II rRNA synthesis (32.35 % of wild-type) (Table 18). Azf1p is a nuclear asparagine-rich zinc finger transcription factor that is involved in the induction of *CLN3* genes in response to glucose in vivo and in vitro (Newcomb, 2002). Azf1p mediates induction of *CLN3* gene by binding to A_2GA_5 sequence within the *CLN3* promoter (Newcomb, 2002). However, deletion of Azf1p reduces but does not abolish glucose based induction of the G1 cyclin *CLN3* thereby indicating that other proteins besides Azf1p are involved (Newcomb, 2002). The binding of Azf1p might enhance binding of a regulatory protein or an activator in response to signals from glucose. The rDNA promoter has a single binding site for Azf1p located downstream from the critical region (-93). Previous studies in lab have shown that the downstream sequences are also vital for Pol II driven rDNA transcription (Jodhka, 2004).

In summary, Pol II rDNA transcription could be cell cycle specific as cell cycle transcription factor Fkh2p has a significant influence on Pol II rDNA expression. However, further studies involving CHIP or Gel Retardation Assays are required to

establish its binding to the rDNA promoter. Moreover, Fluorescent Activated Cell Sorter (FACS) studies will be prudent to pinpoint the stage of the cell cycle where Fkh2p has its maximum effect on Pol II rDNA expression. A similar set of experiments will also confirm the role of other cell cycle specific transcription factors such as Azf1p in Pol II rDNA transcription.

Role of Retrograde Pathway in Pol II rRNA Synthesis

The Retrograde Pathway, a pathway of communication from mitochondria to the nucleus is universally conserved from yeast to humans (Liu and Butow, 2006). In *S. cerevisiae*, it serves as a sensor of mitochondrial dysfunction and is responsive to levels of glutamate/glutamine, the major precursor of cellular nitrogen and amino acid synthesis (Butow and Avadhani, 2004). Upon activation, the retrograde pathway enhances expression of retrograde responsive genes such as peroxisomal isoform of citrate synthase (*CIT2*) and D-lactate dehydrogenase (*DLD3*) thereby promoting synthesis of enzymes leading to increased glutamate/glutamine levels (Sekito et al., 2002; Small et al., 1995).

The retrograde pathway is activated by two known triggers, a loss of mitochondrial DNA as in ρ^0 cells or low levels of extracellular amino acids such as glutamate and glutamine. Low levels of glutamate and glutamine are sensed by SPS sensor and transduced to Rtg2p, causing the hypophosphorylated form of Mks1p to associate with Rtg2p (Liu and Butow, 2006) (Figure 26). The Rtg2p-Mks1p complex then promotes nuclear translocation of basic Helix Loop Helix (bHLH) transcription factors Rtg1p and Rtg3p (Sekito et al., 2002). The nuclear transcriptional factors, Rtg1p and Rtg3p bind to

the promoter of retrograde responsive genes such as *CIT2* and *DLD3* to activate transcription (Liu and Butow, 1999).

