DISSERTATION

DYNAMIC INTERPLAY OF TRANSCRIPTION FACTORS DURING THE RESPONSE TO OXIDATIVE STRESS

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2006

COLORADO STATE UNIVERSITY

October 31, 2006

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DAVID ALLEN GOLDSTROHM ENTITLED DYNAMIC INTERPLAY OF TRANSCRIPTION FACTORS DURING THE RESPONSE TO OXIDATIVE STRESS BE ACCEPTED AS FULLFILING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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Complex biological phenomena such as cell growth, response to environmental change, development of multicellular organisms, and disease, are all directly influenced by transcriptional regulatory mechanisms. These mechanisms are fundamentally similar in all eukaryotic organisms. Therefore, understanding transcriptional regulatory mechanisms in less complex eukaryotic organisms such as yeast, will lead to a greater knowledge of similar processes in human cells.

Oxidative stress is linked to numerous deteriorating conditions including cancer, neurodegenerative diseases, atherosclerosis, alcohol-induced liver damage, and aging. In yeast, as in humans, the oxidative stress response is regulated at the level of transcription, thus making yeast a useful model system of study. Here we propose a model for transcriptional activation of RNA polymerase II (Pol II) gene expression during the response to oxidative stress. This work reveals the oxidative stress inducible gene *FLR1* functions through a noncanonical core that contains a relatively complete inert preinitiation complex (PIC) prior to gene activation. Further studies show that phosphorylation of the carboxy-terminal domain (CTD) of Pol II is not sufficient for PIC activation, which is ultimately

achieved by activator-dependent recruitment of Mediator to the *FLR1* promoter. In addition, the essential subunits of Mediator required for cell growth during non-optimal conditions are characterized and grouped into novel Mediator modules. Overall, these findings reveal a two-step mechanism for transcriptional activation involving the combinatorial function of sequentially acting activators. This model suggests the activation of gene expression during oxidative stress is more complex than classical models demonstrate and supports a cooperative role for the function of activator proteins.

Collectively, this entire body of work contributes significantly to a greater understanding of the transcriptional regulatory mechanisms for cell survival during oxidative stress conditions. Ultimately, with this knowledge treatments may be developed to combat the harmful diseases that result from the misregulation of similar stress response processes in human cells.

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ACKNOWLEDGMENTS

First and foremost I would like to thank my advisor Dr. Laurie Stargell who has been an outstanding mentor and friend throughout my entire graduate career. Laurie understands the steps needed to become a successful scientist because she is truly one herself. She has provided me with continual support and has been a joy to work with. Laurie has given me the freedom to solve problems on my own, but her door has always been open for in-depth discussions of my work. Not only has Laurie taught me how to interpret and analyze my work, but also how to present it effectively, which is an essential asset to possess to be successful in the field of science.

Secondly, I would like to thank my committee members Dr. Paul Laybourn, Dr. Karolin Luger, Dr. Marvin Paule, and Dr. Donald Mykles for all of their time and effort throughout the years. I would like to thank Paul who has always taken the time to understand my goals and for the stimulating conversations about my work over a couple of beers we had at this years Keystone conference. I would like to thank Karolin who always brings incredible energy to our meetings and who has greatly supported my efforts along the way. I would also like to thank Marv for his helpful critiques of my work and for pushing me to become a better scientist and scientific writer. I would also like to thank my outside committee member Don Mykles who was gracious enough to replace my initial outside member and for always showing a genuine interest in my work and scientific pursuits.

I would also like to thank all of the current members of the Stargell Lab including, Cathy, Xu, Aaron, Monali, Sarah, Mallory, Judy and Adam. They have been a superb group to work with and they have not only been outstanding colleagues, but friends as well. They have allowed for a productive work environment and have provided me with many stimulating discussions of my work. I would especially like to acknowledge Monali Babre and Sarah Lee for their hard work and interpretation of results for the Mediator story, which they will both be coauthors on this year. They have been dedicated to the project and have been fantastic to work with.

In addition, I would like to acknowledge past lab members for their help with my work. I would especially like to thank Dr. Sue Kraemer for her collaboration with the TFIIA story I published earlier this year, which set the foundation and direction for my current endeavors. I would also like to thank Dr. Mary Robinson for taking me under her wing when I entered the lab and teaching me the "ropes" of the Stargell Lab. She is a great mentor and friend.

I would like to thank two of my collaborators Dr. Scott Moye-Rowley and Dr. Larry Parkhurst. I would like to thank Scott for his efforts with the TFIIA story we published earlier this year and all of his help with the GFP-Yap1 localization studies. I would like to thank Larry for allowing me to contribute to his work concerning the kinetic and thermodynamic effects of TFIIA-TBP-DNA interactions, which will also be published this year.

Next, I would like to thank all of my friends both inside and outside of the department who have provided great release from the everyday stresses of graduate school and science. I especially want to thank Andy Vendel and his wife Natalie who have become like family to me throughout the years. We have had a lot of fun times together and they have really supported me along the way. I also want to thank Jessica Kasel for all of her love and support especially through the last couple of stress-filled months. Just seeing her smiling face, made the struggles and worries of graduate school disappear. She is a real joy to be around. She is an awesome person and it is a dream come to true to have her in my life.

Finally, I would like to thank my family for all of their love and support. I would especially like to thank my Mom Sharon and Dad Don who have been the main support group in my life. They have been outstanding role models to follow and I owe all of my success to their love as parents as well as friends. They have taught me to always fight for what I believe in and to never give up, which has allowed me to succeed in life. They have sacrificed continually along the way to provide me with an outstanding education. Although, they are several miles away, they are really just a phone call away. I am honored and thrilled to dedicate all of my success as a scientist, and more importantly as a person, to them. I love you both!

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CHAPTER 1

Transcriptional activation of RNA polymerase II gene expression during the response to oxidative stress

1.1 Significance of RNA polymerase II mediated transcription

A new era in biology and medicine commenced upon the complete sequencing of the entire human genome. However, the sequence identification of every human gene is of limited value without a description of the function, regulation, and expression of the gene products. Ultimately, it is with this knowledge that true insights into normal human function can be obtained, as well as, an understanding of the many human diseases that result from genetic defects in gene regulation processes.

1.1a Accurate gene regulation is essential for cellular homeostasis

Complex biological phenomena such as cell growth, response to environmental change, development of multicellular organisms, and disease, are all directly influenced by transcriptional regulatory mechanisms (Maniatis et al., 1987; Mitchell and Tjian, 1989; Sauer and Tjian, 1997). These mechanisms are fundamentally similar in all eukaryotic organisms (McKnight and Yamamoto Eds., 1992). The conservation of transcriptional regulatory mechanisms is so great that factors involved in such mechanisms, in many cases, are functionally interchangeable. More than 60 human diseases are due to genetic defects in

transcription factors and their functions. Therefore, understanding the mechanisms of gene regulation in less complex eukaryotic organisms such as yeast, will ultimately lead to a greater understanding of similar processes in human cells.

1.1b Importance of the yeast *Saccharomyces cerevisiae* as a model system

To gain insight into the molecular mechanisms underlying human gene regulation and how defects in such regulation lead to human disease, one must utilize many different types of systems. Fortunately, organisms as diverse as yeast, worms, flies, mice, and humans have homologous genomes. This similarity allows for the ability to understand human gene function through the analysis of genes in these so called "model organisms". Even a slight similarity between a human gene and one from a model organism is often enough to determine a role for the gene in human health and disease. The major strengths of model organisms are that they are much easier to manipulate in a laboratory setting and have rapid reproduction rates, thus making them of even greater scientific significance.

One of the most widely used eukaryotic model organisms in science today is the yeast *Saccharomyces cerevisiae*. Yeast have been established in the industries of beer, bread, wine fermentation, and ethanol production for many years. In 1996, the yeast *Saccharomyces cerevisiae* became the first eukaryote to have its entire genome sequenced. The complete sequencing of the yeast genome led to the development of the yeast genome database (http://www.yeastgenome.org),

which is highly annotated and remains an important tool for mining basic knowledge about the function and organization of eukaryotic cell genetics and physiology. It is with this work that many proteins important in human biology were first discovered by studying their orthologs in yeast. The conservation of yeast and human genes is so great that human genes can often be substituted for yeast genes with no resulting differences in cell function. In addition, genetic manipulations in yeast are relatively straightforward and cheap, whereas such manipulation, even when possible in mammalian systems, is neither simple nor cheap.

Studies utilizing yeast as a model organism have developed powerful approaches in biochemistry, molecular biology, and genetics that are not available in other eukaryotic organisms. Yeast is particularly amenable for analyzing transcriptional regulatory mechanisms in vivo, under true physiological conditions. Classical and molecular yeast genetics have permitted the discovery and functional characterization of transcriptional regulatory proteins that were not identified in biochemical studies. Thus, genetic analysis in yeast has often generated information complementary to that obtained from biochemical studies of transcription in vitro and has provided unique insights into mechanisms of transcriptional regulation in higher eukaryotes. It is with these advantages that yeast are successfully utilized in this study to further our understanding of transcriptional activation mechanisms during the response to oxidative stress.

1.2 Fundaments of RNA polymerase II mediated transcriptional activation

A major focus in biological research is understanding the mechanisms of RNA polymerase II (Pol II) gene regulation responsible for normal function as well as the misregulation in the disease state, which results from genetic defects in transcription factors and their regulatory mechanisms. Current knowledge of gene regulation mechanisms is the result of combinatorial efforts from biochemists, geneticists, and biophysicist, which have utilized a wide variety of genes, promoters, proteins, organisms, and experimental approaches. Due to the vast amount of information regarding transcriptional regulatory mechanisms, this section focuses on only the key aspects that pertain to my doctoral studies utilizing the eukaryotic yeast *Saccharomyces cerevisiae* in the study of Pol II transcriptional activation. Notably, a high level of conservation exists between all eukaryotic Pol II promoters, Pol II machinery, transcription factors, and mechanisms. Thus, studies in yeast will allow for a greater understanding of similar processes in humans cells.

1.2a Basic promoter architecture

Eukaryotic Pol II promoters contain three main features important to this work: the initiator element (Inr), the core promoter, and upstream activation sequences (UASs) (Blackwood and Kadonaga, 1998; Struhl, 1989) (Figure 1.1A). The Inr is located near the transcription initiation site (*tis*) and is the primary determinant of where transcription begins. Various factors are known to bind the Inr and this binding may facilitate recruitment of the preinitiation complex (PIC) to promoter

DNA (Aso et al., 1994; Carcamo et al., 1991). In yeast, the core promoter is important in TATA-binding protein (TBP) promoter recognition and may contain an AT-rich site called the TATA box that is located 40-120 bp upstream of the *tis* (Singer et al., 1990). The binding of TBP to promoter DNA is thought to nucleate the assembly of the PIC to promoter DNA. Recent data suggests that approximately 80% of genes do not contain a canonical TATA element (Basehoar et al., 2004). Therefore, in core promoter elements lacking recognizable TATA elements, TBP binds to the core promoter in a manner stabilized by protein-protein interactions with other transcription factors (Burke and Kadonaga, 1997). The UAS is defined as short (10-30 bp) promoter-specific DNA element that is located 50-500 bp upstream of the *tis* (Guarente et al., 1984). Sequence-specific activator proteins bind UAS elements, thus enhancing gene expression (Blackwood and Kadonaga, 1998). Binding of activators to the UAS can also stimulate the assembly of the PIC by direct or indirect contacts (Buratowski, 2000).

1.2b Transcription cycle events and required factors

A single round of the eukaryotic Pol II transcription cycle encompasses five main events important to this work: PIC assembly, transcriptional initiation, promoter clearance, elongation, and termination (Figure 1.1B). The first step of the transcription cycle involves the recruitment of the PIC to the core promoter (Lee and Young, 2000). Initially, the general transcription factor TFIID, comprised of TBP and its associated factors (TAFs), is recruited to the core promoter

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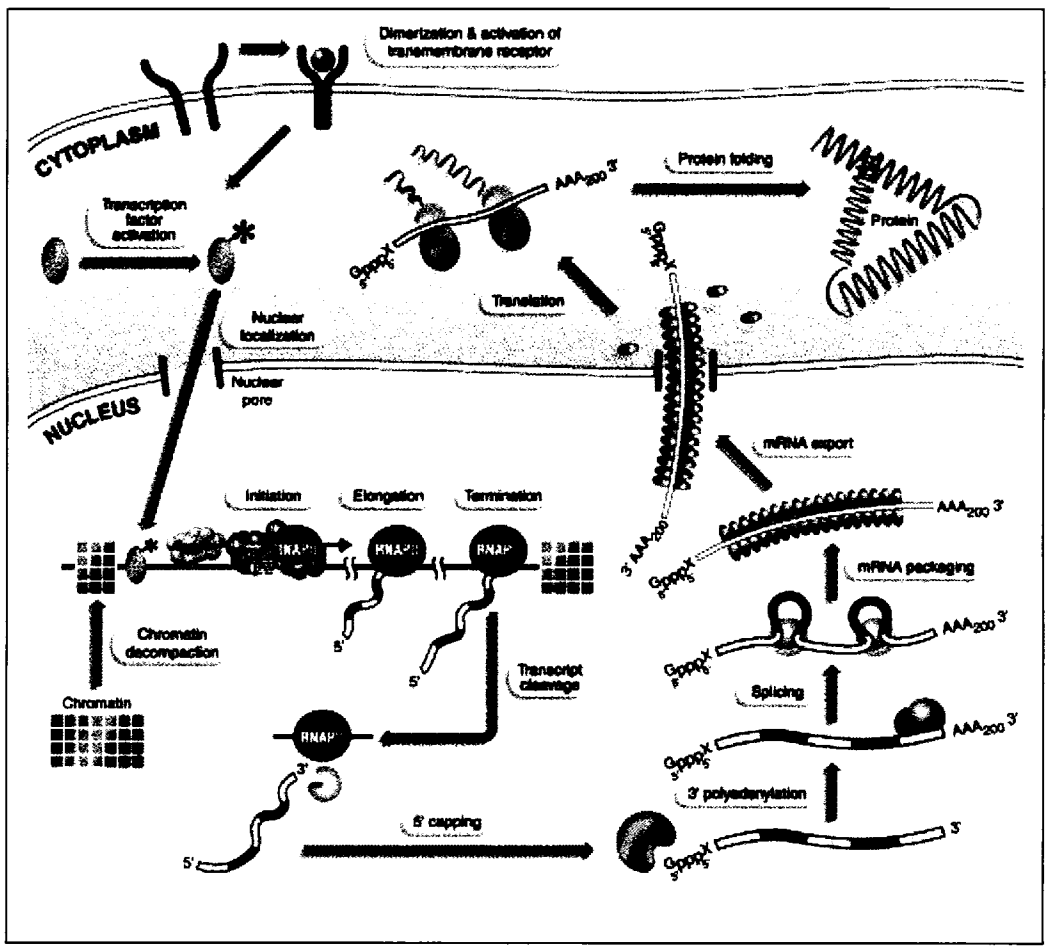

Figure 1.1 Eukaryotic Pol II promoter architecture and transcription cycle events
(A) Basic eukaryotic Pol II promoters contain three common features: the initiator element (Inr), the core promoter, and upstream activation sequences (UAS). These elements are utilized to regulate gene expression during the transcription cycle. (B) The eukaryotic Pol II transcription cycle encompasses five main events: PIC assembly, transcriptional initiation, promoter clearance, elongation, and termination.

(Hernandez, 1993; Struhl, 1994). TFIID binding to the core promoter nucleates the ordered recruitment of the additional factors TFIIA, TFIIB, TFIIF, Pol II, TFIIE, and TFIIH (Hampsey, 1998; Kornberg, 2001; Kuras and Struhl, 1999; Lee and Young, 2000) to form a transcriptionally competent PIC. Coactivator complexes, such as Mediator can also be recruited to the core promoter and aid in gene transcription (Fan et al., 2006; Kornberg, 2005).

Once the PIC is recruited to the core promoter, transcription is initiated by the melting of the double-stranded DNA to form an open promoter complex. This melting of DNA into a single-stranded form involves the ATP-dependent helicase subunit of the transcription factor TFIIH (Goodrich and Tjian, 1994; Kim et al., 2000). Melting of the double-stranded DNA leads to the formation of a single-stranded transcription bubble where the first phosphodiester bond of the nascent RNA strand is formed. The RNA strand is formed upon the addition of the first two nucleoside triphosphates (NTPs), which is dependent on the particular DNA sequence. Even though a RNA transcript has been made, Pol II must pass the promoter clearance stage before it can enter the productive stage of transcriptional elongation (Dvir et al., 1996).

The promoter clearance step of the transcription cycle involves the disassembly of the PIC on the core promoter. Disassembly of the PIC allows Pol II and TFIIF to escape the promoter, leaving a subset of factors behind, which serve as a scaffold for PIC complex formation during transcriptional reinitiation (Yudkovsky

et al., 2000; Zawel and Reinberg, 1995). A hallmark of the transition from transcriptional initiation to elongation is the phosphorylation of the carboxy-terminal domain (CTD) of Pol II, which is thought to aid in release of a substantial part of the PIC (O'Brien and Lis, 1991). In yeast, the CTD of Pol II consists of 26 repeats of the heptapeptide sequence (Tyr-Ser-Pro-Thr-Ser-Pro-Ser), which is phosphorylated by the kinase subunit of TFIIH on serine residues 5 and 2, respectively (Dahmus, 1996). Phosphorylation of the CTD is also thought to enhance interactions with numerous elongation factors including the 5' capping enzyme Ceg1 (Yue et al., 1997), pre-mRNA processing factors (Proudfoot et al., 2002), and even RNA exportation factors (Luo and Reed, 1999).

Once Pol II has cleared the promoter, the transcriptional elongation step can occur as Pol II transcribes the coding region of the gene. The progression of Pol II into the coding region allows for reinitiation of transcription at the promoter region of the gene by additional Pol II proteins. The transcriptional reinitation phase is more efficient than the initial round of transcription and is responsible for the majority of transcription in vivo (Jiang and Gralla, 1993; Orphanides and Reinberg, 2000; Ranish et al., 1999). CTD phosphorylation is thought to aid in the transition into the elongation phase of the transcription cycle by disrupting interactions between Pol II and factors involved in the initiation process. The phosphorylation state of the CTD is also believed to concomitantly establish interactions with specific elongation factors that interact with Pol II during the transcriptional elongation process. These factors include but are not limited to

TFIIF, TFIIS, Elongins, and Elongator (Elmendorf et al., 2001; Jeon and Agarwal, 1996; Krogan and Greenblatt, 2001; Tan et al., 1995). These elongation factors allow Pol II to overcome certain disruptions in the elongation process such as pausing, arresting, as well as function to stimulate the rate of transcription (Reines et al., 1999).

After efficient elongation of the coding region of Pol II genes, the process of transcriptional termination occurs. At this stage, the mRNA is cleaved, spliced, polyadenylated, and transported to the cytoplasm where it can be translated into protein by the ribosome (Proudfoot et al., 2002). The mechanism of Pol II termination is thought to be coupled to the maturation of 3' mRNA ends at the poly(A) site (Proudfoot, 2004). This is supported by studies illustrating that the poly(A) consensus sequence is essential for triggering the transcriptional termination process (Logan et al., 1987; Zaret and Sherman, 1982). Recent studies also suggest that the transcribing Pol II is "torpedoed" by exonucleases, which triggers transcriptional termination (Kim and Iyer, 2004; West et al., 2004). It is believed that as the nascent RNA is cut at the polyadenylation site, the exonuclease recruited by 3' processing factors associated with phosphorylation of the CTD is loaded onto the free end of downstream RNA and acts to rapidly degrade the RNA until it catches up with the transcribing Pol II, thus terminating transcription.

1.2c Recruitment regulated gene activation

The expression of eukaryotic genes by Pol II is regulated at the level of transcription (Iyer and Struhl, 1996; Klein and Struhl, 1994; Kuras and Struhl, 1999). The activation of Pol II gene expression occurs by DNA-binding transcriptional activator proteins that specifically recognize upstream promoter regions via typical eukaryotic DNA-binding motifs (homeodomains, zinc finger, bZIP, bHLH etc.). While transcriptional activation of Pol II gene expression can occur by the binding of a single activator protein to an upstream element, some activators (AP-1, ATF/CREB, Swi5/Ace2) require the binding of multiple activator proteins (McNabb et al., 1995; Primig et al., 1992). Once activator proteins bind to the UAS located in the upstream promoter region, they act to stimulate gene expression via a transcriptional activation domain that is functionally distinct, and usually physically separable, from the DNA-binding domain (Ptashne and Gann, 1997).

