DISSERTATION

TATA BINDING PROTEIN DYNAMICS WITHIN

THE CELLULAR CHROMATIN LANDSCAPE

Submitted by

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ABSTRACT

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LANDSCAPE

RNA polymerase II (RNAPII) is a twelve subunit enzyme that catalyzes messenger (mRNA) in eukaryotic organisms. A number of essential transcription factors associate with RNAPII to form the pre-initiation complex (PIC) at gene promoter regions. TATA binding protein (TBP) is one member of the transcription machinery indispensable for transcription. At some genes, the formation of the PIC correlates strongly with the transcription output (Ptashne, 2005). These genes have a low occupancy of TBP and other PIC components prior to activation. Upon activation, these factors assemble onto the promoter and transcriptional output increases. Genes that become active upon PIC formation are termed recruitment regulated because their transcription is regulated at the level of recruitment of the PIC to the promoter. While recruitment of the PIC is required for transcription, in many cases promoter-occupancy is not correlated with transcription output. Post-recruitment gene regulation has been conserved across evolution from prokaryotes to humans (Choy et al., 1997; Guenther et al., 2007). At these genes, TBP and RNAPII and other transcription-related factors occupy the promoter region regardless of whether transcription is occurring. Upon gene activation, the occupancy increases only slightly when compared to the increase in transcript level. These genes are described as being poised. At poised genes, these transcription proteins constitutively occupy the promoter region, but it is unknown if the promoter interaction is stable or dynamic. One principal objective of my work was to investigate TBP-promoter dynamics at the poised *CYC1* gene in yeast. Due to the genetic and biochemical amenability of the yeast system, studies of the transition of poised *CYC1* gene to the active form have provided key insights into the sophisticated molecular

requirements involved in this post-recruitment process. To describe the dynamics of the

transcription complex bound at the *CYC1* promoter I developed a TBP exchange assay. The

results suggest that the TBP within the RNAPII transcription complex exists in a relatively stable

configuration at the poised gene prior to activation. Upon induction, TBP-promoter dynamics

increased at the *CYC1* gene promoter. Rapid exchange during activated transcription was also

observed at other genes, including at recruitment regulated gene promoters. Overall, we found

rapid TBP-promoter exchange to be associated with active transcription. From my findings I

propose a model where frequently clearing the promoter offers a functional advantage to support

activated transcription.

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CHAPTER 1. INTRODUCTION[1]

1.1 TBP: AN ESSENTIAL COMPONENT OF THE TRANSCRIPTION MACHINERY

RNA polymerase II (RNAPII) is a twelve subunit enzyme that catalyzes messenger (mRNA) in eukaryotic organisms. A number of essential transcription factors associate with RNAPII to form the pre-initiation complex (PIC) at gene promoter regions. TATA binding protein (TBP) is one member of the transcription machinery indispensable for transcription (FIGURE 1.1). TBP is a saddle shaped protein with an underside comprised largely of hydrophobic residues. This hydrophobic patch has been highly conserved among eukaryotes, sharing nearly eighty-percent sequence identity between yeast and humans. Crystallographic studies have captured the TBP-DNA interaction and have found TBP binds to the minor groove of DNA through this highly conserved hydrophobic patch. The TBP-DNA interaction compresses the major groove causing the DNA to bend to a near ninety degree angle. This TBP-induced DNA bend has been observed when the TBP-DNA complex is in association with RNAPII and other members of the PIC. Thus, TBP is a major architectural feature contributing to pre-initiation complex structure. The region of the promoter associated with TBP is traditionally called the TATA box due to the

FIGURE 1.1 Structure of TBP and the TBP-DNA interaction. (**A**) Structure of TBP with the hydrophobic residues highlighted in red. (**B**) The TBP-DNA interaction along the hydrophobic pocket**.** (**C**) TBP contorts DNA upon binding (Jmol) (Kim et al., 1993).

consensus for the DNA sequence. Like all consensus sequences, variation exists and in actuality most gene promoters do not contain a canonical TATA sequence in the region of TBP binding (Rhee and Pugh, 2012). Thus, classes of RNAPII promoters have been assigned to more accurately describe TBP-promoter region. A single base pair change from the canonical sequence is described as TATA-like, while two or more substitutions are described as being TATA-less (Rhee and Pugh, 2012). This is a nomenclature used to describe TATA boxes across eukaryotes. In crystallographic studies, TBP-induced DNA bend was not substantially changed when TBP interacts with either canonical, TATA or non-canonical, TATA-like promoters.

1.2 THE TBP-DNA INTERACTION

In vitro, the TBP-DNA interaction is strongly influenced by the sequence of promoter DNA as well as the presence or absence of transcription factors (Hoopes et al., 1992). FRAP assays (fluorescent recovery after photobleaching) in both yeast and human cells have shown most transcription factors recover as a single fraction, but TBP has a biphasic recovery (de Graaf et al.; Sprouse et al., 2008). The unique two-component recovery observed for TBP suggests that two distinct populations of TBP exist in living cells − a dynamic and a quiescent fraction. Understanding the TBP-DNA interaction is extremely important since there are at least forty documented human diseases that arise due to single nucleotide polymorphisms (SNPs) within the "TATA box". Although the mutation occurs within the TATA box, these genes do not necessarily contain the canonical TATA sequence. Strictly speaking, these mutations alter the wildtype sequence of DNA in the region of TBP binding (Savinkova et al., 2009). In these cases, the single base mutation in this region is sufficient to alter transcription and lead to the diseased state.

Early studies in *Drosophila* (Gilmour and Lis, 1986) and more recent genome-wide analyses in both flies and humans have revealed that thousands of genes contain TBP and RNAPII at their promoters independent of transcription output (Guenther et al., 2007; Kim et al., 2005; Min et al., 2011; Muse et al., 2007). Thus, recruitment of TBP to a promoter is necessary for productive gene expression, although it is not correlated in many cases. This form of regulation is termed post-recruitment regulation, since regulation of the transcription-related proteins occurs after their recruitment to the gene. Genes that contain transcription-related proteins independent of transcription can be referred to as poised. One function of these poised promoters is to allow for rapid and synchronous activation, thereby providing the precise timing of gene expression critical for developmental processes (Saha et al., 2011; Zeitlinger et al., 2007). Indeed, post-recruitment events necessary to convert RNAPII into a productively elongating form are increasingly considered general regulatory features of transcription in higher eukaryotes (Levine, 2011; Nechaev and Adelman, 2011; Selth et al., 2010).

The *Drosophila* heat shock genes serve as an archetype for a specific form of post-recruitment regulation known as pausing [reviewed in (Adelman and Lis, 2012)]. At paused genes, RNAPII has initiated a transcript, but has paused at a promoter proximal region. The paused polymerase can then resume transcription elongation upon receiving signals for activation. The mechanism for activation of these genes involves signals from additional transcription factors. In the paused state, RNAPII complex is in association with NELF (negative elongation factor) and DSIF (DRB sensitivity inducing factor). At this stage of transcription, RNAPII is phosphorylated on serine 5 of its carboxyl-terminal domain (CTD) of the largest subunit of the enzyme, Rpb1. Upon heat shock, P-TEFb (positive transcription elongation factor b) is recruited to the paused polymerase

complex, which phosphorylates serine 2 of the CTD, NELF and DSIF through the activity of the Cdk9 kinase subunit. The phosphorylated NELF is released from the paused polymerase, which correlates with a release from transcriptional pausing. A gene is only termed "paused" if a nascent transcript has initiated and halted transcription at the promoter proximal region. This paused transcript must also be able to resume elongation upon induction. Any post-recruitment regulated gene where a paused transcription has not been identified is generally described as being poised.

In yeast cultured to stationary phase, approximately 40% of the genes in the genome show association of RNAPII in their inactive state (Radonjic et al., 2005a). These polymerases are thought to be primed for rapid and concerted activation upon transition to more opportunistic growth conditions. In actively growing yeast cultures, genome-wide studies indicate that partial but inactive pre-initiation complexes (PIC's) are a widespread phenomena across the genome (Zanton and Pugh, 2006) and a majority of bound RNAPII may be in an inactive state (Pelechano et al., 2010; Radonjic et al., 2005a). Regulation at critical post-recruitment steps in *S. cerevisiae* is supported by differences in 5' to 3' RNAPII occupancy within specific genes (Venters and Pugh, 2009), and the frequent pausing of RNAPII across gene bodies (Churchman and Weissman, 2011). In addition, accumulation of inactive RNAPII within the coding region of ribosomal protein genes (Pelechano et al., 2009) and at the promoter of the uninduced *CYC1* gene (Martens et al., 2001) provide further support for post-recruitment transcriptional regulation in yeast. Due to the genetic and biochemical amenability of the yeast system, studies of the transition of poised RNAPII to the active form have provided key insights into the sophisticated molecular requirements involved in this post-recruitment process.

1.4 THE YEAST *CYC1* GENE: A MODEL FOR POST-RECRUITMENT REGULATION VIA POISED TBP AND RNAPII

The yeast *CYC1* gene encodes iso-1-cytochrome c, a nuclear-encoded protein involved in the electron transport chain in the mitochondria (Sherman et al., 1966). In the presence of a fermentable carbon-source (such as dextrose), *CYC1* gene expression is extremely low (Guarente et al., 1984; Guarente and Mason, 1983). When cells are grown on a non-fermentable carbon-source (such as lactate or ethanol), *CYC1* is activated and transcript levels increase 10-fold. In contrast to the change in transcriptional output, the occupancy of TBP, RNAPII, and other related factors, is maintained during the carbon-source change (FIGURE 1.2) (Lee et al., 2010; Zhang et al., 2008),(Kuras and Struhl, 1999; Martens et al., 2001).

The *CYC1* promoter contains preloaded RNAPII, the general transcription factors TATA-binding protein and TFIIH, the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex, and Spn1, a highly conserved chromatin-associated transcription factor (Lee et al., 2010; Zhang et al., 2008). Intriguingly, RNAPII is Serine 5 phosphorylated on the C-terminal domain (CTD) of Rpb1 prior to activation (Zhang et al., 2008). At recruitment-regulated genes, the CTD is hypo-phosporylated prior to initiation and typically becomes Serine 5 hyper-phosphorylated during the transition from initiation to elongation (Cheng and Sharp, 2003; Komarnitsky et al., 2000). The phosphorylation of the CTD at *CYC1* prior to activation is consistent with TFIIH occupancy, since TFIIH has CTD-kinase as well as DNA unwinding activities (Feaver et al., 1991; Lu et al., 1992; Schaeffer et al., 1993; Serizawa et al., 1993; Serizawa et al., 1992). Under inducing conditions for *CYC1,* a number of new factors are recruited to the promoter including the Mediator complex, and the chromatin regulatory factors Swi/Snf and Spt6 (Lee et al., 2010).

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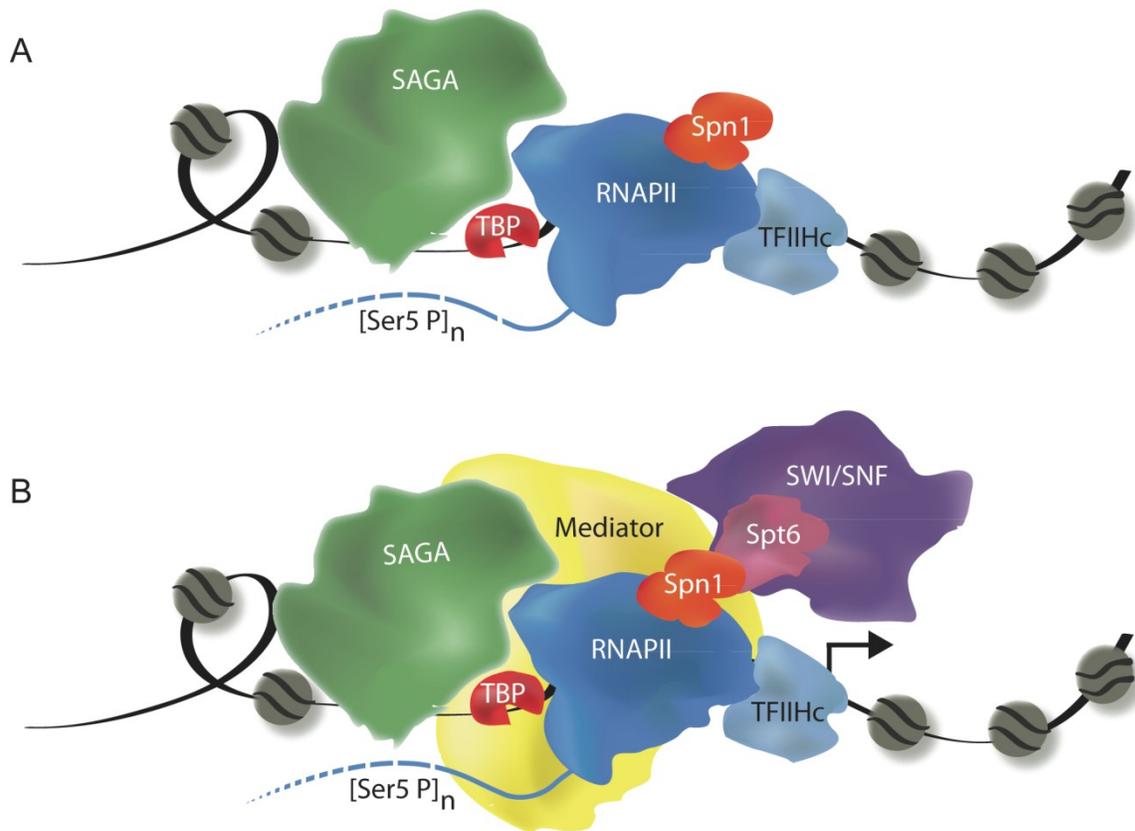
FIGURE 1.2 The poised *CYC1* promoter contains preloaded transcription components.
(**A**) Prior to activation, the preloaded *CYC1* promoter contains TATA binding protein (TBP), RNA polymerase II (RNAPII), the core TFIIH complex (TFIIHc), Spt-Ada-GCN5 acetyltransferase (SAGA) and the transcription factor Spn1. The CTD, shown by the hashed line trailing RNAPII exhibits serine 5 phosphorylation potentially on multiple repeats (denoted by 'n'). These components occupy the promoter prior to high levels of transcriptional output. (**B**) The occupancy of the preloaded factors is maintained under inducing conditions and Mediator, Spt6 and Swi/Snf are recruited, leading to an increase in transcriptional output (indicated by the arrow) image adapted from (Yearling et al., 2011).

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This poised promoter could be advantageous in the native environment, allowing for rapid induction due to changing nutritional needs (Johnston, 1999).

Activation of *CYC1* through an alternative carbon-source requires the activity of the Hap complex of activators, which is comprised of the Haps 2, Hap3, Hap4 and Hap5 proteins (Lee et al., 2013)(Forsburg and Guarente, 1989a). Three of the subunits, Haps 2, 3, and 5, contain DNA interaction domains and are required for promoter recognition. The Hap4 subunit contains the activation domain. During activated transcription, the Hap4 subunit binds to the DNA-bound Hap complex to induce transcription. The activity of the Hap complex is regulated through gene expression. The DNA binding Hap subunits (2,3 and 5) are constitutively expressed, while Hap4 is induced upon shift to an alternative carbon-source such as ethanol (De Risi *et al*., 1997). *CYC1* transcript levels accumulate over hours after the shift to an alternative carbon-source and reach peak level after 4 to 6 hours. This level of expression is maintained as long as ethanol remains the sole carbon-source.

CYC1 can also be induced in response to oxidative stress. An intricate and vital balance between pro-oxidants and reactive-oxygen species (ROS) is maintained in healthy aerobically growing cells. Upon cellular oxidative stress, this balance is disrupted and the concentration of ROS increases. Damage to proteins, nucleic acids and lipids can occur due to an overwhelming concentration of ROS in the cell (Halliwell and Gutteridge, 1984). This damage is known to lead to cancer and contribute to the aging process and related diseases (Finkel and Holbrook, 2000). Oxidative stress can arise endogenously through metabolic processes as well as through exposure to UV, chemical agents and metals (Storz and Imlay, 1999). To combat the harmful effects of oxidative stress, the cell's transcriptional program is modified. The treatment of *Saccharomyces cerevisiae* with a potent producer of ROS, hydrogen peroxide (H_2O_2), results in the activation of

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approximately 800 oxidative-stress response genes, including *CYC1* (Gasch et al., 2000; Lee et al., 2013). Many of these genes encode for various detoxifying proteins and small antioxidants molecules to cope with the increased concentration of ROS in the cell (Godon et al., 1998).