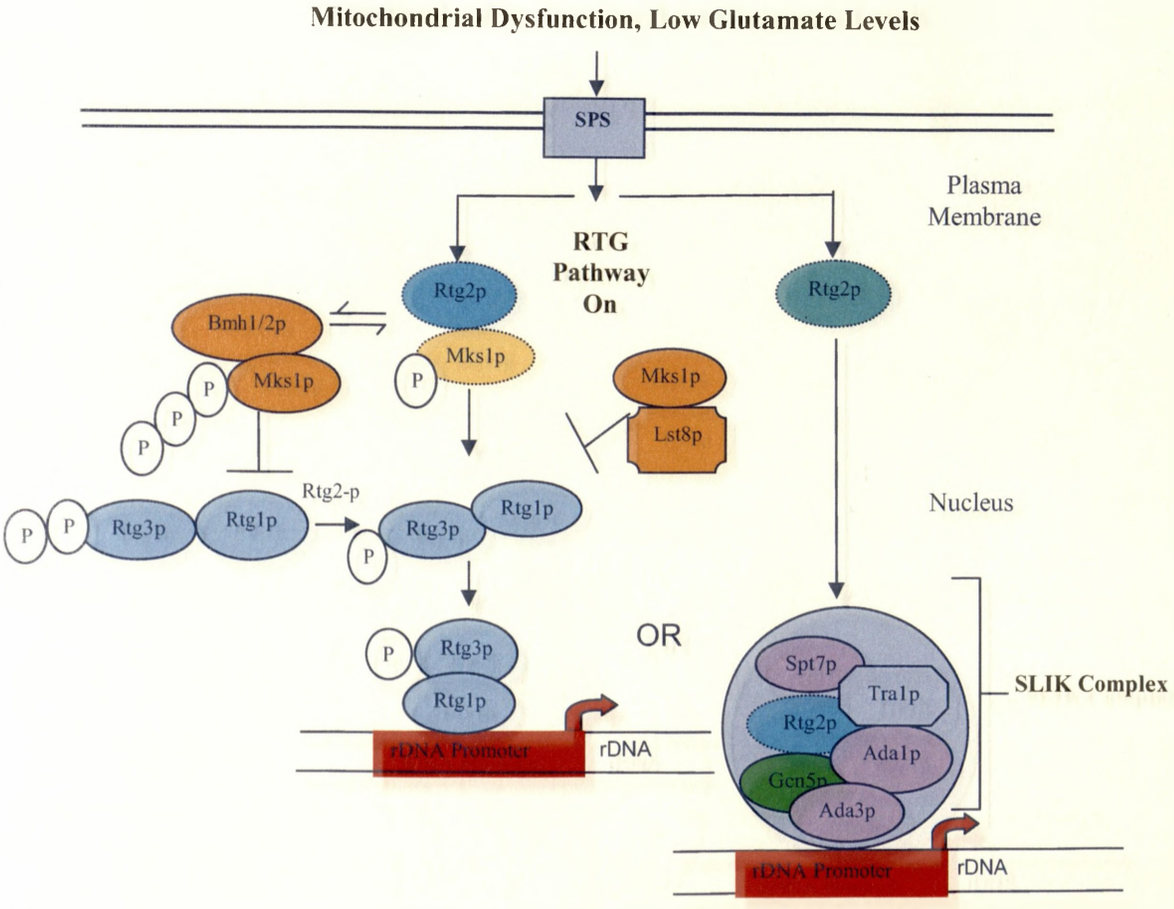


Figure 26. Activation of rDNA Genes by Rtg2p as a Part of SLIK Complex.
In response to low levels of glutamate or mitochondrial dysfunction, retrograde pathway is activated leading to activation of Rtg2p. Upon activation Rtg2p activates rDNA expression by activating nuclear translocation of Rtg1p or Rtg3p and/or activates rDNA expression as a part of SLIK complex (Modified from Liu and Butow, 2006).

Contrary to most bHLH-type transcription factors, Rtg1p and Rtg3p bind to an unusual promoter sequence GTCAC, referred to as R box instead of E box consensus sequence (CANNTG) (Butow, 2002; Liu and Butow, 2006; Robinson and Lopes, 2000). Thus, Rtg2p serves as a relay protein that transmits signals downstream of SPS to nuclear transcription factors Rtg1p and Rtg3p and activates retrograde responsive genes.

Retrograde pathway is repressed in the presence of high levels of glutamate. Under this condition Mks1p is released from the positive regulatory Mks1p-Rtg2p complex. The released Mks1p can either associate with Bmh1/2p or Lst8p proteins to from Mks1p-Bmh1/2p or Mks1p-Lst8p repressor complexes respectively. The formation of either complex prevents nuclear translocation of Rtg1p and Rtg3p and leads to repression of retrograde responsive genes (Dilova et al., 2002; Sekito et al., 2002) (Figure 26). Thus Mks1p plays a dual role in transcription of retrograde responsive genes.

Rtg2p also facilitates retrograde response independent of Rtg1p and Rtg3p as a part of a transcriptional co-activator SAGA (Spt-Ada-Gcn5)-like histone acetylase complex, SLIK (Pray-Grant et al., 2002). SAGA and SLIK share the majority of common subunits except for Spt8p that is unique to SAGA and Rtg2p that is unique to SLIK (Pray-Grant et al., 2002). In addition, SLIK contains posttranslational modified shortened form of Spt7p (Pray-Grant et al., 2002). As a part of SLIK complex, Rtg2p binds to chromatin at the promoters of retrograde- responsive genes and extends SAGAs function to metabolic genes in addition to stress responsive genes (Jazwinski, 2005). Thus, as a part of SLIK, Rtg2p exerts its chromatin dependent gene functions and helps in the propagation of retrograde response (Pray-Grant et al., 2002).

