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Transcriptional regulation in stationary phase of the yeast *Saccharomyces cerevisiae*

by

Dean B. Zaragoza

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry

Edmonton, Alberta

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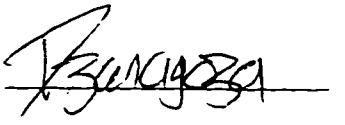

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Transcriptional Regulation in Stationary Phase of the Yeast *Saccharomyces cerevisiae* submitted by Dean B. Zaragoza in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

The yeast *Saccharomyces cerevisiae* responds to the stress of starvation by arresting growth and entering into a period of dormancy known as stationary phase. During entry into stationary phase, distinct changes in the pattern of transcription occur. While transcription by RNA polymerases I (pol I) and III (pol III) is repressed, and the majority of RNA polymerase II (pol II) genes are shut down during starvation, a small subset of polymerase II genes is actually induced in response to starvation. This study examines mechanisms responsible for both the inhibition of pol III transcription and the induction of transcription of the pol II gene *SSA3* during entry into stationary phase. We demonstrate that either of two closely related zinc finger proteins, Msn2p and Msn4p, is required for the stationary induction of *SSA3*. A sequence found upstream of *SSA3*, the PDS element, has previously been shown to mediate transcriptional induction during entry into stationary phase. We find that the PDS element binds to an activity in wild type extracts that is missing in *msn2* and *msn4* null extracts, suggesting that Msn2p and Msn4p function by directly binding to the PDS element. This study also demonstrates the use of the drug rapamycin to induce the starvation response. Treatment of yeast cells with rapamycin results in the inhibition of transcription by pol I and III. We show that pol III inhibition results from interference with a signaling pathway, the so called TOR pathway, that controls translation. We also present evidence that the TOR pathway signals directly to the pol III transcription machinery.

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List of Abbreviations

A_{260} - Absorbance at 260 nm

ARE - AP-1 responsive element

ATP - Adenosine triphosphate

BSA - Bovine serum albumin

CKII - Casein kinase II

CDK - Cyclin dependent kinase

CTP - Cytidine triphosphate

dCTP - Deoxycytidine triphosphate

DNA - Deoxyribonucleic acid

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic acid

EGTA - bis(aminoethyl)glycolethertetraacetic acid

Fig. - Figure

FKBP - FK506 binding protein

GTP - Guanosine triphosphate

HOG pathway - High osmolarity glycerol pathway

hrs - Hours

HSE - Heat shock element

HSP - Heat shock protein

kDA - Kilodalton

M2 - Mutation 2

M3 - Mutation 3

MET^- media - Methionine minus media

mFKBP - Mammalian FKBP

mRNA - Messenger RNA

OD_{600} - Optical density measured at 600 nm

PCR - Polymerase chain reaction

PCs - Positive cofactors

PDS - Post-diauxic shift

PI-3 kinase - Phosphatidylinositol-3 kinase

PKA - Protein kinase A

PMSF - Phenylmethylsulfonyl fluoride

Pol I - RNA polyemerase I

Pol II - RNA polymerase II

Pol III - RNA polymerase III

RAFT1 - Rapamycin and FKBP12 target

RNA - Ribonucleic Acid

rRNA - Ribosomal RNA

SDS - Sodium dodecyl sulfate

snRNA - Small nuclear RNA

Srb - Suppressors of RNA polymerase B

STRE - Stress response element

TAFs - TBP-associated factors

TBP - TATA binding protein

TFIIA - Transcription factor IIA

TFIIB - Transcription factor IIB

TFIID - Transcription factor IID

TFIIE - Transcription factor IIE

TFIIF - Transcription factor IIF

TFIIH - Transcription factor IIH

TFIIIA - Transcription factor IIIA

TFIIIB - Transcription factor IIIB

TFIIIC - Transcription factor IIIC

TIF-IB - Transcription initiation factor IB

TOR - Target of rapamycin

TPCK - L-1-chloro-3-(-4-tosylamido)-4-phenyl-2-butanone

tRNA - Transfer RNA

UAS - Upstream activating sequence

UTP - Uridine triphosphate

WT - Wildtype

YDBI - Yeast dialysis buffer I

yFKBP - Yeast FKBP

YPD - Yeast/peptone/dextrose

INTRODUCTION

The stress response and transcriptional regulation

Stress in nature

In order to survive in nature, it is important for living organisms to sense and properly respond to changes in the environment. It is often the case that changes in the environment have the potential to be harmful, so it is imperative that living organisms have ways to defend against such stress. Because of the existence of these defense mechanisms, organisms can withstand constantly changing environmental factors such as nutrient availability and temperature.

At the cellular level there are two lines of defense that protect cells against stress. The first line of defense, the primary or immediate response, consists of the activation of specific proteins required for immediate survival. These proteins are pre-existing and are stress-activated; they provide minimal protection against the activating stress. Such proteins produce changes in metabolism and membrane transport in order to adapt cells to their environment. These pre-existing proteins also activate a second line of defense, which consists of signal transduction pathways that activate more proteins and genes with protective functions. This is known as the delayed response. Together, these immediate and delayed reactions make up the stress response and allow cells to survive stressful conditions.

Studies of the stress response have important implications in both medicine and agriculture. Understanding the factors involved in the stress response may be helpful in agriculture by making plants more resistant to heat, drought or other types of stress. In addition, understanding these factors may help in development of agents to fight infection and cancer. During infection, the stress proteins of invading bacteria or parasitic microorganisms are often the major antigens that the immune system uses to synthesize antibodies. Stress proteins from these pathogens (made by recombinant DNA techniques) may therefore have potential as vaccines for preventing infections. Furthermore, the finding that tumors are often more thermally sensitive than normal tissues suggests that elevating tissue temperature may eradicate tumors (reviewed in Welch, 1993).

The general stress response in yeast

The stress response in eukaryotes has been extensively examined in the yeast *Saccharomyces cerevisiae* because the environment of yeast cells grown in liquid culture is easily manipulated and because yeast is amenable to detailed molecular genetic analysis. In the study of the yeast stress response, many types of stress have been analyzed (reviewed in Mager and Varela, 1993; Mager and Morasdus-Ferreira, 1993; Piper, 1993). These stresses include nutrient starvation, heat shock, osmotic stress, drastic changes in pH, and exposure to heavy metal ions. While specific responses to stress have been identified (Mager and Varela, 1993; Chang and Fink, 1994), studies indicate that yeast cells exposed to a specific stress become resistant to many types of stress (Mager and Varela, 1993; Chang and Fink, 1994). This phenomenon, known as cross resistance, suggests that cells have one system or a limited number of systems that can be activated by different stresses. Once activated, the system or systems will protect cells against a wide variety of stresses.

The yeast stress response involves the activation or inactivation of signal transduction pathways. This results in responses at the transcriptional, translational, and post-translational level. Of particular importance is the transcriptional stress response since activation of gene transcription plays a pivotal role in the induction of genes that repair stress-induced cellular damage and offer protection from stress. Because this work focuses on the transcriptional regulation in response to stress, the regulatory mechanisms of RNA polymerases I, II, and III will be briefly reviewed. More specifically, the following sections examine regulation of transcriptional initiation since this is the most studied aspect of transcriptional regulation. In addition two signaling pathways that have been shown to play either a direct or indirect role in the transcriptional stress response will be reviewed. These pathways are the HOG (high osmolarity glycerol) pathway and the RAS-protein kinase A pathway.

The high osmolarity glycerol (HOG) pathway

An increase in external osmolarity is stressful to cells because it results in loss of an osmotic gradient across the plasma membrane and also causes cell shrinkage (Mager

and Varela, 1993). Yeast cells adapt to high external osmolarity by increasing the internal osmolarity. This is accomplished by the accumulation of glycerol. Glycerol accumulation in response to high external osmolarity has been shown to require two genes, *HOG1* and *PBS2* (Boguslawski, 1992; Brewster et al., 1993). The HOG pathway is defined by these two genes, which respectively encode members of the MAP kinase and MAP kinase kinase family. This signaling pathway appears to signal specifically increases in external osmolarity (Brewster et al., 1993). The fact that the pathway is specific for one type of stress suggests that there may be multiple pathways involved in signaling different stresses (Schuller et al., 1994).

It has been demonstrated that the HOG pathway signals to a specific cis-acting sequence known as the stress response element (STRE) found upstream of genes that are induced in response to a variety of stresses (Marchler et al., 1993). Mutations in the HOG pathway abolish the osmotic transcriptional stress response of STRE controlled genes (Schuller et al., 1994). Furthermore, phosphorylation of Hog1p occurs rapidly after an increase in osmolarity. This is followed by an increase in transcription by STRE controlled genes (Schuller et al., 1994). These results suggest that the HOG pathway transmits a specific stress signal (high osmolarity) to the STRE.

The RAS-protein kinase A pathway

The RAS-protein kinase A pathway has also been demonstrated to be involved in the stress response but it is not known whether it directly transmits stress signals. Upstream activating sequences like the STRE and the post-diauxic shift element (PDS element; see chapter 1 introduction) are negatively regulated by protein kinase A (PKA) (Marchler et al., 1993; Boorstein and Craig, 1990b; Belazzi et al., 1991; Engelberg et al., 1994). Marchler et al. (1993) and Boorstein and Craig (1990b) have demonstrated that *bcy1* mutants (high PKA activity) have reduced levels of STRE and PDS element dependent transcription while *ras2* mutants (low PKA activity) have induced levels of STRE and PDS element dependent transcription. Interestingly, although they both appear to be controlled by protein kinase A, the STRE and PDS elements respond to different stresses. That is, the STRE activates transcription in response to stress

conditions such as nitrogen starvation, heat shock, osmotic and oxidative stress (Marchler et al., 1993), whereas the PDS element has thus far only been shown to respond to gradual nutrient depletion (Boorstein and Craig, 1990b). In summary, the available evidence suggests that the RAS-protein kinase A pathway may regulate transcription from both STRE and PDS element controlled genes. However, there is no evidence to indicate that PKA directly transmits stress signals, as is the case for Hog1p.

Regulation of transcription by RNA polymerase I

Transcription by RNA polymerase I (pol I) gives rise to the large ribosomal RNAs (rRNA) and 5.8S rRNA. The regulation of pol I transcription is known to be coupled to growth regulation. Several studies have shown that transcription by pol I responds to treatments that affect cellular growth (reviewed in Sollner-Webb and Tower, 1986). Inhibition of pol I transcription has been observed when yeast cells enter into stationary phase (Schultz et al., 1991), when mouse cells are serum starved (Tower and Sollner-Webb, 1987), when cells are treated with protein synthesis inhibitors (Mishima et al., 1979; Tower and Sollner-Webb, 1987) and when cells are treated with glucocorticoids (Cavanaugh and Thompson, 1985). Stimulation of pol I transcription has been observed when cells are treated with phorbol ester or serum (Vallett et al., 1993; Chao and Pellegrini, 1993). The data in all of the above experiments suggest that the effects on pol I transcription are due to effects on transcription initiation. Studies have identified a factor, distinct from pol I transcription factors TIF-IB and UBF, that can rescue transcription in serum-starved cells. This factor has been termed factor C (also known as TFIC and TFIA) and may be an activated subform of pol I (Tower and Sollner-Webb, 1987). It is thought that down regulation of pol I activity observed in the above cases is due to an inactivation of factor C (Mahajan and Thompson, 1990; Tower and Sollner-Webb, 1987). Factor C appears to be important only for regulation of the polymerase itself as stable initiation complexes can form in the absence of factor C (Cavanaugh and Thompson, 1985).

Recent studies have identified a growth regulated pol I transcription factor termed E1BF (enhancer 1 binding factor) (Niu and Jacob, 1994). This factor consists of two

polypeptides (72 and 85 kDa) that bind to several DNA sequences involved in the regulation of pol I transcription (Hof and Jacob, 1993). E1BF has both repressive and stimulatory effects on pol I transcription depending on post-translational modifications. E1BF is post-translationally modified following serum starvation of cells. This results in repression of pol I activity (Niu and Jacob, 1994). On the other hand E1FB from serum enriched cells functions as a positive factor for pol I. These results suggest an important role of E1BF in the regulation of transcription by RNA polymerase I.

Regulation of RNA polymerase II transcription

Transcription by RNA polymerase II (pol II) gives rise to messenger RNA (mRNA) that will be translated into proteins. With regards to transcriptional regulation, one of the most studied aspects of pol II is the mechanism of transcriptional activation. Many activators of pol II transcription function by increasing the recruitment of pol II to the promoter. Once bound to an upstream activating sequence (possibly with the help of chromatin remodeling systems; see below), activators either interact with the components of the transcription machinery or intermediate factors (cofactors) that then contact the transcription apparatus. Proteins with proposed roles as cofactors include the TBP-associated factors (TAFs), the positive cofactors (PCs), and components of the pol II holoenzyme.

The TBP-associated factors (TAFs) are components of transcription factor IID (TFIID), which also contains the TATA-binding protein. *Drosophila* TFIID contains eight TAFs with sizes ranging from 30 to 250 kDa (Chen et al., 1994). In humans, TFIID has at least 12 TAFs with sizes ranging form 30 to 250 kDa, and yeast TFIID contains at least 12 TAFs with sizes ranging from 19 to 130 kDa (reviewed in Tansey and Herr, 1997). These TAFs have been found to play multiple roles in the activation of transcription by pol II. *Drosophila* TAF30, TAF40 and TAF60 are similar in structure to core histones H2B, H3, and H4, respectively (Burley and Roeder, 1996). This suggests that these TAFs may bind DNA like their histone counterparts and therefore may enhance contacts of TFIID with the promoter. In addition, recent studies have identified enzyme activities of certain TAFs such as histone acetylation and phosphorylation of TFIIF,

TFIIE, and TFIIA (Mizzen et al., 1996; Dikstein et al., 1996). Histone acetylation could be important in disrupting chromatin to allow access for transcription factors. Basal factor phosphorylation may be an important mechanism for signaling between components of the transcription machinery. In addition to these roles, TAFs may also be targets for activators.

Studies have demonstrated that addition of TAFs to TBP restored activation *in vitro* (TBP alone cannot support activated transcription). This suggests a role of one or more of the TAFs as coactivators (Tanese et al., 1991). It was later found that the majority of transcriptional activators require TAFs for *in vitro* transcriptional activation (Zhou et al., 1992; Jacq et al., 1994; Chiang and Roeder, 1995). Furthermore, it has been demonstrated that activators and TAFs interact physically and that stimulation of transcription by certain activators such as Sp1 and NTF-1 is dependent on these interactions (Chen et al., 1994; Suaer et al., 1996). While these studies demonstrate an important role for TAFs in transcriptional activation, studies in yeast have shown that TAFs are not required for transcriptional activation (Moqtaderi et al., 1996). This suggests alternate mechanisms for transcriptional activation in yeast.

Other activator targets have also been identified that are not part of the TFIID complex. Positive cofactors (PCs) have been shown to enhance the response to transcriptional activators. Analysis of one of these, PC4, demonstrates that it is capable of interacting with the activation domain of the transcriptional activator VP16 and with TFIIA within the TBP-TFIIA-promoter complex (Ge and Roeder, 1994; Ge et al., 1994). This provides another example of a protein linking an activator to the transcription initiation machinery.

There is also evidence that some activators directly contact the initiation factors themselves. Direct interactions have been observed between activators and TFIIB, and TFIIH. Studies with VP16 have shown that a direct interaction between TFIIB and VP16 is required for transcriptional activation (Lin et al., 1991). Binding of VP16 to TFIIB has been proposed to expose binding sites on TFIIB for TBP and pol II, thus promoting initiation (Roberts and Green, 1994). VP16 has also been shown to interact with TFIIH (Xiao et al., 1994). Furthermore, mutations in VP16 that reduce its transactivation

activity also reduce its ability to interact with TFIIH. This finding suggests that interaction of VP16 with TFIIH is an important aspect of transcriptional activation.

The pol II holoenzyme has also been implicated in conveying signals from activators to the transcription machinery. The holoenzyme is composed of the general transcription factors TFIIB, TFIIH, and TFIIF as well as RNA polymerase II and a multimeric mediator (Koleske and Young, 1994). The mediator is made up of numerous polypeptides including Srb proteins, Sug1, and Gal11 which have previously been shown to be involved in transcriptional regulation (Suzuki et al., 1988; Fassler and Winston, 1989; Himmelfarb et al., 1990; Nishizawa et al., 1990; Vallier and Carlson, 1991; Swaffield et al., 1992; Yu and Fassler, 1993; Thompson and Young, 1995). In addition, the mediator also contains SWI/SNF proteins which allow the holoenzyme to disrupt nucleosomal DNA and facilitate stable binding of various components of the initiation complex at promoters (Wilson et al., 1996). SWI/SNF proteins have been previously shown to be necessary for transcriptional activation of many genes in yeast cells (Peterson and Tamkun, 1995). Previous biochemical studies have shown that the holoenzyme is responsive to activators (Kim et al., 1994; Koleske and Young, 1994) while purified pol II and basal factors are not (Flanagan et al., 1991). This suggests that the holoenzyme contains components necessary for response to transcriptional activators. Recent studies have shown that VP16 binds to the holoenzyme through the mediator complex *in vitro* (Hengartner et al., 1995). Therefore it may be that the pol II holoenzyme can be recruited to a promoter via direct interactions with activators. Once at the promoter, the SWI/SNF components of the holoenzyme may enhance activator-DNA binding by destabilizing nucleosomes.

These various studies on the regulation of transcription by pol II indicate that activation of transcription at the level of initiation may occur through direct contacts of activators with the transcription machinery or through interactions of activators with cofactors. These cofactors have been identified as components of TFIID (TAFs), the pol II holoenzyme (mediator), or positive cofactors (PCs). The ultimate result of either contact of activators with the transcription machinery or interaction of activators with cofactors is the increased recruitment of pol II to the promoter.

Regulation of RNA polymerase III transcription

RNA polymerase III (pol III) is responsible for the synthesis of RNA molecules that are involved in protein synthesis (tRNA and 5S rRNA) and RNA processing (snRNAs). Studies of the regulation of transcription initiation by pol III have demonstrated roles for many different proteins in either activation or repression. Many of these proteins have been shown to function through an effect on the transcription initiation machinery, TFIIIA, TFIIIC, and the TBP containing TFIIIB. For example, Dr1 has been shown to regulate pol III transcription by binding to TBP. This results in the inactivation of TFIIIB and an inhibition of pol III transcription (White et al., 1994).

Recent studies suggest that modification of basic pol III transcription factors may underlie the global regulation of pol III transcription. An unidentified kinase has been shown to phosphorylate TFIIIC in virally infected mammalian cells, resulting activation of pol III transcription (Hoeffler et al., 1988; White et al., 1990). In *Drosophila* protein kinase C is implicated in the induction of pol III transcription when cells are stimulated by phorbol ester. This induction is through an effect on TFIIIB (Garber et al., 1994). Furthermore, in *Xenopus laevis*, $p34^{cdc2}$ can mediate repression of pol III transcription (Gottesfeld et al., 1994; Hartl et al., 1993). Such studies reveal the importance of protein kinases in transcriptional regulation of RNA polymerase III.

A recent study in the regulation of nuclear transcription has identified another protein kinase involved in the regulation of transcription by RNA polymerase III. This enzyme is casein kinase II (CKII) (Hockman and Schultz, 1996). CKII is a ubiquitous and highly conserved serine/threonine kinase found in both the nucleus and cytoplasm of most cells (reviewed in Issinger, 1993). CKII was found to be required for transcription of the 5S rRNA and tRNA genes by RNA polymerase III both *in vivo* and *in vitro*. Furthermore, *in vitro* add-back experiments demonstrated CKII to be directly involved in transcription by pol III. Recent work has implicated TBP, a component of TFIIIB, as the target of CKII regulation (Ghavidel and Schultz, unpublished results). These results suggest that phosphorylation of TBP by CKII may be important for transcription by pol III.

Goals of this work

In this study we examine transcriptional regulation in response to the specific stress of gradual nutrient depletion as the yeast *Saccharomyces cerevisiae* enters stationary phase. At the level of transcriptional regulation there are two components of the yeast starvation response. First, gradual nutrient depletion results in growth arrest which involves the inhibition of transcription by pol I, III, and the majority of pol II genes. Second, entry into stationary phase results in enhanced transcription of a small minority of pol II genes. We examined both aspects of the yeast starvation response by allowing cells to naturally exhaust nutrients from the growth media and by treating cells with rapamycin, a drug previously shown to interfere with the "target of rapamycin" (TOR) signal transduction pathway and to induce the starvation response of the pol II transcription machinery (Barbet et al., 1996).

The present study of transcriptional regulation in stationary phase focused on two goals: 1) to identify trans-acting factors involved in the induction of the pol II gene, *SSA3*, upon entry into stationary phase and 2) to test if interference with TOR signaling by rapamycin induces the typical stationary phase response of the pol III transcription machinery. Each of these studies will be dealt with in turn.

CHAPTER ONE

Identification of factors involved in the induction of *SSA3* in stationary phase

Introduction

The yeast *Saccharomyces cerevisiae* responds to some environmental stresses by arresting growth and entering into a period of dormancy known as stationary phase. This non-proliferative state allows yeast cells to survive long periods of time with no nutrients and is analogous to G_0 in mammalian cells (Pringle and Hartwell, 1981; Iida and Yahara 1984). Of the many environmental stresses encountered by cells, starvation is one of the most common. For this reason, the mechanisms regulating entry into stationary phase as a result of starvation have been the focus of many studies.

Yeast grown in rich medium (YPD) exhibit different phases of growth. When nutrients are abundant, yeast cells progress through the cell cycle in about 90 minutes to 2 hours. This phase of growth is referred to as logarithmic phase because the cells grow rapidly and increase their mass exponentially. During this phase yeast cells grow using energy provided by fermentation. Eventually the nutrients become exhausted from the media and cells stop growing and switch to respiratory metabolism using the products of fermentation as a carbon source. The period of change from anaerobic metabolism to aerobic metabolism is referred to as the diauxic shift. After the diauxic shift, cells resume growth at a much slower rate (post-diauxic phase). When the products of fermentation are used up cells enter into stationary phase and stop growing (Fig. 1.1a).