Yeast AP-1 protein (Yap1) is the master regulator of oxidative stress response genes in yeast and is a member of the basic leucine zipper family of transcription factors (Moye-Rowley, 2002). The human homolog of Yap1, AP-1, regulates oxidative stress response in human cell lines and is a proto-oncogene involved in the development of some tumors (Ikner and Shiozaki, 2005). Yap1 is a REDOX-sensitive protein whose activity is regulated mainly through its cellular localization (Delaunay et al., 2000; Kuge et al., 1997). In reducing conditions, a nuclear export sequence near the carboxyl-terminal domain of Yap1 is exposed for interaction with the nuclear export factor Crm1 (Kuge et al., 1998). This interaction maintains cytoplasmic Yap1 localization in non-oxidative conditions. Upon oxidative stress, intramolecular disulfide bond linkage between cysteine residues of Yap1 masks the nuclear export sequence. This inhibits the Yap1-Crm1 interaction and Yap1 accumulates in the nucleus (Kuge et al., 2001). The nuclear localized form of Yap1 binds to Yap1-responsive DNA elements (YREs) and activates transcription of the genes required to manage oxidative stress (Coleman et al., 1999). Chromatin immunoprecipitation assays (ChIP) have been used to demonstrate that upon treatment with H_2O_2, Yap1 is recruited to the promoter region of *CYC1 in vivo* (Lee et al., 2013). The deletion of Yap1 blocks the ability of *CYC1* to activate under oxidative stress conditions, but does not impact TBP or RNAPII occupancy at the promoter (Lee et al., 2013).

1.5 Chromatin components with critical roles in the transition to active RNAPII

DNA is organized with proteins in the eukaryotic nucleus to form chromatin. The basic repeating unit of chromatin is the nucleosome, which consists of 147 basepairs of DNA wrapped around a

protein octamer (Luger et al., 1997; Luger and Richmond, 1998). This octamer contains two copies of the four core histones, H2A, H2B, H3 and H4. To form a nucleosome, a $(H3-H4)_2$ tetramer associates with approximately 80 base pairs of DNA. The tetramer core is then capped on either side by two (H2A-H2B) dimers, which each wrap an additional 30 base pairs of DNA to create the complete nucleosome. The DNA is repeatedly wrapped around histone octamers to create nucleosomal arrays. The organization of DNA into these arrays in conjunction with DNA-associated non-histone proteins creates chromatin fibers. Histone chaperone proteins maintain chromatin architecture through their ability to facilitate nucleosome assembly and disassembly.

Assembly of DNA into chromatin is repressive to transcription, since nucleosome formation can block the recruitment of transcription proteins to gene regulatory elements. Thus, nucleosome depleted regions at promoters and upstream activator locations are often associated with activated transcription (Boyle et al., 2008; Erkina et al., 2008; Fu et al., 2008; Heintzman et al., 2007; Mavrich et al., 2008; Petesch and Lis, 2008; Schones et al., 2008; Wang et al., 2008; Zhao et al., 2005). DNA regions that lack nucleosomes can occur passively though DNA sequences that inherently disfavor nucleosome formation (Segal et al., 2006; Zhang et al., 2009). They can also occur actively though proteins that function to remove histones from DNA (for a review see (Hansen et al., 2010). Therefore, nucleosome location and occupancy serves to regulate gene expression by mediating access of the pre-initiation complex and activator proteins to gene regulatory elements.

Chromatin can also regulate transcription through post-translation modification of histone residues. A prominent example is histone acetylation, which occurs through the activity of histone acetyl transferase (HAT) enzymes. Acetylation of histone residues can regulate gene expression by functioning to recruit specific transcription regulatory proteins (Mujtaba et al.,

2007). These proteins contain bromodomains, which specifically bind and recognize acetylated histone residues. The large, multi-subunit co-activator complex, SAGA is a principle example of a bromodomain-containing transcription regulatory complex (Daniel and Grant, 2007; Grant et al., 1999; Kuo et al., 2000). Upon recruitment to gene promoters, SAGA can function to activate transcription through many avenues, including TBP deposition onto promoters (Belotserkovskaya et al., 2000; Bhaumik and Green, 2001; Dudley et al., 1999; Larschan and Winston, 2001; Qiu et al., 2005). The histone acetylation mark also functions to regulate transcription by altering the physical properties of the nucleosome (Annunziato and Hansen, 2000; Robinson et al., 2008; Shogren-Knaak et al., 2006; Tse et al., 1998). The addition of an acetyl group to a histone residue neutralizes the positive charge of the amino acid. This reduces inter-nucleosomal interaction and decondenses chromatin structure. Unfolding the chromatin fiber enhances access of the transcription-related proteins and activators. As a result, histone acetylation is widely correlated with transcription activation (Shahbazian and Grunstein, 2007).

The transition of the poised *CYC1* gene from the uninduced state to activated transcription is highly dependent on a number of chromatin regulatory factors (Lee et al., 2010; Martens et al., 2001; Zhang et al., 2008). One of these is the large multi-subunit coactivator SAGA, which can facilitate gene expression at multiple steps within the transcription cycle (Koutelou et al., 2010; Rodriguez-Navarro, 2009). Deletion of the SAGA-integrity subunits blocks SAGA complex formation in the cell (Lee et al., 2010). Under these conditions *CYC1* fails to activate (Lee et al., 2010). Thus, the poised *CYC1* promoter requires SAGA for the transition from a preloaded complex to an actively transcribing unit (Lee et al., 2010). Several well-characterized functions of SAGA are not relevant to this transition. For example, SAGA contains a TBP interaction module essential for delivering TBP to certain promoters (Belotserkovskaya et al., 2000;

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Bhaumik and Green, 2001; Dudley et al., 1999; Larschan and Winston, 2001; Qiu et al., 2005). Since the preloaded promoter has both TBP and SAGA present under non-inducing conditions, a functional connection between the two seemed likely. Surprisingly, although abolishing the SAGA complex results in loss of activated transcription, it does not alter TBP occupancy measured though chromatin immunoprecipitation assays (ChIP) (Lee et al., 2010). SAGA also has two known histone modifying enzymatic-capabilities, a histone acetyltransferase (HAT) module responsible for acetylation events involved in facilitating active transcription (Brownell et al., 1996; Grant et al., 1997; Grant et al., 1998; Utley et al., 1998) and a histone deubiquitinase (DUB) module known to aid in elongation (Daniel et al., 2004; Henry et al., 2003). Histone deubiquitination activity is not required for transcription of *CYC1*, since this gene was competent for activation in a strain containing a deletion of the SAGA DUB module. The HAT module of SAGA however, is required for activation of *CYC1* and suggests that acetylation of histone is important for *CYC1* transcriptional output.

Activation of *CYC1* also requires the activities of additional chromatin regulatory factors, including Spn1/Spt6 (Fischbeck et al., 2002; Pujari et al., 2010) complex and the Swi/Snf complex (Zhang et al., 2008). Notably, RNAPII and Spn1 occupy the poised promoter in the uninduced state, whereas Spt6 and Swi/Snf are recruited upon activation (FIGURE 1.2). Spn1 interacts with both RNAPII and Spt6 (Gavin et al., 2002; Krogan et al., 2002; Li et al., 2010; Lindstrom et al., 2003; Tarassov et al., 2008; Yoh et al., 2007; Zhang et al., 2008), thereby linking the regulation of the poised promoter to the chromatin architecture. The loss of Spn1 at *CYC1* under non-inducing conditions results in a failure to recruit Spt6 under inducing conditions (Zhang et al., 2008), and leads to an increase in basal level of transcription. Specifically at *CYC1*, the absence of promoter binding by Spn1 results in constitutive

recruitment of the Swi/Snf complex (Zhang et al., 2008). Thus, the binding of Spn1 blocks the recruitment of the Swi/Snf complex in the uninduced state, and also serves as a platform for recruiting Spt6 during the activated state. These studies highlight the importance of chromatin architecture for proper regulation of the poised *CYC1* gene.

1.6 GAPS TO BE FILLED

It is intriguing that these poised transcription complexes constitutively coexist with surrounding nucleosomes to shape the chromatin landscape into a polymerase-rich, yet transcription-deficient environment. The occupancy of the transcription machinery at the poised *CYC1* gene could be explained by two contrasting molecular mechanisms. First, it is possible the transcription complex is stably engaged, creating an inert and static landscape. It is also possible the complex is undergoing perpetual assembly and disassembly at the gene. Both could be refractory to activation, but the local environment created by these two models is fundamentally different. Determining which model accurately describes the poised gene will shape our understanding of the interplay between the poised complex and the surrounding chromatin landscape.

Our previous work highlights the importance of chromatin-related factors for proper regulation of the poised *CYC1* gene, but precisely how these activities are related to the poised RNAPII in the uninduced state remains to be investigated. Due to the constitutive occupancy of SAGA at *CYC1*, it is possible the required HAT activity occurs prior to activation at the poised, uninduced locus. However, at the majority of yeast genes, Gcn5-mediated histone acetylation is strongly correlated with activation (Pokholok et al., 2005; Reinke and Horz, 2003). Thus, it is also possible that the HAT enzymatic activity lies dormant until *CYC1* activation. Both would result in a Gcn5 requirement for activation, but have different implications for the status of the histones surrounding the poised complex.

Based on accumulating evidence it is tempting to speculate that the interplay between RNAPII and the nucleosomal architecture contributes to the inactive state of *CYC1*. In Chapter 3, we investigate this further by mapping the poised RNAPII transcription complex and the surrounding nucleosomes at *CYC1*. We also describe the dynamics of TBP within the poised transcription complex and the histones within the neighboring nucleosomes to examine the interplay between preloaded transcription machinery and local chromatin.

As previously described, numerous genetic diseases have been linked to mutations within the TATA box region, but it is unclear how these mutations impact transcriptional output to causes the related diseases(Savinkova et al., 2009). Reconstituted systems have significantly contributed to our understanding of TBP-DNA interactions. For example, a well-characterized interaction exists *in vitro* between TBP and TATA elements (Hoopes et al., 1998; Stewart and Stargell, 2001; Stewart et al., 2006). These experiments provide detailed thermodynamic analyses of TBP-DNA interactions; however, they lack many of the complex chromatin components and transcription factors present in living cells that influence TBP dynamics. To address this issue, FRAP experiments have been performed to investigate the turnover of TBP *in vivo* (de Graaf et al.; Sprouse et al., 2008). Nevertheless, FRAP experiments are limited to measure only bulk protein dynamics and cannot recognize gene-specific turnover. More recently, a method to measure gene-specific TBP dynamics *in vivo* was developed (van Werven et al., 2009). From these studies, we learned that the RNAPII-transcribed genes have the fastest TBP turnover. Furthermore, within this class of genes the TBP-promoter dynamics can vary. Although these studies provide an important advancement in our understanding, it is still unclear how DNA sequence impacts TBP-promoter dynamics and its relationship to transcriptional output. In Chapter 4, we work to address this by developing a system to observe TBP dynamics in living

cells. We then compare the TBP-promoter dynamics before and after altering the TATA sequence to determine if a relationship exists between TBP-promoter dynamics and transcriptional output *in vivo*. These studies have a broader impact since they will help us to understand how mutations in the TATA box, which can alter transcription and lead to human disease, impact TBP dynamics.

CHAPTER 2. MATERIALS AND METHODS

2.1 YEAST STRAINS

The strains used in these studies are listed in TABLE 2.1. The parental strain BY4741, (*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*) was purchased from Research Genetics, while the TAP tagged strains were purchased from Thermo Scientific. The W303a and W303α (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 phi$^+$*) strains were a gift from Tingting Yao.

For the TBP exchange assays, the ^{myc}TBP (3xmyc) construct previously created in our lab (Campbell et al., 2000) was inserted into the doxycycline-regulated (dox) vector pCM188 (Gari et al., 1997) and transformed into the BY4741 background. Briefly, the entire fragment of ^{myc}TBP including the surrounding untranslated regions was amplified through polymerase chain reaction (PCR) with Pfu Turbo. The resulting product was digested with BAMHI and NOT1, restriction enzymes compatible for insertion into the dox-regulated vector pCM188. The plasmid was sequenced to confirm that no mutations were introduced during this process.

For the histone exchange assays, the plasmids with dox-regulated ^{HA}H2B and H3^{HA} were generated from cloning as follows. The plasmids p*GAL1* ^{HA}H2B and p*GAL1* H3^{HA} (3XHA) were generously provided by M. Strubin (Jamai et al., 2007) A Not1-BamH1 DNA fragment from p*GAL1* ^{HA}H2B that encodes ^{HA}H2B was cloned into the pCM188 plasmid (ATCC, 87660) using standard techniques (Current Protocols). The H3^{HA} gene was amplified from the p*GAL1* H3(3XHA) plasmid via PCR with Pfu turbo. The primers used in the PCR were designed to incorporate a BamH1 or Eag1 site near the ends of the PCR product. After amplification, the H3^{HA} encoding DNA was cleaved with BamH1 and Eag1, and cloned into pCM188. The H3^{HA} gene in the pCM188 plasmid was sequenced to ensure no mutations were introduced during the

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TABLE 2.1 *S. cerevisiae* strains used in these studies

Strain	Genotype	Source
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
W303	MATα *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15* phi[+]	Gift from Yao lab[a]
W303	MATa *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15* phi[+]	Gift from Yao lab[a]
HHY209	W303α MATα *tor1-1 fpr1::NAT RPL13A-2xFKB12::TRP1 TBP1-FRB-GFP::kanMX6*	Euroscarf
HHY168	W303α *MATα tor1-1 fpr1::NAT RPL13A-2×FKBP12::TRP1*	Euroscarf
Rpb3-tap	BY4741 MATa *leu2Δ0 met15Δ0 ura3Δ0 Rpb3-tap::his3Δ1*	Thermo Scientific
Rpb7-tap	BY4741 MATa *leu2Δ0 met15Δ0 ura3Δ0 Rpb7-tap::his3Δ2*	Thermo Scientific
Dox mycTBP	BY4741 MATa pCM188-mycTBP::*URA3*	(Campbell et al., 2000)
Dox HA-H2B	BY4741 MATa pCM188[HA]H2B::*URA3*	
Dox H3-HA	BY4741 MATa pCM188 H3[HA]::*URA3*	
TATA TBP dynamics	HHY209 MATα *tor1-1 fpr1::NAT RPL13A-2xFKB12::TRP1 TBP1-FRB-GFP::kanMX6 pJS3801::URA3 wtTBP::LEU*	(Stewart and Stargell, 2001)
CATA TBP dynamics	HHY209 MATα *tor1-1 fpr1::NAT RPL13A-2xFKB12::TRP1 TBP1-FRB-GFP::kanMX6 pJS3803::URA3 wtTBP::LEU*	(Stewart and Stargell, 2001)

[a] These strains were a gift from Tingting Yao at Colorado State University.

PCR amplification. The tagged histones inserted into the dox vector were transformed into BY4741 *BAR1* deletion background. This deletion was required to inhibit the natural degradation of the mating pheromone alpha factor. We used alpha factor for cell-cycle arrest to examine histone dynamics independent of replication. The *BAR1* gene deletion was generated based on the established Longtine protocol (Longtine et al., 1998). In summary, HA-tagged H2B or H3 were inserted in the dox-regulated vector and transformed into BY4741 background to create the dimer (H2B) and tetramer (H3) exchange strains, respectively.

The dox-regulated vector pCM188 used for these studies was a low copy number vector. The plasmid constitutively expressed the tet transactivator fusion protein comprised of a tet binding protein connected to the Gal4 activation domain through a flexible linker region. Two tet operator sequences lie upstream of the ^{myc}TBP, ^{HA}H2B or H3^{HA} insert and drive their expression. In the absence of dox, the tet transactivator interacts with the operators and expression of the insert is induced via the Gal4 activation domain. In the presence of dox, the tet transactivator is bound by dox resulting in a conformational change in the DNA binding domain. This inhibits transactivator binding and results in rapid shut-off in expression. The tetracycline derivative doxycycline, was previously shown to be superior in its ability to inhibit and maintain the uninduced state of the pCM188 vector and was thus, chosen instead of tet for the exchange assays (Gari et al., 1997).

2.2 YEAST MEDIUM

Yeast media were made as described in Hampsey *et al.* (Hampsey, 1997). Multiple carbon-sources were used for growth at the indicated final amount: glucose (2%), ethanol (3%), galactose (2%), raffinose (2%). When indicated hydrogen peroxide concentrations would vary between 0.3mM and 1.0mM and was added to actively growing cultures in glucose containing

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medium. Dox was added to actively growing cultures to a final concentration between 1 and 2µg/mL and rap was added to a final concentration of 1 µg/mL for anchor-away experimentation.

2.3 CELL CULTURING CONDITIONS

For inducing *CYC1* via growth in an alternative carbon-source, yeast cultures were grown overnight, then diluted and allowed to undergo at least 2 cell doublings in 2% glucose-containing medium. Cells were washed three times in media lacking carbon-source and diluted into medium containing 3% ethanol or 2% galactose as the sole carbon-source. Cells were cultured at 30C, shaking for at least 4 hours before experimental analysis. For uninduced samples, cells were grown in 2% glucose media for at least 4 hours at 30C.

Induction of yeast through hydrogen peroxide (H_2O_2) treatment was performed by diluting overnight yeast cultures into 2% glucose medium. After at least two cell doublings, H_2O_2 was added at a final concentration of either 0.3 or 1.0 mM and cultures were incubated, shaking at 30C until harvested at indicated times following treatment. For uninduced samples, cultures were treated identically, but were not induced with H_2O_2.