In *rtg2*Δ, Pol II rRNA expression is reduced to 33.2% of the wild-type implicating the involvement of Rtg2p in Pol II rRNA synthesis (Table 8). To confirm the role of the retrograde pathway in Pol II rRNA synthesis, β-galactosidase activity per copy number was examined in *mks1*-Δ cells. Since in *mks1*-Δ strains the transcriptional activity of retrograde responsive genes *CIT2* and *DLD3* is several folds higher than the wild-type (Sekito et al., 2002), a similar result would be expected for Pol II rDNA reporter expression. However, activity of Pol II rDNA promoter expression in *mks1*-Δ ρ^+ was wild-type, suggesting that Rtg2p is not acting through Rtg1p and Rtg3p (Sagar and Conrad-Webb, 2008).

To confirm that Rtg2p does not regulate Pol II rDNA expression through Rtg1p and Rtg3p, Shenbaga Pandyaraj (2008) compared β-galactosidase activity per copy number of *rtg1*-Δ, *rtg2*-Δ and *rtg3*-Δ with the wild-type strain. The *rtg1*-Δ and *rtg3*-Δ deletion strains were 154% and 130.5% of wild-type whereas *rtg2*-Δ was 73.4% of wild-type. Since the activities in *rtg1*-Δ and *rtg3*-Δ deletion strains were higher than wild-type, it suggests that Rtg1p and Rtg3p are not involved in Pol II rRNA synthesis and Rtg2p might be exerting its effect independent of Rtg1p and Rtg3p (Pandyaraj et al., 2008). This further reinforces that conclusion that Rtg2p does not activate rDNA transcription by activating nuclear translocation factors Rtg1p and Rtg3p as a part of Mks1p-Rtg2p complex, but may activate rDNA transcription as a part of SLIK complex.

Potential Binding Sites for Pol II Transcription Factors in the rDNA

An analysis of rDNA promoter region by YEASTRACT and SGD revealed binding sites for transcription factors regulating metabolism, cell cycle progression and stress

response. The factors can be grouped into three major categories on basis of their role in energy metabolism and combating stress response.

Metabolic Transcription factors identified by screening the Pol II rDNA promoter sequence are primarily involved in carbon source metabolism and respiration including maltose fermentation (Mal63) (Chang et al., 1988), glycolytic (*GCR1)* pathways, glycerol metabolism (ADR1) and Mitochondrial respiration (Hap1, Hap 2, Hap4). It is intriguing that Adr1p, Hap2p and Hap4p are also linked to the retrograde pathway. Adr1p serves as a key regulator of approximately 20 glucose repressed genes during diauxic phase of growth (Young et al., 2003). Interestingly, there is a considerable overlap between the genes activated by Adr1p and the genes involved in retrograde pathway, implicating a link between Adr1p and retrograde transport. Young et al. (2003) demonstrated that out of 43 genes induced by at least 2-fold in ρ^0 cells 17 of them were also *ADR1* dependent. Since the β-galactosidase activity per copy number of *adr1*-Δ strain was 78.13% of wild-type its role as an activator of Pol II rDNA transcription is less likely. However, Adr1p is activated in the absence of glucose and Pol II rDNA expression should be tested under low glucose or under non-fermenting conditions.

The Pol II rDNA promoter also bears a single Hap2/4p binding site upstream of the critical region (-535) as well as a Hap1 site within the critical region (-261).Hap2p and Hap4p are heme activated transcription factors that regulate expression of mitochondrial respiratory genes such as *CIT1*, *ACOI*, *IDH1*, and *IDH2* in respiratory competent cells (ρ^+) (Liu and Butow, 1999). However, the expression of these genes is under the control of *RTG* genes in respiratory deficient (ρ^0) cells (Liu and Butow, 1999). Since β-

galactosidase activity per copy number of *hap2*-Δ and *hap4*-Δ ρ^+ mutants was 375.3% and 285.4% of wild-type respectively, it suggests that Hap2 and Hap4p may be negatively regulating Pol II driven rDNA expression in ρ^+ cells.