There are many characteristics that distinguish stationary phase cells from logarithmically growing cells. Stationary cells show a reduction in protein synthesis (Fuge et al., 1994), accumulate glycogen (Panek, 1991; Entian, 1986), and are thermotolerant (Finley et al., 1987). In addition stationary cells exhibit changes in the patterns of transcription. As nutrients are exhausted from the growth media transcription by pol I and III is shut down (Sethy et al., 1995; Schultz et al., 1991). In addition many mRNAs that are abundant during log phase are barely detectable after cells pass the diauxic shift. This indicates that the majority of pol II transcription is also shut down (Werner-Washburne et al., 1989; Choder, 1991). Despite the decrease in abundance of most pol II transcripts after the diauxic shift, steady-state levels of some transcripts do not fluctuate with growth phase. Examples of these are the transcripts encoded by *UBI4*

(Finley et al., 1987; Tanaka et al., 1988), *ENO1* (McAlister and Holland, 1985), and *BCY1* (Werner-Washburne et al., 1993). In addition to the persistent transcripts are those that are undetectable during log phase and accumulate after the diauxic shift as cells enter into stationary phase. Examples of these are the transcripts encoded by *CTT1* (Bissinger et al., 1989), *HSP26* (Choder, 1991; Petko and Lindquist, 1986), and *SSA3* (Choder, 1991; Boorstein and Craig, 1990b).

The importance of both the persistent and stationary induced gene products for survival of cells under stresses such as the absence of nutrients is currently unknown but some of these, such as the *UBI4* transcript, have been shown to be essential for survival after exhaustion of nutrients (Finley et al., 1987). The *SSA3* transcript is the only one of the eight members of the yeast multigene HSP70 (heat shock protein; molecular weight 70 kDa) family whose mRNA levels increase after the diauxic shift. How these pol II genes escape repression and are induced following the diauxic shift has been the focus of many studies on stationary phase in yeast. Studies aimed at answering this question have identified many transcriptional control elements that are activated by stress conditions. Examples of these are the well characterized heat shock element (HSE) (Jakobsen and Pelham, 1988; Parsell and Lindquist, 1994), the AP-1 responsive elements (ARE) (Kuge and Jones, 1994), the stress response element (STRE), (Schmitt and McEntee, 1996; Martinez-Pastor et al., 1996), and the post-diauxic shift element (PDS) (Boorstein and Craig, 1990b). Only the STRE and the PDS element are implicated in the response to nutrient starvation since genes controlled by HSEs have been shown to be non-essential for protection from general stresses such as nutrient deprivation (Lindquist and Craig, 1988). Furthermore the ARE only controls expression of genes required for toxic responses to heavy metal ions and hydrogen peroxide. The ARE also controls expression of genes induced under conditions of oxidative stress (Schnell and Entian, 1991; Schnell et al., 1992).

The STRE element, which has the consensus core sequence AGGGG, has been found upstream of many genes with protective functions, for example *CTT1*, *DDR2*, *HSP12*, *TPS2*, *GSY2* and *GPH1* (Ruis and Schuller, 1995; Kobayashi and McEntee, 1993). This element mediates the induction of these genes in response to a variety of

severe stresses such as immediate nutrient depletion. Two proteins that bind to STRE sequences have been identified as the homologous transcriptional activators Msn2p and Msn4p (Schmitt and McEntee, 1996; Martinez-Pastor et al., 1996). It has been demonstrated that a yeast strain in which both of these proteins have been knocked out is unable to induce transcription of *HSP26*, *HSP12*, *CTT1*, and *DDR2* in response to these severe stresses. This result suggests that Msn2p and Msn4p likely act as positive transcription factors for STRE-controlled genes. The PDS element, with a consensus core sequence AGGGA similar to the STRE, has been found upstream of *SSA3* (reviewed in Werner-Washburne et al., 1993). This element is required for the induction of *SSA3* transcription following the diauxic shift (Boorstein and Craig, 1990b). While the PDS-binding factors responsible for the induction of these genes following the diauxic shift have not been identified, gel mobility shift experiments suggest that both Msn2p and Msn4p are capable of binding to the PDS element (Marchler et al., 1993, Martinez-Pastor et al., 1996). However these two proteins are not required for the induction of *SSA3* in response to severe stresses such as immediate nutrient depletion (Martinez-Pastor et al., 1996). The goal of this study was to identify proteins that could bind to the PDS element *in vitro*. Such proteins might be involved in the transcriptional induction of *SSA3* after the diauxic shift.

We propose that the induction of *SSA3* following the diauxic shift is due to binding of a transcriptional activator to the PDS element upstream of *SSA3*. Three possible mechanisms could underlie the induction of *SSA3* after the diauxic shift: 1) the putative transcriptional activator that binds the PDS element is present only after the diauxic shift, 2) the activator is always bound to the PDS element and is modified following the diauxic shift to cause activation of *SSA3* transcription, and 3) the activator is present during all stages of growth and is only able to bind the PDS element after the diauxic shift. In the latter case the factor may be modified to allow binding or translocated into the nucleus after the diauxic shift. In order to test these possibilities we developed a gel mobility shift assay to detect PDS binding activities in yeast whole cell extracts. This assay utilized a 33 nucleotide probe which contained the PDS element. We assayed for PDS binding activities in extracts derived from log, PDS, and stationary

phase cells. We expected to detect a binding activity in PDS and stationary extracts that was not present in log extracts. Such a result would be consistent with the hypothesis that PDS and stationary phase cells contain a transcription factor that can bind to the PDS element and activate transcription of *SSA3*. However we found that a PDS-specific binding activity is present in log, PDS, and stationary phase extracts. We demonstrate that this activity is present in nucleus of stationary cells, consistent with its proposed role as a transcriptional activator. Furthermore we demonstrate that the induction of *SSA3* transcription following the diauxic shift requires Msn2p and Msn4p and that the PDS-specific binding activity is dependent on the presence of either of these two proteins.

Results

Development of a gel mobility shift assay to detect activities that can bind specifically to the PDS element

Because we were interested in detecting activities involved in the regulation of pol II transcription, we chose to prepare extracts by a method previously shown to support accurate transcription initiation on a yeast pol II promoter (Schultz et al., 1992). This method involves the breakage of yeast cells while they are frozen, an approach that is likely to limit the activation of inhibitory proteases. Extracts prepared by this method also preserve the stationary repression of pol I transcription (Schultz et al., 1991). Figure 1.1b further demonstrates that the repression of pol III transcription in stationary phase is reproduced in the extract. This result encouraged us to test the extract for DNA binding activities possibly involved in the growth regulation of *SSA3* transcription.

In order to pursue this approach it was necessary to develop a gel mobility shift assay for activities that could bind specifically to the PDS element of the *SSA3* gene (Fig. 1.2a). Therefore a double-stranded DNA probe corresponding to the PDS element was end labeled with ^{32}P and used as the target for binding using extracts from stationary cells. The assay was developed in three steps: 1) design of a gel system capable of resolving shifts using the PDS probe, 2) experimental determination of optimal conditions for DNA binding, and 3) establishment of DNA binding specificity. For the first two steps we used unlabeled pBluescript KS^+ cut with HpaII, or poly (dI-dC) · poly (dI-dC) as a non-specific competitor to attempt to eliminate shifts due to non-specific DNA binding activities. This approach was sufficient for the initial development of the assay, but was refined in order to establish binding specificity.

Our initial attempts to identify activities in stationary phase extracts that could bind to the PDS element involved a high ionic gel system. We performed binding reactions essentially as described in Wahls et al. (1991). The reactions were loaded on a 5% non-denaturing polyacrylamide in Tris-glycine buffer. We reasoned that under these high ionic conditions we could reduce the number of non-specific interactions with the probe. We detected multiple DNA binding activities even in the presence of pBluescript

KS$^-$/HpaII or poly (dI-dC) · poly (dI-dC) (data not shown). This result is not surprising, considering that multiple DNA-dependent activities are supported in the extract. These activities include transcription by all three nuclear RNA polymerases and mitochondrial RNA polymerase (Schultz et al., 1991; 1992), as well as second strand synthesis of DNA and chromatin assembly (Schultz et al., 1997).

Using the high ionic gel system, we tried to optimize the binding conditions. The first variable examined was the amount of extract used in the binding reaction. We found that although there was a parallel increase in the intensity of each shift with increasing amounts of extract (1-20 μg), there was no change in the pattern of shifts. We chose an intermediate amount of 6 μg to use in subsequent binding reactions. We then tested the ability of different competitors to eliminate non-specific binding activities. Among the competitors tested was pBluescript KS$^+$/ HpaII, poly (dI-dC) · poly (dI-dC) and poly (dA-dT) · poly (dA-dT). Of these compounds poly (dI-dC) · poly (dI-dC) was found to be the most potent in competing out binding to the probe. 100 to 1100 ng of poly (dA-dT) · poly (dA-dT) per binding reaction had no effect on any of the binding activities (data not shown). We also tested the dependence of the multiple binding activities on ATP. None of the shifts obtained was dependent on ATP and they were all observed whether or not $MgCl_2$ was present in the binding reaction. Other variables tested were the reaction time and temperature. Binding reactions were performed for 5 to 60 minutes at 0°C, 22°C, 30°C, or 37°C. Results from these experiments indicated that the binding of all activities detected occurred within 10 minutes and was not dependent on temperature. Subsequent assays were performed at 22°C for 15 minutes. Further experiments revealed that preincubation of the extract with the non-specific competitor for 15 minutes reduced the number of non-specific interactions. This step was adopted for all subsequent experiments.

Despite all the refinements made to the DNA binding reactions we were unable to significantly reduce the number of activities that could bind to the probe. This made it difficult to determine which activity might be important for regulation of *SSA3* in stationary phase. We therefore tested if the large number of shifts obtained was an

artifact of the gel system we were using. We reasoned that under the stringent conditions of electrophoresis in high ionic strength buffer, a DNA bound protein might dissociate from the probe as it migrates through the gel. This could result in the multiple shifts that are detected under high-ionic conditions. To test this we performed binding reactions using the optimal conditions determined above, and resolved the protein-probe complexes under low-ionic conditions (see Materials & Methods). Under these conditions the number of binding activities obtained was dramatically reduced. Therefore we continued to use the low-ionic gel system in our attempts to detect DNA binding specific to the PDS element within our probe.

Previous *in vivo* studies demonstrated that constructs containing one of two mutations (designated M2 and M3; Fig. 1.2b) within the PDS element were impaired in their ability to induce transcription of a reporter construct upon entry into stationary phase (Boorstein and Craig, 1990b). In an effort to identify binding activities that were specific for the PDS element and dependent on sequences within the M2 and M3 region, gel shifts were performed using probes containing either of these mutations. By comparing shifts obtained using the these mutant probes with shifts obtained using the wild type probe we identified two shifts that were not present when mutant probes were used. This indicates that the missing shifts were dependent on the sequences that were mutated in M2 and M3.

A complementary method for identifying the specific PDS binding activity was to use wild type probe and unlabelled WT, M2, or M3 oligonucleotides as competitors (Fig. 1.2b). When using the mutant oligonucleotides as cold competitors, all non-specific interactions with the probe are eliminated, leaving only the interaction that is dependent on the M2 or M3 sequences. Contrary to the experiments using mutant probes, we obtained only one shift that was dependent upon the presence of the M2 or M3 sequence (designated by the S in Fig. 1.3). This suggested that the second shift obtained using mutant probes may be an artifact of using poly(dI-dC) · poly(dI-dC) as a competitor. We therefore chose to use cold mutant oligonucleotides to detect the specific binding activities in subsequent experiments. From the results in Fig. 1.3, we concluded that it

was possible to detect activities that could bind to the PDS element and respond to mutations that were known to prevent the stationary induction of *SSA3 in vivo*.

An activity that binds specifically to the PDS element is present in log, PDS, and stationary phase extract

As outlined above we used cold competitors containing one of two mutations within the PDS element (M2 or M3) to identify the shifted complex that was specific for the PDS element (Fig. 1.2b). Having identified an activity that was capable of binding to the PDS element and dependent upon the sequences required for induction of *SSA3*, we tested for the presence of this activity at different points of the yeast growth curve (Fig. 1.1a). Specifically, we performed gel mobility shift assays under optimized conditions using yeast extracts derived from log, PDS, or stationary phase cells to test if the activity was present only in stationary extracts. Yeast strain BJ5626 was grown to an OD_{600} of either 0.5 (log cells), 4.5 (PDS cells), or 8.5 (stationary phase cells) at which point the cells were harvested and used to make whole cell extracts. We compared the abilities of these extracts to retard the mobility of the 33 nucleotide probe which contained the PDS element.

Figure 1.3 shows a comparison of the PDS-specific DNA binding activities present in log, PDS, and stationary extracts. Lanes 1, 5, and 9 demonstrate binding activities present when no competitor is used. When a WT oligonucleotide (Fig. 1.2b) is used as a competitor in the binding reaction, virtually no binding activities are observed in stationary extracts (lane 10). However, when oligonucleotides containing either the M2 or M3 mutation are used as competitor a single shifted species is reproducibly observed in this extract (lanes 11 and 12; denoted by S). This activity is therefore dependent on sequences that are mutated in M2 and M3 (Fig. 1.2b). This PDS-specific binding activity is also present in log and PDS extracts (lanes 3, 4, 7, and 8). We conclude that yeast whole cell extracts derived from log, PDS, and stationary phase cells contain an activity that binds to sequences within the PDS element. This activity may be involved in the induction of *SSA3* after the diauxic shift.

The three extracts also contained non-specific binding activities (N in Fig. 1.3). These non-specific shifts did not vary with competitor used (WT, M2, or M3). On the other hand, the presence of these non-specific shifts was to some extent dependent upon the extract used. Log extracts contained a non-specific activity that was not present in PDS or stationary extracts (low mobility band designated by N in Fig 1.3). Again, this shift was not dependent on sequences within the PDS element required for *SSA3* induction. Stationary phase extracts contained an activity that was dependent on M2 sequences (arrowhead beside lane 8). However the detection of this activity was variable (see Fig. 1.4 & Fig. 1.6).

The PDS-binding activity is present in the nucleus of post-diauxic shift cells

Having identified a PDS binding activity that is present in log, PDS, and stationary phase whole cell extracts, we wanted to determine if the activity was present in the nucleus, consistent with it being a transcription factor. We therefore prepared nuclear extracts from post-diauxic shift cells and performed gel-mobility shift assays as described above. Figure 1.4 illustrates that the activity dependent on M2 and M3 sequences is present in nuclear extracts made from post-diauxic shift cells (lanes 1,2, and 3; denoted by S). This demonstrates that the PDS binding activity can be detected in the nucleus, as expected for a transcription factor.

***MSN2* and *MSN4* are required for the induction of *SSA3* after the diauxic shift**

Previous studies on the stress response of yeast *Saccharomyces cerevisiae* have identified two transcriptional activators, Msn2p and Msn4p, that mediate the induction of several genes in response to severe stresses such as carbon starvation, nitrogen starvation, osmotic and oxidative stresses (Martinez-Pastor et al., 1996, Schmitt and McEntee, 1996). It has been demonstrated that a yeast strain in which both of these proteins have been knocked out is unable to induce *HSP26*, *HSP12*, *CTT1*, and *DDR2* in response to these stresses. The only gene not affected was *SSA3*. Therefore *MSN2* and *MSN4* are not required for the induction of *SSA3* in response to severe stress. Since entry into stationary phase is a moderate stress (because nutrients are gradually depleted from the growth media over time), we wanted to determine if *MSN2* and/or *MSN4* were required

for the induction of *SSA3* after the diauxic shift. We compared *SSA3* transcript levels by Northern analysis in log versus PDS cells grown from four isogenic yeast strains. a wild type strain W303-1a, a strain in which *MSN2* has been knocked out (msn2), a strain in which *MSN4* has been knocked out (msn4), and a strain in which both *MSN2* and *MSN4* have been knocked out (msn2msn4). Lanes 1 and 2 in the top of Figure 1.5 show the levels of *SSA3* transcript in log and PDS wildtype cells respectively. This illustrates the dramatic induction of *SSA3* after the diauxic shift, as reported by others (Boorstein and Craig, 1990b; Choder, 1991). *In vivo* studies have demonstrated that the PDS element mediates an increase in transcription of a reporter gene after the diauxic shift (Boorstein and Craig, 1990b). This result argues against the possibility that the increase of *SSA3* transcripts in PDS cells is a result of an increase in the stability of the transcript. Lanes 3-6 show that induction of *SSA3* transcription in PDS cells also occurs in the msn2 and msn4 strains. On the other hand no induction of *SSA3* occurs in the double knock-out in which both *MSN2* and *MSN4* are deleted (lanes 7 and 8). As a loading control the bottom panel of Figure 1.5 shows an identical membrane that was probed with the *PDA1* probe. *PDA1* transcripts, which encode the E1α subunit of the pyruvate dehydrogenase complex from *Saccharomyces cerevisiae*, are stable at all points of a yeast growth curve (Wenzel et al., 1995). These results demonstrate that either *MSN2* or *MSN4* is required for the induction of *SSA3* after the diauxic shift.

The PDS shift is dependent upon *MSN2* and *MSN4*

Since either *MSN2* or *MSN4* is required for *SSA3* induction we tested if the activity we detected in our gel mobility shift experiments was dependent on *MSN2* and *MSN4*. To do this we performed gel-mobility shift assays using PDS whole cell extracts derived from strains msn2, msn4, or msn2msn4. In Figure 1.6 the specific shift is present in msn2 (lanes 2 and 3), and msn4 (lanes 5 and 6) but not in msn2msn4 (lanes 8 and 9). This result suggests that the PDS binding activity is dependent on the presence of either *MSN2* or *MSN4*.

Taken together these experiments suggest that the induction of *SSA3* after the diauxic shift is dependent on either *MSN2* or *MSN4*. Furthermore these two proteins are

required for an activity detected in nuclear and whole cell extracts that can bind to sequences in the PDS element required for transcriptional induction of *SSA3* after the diauxic shift (Boorstein and Craig, 1990b).

Discussion

We have detected an activity in yeast whole cell extracts that is capable of binding to the PDS element found upstream of the *SSA3* gene. This activity was present in extracts derived from log, PDS, and stationary cells. Furthermore we have shown that this PDS binding activity is present in the nucleus of yeast cells. These results are consistent with the hypothesis that the factor which binds to the PDS element is a transcriptional activator of *SSA3*.

Previous studies of the yeast response to severe stress identified two transcriptional activators, Msn2p and Msn4p, that are required for the transcriptional induction of a variety of genes with protective functions (Martinez-Pastor et al., 1996). Among the stresses that cause transcriptional induction of these genes is immediate nutrient depletion. Although these two transcriptional activators are not required for the induction of *SSA3* in response to immediate nutrient depletion, we found that Msn2p and Msn4p are required for *SSA3* induction in response to gradual nutrient depletion (Fig. 1.5). Furthermore, we demonstrate that the PDS-specific binding activity detected in our gel mobility shift assays is dependent on the presence of either Msn2p or Msn4p. Collectively these results suggest that Msn2p and Msn4p may be transcriptional activators for *SSA3* after the diauxic shift. Analysis of the transcript levels of other genes after the diauxic shift in the Msn2p and Msn4p knockouts would be valuable in determining if these proteins play a more general role in transcriptional induction following the diauxic shift.

Functional redundancy of Msn2p and Msn4p

Northern analysis demonstrates that either Msn2p or Msn4p alone is sufficient for *SSA3* transcriptional induction following the diauxic shift (Fig. 1.5). This result is not surprising since Msn2p and Msn4p are very similar to one another, sharing 41% identity in their protein sequences (Estruch and Carlson, 1993). It has been previously reported that the zinc fingers of Msn2p and Msn4p are identical at three key residues that are implicated in DNA sequence recognition (Pavletich et al., 1991). This suggests that the two proteins recognize the same sequence. Functional redundancy has already been

reported for these two proteins in carbon utilization (Pavletich et al., 1991) and transcriptional activation of *DDR2*, *CTT1*, *HSP12*, and *TSP2* following heat shock and DNA damage (Schmitt and McEntee, 1996). This transcriptional activation involves binding of the activator to the STRE upstream of these genes. These results indicate that Msn2p and Msn4p may act as redundant transcriptional activators of *SSA3* by binding to the PDS element which is similar to the STRE. Our results have also shown that either Msn2p or Msn4p is required for the PDS-specific binding activity detected in our extracts (Fig. 1.6). It may be that the probe retardation detected in our assays is directly due to Msn2p and Msn4p binding to the PDS element. The fact that either protein gives a similar shift in our gel mobility shift assays is not surprising since the proteins are of similar molecular weights. Msn2p and Msn4p have molecular weights of 77 kDa and 70 kDa respectively (Estruch and Carlson, 1993). Gel mobility shift assays using antibodies to Msn2p and Msn4p to supershift the PDS-protein complexes would establish if these are the PDS binding proteins detected in our gel mobility shift assays.

Possible mechanisms of transcriptional activation of *SSA3* after the diauxic shift

The result that the PDS-specific binding activity was present in log, PDS, and stationary extracts is somewhat surprising given that *SSA3* transcription is only induced following the diauxic shift (Fig. 1.3). There are two likely explanations for why this would be the case. First, it may be that the transcription factor is bound to the PDS element at all times and is preferentially modified after the diauxic shift to activate transcription of *SSA3*. At present no modifications of Msn2p or Msn4p have been reported but the two proteins each have many potential protein kinase A sites. Second, the transcription factor may be present in the cytoplasm during logarithmic growth and translocated (possibly due to post-translational modification) after the diauxic shift into the nucleus where it can then bind to the PDS element and activate *SSA3* transcription. Such a mechanism has been reported for the SWI5 yeast transcription factor. This factor is excluded from the nucleus by phosphorylation in a cell-cycle dependent manner (Jans et al., 1995). Nuclear translocation of Msn2p and Msn4p may be taking place since we have detected the PDS-specific binding activity in greater abundance in PDS nuclear

extracts than in log nuclear extracts (data not shown). However, since some PDS binding activity was observed in log nuclear extracts (data not shown), it remains possible that a mechanism other than regulated nuclear translocation could account for the transcriptional induction of *SSA3* after the diauxic shift. A careful comparison of the PDS binding activity in the cytoplasmic and nuclear fractions of log and stationary cells should help in determining if nuclear translocation occurs during the growth cycle in liquid culture.

The RAS-protein kinase A pathway and the stress response

Previous studies have suggested that multiple signaling pathways are involved in the yeast response to stress. One signaling pathway in particular is implicated in the general stress response. This is the RAS-protein kinase A pathway. Both the STRE and the PDS element are negatively regulated by protein kinase A (Marchler et al., 1993; Boorstein and Craig, 1990b; Belazzi et al., 1991; and Engelberg et al., 1994). Despite these findings little is known about the mechanism by which this pathway affects transcriptional activation. Protein kinase A activity may either directly or indirectly repress transcriptional activation by the STRE and PDS element. One model suggests that the transcriptional activators (possibly Msn2p and Msn4p) that bind to the STRE and PDS element are inactivated by protein kinase A phosphorylation. Such a mechanism has already been observed for the *ADHII* gene. The transcriptional activator of this gene, ADR1, has been shown to be inactivated by protein kinase A phosphorylation under conditions of high cAMP concentrations (Cherry et al., 1989).