Transcriptional activation occurs through the ability of activator proteins to recruit and therefore increase PIC assembly on promoter DNA (Figure 1.2). Evidence supporting this model has been retained both in vitro (Lin and Green, 1991) and in vivo (Kuras and Struhl, 1999). Studies also suggest that in some instances, the recruitment of TBP to the core promoter can be a rate-limiting step in the transcriptional activation process (Majello et al., 1998). These results are supported further by artificial recruitment experiments that involve fusing a subunit of the general transcription machinery to a DNA-binding domain, and

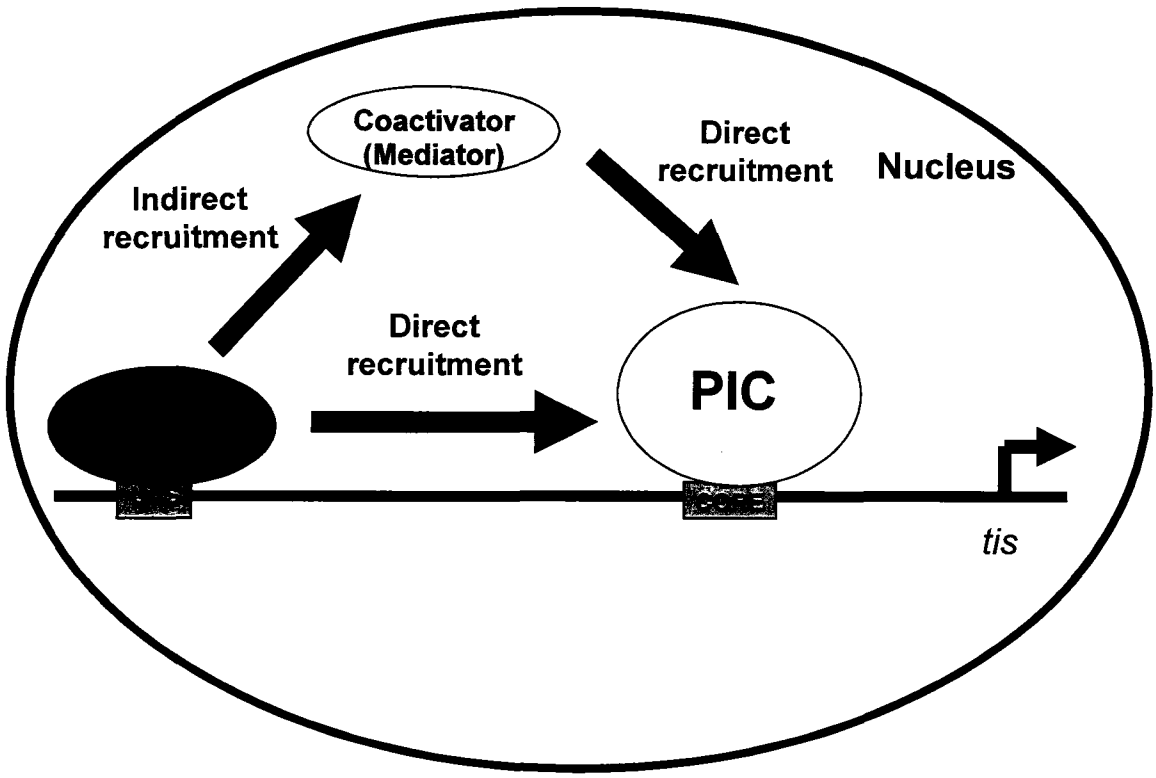

Figure 1.2 Transcriptional activation by recruitment
Generally, transcriptional activation of Pol II gene expression occurs through the direct recruitment of the preinitiation complex (PIC) by activator proteins. Transcriptional activation can also occur through the indirect recruitment of coactivator complex such as Mediator that act to directly recruit the PIC.

testing for its ability to increase transcription. These fusion proteins have been shown to be successful in artificially directing the assembly of the PIC to promoter DNA, thus stimulating transcription (Ptashne and Gann, 1997). Although activator proteins have been shown to stimulate transcription by the recruitment of the PIC to promoter DNA, the mechanism by which these activators increase recruitment of the PIC still remains unclear. It is possible that the activation domain may directly interact with coactivator proteins, thereby stabilizing the association of the PIC with promoter DNA. For example, many studies have shown that gene activation occurs by activator-dependent recruitment of Mediator to promoter DNA (Chapter 2; (Bhoite et al., 2001; Bryant and Ptashne, 2003; Cosma et al., 2001; Cosma et al., 1999; Kuras et al., 2003). Therefore, uncovering the role of Mediator in transcriptional activation will allow for a more complete understanding of gene regulation processes.

1.3 Mediator and its role in RNA polymerase II mediated transcriptional activation

A central goal in the transcription field is to understand the regulation of gene expression as it occurs through both intrinsic genetic programming and extracellular signals. A simplistic view of transcriptional activation mechanisms would entail activator-dependent recruitment of the PIC to promoter DNA. However, an increasing number of studies are finding a required role for coactivator proteins in the activation of gene expression. In particular, the coactivator Mediator is essential for activated forms of transcription where it is

thought to interact with general transcription factors and Pol II. However, the mechanism of Mediator function during transcriptional activation remains unclear. Therefore, understanding Mediator function will allow for a more complete understanding of transcriptional activation processes.

1.3a Discovery of Mediator function

Early biochemical studies in yeast led to the first evidence of Mediator function. It was observed that the over-expression of one activator protein interfered with the activation of Pol II by another activator (Gill and Ptashne, 1988). This effect, termed "squelching", was the result of the competition between activators for a common limiting target. This target was thought to be a component of the Pol II transcription machinery. A direct mechanism of activator function, through the PIC, was later supported by experiments showing activator binding to the general transcription factor TBP as well as TFIIH and Pol II (Brandl and Struhl, 1989; Stringer et al., 1990; Truant et al., 1993). However, the widespread belief in a direct mechanism was challenged by analysis of squelching in a crude yeast Pol II transcription system in vitro (Kelleher et al., 1990). In these studies, the addition of excess amounts of GTFs or Pol II to relieve squelching argued against these proteins being the direct targets of activators. However, the addition of a crude protein fraction from yeast did relieve squelching and the activity of this crude fraction was termed Mediator. Eventually, Mediator was purified to homogeneity (Kim et al., 1994) by the purification of a complete fractionated yeast Pol II transcription system (Flanagan et al., 1991). Mediator

was isolated in two forms, free Mediator and a Mediator/Pol II complex. It was observed that Mediator function was not only required for the response to transcriptional activators, but it also stimulated basal transcription in the absence of an activator (Kim et al., 1994). Overall, the isolation of Mediator united the initial observations of genetic screens with a biochemical entity that is a critical component in the regulation of gene expression.

1.3b Structure of Mediator

Mediator is a large multi-protein complex that consists of 25 subunits with a total mass of greater than one million Daltons (Figure 1.3). Of these 25 total subunits, 15 are nonessential and thus, the cell can live in rich medium. Although no high resolution X-ray structure of Mediator exists, numerous studies involving mapping of protein-protein interactions, as well as biochemical and EM analyses have led to the development of a low resolution structure of Mediator (Asturias et al., 1999; Dotson et al., 2000; Guglielmi et al., 2004; Sato et al., 2003). These experiments have defined Mediator as a complex consisting of four distinct modules termed: Head, Middle, Tail, and Cdk8. Mutations in the Head components of Mediator disrupt Pol II binding (Ranish et al., 1999). The Middle module also contributes to Mediator and Pol II interactions, whereas the Tail module of Mediator is thought to be involved in the interaction with activator proteins. The Cdk8 module of Mediator contains a kinase domain that is thought to participate in the phosphorylation of the CTD of Pol II (Kim et al., 1994).

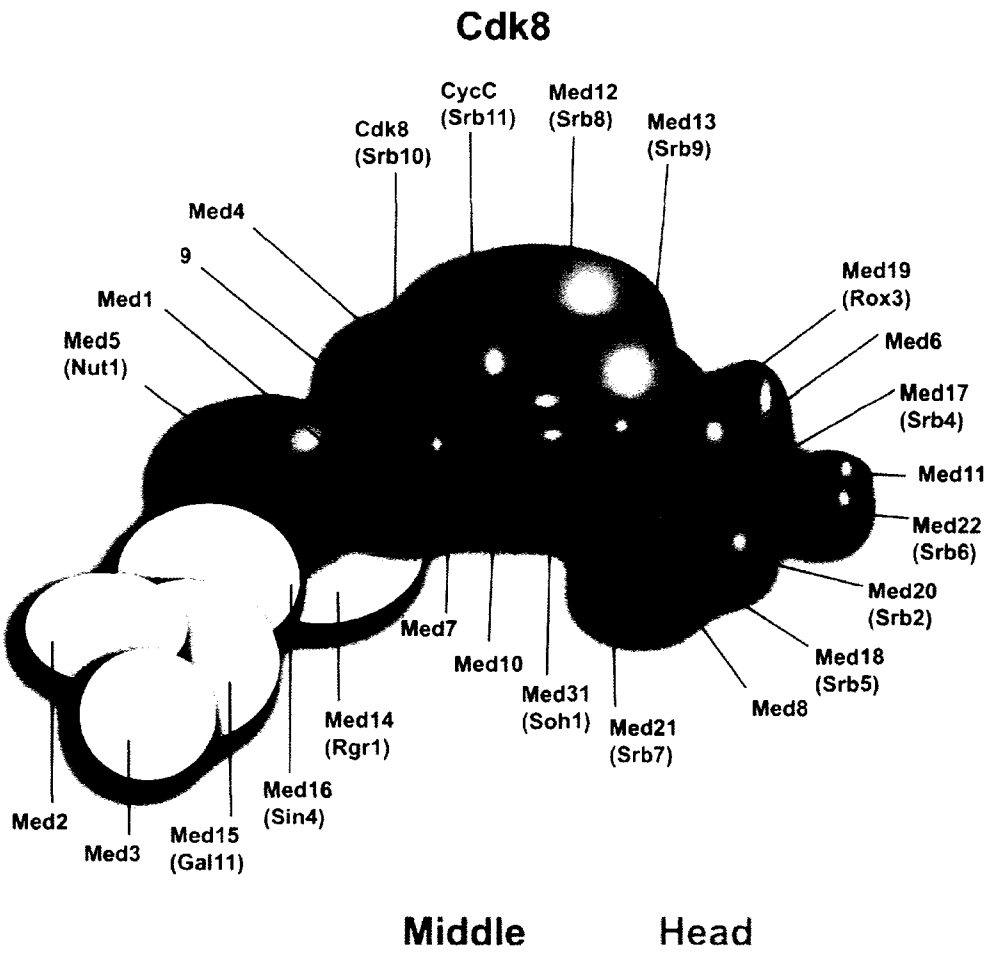

Figure 1.3 Structure of the coactivator Mediator
Mediator is a large multi-protein complex that consists of 25 subunits what a total mass of greater than one million Daltons. These subunits are grouped into modules called Head, Middle, Tail, and Cdk8. The Head and Middle modules interact with Pol II where the Tail module interacts with activator proteins. 15 of the 25 subunits are essential for cell growth on rich medium.

1.3c Mediator is conserved throughout evolution

Mediator is conserved throughout eukaryotes. Although the Mediator genes are the best characterized in the yeast *Saccharomyces cerevisiae* (Borggrefe et al., 2002; Kang et al., 2001; Myers and Kornberg, 2000). Mediator genes have also been studied in the yeast *Schizosaccharomyces pombe*, the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster* (Boube et al., 2000; Gu et al., 2002; Rachez and Freedman, 2001; Spahr et al., 2003). In addition, studies have revealed nearly all yeast Mediator subunits in human cells (Boube et al., 2002; Bourbon et al., 2004; Sato et al., 2004). The close similarity between yeast and mammalian Mediators was also observed in structural studies where the complexes appear similar in size, shape, and internal structural (Davis et al., 2002; Dotson et al., 2000).

1.3d Mediator function in transcriptional activation

It is thought that activators of Pol II gene expression interact with both Mediator and Pol II (Naar et al., 1998). Thus, Mediator likely regulates gene expression through the relaying of vital information between these activator proteins and the PIC, through Pol II (Figure 1.4A). Even though Mediator stimulates both basal and activated transcription, the exact mechanism for this action is still unclear. In dissecting the mechanism of the role of Mediator in gene activation, several studies have observed activator-dependent recruitment of Mediator (Chapter 2; (Bhoite et al., 2001; Bryant and Ptashne, 2003; Cosma et al., 2001; Cosma et al., 1999; Kuras et al., 2003). In eukaryotic cell extracts, Mediator is also found in a

complex with Pol II in solution (Kim et al., 1994; Myers and Kornberg, 2000; Taatjes and Tjian, 2004), suggesting that Mediator and Pol II bind to promoters simultaneously (Figure 1.4B). However, Mediator promoter occupancy does not strictly correlate with that of Pol II occupancy (Cosma et al., 2001; Fan et al., 2006; Park et al., 2001). In addition, there is increasing evidence for the role of Mediator in influencing PIC formation where Mediator can stimulate basal transcription from core promoters in fractionated functional systems even when Pol II is not limiting (Kim et al., 1994). Also, Mediator is critical for core promoter activity in human systems (Baek et al., 2002; Mittler et al., 2001; Wu et al., 2003). Several lines of evidence suggest that Mediator activates through the general transcription factor TFIIH. Mediator has been shown to interact with TFIIH and also stimulates the kinase subunit of TFIIH through the Kin28 subunit of TFIIH (Giot et al., 2003; Sakurai and Fukasawa, 2000). The mechanisms of transcriptional activation by Mediator are presently unclear. However, this process is likely dependent on the core promoter structure and the regulatory context of the gene.

1.4 Defining post-PIC recruitment gene function

Expression of eukaryotic genes by Pol II is controlled primarily at the level of transcription initiation (Kuras, 1999; Klein, 1994; Iyer, 1996). In many instances, the recruitment of TBP to promoter DNA is a rate-limiting step in the transcriptional activation process (Kuras, 1999; Li, 1999). A variety of transcription factors such as activator proteins, GTFs, TAFs, and numerous other

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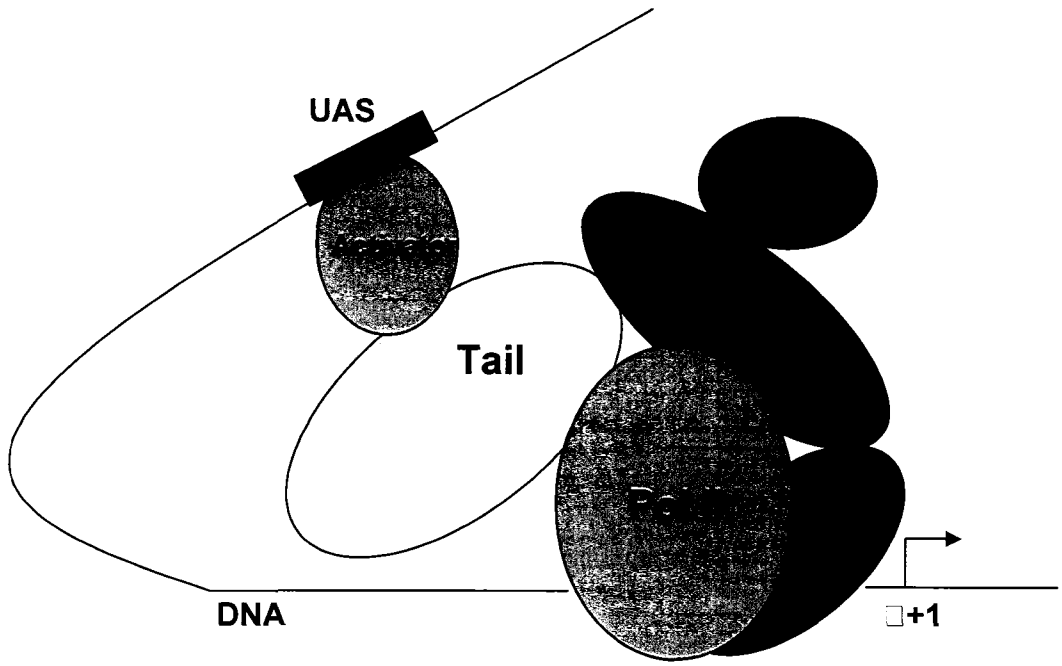

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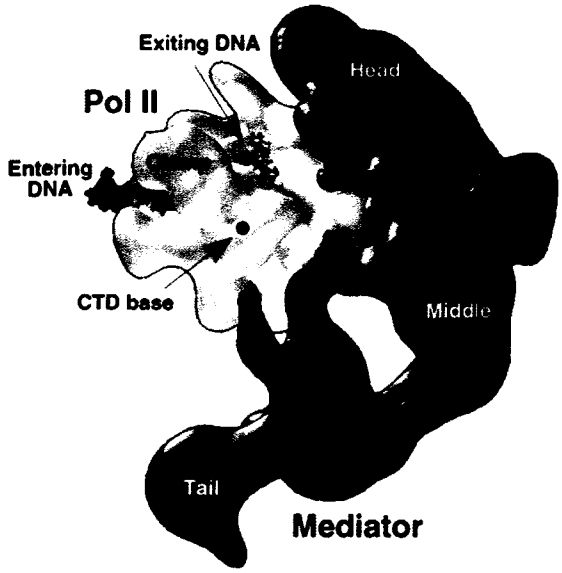


Figure 1.4 The role of Mediator in transcriptional activation
(A) The coactivator Mediator can act as a bridge between activator proteins and the PIC through Pol II to relay critical information important for gene activation. (B) The low resolution EM structure of Mediator (blue) in complex with Pol II (white) shows Mediator mainly contacts Pol II with the Head and Middle domains.

factors, participate in TBP binding to promoters (Hampsey, 1998; Aubel, 1994). Thus, the functions of these factors can be defined as "recruitment regulated" functions. Since the recruitment of TBP to promoter DNA nucleates the formation of the PIC, the TBP recruitment functions can be considered a part of promoting the assembly of the PIC and gene activation. However, for several genes TBP recruitment is not rate limiting. Several of these genes are important in a variety of responses critical to cell survival, such as the heat shock responses and oncogenesis. Therefore, defining the transcriptional regulatory mechanisms of these genes provide valuable information of stress regulate genes, in general.

1.4a Post-PIC recruitment transcriptional activation

Although previous studies demonstrate the ability of activators to stimulate transcription by increasing the recruitment of TBP to promoter DNA, other mechanisms of transcriptional activation exist. Many studies indicate that transcriptional activation occurs at a step after recruitment of the PIC to the promoters of numerous genes, including *CYC1*, *COX5a*, and the *Drosophila* heat shock genes (Kuras and Struhl, 1999; Martens et al., 2001). For example, TBP is bound to the *CYC1* promoter in the absence of upstream activator proteins (Chen et al., 1994) and artificial recruitment of TBP does not result in transcriptional activation (Chatterjee and Struhl, 1995). This suggests that recruitment of TBP or the PIC to promoter DNA is not a rate-limiting step of the transcriptional activation process. These functions are defined as "post-PIC

recruitment" functions as they are required for gene activation at a step after the recruitment of the PIC to promoter DNA. These post-PIC recruitment functions of the PIC are likely to be involved with the recruitment of other factors necessary for the transcriptional activation process or facilitate subsequent steps in the transcription cycle, such as promoter melting, promoter clearance, elongation, termination, or even pre-mRNA processing. In this dissertation, the oxidative stress inducible gene *FLR1* was found to be activated by recruitment of Mediator. However, members of the PIC, including TBP and Pol II were found to occupy the *FLR1* promoter prior to gene activation. Therefore, defining the mechanism of post-PIC recruitment regulation will provide valuable information concerning how genes are regulated during oxidative stress.

1.5 RNA polymerase II mediated transcriptional activation during the response to oxidative stress

Aerobically growing cells have evolved complex and efficient strategies for dealing with variable and harsh environments. A key aspect of cell survival during such conditions involves the regulation of stress responses that transcriptionally activate genes encoding important defense and repair mechanisms. In yeast, the key regulator of the transcriptional response to oxidative stress is the activator Yap1 (yeast AP-1). Importantly, the generation of reactive oxygen species (ROS) and many of the responses are conserved from yeast to humans (Dalton et al., 1999). Therefore, the study of model

organisms such as yeast, will increase our understanding of how cells survive and flourish under oxidative stress.

1.5a Reactive oxygen species, oxidative stress, and cellular damage

Remarkable similarities are observed between yeast and human cells at the macromolecular and organelle level. In addition, a number of yeast proteins have been shown to be functionally interchangeable with those of humans. These findings strongly support yeast as a model system for the study of cellular effects caused by oxidative stress. Since yeast cells can grow aerobically, they are continually bombarded by ROS generated as by-products of cellular metabolism. Under normal conditions, cellular damage is prevented by antioxidant defenses that neutralize harmful ROS (primary defenses) and repair molecular of DNA damage (secondary defenses). However, under stress conditions the levels of ROS exceeds the antioxidant capacity of the cell, leading to oxidative stress. This imbalance can result from a decrease in antioxidants, an increase in ROS, or both.

Formation of ROS, namely superoxide radical, hydroxyl radical, and hydrogen peroxide (H_2O_2) leads to the cellular damage of proteins, DNA, and lipids (Gutteridge and Halliwell, 2000; Halliwell, 1998; Halliwell, 1999a; Halliwell, 1999b; Halliwell and Gutteridge, 1984). For example, enzyme inactivation by H_2O_2 occurs through direct oxidation of essential thiol groups of cysteine residues at the active site. In addition, the oxidation of DNA leads to base and sugar

damage, single strand breaks, and DNA-protein cross-links that can cause lethal or mutagenic effects. For example, H_2O_2 has been shown to induce base oxidation, generate strand breaks, and increase the frequency of intrachromosomal recombination (Brennan et al., 1994; Frankenberg et al., 1993). Lastly, damage to lipids involves the oxidation of polyunsaturated fatty acids by an autocatalytic process, leading to the production of fatty acid hydroperoxides. These hydroperoxides undergo fragmentation, generating a variety of highly reactive products. Overall, it is the accumulation of the above cellular damage that can lead to a variety of deteriorating conditions including atherosclerosis, cancer, alcohol-induced liver damage, and aging (Finkel and Holbrook, 2000; Halliwell, 1992a; Halliwell, 1992b) (Figure 1.5). Therefore, it is the ability of cells to sense these challenges by oxidative stress and mount a response at the molecular level (Figure 1.6). This involves activator proteins who function to increase the expression of genes whose products act to decrease levels of reactive oxygen species within the cell. This likely involves induction of both primary and secondary antioxidant defenses (Jamieson, 1998; Moradas-Ferreira and Costa, 2000).

1.5b Yap1, the key regulator of the oxidative stress response

The key regulator of the oxidative stress response in the yeast *Saccharomyces cerevisiae* is the basic-leucine zipper transcriptional activator Yap1 (Moye-Rowley, 2002) (Figure 1.7A). Yap1 was first identified as a DNA-binding activity present in *Saccharomyces cerevisiae* nuclear extract that bound the SV-40 early

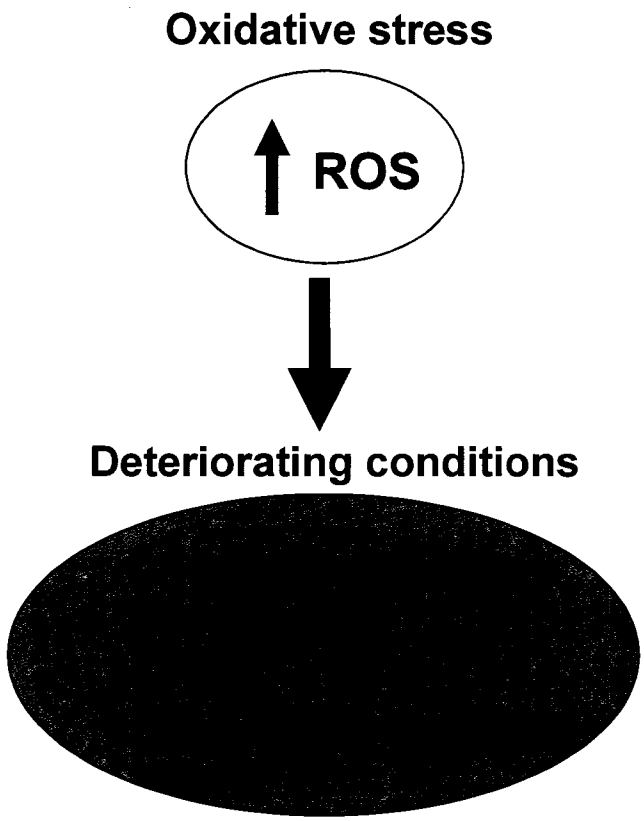


Figure 1.5 Oxidative stress and disease
The accumulation of reactive oxygen species often leads to oxidative stress within the cell. Oxidative stress has been linked to numerous deteriorating conditions and diseases.