Promoter scanning revealed potential binding sites for many transcription factors involved in combating oxidative stress. Sut1p, putative nuclear [ZnII]2Cys6-transcription factor (Ness et al., 2001) is either expressed under anaerobic conditions or in the absence of heme (Bourot and Karst, 1995). Under these conditions Sut1p promotes the uptake of extracellular sterols (Bourot and Karst, 1995). However, Sut1p is negatively regulated by Rox1p under aerobic condition and *SUT1* promoter bears two Rox1p-like binding sequences (Bourot and Karst, 1995). There is a single Sut1p binding site within the critical region rDNA Pol II promoter (-270). In order to elucidate its role in Pol II rDNA transcription the β-galactosidase activity in *sut1*-Δ strain was compared to wild-type. In standard aerobic growth conditions, β-galactosidase activity per copy number in *sut1*-Δ strain was wild-type. However, *sut1*-Δ strain should be tested under anaerobic growth conditions to see the effect of Sut1p on Pol II rDNA expression. Stb5p, a Sin3 binding factor, promotes the recruitment of the Sin3p repressor/activator to the promoter as Sin3p cannot directly bind to DNA (Kasten and Stillman, 1997).

Stb5p activates the genes of pentose pathway by directly binding to their respective promoters upon oxidative stress (Larochelle et al., 2006). In addition, Stb5p mediated induction of *ZWF1* and *GND1/ GND2* results in the production of NADPH (Larochelle et al., 2006). NADPH serves as a cofactor for glutathione and thioredoxin dependent

enzymes which, play a role in combating oxidative stress (Carmel-Harel and Storz, 2000).

In order to examine the role of Stb5p in Pol II rDNA transcription, β-galactosidase activity per copy number in *stb5*-Δ mutant was compared with the wild-type. The *stb5*-Δ was 57.31% and 67% of wild-type in two independent experiments (Tables 15&18). Thus, Stb5p may regulate Pol II rDNA transcription by acting as an activator. In summary, oxidative and anoxic conditions might regulate Pol II rDNA synthesis by activating or deactivating some of these transcription factors. However, future experiments conducted under specific growth conditions would confirm the role of these diverse factors in Pol II rDNA transcription.

Role of Chromatin Remodeling in Transcription

Chromatin remodeling plays a predominant role in regulating transcription. It is manifested by interplay of large number of chromatin modifying proteins such as Histone Acetylases (HATs) and Histone Deacetylases (HDACs) (Kuo and Allis, 1998). HATs activate transcription by acetylating lysine residues at amino terminal domains of histone tails (Kuo and Allis, 1998). A yeast transcriptional regulator, Gcn5p has an intrinsic HAT activity and is a constituent of histone acetyl transferase complex, SAGA and Ada complex (Grant et al., 1997).

In contrast, HDACs repress transcription by deacetylating lysine residues in the amino terminal domains of histone tails (Kuo and Allis, 1998). The HDAC, Rpd3p, also serves as a transcriptional activator of osmostress-inducible genes such as *STL1*, *CTTI*, *ALD3*,

ARO9, *ENA1* and *GRE2* and heat shock gene such as *HSP12* (De Nadal et al., 2004) and DAN/TIR .

The screen of the homozygous deletions for candidate genes that influence Pol II rDNA transcription revealed three major pathways of potential regulation: the cell cycle, osmotic stress and the retrograde regulation. Each of these pathways implicate role of chromatin structures as an essential regulator of Pol II rRNA synthesis. Rtg2p is a unique component of SLIK, a SAGA like histone acetylase complex. Rtg2p as a component of SLIK is known to regulate transcription of *RTG* regulated genes such as *CIT2* in respiratory deficient cells (Pray-Grant et al., 2002). In the HOG pathway, activated Hog1p recruits Rpd3p HDAC to osmoresponsive gene promoter of *CTT1* and heat stress promoter of *HSP12* upon osmostress (De Nadal et al., 2004). The recruitment of Rpd3p-Hog1p enhances *CTT1* and *HSP12* gene expression leading to increased production of cytosolic catalase T and heat shock protein. Rpd3p-Sin3p complex is involved in the regulation of cell cycle genes in association with Fkh2p. During G1 phase the Rpd3p-Sin3p complex represses *CLB2* transcription by binding to the promoter and this coincides with its peak levels during G1 phase. However, during S phase there is increased *CLB2* expression coinciding with a drop in Rpd3p levels. The Rpd3p-Sin3p complex mediates its repressive action only in the presence of its effector, Fkh2p, suggesting that Rpd3p-Sin3p complex directly associates with Fkh2p. Moreover, the N terminal residues (1-194) of Fkh2p are important for its interaction with Rpd3p-Sin3p.