Complex roles of Msn2p and Msn4p in response to stress

Given the result that both the PDS element and the STRE are negatively regulated by the RAS-protein kinase A pathway and the possibility that they both bind the transcriptional activators Msn2p and Msn4p, how do these elements differ in the yeast stress response? Msn2p and Msn4p are absolutely required for the induction of STRE containing genes (such as *CTT1*) in response to severe stresses such as immediate carbon depletion (Martinez-Pastor et al., 1996). However, Msn2p and Msn4p are dispensable

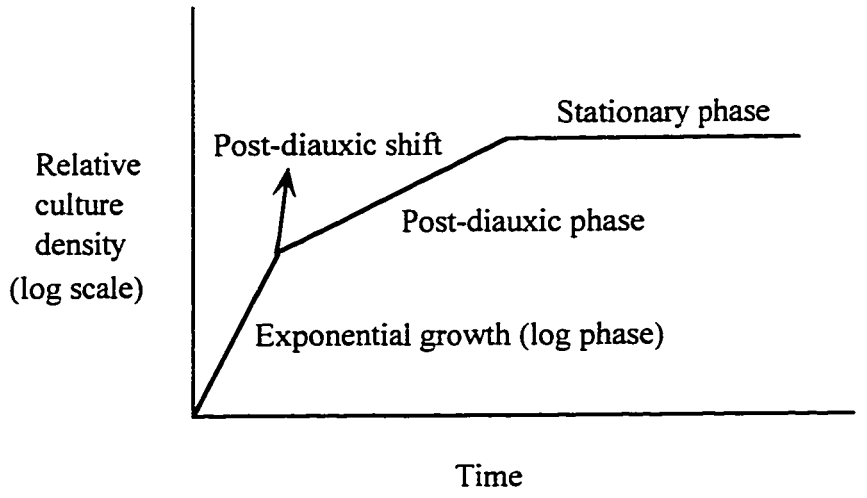
for the induction of *SSA3* in response to severe stresses like immediate carbon depletion. Our results show that these two proteins are necessary for the PDS element mediated induction of *SSA3* in response to gradual nutrient depletion. This implies that Msn2p and Msn4p play complex roles in the yeast stress response. They are clearly required for induction of STRE controlled genes such as *CTT1* in response to severe stresses but may only be required by PDS controlled genes such as *SSA3* in response to moderate stress. If Msn2p and Msn4p are only required for transcriptional induction of PDS element containing genes in response to moderate stress, what mediates the induction of these genes in response to severe stresses?

SSA3 induction in response to severe stress, a possible role for the HSE

It may be that in the absence of Msn2p and Msn4p other cis-acting elements upstream of the *SSA3* gene (or other PDS element containing genes) can mediate transcriptional induction in response to severe stress. The HSE (heat shock element), located 13 nucleotides downstream of the PDS element may serve such a purpose. This element has been shown to be dispensable for *SSA3* transcriptional induction following the diauxic shift (Boorstein and Craig, 1990b). It is required for induction in response to heat shock (Boorstein and Craig, 1990a) but it has not been tested for a possible role in response to other severe stresses such as those studied by Martinez-Pastor et al. (1996). *UBI4*, another gene whose transcripts are present after the diauxic shift, also does not require Msn2p and Msn4p for induction following severe stress (Martinez-Pastor et al., 1996). This gene has the putative STRE element and an upstream HSE element which could possibly mediate *UBI4* transcriptional induction in the absence of Msn2p and Msn4p. Therefore, it may be that the HSE element can mediate transcriptional induction of *SSA3* and *UBI4* in response to severe stress. In STRE containing genes that do not contain HSEs, Msn2p and Msn4p and the STRE may respond to both severe and moderate stresses. It has already been demonstrated that the STRE element mediates heat shock induction (severe stress) of *CTT1*, a gene that does not have a HSE (Marchler et al., 1993). Furthermore, heat shock induction of *CTT1* requires Msn2p and Msn4p (Martinez-Pastor et al., 1996). Further studies will be necessary to determine if the above

scenario holds true. Mutational analysis of both the *SSA3* and *UBI4* HSE would be valuable in assessing its role in severe stress response. Also, it would be interesting to know what prevents Msn2p and Msn4p from responding to severe stresses (as they do in STRE containing genes) in the case of *SSA3*. In addition, an analysis of the role of the STRE in response to moderate stresses will be important in determining if the STRE is important for all stresses.

A.



B.

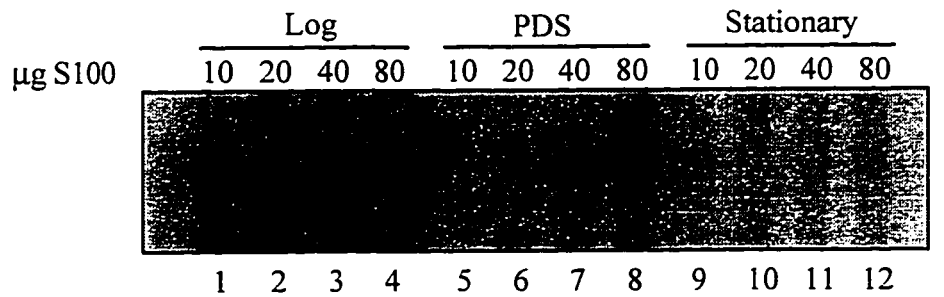
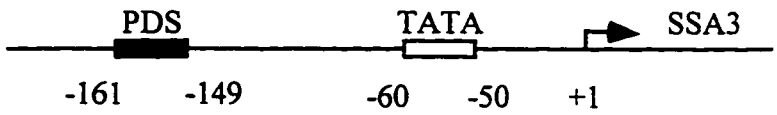


Figure 1.1. A. Typical growth curve of yeast *Saccharomyces cerevisiae*. Cell density measured by OD_{600} is plotted versus time. **B.** Transcription by RNA polymerase III is inhibited as yeast cells enter into stationary phase. Yeast strain RS188 was grown to an OD_{600} of 0.1 for log cells, 4.5 for PDS cells and 8.5 for stationary phase cells and used in the preparation of whole cell extracts. The extracts were assayed for RNA polymerase III transcription using a template which contains the 5S rRNA gene (pY5S). Lanes 1-4 show transcription products using 10, 20, 40, and 80 µg of log extract. Lanes 5-8 show products using the same amounts of PDS extract, and lanes 9-12 show products using the same amounts of stationary phase extract.

A.



B.

Oligonucleotides (33mer) used for gel shifts assays:

WT: TGGGTGCCCTTAATTAGGGATCGCTGTGGAAAG
ACCCACGGGAATTAATCCCTAGCGACACCTTTC

M2: TGGGTGGTCGACATTAGGGATCGCTGTGGAAAG
ACCCACCAGCTGTAATCCCTAGCGACACCTTTC

M3: TGGGTGCCCTTAATGTCGCTGTGGAAAG
ACCCACGGGAATTACAGCGACACCTTTC

Figure 1.2. **A.** Diagram of the major regulatory elements of the *SSA3* gene. The PDS element (shaded box) is located at position -161. The TATA box (open box) is located at -60. **B.** Sequences of the three double stranded oligonucleotides used for gel mobility shift assays. The WT oligonucleotide contains the PDS element (underlined sequences). M2 and M3 contain 5 point mutations each (shaded boxes) which have been found to impair the cells ability to induce transcription of *SSA3* in stationary phase (Boorstein and Craig, 1990b). These three oligonucleotides were either end-labeled and used as probes in gel mobility shift assays or used as cold competitors.

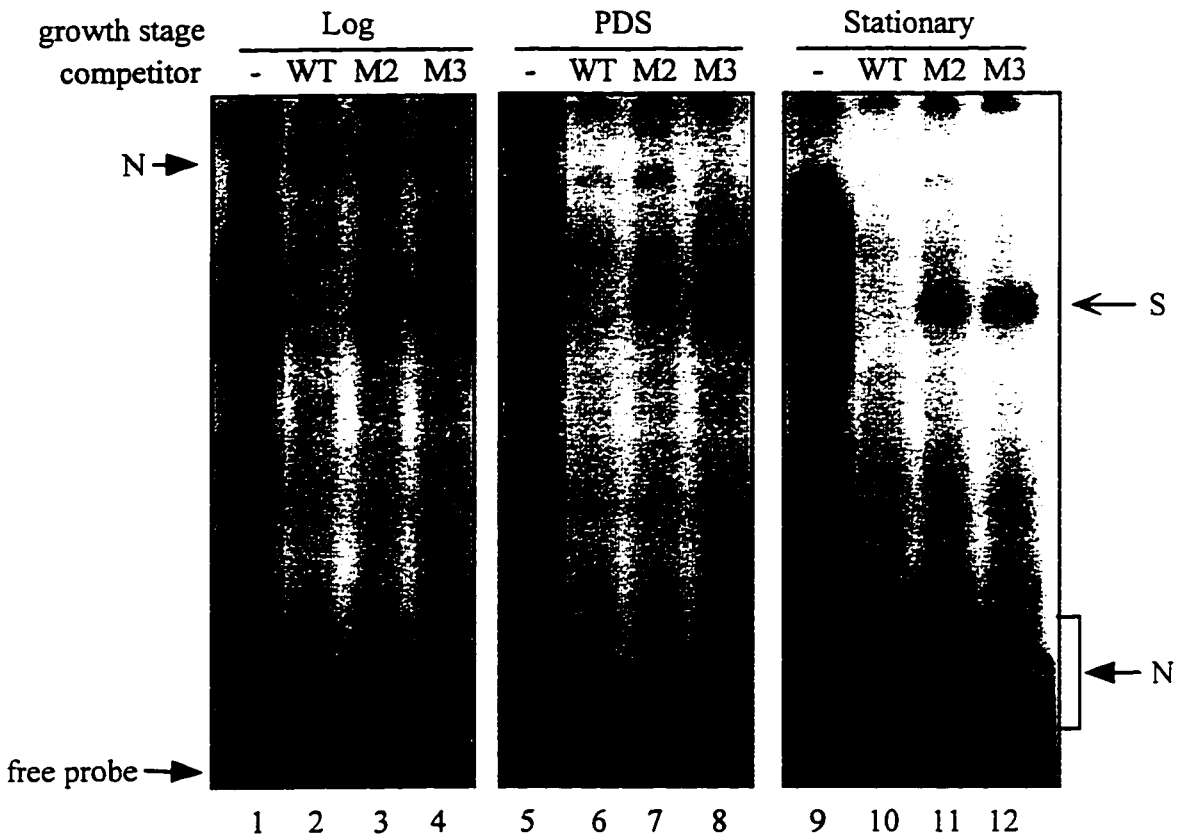


Figure 1.3. Comparison of the PDS-specific DNA binding activities present in log, PDS, and stationary extracts. Gel mobility shift assays were performed using the indicated whole cell extract and the WT oligonucleotide as probe (Fig. 1.2b). Whole cell extracts were prepared as described in Materials and Methods. Binding reactions containing the indicated unlabeled competitor were loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed under low ionic strength conditions. Lanes 1-3 illustrate activities from log extracts that can bind to the probe. Lanes 4-6 illustrate binding activities from PDS extracts and lanes 7-9 illustrate stationary binding activities. N denotes non-specific interactions with the probe. S designates PDS-specific DNA binding activities that are dependent on sequences mutated in M2 and M3. The arrowhead in lane 9 indicates an M2-specific complex present only in some stationary extracts (see text).

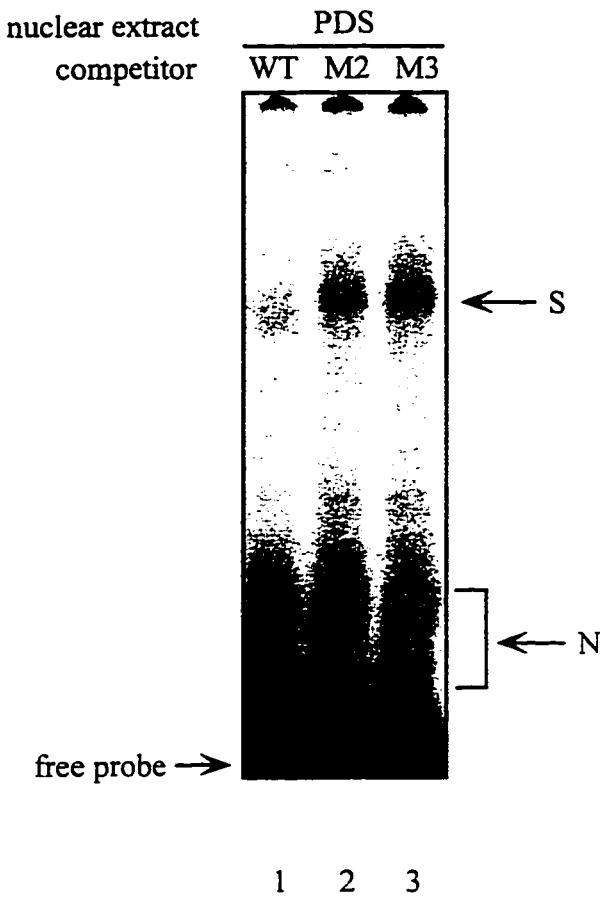


Figure 1.4. The PDS-specific DNA binding activity is present in nuclear extracts prepared from post-diauxic shift cells. Gel mobility shift assays were performed as in Fig. 1.3 except that nuclear extracts were used in the binding reactions. Nuclear extracts were prepared as described in Materials and Methods. Lanes 1-3 illustrate activities present in nuclear extracts that can bind to the WT probe. N denotes non-specific interactions with the probe. S denotes PDS-specific DNA binding activities that are dependent on sequences mutated in M2 and M3.

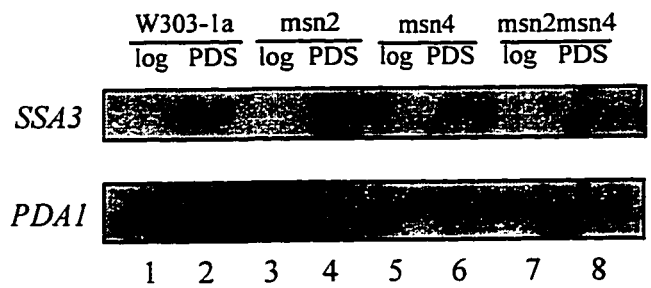


Figure 1.5. Effect of deletion of *MSN2* and *MSN4* on the induction of the *SSA3* transcript after the diauxic shift. Northern analysis was performed on RNA isolated from log and post-diauxic shift cells derived from a wild type strain W303-1a (lanes 1 and 2), msn2 (lanes 3 and 4), msn4 (lanes 5 and 6), and the double knockout strain msn2msn4 (lanes 7 and 8). The top panel illustrates a membrane probed for *SSA3* transcripts and the bottom panel illustrates an identical membrane probed for *PDA1* transcripts as a loading control (see Materials and Methods for details).

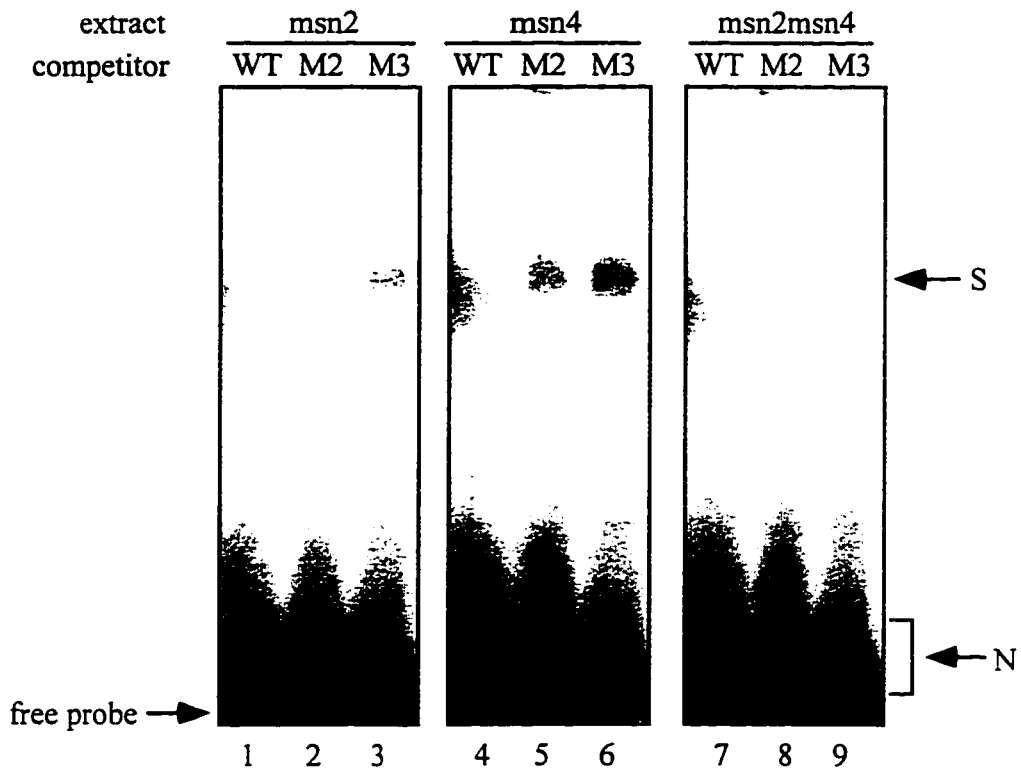


Figure 1.6. Effect of *MSN2* and *MSN4* deletion on PDS-specific DNA binding activities. Whole cell extracts were prepared from yeast strains msn2, msn4 and msn2msn4 grown to just after the diauxic shift (OD_{600} = 4.5). Gel mobility shift assays were performed as in Fig. 1.3 except that either the msn2 extract (lanes 1-3), the msn4 extract (lanes 4-6), or the msn2msn4 extract (lanes 7-9) was used in the binding reactions. N denotes non-specific binding activities. S denotes the PDS-specific DNA binding activity that is dependent on sequences mutated in M2 and M3.

CHAPTER TWO

Effects of the TOR signaling pathway on translation initiation and transcription by RNA polymerase III

Introduction

The immunosuppressant rapamycin

The macrolide antibiotic rapamycin has been the subject of intense study because of its potential as an immunosuppressant (Fruman et al., 1994; Heitman et al., 1992). This immunosuppression is the result of inhibition of interleukin-2 signaling in T-cells (Bierer et al., 1990a, 1990b; Dumont et al., 1990; Schreiber, 1991). In addition to its effects in T-cells, rapamycin also inhibits growth-factor stimulated proliferation of other cell types (Fruman et al., 1994). This suggests that the drug operates by interfering with signaling pathways triggered by growth factors. Much of the work in this field involved attempts to identify the components of these signaling pathways. In the yeast *Saccharomyces cerevisiae* phosphatidylinositol-3 kinases (PI-3 kinases) have been found to mediate rapamycin toxicity (Kunz et al., 1993). Mammalian homologs of the PI-3 kinases have also been found to interact with rapamycin (Sabatini et al., 1994). In addition to these proteins, other signaling molecules have also been found to be affected by rapamycin. The p70 S6 kinase (Kuo et al., 1992; Chung et al., 1992; Price et al., 1992; and Calvo et al., 1992) and the mitotic kinase $p34^{cdc2}$ (Morice et al., 1993) are inactivated by rapamycin treatment. Rapamycin also inhibits the activation of cyclin-dependent kinase-2 (cdk-2) and the formation of cyclin D/cdk-2 complexes (Albers et al., 1993). Currently, there are two models to account for the wide range of effects caused by rapamycin. One model proposes that rapamycin interacts with one critical intracellular target and that all of the above effects occur in response to this complex. Another model suggests that there are many different cellular targets of rapamycin and that the wide range of effects is due to interference with different signaling pathways.

FK-506 Binding proteins (FKBPs)

Rapamycin exerts its toxic effects by binding to a family of proteins called FKBPs. These proteins have *cis-trans* peptidyl-prolyl isomerase activity involved in protein folding. There are multiple FKBP isoforms each named by molecular weight. Each of these has a distinct subcellular localization. For instance FKBP12 (12 kDa) is found in the cytoplasm, FKBP13 (13 kDa) is found in the ER, and FKBP25 (25 kDa) is

found in the nucleus (Fruman et al., 1994). Rapamycin binds to the isomerase active site by mimicking the amide transition state of a peptidyl-prolyl bond (Michnick et al., 1991). This results in inhibition of isomerase activity. However, loss of isomerase activity does not cause the anti-proliferative activity observed when cells are treated with rapamycin, since other drugs that inhibit the isomerase reaction do not suppress the immune response (Schreiber et al., 1991; Bierer et al., 1990b). Furthermore, in yeast the FKBPs are nonessential yet they are still the intracellular receptors for rapamycin (Heitman et al., 1991a; Koltin et al., 1991; and Wiederrecht et al., 1991). It is now known that the effects of the drug are the result of a complex formed by binding of rapamycin to an FKBP. It is this complex that has toxic activity in the cell. The identification of the target of this rapamycin/FKBP complex as well as the signaling pathway this target is involved in has been the focus of much interest in the signal transduction field.

Targets of the rapamycin/FKBP complex

Studies analyzing signaling pathways affected by rapamycin in yeast have identified two PI-3 kinase homologs, TOR1 and TOR2, as targets of the rapamycin/FKBP12 complex (Kunz et al., 1993). Association of a TOR (target of rapamycin) protein with the rapamycin/FKBP12 complex likely inhibits the PI kinase activity, which in turn causes cell cycle arrest. Similar work done in mammalian cells also identified a PI-3 kinase homolog as an important target for the rapamycin/FKBP12 complex (Sabatini et al., 1994). Subsequent studies in yeast revealed downstream components of the signaling pathway that includes the TOR proteins. TOR2 and its nonessential homolog TOR1, are part of a signaling pathway that activates eIF-4E-dependent protein synthesis in response to nutrient availability. This activation results in G1 progression (Barbet et al., 1996). Thus by targeting the TOR proteins, rapamycin/FKBP12 inhibits the activation of eIF-4E-dependent protein synthesis and prevents progression through the cell cycle. More specifically, rapamycin/FKBP12 exerts its inhibition of translation by affecting the eIF-4E binding protein 4E-BP1 (Beretta et al., 1996). Rapamycin blocks the phosphorylation of 4E-BP1. This unphosphorylated form of 4E-BP1 has a high affinity for eIF-4E and its binding causes

translational inhibition (Lin et al., 1994; Pause et al., 1994). These studies have therefore identified a TOR signaling pathway that controls translational initiation in response to nutrient availability.