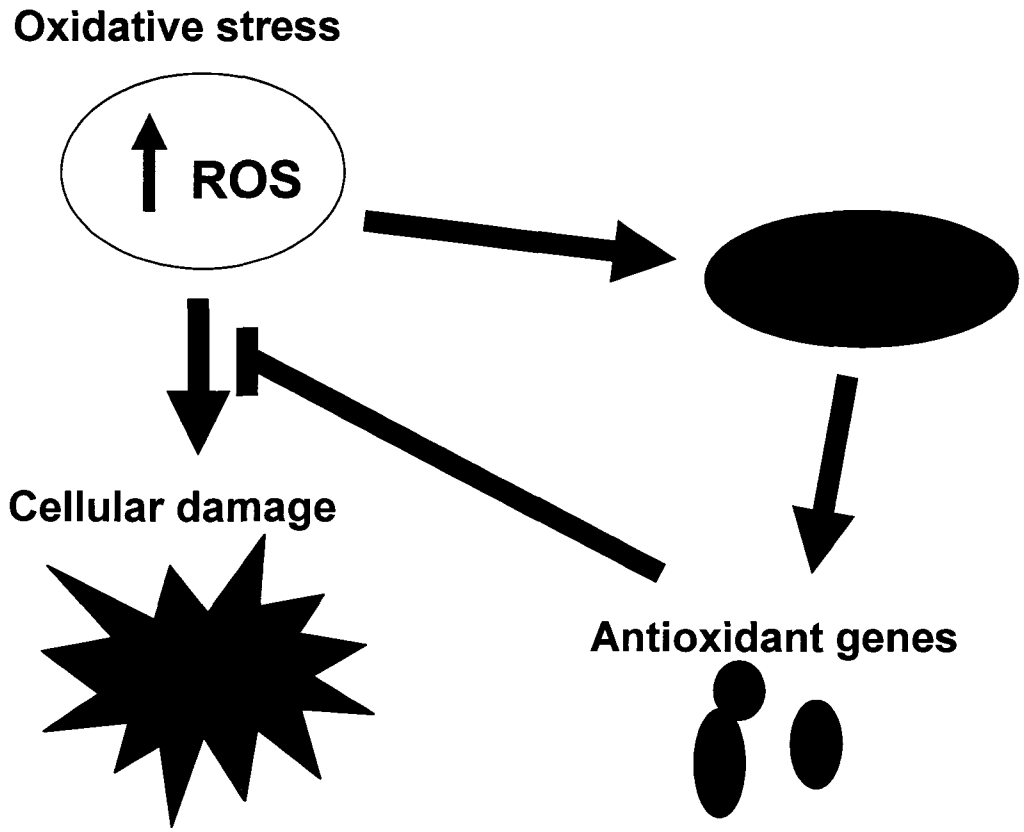
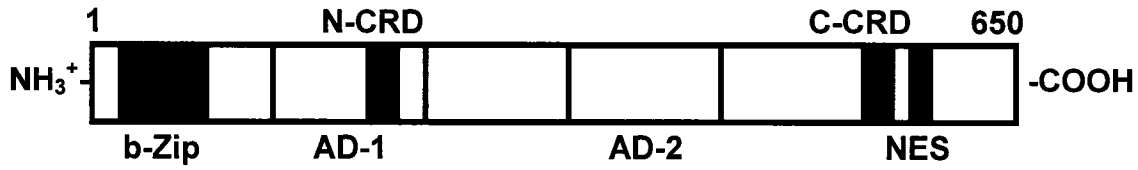


Figure 1.6 The oxidative stress response
Activator proteins function to increase the expression of gene whose products act to decrease the levels of reactive oxygen species within the cell, thus diminishing cellular damage.

enhancer in a manner closely resembling the mammalian activator protein-1 (AP-1) transcriptional activator (Harshman et al., 1988). In addition, studies using an artificial AP-1 recognition element from the SV-40 early enhancer demonstrated that Yap1 was capable of activating transcription from this viral DNA element in human cells (Harshman et al., 1988). The role of Yap1 in the regulation of enzymes that protect against oxidative stress is supported by the observation that strains deleted for Yap1 are hypersensitive to H_2O_2 as well as other oxidants (Schnell and Entian, 1991; Schnell et al., 1992). In addition, cells over-expressing Yap1 have an increased stress tolerance to numerous oxidants including H_2O_2 (Kuge et al., 1997; Vido et al., 2001; Wemmie et al., 1997). Recent studies utilizing global analysis techniques have added a growing number of Yap1 gene targets that are involved in the detoxification of harmful ROS (Carmel-Harel et al., 2001; Dumond et al., 2000; Gasch et al., 2000; Lee et al., 1999). The regulation of Yap1 gene targets is accomplished through the cellular localization of Yap1 protein (Kuge et al., 1997) (Figure 1.7B). Under normal conditions, the majority of Yap1 is exported out of the nucleus by the nuclear export factor Crm1, leading to low levels of transcription of Yap1-regulated genes. However, under oxidative stress conditions, the interaction between Crm1 and Yap1 is inhibited. This inhibition leads to nuclear accumulation of Yap1, binding of Yap1 to YREs (Yap1 response elements), and the activated transcription of genes involved in the oxidative stress response (Kraemer et al., 2006; Kuge and Jones, 1994; Lee et al., 1999; Nguyen et al., 2001; Wu and

A



B

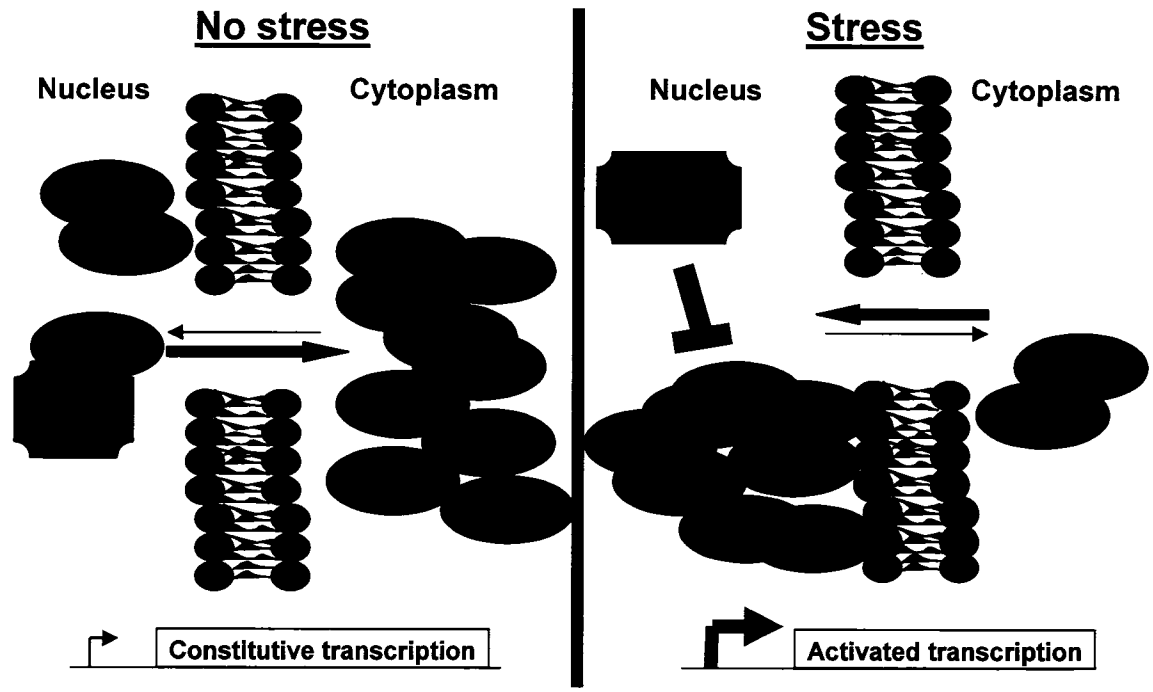


Figure 1.7 Yap1 is involved in cellular detoxification
(A) Yap1 is a 650 amino acid activator protein that is the key regulator of the oxidative stress response. Yap1 contains a b-ZIP (basic leucine zipper) DNA binding domain, two AD (activation domains), two CRD (cysteine rich domains), and a NES (nuclear export sequence). (B) During normal (no stress) conditions, Yap1 remains in the cytoplasm due to interactions with the nuclear export factor Crm1. Upon oxidative stress, the interaction with Yap1 and Crm1 is inhibited, thus leading to nuclear accumulation of Yap1 and the activated transcription of Yap1-regulated genes.

Moye-Rowley, 1994). In particular, the gene *FLR1* (Fluconazole Resistance 1), which encodes a multi-drug transporter of the major facilitator superfamily, is up-regulated by over-expression of Yap1 (Alarco et al., 1997), contains three YREs in its upstream promoter region (Kuge and Jones, 1994; Lee et al., 1999; Nguyen et al., 2001; Wu and Moye-Rowley, 1994), and is induced by oxidative stress (Broco et al., 1999; Nguyen et al., 2001; Tenreiro et al., 2001), thus making the *FLR1* gene an ideal gene to study Yap1-dependent transcriptional regulation during oxidative stress. Despite these observations, the mechanism of transcriptional activation by Yap1 during the oxidative stress response is unknown. Since Yap1 and the oxidative stress response are conserved from yeast to humans, understanding the mechanism of activation by Yap1 will allow for a greater understanding of similar regulation mechanisms in human cells.

1.6 Significance and thesis layout

Throughout the duration of my doctoral studies, I have had the opportunity to address and answer several important questions regarding the mechanisms of activator-dependent gene regulation, utilizing the yeast *Saccharomyces cerevisiae* as a model system. In Chapter 2, I utilized the oxidative stress inducible gene *FLR1* to develop a model of activated transcription during oxidative stress. My findings reveal a two-step mechanism for transcriptional activation involving the combinatorial function of sequentially acting activators. In the first step, one activator functions to recruit an inactive remarkably complete

PIC. In the second step, another regulator "activates" the PIC via the recruitment of Mediator. This model suggests the regulation of gene expression during oxidative stress is more complex than classical models and supports a cooperative role for the function of multiple activator proteins during the response to oxidative stress.

In the above study, the coactivator Mediator was found to play a role in the activation of *FLR1* gene expression. In Chapter 3, a fruitful collaboration with Monali Babre and Sarah Lee in the lab was successful in dissecting the essential subunits of Mediator involved in the regulation of gene expression during the response to oxidative stress. We focused on the nonessential subunits of these modules and determined how deletion of these subunits affects cell survival during various stress conditions. Overall, this study uncovered novel mediator modules that are uniquely involved in cell survival during various stress conditions.

My previous findings in Chapter 2 demonstrate that, prior to gene activation by oxidative stress, the promoter of the *FLR1* gene was already occupied by members of the PIC, including TBP and Pol II. However, activation of the PIC occurs by Yap1-dependent recruitment of Mediator to the *FLR1* promoter upon oxidative stress. These results reveal that the regulation of *FLR1* gene activation occurs through a post-PIC recruitment mechanism. In Chapter 4, I present work to define the unique aspects of the promoter of the post-PIC recruitment

regulated gene *FLR1* and reveal the components of the PIC involved in establishing an inactive PIC on promoter DNA. Overall, these results strongly suggest the *FLR1* gene functions through a noncanonical core promoter and that Pol II CTD phosphorylation is not sufficient for PIC activation on post-PIC recruitment regulated promoters. This work also suggests the coupling of transcriptional initiation and pre-mRNA processing events.

In addition, I had the pleasure of collaborating with Dr. Sue Kraemer in the lab in characterizing the role of the general transcription factor TFIIA in the regulation of gene expression by Pol II. Taken together, these studies demonstrate a dependence of Yap1 on TFIIA function and highlight a new role for TFIIA in the cellular mechanism of defense against reactive oxygen species (Appendix I).

Lastly, I was fortunate to participate in an exciting collaboration with Sarah Williams from the Parkhurst Lab at the University of Nebraska-Lincoln analyzing ternary complex formation of TBP-TFIIA-DNA interactions (Appendix II). These studies were successful in determining the kinetic and thermodynamic propterties of TFIIA-TBP-DNA complex assembly.

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CHAPTER 2

Activation of gene expression during oxidative stress

The oxidative stress response is the phenomenon by which cells respond to alterations in their redox state due to the generation of ROS. ROS are formed by the incomplete reduction of O_2 to H_2O during cellular respiration as well as the environmental exposure to a variety of chemicals and metals. In higher eukaryotes such as humans, oxidative stress is linked to numerous deteriorating conditions including cancer, neurodegenerative diseases, atherosclerosis, alcohol-induced liver damage, and aging. In yeast, as in humans, the oxidative stress response is regulated at the level of transcription, thus making yeast a useful model system of study. Here we utilize the yeast *Saccharomyces cerevisiae* to uncover the mechanisms and important players involved in the regulation of the oxidative stress response at the transcriptional level. This study was performed entirely by me. I have created all figures and have written this work in manuscript format, which is currently being submitted to the professional journal entitled Molecular and Cellular Biology. The literature citation reads as follows:

Combinatorial regulation of gene expression during oxidative stress

Goldstrohm, D.A. and Stargell, L.A.

2.1 Abstract

Oxidative stress is linked to numerous deteriorating conditions including cancer, alcohol-induced liver damage, atherosclerosis, neurodegenerative diseases, and aging. Here we use *Saccharomyces cerevisiae* and the *FLR1* gene to develop a model for transcriptional activation during oxidative stress. *FLR1* gene activation is maximal upon 30 minutes of oxidative stress treatment, and is dependent on the recruitment of the transcriptional activator Yap1. Yap1 appearance at *FLR1* correlates kinetically with the increase in *FLR1* gene expression. Interestingly, prior to the induction by oxidative stress, the *FLR1* promoter is already occupied by components of the PIC, including TBP and Pol II. Occupancy of the PIC is dependent upon the constitutive activator Pho2. However, activation of the PIC occurred by Yap1-dependent recruitment of Mediator to the *FLR1* promoter upon oxidative stress. We propose a model whereby Yap1-dependent recruitment of Mediator during oxidative stress is required for PIC transition, resulting in elevated levels of *FLR1* gene expression. Our findings reveal a two-step mechanism for transcriptional activation involving the combinatorial function of sequentially acting activators. In the first step, one activator functions to recruit an inactive PIC. In the second step, another regulator "activates" the PIC via the recruitment of Mediator. This model suggests the regulation of gene expression during oxidative stress is more complex than classical models demonstrate and supports a cooperative role for the function of activator proteins during the response to oxidative stress.

2.2 Introduction

All aerobically growing organisms need oxygen to survive. However, harmful forms of oxygen called reactive oxygen species (ROS) damage DNA, proteins, and lipids (Freeman and Crapo, 1982; Halliwell and Gutteridge, 1984; Moradas-Ferreira and Costa, 2000). ROS are generated by the incomplete reduction of O_2 during respiration as well as exposure to light, chemicals, and metals (Storz and Imlay, 1999). Accumulation of ROS often leads to oxidative stress within the cell and oxidative stress is linked to numerous deteriorating conditions including cancer, neurodegenerative diseases, atherosclerosis, alcohol-induced liver damage, and aging (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 1984). Unfortunately, little is known regarding how oxidative stress leads to such deteriorating conditions. In yeast, as in humans, the oxidative stress response is regulated at the level of transcription, thus making yeast a useful model system of study (Collinson and Dawes, 1992; Dalton et al., 1999; Jamieson et al., 1994; Stephen and Jamieson, 1996; Stephen et al., 1995).

In the yeast, *Saccharomyces cerevisiae*, the bZIP DNA-binding transcriptional activator Yap1 is the key regulator of genes whose products are critical for cell survival during oxidative stress (Moye-Rowley, 2002). *YAP1* mutants are hypersensitive to numerous oxidants including H_2O_2 (Schnell and Entian, 1991; Schnell et al., 1992) and cells over-expressing Yap1 have an increased stress tolerance to H_2O_2 as well as other oxidants (Kuge et al., 1997; Vido et al., 2001; Wemmie et al., 1994; Wu and Moye-Rowley, 1994). Activation of Yap1 is

controlled by nuclear localization (Kuge et al., 1997). Yap1-GFP fusion studies illustrate that Yap1 is localized to the nucleus upon treatment with oxidative stress conditions (Kraemer et al., 2006). Once in the nucleus, Yap1 binds Yap1-responsive elements (YREs) (Kuge and Jones, 1994; Lee et al., 1999a; Nguyen et al., 2001; Wu and Moye-Rowley, 1994) in the upstream promoter of numerous genes essential to cell survival during stress conditions (Carmel-Harel et al., 2001; Dumond et al., 2000; Gasch et al., 2000; Lee et al., 1999b). The gene *FLR1* (Fluconazole Resistance 1), which encodes a multidrug transporter of the major facilitator superfamily, is up-regulated by over-expression of Yap1 (Alarco et al., 1997), contains three Yap1-response elements (YREs) in its upstream promoter region (Kuge and Jones, 1994; Lee et al., 1999a; Nguyen et al., 2001; Wu and Moye-Rowley, 1994), and is induced by oxidative stress (Broco et al., 1999; Nguyen et al., 2001; Tenreiro et al., 2001), thus making the *FLR1* gene an ideal gene to study Yap1-dependent transcriptional regulation during oxidative stress. Although much is known regarding Yap1 nuclear localization and specific gene targets, the transcriptional mechanism by which Yap1 regulates important genes involved in the oxidative stress response remains unclear. Therefore, since Yap1 is the key regulator of the oxidative stress response and this response is conserved from yeast to humans, dissecting Yap1 gene regulation at oxidative stress inducible genes like *FLR1* is critical to our understanding of how cells survive in an oxidative stress environment.

Transcriptional activation of Pol II gene expression is regulated by environmentally responsive activator proteins that commonly bind DNA in a sequence-specific manner. Once bound to promoter DNA, typical activators act to recruit the general transcription machinery (TFIID (TBP + TAFs), TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH) to the core promoter to form the PIC, which is involved in directing Pol II to the transcription initiation site (Choy and Green, 1993; Drysdale et al., 1998; Ingles et al., 1991; Lin et al., 1991). Current models of transcriptional regulation suggest that recruitment of TBP, and therefore the PIC, to promoter DNA is a rate-limiting step in the transcriptional activation process (Malik et al., 2005; Orphanides and Reinberg, 2002). Therefore, in developing a model for Yap1-dependent gene regulation during oxidative stress it was hypothesized that oxidative stress induced nuclear localization of Yap1 leads to Yap1-dependent recruitment of the PIC to promoter DNA, thus allowing for the activation of gene expression during oxidative stress. The development of an accurate model of transcriptional activation during oxidative stress will allow for a better understanding of how cells survive in an oxidative stress environment.

In this study we develop a model for Yap1-dependent transcriptional activation during oxidative stress. Initial experiments demonstrate the gene *FLR1* is maximally induced upon 30 minutes of oxidative stress in a Yap1-dependent manner. In addition, chromatin immunoprecipitation (ChIP) assays show that Yap1 promoter occupancy at the *FLR1* promoter increases significantly upon oxidative stress, which correlates kinetically with the increase in *FLR1* gene

expression. Occupancy of an inactive remarkably complete PIC at the *FLR1* promoter occurs prior to oxidative stress and was dependent on the constitutive activator Pho2. However, activation of the PIC occurs by Yap1-dependent recruitment of Mediator to the *FLR1* promoter upon oxidative stress. Overall, this work has lead to the development of a model of transcriptional activation during oxidative stress whereby Yap1-dependent recruitment of Mediator is required for PIC transition, resulting in elevated levels of *FLR1* gene expression. This model illustrates a combinatorial role for dual activator function in the regulation of gene expression during oxidative stress.

2.3 Materials and Methods

2.3a Yeast strains and DNA constructs

Yeast wild type and deletion strains utilized in this study were purchased from Invitrogen (BY4741: *MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*). Yeast strains utilized in ChIP assays involved tagging of Yap1 and MED19 with a myc-epitope (Longtine et al., 2002). The DNA element utilized for primer extension assays contained the 21-base pair reverse oligonucleotide 5'-CTTCACGGGCACTCTGTAAAG-3', which was end-labeled using T4 polynucleotide kinase with 150 μCi of [γ-^{32}P]ATP.

2.3b Yeast medium and plates

Yeast medium and plates utilized throughout this study were made according to (Hampsey, 1997) with the exception of H_2O_2 plates, which were made by the addition of H_2O_2 to the cooled liquid medium before pouring the plates to a final concentration of 3.5 mM.

2.3c Phenotypic studies

For phenotypic studies, 10-fold serial dilutions of strains were applied as spots to plates containing rich medium (YPD) and incubated at 30°C. Stock solutions of H_2O_2 (200 mM) were made and appropriate dilutions were used for making plates. Plates were spotted within 36 hours of pouring.

2.3d Primer extension analysis

5' end-labeling of a *FLR1*–specific primer with polynucleotide kinase was performed and primer extension assays were conducted as described (Imboden et al., 1992). The primer was the same as positions 100 (5') to 80 of *FLR1*, which should yield a 130-nucleotide product. For *FLR1* induction, H_20_2 was added for 30 minutes to a final concentration of 0.3 mM. RNA was prepared by hot phenol extraction and quantified spectrophotometrically at 260 nm.

2.3e Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation assays were performed as described (Frank, 2001) with the following modifications. Oligos were designed for PCR analysis of

the *FLR1* promoter at positions -221 (5') to -200 and 80 (3') to 100. For *FLR1* induction, H_2O_2 was added to a final concentration of 0.3 mM for 30 minutes. Antibodies used in immunoprecipitations (Covance Inc.) were to that of TBP, Pol II, myc (Yap1), and HA (MED19). Standard curves were generated using 10-fold serial dilutions of input DNA. PCR efficiencies ranged from 90-100%, with a correlation coefficient of 0.990 or greater. Threshold cycle data were quantified relative to the input, as described (Frank et al., 2001). The relative occupancy for each factor was compared to an arbitrary value of 10, which was assigned to the occupancy levels of the tagged induced strains. Background levels were calculated as the relative occupancy of untagged strains and these values were subtracted from that of the tagged strains. The majority of experiments were performed in triplicate with the exception of a few in duplicate.