To examine strain phenotypes, overnight cultures of yeast were diluted into medium containing 2% glucose. After at least two doublings, actively growing yeast cultures an OD_{600} of approximately 1.0 were pelleted using centrifugation. The pellets were resuspended in sterile deionized water to a final concentration equal to an OD_{600} of 1.0 in 1mL. In other words, if the OD_{600} was 0.9, the pellet was resuspended in 900uL of water. Then, 5, ten-fold serial dilutions were performed on the resuspended cultures. The cultures were then spotted in succession in 10µL aliquots onto indicated medium. Before spotting, each culture was gently vortexed to mix.

Spots were allowed to dry before turning upside-down and stored at 30C. For temperature sensitive and cold sensitive phenotypes, the cultures were immediately placed in the appropriate incubator and were not removed until images were acquired.

2.4 CHROMATIN IMMUNOPRECIPITATION ASSAYS

For chromatin immunoprecipitation assays (ChIP), 100mL aliquots of actively growing yeast cultures were placed in 500mL flasks. Fresh 37% formaldehyde was added to a final concentration of 1% and mixed thoroughly. The flasks were incubated at room temperature for 15 minutes, and were occasionally swirled. Immediately following, 2.5M glycine was added to final concentration of 125mM for 5 minutes to quench formaldehyde crosslinking. Unless stated, the remainder of the experiment was performed on ice with pre-chilled reagents and buffers to maintain crosslinking. The crosslinked cell suspension was pelleted and washed twice, once in tris buffered saline and once in FA lysis buffer (50mM Hepes pH 7.5, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 1x protease inhibitor cocktail: PMSF, benzamidine, pepstatin, leupeptin, chymostatin). The pellets were frozen at -80C overnight. The following day the pellets were resuspendend in FA lysis buffer and an equal volume of acid washed glass beads. Cells were lysed through bead beating for 1 minutes followed by 1 minute incubation on ice. This process was repeated 15 times. The lysed cells were pelleted and the supernatant discarded. The remaining pellet was washed in FA lysis buffere before sonication. The chromatin was sheared using a Branson W-350 model sonifier. Each sample was treated 10 times for 10 seconds on continuous plus at a microtip power setting of 6, with at least 1 minute ice incubation between each round. The sheared chromatin was then incubated with 5-10µL of antibody rotating overnight at 4C. The antibodies used are as follows: anti-serine5 phosphorylation Rpb1 CTD (Active Motif, #39749), anti-TBP, anti-HA (Santa Cruz, sc-7392),

anti-myc (Milipore, #05-419), anti-H2A (Active Motif #39235), anti-H2B (Active Motif, #39237), anti-H3 (Abcam, ab1791), anti-H3K9 acetylation (Millipore, #07-352) or anti-H3K14 acetylation (Millipore, #07-353) anti-FRB (Enzym Life Sciences, # AXL-215-065). For precipitation of TAP tagged proteins, antibodies were not required. For immunoprecipitation, IgG Sepharose 6 fast flow (GE, Cat#: 17-0969-01) were incubated, rotating at room temperature for three hours. The beads were washed and the eluted in buffer (50mM Tris, 10mM EDTA) containing 1% SDS at 65C for 15 minutes. The elution was collected in fresh tubes and the proteins were degraded using proteinase K enzyme. The crosslinking was reversed through overnight incubation at 65C followed by RNAase treatment at 37C for 30 minutes. The resulting DNA was purified through phenol-chloroform extraction and ethanol precipitated overnight at -20C. The purified DNA was resuspended in 100-750µL of sterile deionized water and stored at 4C during quantitative PCR analysis. Long term storage of ChIP samples was at -80C.

2.5 QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Quantitative PCR analysis of ChIP DNA was performed in a 20 to 25uL reaction volume using the CFX96 real-time PCR detection system from BioRad with SYBR super mix (BioRad). Two-step PCR reactions were performed starting with a 3 minute heat start treatment and followed with at least 30 cycles of 95C for 30 seconds and 1 minute annealing, extension reactions (TABLE 2.2). All reaction temperatures and primer concentrations were optimized for each primer set. A 10-fold serial dilution of input DNA was used for standard curve creation and all PCR reaction efficiencies were between 90-110% with a correlation coefficient of 0.98 or greater. PCR reactions displayed a single melt curve to confirm each primer set amplifies a single DNA target. Critical threshold values for each sample were quantified relative to input control, and telomere

TABLE 2.2 QUANTITATIVE PCR PRIMER SEQUENCES AND CONDITIONS

Gene	Location	Cat. #	Sequence (5' to 3')	Conditions
CYC1	*Upstream A*	STA 623	GGT GTA TGT TTT CTT TTT GCT	63C[a], 100nM[b]
		STA 624	TCA TTG TTG TCG GAA CTC AG	
	Upstream B	STA 625	TGG ATT AGG AAA CTT TGA ACG	60.5C, 100nM
		STA 626	CTT TCT GCA TTC TTT TCT GTG	
	Upstream C	STA 627	AAG GTG AAC ACA GGA AAA AAA	63C, 100nM
		STA 628	ATG TCG TCT CAC ACG GAA AT	
	Upstream D	STA 629	TCG TCG AAT ATG ATT CAG GG	63C, 100nM
		STA 630	AAC GCT CGC CAA ATG AAC T	
	UAS	STA 631	AGC GTT GGT TGG TGG ATC AA	67C, 100nM
		STA 632	AGC ACA TGC ATG CCA TAT GA	
	Promoter	STA 633	TCA TAT GGC ATG CAT GTG CT	63C, 100nM
		STA 634	CGG CCT TGA ATT CAG TCA TT	
	ORF	STA 635	AAG GCC GGT TCT GCT AAG AA	67C, 100nM
		STA 636	ACG AAT ACC CTT CAG CTT GAC	
	3' ORF	STA 637	AAA AAC GTG TTGTGG GAC GA	62C, 100nM
		STA 638	CAC AGG CTT TTT TCA AGT AGG	
RPL11a	Promoter	STA 529	TCA CAT CCA CGT GAC CAG TT	65.3C, 200nM
		STA 530	AAC TTT CGC ATA GCT GAG TGG	
TATA/ CATA	Promoter	STA 457	TCA GGA ACG CGA CCG GTG AAG A	71.4C, 100nM
		STA 364	GCA CTC AAC GAT TAG CGA CCA GCC GG	
RDN37-1	Promoter	STA 698	TAGTTAAGGCAGAGCGACAGA	60C, 100nM
		STA 699	CCTACTCGAATTCGTTTCCAA	

[a]the annealing and extension temperature for quantitative PCR
[b]final concentration of indicated primer

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normalized. All results are the average of at least three independent biological replicates and error bars represent biological variation between samples.

2.6 MESSENGER RNA ABUNDANCE

S1 nuclease assays were carried out to quantify messenger RNA abundance. Total RNA from cultures of actively growing yeast cultures were harvested using the standard hot-acid phenol extraction technique. The purified RNA samples were quantified using absorbance at 260nm and 30μg of RNA was used for S1 nuclease protection assays. DNA probes specific to mRNA transcript of interest were labeled with radioactive ^{32}P on the gamma phosphate of the 5' end of the probe. RNA was incubated overnight with labeled probe at 55C to allow hybridization of the probe to the specific target RNA (TABLE 2.3). The following morning the samples were digested with S1 nuclease at 37C for 30 minutes to degrade all single-stranded nucleic acids. Reactions were halted using EDTA and precipitated with ethanol using ssDNA and linear polyacrylamide as carrier agents. The remaining nucleic acid was then separated on a 10% sequencing gel in 1x Tris Buffered EDTA at 10mApms for 1 to 2 hours. Gels were dried at 80C for 1 hour in a vacuum seal. The gel was exposed to a phosphorimager screen overnight and visualized using Storm Molecular Imager or Typhoon FLA 9500 (GE Healthcare).

2.7 IMMUNOBLOTTING ANALYSIS

Protein extracts of actively growing yeast cultures were harvested through bead beating. Following protein extraction, 20-25μg of protein samples were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. The following antibodies were incubated with the membrane at the given dilutions: anti-TBP (1:5,000), anti-spn1 (1:10,000). Fluorescently labeled secondary goat anti-rabbit (LI-COR) antibodies at 1:15,000 dilutions were used to visualize

TABLE 2.3 S1 nuclease digestion probes used in these studies

Primer Name	mRNA Target	Probe Sequence (underlined sequence is complimentary)
STA 297	*CYC1*	5' <u>GTA GCA CCT TTC TTA GCA GAA CCG GCC TTG AAT TCA GTC ATT ATT AAT TTA GRG TGT GTA TTT GT</u>A CCG TA 3'
STA 497	*FLR1*	5' <u>GGG GCC AGT TTT GTG GGT TCT CAG GAT CAC TGG GGC CGT TCC AAT CCA CCC TGA AAG GAT C</u>TA AAA A 3'
STA 683	*GTT2*	5' <u>GTG CTC TCC CTT CCA GAG GTT GAT CCT CAC AAA TTG CAC ACT TGA TAG C</u>TA CAA G 3'
STA 656	*mycTBP*	5' <u>CGT TCC TCA TCG GCC ATA ATT AAA AAA GGA ATT CTT CCG TTC AAG TCT TCT</u> AGA CTC 3'
STA 303	*tRNA*	5' <u>GGA ATT TCC AAG ATT TAA TTG GAG TCG AAA GCT C</u>GC CTT A 3'

[a]The annealing and extent ion temperature for quantitative PCR.

[b]Final concentration of the indicated primer.

secondary goat anti-rabbit (LI-COR) antibodies at 1:15,000 dilutions were used to visualize protein bands and imaged on the Odyssey imaging system (LI-COR).

<center>2.8 TBP DYNAMICS USING THE DOX SYSTEM</center>

For the TBP exchange assays, cells were grown overnight in either 2% glucose or 3% ethanol. The following morning the cultures were diluted and allowed to reach log-phase growth before dox addition to a final concentration of 1µg/mL. Samples were removed after the indicated time in dox. The volume of the samples removed were adjusted to obtain cell weight similar to that achieved at OD=0.8-1.0. Each sample was processed for subsequent ChIP, immunoblotting and S1 nuclease protection assays as described in above sections. The decay of the ^{myc}TBP signal was observed throughout the dox time-course using quantitative PCR analysis to yield an exchange curve. The ^{myc}TBP signal for each time-point was normalized to input and the three independent biological replicates were averaged. After 5 hours in dox, no ^{myc}TBP protein was detected so the signal was used for background subtraction of the exchange curve. The sample lacking dox treatment was set to 100 and the other values of the exchange curve were expressed proportionately.

<center>2.9 HISTONE DYNAMICS USING THE DOX SYSTEM</center>

For the histone exchange assays, wildtype (in bar1Δ background) strains were cultured in glucose or galactose media to the early log phase (OD$_{600}$=0.2). To arrest cell cycle division, alpha factor was added to the media to a final concentration of 5µM. After 90-120 minutes of alpha factor treatment cell shmooing was observed under the microscope, which confirms arrest. Then, the dox was added to the media (1µg/ml in glucose, 3µg/ml in galactose) to turn off the expression of the tagged histones. During the five-hour dox treatment, cells were collected every

<center>25</center>

hour for the western blots and chromatin immunoprecipitation as described in above sections. The raw occupancy of the tagged histone at T5 is set as the background value and is subtracted from each time point. The ratio of tagged histone to the endogenous histone at each time point of doxycycline treatment was calculated. The sample lacking dox treatment was set to 100 and the other values of the exchange curve were expressed proportionately.

2.10 TBP DYNAMICS USING THE ANCHOR-AWAY SYSTEM

All strains generated for the anchor-away approach were derived from the Lamelli laboratory and purchased through Euroscarf. For TBP exchange using anchor-away, the TBP was endogenously tagged with FRB and purchased as such. Briefly, the purchased strain contains the *tor1-1* point mutation to allow for growth on rapamycin (rap). The abundant ribosomal protein gene, RPB13a was tagged with FKBP12, which binds to rap. In addition, the *FPR1* gene was deleted since it competes with FKBP12 for rap binding. To examine the TBP exchange at the TATA or CATA promoter, the pJS3801 (TATA) or pJS3803 (CATA) previously created in our lab and both *URA3* marked were transformed into the anchor-away strains, respectively (Stewart and Stargell, 2001). The pBR336 containing full length wildtype TBP expressed from endogenous promoter was used to supplement TBP^{FRB} removed from nucleus during anchor-away experimentation.

Actively growing cultures in 2% galactose were allowed to reach an approximate OD_{600} of 0.7 and one aliquot was removed for ChIP analysis. Then, rap was added to a final concentration of 1µg/mL, and the cultures were allowed to incubate at 30C, shaking. Samples were removed at the indicated time points for ChIP experimentation. The loss of the FRB-tagged TBP molecule at promoter regions was observed throughout a rap timecourse using qPCR. The TBP^{FRB} signal for each time-point was normalized to input and the three independent biological replicates were

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averaged. The assay was stopped after 15 minutes in rap since the tagged TBP were unchanged at later time-points. Each biological replicate was normalized to the average loss of telomere signal. The sample lacking rap treatment was set to 100 and the other values of the exchange curve were expressed proportionately.

2.11 CHROMATIN MAPPING VIA MNASE AND INDIRECT ENDLABELING

The preparation of spheroplast, micrococcal nuclease (MNase) digestion, purification of genomic DNA, and detection of products by indirect end-labeling were carried out as described in Reese *et al.* with a few exceptions (Zheng et al., 1995). Actively growing yeast cultures were crosslinked for 15 minutes in 1% formaldehyde, quenched for 5 minutes in 125mM glycine. MNase digestion (0, 20, 160, 320 units) was carried out for 30 minutes at 37C. DNA was harvested using standard phenol extraction methods and ethanol precipitated. RNA was digested using RNAase and DNA was purified again using phenol extraction followed by ethanol precipitation. The extent of MNase digestion was analyzed by separation on a 1.5% agarose gel and visualized through ethidium bromide staining.

For detection of *CYC1* digestion products, MNase digested DNA was also digested with HindIII (downstream probe) or SalI (upstream probe) restriction enzymes. The probes were created by PCR of genomic DNA and anneal near their respective restriction cut-site. For the markers, purified genomic DNA was digested with Hind III (+677), EcoRI (+78), NdeI (-78), SmaI (UAS) or SalI (-1011). All digestion products were ethanol precipitated and resuspendend in 10 to 15µL of 1mM Tris-HCL and 0.1mM EDTA. Detection of *CYC1* digestion products was carried out as described in (Cavalli and Thoma, 1993). The digestion samples were run on a 1.5% agarose gel prepared in Tris-buffered EDTA and run at 5.5V/cm for at least 4 hours. The gel was then cut 2cm below the bromophenol blue dye and along the wells. To aid in the transfer of the larger

DNA fragments, the gel was soaked in 0.2 M HCl for 10 min with gentle shaking. Briefly, the gel was rinsed with deionized water and transfer to denaturing buffer (1.5M NaCl, 0.5M NaOH), and incubated for 45 min with gentle shaking before washing again in deionized water. The gel was transfered to renaturing buffer (1.5M NaCl, 1M Tris-HCL pH 7.4) and incubated for 45 minutes with gentle shaking (the renaturing buffer was replaced after 20 minutes). The DNA was transferred from the agarose gel to the Nylon membrane (Gene Screen) using capillary transfer in 10x SSC (1.5M NaCl, 150mM Sodium Citrate). The DNA was fixed to the membrane using ultra-violet light exposure for 5 minutes while the membrane was still wet. The membrane was dried and placed in a glass tube for prehybridization in 10mL of 2x SSC for at least 1 hour at 65C. The radiolabled probed was created using a random labeling kit (Takara) following kit instructions. The entire volume of probe was added to the glass tube containing the membrane and incubated, rotating overnight at 65C. The following morning, the membrane was washed twice in blot washing buffer (2xSSC, 0.1%SDS) for 15 minutes at 65C. Then, the membrane was placed in a sealed container and washed twice more in blot washing buffer before exposing to phosphorimager screen overnight. Images were acquired using Typhon FLA 9000 (GE Healthcare) and quantified using imagequant. The well-positioned nucleosomes and hypersensitive cut-sites at *CYC1* were observed on three independent blots from two separate biological replicates.

2.12 GFP MICROGRAPHS

Exponentially growing cultures were treated with rapamycin to a final concentration of 1µg/mL. At the indicated time after treatment, cells were collected by centrifugation to completely remove supernatant. The cells were resuspended in approximately 30µL of sterile deionized water and fixed by adding 1mL of 100% methanol at -20C for 6 minutes. The cells were then collected by

centrifugation and rehydrated in 1mL phosphate buffered saline containing 0.2% Tween 20 and 20ng/mL of DAPI and incubated for 5 minutes at room temperature. After collecting the cells through centrifugation, cells were immobilized by adding 1% agarose to a final concentration of 0.5%. to a glass slide and cover with a coverslip. Fluorescent microscopy was performed using an Olympus IC81 spinning-disk confocal microscope with Photometrics Cascade II camera (Tucson, AZ) and a 100x/1.40 numerical aperture objective.