Several other proteins besides PI-3 kinases have been found in association with FKBPs (Fruman et al., 1994). Of these proteins, only the TOR proteins and one other enzyme, casein kinase II (CKII), are potential signaling kinases. CKII is a serine/threonine protein kinase (reviewed in Issinger, 1993; Litchfield and Luscher, 1993). *In vitro* experiments with mammalian lysates demonstrate that FKBP25 forms a complex with CKII (Jin and Burakoff, 1993). Jin and Burakoff (1993) also demonstrated that FKBP25, a high affinity receptor for rapamycin, is phosphorylated by CKII. However, rapamycin does not disrupt the FKBP25/CKII complex or inhibit phosphorylation. Therefore, it is not clear from this work if FKBP25/CKII is a target of rapamycin. Several findings do however suggest that CKII is involved in cellular activities that are affected by rapamycin treatment. This enzyme has been shown to play an important role in cellular proliferation (Pinna, 1990; Issinger, 1993; ole-MoiYoi, 1995) and its activity is elevated when cells are stimulated with epidermal growth factor (Sommercorn et al., 1987). Such findings implicate CKII as a signaling kinase involved in growth control. These results led us to postulate that the anti-proliferative effects of rapamycin could be partly due to an effect on CKII function, that would inhibit critical cellular activities controlled by CKII.

As described in the introduction (Regulation of RNA polymerase III transcription), CKII is required for transcription by pol III (Hockman and Schultz, 1996). The major products of pol III transcription are the 5S rRNAs and the tRNAs. These gene products are essential for protein synthesis and therefore are required for growth. It has been demonstrated that the production of these transcripts is under stringent growth control (Tower and Sollner-Webb, 1988). Thus, the levels of pol III initiation is directly proportional to the growth rate of cells. In summary, pol III transcription is essential for growth and requires CKII activity.

Because CKII exists in a complex with the rapamycin binding protein FKBP25 in mammalian cells, we postulated that CKII could interact with the yeast homolog of

FKBP25, yFKBP47. This postulate is supported by two further observations. First, CKII is highly conserved between yeast and humans (Pinna, 1990). Second, yFKBP47 is also phosphorylated by CKII *in vitro* (J. Heitman, personal communication). These observations suggest that CKII might be a target of rapamycin/FKBP47 in yeast.

Based on the above observations we predicted 1) that rapamycin would affect pol III transcription, and 2) that the transcriptional effect of rapamycin would occur through CKII. We found that extracts made from cells treated with rapamycin were defective in transcription by pol III and that this defect was not due to the presence of an inhibitory factor in rapamycin extracts. We also demonstrate that CKII is able to partially rescue this transcriptional defect. However, contrary to our hypothesis, CKII activity is not affected in extracts derived from cells treated with rapamycin. Instead our results show that the inhibition of pol III transcription by rapamycin acts through FKBP12 and therefore the TOR pathway. The transcriptional defect is not unique to pol III as transcription initiation by pol I is also affected. Furthermore the defect in pol III transcription is largely to due an inhibition of protein synthesis. We also demonstrate that while pol III transcription is inhibited as a result of interference with signaling to the translation initiation machinery through the TOR pathway, there may be a TOR-dependent pathway that signals directly to the transcription machinery.

Results

Yeast whole cell extracts derived from rapamycin treated cells are deficient for transcription by RNA polymerase III

In order to test the possibility that rapamycin affects pol III transcription we performed *in vitro* transcription reactions using pol III templates and yeast whole cell extracts derived from cells treated with either rapamycin or a placebo. Yeast strain RS188 was grown in complete media to an OD_{600} = 0.1 at which point rapamycin or a placebo was added to a final concentration of 1 μg/ml. Cells were grown for 24 hours, when >90% of the rapamycin treated cells were large and unbudded, characteristic of G1 arrest. Cells were then harvested to make whole cell extracts.

In vitro transcription of a plasmid containing either the yeast 5S ribosomal RNA gene or the leucine tRNA gene was compared in the two extracts. Transcription of the 5S rRNA template gives rise to a 118 nucleotide transcript and the leucine tRNA gene gives rise to a 135 nucleotide transcript which is quickly processed (Fig. 2.1b; lower band). Figure 2.1a shows the result when increasing amounts of extract were used in the transcription reaction. There is a clear inhibition of transcription of the 5S gene at all points of a protein titration in the extract derived from rapamycin treated cells as compared to extracts from placebo treated cells (Fig. 2.1a; compare lanes 1-5 with 6-10). This result is a global effect on pol III transcription, since inhibition was also observed when the leucine tRNA gene was used as a template (Fig. 2.1b). In addition the defect is unlikely to be an indirect result of G1 arrest, given that extracts derived from a cdc28 ts mutant, which arrests at G1 when shifted to the non-permissive temperature, are not deficient in transcription (Fig. 2.2a). Furthermore the decrease in transcription is not due to cell death. This possibility was ruled out in a plating experiment where an equal number of cells from placebo and rapamycin treated cultures were grown on rich YPD plates. Both conditions gave rise to the same number of colonies (Fig. 2.2b) indicating that rapamycin treated cells are still viable; we conclude that the transcription effect cannot be an indirect result of cell death. In addition, other activities in these extracts such as chromatin assembly are not affected (data not shown). Taken together these

results demonstrate that yeast whole cell extracts derived from rapamycin treated cells are deficient for transcription by pol III.

The deficiency in polymerase III transcription is not due to an inhibitory factor present in rapamycin extracts

We subsequently tested if the defect in transcription was due to the presence of an inhibitory factor in rapamycin treated extracts. We performed a mixing experiment where an equal amount of extract from placebo cells was mixed with an equal amount of extract from rapamycin cells and used in a transcription reaction. If there was an inhibitory factor present in the rapamycin extracts, mixing with placebo extract should cause a decrease in transcription as compared to the placebo alone. Figure 2.3 shows the results of this mixing experiment. Combining 10 μg of rapamycin extract with 10 μg of placebo extract resulted in an increase in the levels of leucine tRNA transcript greater than the 10 μg of placebo condition alone and comparable to the 20 μg placebo condition (Fig. 2.3; compare lane 3 with lanes 1 and 2). Since the treated extract does not inhibit the placebo extract, the treated extract is unlikely to contain an inhibitory factor. The fact that the levels of leucine tRNA transcript obtained when the extracts were mixed was comparable to the condition in which 20 μg of placebo was used suggests that treated extracts may be missing a stimulatory factor for pol III transcription.

Inhibition of polymerase III transcription by rapamycin acts through FKBP12 and therefore the TOR pathway

Previous work in yeast demonstrated that cell cycle arrest caused by rapamycin requires the proline rotamase FKBP12. The complex formed by binding of rapamycin to this protein acts as the toxic agent and inhibits a signaling pathway involving TOR proteins. Rapamycin when complexed with the FKBP12 binds to TOR, resulting in cell cycle arrest (Heitman et al., 1991b). This finding lead us to speculate that the TOR pathway may signal to the pol III machinery. To test this possibility we examined the effect of rapamycin treatment on pol III transcription in two different yeast strains, a wild type strain (FPR1) and a strain in which FKBP12 is knocked out (fpr1). Cells from these

two strains were treated with a placebo or with rapamycin and the corresponding whole cell extracts were assayed for pol III transcription as before. The top panel of Figure 2.4 illustrates that the FPR1 strain is deficient for pol III transcription as it was for RS188 at all points of a protein titration (Fig. 2.4, top; compare lanes 1-4 with 5-8). On the other hand, pol III transcription in the fpr1 strain is unaffected by rapamycin treatment (Fig. 2.4, bottom; compare lanes 1-4 with 5-8). From this result we conclude that the defect in pol III transcription is due to inhibition of signaling through the TOR pathway.

Casein kinase II is able to partially rescue the transcription defect caused by rapamycin but not to placebo levels

Because CKII is required for transcription by pol III (Hockman and Schultz, 1996) and has been reported to exist in a complex with a rapamycin binding protein (Jin and Burakoff, 1993), we tested the hypothesis that the rapamycin effect on pol III transcription is due to an effect on CKII. This hypothesis was tested in two steps. First, we tested if purified wildtype CKII could rescue pol III transcription in extracts derived from rapamycin treated cells. Second, we tested if rapamycin affected bulk CKII activity in whole cell extracts. Figure 2.5 shows the result of adding back increasing amounts of CKII to an extract derived from rapamycin treated cells. While 12.5 ng to 50 ng of CKII is able to stimulate transcription in these extracts, the effect is modest and the maximal level of transcription remains far below that of placebo extracts. Therefore while CKII can stimulate transcription in rapamycin extracts, the degree of inhibition of pol III transcription caused by rapamycin cannot solely be due to an inhibition of CKII. Consistent with this interpretation, we have also observed that CKII is limiting in some placebo extracts (data not shown).

The activity of casein kinase II is not affected in cell extracts derived from cells treated with rapamycin

In order to determine if CKII activity is responsive to the TOR signaling pathway, we measured the activity of CKII in whole cell extracts derived from rapamycin or placebo treated cells. CKII activity was measured by a filter binding assay in which a

specific peptide substrate of CKII is incubated with extract in the presence of [γ-^{32}P] ATP. The reaction is spotted onto P-81 paper, washed and counted. Figure 2.6 shows the results of this experiment; the blanks are performed without the peptide substrate and measure background activity. The level of background in both extracts is the same, as is the amount of phosphorylation of the CKII peptide substrate. Thus the activity of CKII is not affected in cell extracts derived from cells treated with rapamycin. This result argues against the hypothesis that CKII is inhibited by rapamycin treatment and that CKII is downstream of TOR. The partial stimulation of transcription obtained by adding back CKII to rapamycin extracts (Fig. 2.4) indicates that CKII activity is limiting but not inhibited in the whole cell extracts.

Transcription by RNA polymerase I is also inhibited by rapamycin

For two reasons we originally envisaged that the effect of rapamycin on transcription would be specific for pol III. First, previous work had demonstrated that CKII was required for transcription by pol III but not pol I or II (Hockman and Schultz, 1996). Second, previous work raised the possibility that rapamycin effects might include inhibition of CKII. However, in the light of the findings that rapamycin treatment does not inhibit bulk CKII activity and that purified CKII cannot rescue transcription in extracts derived from rapamycin treated cells, we decided to investigate the possibility that rapamycin has a more global (CKII-independent) effect on transcription. We therefore tested if pol I transcription was affected by rapamycin treatment. The activity of pol I in extracts derived from placebo or rapamycin treated cells was assayed by *in vitro* transcription using a pol I promoter construct that directs the synthesis of a uniquely tagged primary transcript. The synthesis of this tagged transcript was measured by an S1 nuclease protection assay in which a 50 residue oligonucleotide probe is hybridized to the products of the reaction and subsequently digested with S1 nuclease. The probe recognizes the first 35 nucleotides of the 35S rRNA primary transcript including the unique tag. The amount of this protected 35 nucleotide fragment remaining after S1 nuclease digestion is taken as a measure of pol I activity. As shown in Figure 2.7, transcription by pol I is inhibited at all points of a protein titration in rapamycin extracts,

as was the case for pol III transcription. Thus transcription by pol I is also inhibited by rapamycin.

Treatment with rapamycin causes an inhibition of translation

Recent work has demonstrated that the TOR pathway controls translation initiation and early G1 progression in yeast (Barbet et al., 1996). We examined the effect of rapamycin treatment on protein synthesis in the FPR1 wild type strain used for our analysis of transcription. Cells treated with rapamycin or a placebo were labeled with [^{35}S] methionine and the level of incorporation into protein was measured. Treatment of cells with rapamycin resulted in an approximate 80% reduction in incorporation of label (Fig. 2.8). The large effect on protein synthesis was obtained with the same concentration of rapamycin used in the *in vitro* transcription experiments but with a shorter treatment time (1 hr as opposed to 24 hrs in transcription experiments). This result suggests that the effect of rapamycin on transcription may be an indirect result of inhibiting protein synthesis. To test this we treated yeast cells with the translation inhibitor cycloheximide, prepared whole cell extracts, and assayed for pol III transcription. Treatment with cycloheximide resulted in an inhibition of pol III transcription at all points of a protein titration (Fig. 2.9). This suggests that the effect of rapamycin on transcription is a result of an inhibition of translation, possibly because short lived transcription factors are no longer being made.

To further compare the effect of rapamycin treatment on transcription and on translation, we also performed transcription reactions using extracts derived from cells treated for 1 hour with rapamycin. Treatment of cells for this amount of time did not result in any detectable cell cycle arrest. Even in the absence of cell cycle arrest, transcription was defective at all points of a protein titration (Fig. 2.10). However the degree of inhibition was not as pronounced as it was with 24 hours of treatment, in which cell cycle arrest was observed. Barbet et al. (1996) demonstrate that the effect of rapamycin on protein synthesis prevents the translation of a G1 cyclin, thereby resulting in cell cycle arrest. Whether the effect on transcription is also due to an inhibition of translation or whether there is a separate rapamycin sensitive pathway that signals to the

transcription machinery is unknown. Taken together these results indicate that some or all of the defect in pol III transcription caused by rapamycin is due to an inhibition of translation.

***In vitro* treatment with rapamycin inhibits transcription by RNA polymerase III and this inhibition is specific for the TOR pathway**

In order to directly test if the transcription defect is separate from the translation defect, we treated whole cell extracts with rapamycin and assayed for transcription by pol III. The experiment is based on the observation that the extracts are not capable of supporting translation as determined by measuring [^{35}S] methionine incorporation. As shown in Fig. 2.11a we observe a significant and reproducible inhibition of transcription (0.4 to 2 fold) when extract is incubated with rapamycin prior to addition of the DNA template. This suggests that rapamycin can affect transcription separate from an effect on translation. We also tested whether this *in vitro* effect was a result of inhibiting the TOR pathway. Rapamycin was added to extracts derived from both the FPR1 wildtype strain and the fpr1 strain in which FKBP12 is knocked out. If the *in vitro* treatment with rapamycin was affecting a signaling pathway other than TOR, the defect in transcription should be observed in both strains. Fig. 2.11b shows that inhibition of transcription was observed in the FPR1 strain (40% reduction) but not the fpr1 strain (compare lanes 1 and 2 with 3 and 4). This suggests that the TOR pathway may signal to both the translation machinery and to at least the pol III machinery. The effect of adding rapamycin directly to the transcription extract is not as dramatic as treating the cells with rapamycin (compare Fig. 2.5 and Fig. 2.11b). The huge decrease in transcription observed in treating cells with rapamycin is probably due to the inhibition of translation and a separate effect on transcription (Fig. 2.11), both of which are due to an inhibition of the TOR pathway.

Discussion

We have shown that treatment of yeast cells with the macrolide antibiotic rapamycin results in the inhibition of transcription by pol III. This effect was not an indirect result of cellular necrosis and it was not due to the presence of an inhibitor in extracts derived from rapamycin treated cells. Furthermore, it was not an indirect effect of cell cycle arrest because cdc28 cells showed no defects in pol III transcription and cells treated with rapamycin for 1 hour did not show cell cycle arrest but still showed a defect in pol III transcription. Contrary to our initial hypothesis, the effect on pol III transcription was not due to an effect on CKII since bulk CKII activity was not affected by rapamycin treatment and purified CKII was not able to completely rescue the pol III transcription defect. The defect in pol III transcription was subsequently found to be caused by inhibition of signaling through the TOR pathway, a pathway shown to control translation initiation and early G1 progression in yeast (Barbet et al., 1996). Furthermore the transcription defect was not specific to pol III as transcription by pol I was also affected. Translation experiments suggest that the observed inhibition of transcription is due to the translation inhibition caused by rapamycin treatment. However, we also show that the TOR proteins may signal directly to the transcription machinery as a TOR-dependent transcription defect in pol III was also observed when whole cell extracts were treated with rapamycin.

Inhibition of RNA polymerase III transcription by rapamycin is a result of inhibition of the TOR pathway

Contrary to our initial expectation, the observed defect in pol III transcription was not due to a rapamycin/yFKBP47 complex and most likely does not act through CKII. Instead our experiments demonstrate that the pol III defect occurs through interference of the TOR pathway. This was shown by examining pol III transcription in extracts from the fpr1 mutant. *FPR1* encodes FKBP12, a member of the FKBP family already shown to be required for rapamycin induced cell cycle arrest (Heitman et al., 1991b). The inhibition of pol III transcription by rapamycin is not observed in a strain in which FKBP12 is deleted. Due to the findings that rapamycin forms a complex with FKBP12

and that this complex interacts with the TOR proteins (Lorenz and Heitman, 1995; Stan et al., 1994) we believe that the pol III defect is due to interference with the TOR pathway.

Although FKBPs interact with many proteins in the cell, we are confident that the inhibition of transcription by pol III by rapamycin is due interference with the TOR pathway and not because of interference with the function of some other FKBP/protein complex. While FKBP12 has been reported to bind to the ryanodine receptor in the sarcoplasmic reticulum of skeletal muscle (Jayaraman et al., 1992), no other endogenous substrates of FKBP12 have been described. Although it is possible that FKBP12 may interact with some other protein, it only interacts with the TOR proteins in a rapamycin dependent manner. It has been demonstrated that interaction of TOR with FKBP12 requires a conserved Serine residue and that this association is dependent upon rapamycin (Stan et al., 1994). There is also evidence that RAFT1 (Rapamycin and FKBP12 target), a mammalian homolog of the yeast TOR proteins, directly interacts with mFKBP12 in a rapamycin dependent manner (Sabers et al., 1995; Sabatini et al., 1994; Chui et al., 1994). In these studies, no other proteins were detected that could interact with rapamycin/FKBP12. These results suggest that only the TOR proteins and their mammalian homologs can bind to rapamycin/FKBP12, and further that rapamycin/FKBP12 inhibits the TOR pathway exclusively. Because of this, we believe that the inhibition of pol III transcription by rapamycin is due to a rapamycin/FKBP12 complex that targets and interferes with the TOR pathway.

While it is possible that the defect in pol III transcription is a result of rapamycin preventing FKBP12 from interacting with a critical effector of pol III transcription, this is unlikely since FKBP12 is not essential in yeast and is not required for pol III transcription. None-the-less, experiments with TOR mutant yeast strains could help to confirm that the inhibition of pol III transcription is through interference with the TOR signaling pathway. For example if transcription inhibition does result from interference with TOR signaling then TOR mutants treated with rapamycin should show no defect in pol III transcription, as was the case for the FKBP12 deleted strain. TOR mutants have

already been shown to be rapamycin resistant with respect to cell cycle arrest (Lorenz and Heitman, 1995; Zheng et al., 1995; Kunz et al., 1993).

The TOR pathway controls G1 progression and translation

Recent studies have established that the TOR pathway controls translation initiation and early G1 progression in yeast (Barbet et al., 1996). Barbet et al. demonstrate that loss of TOR function (TOR knockouts or rapamycin treatment) results in the starvation response and sends yeast cells into stationary phase. Entry into stationary phase by TOR deletion or rapamycin treatment is the result of an inhibition of translation initiation. Translation is inhibited by preventing the phosphorylation of 4E-BP1, the repressor of the cap-binding protein, eIF-4E (Beretta et al., 1996). Unphosphorylated 4E-BP1 remains associated with eIF-4E and this results in the specific inhibition of cap-dependent translation (Beretta et al., 1996; Pause et al., 1994). Our own experiments support the results of Barbet et al. (1996) (rapamycin causes an inhibition of translation) since treatment of yeast cells with rapamycin results in ~80% reduction in the incorporation of [^{35}S] methionine into protein (Fig. 2.8).

The inhibition of transcription by pol III may be a direct result of the inhibition of translation caused by rapamycin. It may be that a labile transcription factor is no longer being made upon treatment with rapamycin and this results in the inhibition of pol III transcription observed. This is supported by the finding that yeast cells treated with the translation inhibitor cycloheximide also show a defect in transcription by pol III (Fig. 2.9). This decrease in pol III transcription as a result of an inhibition of protein synthesis has also been reported in mammalian cells (Tower and Sollner-Webb, 1988). Also, transcription by pol III is inhibited as yeast cells enter into stationary phase (Fig. 1.1a). This inhibition may also be due to a reduction in protein synthesis since global protein synthesis declines as cells enter stationary phase (Fuge et al., 1994). These findings suggest that the inhibition of transcription by pol III with rapamycin treatment may be completely or partly due to the inhibition of translation that occurs as a result of interference with signaling through the TOR pathway.

The TOR pathway may signal to both the translation machinery and the transcription machinery

The observation that rapamycin inhibits pol III transcription when added directly to the transcription extract suggests that the TOR pathway may also signal directly to the pol III machinery. Considering that whole cell extracts are incapable of supporting translation, our *in vitro* results suggest that the transcription response is dissociable from the inhibition of protein synthesis. The inhibition of pol III transcription obtained when the extract is treated with rapamycin is small in comparison to the defect when cells are treated. The massive decline in pol III transcription when cells are treated with rapamycin may be an indirect result of translation inhibition (loss of a labile transcription factor) and a direct result of inhibition of a component of the TOR pathway that signals to the transcription machinery.

Rapamycin may cause a global repression of transcription

We have also demonstrated that treatment of yeast cells with rapamycin results in an inhibition of pol I transcription. This suggests that the effect of rapamycin on transcription may be global and not restricted to pol III. It is not known whether the effect on pol I is due to interference with the TOR pathway and whether the effect is completely or partly due to an inhibition of translation. Previous studies have shown that pol I transcription is inhibited as a result of inhibition of protein synthesis (Tower and Sollner-Webb, 1987). Assaying for pol I and II transcription in cell extracts derived from TOR mutants treated with rapamycin would establish if the transcription effect is global and whether the defect in transcription is due to interference with the TOR pathway.

An inhibition of all three polymerases that is separate from an inhibition of protein synthesis would suggest that the TOR pathway signals to a shared component of the pol I, II, and III transcription machinery. Likely candidates for this component would be the TATA binding protein (TBP), which is required for transcription of all nuclear genes (Schultz et al., 1992), and the 27, 23, 14.5, and 10 kDa subunits shared by polymerases I, II, and III (Mosrin and Thuriaux, 1990; Young 1991; Thuriaux and Sentenac, 1992). Future experiments will determine if TBP or a polymerase subunit is

regulated by the TOR pathway, or if this signaling pathway affects a unique component of the pol III machinery.