2.4 Results

2.4a *FLR1* gene expression is maximally induced upon 30 minutes of oxidative stress

To develop a model of Yap1-dependent gene regulation during oxidative stress, the *FLR1* gene was analyzed. Previous studies utilizing DNA microarray technology show *FLR1* gene activation to be maximal approximately 30 minutes post-treatment with oxidative stress followed by a decrease similar to that of no stress conditions by 60 minutes (Gasch et al., 2000). Therefore, it was predicted that a similar *FLR1* expression profile would be observed for the strains used in

this study. To confirm these results, a time course of *FLR1* gene activation was performed in which cells were treated with H_2O_2 (0.3 mM final concentration) for 0, 2, 5, 10, 15, 30, 45, and 60 minutes. Following induction of oxidative stress, total RNA was extracted and utilized in primer extension assay to measure relative amounts of *FLR1* gene expression. As expected, these results show that *FLR1* gene expression is maximally activated approximately 30 minutes post-treatment with oxidative stress with a decrease to that of basal levels following 60 minutes of treatment with oxidative stress (Figure 2.1A). In addition, *FLR1* gene activation was abolished in strains deleted for the activator Yap1, thus confirming *FLR1* gene activation occurs in a Yap1-dependent manner (data not shown).

2.4b Yap1 occupancy on the *FLR1* promoter correlates kinetically with an increase in *FLR1* gene expression

Yap1 is localized to the nucleus upon treatment with oxidative stress (Kraemer et al., 2006). Therefore, it was hypothesized that Yap1 occupancy at the *FLR1* promoter should increase upon treatment with oxidative stress. To test this, ChIP assays were used to analyze Yap1 occupancy at the *FLR1* promoter during oxidative stress. In these studies, yeast strains containing a myc-tagged derivative of Yap1 or an untagged control were grown to log phase and induced with oxidative stress by the addition of H_2O_2 (0.3 mM final concentration) for 30 minutes. Protein-DNA interactions were cross-linked with formaldehyde, the DNA sheared, immunoprecipitated for Yap1, cross-links were reversed, and Yap1 factor occupancy at the *FLR1* promoter was analyzed by quantitative (real-

time) PCR. The region of the *FLR1* promoter analyzed spans –221 to +100 from the ATG start codon (Figure 2.1B). As expected, Yap1 occupancy increased significantly at the *FLR1* promoter following treatment with oxidative stress and correlated kinetically with the observed increase in *FLR1* gene expression (Figure 2.1C).

2.4c PIC component assembly on the *FLR1* promoter occurs prior to oxidative stress

To further develop a model of activated transcription during oxidative stress, PIC component formation at the *FLR1* promoter during oxidative stress was analyzed. Since the activator Yap1 was observed to occupy the *FLR1* promoter upon oxidative stress conditions and classical models of gene activation suggest activator-dependent PIC recruitment to promoter DNA (Malik et al., 2005; Orphanides and Reinberg, 2002), it was predicted that PIC component occupancy would increase upon oxidative stress conditions. To examine this, ChIP assays were performed to analyze the occupancy of TBP and Pol II as representative components of the PIC. Interestingly, both TBP and Pol II occupancy at the *FLR1* promoter occurred prior to oxidative stress and occupancy levels did not change significantly upon treatment with oxidative stress (Figure 2.2A), which suggest that *FLR1* gene activation is likely regulated in a post-PIC recruitment manner.

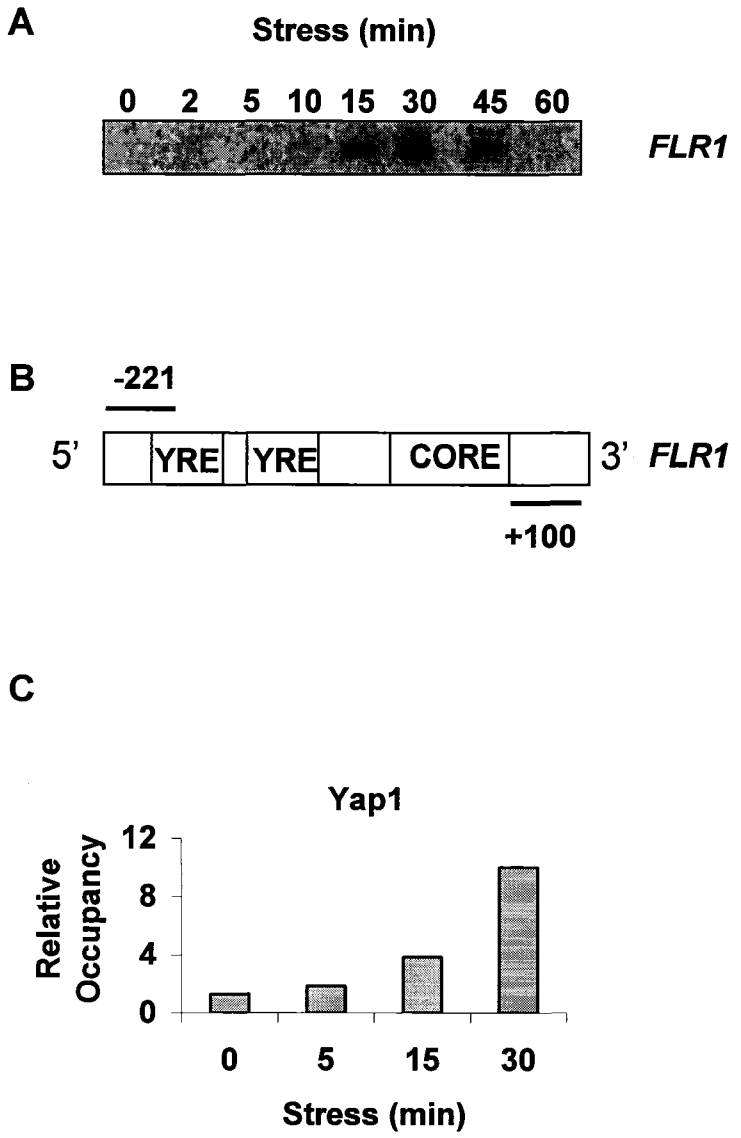

Figure 2.1. Yap1 promoter occupancy at *FLR1* correlates positively with transcriptional output (A) Log phase cells were induced with oxidative stress by the addition H_2O_2 (0.3 mM final concentration) for the following time course (0, 2, 5, 10, 15, 30, 45, 60 and min.). RNA was extracted at each time point to measure *FLR1* gene expression by primer extension analysis, which illustrates maximal *FLR1* gene activation at 30 min. post-treatment with H_2O_2. (B) Diagram of primer design for ChIP assays on the *FLR1* promoter. Dark bars represent region of the *FLR1* gene where each primer was designed. (C) Log phase cells were induced with oxidative stress by the addition of H_2O_2 (0.3 mM final concentration) for 30 min. Cells were crosslinked with formaldehyde and protein-DNA crosslinks were analyzed by quantitative PCR. ChIP anaysis reaveal a significant increase in Yap1 occupancy at the *FLR1* promoter upon oxidative stress conditions.

2.4d The transcriptional activator Yap1 is not required for PIC assembly on the *FLR1* promoter

A common model of gene activation is activator-dependent recruitment of the PIC to promoter DNA (Malik et al., 2005; Orphanides and Reinberg, 2002). Therefore, it was interesting to find that PIC component assembly at the *FLR1* promoter occurs prior to oxidative stress, where occupancy levels of the transcriptional activator Yap1 are relatively low. It was predicted that these low levels of Yap1 occupancy at the *FLR1* promoter prior to oxidative stress might be sufficient for PIC component recruitment. In support of this, previous work demonstrates that Yap1 has differing binding affinities (YRE3>YRE2>YRE1) for each YRE in the upstream promoter region of *FLR1* (Nguyen et al., 2001). Therefore, it is possible that prior to oxidative stress, occupancy levels of Yap1 are only sufficient to contact the highest affinity binding site and this in-turn is sufficient to recruit components of the PIC to the *FLR1* promoter. Since Yap1 would only be binding to a single YRE, this would be observed as low level occupancy in the ChIP assays as compared to full Yap1 occupancy when all YREs are occupied as during oxidative stress. To test this hypothesis, strains both containing and deleted for Yap1 were utilized in ChIP assays to analyze PIC component occupancy at the *FLR1* promoter upon oxidative stress conditions. These results reveal the ability of the Pol II to assemble at the *FLR1* promoter in the absence of the activator Yap1 and Pol II promoter occupancy levels did not change significantly upon treatment with oxidative stress (Figure 2.2B). This suggests that Yap1 is not required for PIC component assembly on the *FLR1*

promoter prior to oxidative stress, and its requirement for gene activation likely occurs at a step after PIC assembly.

2.4e The constitutive activator Pho2 recruits an inactive PIC to the *FLR1* promoter

Since PIC component assembly at the *FLR1* promoter occurs prior to oxidative stress in a manner independent of the activator Yap1, it was predicted that another activator protein is required for PIC component assembly at the *FLR1* promoter. To identify other possible factors responsible for PIC recruitment on the *FLR1* promoter, the sequence of the *FLR1* promoter was analyzed for known factor DNA-binding sites. This search revealed multiple DNA-binding sites for the constitutive activator Pho2 (data not shown). Pho2 is known to play a role in the activation of numerous genes (Oshima, 1997). Therefore, it was hypothesized that the activator Pho2 may play a role in *FLR1* gene activation during oxidative stress. To test this, yeast strains containing and deleted for Pho2 were utilized in ChIP assays to analyze Pol II occupancy on the *FLR1* promoter during oxidative stress. These results demonstrate that strains deleted for the activator Pho2 have a dramatic decrease in Pol II occupancy at the *FLR1* promoter both prior to and after oxidative stress conditions (Figure 2.2C). These findings suggest a role for the activator Pho2 in PIC occupancy to the *FLR1* promoter, prior to oxidative stress. Also, since Pho2-dependent recruitment of components of the PIC prior to oxidative stress does not result in *FLR1* gene

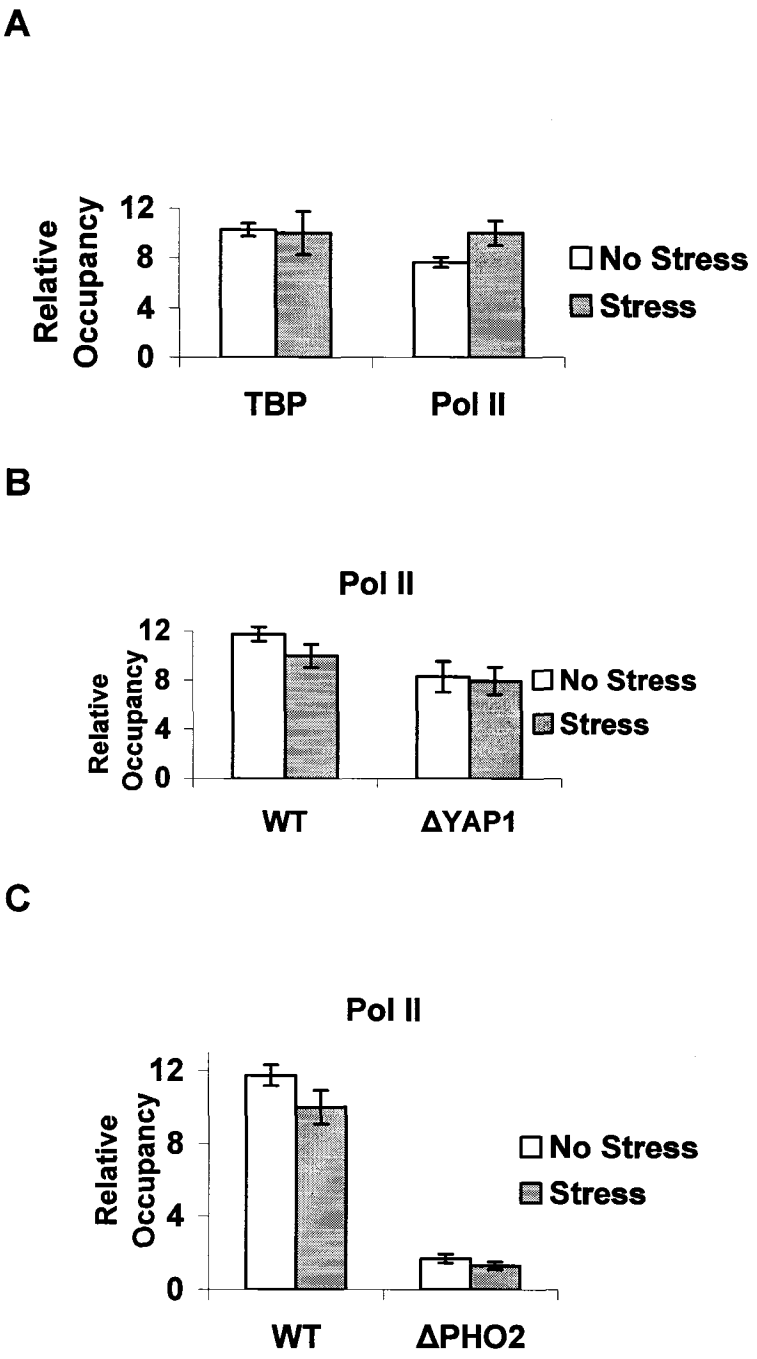

Figure 2.2. PIC component occupancy at the *FLR1* promoter prior to oxidative stress
Log phase cells were induced with oxidative stress was induced by the addition of H_2O_2 (0.3 mM final concentration) for 30 min. and ChIP assays performed to analyze PIC component occupancy at the *FLR1* promoter as described previously. (A) ChIP analysis reveals Pol II occupancy at the *FLR1* promoter, prior to oxidative stress. (B) ChIP analysis shows deletion of the activator Yap1 does not diminish Pol II occupancy at the *FLR1* promoter. (C) ChIP analysis demonstrates deletion of Pho2 abolishes Pol II occupancy at the *FLR1* promoter, prior to oxidative stress.

activation, the recruited PIC is thought to be inactive. Notably, additional ChIP assays confirm Yap1 occupancy at the *FLR1* promoter during oxidative stress in strains deleted for Pho2.

2.4f Mediator occupancy at the *FLR1* promoter increases significantly upon oxidative stress

We show the regulation of *FLR1* gene activation likely occurs in a post-PIC recruitment manner since PIC component occupancy at *FLR1* prior to oxidative stress where levels of *FLR1* transcription remain relatively low. These observations suggest a rate-limiting step of *FLR1* gene activation is not the recruitment of the PIC to the promoter, but occurs at a step after PIC assembly. Since Yap1 is required for the activation of *FLR1* gene expression, but does not act to recruit components of the PIC to the *FLR1* promoter prior to oxidative stress, it is hypothesized that Yap1 may instead act to recruit factors critical for *FLR1* gene activation. To examine this, ChIP assays were performed in strains both containing a HA-tagged Mediator component (MED19) and an untagged control during no stress and oxidative stress conditions. Mediator occupancy at the *FLR1* promoter increased significantly upon induction with oxidative stress. Importantly, Mediator occupancy correlates positively with the occupancy observed for the activator Yap1 as well as the transcriptional output observed upon oxidative stress, but is in contrast to occupancy levels observed for the PIC (Figure 2.3A). These findings are supported by recent findings of Mediator

recruitment upon oxidative stress to other genes important in the oxidative stress response (Gulshan et al., 2005).

2.4g Mediator recruitment to the *FLR1* promoter allows for the transition of *FLR1* gene expression to the activated state

The above study demonstrates that Mediator is recruited to the *FLR1* promoter upon oxidative stress and that Mediator may play a role in the transition of *FLR1* gene expression to the activated state. To confirm a role for Mediator in the activation of *FLR1* gene activation during oxidative stress, strains both containing and deleted for Mediator were induced with oxidative stress by the addition of hydrogen peroxide (0.3 mM final concentration) for 0 or 30 minutes. Following exposure to oxidative stress-inducing conditions, total RNA was extracted and utilized in primer extension assays to measure relative amounts of *FLR1* gene expression. These results show *FLR1* gene activation during oxidative stress is decreased significantly in strains deleted for Mediator (Figure 2.3B). In addition, strains deleted for Mediator display an oxidative stress sensitive phenotype when grown under oxidative stress conditions as compared to wild type cells (Figure 2.3C). Collectively, these results demonstrate Mediator is involved in the transition of *FLR1* gene expression to the activated state and Mediator is essential for cell survival during oxidative stress.

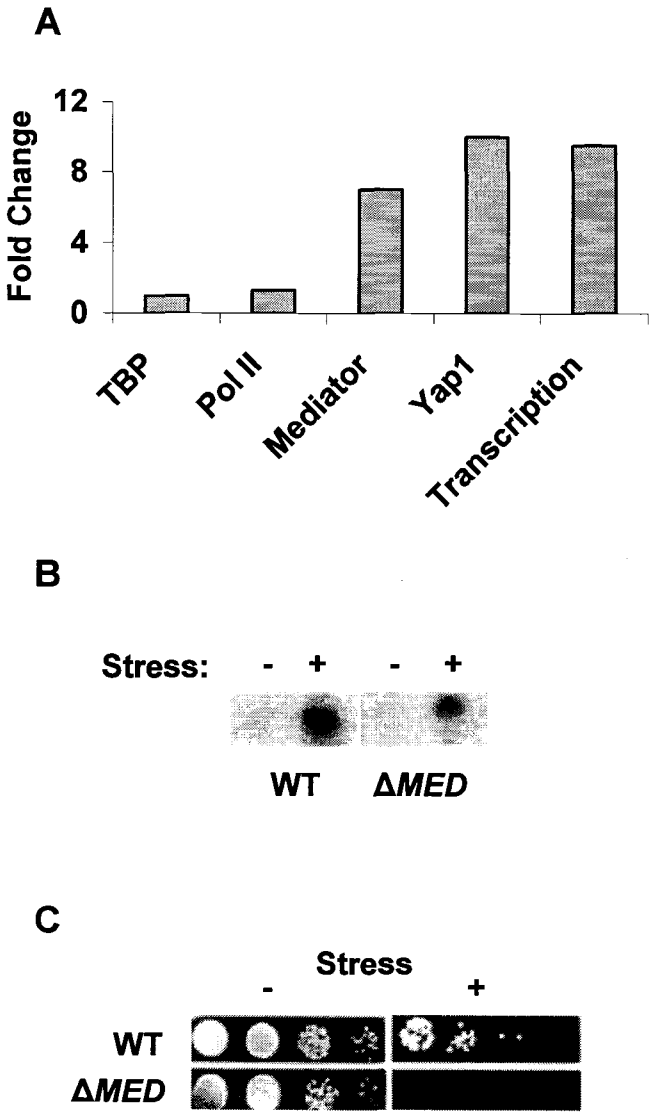

Figure 2.3. The role of Mediator in *FLR1* gene activation
Log phase cells were induced with oxidative stress by the addition of H_2O_2 (0.3 mM final concentration) for 30 min. and ChIP assays were performed as described previously. (A) ChIP analysis illustrates Mediator occupancy at the *FLR1* promoter correlates positively with that of the activator Yap1 as well transcriptional output. (B) Primer extension analysis reveals *FLR1* gene expression is decreased in strains deleted for multiple Mediator components. (C) 10-fold serial dilutions of wild type and Mediator deletion strains were spotted to YPD and oxidative stress conditions (0.3 mM H_2O_2). Phenotypic analysis reveals the requirement for Mediator suburnits for cell growth during oxidative stress.

2.5 Discussion

Aerobically growing organisms are continuously bombarded by ROS that damage DNA, protein, and lipids (Freeman and Crapo, 1982; Halliwell and Gutteridge, 1984; Moradas-Ferreira and Costa, 2000). The oxidative stress response is the phenomenon by which cells respond to alterations in their redox status due to the generation of ROS. This response is essential for a cells ability to combat reactive oxygen species and survive in an oxidative stress environment. In higher eukaryotes, defects in the oxidative stress response can lead to many deteriorating conditions including cancer, neurodegenerative diseases, atherosclerosis, alcohol-induced liver damage, and aging (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 1984). In yeast, as in humans, the oxidative stress response is regulated at the level of transcription, thus making yeast a useful model system of study (Collinson and Dawes, 1992; Dalton et al., 1999; Jamieson et al., 1994; Stephen and Jamieson, 1996; Stephen et al., 1995).

The transcriptional activator Yap1 is the key regulator of the oxidative stress response in the yeast *Saccharomyces cerevisiae* (Moye-Rowley, 2002). Therefore, the activator Yap1 was chosen to develop a model of activated transcription during oxidative stress. Our initial model predicted that oxidative stress induced nuclear localization of Yap1 leads to Yap1-dependent recruitment of the PIC to promoter DNA, thus allowing for the activation of gene expression during oxidative stress. This is supported by evidence showing that Yap1 is localized to the nucleus upon oxidative stress conditions (Kraemer et al., 2006)

and by classical transcriptional activation models suggesting the rate-limiting step in gene activation is the recruitment of Pol II to promoter DNA (Orphanides and Reinberg, 2002; Parada and Roeder, 1999).

Here it is shown that activators do not play a direct role in activating gene expression during oxidative stress. It was found that Yap1 does not function as a classical activator in directly recruiting components of the PIC to promoter DNA upon oxidative stress. Interestingly, it was found the PIC components are recruited to promoter DNA in an inactive form and that this recruitment of PIC components is not a rate-limiting step in the activation of gene expression since prior to oxidative stress gene expression levels are relatively low. Studies using genome-wide analyses reveal the ability of Pol II to be located upstream of numerous genes prior to their activation (Kim et al., 2005; Radonjic et al., 2005) and support the findings in this study. In addition, experiments analyzing the activation of numerous heat-shock and oncogenes illustrate the ability of genes to establish a poised Pol II prior to gene activation (Krumm et al., 1992; Lis and Wu, 1993; Meulia et al., 1992; Rasmussen and Lis, 1995) giving further support to our current model of an occupied Pol II at promoter DNA prior to transcriptional activation.

Furthermore, Yap1 was shown to recruit Mediator at a step after PIC component recruitment to promoter DNA. Mediator was found to be required for the transition of gene expression to the activated state. Recent studies in other

experimental systems support these findings and show that Mediator and Pol II association with promoter DNA is not always strictly correlated (Fan et al., 2006; Struhl, 1995) where numerous genes have Pol II bound in the absence of Mediator (Park et al., 2001). Also, Mediator and Yap1 are known to interact by the utilization of both in vitro and in vivo methods (Gavin et al., 2002; Ito et al., 2001) and Mediator has also been shown to play a direct role in activation by the acetylation of histones (Lorch et al., 2000). Therefore, we hypothesize that Yap1-mediated recruitment of Mediator to the PIC plays a direct role in the transition of gene activation during oxidative stress by the ability of Mediator to acetylate histones. Notably, this model is in contrast to classical models of Mediator function that suggests a role for Mediator in the recruitment of Pol II to promoter DNA (Biddick and Young, 2005; Himmelfarb et al., 1990; Jiang and Stillman, 1992; Song, 1996).