Thus, chromatin remodeling appears to be necessary for the polymerase switch in response to cellular cues such as osmotic stress, activation of the retrograde pathway or cell cycle.

The hypothesis that Pol II rRNA switch may be a backup mechanism for cells undergoing stress is supported by the involvement of retrograde and HOG pathways in triggering the Pol I to Pol II switch. Rtg2p, a member of retrograde pathway and Hog1p, a member of HOG are required for the polymerase switch in ρ^+ cells of *S. cerevisiae* suggesting that osmotic shock, mitochondrial dysfunction and low levels of nitrogen trigger the polymerase switch (Figure 27). Since the entire collection of homozygous deletions mutants were not screened for defects in Pol II rRNA synthesis, other stress pathways may also be implicated. Surprisingly, Fkh2p, a transcription factor regulating transcription of cell cycle genes may also play a role in Pol II driven rDNA expression during the G2 phase. This suggests that Pol II rRNA synthesis may also be regulated in a cell cycle dependent manner. Thus, Pol II driven rDNA transcription besides serves as a compensatory mechanism under conditions when rDNA transcription by Pol I is suppressed. These stressors result in the activation of HOG or Retrograde pathway to ensure sufficient production of rRNA for formation of functional ribosomes.

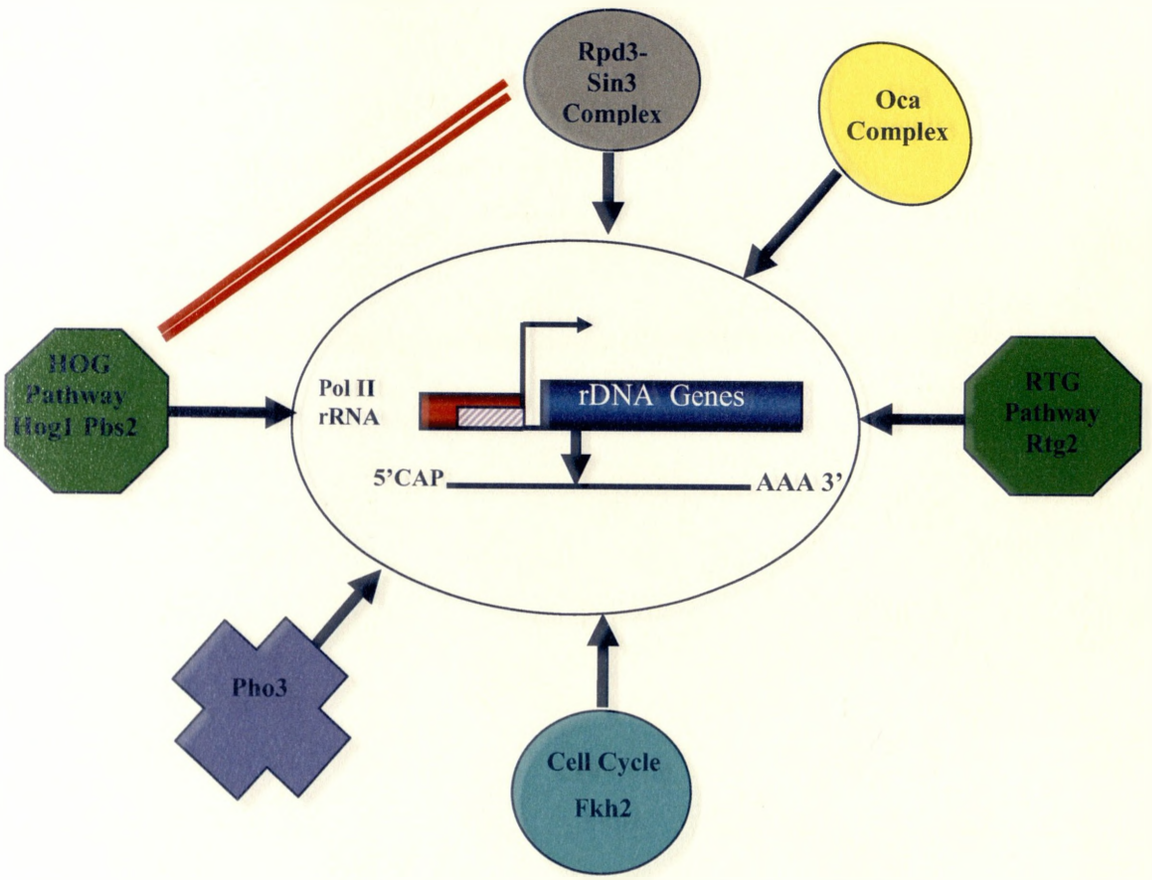


Figure 27. Pathways and Stressors Activating Pol II Switch.
The members of Retrograde (Rtg2p) and HOG pathway (Hog1p and Pbs2p) are involved in Pol II rDNA transcription implicating that under the conditions activating these pathways Pol II rDNA transcription may serve as a back up mechanism. In addition cell cycle factor, Fkh2p, members of Oca complex and Pho3p, a phosphatase also regulate Pol II rDNA transcription. Rpd3-Sin3 complex, a chromatin remodeling complex effects Pol II rDNA transcription under high salt stress.

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APPENDIX A

List of Abbreviations

List of Abbreviations

ABC- ATP Binding Cassette

ADB-Agarose Dissolving Buffer

ARS-Autonomously Replicating Sequence

BMV-Brome Mosaic Virus

C-Critical Region

CE-Core Element

CEN-Centromere

CF-Core Factor

CHIP-Chromatin Immunoprecipitation

D-Downstream Region

DTT-Di-Thiothreitol

FACS-Fluorescent Activated Cell Sorter

F.P- Forward Primer

F.S-Fail Safe