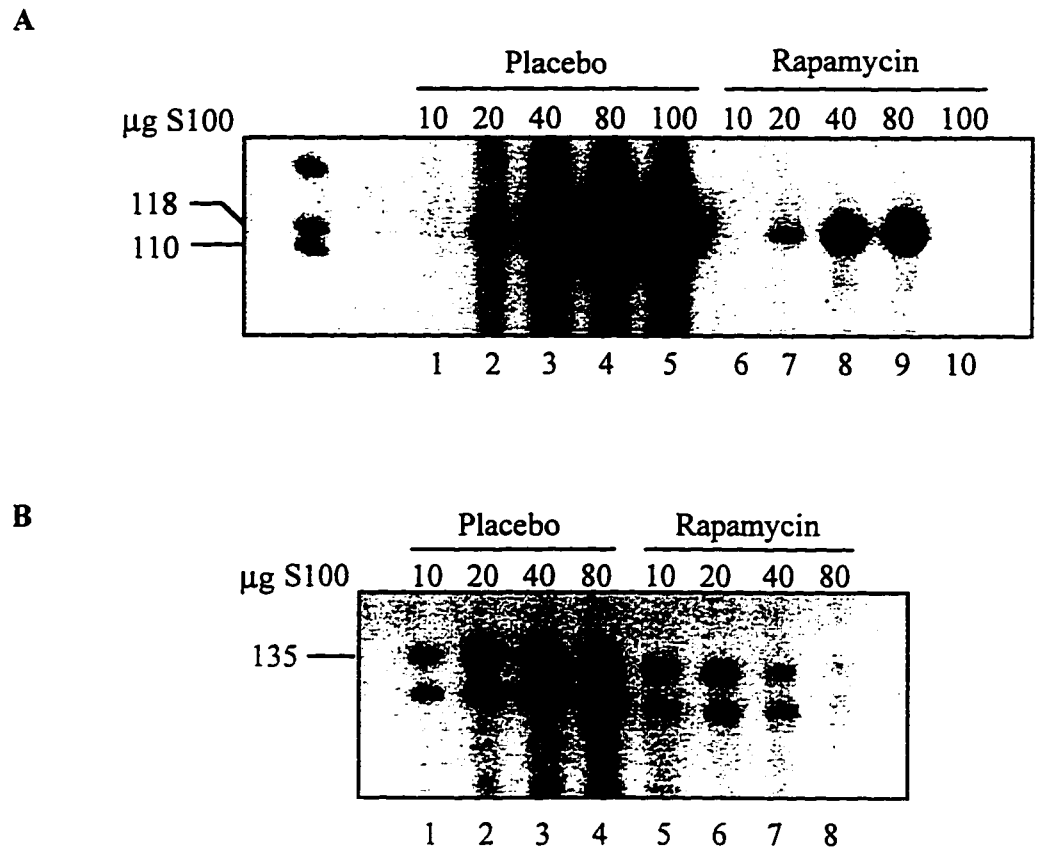
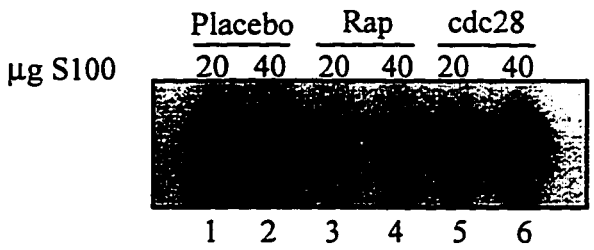


Figure 2.1. Effect of rapamycin treatment in yeast cells on transcription by RNA polymerase III. Yeast strain RS188 was grown to an OD_{600} of 0.1 and treated with either 1 µg/ml of rapamycin for 24 hours (reached OD_{600} = 3.0) or with an equal amount of placebo. The placebo treated culture was also grown to an OD_{600} = 3.0. Cells were harvested and used to make whole cell extracts. The corresponding extracts were assayed for RNA polymerase III transcription. **A.** Pol III transcription reactions using a plasmid which contains the 5S rRNA gene as a template. 10-100 µg of placebo (lanes 1-5) or rapamycin extract (lanes 6-10) were used in the transcription reactions (see Materials and Methods for details). **B.** Pol III transcription reactions using a plasmid which contains the leucine tRNA gene as a template. 10-80 µg of placebo (lanes 1-4) or rapamycin extract (lanes 6-10) were used in transcription reactions (see Materials and Methods for details).

A



B

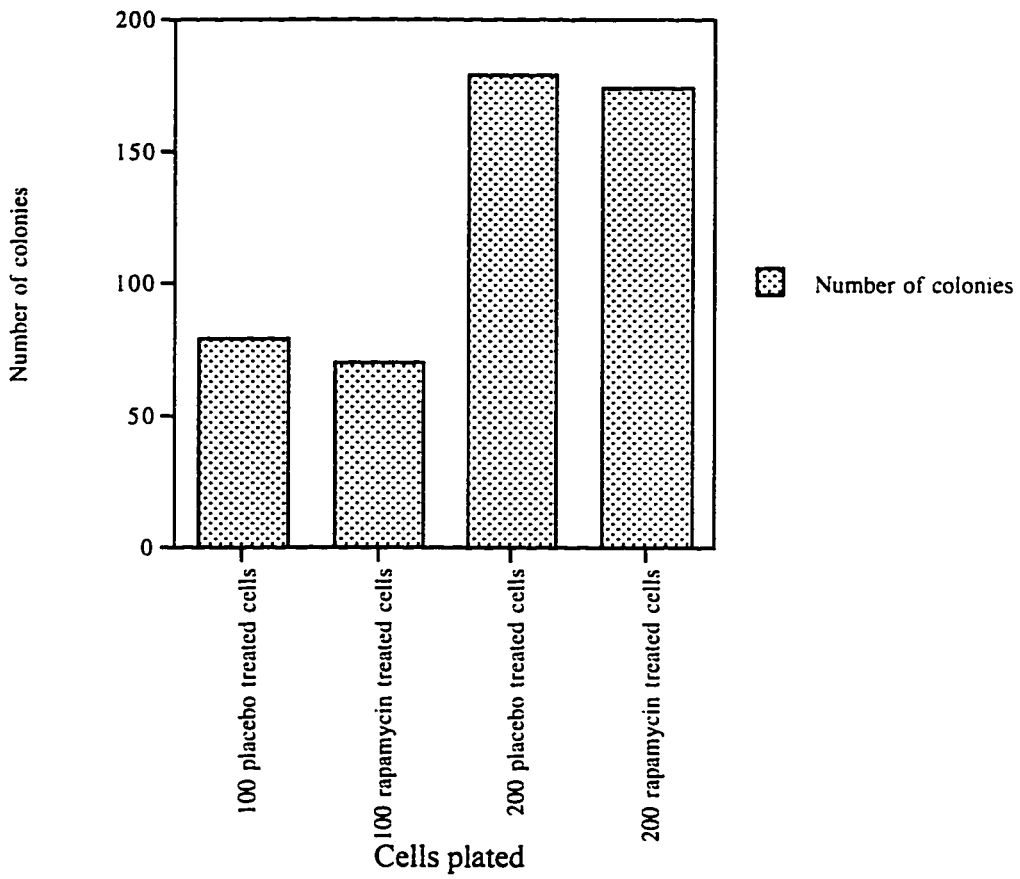


Figure 2.2. Effect of rapamycin treatment on polymerase III transcription is not due to apparent G1 arrest or cell death. **A.** Transcription reactions using the 5S gene as a template were performed using 20 or 40 µg of either placebo extract (lanes 1 and 2), rapamycin extract (lanes 3 and 4), or cdc28 extract (lanes 5 and 6). G1 arrest of cdc28 cells prior to extract preparation was confirmed by microscopic assessment of budding pattern. **B.** Yeast cells were treated with either a placebo or with 1 µg/ml of rapamycin as in Figure 2.1. After the 24 hour treatment either 100 or 200 cells from each sample were plated on YPD and the number of colonies on each plate were counted.

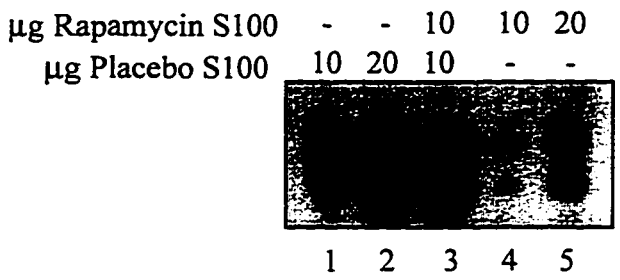


Figure 2.3. Mixing experiment using equal amounts of rapamycin and placebo extracts. 10 µg of placebo derived extract was mixed with 10 µg of rapamycin derived extract and assayed for pol III transcription using a leucine tRNA template (lane 3). The amount of transcription was compared to conditions in which either 10 or 20 µg of placebo extract was used in the transcription reaction (lanes 1 and 2), and which either 10 or 20 µg of rapamycin extract was used in the transcription reaction (lanes 4 and 5).

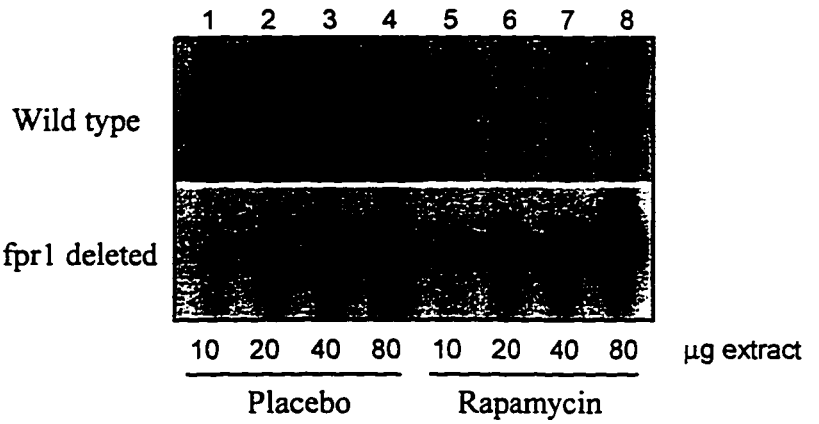


Figure 2.4. Effect of rapamycin treatment on pol III transcription in a FKBP12 (FPR1) deleted strain. Yeast strains FPR1 and fpr1 were treated with either rapamycin or a placebo as in Fig. 2.1 and assayed for pol III transcription using the 5S rRNA gene as a template. 10-80 µg of either placebo (lanes 1-4) or rapamycin extract (lanes 5-8) was used in transcription reactions. The top panel illustrates transcription reactions using FPR1 wildtype extracts and the bottom panel illustrates reactions using fpr1 extracts in which FKBP12 is deleted. The gel in the top panel was overexposed in order to detect the signal in the rapamycin treated extract.

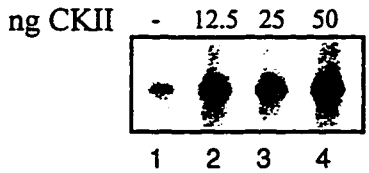


Figure 2.5. Effect on pol III transcription when adding purified casein kinase II to rapamycin extracts. 0 ng (lane 1), 12.5 ng (lane 2), 25 ng (lane 3), or 50 ng (lane 4) of CKII was added to extracts derived from rapamycin treated cells and polymerase III transcription was assayed using the 5S rRNA gene as a template.

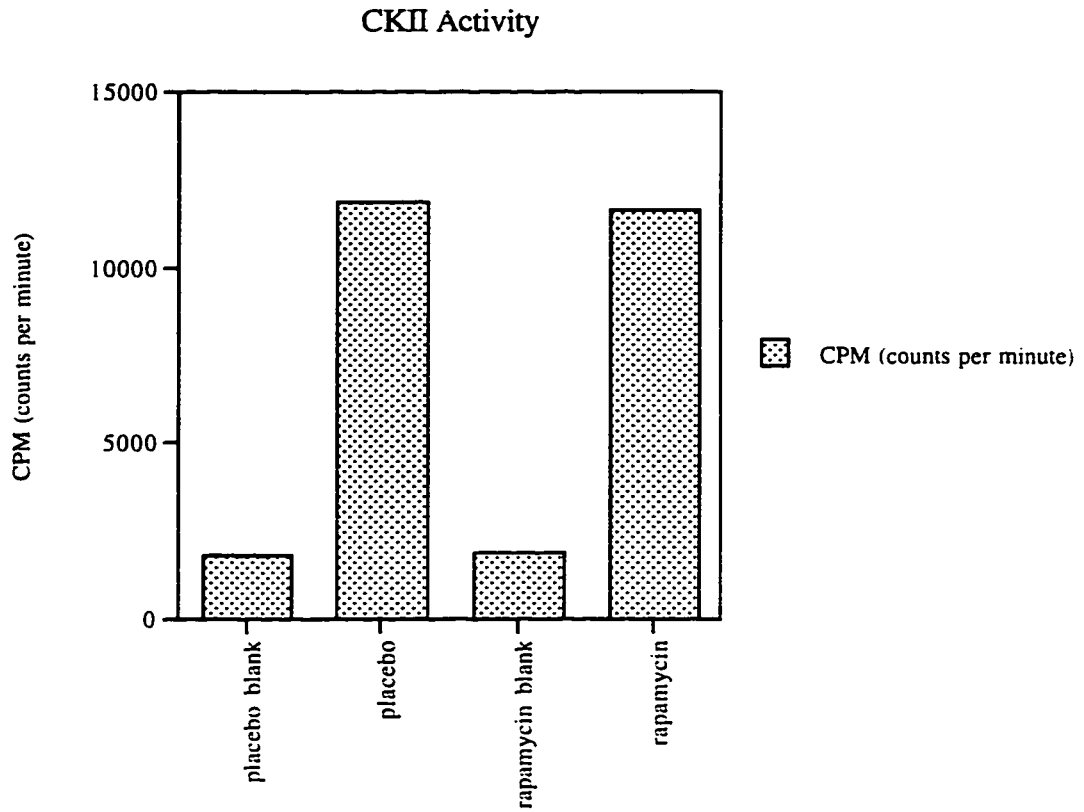


Figure 2.6. Effect of rapamycin treatment on CKII activity. Rapamycin or placebo extracts were assayed for their ability to phosphorylate a peptide specific for CKII. The specific peptide was incubated with extract in the presence of [γ-^{32} P] ATP (3000 μCi/ml. NEN). The reaction was spotted onto P-81 paper, washed, and counted in a liquid scintillation counter (see Materials and Methods for details). The graph also indicates the amount of phosphorylation in the extracts as determined by ^{32}P incorporation in reactions that did not receive the peptide substrate.

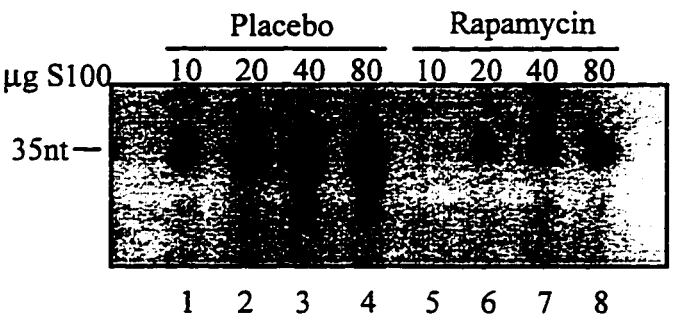


Figure 2.7. Effect of rapamycin treatment on transcription by RNA polymerase I. 10-80 µg of either placebo (lanes 1-4) or rapamycin extracts (lanes 5-8) were assayed for RNA polymerase I transcription using an S1 nuclease protection assay. Polymerase I transcription reactions were carried out using a template containing the promoter region of 35S rRNA gene. Nucleic acids were isolated from the reaction mix and hybridized to a 50 nt end-labeled probe which recognizes 35 nt of the tagged primary 35S rRNA transcript. The hybridization reaction was digested with S1 nuclease and products were resolved on an 8% denaturing gel (see Materials and Methods for details).

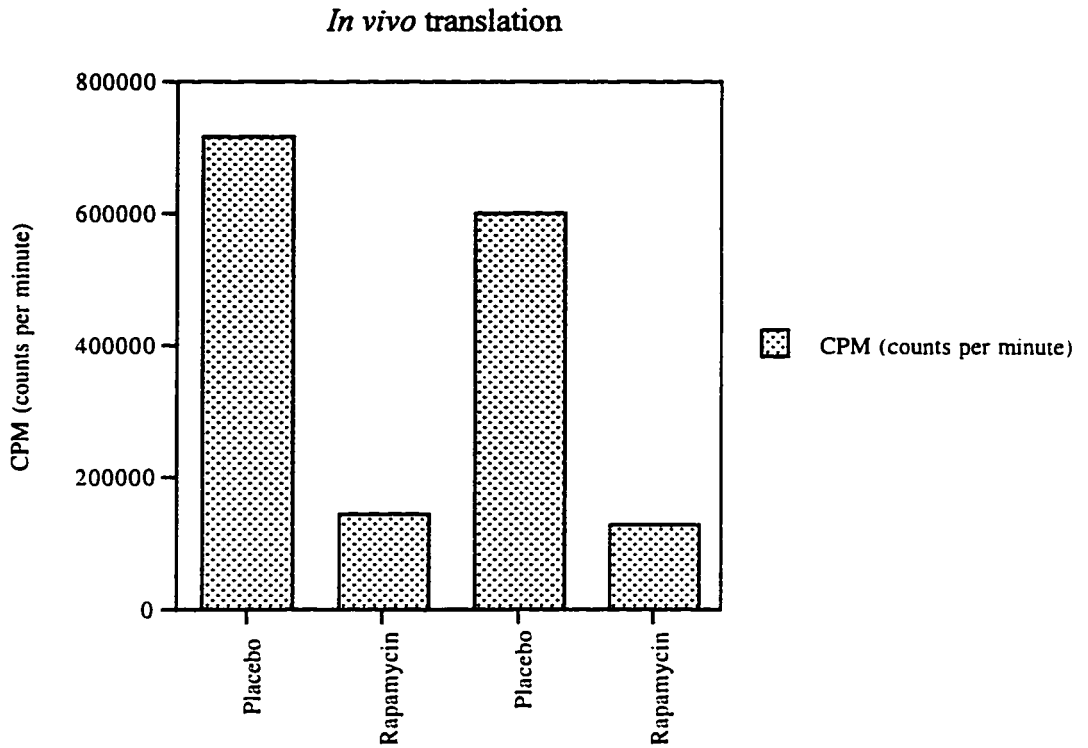


Figure 2.8. Effect of rapamycin treatment on protein synthesis *in vivo*. Yeast cells were treated with either a placebo or 1 μg/ml rapamycin for 1 hour. After 1 hour the cells were centrifuged and resuspended in MET^- media containing L-[^{35} S] methionine and fresh rapamycin. The cells were labeled for 20 minutes and the level of incorporation into protein was measured (see Materials and Methods). The graph illustrates two independent experiments.

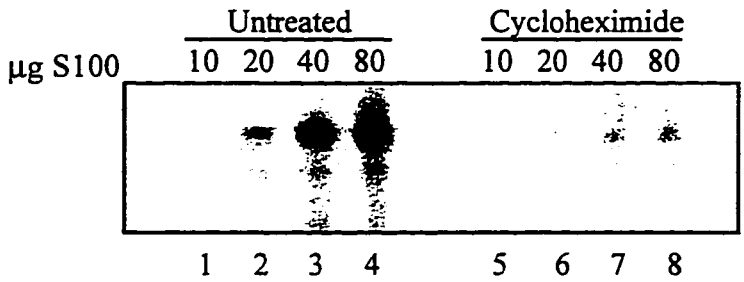


Figure 2.9. Effect of cycloheximide treatment on transcription by RNA polymerase III. Yeast cells were grown to an OD_{600} of 1.0 and treated with either water or cycloheximide for 30 minutes. After treatment the cells were harvested and used to prepare whole cell extracts. 10-80 µg of either an untreated (lanes 1-4) or cycloheximide extract (lanes 5-8) were used in pol III transcription reactions using the 5S rRNA gene as a template.

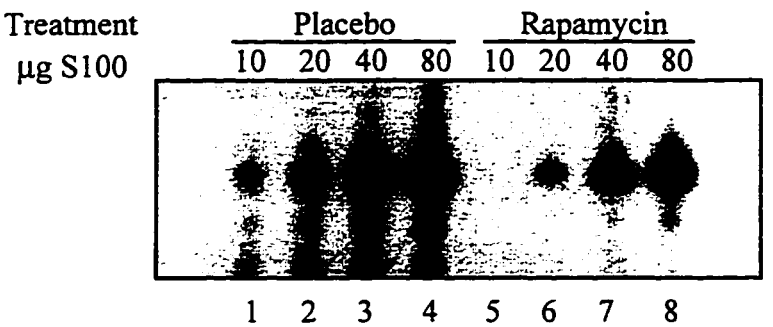


Figure 2.10. Effect of 1 hour treatment of yeast cells on transcription by RNA polymerase III. Yeast strain FPR1 was grown to an OD_{600} of 1.0 and treated with either a placebo or rapamycin to 10 μg/ml for one hour. After treatment the cells were harvested and used to prepare whole cell extracts. 10-80 μg of either placebo (lanes 1-4) or rapamycin extract (lane 5-8) were used in pol III transcription reactions, with the 5S rRNA gene as a template.

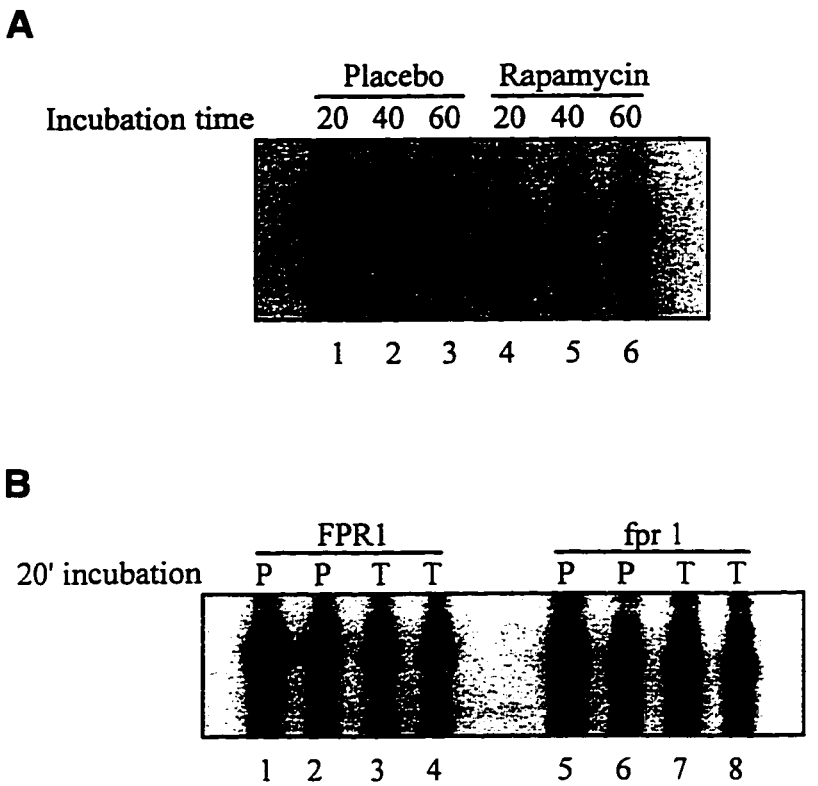


Figure 2.11. Effect on pol III transcription when adding rapamycin directly to whole cell extracts. **A.** Extracts from untreated, wild type cells were incubated with a placebo (lanes 1-3) or rapamycin (lanes 4-6) for either 20, 40 or 60 minutes. After the preincubation the extracts were assayed for transcription for pol III using the 5S rRNA gene as a template. **B.** Whole cell extracts derived from either FPR1 (lanes 1-4) or fpr1 (lanes 5-8) were incubated with a placebo (P) or with rapamycin (T, treated) for 20 minutes, after which they were assayed for pol III transcription using the 5S rRNA gene as a template. Each treatment was performed in duplicate.