Collectively, these findings reveal a two-step mechanism for transcriptional activation during oxidative stress involving the combinatorial function of sequentially-acting activators. In the first step, one activator functions to recruit an inactive PIC, and in the second step, another activator "activates" the PIC via the recruitment of Mediator. This model suggests the regulation of gene expression during oxidative stress is more complex than classical models and suggests a cooperative role for the function of activator proteins during the response to oxidative stress.

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CHAPTER 3

Identifying Mediator subunits required for cell viability under non-optimal growth conditions

In previous work we demonstrated that activation of the *FLR1* gene expression occurs by Yap1-dependent recruitment of Mediator upon treatment with oxidative stress. Thus, we uncovered an essential role for Mediator in gene regulation during oxidative stress. Here we expand this work to determine the requirement of Mediator under additional stress-inducing and growth-compromising conditions. This study was performed in collaboration with two additional members of the Stargell lab: Monali Babre and Sarah Lee. The following are a description of my contributions to this work: I spearheaded the entire project, created the figures, and wrote the findings into manuscript format to be submitted to the professional journal entitled Eukaryotic Cell. Once published, the literature citation will read:

Differing roles of Mediator subunits in the response to stress

Goldstrohm, D.A., Babre, M., Lee, S.K., and Stargell, L.A.

3.1 Abstract

Mediator is a large multi-subunit protein complex that is evolutionarily conserved from yeast to humans. Mediator plays an important role in the activation of Pol II gene expression and functions to relay regulatory signals from activator proteins to the general transcription machinery. In the yeast, *Saccharomyces cerevisiae*, Mediator is comprised of 25 subunits that are grouped into subcomplexes termed Head, Middle, Tail, and Cdk8 modules. Of the 25 total subunits, 15 are nonessential for cell growth on rich medium and have not been well-characterized for roles in the gene regulation process. However, the majority of these 15 subunits are conserved throughout evolution, suggesting critical functions. Here we focus on Mediator subunits nonessential for growth on rich medium and determine how deletions of these subunits affect cell survival under adverse growth conditions. Mediator deletion strains were grown in media containing agents that induce the oxidative stress response (hydrogen peroxide), serve as anti-fungal agents (fluconazole), inhibit protein synthesis (cycloheximide), inhibit tumor growth in mammals (methotrexate), and induce the heat shock response (38°C). Overall, this study has uncovered essential functions for additional Mediator subunits and has defined Mediator modules involved in cell survival under various growth-compromising conditions.

3.2 Introduction

All organisms are challenged or "stressed" by unfavorable growth conditions. Unfavorable conditions can be produced by normal cellular processes and/or can be the result of environmental influences. To combat the damaging effects of such conditions, cells activate numerous response pathways. These pathways are important in reducing stress levels within the cell and the regulation of these pathways is critical to overall cell survival. Stress response pathway activation leads to alteration in global cellular transcription. This results in the down-regulation of housekeeping genes and activation of genes whose products are critical in cell detoxification processes. Importantly, the majority of the transcription pathways and the factors involved are conserved from yeast to humans (Collinson and Dawes, 1992; Dalton et al., 1999; Jamieson et al., 1994; Stephen and Jamieson, 1996; Stephen et al., 1995).

Activation of genes transcribed by Pol II requires assembly of numerous GTFs, including TFIID (TBP + TAFs), TFIIA, TFIIB, TFIIF, TFIIH, TFIIE to promoter DNA (Martinez, 2002; Woychik and Hampsey, 2002). GTF binding to promoter DNA is accompanied by Pol II promoter recognition. The assembly of the GTFs and Pol II at the promoter can be enhanced by activator proteins, which bind to upstream sequences. Mediator is a multi-subunit factor that is thought to play a key role in gene activation by relaying critical information between GTFs and activator proteins (Kornberg, 2005). In the yeast *Saccharomyces cerevisiae*, Mediator is critical for stimulation of Pol II gene expression (Flanagan et al., 1991; Kelleher et

al., 1990; Kim et al., 1994). Mediator stimulates transcription through interactions with both activator proteins and Pol II and these interactions are critical for Mediator function (Myers and Kornberg, 2000). Mediator consists of 25 subunits of which 15 are nonessential for growth on rich medium and have not yet been well-characterized for roles in gene expression. However, the majority of these 15 subunits are conserved throughout evolution, suggesting critical functions. Studies utilizing both biochemical and genetic approaches have classified Mediator into four distinct subcomplexes termed: Head, Middle, Tail, and Cdk8 modules (Guglielmi et al., 2004). The Head and Middle modules of Mediator are important for interactions with Pol II, whereas the Tail module is critical for the interaction of Mediator with different activator proteins. The Cdk8 module of Mediator contains a cyclin-dependent kinase that plays a repressive role in the gene regulation process (Kim et al., 1994).

Here we utilize the yeast *Saccharomyces cerevisiae* to analyze the role of Mediator in response to compromised growth conditions. We focus on Mediator subunits nonessential for growth on rich medium and determine how deletions of these subunits affect cell survival during various growth conditions, including cell survival during oxidative stress (H_2O_2), as well as examining the effects of a number of other agents: the anti-fungal agent (fluconazole), the protein synthesis inhibiter (cycloheximide), the tumor growth inhibiter in mammals (methotrexate), and heat shock (38°C). Overall, this study has uncovered essential functions for

additional Mediator subunits and has defined unique Mediator modules involved in cell survival during various challenging conditions.

3.3 Materials and Methods

3.3a Yeast strains and medium

Yeast wild type and deletion strains were purchased from Invitrogen (BY4741: *MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*). Yeast medium utilized to analyze phenotypic changes were made as described (Hampsey, 1997). With the exception of heat shock stress, the resulting stress plates were made by supplementing YPD medium with H_2O_2, fluconazole, cycloheximide or methotrexate to the liquid medium before pouring to the final concentrations of 3.5 mM, 20 µg/mL, 0.1 µg/mL, and 50 µg/mL, respectively. Heat shock stress plates were made with YPD medium and placed at 38 °C.

3.3b Phenotypic studies

For phenotypic studies, cells were grown to log phase and 10-fold serial dilutions were applied in 10 uL spots to plates containing rich medium (YPD) and various challenging growth conditions and incubated at 30 °C with the exception of heat shock stress, which were incubated at 38 °C. Plates were spotted within 36 hours of pouring.

3.4 Results

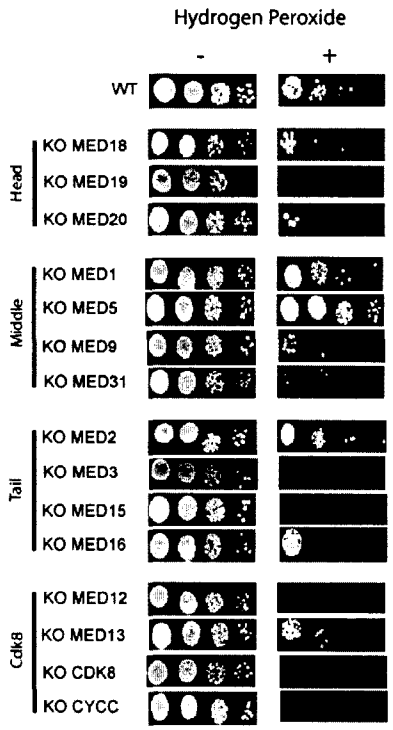
3.4a Defining the hydrogen peroxide module of Mediator

Mediator subunits nonessential for cell growth on rich medium were deleted and the resulting strains were grown under oxidative stress conditions and compared to wild type cells. Since Mediator has been implicated in the response to oxidative stress (Chapter 2), we anticipated that additional subunits might be required under these conditions compared to growth in rich medium. Remarkably, 80% (12 of 15) of the total Mediator subunits tested were essential for cell growth during oxidative stress, including MED3, MED9, MED12, MED13, MED15, MED16, MED18, MED19, MED20, MED31, CDK8, and CYCC (Figure 3.1A). These subunits are contained in all 4 Mediator modules (Figure 3.1B). These results are consistent with an absolute dependence on the entire Head (100% of the subunits) and Cdk8 (100%) modules for growth under oxidative stress conditions.

3.4b Defining the fluconazole module of Mediator

In addition to oxidative stress, we further examined the effects of Mediator subunit deletions on stress induced by the anti-fungal agent fluconazole. Fluconazole is a triazole anti-fungal drug used in the treatment and prevention of superficial and systemic fungal infections. Like other imidazole- and triazole-class anti-fungals, fluconazole inhibits the fungal cytochrome P450 enzyme 14α-demethylase. This inhibition prevents the conversion of lanosterol to ergosterol,

A



B

Hydrogen Peroxide

HEAD	MIDDLE	TAIL	CDK8
MED18	MED1	MED2	MED12
MED19	MED5	MED3	MED13
MED20	MED9	MED15	CDK8
---------	MED31	MED16	CYCC
100%	50%	75%	100%

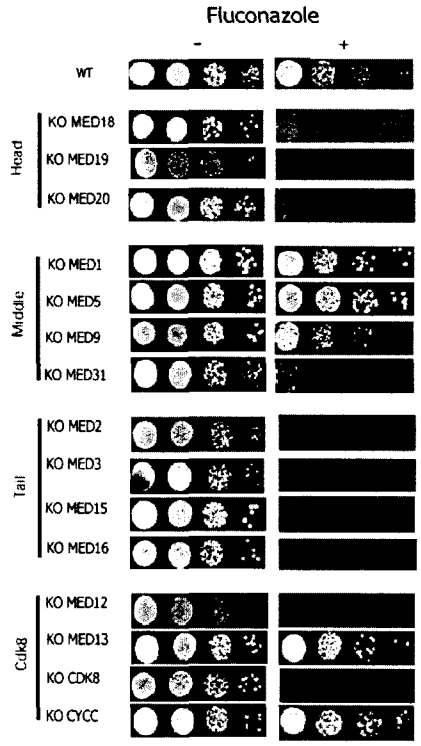
Figure 3.1. Mediator subunits required for growth on hydrogen peroxide
(A) Wild type strains and Mediator deletion strains were grown to log phase and 10-fold serial dilutions were spotted to YPD and H_2O_2 (3.5 mM final concentration). Mediator subunits MED3, MED9, MED12, MED13, MED15, MED16, MED18, MED19, MED20, MED31, CDK8, and CYCC are all required for cell growth during oxidative stress. (B) Mediator subunits required for growth on H_2O_2 grouped into all 4 Mediator modules. Gray boxes indicate Mediator subunits required for cell growth. Numbers indicate percent required of total subunits tested for each module.

an essential component of the fungal cell wall, and subsequent accumulation of 14α-methyl sterols. As such, fluconazole inhibits lipid biosynthesis. To test the effects of fluconazole treatment, we compared the growth of wild type strains and strains deleted for Mediator subunits on fluconazole. Strikingly, 67% (10 of 15) of the total Mediator subunits tested were essential for cell growth on fluconazole, including MED2, MED3, MED12, MED15, MED16, MED18, MED19, MED20, MED31, and CDK8 (Figure 3.2A). These subunits are contained in all 4 Mediator modules (Figure 3.2B), and indicate a requirement for the entire Head and Tail modules for cell viability on fluconazole.

3.4c Defining the cycloheximide module of Mediator

We next tested the effects of Mediator subunit deletions on growth challenges induced by cycloheximide. Cycloheximide, an inhibitor of protein biosynthesis in eukaryotic organisms, is produced by the bacterium *Streptomyces griseus*. It exerts its effects by interfering with peptidyl transferase activity of the 60S ribosomal subunit, thus blocking translational elongation. To test the effects of cycloheximide treatment, yeast wild type strains and strains deleted for particular Mediator subunits were grown under conditions that induce stress by the inhibition of protein biosynthesis. Identical to the results observed with fluconazole treatment, 67% (10 of 15) of the total Mediator subunits tested were essential for cell growth during cycloheximide treatment, including MED2, MED3, MED12, MED15, MED16, MED18, MED19, MED20, MED31, and CDK8 (Figure

A



B

Fluconazole

HEAD	MIDDLE	TAIL	CDK8
MED18	MED1	MED2	MED12
MED19	MED5	MED3	MED13
MED20	MED9	MED15	CDK8
---------	MED31	MED16	CYCC
100%	25%	100%	50%

Figure 3.2. Mediator subunits required for growth on fluconazole
(A) Wild type strains and Mediator deletion strains were grown to log phase and 10-fold serial dilutions were spotted to YPD and fluconazole (20 µg/mL). Mediator subunits MED2, MED3, MED12, MED15, MED16, MED18, MED19, MED20, MED31, and CDK8 are all required for cell growth on fluconazole. (B) Mediator subunits required for growth on fluconazole grouped into all 4 Mediator modules. Gray boxes indicate Mediator subunits required for cell growth. Numbers indicate percent required of total subunits tested for each module.

3.3A). These subunits are contained in all 4 Mediator modules with the Head and Tail domains being absolutely required for growth (Figure 3.3B).

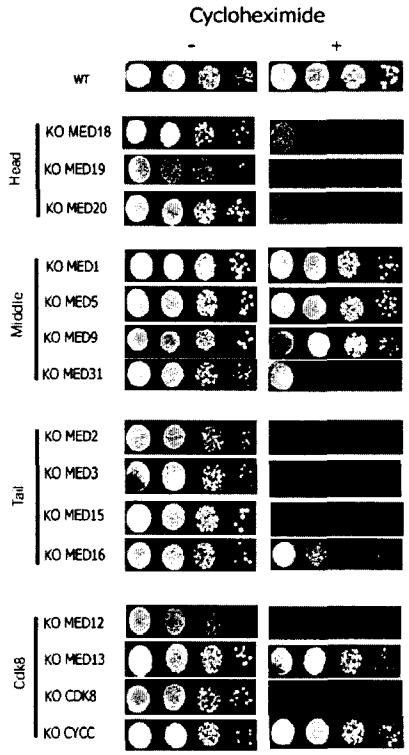
3.4d Defining the methotrexate module of Mediator

Here we test the effects of Mediator subunit deletions on growth in the presence of methotrexate, an inhibitor of folic acid metabolism (Johnston et al., 2005; Klareskog et al., 2004). Methotrexate is used as an anti-metabolite drug in treatment of cancer and autoimmune diseases. To test the effects of methotrexate treatment, yeast wild type strains and Mediator strains deleted for particular subunits were grown on methotrexate. We found that 40% (6 of 15) of the total Mediator subunits tested were essential for cell growth during methotrexate treatment, including MED1, MED18, MED19, MED20, MED31, and CYCC (Figure 3.4A). Notably, these subunits are contained in only 3 of the 4 Mediator modules (Figure 3.4B), and here, only the intact Head module was absolutely required.

3.4e Defining the heat shock module of Mediator

Here we test the effects of Mediator subunit deletions on stress induced by heat shock. During exposure to elevated temperatures, cells increase the expression of heat shock proteins. This increase in expression is transcriptionally regulated and the dramatic up-regulation of the heat shock proteins is a key part of the heat shock response. Production of high levels of heat shock proteins can also be triggered by exposure to different kinds of environmental stress conditions, such

A



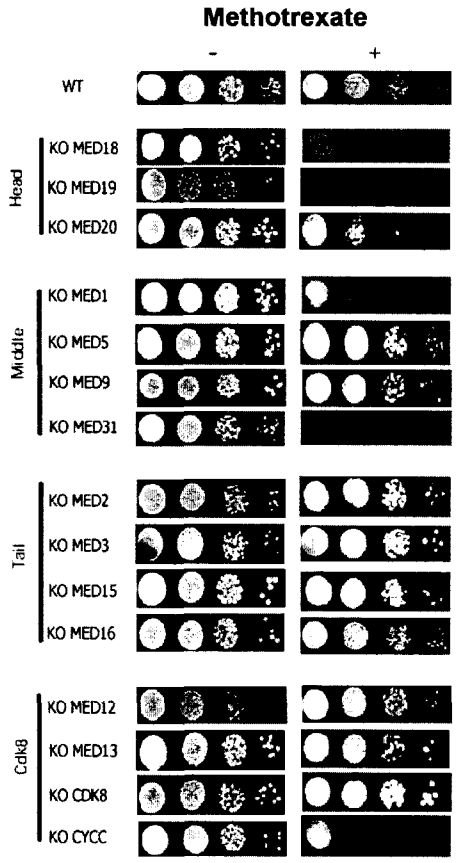
B

Cycloheximide

HEAD	MIDDLE	TAIL	CDK8
MED18	MED1	MED2	MED12
MED19	MED5	MED3	MED13
MED20	MED9	MED15	CDK8
---------	MED31	MED16	CYCC
100%	25%	100%	50%

Figure 3.3. Mediator subunits required for growth on cycloheximide
(A) Wild type strains and Mediator deletion strains were grown to log phase and 10-fold serial dilutions were spotted to YPD and cycloheximide (0.1μg/mL). Mediator subunits MED2, MED3, MED12, MED15, MED16, MED18, MED19, MED20, MED31, and CDK8 are all required for cell growth on cycloheximide. (B) Mediator subunits required for growth on cycloheximide grouped into all 4 Mediator modules. Gray boxes indicate Mediator subunits required for cell growth. Numbers indicate percent required of total subunits tested for each module.

A



B

Methotrexate

HEAD	MIDDLE	TAIL	CDK8
MED18	MED1	MED2	MED12
MED19	MED5	MED3	MED13
MED20	MED9	MED15	CDK8
---------	MED31	MED16	CYCC
100%	50%	0%	25%

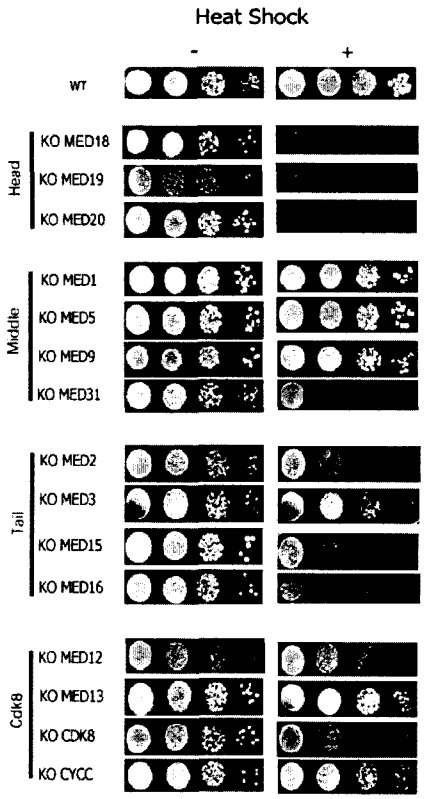
Figure 3.4. Mediator subunits required for growth on methotrexate
(A) Wild type strains and Mediator deletion strains were grown to log phase and 10-fold serial dilutions were spotted to YPD and methotrexate (50 µg/mL). Mediator subunits MED1, MED18, MED19, MED20, MED31, and CYCC are all required for cell growth on methotrexate. (B) Mediator subunits required for growth on methotrexate grouped into all 4 Mediator modules. Numbers indicate percent required of total subunits tested for each module.

as infection, inflammation, exposure of the cell to toxins (ethanol, arsenic, trace metals and ultraviolet light, among many others), starvation, hypoxia (oxygen deprivation), nitrogen deficiency (in plants), or water deprivation (Calabrese et al., 2006). Since Mediator and the heat shock response are conserved from yeast to humans, this information will allow for a better understanding of these processes in higher eukaryotes. To test the effects, yeast wild type strains and Mediator strains deleted for subunits nonessential for cell growth on rich medium were grown on conditions that induce stress by heat shock (38°C). In this study, 60% (9 of 15) of the total Mediator subunits tested were essential for cell growth during heat shock, including MED2, MED12, MED15, MED16, MED18, MED19, MED20, MED31, and CDK8 (Figure 3.5A). These subunits are contained in all 4 Mediator modules (Figure 3.5B), and indicate that the Head subunits are all essential for growth in response to elevated temperatures

3.5 Discussion

Every organism experiences various non-optimal states of growth. This can be the result of various forms of stress produced through normal metabolic processes or from the environment (Storz and Imlay, 1999). Our previous work indicated that Mediator, a large multi-subunit complex, plays an important role during the transcriptional response to oxidative stress (Chapter 2), thus establishing a link between Mediator and stress response pathways. Here we extend this analysis and examine the role of individual subunits of the Mediator in the response to oxidative stress. We utilized 15 different strains, each containing

A



B

Heat Shock

HEAD	MIDDLE	TAIL	CDK8
MED18	MED1	MED2	MED12
MED19	MED5	MED3	MED13
MED20	MED9	MED15	CDK8
---------	MED31	MED16	CYCC
100%	25%	75%	50%

Figure 3.5. Mediator subunits required for growth during heat shock
(A) Wild type strains and Mediator deletion strains were grown to log phase and 10-fold serial dilutions were spotted to YPD and conditions that induce heat shock (38 °C). Mediator subunits MED2, MED12, MED15, MED16, MED18, MED19, MED20, MED31, and CDK8 are all required for cell growth during heat shock. (B) Mediator subunits required for growth during heat shock grouped into all 4 Mediator modules. Gray boxes indicate Mediator subunits required for cell growth. Numbers indicate percent required of total subunits tested for each module.

a deletion for a gene encoding a subunit that is not required for growth under optimal conditions. These strains were compared to wild type cells for growth on hydrogen peroxide, an agent that induces oxidative stress. Remarkably, 80% (12 of 15) of the Mediator subunits tested were essential for cell growth during oxidative stress. This result indicates a global requirement for a majority of the Mediator complex to combat the harmful effects of reactive oxygen species.

We next characterized the requirement for these Mediator subunits for growth under additional unfavorable growth conditions. Cell growth on methotrexate, a potent anti-cancer drug and inhibitor of folic acid metabolism, was dependent upon 40% of the Mediator subunits tested. It was also observed that 60% of the total Mediator subunits tested were critical for cell growth during stress caused by elevated temperatures along with 67% required for cell growth on stress caused by anti-fungals and protein biosynthesis inhibitors. These results clearly demonstrate that Mediator is important in cell survival during metabolically challenging conditions and highlight a key finding that Mediator subunits not required for cell growth on rich medium become essential for cell growth during growth-compromising conditions.