CHAPTER 3. A STABLY POISED TRANSCRIPTION COMPLEX MARKS A BOUNDARY FOR HISTONE DYNAMICS[2]

3.1 INTRODUCTION

Transcription of messenger RNA by RNA polymerase II is a highly regulated process that requires a coordinated effort from a host of factors. Recruitment of these factors is a necessary requirement for activation. However, it is oftentimes not correlated with transcription. These genes can be described as being poised. In yeast, the extent to which poising occurs as a regulatory scheme is variable. When cultured to stationary phase, approximately 40% of the genes in the genome contain RNAPII, but lack transcriptional output (Radonjic et al., 2005b). Others have shown partial preinitiation complex formation at inactive genes is a common feature of actively growing cultures (Zanton and Pugh, 2006).

Poising shapes the chromatin landscape into a polymerase-rich, yet transcription deficient environment. Investigating chromatin at an uninduced gene containing transcription machinery is distinct from the majority of studies, which focus on chromatin in the context of repressed or activated transcription. Since poising is prominent gene regulatory feature (Adelman and Lis, 2012), it's important to further our understanding of the relationship between transcription machinery and chromatin at poised genes. Specifically, how does the transcription machinery at poised genes coexist with the local histones? Is there a dynamic exchange of the transcription

proteins and histones from the gene or are they relatively stable? Distinguishing between these two models will shape our understanding of the interplay between the transcription complex and the surrounding chromatin at poised genes.

One example of poising in yeast is at the *CYC1* (iso-1-cytochrome C) gene (Lee et al., 2010; Martens et al., 2001; Zhang et al., 2008). This gene has low levels of transcription when grown in the presence of a fermentable carbon-source such as dextrose (Guarente, 1983, 1984). When grown on an alternative carbon-source, such as galactose or ethanol, *CYC1* is activated resulting in a significant fold change in transcript levels (Guarente, 1983, 1984; Lai et al., 2008). In contrast to the significant increase in transcription output upon induction, the promoter occupancy of RNAPII and TBP (among others) is unchanged between the two conditions (Lee et al., 2010; Martens et al., 2001; Zhang et al., 2008). This finding is a hallmark of genes regulated in a post-recruitment manner since poised genes possess constitutive occupancy of TBP, RNAPII and other related transcription proteins independent of transcription output [reviewed in (Adelman and Lis, 2012)].

The occupancy of the transcription machinery at the poised *CYC1* gene could occur by two contrasting molecular mechanisms. First, it is possible that the transcription complex is stably engaged, creating an inert and static landscape. It is also possible the complex is undergoing perpetual assembly and disassembly at the gene. Both could be refractory to activation, but the local environment created by these two models is fundamentally different. Through exchange assays we measured the dynamics of TBP within the transcription complex at the poised *CYC1* gene.

To examine the interplay between preloaded transcription machinery and chromatin, we mapped the RNAPII transcription complex and the surrounding nucleosomes at the *CYC1* gene containing transcription machinery, but lacking activated transcription. We also examined the dynamics of TBP within the transcription complex at the poised gene and the histones within the neighboring nucleosomes. We found the preloaded transcription machinery at the promoter to be stably poised within an open chromatin landscape. This stably poised complex lies within the boundary between two nucleosomes with contrasting histone exchange patterns.

3.2 RESULTS

RNAPII occupies the poised *CYC1* promoter and ORF independent of transcription output. To examine the interplay between an inactive transcription complex and the local chromatin environment we investigated *CYC1* (iso-1-cytochrome C), which contains TBP, RNAPII and other transcription components at the promoter prior to transcription activation (Lee et al., 2010; Martens et al., 2001; Zhang et al., 2008). RNAPII occupancy was mapped across the *CYC1* locus using chromatin immunoprecipitation (ChIP) assays. Since RNAPII is a 12 subunit enzyme consisting of a 10-subunit core and a 2-subunit dissociable heterodimer (Armache et al., 2003; Bushnell and Kornberg, 2003), we investigated formation of these two components at *CYC1*. For this, Rpb3, a member of the core enzyme, and Rpb7, a subunit within the dissociable heterodimer, were chosen. We found that in the uninduced condition, both Rpb3 and Rpb7 occupy *CYC1* with a significant enrichment of RNAPII along the core-promoter and ORF when compared to the UAS (FIGURE. 3.1A and B). Upon induction, the levels of RNAPII are unchanged despite a significant increase in transcription output (Lee et al., 2010). These observations at *CYC1* are characteristic of a post-recruitment regulatory scheme.

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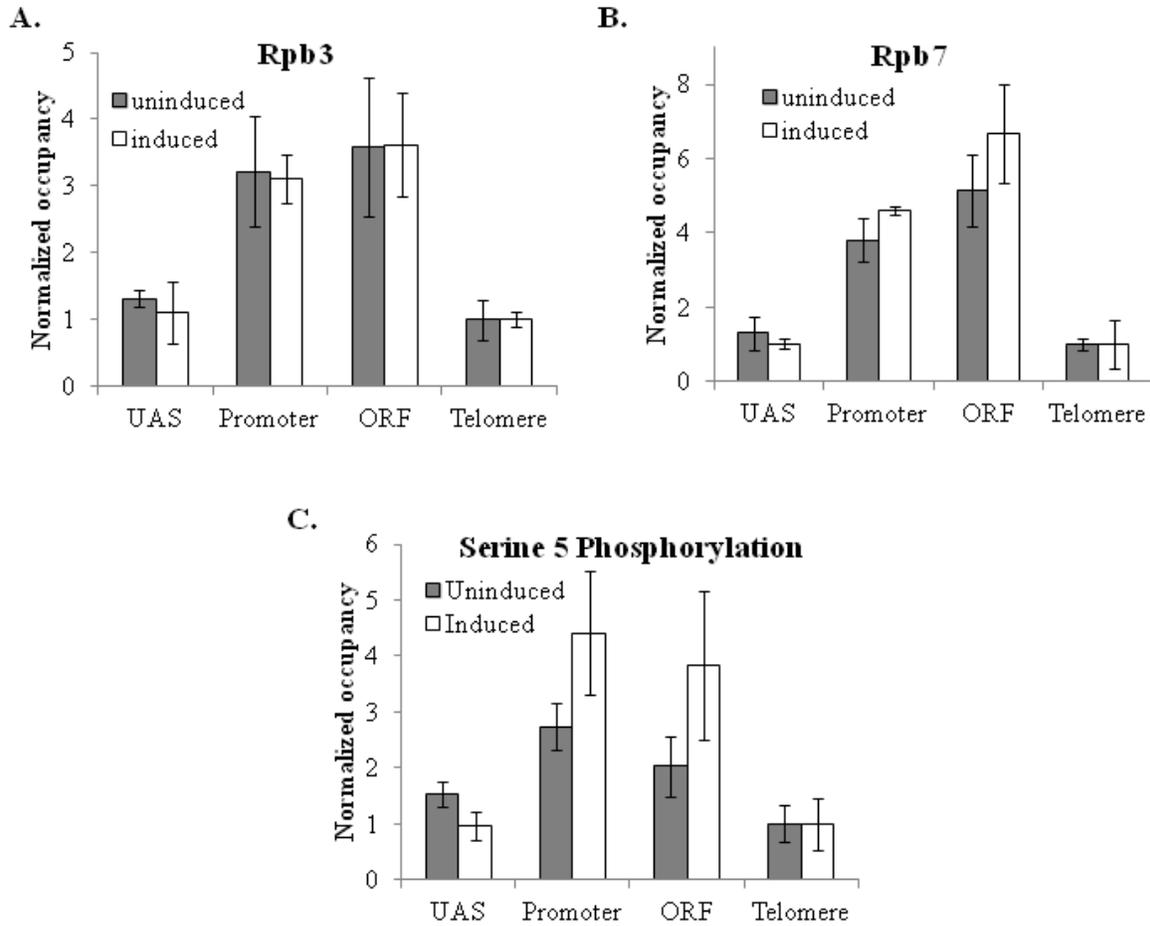
FIGURE 3.1. The Rpb3 and Rpb7 subunits of RNAPII occupy the *CYC1* locus independent of activation. Occupancy of Rpb3 (**A**), Rpb7 (**B**) and serine 5 phosphorylation of the CTD of Rpb1 (**C**) across the *CYC1* locus in the uninduced (grey bars) and induced conditions (white bars) using ChIP assays. The location of the region of *CYC1* probed is indicated along the x-axis. The occupancy is normalized to the telomere and error bars represent the standard deviation of three independent biological replicates. Telomere is graphed to show enrichment of signal above background. RNAPII occupies the promoter prior to activation to a degree sufficient for activated transcription output.

To investigate the status of preloaded RNAPII, we measured serine 5 phosphorylation of the Rpb1 carboxyl terminal domain, a feature of transcription initiation [reviewed in (Phatnani and Greenleaf, 2006)]. Prior to activation, serine 5 phosphorylation was observed with significant enrichment at the core-promoter and ORF region (FIGURE. 3.1C). Upon activation, the level of serine 5 phosphorylation increased slightly along these regions. Since RNAPII occupancy does not increase upon induction, these results suggest that serine 5 phosphorylation is not completely saturated prior to activation.

TBP within the preloaded transcription complex is stably bound at the poised *CYC1* promoter. To describe TBP-promoter dynamics within the preloaded transcription complex, we developed a TBP exchange assay. TBP was chosen for these studies since it exists as a single subunit and is an essential transcription component highly conserved among eukaryotes (Kojima et al., 1995; Sprouse et al., 2008). TBP is preloaded at the promoter of *CYC1* prior to activation and the levels of TBP do not increase upon induction, a result comparable to our observations of RNAPII occupancy at the promoter (Lee et al., 2010)

For the exchange assay, we monitored the loss of tagged [myc]TBP through ChIP assays following shut-off of [myc]TBP expression. This was performed in the presence of an endogenously expressed untagged TBP. The [myc]TBP molecule was expressed from a doxycycline (dox)-repressible promoter to allow for [myc]TBP conditional shut-off (Gari et al., 1997). A similar approach has also been used to examine TBP dynamics *in vivo* by a galactose inducible system (van Werven et al., 2009). However, the galactose system could not measure TBP dynamics at the poised, uninduced *CYC1* promoter since under galactose conditions *CYC1* is activated (FIGURE 3.2A) (Lai et al., 2008). In this system, we confirmed that *CYC1* expression is
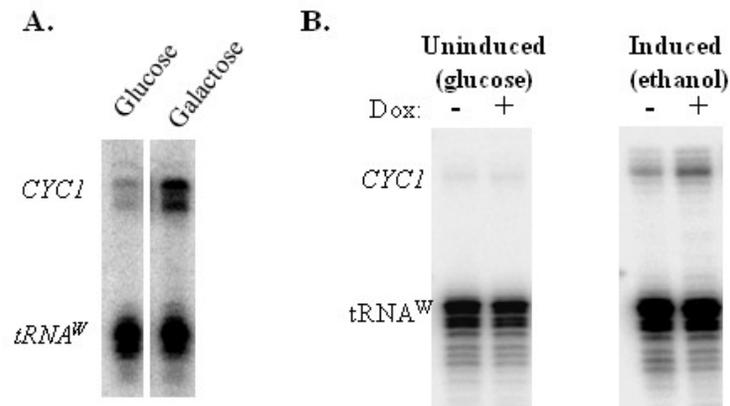
Figure 3.2. Validation of the dox-regulated expression system to measure TBP dynamics in the uninduced and induced conditions. (**A**) *CYC1* transcript levels in medium containing glucose or continuous culture in galactose shown using S1 nuclease protection assays. Galactose-containing medium activates *CYC1*. (**B**) *CYC1* transcript levels before and after dox addition (1 hour) in the uninduced (glucose) and induced (continuous culture in ethanol) conditions. Dox does not impact *CYC1* transcript levels and allows analysis of TBP dynamics in both the uninduced and induced conditions. For both A and B, the *tRNA^W* intron serves as a loading control.

independent of dox, which allows TBP dynamics to be measured in the uninduced and induced conditions (FIGURE 3.2B).

To monitor dox control of ^{myc}TBP expression, we examined ^{myc}TBP levels before and after dox repression. As anticipated, prior to dox addition ^{myc}TBP was expressed in the cell and following dox addition ^{myc}TBP protein levels in the cell declined (FIGURE 3.3A and B). Concomitantly, when ^{myc}TBP was expressed (in the absence of dox) we observed ^{myc}TBP-promoter occupancy (FIGURE 3.3C). Conversely, following dox addition, ^{myc}TBP was lost from promoter regions (FIGURE 3.3C). The time required to replace ^{myc}TBP with untagged TBP at promoter regions can thus be related to the exchange of the promoter-bound TBP.

We also examined total TBP-promoter occupancy through ChIP assays and found the levels to be independent of dox (FIGURE 3.3C). This indicates that ^{myc}TBP and untagged TBP compete for promoter binding. It also shows that the maximum occupancy of TBP at the promoter is unaffected by the level of TBP expressed in the cell. Consistent with this, we did not observe any mutant phenotypes for this strain (FIGURE 3.4).

To investigate TBP dynamics at the poised promoter we compared the time ^{myc}TBP resides at the uninduced promoter to the residency time at the induced *CYC1* promoter (FIGURE 3.5A). We found that the exchange of TBP at the poised promoter is slower than when the *CYC1* gene is induced and actively transcribing. In contrast, the constitutively active *RPL11a* gene, (Knijnenburg et al., 2009) TBP-promoter exchange is unchanged between the two conditions and mirrors the loss of ^{myc}TBP protein levels in the cell (FIGURE 3.5B). These observations place the poised promoter in the uninduced condition in a class with slow TBP dynamics.

A. mycTBP protein levels

Time (hours) in dox

0   1   2   3   4

Spn1

mycTBP
TBP

B. mycTBP transcript levels

Dox   −   +

mycTBP

tRNA^W

C. Promoter occupancy

□ − dox   ■ + dox
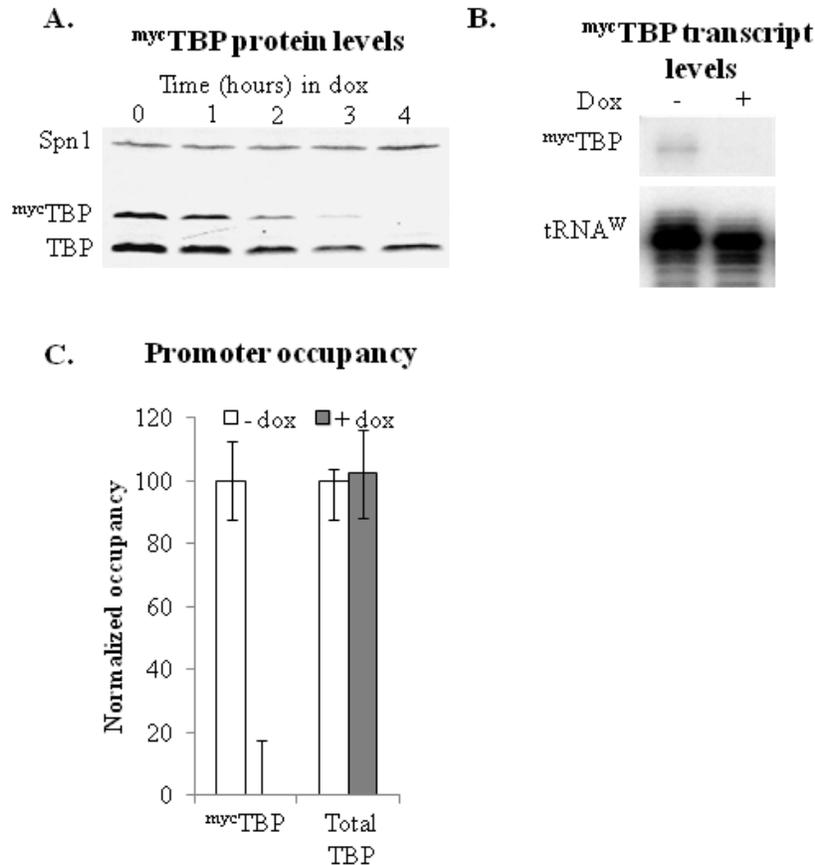
FIGURE 3.3. Dox shut-off of ^{myc}TBP reduces protein levels and results in a loss of promoter bound ^{myc}TBP. (**A**) Protein extracts harvested before and every hour following dox addition up to four hours. Samples were visualized on an immunoblot probed for TBP and the transcription factor Spn1, which was used as a loading control. The TBP antibody recognizes total TBP (untagged TBP and ^{myc}TBP). The ^{myc}TBP protein levels decline following dox addition. (**B**) The ^{myc}TBP transcript levels shown using S1 nuclease protection assays before (−) and one hour after (+) dox addition to demonstrate the rapid repression of ^{myc}TBP following dox addition. The tRNA^W intron was used a loading control. (**C**) ChIP assays using either anti-myc or anti-TBP to show ^{myc}TBP and total TBP occupancy, respectively. Samples were taken before (grey bars) and 5 hours after (white bars) dox addition for three independent biological replicates. Error bars represent biological variation. The occupancy of ^{myc}TBP is dependent on dox, while total TBP is independent of dox addition.
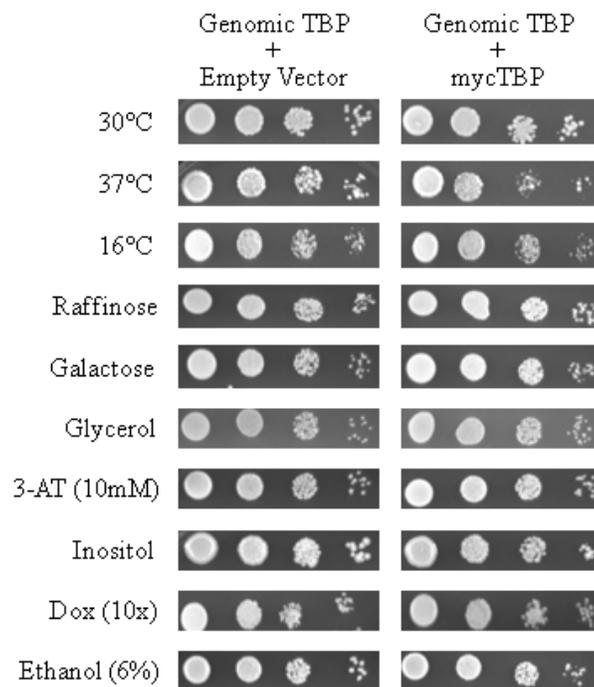
FIGURE 3.4. There are no mutant phenotypes observed for the TBP-promoter exchange strain. Growth of a strain containing genomic TBP and an empty vector compared to a strain with genomic DNA and ^{myc}TBP (TBP-promoter dynamics strain) on indicated medium. No mutant phenotypes were observed for the merodiploid.