Conclusions

The starvation response is extremely complex and involves the induction of some pol II genes and the repression of pol I and pol III transcription.

The sequence of events that occurs as yeast cells are gradually deprived of nutrients is just beginning to be understood. It is clear that entry into stationary phase is a very complex process. At the level of transcription, gradual nutrient depletion results in the induction of a small subset of pol II genes and the repression pol I and pol III transcription. The present work analyzed mechanisms responsible for both the induction and repression of transcription in response to gradual nutrient depletion.

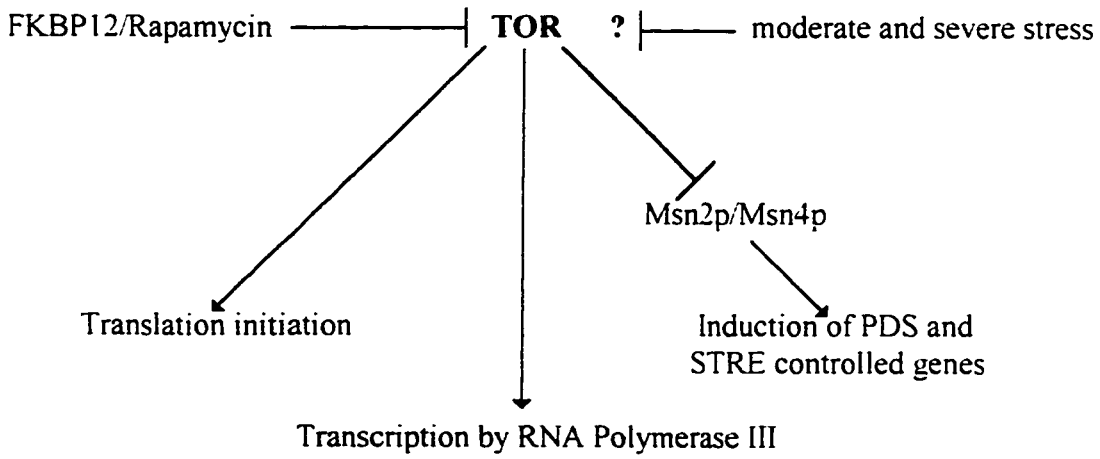
Our work has established that the related zinc finger proteins Msn2p and Msn4p are important for the induction of *SSA3* in response to slow nutrient withdrawal that occurs during entry into stationary phase. Msn2p and Msn4p are not required for the induction of *SSA3* in response to severe stress applied over a short period of time, although these same stresses induce some genes via a mechanism that is dependent on Msn2p and Msn4p (Martinez-Pastor et al., 1996). Based upon this observation and the work presented here, we conclude that while Msn2p and Msn4p are involved in the general stress response, their functions must be modulated by the type of stress signal (severe vs. moderate). Thus, the regulation of Msn2p and Msn4p is very sophisticated, such that *SSA3* induction is Msn-dependent in stationary phase but not in response to short term severe stress.

This work also demonstrates the use of the macrolide antibiotic rapamycin to induce the starvation response and repress pol I and pol III transcription. The defect in pol III transcription is a result of inhibiting signaling through the TOR pathway. Although the TOR pathway controls G1 progression (Barbet et al., 1996), the defect in pol I and pol III transcription is dissociable from cell cycle arrest. The work presented here suggests that TOR controls the synthesis of a labile pol III transcription factor as well as signals directly to the pol III machinery. From these results we conclude that while the TOR pathway directly effects pol III transcription, it mostly signals to the pol III machinery indirectly through an effect on translation.

The yeast transcriptional stress response, a possible central role for TOR.

Because inhibition of TOR also results in the pol II transcriptional stress response (Barbet et al., 1996), the TOR pathway may be affected by stress conditions. The above data are consistent with the following speculative model for the yeast transcriptional stress response (Fig. C.1). Interference with TOR results in the inhibition of translation and the induction of PDS and STRE containing genes (Barbet et al., 1996). We have shown that interference with TOR signaling also affects transcription by pol III and that the moderate stress of gradual nutrient depletion results in Msn2p/Msn4p dependent induction of *SSA3*. It may be that severe and moderate stresses interfere with the TOR pathway by independent mechanisms which have different requirements for Msn2p/Msn4p. Further studies will be necessary to determine whether such mechanisms exist.

Figure C.1. Model of yeast transcriptional stress response



Materials and methods

Materials

Yeast extract and Bacto Peptone were from Difco, S1 nuclease, α-amanitin, and cycloheximide were from Sigma, zymolyase was from Seikayaku Corporation (PDI Scientific), rapamycin was a gift from Dr. B. Leduc and S. Sehgal at Wyeth-Ayerst Research Laboratories, poly (dI-dC)·poly (dI-dC) was from Pharmacia, and TRIzol reagent was from BRL. The CKII peptide substrate was prepared by the Alberta Peptide Institute. It was purified to >95% homogeneity by high-pressure liquid chromatography and checked by amino acid analysis and mass spectrometry. Oligonucleotides were synthesized by the DNA Core Facility of the Biochemistry Department, University of Alberta, and gel purified. CKII (gift of C. V. C. Glover, University of Georgia) was purified to 95% homogeneity from commercial yeast as described by Bidwai et al. (1994). This protocol includes the use of high-salt buffers to prevent the formation of CKII filaments and α-subunit aggregates (Bidwai et al. 1992).

Yeast strains, Growth, and in vivo drug treatments

Yeast strain BJ5626 (MATa *ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3Δ200 prb1Δ1.6Rcan1 GAL*) was provided by S. Hahn, Hutchinson Cancer Research Center Seattle, WA). Strain RS188/W303-1A (MATa *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15* [*phi*$^+$]) is described in Rothstein et al. (1983). Strain cdc28 (MATα *cdc28-1, ade1 gal1 lys2 met14 his7 tyr1 A 364A*) is a gift of L. Hartwell and is described Hereford and Hartwell (1974). The FPR1 wildtype strain JK9-3Dα (MATα *ura3-52 leu2-3,112 trp1-1 his4 rme1 HMLa*) and fpr1 knockout strain JHY3-3B (JK9-3Dα *fpr::URA3*) were a gift from J. Heitman, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina (Cardenas and Heitman, 1995). Yeast strains msn2 (W303-1A *msn2-Δ3::HIS3*), msn4 (W303-1A *msn4-1::TRP1*) and msn2msn4 (W303-1A *msn2-Δ3::HIS3 msn4-1::TRP1*) were a gift form P. Estruch, Department of Biochemistry and Molecular Biology, University of Valencia, Spain and are described in Estruch and Carlson (1993).

Cells were inoculated into YPD media (2% yeast extract, 1% bactopeptone, and 2% dextrose) and cultured at 30°C with vigorous shaking. Cells were grown to the indicated OD_{600} and used to make whole cell extracts. Cell density measurements were made after making the growth culture 20 mM EDTA to disperse clumped cells (Padmanabha et al., 1990). For treatment of cells with rapamycin or placebo, cells of the indicated strain were inoculated into YPD as above and grown to an OD_{600} = 0.1 at 30°C with shaking. Rapamycin was prepared as instructed by Dr. B. Leduc and S. Sehgal at Wyeth-Ayerst Research Laboratories. Briefly a 2 mM solution was made in 90% ethanol/10% Tween-20. This solution was stored at -70°C until further use. Rapamycin was added to a final concentration of 1 μg/ml, or an equal volume of placebo (90% ethanol/10% Tween-20) was added to the yeast culture. Treatment with rapamycin was for either 24 hours or 1 hour (10 μg/ml) as indicated, at which point OD_{600} measurements were made and the cells harvested and used to make whole cells extracts. For the placebo condition, cells were grown to the same OD_{600} as the rapamycin treated cells, harvested and used to make whole cell extracts. Yeast strain cdc28 was grown in YPD at 30°C to an OD_{600} = 1.0. The cells were then incubated at 37°C for 4 hrs. After 4 hours at 37°C, ~90% of the cells were arrested in G1. The cells were then harvested and used to make whole cell extracts. Cells were treated with cycloheximide as in Grossman et al. (1969). One litre of cells was grown in YPD to an OD_{600} of 2.0 at which point the culture was split into 500 ml aliquots. 10 ml of water was added to one flask, and 10ml of a 10 mg/ml solution of cycloheximide in filter sterilized water was added to the other (200 μg/ml final concentration). The cultures were incubated at 30°C for 30 minutes with vigorous shaking. After incubation the cells were harvested and used to make whole cell extracts.

Preparation of whole cell extracts

Whole cell extracts were prepared according to Schultz et al. (1991) with modifications. Cells grown as above were cooled by pouring over crushed ice and then spun at 4000 rpm for 4 minutes at 4°C. The cell pellet was weighed and washed successively in ice-cold distilled water (2 vol/g of cells) and 1.3 vol of extraction buffer (100 mM Hepes-KOH,

pH 7.9, 245 mM KCl, 5 mM EGTA, 1 mM EDTA, 2.5 mM dithiothreitol (added fresh)). The cell pellet was then resuspended in 1.3 vol of extraction buffer containing 0.2 mM PMSF (phenylmethylsulfonyl fluoride), 10 mM benzamidine-HCl, 25 ug/ml TPCK (L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone), 5 µg/ml leupeptin, 3.5 µg/ml pepstatin and 10 µg/ml aprotinin and pelleted as above. The resulting cell paste was loaded into a syringe, extruded into liquid nitrogen, and then stored at -70°C.

Cells were broken open in a Moulinex coffee mill using dry ice as the coolant. An amount of dry ice was reduced to a powder in the coffee mill (sufficient to cover the blades but not the top of the pin that supports the blades). 2.5 g of frozen yeast noodles was added to the mill. The mill was run for 5-6 minutes producing a frozen powder. The powder was transferred to an ice-cold beaker and slightly warmed to speed thawing. Once the powder began to thaw, 1.3 vol of extraction buffer with protease inhibitors was added. The suspension of broken cells was briefly mixed by pipetting and then spun in a Beckman ultracentrifuge at 100 000 × g for 2 hours at 4°C with a Beckman SW-55 rotor. The supernatant was collected by tube puncture and dialyzed for 4 hr against 50 vol of yeast dialysis buffer (20 mM Hepes-KOH, pH 7.9, 50 mM KCl, 0.05 mM EDTA, 5 mM EGTA-KOH, 20% glycerol, 2.5 mM dithiothrietol (fresh), 2 mM phenylmethysulfonyl fluoride (fresh), and 0.5 µg/ml leupeptin (fresh)). After dialysis the protein concentration was measured by the method of Bradford (1976). The sample was stored in 30 µl aliquots at -70°C.

Preparation of yeast nuclear extracts

Yeast nuclear extracts were prepared essentially a described in Dunn and Wobbe (1993). Yeast strain BJ5626 was gown to the indicated OD_{600} as described above. The cells were rapidly cooled by pouring them over ice over into preweighed centrifuge bottles. The cells were then spun at 3000 rpm in a Sorvall centrifuge with a GSA rotor for 5 minutes at 4°C. The cell pellet was weighed and resuspended in 4 volumes of ice cold water (ml/g) and transferred to plastic Oakridge tubes. Cells were spun at 1500 × g with an SA-600 rotor in a Sorvall centrifuge. Cells were resuspended in 1 volume of zymolyase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 M sorbitol) containing 30 mM DTT

and incubated for 15 minutes at room temperature. The suspension was spun for 5 minutes at 4°C at 1500 × g in an SA-600 rotor. The cell pellet was then resuspended in 3 volumes of zymolyase buffer containing 1 mM DTT plus 200 U of zymolyase/ml of cell volume. The mixture was placed on a rotating wheel for 45 minutes at 30°C. Completeness of spheroplasting was checked by dispensing a 10 µl sample onto a microscopic slide and adding one drop of water to the edge of the coverslip and observing the number of cells that were lysed under a Zeiss microscope. Typically 70-90% of the cells were converted to spheroplasts by this method. After spheroplasting, the mixture was spun for 5 minutes at 4°C at 1500 × g in an SA-600 rotor. Cells were resuspended in 200 ml of YPD containing 1 M sorbitol and incubated at 30°C for 60 minutes with very gentle shaking. This step was to allow metabolic recovery of the spheroplasts. Spheroplasts were spun for 5 minutes at 4°C at 3000 rpm in a GSA rotor. All subsequent manipulations were performed at 4 °C. The spheroplasts were then washed 3 times in 2 volumes of ice-cold zymolyase buffer containing 1 mM DTT followed by centrifugation at 1500 × g for 5 minutes in an SA-600 rotor. After the final wash the spheroplasts were resuspended in 0.5 volumes of zymolyase buffer containing 1 mM DTT. This suspension was pipetted drop by drop into a beaker containing ice-cold ficoll buffer (18% (w/v) ficoll, 10 mM Tris-HCl, pH 7.5, 20 mM KCl, 5 mM $MgCl_2$, 3 mM DTT, 1 mM EDTA, 1 mM PMSF, 2 µg/ml aprotinin, 1 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 0.1 mM benzamidine, 25 µg/ml TPCK (DTT and protease inhibitors were added fresh)) with continuous stirring. The mixture was stirred at 4 °C for an additional 15 minutes. The suspension was then transferred to Oakridge tubes an spun for 10 minutes at 5000 rpm in an SA-600 rotor. The supernatant was transferred to new tubes and spun for 20 minutes at 20000 × g in a Beckman Avanti table top centrifuge with an FO650 avanti rotor. The pellet (nuclei) was then resuspended in 1 volume of lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$, 1 mM EDTA, 10 mM KOAc, 1mM DTT, 1mM PMSF, 2 µg/ml aprotinin, 1 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 0.1 mM benzamidine, 25 µg/ml TPCK (DTT and protease inhibitors were added fresh)). The nuclei were lysed with 30-40 stokes of a tight-fitting pestle (clearance 1 to 3 µm) in a

Dounce homogenizer. The lysate was transferred to an ultracentrifuge tube and an equal volume of extraction buffer (0.8 mM ammonium sulfate, 20% glycerol in lysis buffer with DTT and protease inhibitors) was added. The tubes were sealed and placed on a rotating wheel for 30 minutes at 4°C. The tubes were centrifuged at 100 000 × g for 90 minutes with a 70.1 Ti rotor in a Beckman ultracentrifuge. The supernatant was collected by tube puncture and dialyzed against 100 volumes of freshly made storage buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol, 100 mM KCl, 1 mM DTT, 1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 0.1 mM benzamidine, 25 μg/ml TPCK) for 2 to 4 hours. The dialysis bag was transferred to 100 volumes of fresh storage buffer and dialyzed for another 2 to 4 hours. Dialysis was continued until the conductivity of the sample was equal to that of the storage buffer. The dialysate was centrifuged at 10 000 × g for 10 minutes at 4°C with an FO650 rotor in a Beckman Avanti table top centrifuge. This step was performed to remove a flocculent precipitate that forms during the dialysis. This precipitate contains negligible amounts of most protein factors. The supernatant was collected and frozen in small aliquots in liquid nitrogen and stored at -70°C. The protein concentration was measured by the method of Bradford (1976).

Preparation of yeast genomic DNA

Yeast genomic DNA was obtained as described in Adams et al. (1996). A 5 ml culture of strain BJ5626 was grown overnight at 30°C with shaking. The culture was transferred to a 13 x 100 mm glass tube and centrifuged at 1500 rpm for 5 minutes. The cells were washed with 3 ml of sterile water and centrifuged as above. The pellet was resuspended in 500 μl of lysis buffer (0.1 M Tris-HCl, pH 8.0, 50 mM EDTA, 1% SDS). To the suspension was added ~800 μl of glass beads and 25 ul of 5 M NaCl. The tube was vortexed on the highest setting for 1 minute and centrifuged at 2000 rpm for 2 minutes. The liquid was transferred to a 1.5 ml eppendorf tube containing 500 μl of phenol. The sample was spun for 1 minute at 12 000 × g and the aqueous layer was transferred to a clean tube. 500 μl of 24:1 chloroform:isoamylalcohol was added and the sample was vortexed, spun and extracted as above. 1 ml of cold 95% ethanol was added and the

sample was allowed to precipitate at -20°C for 1 hour. The DNA was pelleted by spinning at 12 000 × g for 5 minutes and the pellet was washed with 70% ethanol. The DNA pellet was resuspended in 250 µl of TE buffer and 25 µl of EDTA-Sark. (0.4 M EDTA, pH 8.0, 2% N-lauryl sarcosine) and 5 µl of proteinase K (10 mg/ml) were added. The solution was incubated at 37°C for 30 minutes. 250 µl of 5 M NH_4Ac was added and extracted with 500 µl of phenol and 500 µl of 24:1 chloroform:isoamyl alcohol as described above. Again the solution was precipitated with 1 ml of 95% ethanol for 1 hour at -20°C. The DNA was pelleted and washed as described above and resuspended in 100 µl of TE buffer. The concentration of genomic DNA was determined by taking the A_{260}. This genomic DNA was used as a template in PCR reactions to make probes for Northern analysis.

Preparation of DNA probes and non-specific competitors

For gel-mobility shift assays, double stranded oligonucleotides were prepared and either end-labeled or used as cold competitors. To make the WT oligonucleotide, 50 pmol of a synthetic 33 base oligonucleotide of the sequence 5′-TGGGTG**CCCTTAAT TAGGGA**TCGCTGTGGAAAG - 3′, and its complementary strand were both end labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP (3000 µCi/mmol; Amersham). This sequence is derived from bases -160 to -192 upstream of the *SSA3* gene (Boorstein and Craig, 1990b) and contains the PDS element which is outlined in bold. The labeling reactions were phenol/chloroform and chloroform extracted and precipitated with 100% ethanol. After a 70% ethanol wash, the DNA pellets were resuspended in water and mixed together to give a final volume of 18 µl. The two strands were annealed by adding 2 µl of 10× kinase buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine HCl, 1 mM EDTA, pH 8.0) and incubating successively for 2 minutes at 88°C, 10 minutes at 65°C, 10 minutes at 37°C and 5 minutes at room temperature. The 20 µl sample was then loaded onto a 1 ml sephadex G50-150 sizing column to separate the double stranded oligonucleotide from the unincorporated nucleotides. The peak fractions were pooled and used as probe. The concentration of probe was determined by dot quantitation (Moore et al., 1993). 4 µl each of DNA standards (0, 1, 2, 4, and 8

ng/μl) was added to 1 μl of 4 μg/ml ethidium bromide and compared to 4 μl of probe plus 1 μl of 4 μg/ml ethidium bromide by spotting the 5 μl samples on Saran wrap on a UV box and comparing the intensities. Probes prepared in this way typically had a concentration of 1 ng/μl. M2 and M3 probes were prepared identically except they each contained five base substitutions (identified by underlined sequences) within the PDS element (bold). The sequence for M2 is 5′-TGGGTG**GTCGACATTAGG GA**TCGCTGTGGAAAG - 3′ and the sequence for M3 is 5′-TGGGTG**CCCTT AATGTCGAC**TCGCTGTGGAAAG - 3′. Cold competitors were prepared by annealing 10 μg of the top DNA 33 base oligonucleotide (WT, M2, M3) with 10 μg of the complementary bottom strand in a 20 μl annealing reaction as outlined above. The sample was precipitated and with 100% ethanol and resuspended in TE buffer. The concentration of the double stranded oligonucleotides was determined by taking the A_{260}. Poly (dI-dC)·Poly (dI-dC) was prepared as instructed by Pharmacia. The lyophilized powder was dissolved to a final concentration of 100 ng/μl in water containing 100 mM NaCl.

The *SSA3* and *PDA1* probes for Northern analysis were obtained by PCR using yeast genomic DNA as a template (see above). The *SSA3* probe was obtained by using the forward PCR primer 5′ - TGTTACCTTTGATATCGACGC - 3′ and the reverse PCR primer 5′ - TAGCACCCGAGTTGGGCAT - 3′. PCR using these primers amplifies a 485 basepair fragment from the 3′ end of the SSA3 gene. The PDA1 probe was obtained by using the forward PCR primer 5′ - TTATGGAATTTGCCCGTCGT - 3′ and the reverse PCR primer 5′ - GAGGCAAAACCTTGCTTTTTG - 3′. PCR using these primers amplifies a 557 basepair fragment from the 3′ end of the PDA1 gene. PCR conditions for both fragments were identical and were performed in a final volume of 100 μl. Reactions contained 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 5 μg of genomic template DNA, 100 pmol of each primer, 2 mM each of dATP, dGTP, dCTP, and dTTP, and 2.5 U of Taq polymerase (BRL). A no primer control reaction was also performed. The 100 μl samples were overlaid with paraffin oil. The PCR conditions were as follows: 3 minutes at 94°C, 30 cycles of 45 seconds at 94°C / 30 seconds at

56°C / 1 minute and 30 seconds at 72°C, and one final 10 minute incubation at 72°C. The reaction was stopped by the addition of 2 µl of 500 mM EDTA and the products were checked on an agarose gel. The *SSA3* and *PDA1* fragments were gel purified using a Geneclean kit (BIO 101 Inc., Bio/Can Scientific) and labeled by random priming using Klenow fragment, [α-^{32}P] dCTP (3000 µCi/mmol; Amersham), and synthetic random hexanucleotides (Feinberg, 1984). The reaction was loaded onto a 5 ml G50-150 sephadex column to separate labeled product from unincorporated nucleotides and the peak fractions (~500 µl, 5×10^7 cpm) were pooled as probe. The entire 500 µl was used in the hybridization to immobilized RNA on nylon membranes (see below).