FHA-Fork Head Associated

FOA-5 Fluoro Orotic Acid

GSR-General Stress Response

HATs-Histone Acetylases

HDACs-Histone Deacetylases

HOG-High Osmolarity Glycerol

HS-Heat Shock

HSV-Herpes Simplex Virus

IC-Initiation Complex

LiAc-Lithium Acetate

MAPKKK- Mitogen Activated Protein Kinase Kinase Kinase

MAPKK-Mitogen Activated Protein Kinase Kinase

MAPK-Mitogen Activated Protein Kinase

MBF-MCB Binding Factor

NTS-Non Transcribed Spacer

ONP-o-Nitrophenyl

ORF-Open Reading Frame

PEG-Poly Ethylene Glycol

PIC-Preinitiation Complex

PKA-3'-5' Cyclic AMP Dependent Protein kinase

PMSF-Phenylmethyl Sulfonyl Fluoride

PTC-Peptidyl Transferase Center

R.P- Reverse Primer

RP-Ribosomal Protein

rRNA-Ribosomal RNA

SAGA- Spt-Ada-Gcn5 Acetylation complex

SBF-SCB Binding Factor

SEM-Standard Error of Mean

SGD-*Saccharomyces* Genome Database

SLIK-SAGA Like Complex

SPSS-Statistical Package for the Social Sciences

SSC-Sodium Chloride and Sodium Citrate

T- Takara

TAP-Tandem Affinity Purification

TBP-TATA Binding Protein

TF-Transcription Factor

TK-Thymidine Kinase

UAF-Upstream Activating Factor

UCE-Upstream Control Element

UPE-Upstream Promoter Element

U-Upstream Region

X-Gal-5-Bromo-4-Chloro-3-Indonyl-Beta-D-Galactosidase

YEASTRACT-Yeast Search for Transcriptional Regulators and Consensus Tracking

YG-Yeast Genome

YKO-Yeast Knock-Out

YPD-Yeast Extract Peptone and Dextrose Media

APPENDIX B

Table 19: Average β-Galactosidase Activity/Copy Number in *mks1*Δ Strain (Trial 1)

Table 19

Average β-Galactosidase Activity/Copy Number in *mks1*Δ Strain (Trial 1)

	Cell Extract 1								
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8	Average
BY4743ρ^+	377.0	379.0	422.0	424.0	416.0	427.0	478.0	482.0	425.6
BY4743ρ^+	406.0	396.0	397.0	401.0	418.0	426.0	425.0	417.0	409.9
BY4743ρ^+	434.0	441.0	424.0	436.0	455.0	436.0	436.0	436.0	437.3
Δ*mks1*ρ^+	351.5	384.9	349.6	345.5	373.5	378.7	436.8	404.6	378.1
Δ*mks1*ρ^+	409.8	392.2	359.0	357.9	433.7	416.0	454.4	461.7	410.6
	Cell Extract 2								
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7		Average
BY4743ρ^+	423.0	442.0	408.0	405.0	418.0	436.0	419.0		421.6
BY4743ρ^+	406.0	400.0	404.0	404.0	390.0	449.0	445.0		414.0
BY4743ρ^+	436.0	431.0	409.0	427.0	455.0	457.0	483.0		442.6
Δ*mks1*ρ^+	377.6	383.9	371.4	363.1	353.8	402.5	402.5		379.3
Δ*mks1*ρ^+	433.7	404.6	381.8	373.5	366.2	429.5	403.6		399.0