Studies utilizing both biochemical and genetic approaches have defined Mediator as having four distinct subcomplexes termed: Head, Middle, Tail, and Cdk8 modules (Guglielmi et al., 2004). The Head and Middle modules of Mediator are important for interactions with Pol II of the PIC, whereas the Tail module is critical

for the interaction of Mediator with different activator proteins. Interestingly, the Cdk8 module of Mediator contains a cyclin-dependent kinase that is suggested to play a repressive role in gene regulation (Kim et al., 1994). Upon analysis of the important Mediator modules required for cell growth during various non-optimal growth conditions, it was observed that cell growth on 4 of the 5 conditions tested (H_2O_2, fluconazole, cycloheximide, and heat shock) required Mediator subunits from all 4 Mediator modules (Head, Middle, Tail, and Cdk8). In contrast, cell growth on methotrexate resulted in a loss of requirement for the subunits of the Mediator Tail module. As stated previously, the Tail module is thought to be critical for the interaction of Mediator with activator proteins. Therefore, the role of Mediator in gene regulation required for cell growth during non-optimal growth conditions caused by tumor growth inhibition is not likely due to the interaction of Mediator with activator proteins, but is thought to involve Mediator function through the Head domain and its interaction with Pol II. In addition, 100% of the subunits tested of the Head module of Mediator are required for cell growth on all non-optimal growth conditions tested. Since the Head module of Mediator is known to interact with Pol II during the gene activation process, these results strongly support the Head module as being required for cell growth during the non-optimal conditions tested. These responses also require a large percentage (>75%) of the subunits tested in the Tail module of Mediator. It was also observed that the majority of Mediator subunits tested of the Middle module are rarely required for cell growth on the various non-optimal growth conditions tested, with the exception of MED31.

To display the individual Mediator subunits essential for cell growth during the various non-optimal growth conditions tested, unique Mediator modules were designed (Figure 3.6). The Mediator subunits highlighted with a checkmark correspond to diminished cell growth when those particular subunits are deleted. Thus, subunits that are checked are essential for cell growth during that stress condition. The following is a list of each module or stress conditions with a grouping of the important Mediator subunits necessary for cell growth: Hydrogen Peroxide module (MED3, MED9, MED12, MED13, MED15, MED16, MED18, MED19, MED20, MED31, CKD8, and CYCC), Fluconazole module (MED2, MED3, MED12, MED15, MED16, MED18, MED19, MED20, MED31, and CDK8), Cycloheximide module (MED2, MED3, MED12, MED15, MED16, MED18, MED19, MED20, MED31, and CDK8), Methotrexate module (MED1, MED18, MED19, MED20, MED31, and CYCC), and Heat Shock induction module (MED2, MED12, MED15, MED16, MED18, MED19, MED20, MED31, and CDK8). Thus, the required subunits of Mediator important for cell growth during various conditions have been determined (Figure 3.7).

Interestingly, there were four subunits, MED18, MED19, MED20, and MED31, that are required for cell growth during every condition tested. These subunits are all part of the Head module of Mediator except for MED31, which is part of the Middle module. Since the Head and Middle modules of Mediator form critical interactions with Pol II, these interactions appear to be vital in maintaining cell growth under non-optimal growth conditions. As such, these subunits play

	YPD	Hydrogen Peroxide Module	Fluconazole Module	Cycloheximide Module	Methotrexate Module	Heat Shock Module
Wild Type						
Head						
MED18		√	√	√	√	√
MED19		√	√	√	√	√
MED20		√	√	√	√	√
Middle						
MED1					√	
MED5						
MED9		√				
MED31		√	√	√	√	√
Tail						
MED2			√	√		√
MED3		√	√	√		
MED15		√	√	√		√
MED16		√	√	√		√
Cdk8						
MED12		√	√	√		√
MED13		√				
CDK8		√	√	√		√
CYCC		√			√	

Figure 3.6. Diagram of unique Mediator modules required for growth during non-optimal conditions Mediator subunits found to be essential for cell growth under non-optimal growth conditions are grouped into unique Mediator modules. Gray boxes indicate Mediator subunits required for cell growth.

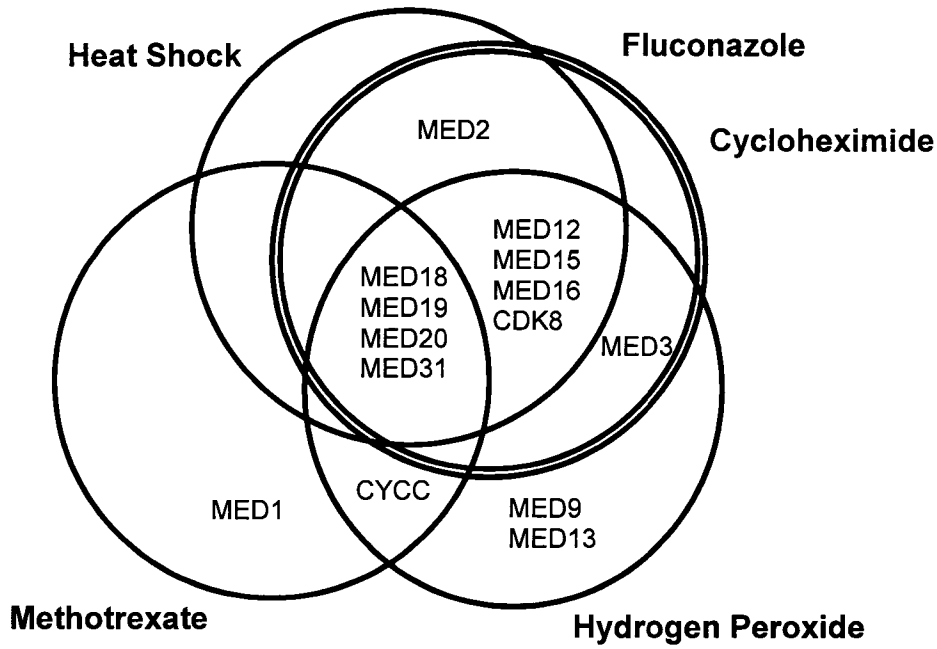


Figure 3.7. Venn diagrams grouping common Mediator subunits
A grouping of the common Mediator subunits essential for growth under non-optimal growth conditions illustrate that MED18, MED19, MED20, and MED31 are essential for cell growth on all conditions tested. MED5 was found to be the only subunit dispensable for growth during all conditions tested. Thus, MED5 was omitted from the diagram.

fundamental role in cell survival under adverse conditions and this blurs the distinction between essential and non-essential annotation. In contrast, MED5 was dispensable for growth on all of the conditions tested. Thus, this Mediator subunit either has specialized roles in undefined pathways, or simply acts to make minor adjustments to Mediator activity. Interestingly, MED5 is not conserved throughout evolution, which further supports its lack of requirement for cell growth under most conditions.

Overall, Mediator is largely required for cell growth on all non-optimal conditions tested. This helps to define Mediator as a key player in cell survival during various non-optimal growth conditions. Since Mediator is conserved from yeast to humans, this gives us a greater understanding of the mechanisms by which cells respond to similar conditions in higher eukaryotes.

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CHAPTER 4

Defining the composition of the preinitiation complex on a post-PIC recruitment regulated promoter

Previous studies demonstrated that components of the PIC, including TBP and Pol II, assemble at the *FLR1* promoter in an inactive form prior to gene activation by oxidative stress. PIC activation occurs by Yap1-dependent recruitment of Mediator upon oxidative stress. This strongly suggests *FLR1* gene activation is regulated in a post-PIC recruitment manner. Importantly, similar post-PIC recruitment mechanisms occur in the regulation of genes involved in the progression of diseases such as cancer and AIDS. Therefore, understanding post-PIC recruitment gene regulation will allow for the development of treatments for such deteriorating conditions. Here, we define the promoter elements of the gene *FLR1* and reveal the state of the PIC involved in maintaining an inactive complex on promoter DNA prior to gene activation by oxidative stress conditions. This study was performed entirely by me. I have created all figures and have written these findings into manuscript format for submission to the professional Journal of Biological Chemistry. Once published, the literature citation will read:

Defining the composition of the preinitiation complex on a post-PIC recruitment regulated promoter

Goldstrohm, D.A. and Stargell, L.A.

4.1 Abstract

The recruitment of the PIC to promoter DNA is often a rate-limiting step in the activation of gene expression. However, recent work demonstrates that prior to gene activation by oxidative stress the promoter of the *FLR1* gene is already occupied by members of the PIC, including TBP and Pol II. The regulation of *FLR1* gene activation occurs at a step after PIC recruitment to promoter DNA. Here, we define the promoter of the gene *FLR1* and determine the composition of the PIC present in the inactive state. Interestingly, we find the regulation of *FLR1* gene expression functions through a noncanonical core promoter, which we show in vitro directly binds members of the PIC. Furthermore, ChIP assays reveal the kinase subunit of TFIIH (Kin28) is associated with the *FLR1* promoter prior to oxidative stress. Consistent with this, phosphorylation of the CTD of Pol II is observed at serine 5 and serine 2 prior to oxidative stress conditions. Thus, CTD phosphorylation is not sufficient for PIC activation on a post-PIC recruitment regulated promoter. In addition, the 5' capping enzyme Ceg1 is observed at the *FLR1* promoter prior to oxidative stress. Thus, a remarkably complete PIC is assembled on this promoter prior to activation by oxidative stress conditions.

4.2 Introduction

Transcriptional initiation of Pol II gene expression requires the interaction between cis-acting promoter elements and trans-acting factors. Generally, the regulation of Pol II gene expression is controlled at the level of transcriptional initiation. During transcriptional initiation of TATA-containing genes, members of

the PIC (including Pol II and a plethora of GTFs including TFIID (TBP + TAFs), TFIIA, TFIIB, TFIIF, TFIIH, TFIIE) are recruited to the TATA element located in the promoter region of the gene (Martinez, 2002; Woychik and Hampsey, 2002). Often, the recruitment of the PIC to promoter DNA is a rate-limiting step in the transcriptional initiation process (Kuras and Struhl, 1999; Lin and Green, 1991; Orphanides and Reinberg, 2002; Roeder, 2005). However, an increasing number of genes are being uncovered that are regulated in a post-PIC recruitment fashion where gene activation occurs at a step after PIC component recruitment to promoter DNA (Chapter 2(Krumm et al., 1992; Lis and Wu, 1993; Meulia et al., 1992; Rasmussen and Lis, 1995).

Following PIC assembly on promoter DNA, an open promoter complex is established by the ATP-dependent helicase subunit of TFIIH (Schaeffer et al., 1993). The formation of this open promoter complex allows transcription to initiate upon the addition of ribonucleoside-triphosphates (NTPs). Once Pol II has transcribed ~20-30 nucleotides, 5' capping of the RNA occurs by the action of the capping enzyme Ceg1. The cap protects the nascent RNA molecule from degradation by nucleases (Woychik and Hampsey, 2002). Following 5' capping of the nascent RNA, Pol II enters the elongation phase where it transcribes the length of the gene until transcription is terminated. After elongation is terminated, several additional pre-mRNA processing events occur such as the splicing of introns within the body of the pre-mRNA and the formation of a 3' end, which is usually modified by the addition of a poly (A) tail (Proudfoot et al., 2002).

Therefore, on post-PIC recruitment regulated genes like *FLR1*, all of the above processes (Pol II promoter clearance, elongation, and termination) are possible rate-limiting steps for the regulation of gene expression.

One of the key factors thought to regulate the coupling of transcriptional initiation with that of transcriptional elongation and pre-mRNA processing is the CTD of the largest subunit of Pol II. In yeast, the CTD contains 26 repeats (52 in humans) of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, which is a direct target of phosphorylation by the kinase subunit of TFIIH (Kin28) (Feaver et al., 1994; Roy et al., 1994; Svejstrup et al., 1995). Generally, phosphorylation of the CTD at serine 5 and serine 2 is thought to aid in the transition from transcriptional initiation to the elongation state (Bartholomew et al., 1986; O'Brien et al., 1994; Zawel and Reinberg, 1993). CTD phosphorylation is also believed to be important in the recruitment of pre-mRNA processing factors to the newly synthesized RNA. For example, the 5' capping enzyme (Ceg1) is recruited by TFIIH-dependent serine 5 phosphorylation of the CTD close to the promoter of Pol II genes (Hirose and Manley, 2000; Komarnitsky et al., 2000; Pei et al., 2001 and this recruitment is required for the further pre-mRNA events of splicing and polyadenylation {Shatkin, 2000 #2845; Proudfoot et al., 2002; Rodriguez et al., 2000). In addition, the coactivator Mediator is known to interact with the CTD of Pol II to aid in transcriptional activation processes (Kim et al., 1994; Myers and Kornberg, 2000) and several lines of evidence suggest that activation of gene expression by Mediator functions through general transcription factor TFIIH.

Mediator has been shown to interact with TFIIH and also activates the kinase activity of TFIIH through its Kin28 subunit (Giot et al., 2003; Sakurai and Fukasawa, 2000).

By observing that members of the PIC, including TBP and Pol II, are present at the promoter prior to oxidative stress inducing conditions, we conclude the *FLR1* gene is activated by a post-PIC recruitment mechanism (Chapter 2). Although many genes are known to be regulated in a post-PIC recruitment manner, little is known about the mechanisms of this regulation. Here, we define the promoter of the post-PIC recruitment regulated gene *FLR1* and analyze the state of the PIC on the promoter DNA prior to activation by oxidative stress. We observe occupancy of the kinase subunit of TFIIH (Kin28) at the *FLR1* promoter and the phosphorylation of the CTD, all prior to oxidative stress inducing conditions. In addition, the 5' capping enzyme Ceg1 is occupied at the *FLR1* promoter prior to oxidative stress. Thus, *FLR1* gene activation occurs at a step after CTD phosphorylation and CTD phosphorylation is not sufficient for PIC activation on this post-PIC recruitment regulated promoter.

4.3 Materials and Methods

4.3a Yeast strains and DNA constructs

Yeast wild type strains utilized in this study were purchased from Invitrogen (BY4741: *MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*). The DNA element used for

the in vitro protein-DNA interaction studies was generated by the using the 23-base pair forward oligonucleotide 5'-CAGTGCGAAAAGGGACATGATAG-3' and the 21-base pair reverse oligonucleotide 5'-CTTCACGGGCACTCTGTAAAG-3' to PCR amplify the genomic *FLR1* promoter from positions –732 to +100, which contains YREs 2 and 1 through 100 base pairs downstream of the ATG. This amplification product was subcloned into the yeast vector pRS316 using the restriction enzymes SpeI-HindIII. These sites were then used to remove the subcloned *FLR1* promoter and Klenow enzyme was used to fill in the single-stranded overhang with 25 µCi of [α-^{32}P]dATP in the presence of 2.0 µM dNTPs. The DNA element utilized for primer extension assays contained the 21-base pair reverse oligonucleotide above, which was end-labeled using T4 polynucleotide kinase with 150 µCi of [γ-^{32}P]ATP.

4.3b Yeast medium and plates

Yeast medium and plates utilized throughout this study were made according to (Hampsey, 1997).

4.3c Protein expression and purification

Untagged, full-length yeast TBP was expressed in *E. coli* strain BL21 (DE3) with a pET11a vector (called pYTBP) as described (Campbell et al., 2000). The soluble fraction was purified over Q, SP, and Heparin HiTrap columns (Amersham Pharmacia Biotech) equilibrated in buffer A (10 mM sodium phosphate, 100 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM dithiothreitol, pH

adjusted to 7.5). TBP flowed through the Q column and was eluted from the SP and Heparin columns using linear NaCl gradients in buffer A. Final purification of TBP was by gel filtration with Sephacryl S-100 equilibrated in 100 mM KCl, 40 mM HEPES (pH 7.9), 20 mM Tris (pH 7.5), 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. The protein was shown to be 90% pure upon staining with Coomassie blue.

Recombinant yeast TFIIA was purified as described (Ranish et al., 1992). This procedure involves expressing each subunit, Toa1 and Toa2, in separate strains of *E. coli* BL21DE3. Isopropyl-β-D-thiogalactopyranoside (final concentration, 0.1 mM) was added at an optical density of 0.6, and after the cells were ruptured by sonication, insoluble material was collected by centrifugation. The proteins were then denatured in a buffer containing 8 M urea and renatured in the presence of the other subunit. The renatured proteins were dialyzed against 100 mM KCl, 40 mM HEPES (pH 7.9), 20 mM Tris (pH 7.5), 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride (EMSA binding buffer without $MgCl_2$). The TFIIA was ~60% pure as determined by Coomassie staining.

4.3d Electrophoretic mobility shift assays

Protein-DNA interactions will be studied by incubation of purified proteins with a ^{32}P-labeled *FLR1* probe. Binding reactions will contain 10 μM poly(dG-dC) nonspecific competitor, 100 mM KCl, 40 mM HEPES (pH 7.9), 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM

phenylmethylsulfonyl fluoride. ^{32}P-labeled *FLR1* promoter probes will be incubated with 13.8 nM TBP at 25 °C for 30 minutes. For EMSA the complexes will be separated on 5% acrylamide gel containing 0.5X Tris borate-EDTA and 2 mM $MgCl_2$ in both the gel and running buffer. Recombinant yeast TFIIA and Yap1 will be isolated and incubated with TBP and probe DNA and treated as above, except $MgCl_2$ was omitted from the gel and running buffer in the experiments shown. Omission of the divalent cations from the binding reaction allows for the stable formation of only the ternary complex, which makes quantification simpler because there is only a single shifted complex. Assays of higher order complex formation will also be performed in the presence of $MgCl_2$ (data not shown). Inclusion of $MgCl_2$ should not alter the results of the experiments. The gels will be transferred to Whatman 3 MM paper, dried under vacuum at 80 °C, and subjected to PhosphorImager screen.

4.3e Chromatin immunoprecipitation analysis

Chromatin Immunoprecipitation assays were performed as described (Frank et al., 2001) with the following modifications. Antibodies used in immunoprecipitations were to that of myc (Kin28, Ceg1), phosphoserine5, and phosphoserine2. Oligos were designed for PCR analysis of the *FLR1* promoter at positions -221 (5') to -200 and 80 (3') to 100. For *FLR1* induction, H_2O_2 was added to a final concentration of 0.3 mM for 30 minutes. Standard curves were generated using 10-fold serial dilutions of Input DNA. PCR efficiencies ranged from 90-100%, with a correlation coefficient of 0.990 or greater. Threshold cycle

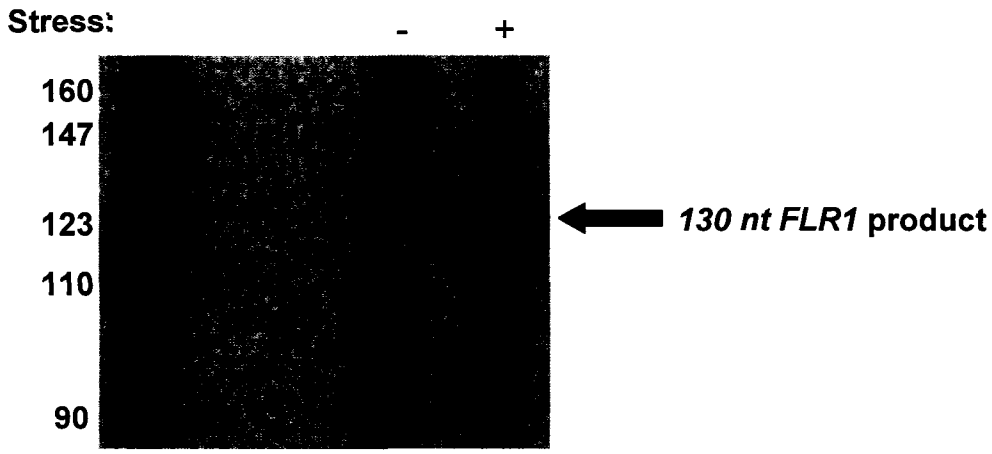
data were quantified relative to the input, as described (Frank et al., 2001). The relative occupancy for each factor was compared to an arbitrary value of 10, which was assigned to the occupancy levels of the tagged induced strains. Background levels were calculated as the relative occupancy of untagged strains and these values were subtracted from that of the tagged strains. The majority of experiments were performed in triplicate with the exception of a few in duplicate.

4.4 Results

4.4a The post-PIC recruitment regulated gene *FLR1* functions through a noncanonical core promoter element

Recent studies demonstrate *FLR1* gene activation is regulated in a post-PIC recruitment manner and activation of the PIC occurs by Yap1-dependent recruitment of Mediator to promoter DNA upon induction of oxidative stress conditions (Chapter 2). Here, we define the promoter element of the *FLR1* gene to try to better understand the regulation of gene expression on post-PIC recruitment promoters. Primer extension was used to map the transcription initiation site of the *FLR1* promoter. A primer extension product of ~130 nucleotides (Figure 4.1A) positions the initiation site ~30 nucleotides upstream of the ATG start codon (Figure 4.1B). *FLR1* promoter sequence analysis reveals this initiation site is located in the middle of the only canonical TATA element in the promoter region (Figure 4.1B). Thus, we conclude that the *FLR1* gene has a noncanonical core promoter element.

A



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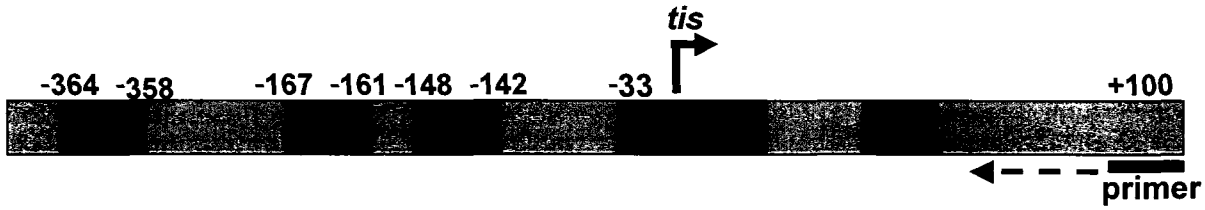

-370 TGGTGA[redacted]TATAGG AATGCCCTGC GCTTTTCTCT ACAATATTGC
GCATCACTAG TTTTTCCGGA AGATTATGAA GATTTCAAAT GAAAGAAGAC
AAATGCAAAG AAAAAAAAAA GGATATTTGC TTAGGTAAGG AGCAATAACA
GTGCGAAAAG GGACATGATA GTACACATTT GAGTAAAATT GGTATCAATC
-170 ATC[redacted] TGGGCGGGAT AAT[redacted] GTAAAAGGGG AACAGGAAAG
AAGACGAGAA TTGCATTTAA ACAGCTCTTT ACGAGGCTAA AATATCTACA
-70 TCCTTATGCC GTCTTTGTCA TTATGGTGAA GCTATT[redacted]GAGTATCC
-20 GTTGAACGAC AATCTCCACT[redacted]TATACA CTTCAACGTA CAGACACACT
ATCGTTGTTG ACCTTTTAGA ATATTTGGGT ATAGTGTCCA ACTTAGAAAC
+90 TTTACAGAGT GCCCGTGAAG

Figure 4.1. *FLR1* functions through a noncanonical promoter
(A) Log phase cells were grown in YPD and oxidative stress was induced by the addition of H_2O_2 (0.3 mM final concentration) for 30 min. RNA was extracted and used in primer extension assays to map the 5' end of the *FLR1* transcript, which resulted in a 130 nt product. (B) Diagram of the *FLR1* promoter illustrating the primer used in the primer extension assays was 100 bp downstream of the ATG start codon and location of canonical TATA element. Red text indicates portion of *FLR1* sequence where primer was designed. Arrow represents the (*tis*).