For S1 nuclease analysis of *in vitro* RNA polymerase I transcription products (see below), a synthetic single stranded 50 base oligonucleotide spanning the promoter region of pBYr11AL from -15 to +35 (Schultz et al. 1991) was prepared (5′ - CAACCCTC CATGAAGTACGCTTTCGTCAACTTCTGTTCAAGCAGTTCTGG - 3′). 12.5 pmoles of the 50mer was end labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP (3000 µCi/mmol; Amersham). The labeling reaction was run over a 1 ml sephadex G50-150 size exclusion column to separate the labeled product from the unincorporated nucleotides. The peak fractions were pooled together and used as probe. 5 µl of probe was used in hybridizations with RNA polymerase I transcription products.

Gel mobility shift assays

Binding reactions were performed essentially as described by Wahls et al. (1991) with slight modifications. 20 µl reactions contained 20 mM Hepes-KOH, 112.5 mM KCl, 1.25 mM EGTA, 0.0125 mM EDTA, 0.625 mM DTT, 5% glycerol, 5 mM $MgCl_2$, 0.3 µg/µl BSA, 400 ng of competitor DNA (either poly dI-dC, WT, M2, or M3), 4 ng of end labeled probe (either WT, M2, or M3), and 6 µg extract protein. The assay was performed as follows: to 400 ng of competitor DNA in 11 µl of water, 2 µl of 10 × binding buffer (150 mM Hepes-KOH, 50 mM $MgCl_2$, 1 M KCl, and 3 mg/ml BSA) was added as well as 5 µl of nuclear or whole cell extract (1.2 µg/µl in YDBI w/o leupeptin and PMSF). The 18 µl reaction was incubated for 15 minutes at room temperature. 2 µl of labeled probe (1 ng/µl) was added to the reaction and incubated for another 15

minutes at room temperature. After the final incubation the entire reaction was electrophoresed under either high-ionic or low-ionic conditions. For high-ionic conditions the reaction was loaded onto a 5% high-ionic strength polyacrylamide gel (50 mM Tris-HCl, pH 7.9, 380 mM glycine, 2 mM EDTA, 5% acrylamide, 0.17% bisacrylamide, and 2.5% glycerol) and run at 120 V for 4 hours at 4°C in 1 × Tris-glycine buffer (50 mM Tris-HCl, pH 7.9, 380 mM glycine, 2 mM EDTA). For low-ionic conditions the reaction was loaded onto a 5% low-ionic strength polyacrylamide gel (20 mM Tris-HCl pH 7.9, 2 mM EDTA, 5 % acrylamide, 0.17% bisacrylamide, and 5% glycerol) and run at 120 V for 4 hours at 4°C in low ionic strength running buffer (20 mM Tris-HCl, 2 mM EDTA). The running buffer was recirculated with Pharmacia P-1 peristaltic pumps set to the 10 × flow rate setting. The gel was dried on a Savant Slab gel drier for 45 minutes and the products visualized by autoradiography after an overnight exposure on Kodac Scientific imaging film.

Northern analysis

RNA was isolated with TRIzol reagent (Chomczynski, 1993) from cultures of the indicated yeast strains grown to early log phase (OD_{600} = 0.6) or the post-diauxic shift (OD_{600} = 4.5). 2.7×10^8 cells were harvested by centrifugation at 4000 rpm for 5 minutes at 4°C. The cell pellet was resuspended with 200 µl of TRIzol reagent and 200 µl glass beads. The mixture was vortexed for 10 minutes at 4°C followed by centrifugation at 3700 rpm for 5 minutes at 4°C. The supernatant was transferred to a new tube containing 40 µl chloroform (0.2 ml/ml of TRIzol used). The mixture was shaken for 3 minutes at room temperature followed by centrifugation for 15 minutes at 12 000 × g at 4°C. The aqueous phase was collected and precipitated with 100 µl isopropanol (0.5 ml/ml of TRIzol used) for 10 minutes at room temperature, followed by centrifugation at 12000 × g for 15 minutes at 4°C. The pellet was washed with 75% ethanol (DEPC treated water) and centrifuged at 7500 × g for 5 minutes at 4°C. The pellet was resuspended in a small volume of DEPC treated water and the concentration of RNA was obtained by taking the A_{260}. The concentration of the RNA was then adjusted to 1 µg/µl with DEPC treated water.

Northern blotting was performed essentially as described in Brown (1993). 5 µl of RNA (5 µg) isolated from the indicated strains grown to the appropriate OD_{600} was mixed with 15 µl of sample buffer (sample buffer was made fresh from 900 µl of formamide, 300 µl of formaldehyde, and 180 µl of 20× borate buffer, see below). The mixture was incubated at 65°C for 5 minutes and cooled on ice. 3 µl of dye mix (0.1% Bromophenol blue, 0.1% Xylene cyanol, and 0.1 M EDTA) was added and the sample was loaded onto a 1% agarose gel made from 100 ml of gel mix (1.0 g agarose, 5 ml 20× borate buffer (0.4 M Boric acid, 4mM EDTA, pH adjusted to 8.3 with NaOH), 9 ml formaldehyde, and 0.5 µg/ml ethidium bromide). The gel was run at 80 volts for 4 hours in 1× borate buffer. After the electrophoresis, the gel was photographed and rinsed in DEPC treated water for 30 minutes with 2 changes of water. The RNA was transferred to Gene Screen Plus nylon membrane by capillary action with 20× SSC (3 M NaCl, 0.3 M $Na_3citrate\text{-}2H_2O$, pH 7.0 (Brown, 1993). The transfer was allowed to take place overnight. After the transfer was complete, the nylon membrane was air dried and the RNA was UV crosslinked to the membrane in a Stratalinker using the autolink setting. The membrane was prehybridized for 4 hours at 65°C with 5× SSC, 1% SDS, 2× Denhart's solution (0.04% Ficoll-400, 0.04% polyvinylpyrrolidone, 400 µg/ml Bovine Serum Albumin), and 100 µg/ml Herring Sperm DNA in hybridization bottles. After the 4 hour prehybridization the solution was poured off and 5 ml of fresh prehybridization solution containing 20×10^6 cpm of SSA3 probe was added. Hybridization was at 65°C for overnight. Following hybridization the membrane was washed 5 times for 5 minutes each in 2× SSC, 0.1 % SDS at room temperature and 2 times for 15 minutes each in 0.1× SSC, 0.1% SDS at 55°C. The membrane was kept moist so it could be reprobed, and was exposed to Kodac film overnight at -70°C with an intensifying screen.

***In vitro* RNA polymerase III transcription assay**

Polymerase III transcription reactions were performed essentially as described by Schultz et al. (1992). Transcription reactions were performed in a final volume of 20 µl containing 10-100 µg whole cell extract, 20 mM Hepes-KOH, 90 mM KCl, 10 mM $MgCl_2$, 2.5 mM DTT, 5 mM EGTA, 0.05 mM EDTA, 10% glycerol, 10 µg/ml α-

amanitin, 500 μM each of ATP, GTP, CTP, 50 μm UTP, 0.5 μl [$\alpha-^{32}$P] UTP (3000 Ci/mmol, NEN), and either 20 ng/μl pY5S, or 1.25 ng/μl of pGE2 (pY5S includes the entire 5S gene cloned into pGEM3 and pGE2 contains the entire Leu3 tRNA gene: Schultz et al. 1992). Reactions were incubated for 30 minutes at 30°C and stopped with 100 μl of stop buffer (7 M urea, 100 mM LiCl, 0.5% SDS, 10 mM EDTA, 10 mM Tris-HCl pH 7.9). After phenol/chloroform and choloroform extraction, the products were precipitated with 100% ethanol for 1 hour at -70°C. The precipitate was washed with 70% ethanol, resuspended in 8 μl of formamide RNA loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophehol blue), boiled for 3 minutes, cooled on ice, and electrophoresed on a 7 M urea, 8% polyacrylamide gel at 20 W for 45 minutes in TBE buffer. RNA transcripts were visualized by autoradiography after an overnight exposure on Fuji X-Ray Medical film with an intensifying screen at -70°C.

CKII add-back experiments were performed as in Hockman and Schultz (1996). The reaction conditions were identical to above, with modifications. Reactions contained 22.5 mM KCl and 75 mM NaCl in place of 90 mM KCl. The maximum amount of rescue by CKII was expected to be achieved by preincubating the whole cell extract with CKII and ATP. This could not be done because the extract contains and ATP-dependent repressor that may mask any CKII effect (Ghavidel and Schultz, unpublished results). For this reason reaction components were mixed as follows. A pY5S template/salts/α-amanitin mix (all components except the nucleotides, CKII and extract) was dispensed into each tube. Separate aliquots of nucleotides and CKII were spotted onto the wall of the tube and washed into the pY5S/salts/α-amanitin mix with 40 μg of extract derived from rapamycin treated cells or placebo treated cells.

For treatment of whole cell extracts with rapamycin and measurement of pol III transcription, reactions were performed as described except the indicated whole cell extracts were pre-heated for 10 minutes at 30°C. We reasoned that pre-heating might break up existing complexes, thereby allowing rapamycin a chance to form inhibitory complexes. After preheating 40 μg of the indicated extract, either 1 μl of rapamycin (0.2 μg/μl) or 1 μl of 90% ethanol/10% Tween-20 was added. The mixture was then

incubated for 20-60 minutes after which the remaining reaction components were added and the polymerase III transcription was carried out for 30 minutes at 30°C. The final concentration of rapamycin was 10 μg/ml. The reaction products were then processed as above.

***In vitro* RNA polymerase I transcription assay**

Transcription by RNA polymerase I was assayed as described by Schultz et al. (1991) with slight modifications. Reaction conditions were identical to those for RNA polymerase III transcription except that UTP was used at a concentration of 500 μM and no $[\alpha-^{32}P]$ UTP was used. Salts, α-amanitin, and 10-80 μg of extract were preincubated with 400 ng of HpaII digested pBluescript KS+ for 10 minutes at room temperature. This preincubation was performed to titrate non-specific DNA binding proteins. 0.4 ng of ScaI-digested pBYr11AL (identical to pBYr11A, which contains the yeast 35S rRNA promoter, except that a single copy of a linker oligo containing a XbaI, SalI, BglII, and a BamHI site was cloned into the large XbaI-BamHI fragment; Schultz et al., 1991) was added to the reaction and incubated for another 5 minutes at room temperature. The nucleotides were then added and the reaction was incubated for 45 minutes at room temperature. The reaction was stopped by addition of 200 μl of 10 mM Tris-HCl, pH 7.5, 1% SDS, 1 mM EDTA. After phenol/chloroform and chloroform extraction the products were precipitated for 1 hour with 100% ethanol at -70°C. The pellet was washed with 70% ethanol and resuspended in 22 μl of DEPC treated water. The RNA products were detected by S1 nuclease analysis using a 50 base oligonucleotide probe spanning the 35S promoter region from -15 to +35 in pBYr11AL (Schultz et al., 1991). Correctly initiated transcripts yielded a 35 nucleotide protected fragment. The S1 nuclease protection assays were performed essentially according to Labhart and Reeder (1986). To the 22 μl RNA sample obtained above, 5 μl of end-labeled probe was added and 3 μl of 10 × hybridization buffer (0.1 M Tris-HCl, pH 7.5, 3 M NaCl, 10 mM EDTA all in DEPC treated water). The mixture was overlaid with 3 drops of paraffin oil and incubated overnight at 65°C. The mixture was cooled quickly on ice and 200 U of S1 nuclease in 270 μl Nuclease Assay Buffer (50 mM NaCl, 30 mM NaAc, 1 mM $ZnSO_4$,

and 5% glycerol) was added. The S1 nuclease mix was spun through the paraffin oil in a microfuge for 5 seconds and the components mixed. The mixture was again spun in a microfuge and incubated for 30 minutes at 37°C. The reaction was cooled briefly on ice and collected from beneath the paraffin oil. 1ml of 100% ethanol and 46 µl of precipitation mix (40 µl of 5 M NH_4OAc, 5 µl of 0.5 M EDTA, 1 µl of 10 mg/ml tRNA) was added and the nucleic acids were precipitated for 1 hour at -70°C. The pellet was washed with 70% ethanol, resuspended in 6 µl of formamide loading buffer, boiled for 3 minutes, cooled on ice, and loaded onto a 7 M urea, 8% polyacrylamide gel. The gel was run at 20 W for 40 minutes in TBE buffer and dried. The products were visualized by autoradiography after an overnight exposure on Kodac Scientific Imaging film with an intensifying screen at -70°C.

Cell viability assays following rapamycin and placebo treatment

Strain RS188 was grown to an OD_{600} = 0.1 and treated with either rapamycin to 1 µg/ml or and equal volume of drug vehicle (90% ETOH/10% Tween-20) for 24 hours. The OD_{600} was taken and either 100 or 200 cells of each culture was plated out on YPD plates. The plates were incubated at 30°C overnight and the number of colonies on each plate was counted.

Casein Kinase II assay

The CKII assay was performed essentially as in Hockman and Schultz (1996). The reactions were 25 µl in volume and used the CKII specific peptide substrate, RRREEETEEE (Kuenzel and Krebs, 1985) at 250 µM. Reactions were performed under the conditions for polymerase I and III transcription (below; Schultz et al., 1992) except that α-amanitin was omitted. Each reaction contained 6.25 µg extract protein and 20 mM Hepes-KOH, 50 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, 0.1mM EDTA, 2.5 mM DTT, 100 µM unlabeled ATP, and 1µl [$\gamma - ^{32}$P] ATP (3000 µCi/ml, NEN). After a 5 min incubation at 22°C, the reaction was made 5% trichloroacetic acid and spun for 2 minutes at 12 000 × g at 4°C to precipitate the large proteins. A 20 µl aliquot from the supernatant (containing the peptide substrate) was spotted onto Whatman P81 paper.

The P81 paper was washed 4 times in 25 mM phosphoric acid for 15 minutes each and allowed to dry. The amount of incorporation was measured by scintillation counting. Reactions were performed in quadruplicate. Data represents the mean of the four reactions.

Incorporation of [^{35}S] methionine into total yeast protein

In vivo labeling of total yeast proteins was performed essentially as described by Barbet et al. (1996) with slight modifications. 1 ml cultures of FPR1 were grown to an OD_{600} = 1.0 and treated with either 10 µg/ml rapamycin or an equal volume of drug vehicle (100% ETOH / 10% Tween-20) for 1 hour at 30°C. After the 1 hour incubation the OD_{600} was measured again to ensure that treated and untreated cells were at equivalent OD_{600}s before labeling. The cultures were centrifuged at 4000 rpm for 4 minutes at 4°C and the cell pellet was washed with ice cold H_2O. The cells were then resuspended in 1 ml of MET^- media plus fresh placebo or rapamycin. 500 µCi of L-[^{35}S] methionine was added and the cells were labeled with shaking for 20 minutes at 30°C. After the incubation 120 µl of each culture was removed and transferred into separate screw capped tubes containing 250 µl of 1 mM NaN_3. The remainder of culture was stored at room temperature. Proteins were isolated as described by Werner-Washburne et al. (1991) with slight modifications. The screw capped tubes were centrifuged for 4 minutes at 4°C at 4000 rpm to pellet the cells. The cells were washed once with 250 µl of ice cold NaN_3 (1 mM) and once with 250 µl ice cold water. The cells were resuspended in 300 µl of sonication buffer (10 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 50 µg/ml RNaseA) plus ~ 0.3 g of glass beads (0.3-0.5 mm). Cells were lysed by vortexing 6 × for 30 seconds on the highest setting of a Fisher scientific vortexer with a 30 second incubation on ice after each burst. The mixture was centrifuged at 2500 × g for 10 minutes at 4°C. 8 volumes of acetone was added to the supernatant and incubated overnight at -20°C. The mixture was spun at 3000 × g for 15 minutes at 4°C and the protein precipitate was resuspended in 50 µl of lysis buffer (9.5 M urea, 2% nonidet P-40 (w/v), 2% ampholines (1.6% pH range 5 to 7 and 0.4% pH range 3 to 10), and 5% β-mercaptoethanol) and

quantitated by scintillation counting. To control for the amount of cell breakage, non-radioactive samples were prepared identically and measured for protein concentration by the method of Bradford (1976). The protein concentrations of samples obtained from rapamycin and placebo treated cultures were found to be similar.

References

Adams, A., Gottschling, D., and C. Kaiser. 1996. In *The yeast genetic course manual.* p. 22. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Albers, M.W., Williams, R.T., Brown, E.J., Tanaka, A., Hall, F.L., and S.L. Schreiber. 1993. FKBP-Rapamycin inhibits a cyclin-dependent kinase activity and a cyclin D1-Cdk association in early G1 of an osteosarcoma cell line. *J. Biol. Chem.* **268:** 22825-22829.

Barbet, N.C., Schneider, U., Helliwell, S.B., Stansfield, I., Tuite, M.F., and M.N. Hall. 1996. TOR controls translation initiation and early G1 progression in yeast. *Mol. Biol. Cell* **7:** 25-42.

Belazzi, T., Wagner, A., Wieser, R., Schanz, M., Adam, G., Hartig, A., and H. Ruis. 1991. Negative regulation of transcription of the *Saccharomyces cerevisiae* catalase T (*CTT1)* gene by cAMP is mediated by a positive control element. *EMBO J.* **10:** 585-592.

Beretta, L., Gingras, A.-C., Svitkin, Y.V., Hall, M.N., and N. Sonenberg. 1996. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J.* **15:** 658-664.

Bidwai, A.P., Hanna, D.E., and C.V.C. Glover. 1992. Purification and characterization of casein kinasse II (CKII) from *Δcka1Δcka2 Saccharomyces cerevisiae* rescued by *Drosophila* CKII subunits. The free catalytic subunit of casein kinase II is not toxic *in vivo. J. Biol. Chem.* **267:** 18790-18796.

Bidwai, A.P., Reed, D.E., and C.V.C. Glover. 1994. Casein kinase II of *Saccharomyces cerevisiae* contains two distinct regulatory subunits, β and β'. *Arch. Biochem. Biophys.* **309:** 348-355.

Bierer, B.E., Somers., P.K., Wandless, T.J., Burakoff, S.J., and S.L. Schreiber. 1990a. Probing immunosuppressant action with a nonnatural immunophilin ligand. *Science* **250:** 556-559.

Bierer, B.E., Mattila, P.S., Standaert, R.F., Herzenberg, L.A., Burakoff, S.J., Crabtree, G., and S.L. Schreiber. 1990b. Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc. Natl. Acad. Sci. USA* **87:** 9231-9235.

Bissinger, P.H., Wieser, R., Hamilton, B., and H. Ruis. 1989. Control of Saccharomyces cerevisiae catalase T gene (CTT1) expression by nutrient supply via the RAS-cyclic AMP pathway. *Mol. Cell. Biol.* **9:** 1309-1315.

Boguslawski, G. 1992. *PBS2*, a yeast gene encoding a putative protein kinase, interacts with the Ras2 pathway and affects osmotic sensitivity of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **138:** 2425-2432.

Boorstein, W.R., and E.A. Craig. 1990a. Transcriptional regulation of *SSA3*, and HSP70 gene from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10:** 3262-3267.

Boorstein, W.R., and E.A. Craig. 1990b. Regulation of a yeast HSP70 gene by a cAMP responsive transcriptional control element. *EMBO J.* **9:** 2543-2553.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Brewster, J.L., deValoir, T., Dwyer, N.D., Winter, E., and M.C. Gustin. 1993. An osmosensing signal transduction pathway in yeast. *Science* **259:** 1760-1763.

Broach, J.R. 1991. RAS genes in *Saccharomyces cerevisiae*: signal transduction in search of a pathway. *Trends Genetics* **7:** 28-32.

Brown, T. 1993. In *Current protocols in molecular biology* (ed. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, K. Struhl), pp. 4.9.1-4.9.13.

Burley, S.K. and R.G. Roeder. 1996. Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* **65:** 769-799.

Calvo, V., Crew, C.M., Vik, T.A., and B.E. Bierer. 1992. Interleukin 2 stimulation of p70 S6 kinase activity is inhibited by the immunosuppressant rapamycin. *Proc. Natl. Acad. Sci. USA* **89:** 7571-7575.

Cardenas, M.E., and J. Heitman. 1995. FKBP12-rapamycin target TOR2 is a vacuolar protein with an associated phosphatidylinositol-4 kinase activity. *EMBO J.* **14:** 5892-5907.

Cavanaugh, A.H. and E.A. Thompson. 1985. Hormonal regulation of transcription of rDNA: glucocorticoid effects upon initiation and elongation *in vitro*. *Nucleic Acids Res.* **13:** 3357-3369.

Chang, A. and G.R. Fink. 1994. Metal metabolism - the copper-iron connection. *Curr. Biol.* **4:** 532-533.

Chao, Y. and M. Pellegrini. 1993. *In vitro* transcription of *Drosophila* rRNA genes shows stimulation by a phorbol ester and serum. *Mol. Cell. Biol.* **13:** 934-941.

Chen, J.-L., Attardi, L.D., Verrijzer, C.P., Yokomori, K., and R. Tjian. 1994. Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. *Cell* **79:** 93-105.

Cherry, J.R., Johnson, T.R., Dollard, C., Shuster, J.R., and C.L. Denis. 1989. Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1. *Cell* **56:** 409-419.

Chiang, C.M. and R.G. Roeder. 1995. Cloning of an intrinsic human TFIID subunit that interacts with multiple transcriptional activators. *Science* **267:** 531-536.

Chiu, M.I., Katz, H., and V. Berlin. 1994. RAPT1, a mammalian homolog of yeast Tor, interacts with the FFKBP12/rapamycin complex. *Proc. Natl. Acad. Sci. USA* **91:** 12574-12578.

Choder, M. 1991. A general topoisomerase I-dependent transcriptional repression in the stationary phase of yeast. *Genes Dev.* **5:** 2315-2326.

Chomczymski, P. 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and protein from cell and tissue samples. *Biotechniques* **15:** 532-534, 536-537.

Chung, J., Kuo, C.J., Crabtree, G.H., and J. Blenis. 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70Kd S6 protein kinases. *Cell* **69:** 1227-1236.

Dikstein, R., Ruppert, S., and T. Tjian. 1996. TAFII250 is a bipartite protein kinase that phosphorylates the base transcription factor RAP74. *Cell* **84:** 781-790.