A.

B.
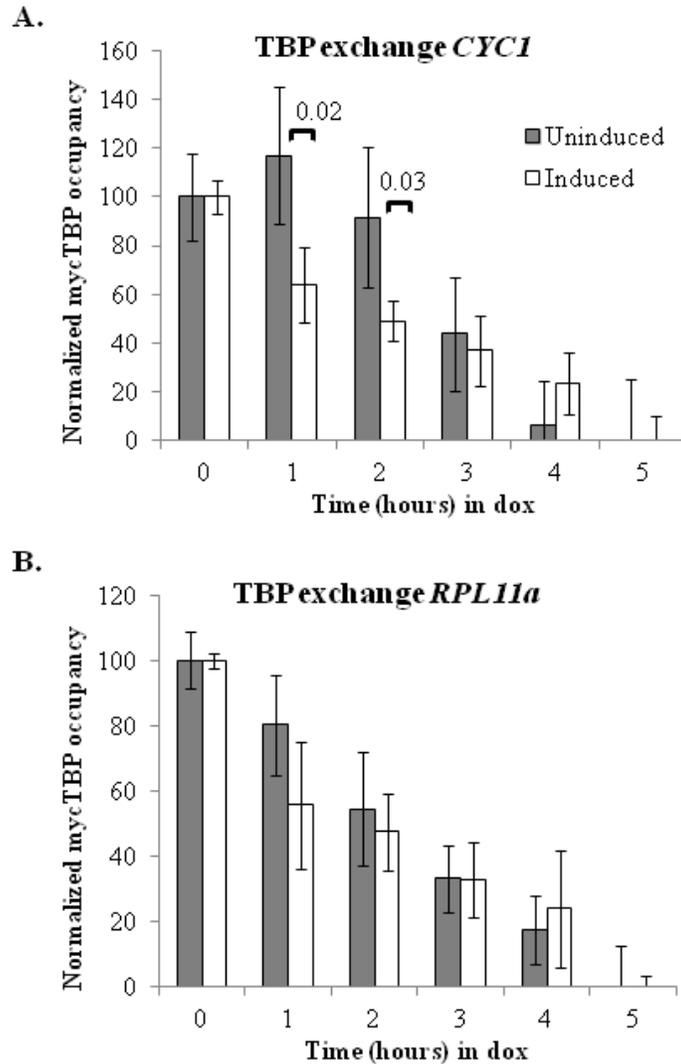
FIGURE 3.5. At the poised, uninduced promoter TBP dynamics are slower than when *CYC1* **is induced and actively transcribing**. (**A**) The occupancy of ^{myc}TBP at *CYC1* in the uninduced (grey) and induced (white) conditions before and every hour after dox addition shown using ChIP assays (**B**) At *RPL11a*, the loss of ^{myc}TBP-promoter occupancy is unchanged between the two conditions. Shown is the average of three independent biological replicates. Error bars represent variation among biological replicates. The p-values calculated using the student T-test are indicated above.

The previous report using a gal-inducible system to investigate TBP exchange also observed slow TBP turnover at Pol I promoters when compared to the other genomic locations (van Werven et al., 2009). We examined the TBP dynamics at the Pol I transcribed 35S locus and confirmed that our system is in agreement with their findings (FIGURE 3.6). It is important to note that slow TBP exchange was not observed at the *CYC1* promoter in the earlier work since *CYC1* was induced under the galactose conditions used for the assay (FIGURE 3.2A). Thus, at the uninduced *CYC1* promoter, we find slow TBP exchange similar to the Pol I gene promoters. This suggests that the TBP within the RNAPII transcription complex exists in a relatively stable configuration at the poised gene prior to activation.

The occupancy of the local histones is maintained upon activation and histone H3 is acetylated prior to activation. After describing the dynamics of TBP we addressed the interplay between the transcription complex and the local chromatin environment at the poised gene. To do this, we measured the histone occupancy for both dimer and tetramer at the *CYC1* locus (FIGURE 3.7A and B). The level of histones bound at *CYC1* in the uninduced condition was comparable to that observed at the nucleosome-rich telomere region. Thus, prior to induction histone occupancy levels at *CYC1* are relatively high. Upon induction, we observed a minimal change in this occupancy. This was somewhat unexpected since histone loss during activation has been a well-document occurrence at many yeast genes (Bernstein et al., 2004; Boeger et al., 2003; Lee et al., 2004; Pokholok et al., 2005; Reinke and Horz, 2003). Indeed, our own observations at the galactose-inducible genes show a substantial change in histone occupancy upon activation (Andrews et al., 2010). These results suggest that transcriptional activation of *CYC1* does not involve nucleosome depletion.
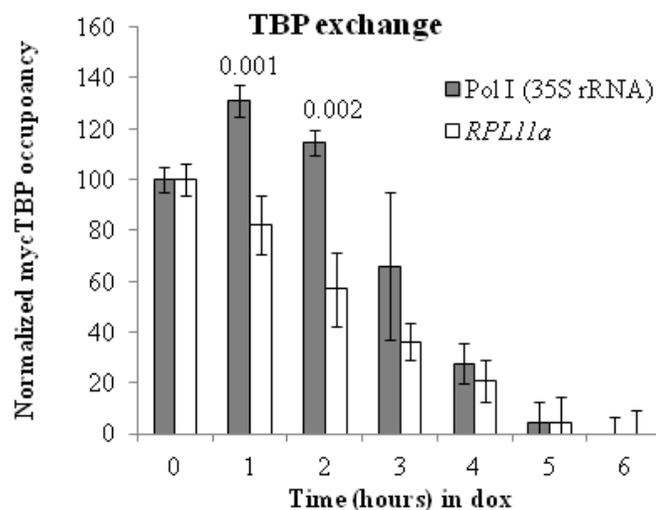
FIGURE 3.6. TBP dynamics at the Pol I gene promoter is slow compared to the Pol II gene, _RPL11a_. The occupancy of ᵐʸᶜTBP at Pol I gene promoter (grey) and _RPL11a_ (white) in glucose-rich conditions before and every hour after dox addition using ChIP assays. Shown is the average of three independent biological replicates. Error bars represent variation among biological replicates. The p-values calculated using the student T-test are indicated above for all significant timepoints.

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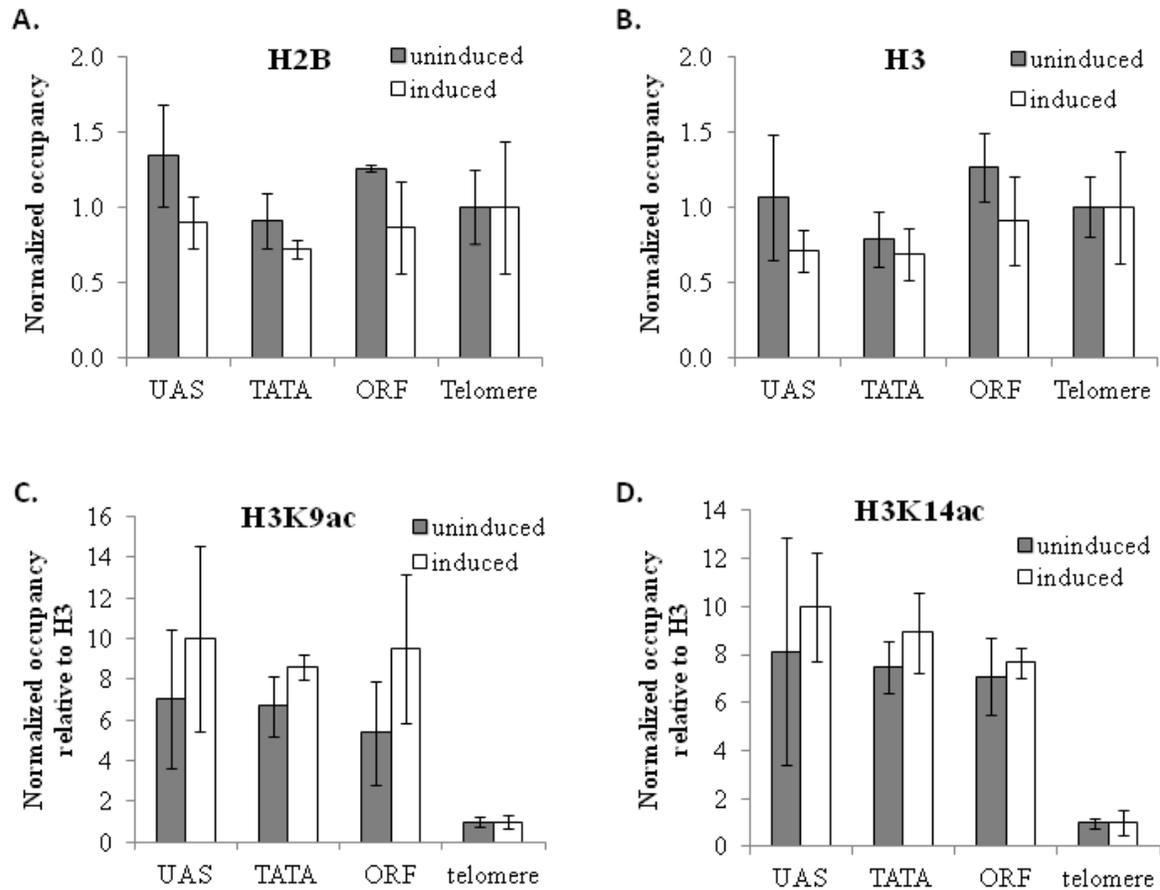
FIGURE 3.7. Histones are pre-acetylated prior to activation and histone occupancy at *CYC1* is maintained upon induction. The histone occupancy across *CYC1* locus using ChIP assays in the uninduced (grey) and induced (white) conditions. H2B shows dimer occupancy levels (**A**), while H3 shows tetramer levels (**B**). Despite a 10-fold increase in transcription output upon induction, the fold change in histone occupancy is minimal. The H3K9 (**C**) and K14 acetylation (**D**) levels along the *CYC1* locus using ChIP assays in the uninduced (grey) and induced (white) conditions. For all data, the average of three independent biological replicates is shown, which were normalized to telomere. For histone acetylation the values are expressed relative to total H3 levels. Telomere is graphed to show occupancy relative to another genomic location. Error bars represent variation among biological replicates.

Prior to activation, the large, multisubunit coactivator SAGA (Spt/Ada/Gcn5 acetyltransferase) is localized to the *CYC1* promoter (Lee et al., 2010; Zhang et al., 2008). The SAGA complex can acetylate histones through the action of the Gcn5 histone acetyl transferase enzyme (Utley et al., 1998; Xue et al., 2013; Zhang et al., 1998), which we have shown to be essential for activation of *CYC1* (Lee et al., 2010). To determine the histone acetylation status at *CYC1*, we examined H3K9ac and K14ac. In the uninduced condition, we observed enrichment of these marks (FIGURE 3.7C and D). We also measured H3K9 and K14 acetylation at the induced and actively transcribing locus and found the acetylation status to be unchanged from the uninduced conditions (FIGURE 3.7C and D). Most genes in yeast have been shown to link histone acetylation to activated transcription (Pokholok et al., 2002; Reinke and Horz, 2003). At *CYC1* however, it appears this process has been uncoupled. Together, these data suggest the histones are primed for activation by pre-acetylation of H3. In addition, an event leading to a change in histone occupancy is not required for activation.

The *CYC1* promoter is surrounded by two positioned nucleosomes in an area highly sensitive to MNase digestion. To characterize the translational positioning of the nucleosomes we used MNase digestion followed by indirect end labeling analysis. Using a probe at the downstream region of *CYC1* we observed protection the size of a nucleosome along the ORF and a large hypersensitive cut-site spanning the core-promoter (FIGURE 3.8A and B). From this digestion pattern we place a nucleosome along the ORF of *CYC1* in the uninduced condition. It was surprising that protection was not observed farther upstream of the ORF since at this low concentration of MNase the genomic DNA exhibits a ladder extending at least 6 nucleosome repeats (FIGURE 3.9). To confirm the presence of a nucleosome ladder we analyzed the *GAL1* gene. Indeed, a ladder extending at least 6 nucleosome repeats was observed and our results are
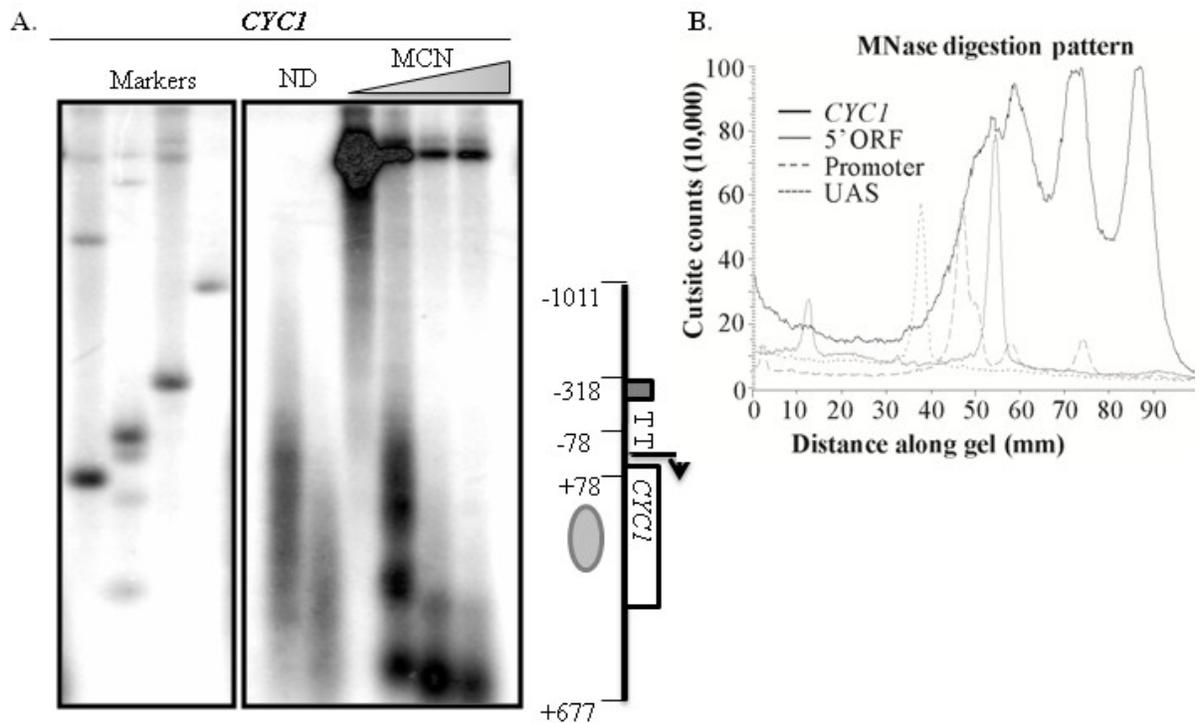
FIGURE 3.8. The poised *CYC1* promoter exists in an open conformation surrounded by two positioned nucleosome prior to activation. (**A**) Chromatin samples were digested with increasing concentration of MNase (MCN) and visualized using indirect endlabling. Restriction enzyme digestion makers map the blot to specific regions of importance along *CYC1*. DNA lacking protein and digested with two concentrations of MNase is also presented (ND). The blot shows a nucleosome-sized protection site along the ORF and a large hypersensitive cut-site spanning the *CYC1* promoter region. The schematic of *CYC1* corresponding to the blot is shown to the right. Included in the schematic is the location of the ORF (white box), transcription start site (arrow), TATA boxes (T) and the upstream activating sequence (grey box). The location of the positioned nucleosome along the ORF is also mapped (grey circle). (**B**) *CYC1* MNase digestion pattern (black line) showing counts relative to the distance from the from top (larges fragments) to bottom (smallest) of the gel. The restriction enzyme digestion sites marking 5' ORF (grey dotted line), promoter (grey hashed line) and the UAS (solid grey line) are overlaid. The peaks indicate cut-sites while the valleys show regions of protection from MNase digestion along. The graph shows the extent of the large hypersensitive cut-site spanning the promoter region as well as the positioned nucleosome along the ORF.