	Average Cell Extracts (1&2)		
Strain	β-Gal/ Copy #	SEM	% WT
BY4743ρ^+	425.1	3.6	100
Δ*mks1*ρ^+	391.7	5.8	92

APPENDIX C

Table 20: Average β-Galactosidase Activity/Copy Number in *mks*1 Δ Strain (Trial 2)

Table 20

Average β-Galactosidase Activity/Copy Number in *mks1*Δ Strain (Trial 2)

	Cell Extract 1											
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8				Average
BY4743ρ^+	512	526	450	467	485	488	488	502				489.7
BY4743ρ^+	478	459	468	472	494	516	459	508				481.6
BY4743ρ^+	445	444	426	429	458	458	461	481				450.1
Δ*mks1*ρ^+	515	518	475	548	509	504	513	526				513.7
	Cell Extract 2											
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8				Average
BY4743ρ^+	545	495	493	507	539	557	518	480	563	532	547	525.1
BY4743ρ^+	495	492	505	484	515	519	534	475	490	504	506	501.8
BY4743ρ^+	444	428	417	427	477	516	500	373	393	466	444	444.2
Δ*mks1*ρ^+	477	466	473	499	504	521	527	400	432	436	463	472.7

	Average Cell Extracts (1&2)		
Strain	β-Gal Copy#	SEM	%WT
BY4743ρ^+	482.1	5.25	100
Δ*mks1*ρ^+	493.2	8.74	102.3

APPENDIX D

Table 21:. β-Galactosidase Activity/Copy Number in *mks1*Δ Strain (Trial 3)

Table 21

β-Galactosidase Activity/Copy Number in *mks1*Δ Strain (Trial 3)

Cell Extract 1													
Strains	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay. 8					Average
BY4743ρ$^+$	370	369	387	385	355	370	319	264					352.4
BY4743ρ$^+$	275	279	287	294	279	280	266	281					280.1
BY4743ρ$^+$	396	413	396	400	380	381	373	265					375.6
Δ*mks1*ρ$^+$	439	431	444	451	434	437	432	338					425.8
Cell Extract 2													
Strains	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8	Assay 9	Assay 10	Assay 11	Assay 12	Average
BY4743ρ$^+$	329.2	338.6	338.8	350.3	357.4	364.6	348.8	357.0	343.8	352.4	333.1	345.2	311.2
BY4743ρ$^+$	285.5	296.2	301.3	308.8	267.2	274.0	272.2	270.0	301.9	311.9	310.0	304.4	292.0
BY4743ρ$^+$	321.3	323.3	326.5	324.8	293.5	290.2	287.7	285.0	279.5	283.6	267.9	258.9	295.2
Δ*mks1*ρ$^+$	391.2	403.8	414.7	412.0	408.0	404.0	425.3	398.5	440.6	433.9	399.4	399.4	410.9
Cell Extract 3													
Strains	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6							Average
BY4743ρ$^+$	290	280	293	310	329	329							304.9
BY4743ρ$^+$	279	273	278	298	300	297							287.7
Δ*mks1*ρ$^+$	410	435	393	392	419	419							411.5

APPENDIX E

Table 22: Average β-Galactosidase Activity/Copy Number in *mks1* Δ Strain

(Trial 3; Cell Extract 1 & 2)

Table 22. Average β-Galactosidase Activity/Copy Number in *mks1*Δ strain (Trial 3; Cell Extract 1 & 2).