Dumont, F.J., Staruch, M.J., Koprak, S.L., Melino, M.R., and N.H. Sigal. 1990. Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK506 and rapamycin. *J. Immunol.* **144:** 1418-1424.

Dunn, B. and Wobbe, C.R. 1993. In *Current protocols in molecular biology* (ed. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, K. Struhl), pp.13.13.1-13.13.9.

Engelberg, D., Zandi, E., Parker, C.S., and M. Karin. 1994. The yeast and mammalian Ras pathways control transcription of heat shock genes independently of heat shock transcription factor. *Mol. Cell. Biol.* **14:** 4929-4937.

Entian, K.D. 1986. Glucose repression: A complex regulation system in yeast. *Microbiol. Sci.* **3:** 366-371.

Estruch, F., and M. Carlson. 1993. Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13:** 3872-3881.

Fassler, J.S. and F. Winston. 1989. The *Saccharomyces cerevisiae SPT13/GAL11* gene has both positive and negative regulatory roles in transcription. *Mol. Cell. Biol.* **9:** 5602-5609.

Feinberg, A.P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137:** 266-267.

Finley, D., Ozaynak, and A. Varshavsky. 1987. The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. *Cell* **48:** 1035-1046.

Flanagan, P.M., Kelleher, R.J. III, Sayre, M.H., Tschochner, H., and R.D. Kornberg. 1991. A mediator required for activation of RNA polymerase II transcription *in vitro*. *Nature* **350:** 436-438.

Fruman, D.A., Burakoff, S.J., and B.E. Bierer. 1994. Immunophilins in protein folding and immunosuppression. *FASEB J.* **8:** 391-400.

Fuge, E.K., Braun, E.L., and M. Werner-Washburne. 1994. Protein synthesis in long-term stationary-phase cultures of Saccharomyces cerevisiae. *J. Bacteriol.* **176:** 5802-5813.

Garber, M.E., Vilalta, A., and D.L. Johnson. 1994. Induction of *Drosophila* RNA polymerase III gene expression by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is mediated by the transcription factor IIIB. *Mol. Cell. Biol.* **14:** 339-347.

Ge, H. and R.G. Roeder. 1994. Purification, cloning, and characterization of a human coactivator, PC4, that mediates transcriptional activation of class II genes. *Cell* **78:** 513-523.

Ge, H., Zhao, Y., Chait, B.T., and R.G. Roeder. 1994. Phosphorylation negatively regulates the function of coactivator PC4. *Proc. Natl. Acad. Sci. USA* **91:** 12691-12695.

Ghavidel, A., and M.C. Schultz. Unpublished data.

Gottesfeld, J.M., Wolf, V.J., Dang, T., Forbes, D.J., and P. Hartl. 1994. Mitotic repression of RNA polymerase III transcription in vitro mediated by phosphorylation of a TFIIIB component. *Science* **263:** 81-84.

Grossman, L.I., Goldring, E.S., and J. Marmur. 1969. Preferential synthesis of yeast mitochondrial DNA in the absence of protein synthesis. *J. Mol. Biol.* **46:** 367-376.

Hartl, P., Gottesfeld, J., and D.J. Forbes. 1993. Mitotic repression of transcription in vitro. *J. Cell Biol.* **120:** 613-624.

Heitman, J., Movva, N.R., Hiestand, P.C., and M.N. Hall. 1991a. FK506 binding protein proline rotamase is a target for the immunosuppressive agent FK506 in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **88:** 1948-1952.

Heitman, J., Movva, N.R., and M.N. Hall. 1991b. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253:** 905-909.

Heitman, J., Movva, N.R., and M.N. Hall. 1992. Proline isomerases at the crossroads of protein folding, signal transduction, and immunosuppression. *New Biologist* **4:** 448-460.

Hengartner, C.J., Thompson, C.M., Zhang, J., Chao, D.M., Liao, S.-M., Koleske, A.J., Okamura, S., and R.A. Young. 1995. Association of an activator with an RNA polymerase II holoenzyme. *Genes Dev.* **9:** 897-910.

Hereford, L.M., and L.H. Hartwell. 1974. Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. *J. Mol. Biol.* **84:** 445-461.

Himmelfarb, H.J., Pearlberg, J., Last, D.H., and M. Ptashne. 1990. GAL11P: A yeast mutation that potentiates the effect of weak GAL4-derived activators. *Cell* **63:** 1299-1309.

Hockman, D.J. and M.C. Schultz. 1996. Casein kinase II is required for efficient transcription by RNA polymerase III. *Mol. Cell. Biol.* **16:** 892-898.

Hoefller, W.K., Kovelman, K.R., and R.G. Roeer. 1988. Activation of transcription factor IIIC by the adenovirus E1A protein. *Cell* **53:** 907-920.

Hof, C.M. and S.T. Jacob. 1993. Characterization of the factor E1BF from a rat hepatoma that modulates ribosomal RNA gene transcription and its relationship to the human ku autoantigen. *Biochem. Biophys. Res. Comm.* **190:** 747-753.

Iida, H. and I. Yahara. 1984. Specific early-G1 blocks accompanied with stringent response in Saccharomyces cerevisiae lead to growth arrest in resting state similar to the G_0 of higher eucaryotes. *J. Cell. Biol.* **98:** 1185-1193.

Issinger, O.-G. 1993. Casein kinases: pleiotropic mediators of cellular regulation. *Pharmacol. Ther.* **59:** 1-30.

Jacq, X., Brou, C., Lutz, Y., Davidson, I., Chambon, P., and L. Tora. 1994. Human TAFII30 is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. *Cell* **79:** 107-117.

Jakobsen, B.K. and H.R. Pelham. 1988. Constitutive binding of yeast heat shock factor to DNA in vivo. *Mol. Cell. Biol.* **8:** 5040-5042.

Jans, D.A., Moll, T., Nasmyth, K., and P. Jans. 1995. Cyclin-dependent kinase site-regulated signal-dependent nuclear localization of the SW15 yeast transcription factor in mammalian cells. *J. Biol. Chem.* **270:** 17064-17067.

Jayaraman, T., Brillantes, A.M., Timerman, A.P., Fleischer, S., Erdjument-Bromage, H., Tempst, P., and A.R. Marks. 1992. FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J. Biol. Chem.* **267:** 9474-9477.

Jin, Y.J., and S.J. Burakoff. 1993. The 25-kDa FK506-binding protein is localized in the nucleus and associates with casein kinase II and nucleolin. *Proc. Natl. Acad. Sci. USA* **90:** 7769-7773.

Kim, Y.-J., Bjorklund, S., Li, Y., Sayre, M.H., and R.D. Kornberg. 1994. A multiprotein mediator of transcriptional activation and it's interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77:** 599-608.

Kobayashi, N., and K. McEntee. 1993. Identification of *cis* and *trans* components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13:** 248-256.

Koleske, A.J. and R.A. Young. 1994. An RNA polymerase II holoenzyme responsive to activators. *Nature* **368:** 466-469.

Koltin, Y., Faucette, L., Bergsma, D.J., Levy, M.A., Cafferkey, R., Koser, P.L, Johnson, R.K., and G.P. Livi. 1991. Rapamycin sensitivity in *Saccharomyces cerevisiae* is mediated by a peptidyl-prolyl cis-trans isomerase related to human FK506-binding protein. *Mol. Cell. Biol.* **11:** 1718-1723.

Kuenzel, E.A., and E.G. Krebs. 1985. A synthetic peptide substrate specific for casein kinase II. *Proc. Natl. Acad. Sci. USA* **82:** 737-741.

Kuge, S. and N. Jones. 1994. YAP1 dependent activation of TRX2 is essential for the response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides. *EMBO J.* **13:** 655-664.

Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N.R., and M.N. Hall. 1993. Target of rapamycin in yeast TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell* **73:** 585-596.

Kuo, C.J., Chung, J., Fiorentino, D.F., Flanagan, W.M., Blenis, J., and G.R. Crabtree. 1992. Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. *Nature* **358:** 70-74.

Labhart, P., and R.H. Reeder. 1986. Characterization of three sites of RNA 3' end formation in the *Xenopus* ribosomal gene spacer. *Cell* **45:** 431-433.

Lin, T.-A., Kong, X., Haystead, T.A.J., Pause, A., Belsham, G., Sonenberg, N., and J.C. Lawrence Jr. 1994. PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science* **266:** 653-656.

Lin, Y.-S., Maldonado, E., Reinberg, D., and M.R. Green. 1991. Binding of general transcription factor TFIIB to an acidic activating region. *Nature* **353:** 569-571.

Lindquist, S. and E.A. Craig. 1988. The heat shock proteins. *Annu. Rev. Genet.* **22:** 631-677.

Litchfield, D.W., and B. Luscher. 1993. Casein kinase II in signal transduction and cell cycle regulation. *Mol. Cell. Biochem.* **127/128:** 187-199.

Lorenz, M.C., and J. Heitman. 1995. TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin. *J. Biol. Chem.* **270:** 27531-27537.

Mager, W.H. and P. Moradas-Ferreira. 1993. Stress response in yeast. *Biochem. J.* **290:** 1-13.

Mager, W.H. and J.C.S. Varela. 1993. Osmostress response of the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **10:** 253-258.

Mahajan, P.B. and E.A. Thompson. 1990. Hormonal regulation of transcription of rDNA: purification and characterization of the hormone-regulated transcription factor TFIC. *J. Biol. Chem.* **265:** 16225-16233.

Marchler, G., Schuller, C., Adam, G., and H. Ruis. 1993. A Saccharomyces cerevisiae UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO J.* **12:** 1997-2003.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., and F. Estruch. 1996. The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO J.* **15:** 2227-2235.

McAlister, L., and M.J. Holland. 1985. Differential expression of the three yeast glyceraldehyde-3-phosphate dehydrogenase genes. *J. Biol. Chem.* **260:** 15019-15027.

Michnick, S.W., Rosen, M.K., Wandless, T.J., Karplus, M., and S.L. Schreiber. 1991. solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin. *Science* **252:** 836-839.

Mishima, Y., Matsui, T., and Muramatsu M. 1979. The mechanism of decrease in nucleolar RNA synthesis by protein synthesis inhibition. *J. Biochem.* **85:** 807-818.

Mizzen, C.A., Yang, X.J., Kokubo, T., Brownell, J.E., Bannister, A.J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S.L., Kouzarides, T., Nakatani, Y., and C.D. Allis. 1996. The TAF(II)250 subunit of TFIID has histone aceyltransferase activity. *Cell* **87:** 1261-1270.

Moore, D., Chory, J., and R.K. Ribaudre. 1993. In *Current protocols in molecular biology* (ed. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, K. Struhl), pp. 2.6.9.

Moqtaderi, Z., Bai, Y., Poon, D., Weil, P.A., and K. Struhl. 1996. TBP-associated factors are not generally required for transcriptional activation in yeast. *Nature* **383:** 188-191.

Morice, W.G., Brunn, G.J., Wiederrecht, G., Siekierka, J.J., and R.A. Abraham. 1993. Rapamycin-induced inhibition of $p34^{cdc2}$ kinase activation is associated with G1/S-phase growth arrest in T lymphocytes. *J. Biol. Chem.* **268:** 3734-3738.

Mosrin, C., and P. Thuriaux. 1990. The genetics of RNA polymerase in yeasts. *Curr. Genet.* **17:** 367-373.

Nishizawa, M., Suzuki, Y., Nogi, Y., Matsumoto, K., and T. Fukasawa. 1990. Yeast GAL11 protein mediates the transcriptional activation signal of two different transacting factors, Gal4 and the general regulatory factor I/repressor/activator site binding protein 1/translation upstream factor. *Proc. Natl. Acad. Sci. USA* **87:** 5373-5377.

Niu, H. and S.T. Jacob. 1994. Enhancer 1 binding factor (E1BF), a ku-related protein, is a growth-regulated RNA polymerase I transcription factor: association of a repressor activity with purified E1BF from serum-deprived cells. *Proc. Natl. Acad. Sci. USA* **91:** 9101-9105.

ole-MoiYoi, O.K. 1995. Casein kinase II in theileriosis. *Science* **267:** 834-836.

Padmanabha, R., Chen-Wu, J.L.-P., Hanna, D.E., and C.V.C. Glover. 1990. Isolation, sequencing, and disruption of the yeast *CKA2* gene: casein kinase II is essential for viability in *Saccharomyces cerevisiael. Mol. Cell. Biol.* **10:** 4089-4099.

Panek, A.D. 1991. Storage carbohydrates. In *The yeasts* (ed. A.H. Rose and J.S. Harrison), pp. 655-678. Academic Press, Inc., New York.

Parsell, D.A. and S. Lindquist. 1994. Heat shock proteins and stress tolerance. In *The biology of heat shock proteins and molecular chaperones* (ed. R.I. Morimoto, A.

Tissieres, C. Georgopoulos), pp. 457-494. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Pause, A., Belsham, G.J., Gingras, A.-C., Donze, O., Lin, T.-A., Lawrence, J.C., Jr and N. Sonenberg. 1994. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* **371:** 762-767.

Pavletich, N.P. and C.O. Pabo. 1991. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. *Science* **252:** 809-817.

Peterson, C.L. and J.W. Tamkun. 1995. The SWI-SNF complex: a chromatin remodeling machine? *Trends Biochem. Sci.* **20:** 143-146.

Petko, L., and S. Lindquist. 1986. Hsp26 is not required for growth at high temperature, nor for thermotolerance, spore development, or germination. *Cell* **45:** 885-894.

Pinna, L.A. 1990. Casein kinase 2: an "eminence grise" in cellular regulation? *Biochim. Biophys. Acta* **1054:** 267-284.

Piper, P.W. 1993. Molecular events associated with aquisition of heat tolerance by the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **11:** 339-356.

Price, D.J., Grove, J.R., Calvo, V., Avruch, J., and B.E. Bierer. 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science* **257:** 973-977.

Pringle, J. and L. Hartwell. 1981. The Saccharomyces cerevisiae cell cycle. In *The molecular biology of the yeast* Saccharomyces: *Life cycle and inheritance* (ed. J.N. Strathern, E.W. Jones, and J.R. Broach), pp. 97-142. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Roberts, S. and M.R. Green. 1994. Activator-induced conformational change in general transcription factor TFIIB. *Nature* **371:** 717-720.

Rothstein, R.J. 1983. One-step gene disruption in yeast. *Methods Enzymol.* **101:** 202-211.

Ruis, H. and C. Schuller. 1995. Stress signaling in yeast. *BioEssays.* **17:** 959-965.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and S.H. Snyder. 1994. RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78:** 35-43.

Sabers, C.J., Marin, M.M., Brunn, G.J., Williams, J.M., Dumont, F.J., Wiederrecht, G., and R.T. Abraham. 1995. Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J. Biol. Chem.* **270:** 815-822.

Sauer, F., Wassarman, D.A., Rubin, G.M. and R. Tjian. 1996. TAF(II)s mediate activation of transcription in the *Drosophila* embryo. *Cell* **87:** 1271-1284.

Schmitt, A.P. and K. McEntee. 1996. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA* **93:** 5777-5782.

Schnell, N. and K.D. Entian. 1991. Identification and characterization of Saccharomyces cerevisiae gene (PAR1) conferring resistance to iron chelators. *Eur. J. Biochem.* **200:** 487-493.

Schnell, N., Krems, B., and K.D. Entian. 1992. The PAR1 (YAP1/SNQ3) gene of Saccharomyces cerevisiae, A c-jun homologue, is involved in oxygen metabolism. *Curr. Genet.* **21:** 269-273.

Schreiber, S.L. 1991. Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science* **251:** 283-287.

Schuller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C., and H. Ruis. 1994. The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae CTT1* gene. *EMBO J.* **13:** 4382-4389.

Schultz, M.C., Choe, S.Y., and R.H. Reeder. 1991. Specific initiation by RNA polymerase I in a whole-cell extract from yeast. *Proc. Natl. Acad. Sci. USA* **88:** 1004-1008.

Schultz, M.C., Hockman, D.J., Harkness, T.A.A., Garinther, W.I., and B.A. Altheim. 1997. Chromatin assembly in a yeast whole-cell extract. *Proc. Natl. Acad. Sci. USA* (in press)

Schultz, M.C., Reeder, R.H., and S. Hahn. 1992. Variants of the TATA-binding protein can distinquish subets of RNA polymerase I, II, and III promoters. *Cell* **69:** 697-702.

Sethy, I., Moir, R.D., Librizzi, M., and I.M. Willis. 1995. In vitro evidence for growth regulation of tRNA gene transcription in yeast. A role for transcription factor TFIIIB70 and TFIIIC. *J. Biol. Chem.* **270:** 28463-28470.

Sollner-Webb, B. and J. Tower. 1986. Transcription of cloned eukaryotic ribosomal RNA genes. *Ann. Rev. Biochem.* **55:** 801-830.

Sommercorn, J., Mulligan, J.A., Lozeman, F.J., and E.G. Krebs. 1987. Activation of casein kinase II in response to insulin and to epidermal growth factor. *Proc. Natl. Acad. Sci. USA* **84:** 8834-8838.

Stan, R., McLaughlin, M.M., Cafferkey, R., Johnson, R.K., Rosenberg, M., and G.P. Livi. 1994. Interaction between FKBP12-rapamycin and TOR involves a conserved serine residue. *J. Biol. Chem.* **269:** 32027-32030.

Suzuki, Y., Nogi, Y., Abe, A., and T. Fukasawa. 1988. GAL11 protein, an auxiliary transcription activator for genes encoding galactose-metabolizing enzymes in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **8:** 4991-4999.

Swaffield J.C., Bromberg, J.F., and S.A. Johnston. 1992. Alterations in a yeast protein resembling HIV Tat-binding protein relieve requirement for an acidic activation domain in GAL4. *Nature* **357:** 698-700.

Tanaka, K., Matsumoto, K., and A. Toh-e. 1988. Dual regulation of the expression of the polyubiquitin gene by cyclic AMP and heat shock in yeast. *EMBO J.* **7:** 495-502.

Tanese, N., Pugh, B.F., and R. Tjian. 1991. Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. *Genes Dev.* **5:** 2212-2224.

Tansey, W.P., and W. Herr. 1997. TAFs: guilt by association. *Cell* **88:** 729-732.

Thevelein, J.M. 1994. Signal transduction in yeast. *Yeast* **10:** 1753-1790.

Thompson, C.M. and R.A. Young. 1995. General requirement for RNA polymerase II holoenzymes *in vivo. Proc. Natl. Acad. Sci. USA* **92:** 4587-4590.

Thuriaux, P., and A. Sentenac. 1992. Yeast nuclear RNA polymerases. In *The molecular and cellular biology of the yeast* Saccharomyces: *Gene expression* (ed. E. Jones et al.). pp. 1-48. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Tower, J., and B. Sollner-Webb. 1987. Transcription of mouse rDNA is regulated by an activated subform of RNA polymerase I. *Cell* **50:** 873-883.

Tower, J., and B. Sollner-Webb. 1988. Polymerase III transcription factor B acitivity is reduced in extracts of growth-restricted cells. *Mol. Cell. Biol.* **8:** 1001-1005.

Vallet, S.M., Brudnak, M., Pellegrini, M. and H.W. Weber. 1993. *In vivo* regulation of rRNA transcription occurs rapidly in nondividing and dividing *Drosophila* cells in response to a phorbol ester and serum. *Mol. Cell. Biol.* **13:** 928-933.

Vallier, L.G. and M. Carlson. 1991. New *SNF* genes, *GAL11* and *GRR1* affect *SUC2* expression in *Saccharomyces cerevisiae. Genetics* **129:** 675-684.

Wahls, W.P., Swenson, G., and P.D. Moore. 1991. Two hypervariable minisatellite DNA binding proteins. *Nucleic Acids Res.* **19:** 3269-3274.

Welch, W.J. 1993. How cells respond to stress. *Sci. Amer.* **268:** 34-41.

Wenzel, T.J., Teunissen, A.W.R.H., and H.Y. Steensma. 1995. PDA1 mRNA: a standard for quantitation of mRNA in Saccharomyces cerevisiae superior to ACT1 mRNA. *Nucleic Acids Res.* **23:** 883-884.

Werner-Washburne, M., Becker, J., Kosic-Smithers, J., and E.A. Craig. 1989. Yeast Hsp70 RNA levels vary in response to the physiological status of the cell. *J. Bacteriol.* **171:** 2680-2688.

Werner-Washburne, M., Braun, E., Johnston, G.C., and R.A. Singer. 1993. Stationary phase in the yeast Saccharomyces cerevisiae. *Microbiol. Rev.* **57:** 383-401.

Werner-Washburne, M., Brown, D., and E. Braun. 1991. Bcy1, the regulatory subunit of cAMP-dependent protein kinase A in yeast, is differentially modified in response to the physiological status of the cell. *J. Biol.Chem.* **266:** 19704-19709.

Wiederrecht G., Brizuela, L., Elliston, K., Sigal N.H., and J.J. Siekierka. 1991. FKB1 encodes a nonessential FK506-binding protein in *Saccharomyces cerevisiae* and contains regions suggesting homology to the cyclophilins. *Proc. Natl. Acad. Sci. USA.* **88:** 1029-1033.

White, R.J., Khoo, B.C.-E., Inostroza, J.A., Reinberg, D., and S.P. Jackson. 1994. Differential regulation of RNA polymerases I, II, and III by the TBP-binding repressor Dr1. *Science* **266:** 448-450.

White, R.J., Stott, D., and P.W.J. Rigby. 1990. Regulation of RNA polymerase III transcription in response to Simian virus 40 transformation. *EMBO J.* **9:** 3713-3721.

Wilson, C.J., Chao, D.M., Imbalzano, A.N., Schnitzler, G.R., Kingston, R.E., and R.A. Young. 1996. RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. *Cell* **84:** 235-244.

Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, J.L., Triezenberg, S.J., Reinberg, D., Flores, O., Ingles, C.J., and J. Greenblatt. 1994. Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. *Mol.Cell. Biol.* **14:** 70137023.

Young, R.A., 1991. RNA polymerase II. *Annu. Rev. Biochem.* **60:** 689-715.

Yu, G., and J.S. Fassler. 1993. *SPT13* (*GAL11*) of *Saccharomyces cerevisiae* negatively regulates activity of the MCM1 transcription factor in Ty1 elements. *Mol. Cell. Biol.* **13:** 63-71.

Zheng, X.F., Fiorentino, D., Chen, J., Crabtree, G.R., and S.L. Schreiber. 1995. TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin. *Cell* **82:** 121-130.

Zhou, Q., Lieberman P.M., Boyer, T.G., and A.J. Berk. 1992. Holo-TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter. *Genes Dev.* **6:** 1964-1974.