4.4b PIC component complex formation on the *FLR1* noncanonical core promoter in vitro

Based on the previous findings, we suggest the *FLR1* promoter functions with a noncanonical TATA element. We predict that members of the PIC can directly bind the *FLR1* promoter in the absence of the canonical TATA element. In this study, the *FLR1* promoter upstream of the transcription start site (thus with the canonical TATA omitted) was utilized in electrophoretic mobility shift assays to analyze PIC component complex formation. A radioactively labeled noncanonical *FLR1* fragment was added to each lane followed by the sequential addition of TBP and TFIIA. These studies illustrate TBP-TFIIA-DNA complex formation on the *FLR1* promoter in the absence of a canonical promoter element (Figure 4.2A). This demonstrates the *FLR1* noncanonical core promoter is functional for PIC component complex.

We next wanted to define the region of the *FLR1* noncanonical promoter responsible for PIC component recognition in vitro. Here footprint experiments were performed to analyze TBP addition on the *FLR1* noncanonical promoter, which was deleted for the canonical TATA element. In these experiments, unprotected DNA was degraded by DNase I digestion and protein-DNA complexes were resolved on an acrylamide gel. It was determined that TBP protects the noncanonical region of the *FLR1* promoter in an area approximately 40-90 base pairs downstream of the YREs (Figure 4.2B). The addition of Yap1 as a control also demonstrates the ability of Yap1 to recognize the YREs on the

synthesized *FLR1* noncanoncial promoter (Figure 4.2B). Collectively, these studies strongly suggest that the *FLR1* promoter functions through a noncanonical promoter.

4.4c TFIIH (Kin28) occupies the *FLR1* promoter prior to oxidative stress

Both TBP and Pol II are occupied at the *FLR1* promoter prior to gene activation by oxidative stress, thus the gene is regulated in post-PIC recruitment of the PIC manner. To better define the mechanism of post-PIC recruitment regulation, experiments were performed to analyze the members of the PIC in the inactive complex on promoter DNA. ChIP assays were used to analyze the occupancy of the kinase subunit of TFIIH (Kin28) at the *FLR1* promoter. Yeast strains containing a myc-tagged derivative of TFIIH or an untagged control were grown to log phase and induced with oxidative stress by the addition of H_2O_2 for 30 minutes. Protein-DNA interactions were cross-linked with formaldehyde, cross-links were reversed, and TFIIH factor occupancy at the *FLR1* promoter was analyzed by quantitative (real-time) PCR. The region of the *FLR1* promoter analyzed spans –221 to +100 from the ATG start codon (Figure 4.3A). Like the results for TBP and RNA Pol II, TFIIH occupancy at the *FLR1* promoter was observed prior to treatment with oxidative stress and these occupancy levels did not increase significantly under oxidative stress conditions (Figure 4.3B). Thus, at the *FLR1* gene, the addition of TFIIH to the complex is not sufficient for high levels of transcription. In addition, these results indicate that a rate-limiting step of *FLR1* gene activation occurs at a step after TFIIH occupancy to the promoter.

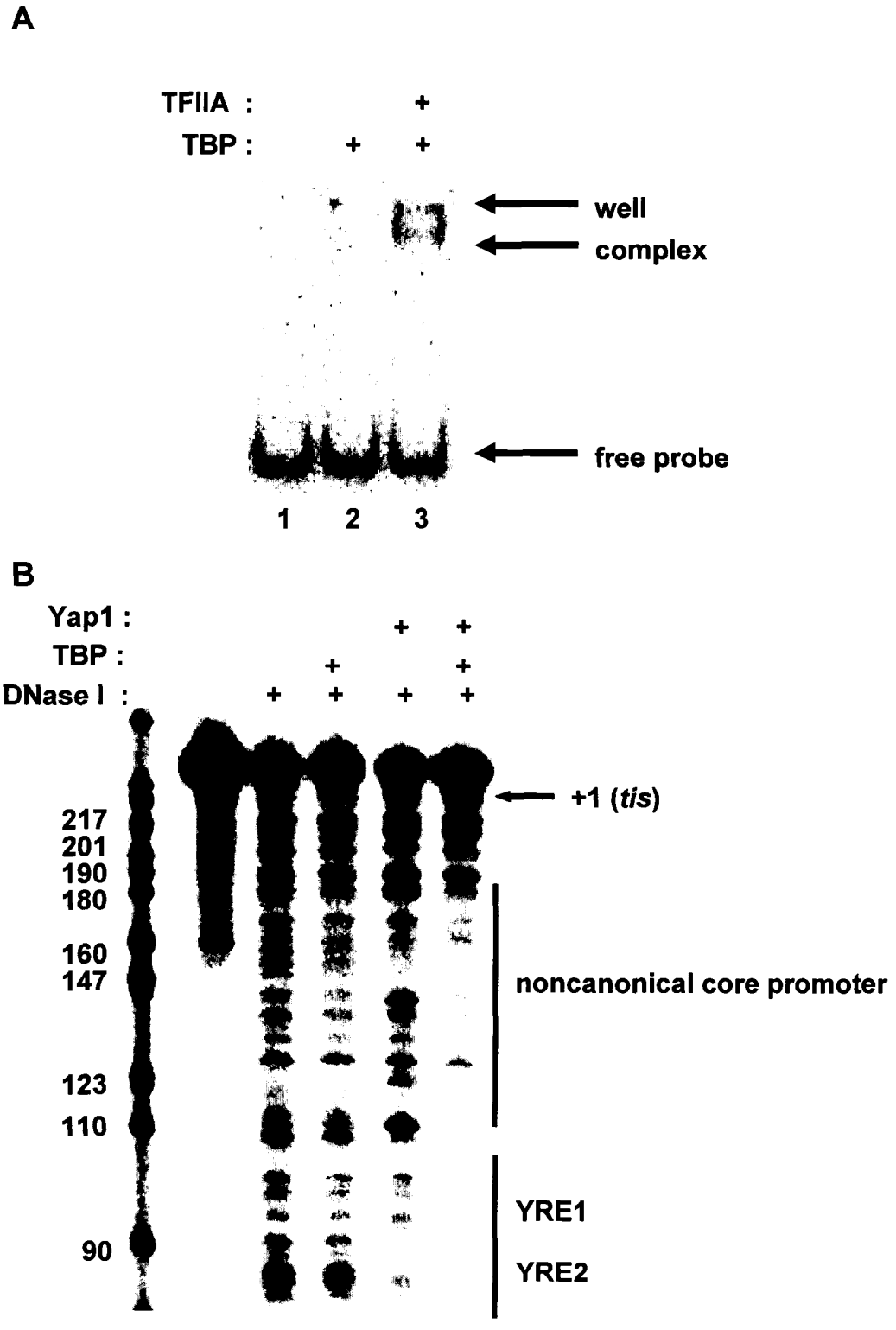

Figure 4.2. PIC component complex formation on *FLR1* noncanonical promoter
(A) EMSAs analyzing TBP-TFIIA-DNA complex formation on the noncanonical promoter element of *FLR1*. (B) Footprint analysis illustrating TBP and Yap1 binding on the noncanonical *FLR1* promoter.

4.4d Pol II CTD phosphorylation occurs prior to *FLR1* gene activation

The above results show the kinase subunit of TFIIH is occupied at the *FLR1* promoter prior to oxidative stress conditions and that these levels do not increase significantly upon gene activation by oxidative stress. Since the kinase subunit of TFIIH is involved in the phosphorylation of the Pol II CTD during gene regulation, we next wanted to analyze the phosphorylation state of the CTD. ChIP assays were performed to analyze the presence of either serine 5 or serine 2 CTD phosphorylation states at the *FLR1* promoter. Strikingly, CTD phosphorylation at both serine 5 and serine 2 was detected at the *FLR1* promoter prior to oxidative stress and occupancy levels did not change significantly upon treatment with oxidative stress (Figure 4.3C). These results indicate that a rate-limiting step of *FLR1* gene activation occurs at a step after phosphorylation of the CTD and that CTD phosphorylation is not always sufficient for PIC activation.

4.4e The 5' capping enzyme Ceg1 is occupied at the *FLR1* promoter prior to oxidative stress

The above findings reveal the *FLR1* promoter contains a phosphorylated form of the CTD of Pol II, prior to gene activation by oxidative stress. This indicates that gene activation of *FLR1* gene expression occurs at a step after phosphorylation of the CTD. As mentioned previously, CTD phosphorylation at serine 5 is required for recruitment of the 5' capping enzyme Ceg1. Thus, to further examine the state of the PIC on the *FLR1* promoter during oxidative stress, ChIP assays were performed to analyze the occupancy of the 5' capping enzyme

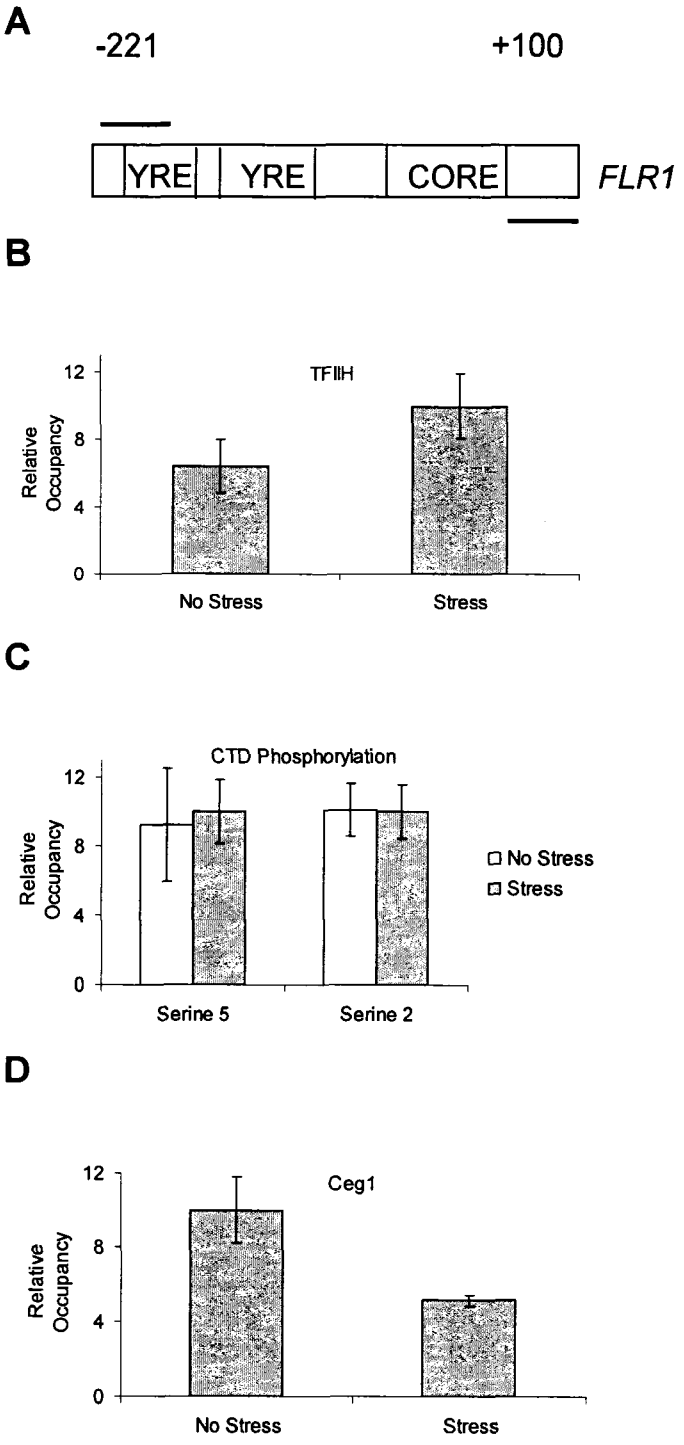

Figure 4.3. A rate-limiting step of *FLR1* gene activation occurs at a step after CTD phosphorylation and recruitment of Ceg1
(A) Diagram of primer design for ChIP assays. (B-D) ChIP analysis of TFIIH, Pol CTD phosphorylation, and Ceg1 occupancy at the *FLR1* promoter and were perfomed as previously described. ChIP analysis reveals all of these occur prior to oxidative stress conditions.

Ceg1. Interestingly, Ceg1 occupancy was observed at the *FLR1* promoter prior to oxidative stress (Figure 4.3D). Since *FLR1* gene transcription is not activated prior to oxidative stress, these results provide supporting evidence that a rate-limiting step of *FLR1* gene activation is at a step after recruitment of the 5' capping enzyme Ceg1 to the promoter.

4.5 Discussion

Transcriptional initiation of RNA polymerase II gene expression involves the ordered recruitment of the PIC to promoter DNA (Martinez, 2002; Woychik and Hampsey, 2002). The recruitment of the PIC to promoter DNA is often observed to be a rate-limiting step in the gene activation process (Kuras and Struhl, 1999; Li et al., 1999; Lin and Green, 1991; Orphanides and Reinberg, 2002; Roeder, 2005). Upon PIC assembly to promoter DNA, an open promoter complex is formed and transcription is initiated by the addition of NTPs (Schaeffer et al., 1993; Serizawa et al., 1993). Once Pol II has transcribed ~20-30 nucleotides, 5' capping of the RNA occurs by the CTD phosphorylation-dependent recruitment of the 5' capping enzyme Ceg1, which protects the nascent RNA molecule from degradation by nucleases (Woychik and Hampsey, 2002). Following 5' capping of the nascent RNA, Pol II proceeds through the coding region of the gene to complete the elongation phase of the transcription process. Importantly, the transition from transcriptional initiation to elongation is thought to be regulated by phosphorylation the Pol II CTD. The different states of CTD phosphorylation are also thought to be important in pre-mRNA processing factors recognition. After

the elongation process, transcription is terminated and pre-mRNA processing events occur including introns splicing and the formation of a 3' end, which is usually modified by the addition of a poly(A) tail (Proudfoot et al., 2002). Recent evidence suggests that the above transcription cycle should not be viewed as biologically distinct steps, but as an overall coupled process (Orphanides and Reinberg, 2002).

Here the *FLR1* gene was used to further our understanding of post-PIC recruitment gene regulation during oxidative stress. Our studies reveal that *FLR1* functions through a noncanonical core promoter. Purified components of the PIC were shown to bind this noncanonical promoter element and the region important for TBP recognition was also defined. The finding that *FLR1* functions through a noncanonical promoter is not surprising since recent evidence suggests approximately 80% of yeast genes function through noncanonical promoters (Basehoar et al., 2004). Indeed, some genes require a noncanonical promoter for proper function (Meyer et al., 1997).

We next determined the composition of the PIC present on the inactive *FLR1* promoter, prior to gene activation by oxidative stress. Collectively, these findings revealed occupancy of the kinase subunit of TFIIH (Kin28), serine 5 and serine 2 phosphorylation of the CTD, and occupancy of the 5' capping enzyme Ceg1 at the *FLR1* promoter, prior to gene activation by oxidative stress. These findings help support a role for TFIIH in CTD phosphorylation, which has been

documented by numerous others (Feaver et al., 1994; Roy et al., 1994; Svejstrup et al., 1995) and also further supports our previous findings that *FLR1* gene expression is regulated in a post-PIC recruitment manner (Chapter 2). However, these results are in contrast to studies suggesting that CTD phosphorylation triggers the transition from transcriptional initiation into elongation (Zawel and Reinberg, 1993). Therefore, our results suggest that CTD phosphorylation is not always sufficient for PIC activation. In addition, the different patterns of CTD phosphorylation are thought to be important in pre-mRNA processing factors recognition. For example, the 5' capping enzyme (Ceg1) is recruited by TFIIH-dependent serine 5 phosphorylation of the CTD at the promoter region or Pol II genes (Komarnitsky et al., 2000; Pei et al., 2001; Rodriguez et al., 2000) and this recruitment is required for the further pre-mRNA events of splicing and polyadenylation (Hirose and Manley, 2000; Proudfoot et al., 2002; Shatkin and Manley, 2000). Our results show that activation of *FLR1* gene expression occurs at a step after 5' capping enzyme Ceg1 occupancy at the *FLR1* promoter and further supports the coupling of the transcriptional initiation process with that of pre-mRNA processing.

Overall, this work furthers our understanding of the regulation of post-PIC recruitment regulated genes. We show that post-PIC recruitment regulated genes can function through a noncanonical core promoter and that the inactive complex is remarkably complete prior to activation. Furthermore, our data supports the coupling of pre-mRNA processes with that of transcriptional

initiation since Ceg1 is occupied on the *FLR1* promoter prior to gene activation by oxidative stress. Importantly, similar post-PIC recruitment mechanisms occur in the regulation of genes involved in the progression of diseases such as cancer and AIDS. Therefore, understanding post-PIC recruitment gene regulation will allow for the development of treatments for such deteriorating conditions.

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CHAPTER 5

Perspectives and future directions

Initially, the focus of my doctoral work was aimed at characterizing the role of the general transcription factor TFIIA in the regulation of gene expression by RNA polymerase II. This study involved examining the transcription profiles of specific TFIIA mutants of *Saccharomyces cerevisiae* using DNA microarray technology. These experiments revealed a group of genes that exhibited altered message levels in these particular TFIIA mutants. Importantly, a search for common motifs in the upstream region of the pool of genes with decreased transcript levels in these mutants yielded the binding site for Yap1, the transcriptional activator that regulates the response to oxidative stress. These findings were not only critical in establishing a connection between the key regulator of the oxidative stress response (Yap1) and the general transcription machinery through the general transcription factor TFIIA, but sparked my interest in developing a model for transcription activation during oxidative stress. In addition, these results were also useful in identifying genes involved in Yap1-dependent gene regulation during oxidative stress that could be utilized to build such a model.

In these experiments, the *FLR1* gene was identified as an ideal gene to study transcriptional regulation during oxidative stress. The *FLR1* gene was found to be down-regulated in the TFIIA mutant strains and further analysis revealed that

it was also regulated in a Yap1-dependent manner. Remarkably, additional experiments showed the *FLR1* promoter was occupied by components of the PIC prior to gene activation during oxidative stress conditions in a Pho2-dependent manner. In addition, phosphorylation of the CTD of Pol II on the *FLR1* promoter was not sufficient for PIC activation upon induction by oxidative stress conditions. These studies revealed supporting evidence for the coupling of transcriptional initiation and pre-mRNA processing events through the occupancy of the 5' capping enzyme Ceg1 at the *FLR1* promoter prior to oxidative stress conditions. Activation of the PIC occurred by Yap1-dependent recruitment of the coactivator Mediator to the *FLR1* promoter upon oxidative stress. Thus, a model was proposed whereby Yap1-dependent recruitment of Mediator during oxidative stress was required for PIC transition, resulting in elevated levels of FLR1 gene expression (Figure 5.1-5.4). Importantly, our findings revealed a two-step mechanism for transcriptional activation involving the combinatorial function of sequentially acting activators. In the first step, one activator functions to recruit an inactive PIC. In the second step, another regulator "activates" the PIC via the recruitment of Mediator. This model suggests the regulation of gene expression during oxidative stress is more complex than described in classical models and supports a combinatorial role for the function of activator proteins during the response to oxidative stress.

The above research also suggests Mediator function as a key regulator of the oxidative stress response. This discovery opened the door to an interest in

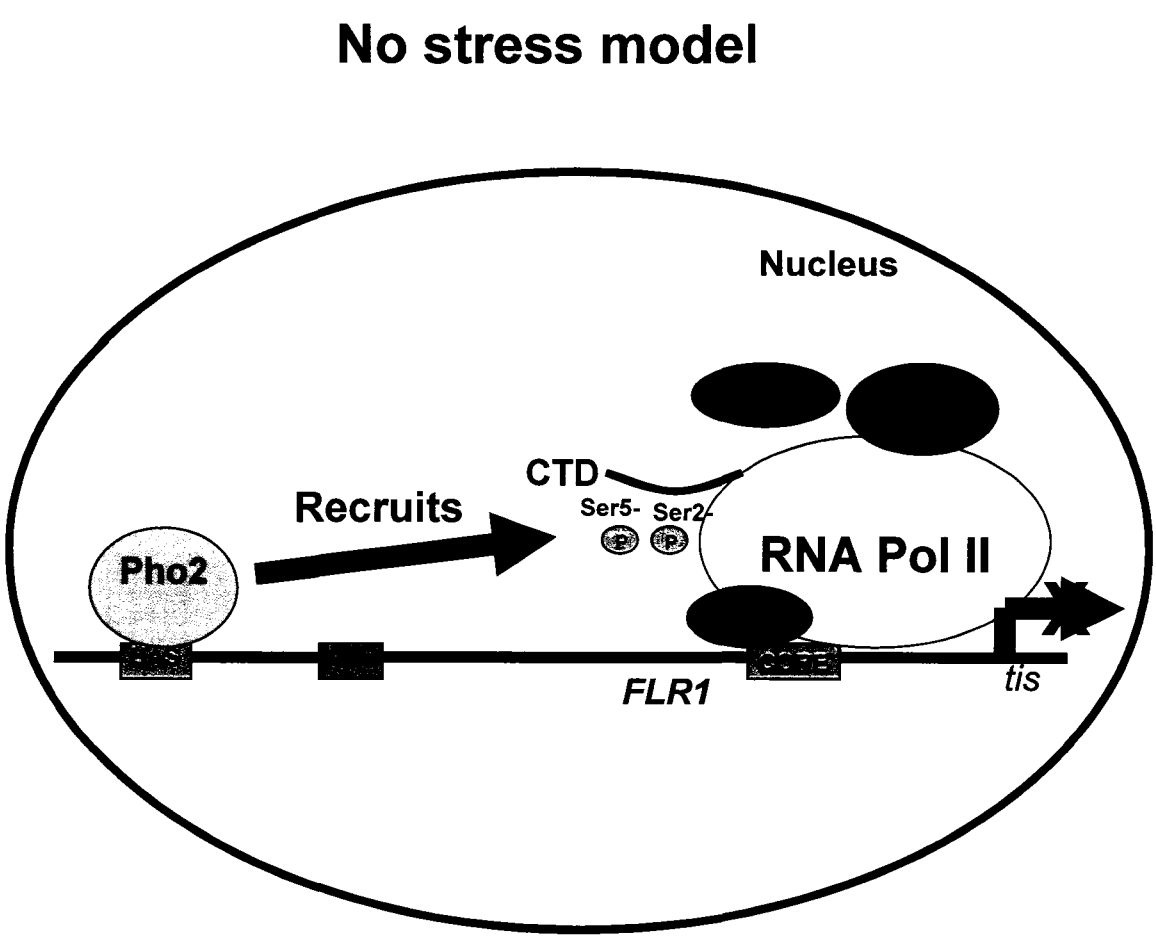

Figure 5.1 Pho2 recruits a relatively complete PIC to the *FLR1* promoter
The current model suggests the constitutive activator Pho2 functions to recruit a relatively complete inactive PIC to the *FLR1* promoter prior to oxidative stress conditions. This relatively complete PIC includes TBP, Pol II, TFIIH, Phosphoserine 2 and 5, and Ceg1.