	Average Cell Extracts (1&2&3)		
Strain	β-Gal/ Copy#	SEM	%WT
BY4743ρ^+	304	4.8	100
Δ*mks1*ρ^+	416	4.68	137

Table 23

β-Galactosidase Activity/Copy Number in Promoter Binding Mutants (Trial 1)

	Cell Extract 1							
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
By4743ρ^+	377.0	379.0	422.0	424.0	416.0	427.0	478.0	482.0
By4743ρ^+	406.0	396.0	397.0	401.0	418.0	426.0	425.0	417.0
Δ*mot3*ρ^+	487.7	506.6	484.9	500.0	528.3	505.7	532.1	545.4
Δ*stb5*ρ^+	231.5	231.5	215.4	231.2	242.9	247.6	242.9	243.5
Δ*gsm1*ρ^+	413.7	403.8	350.1	336.9	449.9	417.0	440.0	439.0
Δ*sut1*ρ^+	417.8	417.0	332.0	326.8	445.6	441.2	466.4	438.6

	Cell Extract 2							
Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Average
By4743ρ^+	423.0	442.0	408.0	405.0	418.0	436.0	419.0	423.7
By4743ρ^+	406.0	400.0	404.0	404.0	390.0	449.0	445.0	412.3
Δ*mot3*ρ^+	499.0	520.8	476.4	536.9	42.0	525.5	541.6	511.5
Δ*stb5*ρ^+	249.6	256.1	227.1	237.9	230.6	248.5	257.0	239.5
Δ*gsm1*ρ^+	437.9	426.9	406.0	378.6	380.8	451.0	437.9	411.3
Δ*sut1*ρ^+	449.0	437.8	416.1	404.8	389.2	432.6	447.3	417.5

APPENDIX G

Table 24: Transformation Summary

Table 24

Transformation Summary

Plate No	Number Transformed	Untransformed Strains	Number Untransformed
301	96		
302	95	E3	1
303	68	A3, A12; C1, C3, C8, C10; D2, D10, D12	28
		E1, E5, E9, E10, E11; G1, G2, G5, G10, G12	
		H-2, 4-10, H12	
304	95	C10	1
305	91	C5, G11, H8	3
306	95	F7	1
307	95	A4	1
308	93	A8, D7, F10	3
309	32	A1-3, A6-10, A12, B1-5, B8, B9, B12 ,C1-3,C10; D1-5, D7-10, D12; E7-9, E12; F1-6, F8-10, F12; G1-10; H2-5; H9-12	59
310	95	A12	1
311	64	A1, A5, A7, A10; B9, B10; D1; E4, E7, E8, E9, E12; F8, F11; G1, G2, G4, G5, G6, G8-12; H1, H2, H4, H5, H9-12	32
312	92	A3, A4; D12; E-9	4
313	95	B1	1
314	85	A2, A6, A8; B2, B8; D4, D11; E8; F5; G2,G10	11
315	93	G12; H3, H4	3
318	55	C2; E1-6; F1-12; G1-12; H2-5, H7-12	41
319	83	B6, B7, C3, C10, C11; D6, D9, D12; E3, E12, F1, F2, H10	13
320	95	B8	1
321	90	C1; D6, D10; F5, F6; H3	6
323	59	A8, A9; B5, B8, B11; C4-6, C8; D3, D5-10, D12; E2, E4, E5; E8-10; F2, F4, F7-12; G9, G11; H4-9	37
324	93	A9; B12; H6	3
326	96		
327	92	C2, C3; D12; E11	4
328	93	A10; B1; C4	3
330	95	B3	1

Plate No	Number Transformed	Untransformed Strains	Number Untransformed
335	69	A5; B3, B5; C6, C11; D2, D3, D6-8, D11, D12, E4, E9; F1, F6, F10, F11; G5, G7, G9, G10, H6, H8, H10, H11,H12	27
336	26	A 6-9, A11, A12; B2, B10-12; C2, C6-8, C11, C12; D1, D2, D4-6, D12, E1-12, F1-12, G1-12, H1-12	70
337	88	A1, A8, B8, C7, C9, D1, E10, F1	8
b338	94	A2, B10	2
340	89	A1, A12, A8, B9, B11, C2, C3	7
341	45	A1, A7-12; B7-12; C2, C3, C7-12; D5, D7; E2-E4, E7-E12; F4, F6-F12; G6-12; H2-4	51
344	39	A2, A4, A7-12; C4, C6-12; D2, D7-12; E7-12; F7-12; G7-12; H6-12	57
370	76	A2; B10, B11; C2, C6, C7; D5, D7, D11; E3, E5, E6, E9, E10, E11; F5, F6, F7, F10, F11	20
	2661		**500**