Genome MNase digestion pattern
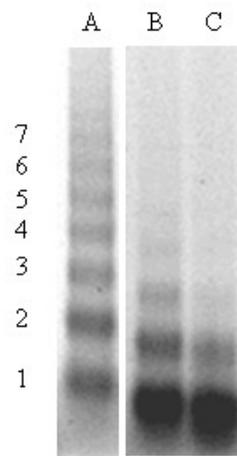
FIGURE 3.9. Genomic MNase digestion pattern. Increasing levels of MNase digested chromatin (A, B, C: 20, 160, 320 U), which was used to probe *CYC1* separated on a 1.5% agarose gel and stained with ethidium bromide. At the low level of MNase treatment a ladder extending at least 6 nucleosome repeats can be observed (1-7). At this concentration of MNase, the *CYC1* locus was considerably more digested, suggesting an open chromatin conformation at *CYC1* when compared to the genome.

consistent with previous findings at *GAL1* (FIGURE 3.10A and B) (Cavalli and Thoma, 1993). This indicates that *CYC1* is substantially more accessible to MNase digestion than *GAL1*, though both genes are transcriptionally inactive under these conditions.

To test whether a nucleosome exists upstream of the hypersensitive cut-site, we used a probe upstream of the *CYC1* locus. From the opposite direction, protection consistent with a positioned nucleosome at the UAS was observed (FIGURE 3.11 A and B). Again, a large hypersensitive cut-site spanning the promoter region was also detected. Thus, from the histone occupancy and chromatin mapping studies it appears that the *CYC1* promoter is highly sensitive to MNase digestion and is surrounded by two positioned nucleosomes.

Histones surrounding the poised promoter have contrasting dynamics. We established the exchange system to measure histone dynamics using an experimental design similar to the TBP exchange assays (FIGURE 3.5). A strain with tagged histone H2B was used to monitor dimer exchange while a strain with tagged histone H3 was used to monitor tetramer exchange. In both strains, untagged histones were endogenously expressed and the dox-regulated vector system was used to shut-off expression of the tagged histone (Chen et al., 2013).

We used the *GAL1* ORF as a reference region and found significant differences in exchange patterns between histones in the uninduced and induced conditions (FIGURE 3.12A and B). To examine histone dynamics at *CYC1*, we compared the exchange profiles in the uninduced and induced conditions at each nucleosome location, which provides information about the relationship between histone dynamics and transcription output. At the UAS nucleosome we observed an increase in dimer exchange upon induction (FIGURE 3.13A). We observed the same trend for tetramer exchange (FIGURE 3.13B). This suggests that the UAS nucleosome becomes

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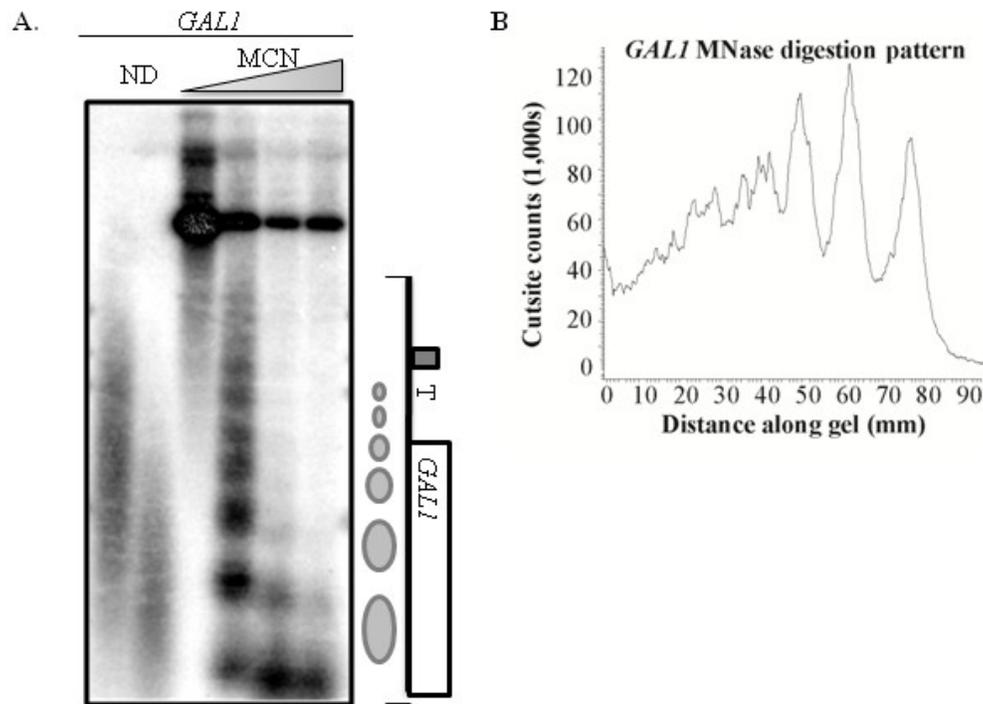
FIGURE 3.10. *GAL1* exhibits a nucleosome ladder. (**A**) The same blot shown for *CYC1* (Figure 9) was reanalyzed using indirect endlabling with a probe specific for *GAL1*. DNA lacking protein and digested with two concentrations of MNase (MCN) is also presented (ND). The schematic of *GAL1* corresponding to the blot is shown to the right including the location of the ORF (white box), TATA box (T) and the upstream activating sequence (grey box). The locations of the positioned nucleosomes along the ORF are also mapped (grey circle). (**B**) MNase digestion pattern (black line) showing counts relative to the distance from the from top (larges fragments) to bottom (smallest) of the gel. The peaks indicate cut-sites while the valleys show regions of protection from MNase digestion along *CYC1* locus.

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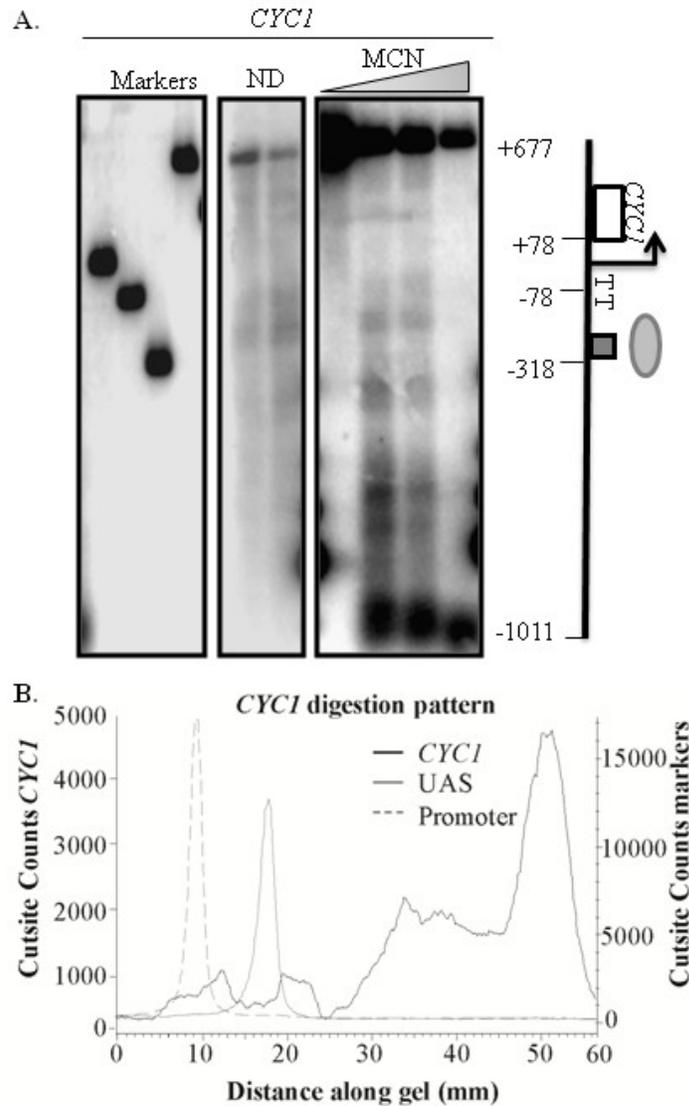
FIGURE 3.11. A positioned nucleosome spans the upstream promoter region of *CYC1*. (A) Chromatin samples were digested using with increasing concentration of MNase (MCN) and visualized using indirect endlabling at *CYC1*. Figure description is identical to **FIGURE 8A** except the probe used against *CYC1* is upstream of the UAS region. (**B**) Quantification of MNase blot visualized using *CYC1* upstream probe (black line). The UAS (marked by restriction enzyme digestion and shown as grey line) is surrounded by two hypersensitive cut-sites (peaks) and spans a region protected from MNase digestion (valley). The protection pattern along the UAS is consistent with a nucleosome at the UAS.
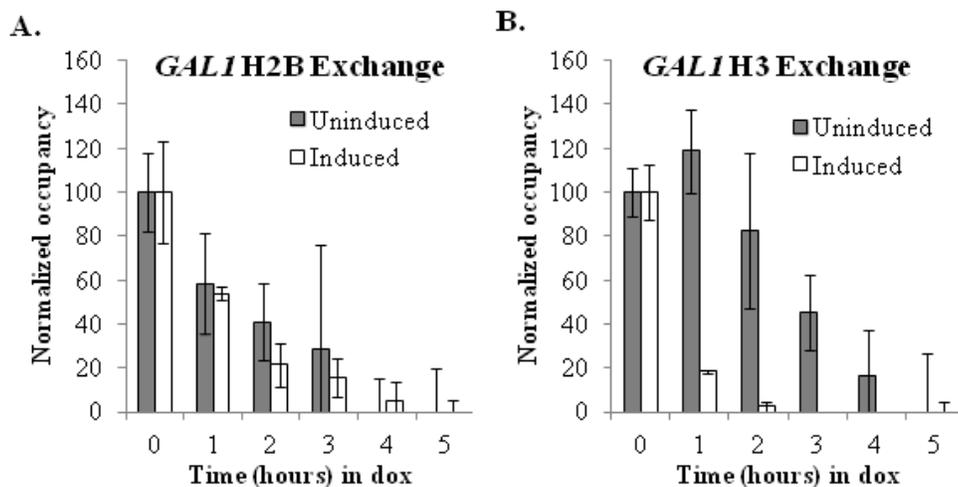
FIGURE 3.12. Histone exchange dynamics at the *GAL1* open reading frame. The occupancy of tagged dimer (**A**) and tagged tetramer (**B**) in the uninduced, glucose (grey) and induced, galactose (white) conditions during replication arrest. Samples were taken before and every hour after dox addition and analyzed through ChIP assays. Shown is the average of three independent biological replicates. Error bars represent variation among biological replicates. Tetramer exchange increased upon induction. In contrast, dimer exchange was unchanged between the uninduced and induced condition, which suggests that dimer dynamics are independent of transcription at the *GAL1* ORF.
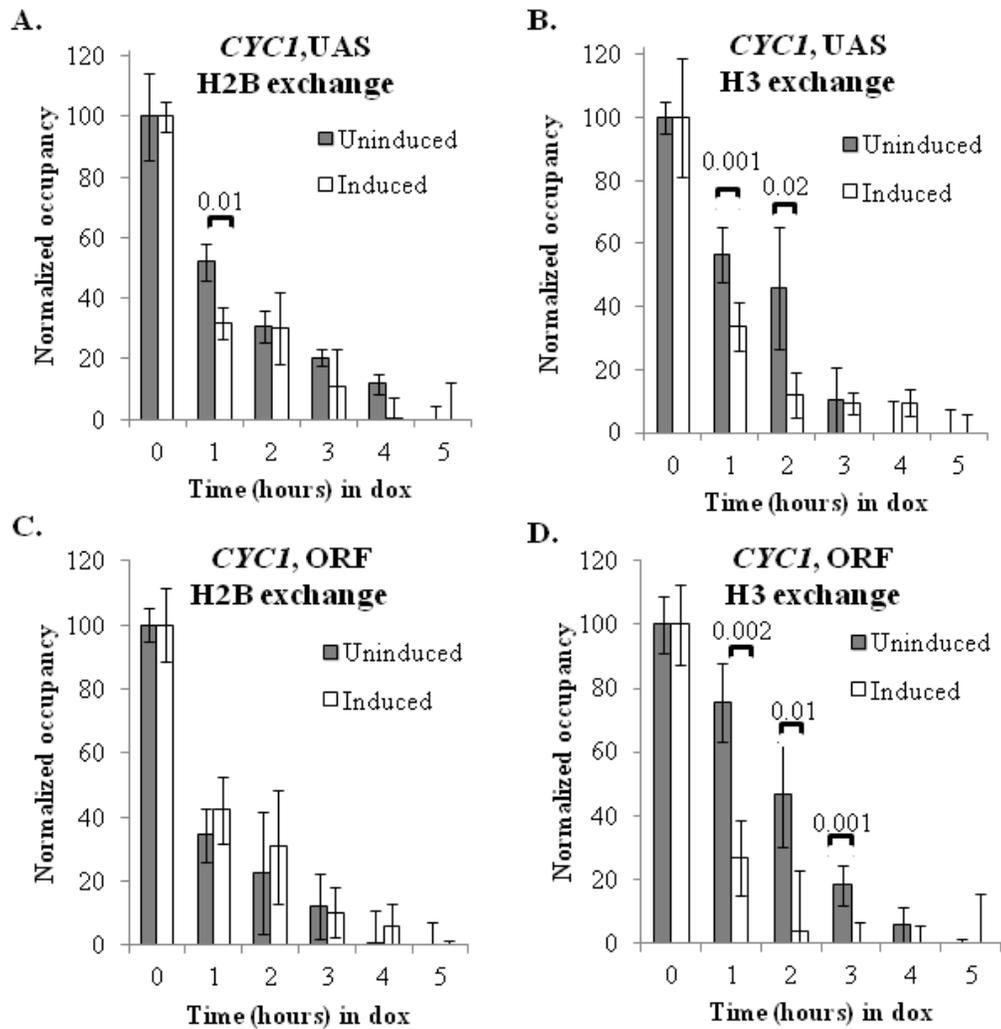
FIGURE 3.13. Distinct histone dynamics exhibited for the UAS and ORF nucleosomes of *CYC1.* The occupancy of tagged dimer (**A, C**) and tagged tetramer (**B,D**) in the uninduced, glucose (grey) and induced, galactose (white) conditions during replication arrest. Samples were taken before and every hour after dox addition and analyzed through ChIP assays. Shown is the average of three independent biological replicates. Error bars represent variation among biological replicates. The P-value calculated using the student T-test is shown for time-points with significant differences. Downstream of the poised promoter, dimer and tetramer have distinct exchange profiles.

more dynamic upon activation of *CYC1*. We also examined the exchange at the ORF nucleosome (FIGURE 3.13C and D). In the uninduced condition, dimer exchange is rapid at this location does not change upon induction. In contrast, tetramer exchange at the ORF increases upon induction, suggesting that tetramer dynamics at the ORF region are associated with transcription. Collectively, the dynamics of the neighboring nucleosomes are not uniformly linked to transcription output.

We also compared the dynamics of dimer and tetramer at the same location, providing information about the dynamics within a nucleosome. We found dimer and tetramer to have similar exchange dynamics in the uninduced condition at the UAS nucleosome (FIGURE 3.13A and B). Upon induction, the increase in exchange for dimer and tetramer was comparable at this location. Thus, at the UAS nucleosome, dimer and tetramer exhibit similar exchange patterns under the two conditions. This is in contrast to the neighboring ORF nucleosome, where dimer exchange was more rapid than tetramer in the uninduced condition (FIGURE 3.13C and D). Upon induction, tetramer exchange increased to match the dynamics of dimer. Therefore, at the ORF nucleosome, dimer and tetramer exchange patterns are different. A comparison of the exchange profiles of the two neighboring nucleosomes revealed tetramer exchange to be slower at the ORF nucleosome than at the UAS location in the uninduced condition (FIGURE 3.13C and D). Taken together, distinct histone dynamics exist at *CYC1* for these two neighboring nucleosomes.

3.3 DISCUSSION

The extent to which poising occurs in yeast seems variable. In cultures grown to stationary phase, 40% of the genome is estimated to be poised (Radonjic et al., 2005a). In actively growing cultures, partial yet inactive transcription components are a common feature of the yeast genome (Zanton and Pugh, 2006). However, it does appear that at many yeast genes transcription output

is largely coordinated with RNAPII recruitment (Steinmetz et al., 2006). For *CYC1*, it is clear RNAPII recruitment and transcription have been uncoupled (Lee et al., 2010; Martens et al., 2001; Zhang et al., 2008). Here we show that dynamics is the feature that best distinguishes the level of transcript produced. In the uninduced condition, TBP within the transcription complex is stably associated at the open promoter. This stability is comparable to that observed for the essential ribosomal RNA genes. Activation of *CYC1* enhances exchange of TBP. Thus, dynamics appear more closely linked to *CYC1* transcription than recruitment of the transcription complex. Interestingly, dynamics have been associated with activation in other work as well. Specifically, the ERα protein functions as a stronger activator when dynamically associated with the target gene than when the binding event is prolonged (Metivier et al., 2003; Reid et al., 2003). The link between dynamics and transcription is not yet clear, but it is possible that frequently clearing the promoter offers some functional advantage to support activated transcription.