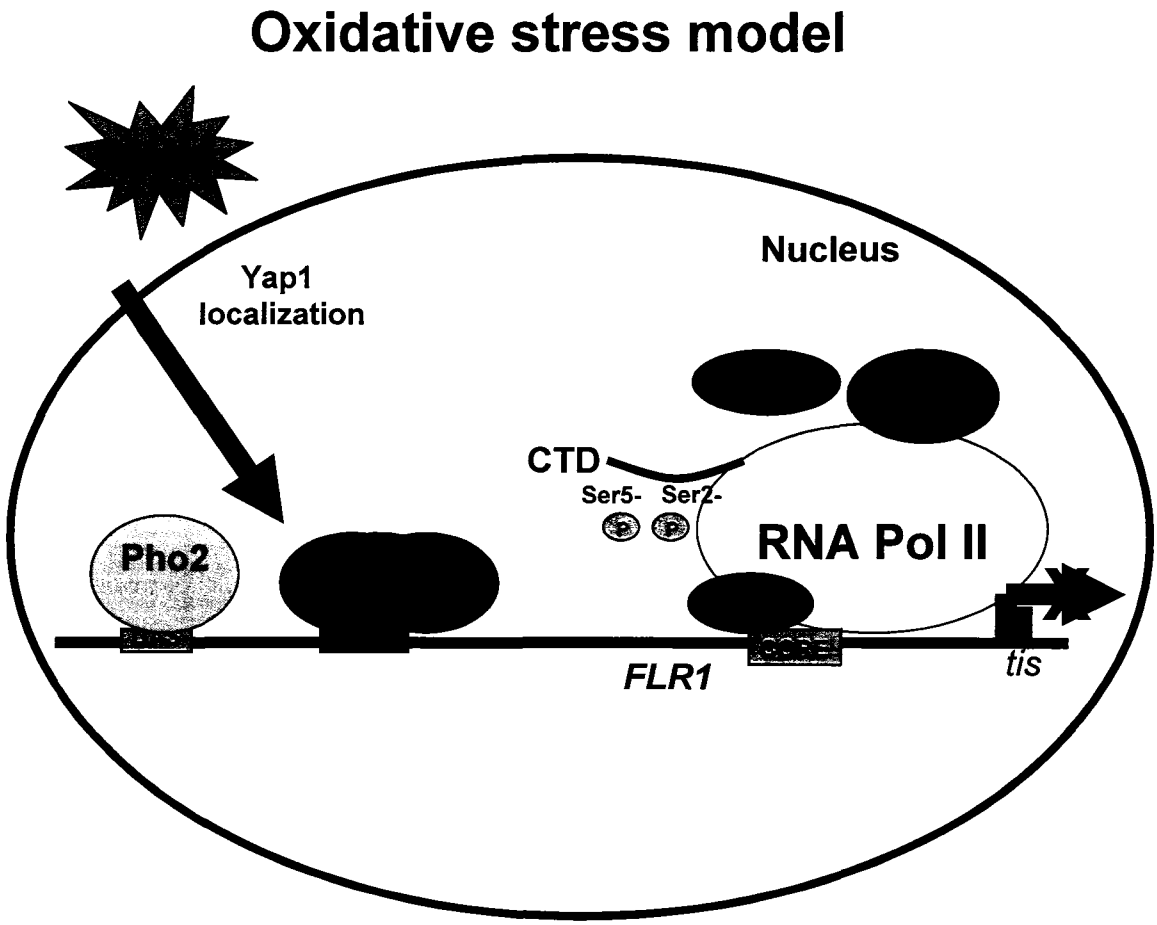

Figure 5.2 Yap1 is localized to the *FLR1* promoter upon oxidative stress
The current model suggests the activator Yap1 is localized to the *FLR1* promoter upon oxidative stress where it recognizes 3 YREs (Yap1-response elements) in the upstream promoter region.

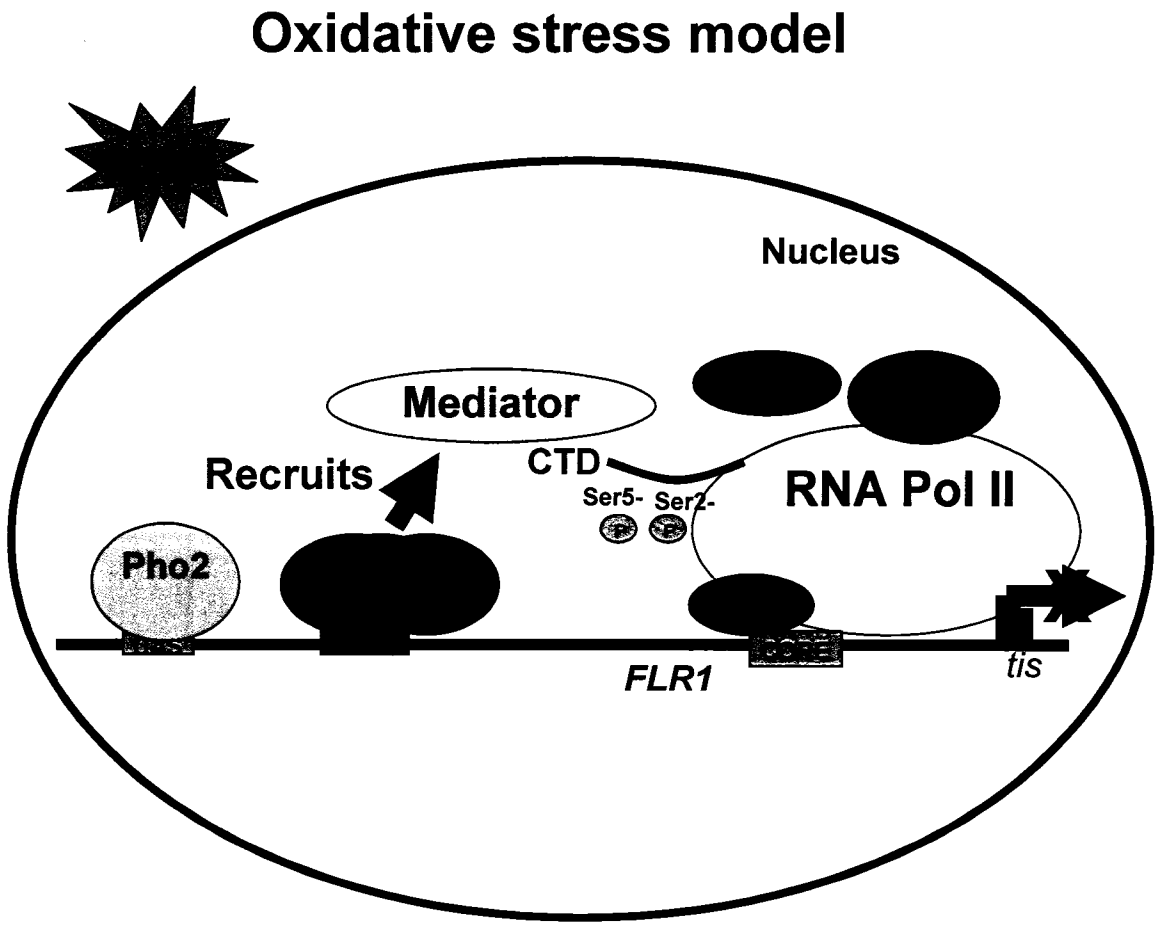

Figure 5.3 Yap1 function to recruit Mediator to the *FLR1* promoter upon oxidative stress
The current model suggests the activator Yap1 functions to recruit the coactivator Mediator to the *FLR1* promoter upon oxidative stress. Mediator is thought to interact with Pol II of the PIC.

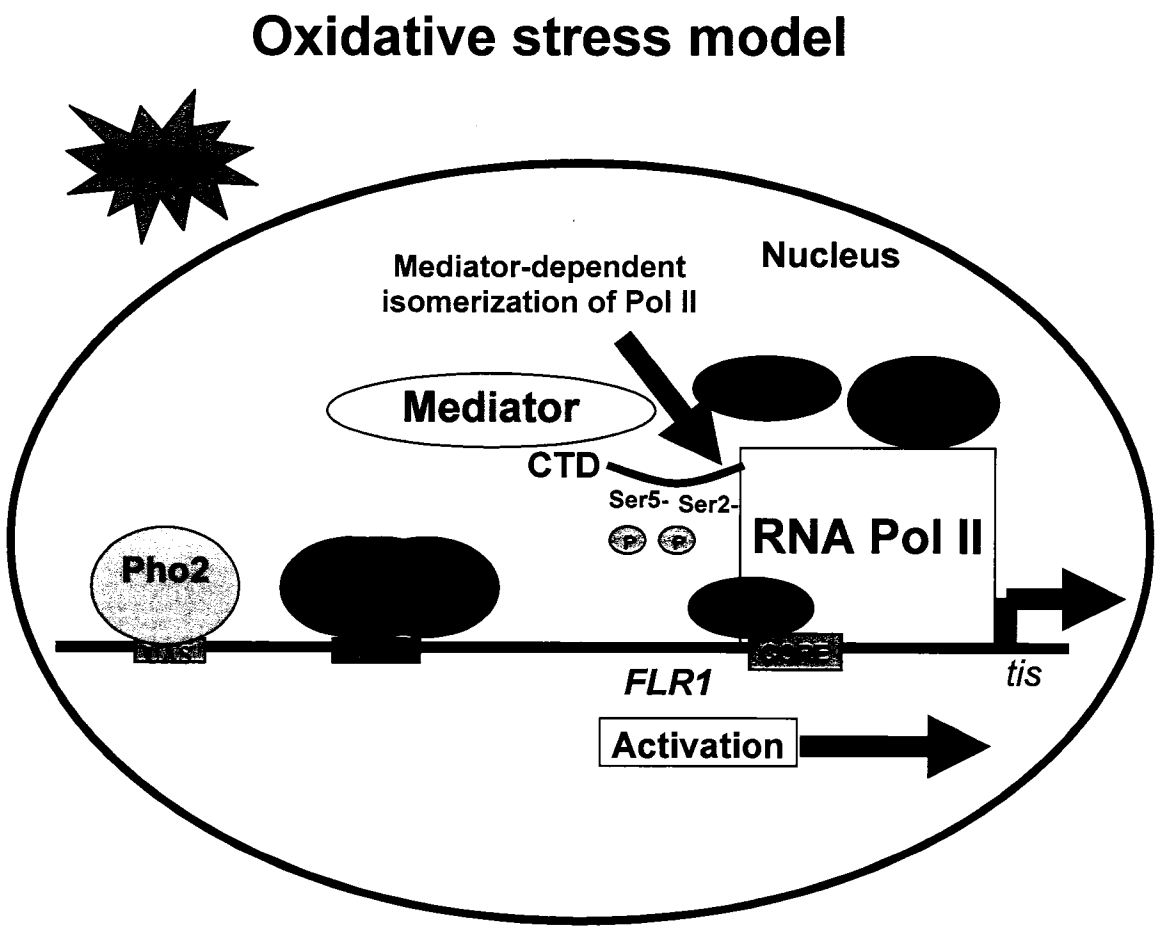

Figure 5.4 Mediator isomerization of Pol II "trigger" *FLR1* gene activation
The current model suggests the activator Mediator functions to isomerizes Pol II of the PIC upon oxidative stress conditions. This isomerization is thought to "trigger" Pol II release from the *FLR1* promoter allowing for transcriptional activation.

defining the requirement for specific Mediator components for cell growth under various non-optimal growth conditions. Mediator consists of 25 subunits of which 15 are nonessential and do not affect cell viability when deleted. This research focused on the subunits of the yeast Mediator modules nonessential for growth on rich medium and determined how deletion of these subunits affects cell survival during various non-optimal conditions. Mediator deletion strains were grown in media containing agents that induce the oxidative stress response (hydrogen peroxide); serve as anti-fungal agents (fluconazole); inhibit protein synthesis (cycloheximide); inhibit tumor growth in mammals (methotrexate); and induce the heat shock response (38°C). These results demonstrated that Mediator is largely required for cell growth during all conditions tested. Remarkably, this suggests that under non-optimal growth conditions, once Mediator subunits that are nonessential during growth at optimal conditions become essential for cell growth during non-optimal conditions. This observation redefines the meaning of nonessential and essential subunits as it relates to cell growth in various growth environments. In addition, it appears that the total number of these nonessential subunits required of Mediator varies based on the condition tested. Therefore, it is likely that the degree of Mediator function during various growth conditions may be directly correlated to the actual number of required Mediator subunits for a particular condition. These findings help to define Mediator as a key regulator of transcription during cell growth on non-optimal conditions. These findings have led to the design of future experiments to better understand the role of Mediator in the response to various stresses. In

particular, only 4 subunits of the 15 total nonessential subunits of Mediator tested were found to be essential for cell growth under every stress condition tested. Interestingly, orthologs of these subunits in humans have been implicated in the metastasis of certain forms of lung cancer. Therefore, determining the roles of these subunits during gene regulation under non-optimal growth conditions will allow for a better understanding of similar functions in human cells.

Overall, this work has contributed significantly to our understanding of how cells survive and flourish in a non-optimal environment. Initially, little was known regarding Yap1 function at the transcriptional level. However, upon completion of these studies, an entire model illustrating Yap1-dependent gene activation of Pol II gene expression during various conditions was developed. Importantly, this work established a new view of transcriptional activation during oxidative stress that differed from previous classical models. In addition, a role for the coactivator Mediator was uncovered as a key regulator of the oxidative stress response. This led to additional experiments that defined the differing roles of Mediator subunits in the response to various non-optimal growth conditions and helped to redefine conventional terminology. Collectively, this entire study brings us closer to fully understanding how cells survive in a stress environment. Understanding this at the molecular level will give us the tools to develop treatments to combat the harmful diseases that result from the misregulation of similar processes.

Excitingly, the above model suggests the possibility of promoter-proximal pausing of Pol II in yeast. In higher eukaryotes, promoter-proximal pausing is an important mechanism in the regulation of genes, such as the oncogenes *c-myc, c-jun* and the *HIV-1* gene. Therefore, understanding the mechanisms of this regulation is critical in the development of treatments for diseases that result from the misregulation of these processes. However, to this date, promoter-proximal pausing in the regulation of gene expression in yeast has not been uncovered. Therefore, future work is aimed at identifying promoter-proximal pausing in yeast by further analyzing *FLR1* gene regulation. In particular, these studies will involve analyzing the architecture of the *FLR1* promoter prior to gene activation by potassium permanganate footprinting to determine if the promoter is melted prior to oxidative stress conditions and also utilizing assays to detect initiation of transcription by Pol II prior to gene activation, such as nuclear run-on experiments. The results from these experiments will provide strong evidence for the ability of yeast cells to establish a paused Pol II on promoter as a key regulatory mechanism of activated transcription during oxidative stress conditions.

APPENDIX I

Characterizing the role of TFIIA in the regulation of gene expression by RNA polymerase II

This focus of this work was directed at characterizing the role of the general transcription factor TFIIA in the regulation of gene expression by RNA polymerase II. These findings were critical in linking the transcription machinery with Yap1 and the oxidative stress response, which sparked my interest in developing a model for transcription activation during oxidative stress. Thus, they laid the ground and foundation for the subsequent finding in Chapters 2, 3, and 4. This work is the result of a fruitful collaboration with Dr. Susan Kraemer from the Stargell Lab, Ann Berger, Susan Hankey, and Sherry Rovinsky from the Pharmacia Corporation, and Dr. Scott Moye-Rowley from the University of Iowa. This collaboration resulted in the following publication in the journal of Eukaryotic Cell. My contributions to this work included the following: Figure 1B, Figure 4, Figure 5A, data analysis and construction of Figure 1A, Figure 3A, Figure 3B, Figure 3C, Figure 6, writing the majority of our findings into manuscript format, and peer reviews. Here I have attached the abstract of the publication entitled:

TFIIA plays a role in the response to oxidative stress

Kraemer, S.M., Goldstrohm, D.A., Berger, A., Hankey, S., Rovinsky, S.A., Moye-Rowley, S.W., Stargell, L.A.

TFIIA plays a role in the response to oxidative stress

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A.1 Abstract

To characterize the role of the general transcription factor TFIIA in the regulation of gene expression by RNA polymerase II, we examined the transcriptional profiles of TFIIA mutants of Saccharomyces cerevisiae using DNA microarrays. Whole-genome expression profiles were determined for three different mutants with mutations in the gene coding for the small subunit of TFIIA, TOA2. Depending on the particular mutant strain, approximately 11 to 27% of the expressed genes exhibit altered message levels. A search for common motifs in the upstream regions of the pool of genes decreased in all three mutants yielded the binding site for Yap1, the transcription factor that regulates the response to oxidative stress. Consistent with a TFIIA-Yap1 connection, the TFIIA mutants are unable to grow under conditions that require the oxidative stress response. Under-expression of Yap1-regulated genes in the TFIIA mutant strains is not the

result of decreased expression of Yap1 protein, since immunoblot analysis indicates similar amounts of Yap1 in the wild-type and mutant strains. In addition, intracellular localization studies indicate that both the wild-type and mutant strains localize Yap1 indistinguishably in response to oxidative stress. As such, the decrease in transcription of Yap1-dependent genes in the TFIIA mutant strains appears to reflect a compromised interaction between Yap1 and TFIIA. This hypothesis is supported by the observations that Yap1 and TFIIA interact both in vivo and in vitro. Taken together, these studies demonstrate a dependence of Yap1 on TFIIA function and highlight a new role for TFIIA in the cellular mechanism of defense against reactive oxygen species.

APPENDIX II

Kinetic and thermodynamic analysis of TBP-TFIIA-DNA assembly

Throughout the duration of my doctoral career, I was fortunate enough to participate in an additional collaborative effort with Sarah Williams and Dr. Larry Parkhurst from the University of Nebraska-Lincoln. This work was aimed at studying the kinetics and thermodynamics of the first steps in the assembly of the eukaryotic transcription complex formed from DNA, TBP, and TFIIA. This collaboration will result in authorship and my contribution to this work includes the following: purification of the protein TFIIA for the entire study and partial writing and reviewing of the manuscript. In addition to studies involving TFIIA, I will also purify the protein TFIIB for use in additional experiments, which will likely result in a total of 4 authorships throughout this collaboration. Here I have attached the abstract for the publication entitled:

The assembly of the preinitiation complex in eukaryotic transcription: thermodynamic and kinetic characterization

Williams, S., Goldstrohm, D.A., Delgadillo, R., Stargell, L.A., and Parkhurst, L.

The assembly of the preinitiation complex in eukaryotic transcription: thermodynamic and kinetic characterization

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A.2 Abstract

We have been studying the kinetics and thermodynamics of the first steps in the assembly of the eukaryotic transcription complex formed from TBP (TATA Binding Protein), DNA, and TFIIA (Transcription Factor IIA). The binary complexes of TBP with DNA have been well characterized using FRET probes linked to the termini of appropriate DNA oligonucleotides bound by TBP. TFIIA binds to the DNA 3-5 bases (and perhaps even further) upstream of the TATA box, and to eleven amino acids on TBP in an outer beta strand that leads to the N-terminal stirrup. That stirrup contains the F99 and F116 intercalating Phe's that kink the DNA. We have also employed oligonucleotides with a single fluorescein linked to the 3' terminus of the top strand, and very far removed from possible interactions with TFIIA for anisotropy studies. Using short, 14-mer ds oligonucleotides, 3' labeled, we find no evidence for interactions with TFIIA in the absence of TBP, thus excluding the TFIIA + DNA binary interaction from further consideration. It has been shown in the Brenowitz lab that when DNA binds to yTBP, the N-terminus of the TBP moves to a different position with respect to the

TATA box. Titrations of TBP with a 14-mer DNA labeled for FRET shows that TFIIA does not affect that binary (TBP + DNA) equilibrium, consistent with the x-ray structure for the ternary complex showing no interactions of TFIIA with such a short oligonucleotide. On the other hand, there are interactions between TFIIA and TBP, and thus the invariance of the binary equilibrium with respect to TFIIA shows that TFIIA does not interact with either the N-terminus or with the sites on TBP involved in DNA dependent interactions with the N-terminus. Fluorescence anisotropy titrations using the 14-mer oligonucleotide shows that TFIIA binds to the TBP moiety with a Kd around 1-4μM over the temperature range 15-25°C. Fluorescence anisotropy stopped-flow shows that the association proceeds with a rate constant $\approx 10^6 M^{-1}s^{-1}$. On the other hand, when a 31-mer ds oligonucleotide, 3' labeled for anisotropy measurements, is employed that contains the TFIIA binding region, the affinity increases some 100 fold. That oligonucleotide contains 12 bases upstream from the TATA box. This decrease in $\Delta G°$ of 2.7 kcal/mole must correspond to the enhancement in forming the ternary complex and derives primarily from interactions of TFIIA with the DNA upstream from the TATA box. Stopped-flow studies also show rapid binding to form the ternary complex, thus, at the concentrations of transcription factors present in the nucleus, TFIIA must be able to bind very tightly and within a few seconds to TBP bound to native TATA boxes.