The histone exchange assays revealed different exchange patterns for two neighboring nucleosomes located at *CYC1* (FIGURE 3.13A). Specifically, the UAS nucleosome becomes more dynamic upon induction, while at the ORF nucleosome, dimer is already actively exchanging prior to induction. In the uninduced condition, tetramer exchange at the UAS nucleosome is faster than tetramer exchange at the ORF nucleosome. Furthermore, the dynamics of dimer and tetramer are similar at the UAS, but differ at the ORF nucleosome in the uninduced condition. It is possible that the low level of RNAPII escape, typical of poised genes, is responsible for the different exchange patterns for these two neighboring nucleosomes (Gilchrist et al., 2012). Despite the difference in histone exchange patterns, upon induction rapid exchange was observed for all histones. From previous work, we found a host of chromatin-related factors are recruited to *CYC1* upon induction including, Mediator, Spt6 and Swi/Snf (Lee et al., 2010; Zhang et al.,

2008). In the absence of these chromatin regulators, *CYC1* transcription is compromised. Together, it seems likely that the increase in histone dynamics is linked with the arrival of these required chromatin-related factors.

The large, multisubunit coactivator SAGA (Spt/Ada/Gcn5 acetyltransferase) is localized to the *CYC1* promoter prior to activation and upon induction occupancy is maintained (Lee et al., 2010; Zhang et al., 2008). The SAGA complex can acetylate histones through the action of the Gcn5 enzyme, which we have shown to be essential for activation of *CYC1* (Lee et al., 2010).Due to the constitutive occupancy of SAGA, it was possible the required activity occurs prior to activation at the poised, uninduced locus. However, at the majority of yeast genes, histone acetylation is strongly correlated with activation (Pokholok et al., 2005; Reinke and Horz, 2003). Through ChIP assays we observed pre-acetylation of H3K9 and K14 at the poised, uninduced gene. We also found the acetylation level of these marks to be unchanged upon induction. Thus, the histone H3K9, K14 pre-acetylation is sufficient to support activated transcription and is triggered before induction at the transcriptionally inactive gene. Noteable, upon induction of *CYC1*, we observed an increase in histone exchange. Therefore, in order to maintain the histone acetylation status at *CYC1*, not only are the histones pre-acetylated at the uninduced locus, but these levels must be actively maintained during induced transcription.

Our MNase digestion studies revealed a large hypersensitive region within the *CYC1* promoter. This suggests the transcription complex bound at the poised promoter resides within a nucleosome-depleted region. Surrounding this region, we observed protection consistent with the occupancy of two positioned nucleosomes. The repressive nature of the chromatin has often been associated with the ability to block recruitment of transcription factors. In yeast, most commonly this barrier is overcome by nucleosome depletion to allow access to the gene (Bernstein et al.,

2004; Boeger et al., 2003; Lee et al., 2004; Pokholok et al., 2005; Reinke and Horz, 2003). At *CYC1* however, high histone occupancy is maintained upon activation. This suggests that nucleosome depletion is not a requirement for productive transcription at *CYC1*. We found that *CYC1* may bypass this requirement by enhancing histone dynamics to allow for a substantial increase in transcript output. Work from Jamai *et al* suggests that nucleosome loss upon activation is counterbalanced by histone deposition at other yeast genes (Jamai et al., 2007). *CYC1* is unique in that the increased exchanged is not associated with reduced histones levels as seen at most active yeast genes.

Recruitment of the RNAPII transcription machinery is necessary, but in many cases not sufficient for productive transcription. This finding was initially uncovered in eukaryotes at the *Drosophila* heat shock genes (Rougvie and Lis, 1988) and through the advancement of genome-wide technologies is now recognized as a general regulatory feature in metazoans (Adelman and Lis, 2012). It is not yet entirely clear how poised genes in yeast relates to pausing in metazoans (Yearling et al., 2011), but it seems there are some differences. For example, yeast lack a negative elongation factor (NELF) homolog. In addition, paused genes have halted transcription of nascent RNA, but in a manner conducive for resuming transcription upon induction. In yeast, there is no evidence for initiated transcripts that have paused (Mayer et al., 2010; Pelechano et al., 2010). Despite these differences we have found some striking similarities including, the occupancy of SAGA (Lebedeva et al., 2005), a requirement for Mediator (Meyer et al., 2010; Park et al., 2001; Wang et al., 2005), a dependency on Spt6 (Ardehali et al., 2009), the involvement of Spn1 (Yoh et al., 2008) and the chromatin architecture (Gilchrist et al., 2010).

The transcription complex bound in the open promoter region of *CYC1* also resembles findings in metazoans, such as the observation of serine 5 phosphorylation independent of transcription

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activation. As a general feature, this RNAPII mark enables recruitment of the nascent RNA capping proteins. Our previous work has identified the Ceg1 capping enzyme at the uninduced gene promoter of *CYC1*, which is also a feature of paused genes in metazoans (Nechaev and Adelman, 2011; Rasmussen and Lis, 1993; Zhang et al., 2008). Interestingly, pausing has been speculated to provide a potential advantage in coordinating the 5' capping activity with productive elongation (Adelman and Lis, 2012).

Histone modifications prior to activation have also been associated with poising in other eukaryotes. For example, gene promoters of embryonic stem cells containing RNA polymerase occupancy, but lacking activated transcription, are also associated with histone H3K9 and K14 acetylation (Guenther et al., 2007). The large, hypersensitive cut-site at the *CYC1* promoter is also reminiscent of the initial finding in *Drosophila,* where the heat shock genes were shown to be hypersensitive to DNAse I treatment (Cheng et al., 1995; Wu, 1980). In recent years, genome wide studies have confirmed this finding as a general feature of paused gene promoters, which are described as being nucleosome-depleted, independent of productive transcription (Gilchrist et al., 2010).

The term "paused" was chosen to describe the heat shock genes in *Drosophila* due to the similarity to a paused polymerase that had been previously observed in *Escherichia coli* (Chen et al., 1995a; Rougvie and Lis, 1988). Thus, some form of post-recruitment gene regulation has been conserved across the evolutionary spectrum, which makes it interesting to question the relationship between the poised *CYC1* gene and pausing in metazoans.

CHAPTER 4. MARKED DIFFERNCE IN STRESS RESPONSE BETWEEN TWO COMMONLY USED YEAST STRAINS

4.1 INTRODUCTION

An intricate and vital balance between pro-oxidants and reactive-oxygen species (ROS) is maintained in healthy aerobically growing cells. Upon cellular oxidative stress, this balance is disrupted and the concentration of ROS increases. Damage to proteins, nucleic acids and lipids can occur due to an overwhelming concentration of ROS in the cell (Halliwell and Gutteridge, 1984). This damage is known to lead to cancer and contribute to the aging process and related diseases (Finkel and Holbrook, 2000). Oxidative stress can arise endogenously through metabolic processes as well as through exposure to UV, chemical agents and metals (Storz and Imlay, 1999). To combat the harmful effects of oxidative stress, the cell's transcriptional program is modified. The treatment of *Saccharomyces cerevisiae* with a potent producer of ROS, hydrogen peroxide (H_2O_2), results in the activation of approximately 800 oxidative-stress response genes (Gasch et al., 2000). Many of these genes encode for various detoxifying proteins and small antioxidants molecules to cope with the increased concentration of ROS in the cell (Godon et al., 1998).

Yap1 is a REDOX-sensitive protein whose activity is regulated mainly through its cellular localization (Delaunay et al., 2000; Kuge et al., 1997). In reducing conditions, a nuclear export sequence near the carboxyl-terminal domain of Yap1 is exposed for interaction with the nuclear export factor Crm1 (Kuge et al., 1998). This interaction maintains cytoplasmic Yap1 localization in non-oxidative conditions. Upon oxidative stress, intramolecular disulfide bond linkage between cysteine residues of Yap1 masks the nuclear export sequence. This inhibits the Yap1-Crm1 interaction and Yap1 accumulates in the nucleus (Kuge et al., 2001). The nuclear

localized form of Yap1 binds to Yap1-responsive DNA elements (YREs) and activates transcription of the genes required to manage oxidative stress (Coleman et al., 1999).

One gene activated in response to oxidative stress through the activity of Yap1 is the gene *CYC1* (Lee et al., 2013). This gene encodes iso-1-cytochrome c, a nuclear-encoded protein involved in the electron transport chain in the mitochondria (Sherman et al., 1966). Under non-oxidizing conditions, *CYC1* gene expression is extremely low (Guarente et al., 1984; Guarente and Mason, 1983). Under cellular oxidative stress, *CYC1* is activated and transcript levels increase dramatically (Lee et al., 2013). In contrast to the change in transcriptional output, the occupancy of TBP, RNAPII, and other related factors, is maintained during the carbon-source change (Kuras and Struhl, 1999; Lee et al., 2010; Martens et al., 2001; Zhang et al., 2008).

Previously we described a TBP exchange assay using a doxycycline (dox) sensitive expression system to monitor TBP dynamics (Chapter 3). With this system, we described TBP-promoter dynamics within a preloaded transcription complex and viewed poising in an endogenous, chromatin environment. From these studies we found TBP to be stably associated with the promoter of *CYC1,* persisting for hours before exchanging in the uninduced condition (FIGURE 3.5). This stable conformation is found in complex with RNAPII in a low activity state, despite the presence of coactivators and other related transcription machinery (Lee et al., 2010; Martens et al., 2001; Zhang et al., 2008). Upon induction, RNAPII takes on a highly active conformation (Lee et al., 2013), which is associated with dynamic TBP-promoter exchange (FIGURE 3.5). The most likely candidate for controlling the switch between the stable and dynamic promoter configuration is SAGA. This large, multi-subunit coactivator is known to directly interact with TBP to deliver it to promoters (Belotserkovskaya et al., 2000; Bhaumik and Green, 2001; Dudley et al., 1999; Larschan and Winston, 2001; Qiu et al., 2005) and is present at the *CYC1* promoter

in both the uninduced and induced conditions (Lee et al., 2010; Zhang et al., 2008). Previous work in our lab has revealed a transcriptional requirement for the TBP-interacting subunits of SAGA at *CYC1* (Lee et al., 2010). Interestingly, these subunits are not required to deliver TBP to the promoter and thus, may be required to modulate TBP-promoter dynamics at *CYC1* (Lee et al., 2010). To investigate the connection between SAGA and TBP-promoter stability we made several SAGA subunit deletion strains in the dox-regulated TBP exchange background. However, SAGA is required for the expression of tagged TBP from the dox-regulated promoter (M. Yearling, unpublished data). Therefore, the TBP-exchange assay cannot operate without a fully functional SAGA complex.

Other studies have successfully analyzed TBP-promoter dynamics at rapidly exchanging loci using a galactose induction system (van Werven et al., 2009). However, those studies can only be performed in galactose, which limits the pool of genes available for analysis. In addition, *CYC1* is induced under galactose conditions (FIGURE 3.2 A). Therefore, TBP-promoter exchange cannot be observed in the uninduced condition using the gal-inducible system. An enticing alternative for rapid depletion of TBP without carbon-source limitations is the anchor-away method developed by the Laemmli laboratory (Haruki et al., 2008). Thus, we modified the anchor-away technique to examine the requirement of SAGA for TBP-exchange at the poised *CYC1* gene. We found that in the anchor-away strain *CYC1* transcription is alternatively regulated and as a result TBP dynamics at *CYC1* in the SAGA deletion background could not be observed. We show that the difference in *CYC1* stress response is not the result of the anchor-away modifications, but rather due to intrinsic differences in the W303 parental strain.

Characterization of the *CYC1* gene in the anchor-away strain. In our typical lab strain BY4741, the *CYC1* gene exhibits low levels of expression under non-oxidizing conditions and becomes rapidly activated in response to the oxidant producing chemical, hydrogen peroxide (H_2O_2) (FIGURE 4.1 A) (Lee et al., 2013). However, we did not observe this transcription response in the anchor-away strain. In fact, prior to treatment with oxidant, *CYC1* exhibited about a 20-fold higher level of transcription output in the anchor-away strain (FIGURE 4.1 A). These findings were independent of rap treatment. Therefore, *CYC1* is already highly expressed in the anchor-away strain under non-oxidizing conditions. Under these same conditions, *CYC1* expression in the BY4741 strain is very low. In addition, treatment with H_2O_2 did not increase *CYC1* transcription in the anchor-away strain.

Since *CYC1* was not further activated in the anchor-away strain with H_2O_2 treatment, we tested if the concentration of H_2O_2 was too low to elicit an additional transcriptional response in this strain. Others have used a 3x higher concentration of H_2O_2 to investigate oxidative stress in the W303 parental strain used to create the anchor-away technique (Morgan et al., 1997; Raitt et al., 2000). As such, we increased the H_2O_2 concentration and compared the transcriptional response of *CYC1* in the BY4741 strain to the anchor-away strain (FIGURE 4.1 B). Despite the increased concentration of H_2O_2, the transcriptional response of *CYC1* was not further increased upon H_2O_2 treatment in the anchor-away strain. As seen earlier, the level of *CYC1* transcription in the non-oxidizing condition was remarkably higher for the anchor-away strain when compared to BY4741.
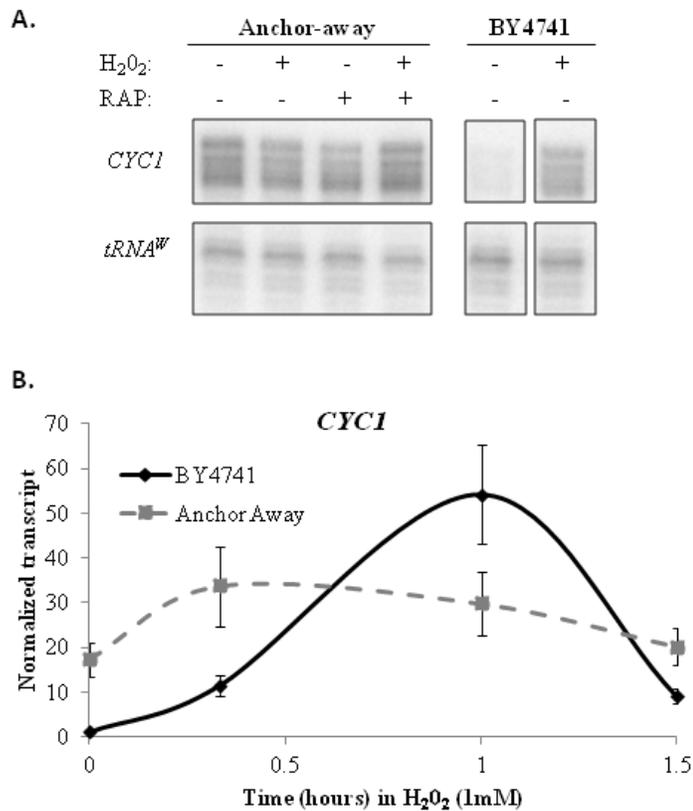
FIGURE 4.1. Anchor-away strain lacks H_2O_2 induction. *CYC1* transcript levels shown using S1 nuclease protection assays in the anchor-away and BY4741 strains. (**A**) Cultures were taken before or 20 minutes after H_2O_2 addition both in the presence and absence of rapamycin. As a loading control $tRNA^W$ intron is used. *CYC1* levels are higher in the anchor-away strain than in BY4741 prior to H_2O_2 treatment and do not increase upon oxidant addition. The results are independent of rapamycin. (**B**) Increased levels of H_2O_2 do not increase *CYC1* transcript levels in the anchor-away strain. Cultures were taken before and at the indicated time after H_2O_2 addition. Shown is the average of three technical replicates using $tRNA^W$ intron as a loading control.

Additional oxidative stress response genes do not induce transcription upon H_2O_2 treatment. To determine if the altered oxidative stress response in the anchor-away strain was unique to *CYC1*, additional genes within the oxidative stress regulon were examined. *FLR1* and *GTT2* were chosen since their activation upon H_2O_2 treatment had been well-characterized in our lab within the BY4741 strain (Lee et al., 2013). In the anchor-away strain, both genes exhibited a weak transcription fold-change in response to oxidant treatment (FIGURE 4.2 A and B). Neither gene displayed a high level of transcription prior to treatment as was observed for *CYC1*.

Anchor-away modifications are not responsible for lack of stress induction. The anchor-away technique requires some genetic manipulation to function (Haruki et al., 2008). Specifically, the nonessential yeast *FPR1* gene is deleted since this protein competes for binding to rapamycin in yeast. In addition, the anchor-away technique requires the *TOR1-1* point mutation to confer resistance to the antifungal drug rap. In view of the fact that three genes did not further activate in H_2O_2, the regulation of the oxidative stress regulon might be altered in the anchor-away strain as a result of these mutations. Therefore, we examined the parental strain (W303) used to create the anchor-away technique (Haruki et al., 2008). Since the mating type of the anchor-away strain also differs from BY4741, we examined both mating types of W303. In the W303 strain, a weak transcriptional response to H_2O_2 was observed for both *CYC1* and *GTT2* (FIGURE 4.3 A and B). The results were similar to observations in the anchor-away strain and were independent of mating type (FIGURE 4.3 C and D). Thus, neither the anchor-away genetic modifications nor the alternate mating type is responsible for the low transcription response of this strain to oxidative stress treatment. It is important to note, both W303 and BY4741 are *S. cerevisiae* strains commonly used in laboratory research (Cherry et al., 2012).
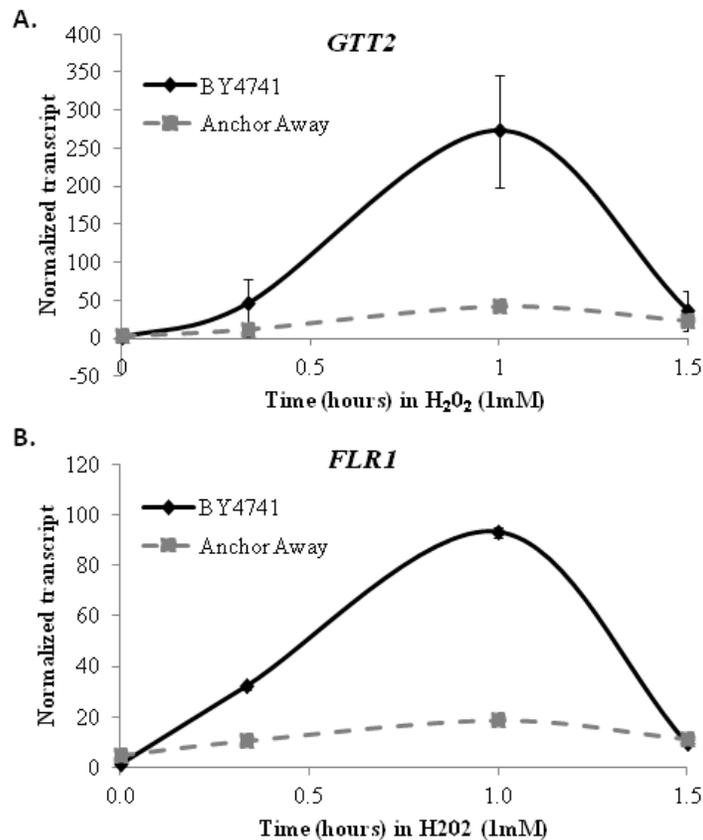
FIGURE 4.2. ***GTT2* and FLR2, additional oxidative stress response genes, also lack transcript induction upon treatment with H_2O_2.** (**A** and **B**) S1 nuclease protection assays were used to quantify *GTT2* and *FLR1* transcript levels in the anchor-away and BY4741 strains. Cultures were taken before and at the indicated time after H_2O_2 addition. Shown is the average of three technical replicates using tRNA[W] intron as a loading control. Starting levels in both genes are similar between the two strains. Upon treatment with H_2O_2, the fold change is minimal in the anchor-away strain when compared to BY4741.
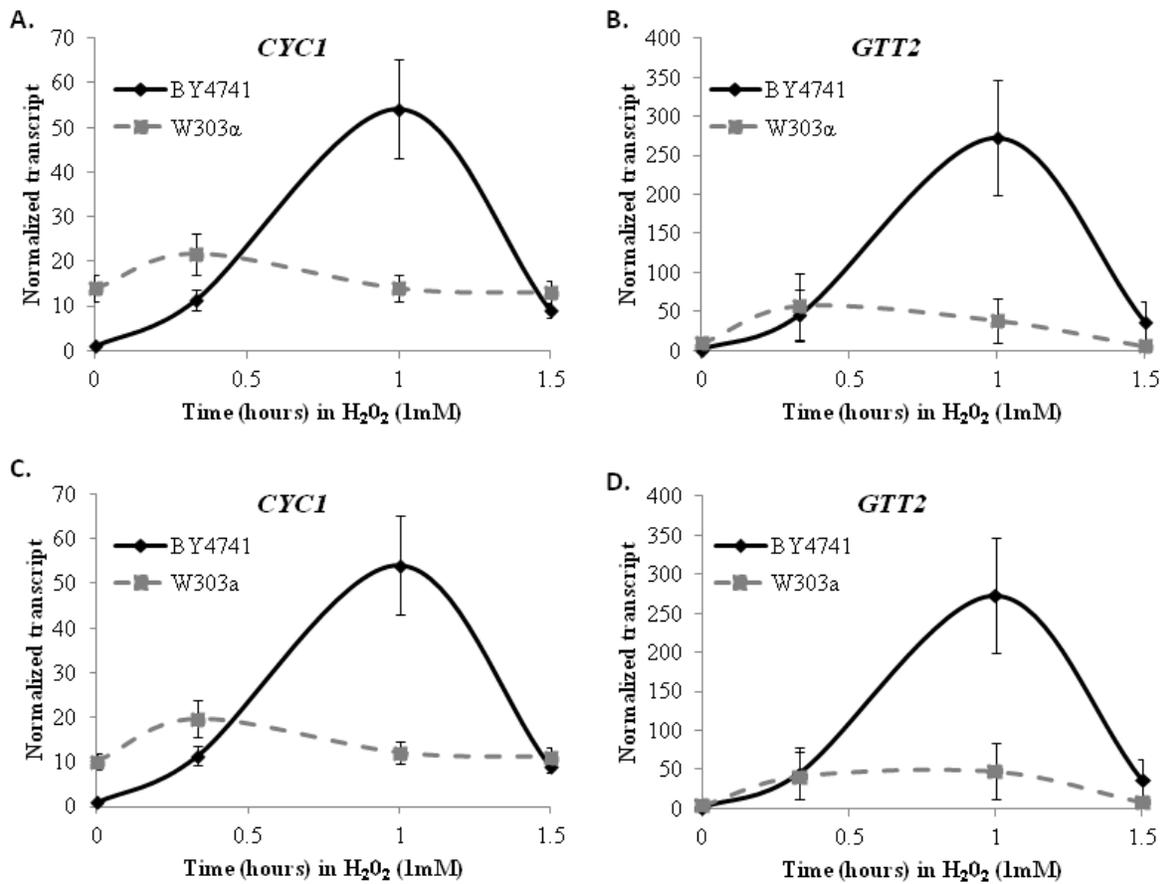
FIGURE 4.3. Parental strain to anchor-away does not response to H₂0₂, which is independent of mating type. (**A** and **B**) S1 nuclease protection assays were used to quantify *CYC1* and *GTT2* transcript levels in the W303a strain (parent to the anchor-away) and BY4741 strain. Cultures were taken before and at the indicated time after H₂0₂ addition. (**C** and **D**) In addition, transcript levels in the W303α strain (same mating type as BY4741) and BY4741 strain were compared for *CYC1* and *GTT2*. Shown is the average of three technical replicates using tRNA^W intron as a loading control. The modifications made in anchor-away are not responsible for differences in the response to H₂0₂ for these genes.

An alternative activator complex does not enhance transcription response to stress. Our lab has previously determined that *CYC1*, *GTT2* and *FLR1* require the transcription factor Yeast AP-1 protein (Yap1) for oxidative stress induction (Lee et al., 2013). This transcription activator is a basic leucine zipper protein, known to be essential for oxidative stress response in yeast (Ikner et al 2005). To determine if the low degree of induction was a Yap1-specific effect, we investigated *CYC1* response to ethanol treatment, which we know to be independent of Yap1 (Lee et al., 2013). Similar to the oxidative stress treatment though, only a minimal fold change in *CYC1* transcript level was observed in both the anchor-away strain and the parental W303 strains (FIGURE 4.4 A and B). Consequently, the lack of *CYC1* induction is independent of the transcription activator.

4.3 DISCUSSION

An important finding derived from these studies is the marked difference in transcription output between these two commonly utilized yeast strains. We found that *CYC1* exhibited nearly 20-fold higher levels of transcript under non-oxidizing conditions in the W303 background. In addition, upon treatment with H_2O_2, *CYC1* transcript levels did not increase. The *CYC1* transcription output remained weak compared to BY4741 even after a 3-fold increase in H_2O_2. If we take into account the higher level of *CYC1* transcript prior to H_2O_2 treatment, the level of transcription output in response to H_2O_2 is still only about half of that observed for BY4741. The lack of further induction however, is not a *CYC1*-specific defect since the response of two other genes (*GTT2* and *FLR1*) was only 20% of that observed in BY4741. These studies also ruled out poising as a potential cause for induction differences since the gene *GTT2* lacks a poised transcription complex prior to activation (Lee et al., 2013). We determined that the anchor-away modifications and mating type differences were not responsible for the weak transcriptional
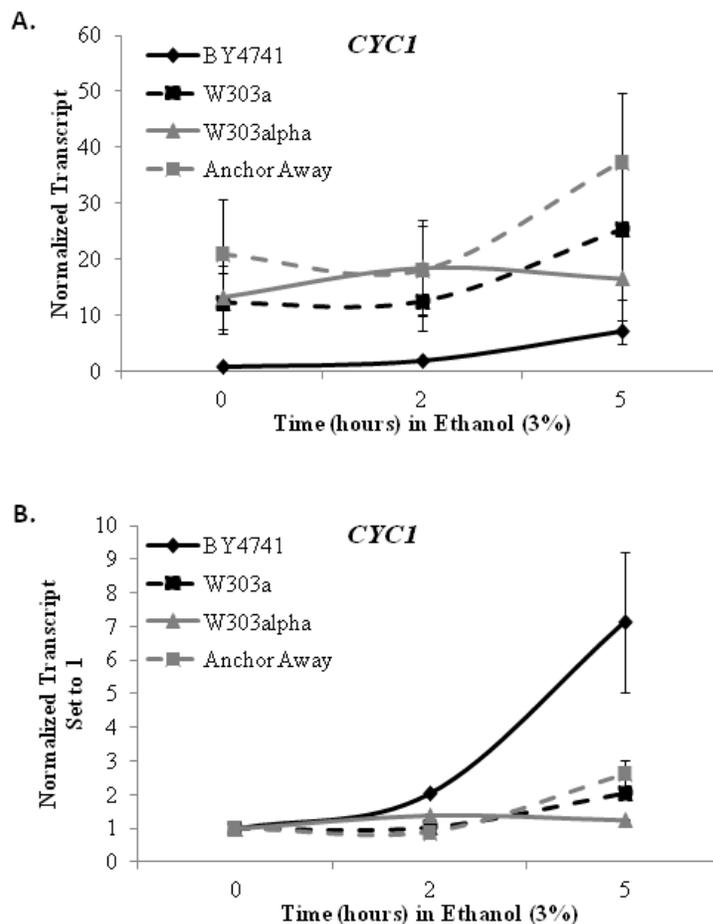
FIGURE 4.4. Ethanol induction of *CYC1* does not elicit as strong of a transcript increase in anchor-away or the parental strains. (**A**) S1 nuclease protection assays were used to quantify *CYC1* transcript levels before and at the indicated time after H_2O_2 addition. BY4741 response was compared to the anchor-away strain, W303a and W303α (parental strain to anchor-away). Although the total transcript levels are higher than in BY4741, the fold change is significantly less as shown in (**B**). For each strain the samples lacking treatment were set to 1 to show fold change upon induction with ethanol. Shown is the average of three technical replicates using tRNA[W] intron as a loading control.

response, which is consistent with previous findings that the anchor-away modifications have minimal impact on global transcription. Furthermore, we found transcription induction defects to be independent of rap addition. We also confirmed that the lack of induction was not a Yap1 specific defect (Lee et al., 2013). Taking all these results together suggests a more universal difference exists between BY4741 and W303 in stress response.

In the past, it has been difficult to characterize differences between commonly used yeast strains. Many can be traced back decades to a parental strain created in the early days of yeast genetics, when scientists worked to create a hardy yeast strain that could survive with minimal nutrients and was non-flocculent [reviewed in (Mortimer and Johnston, 1986)]. Since then, the strains have undergone numerous crosses, some more than others (Cherry et al., 2012). For instance, the BY4741 strain is closely derived from S288C, while W303 was created from a series of strain-crosses, one of which was a descendent of S288C (Cherry et al., 2012). The S288C strain is one of the original yeast strains used for genetics in the 1930's, from which many laboratory yeast strains share homology (Mortimer and Johnston, 1986).

With the advancement of genome-wide technologies we are now beginning to dissect the genetic variation between commonly used yeast strains. A recent genomic comparison of S288C and W303 revealed that W303 is approximately 85% derived from S288C (Ralser et al., 2012). Interestingly, the unique portions of W303 appear similar to West African yeast strains, European strains and even resemble Japanese sake strains (Ralser et al., 2012). In total, some 800 of the approximately 6,400 genes in yeast differ between the two strains, but in most cases only by one or two amino acid residues (Ralser et al., 2012). It has been speculated that these genetic variations account for the physiological differences between BY4741 and W303 (Ralser et al., 2012). Thus, it is possible these genetic differences also are responsible for the altered

transcription stress response we observed. With the continued advancement of genome-wide technologies it will be important to note strain variation when applying these findings to one's own work.

CHAPTER 5. A CORE-PROMOTER POINT MUTATION REDUCES TRANSCRIPTIONAL OUTPUT INDEPENDENT OF TBP OCCUPANCY AND TBP-PROMOTER DYNAMICS[3]

5.1 INTRODUCTION

There are forty documented human diseases that arise due to single nucleotide polymorphisms (SNPs) within the promoter binding region of TBP [reviewed in (Savinkova et al., 2009)]. These pathologies are coupled to the dysregulation of gene transcription. Specifically, polymorphisms that weaken the transcriptional output are linked to β-thalassemia, excessive body weight, lung cancer, hypertension and nearly twenty other diseases. Single base mutations within the TBP-promoter region can also cause excessive transcriptional output leading to multiple sclerosis, diabetes, leukemia, cataracts, among others. Currently, there is no clear pattern connecting the promoter mutation with the impact on transcription and thus, it is unknown how the TBP-DNA interaction directly affects transcriptional output in living cells.

In vitro, the TBP-DNA interaction is strongly influenced by the presence or absence of other transcription factors as well as the promoter DNA sequence itself (Hoopes et al., 1992). FRAP assays (fluorescent recovery after photobleaching) in both yeast and human cells have shown most transcription factors recover as a single fraction, but TBP has a biphasic recovery (de Graaf et al.; Sprouse et al., 2008). The unique two-component recovery observed for TBP suggests that two distinct populations of TBP exist in living cells – a dynamic and a quiescent fraction.

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Our lab has previously demonstrated that a SNP within the TATA sequence dramatically reduces transcription output of a gal-inducible reporter gene (Stewart and Stargell, 2001). Specifically, the canonical TATA core-promoter sequence was mutated to a non-canonical CATA sequence in the TBP binding region of the promoter. Using an *in vitro* approach, we found that the reduced output correlated with an altered TBP-promoter stability (Stewart and Stargell, 2001). Interestingly, this change in the *in vitro* TBP-promoter stability was only observed in the presence of the general transcription factor TFIIA (Stewart and Stargell, 2001). This indicates that additional TBP associated factors are critical for assessing the biological impact of TATA box mutations on TBP-promoter dynamics. Thus, the ability to observe changes in TBP-promoter dynamics *in vivo* is critical for accurately describing the relationship between core-promoter DNA sequence and TBP dynamics. Here describe a system to test TBP-promoter dynamics and show that the reduced transcription output in the mutant strain is independent of TBP-promoter occupancy levels and TBP-promoter dynamics *in vivo*.

5.2 RESULTS

Implementation of the anchor-away technique to assay TBP-promoter dynamics at rapidly exchanging loci. The initial study examined phenotypes upon nuclear depletion of TBP and nearly 40 other individual proteins through the anchor-away technique (Haruki et al., 2008). In this assay, TBP was depleted from the nucleus within 30 minutes. This is similar to the time-scale used for the galactose induction system to measure TBP dynamics. Thus, it seems likely the anchor-away system could be used for fast depletion of TBP to allow observation of the TBP-promoter dynamics at rapidly exchanging loci without carbon-source limitations (FIGURE 5.1). In brief, the strain consists of a fusion of the human 12 kDa, FK506 binding protein (FKBP12) to the abundant ribosomal protein *RPL13a*. TBP was also fused with the 11 kDa,

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FKBP12-rapamycin-binding-domain (FRB) of human mTOR at the endogenous locus. The system works to remove TBP by anchoring it to the ribosomal protein in the cytoplasm through an interaction between the two tags (FKBP12 on the ribosomal protein and FRB on TBP). This can be conditionally controlled given that FKBP12-FRB interaction only occurs in the presence of rapamycin (rap). The tagged ribosomal protein has the ability to interact with the tagged TBP in the nucleus due to a natural nuclear import/export process that occurs in the cell. Specifically, the ribosomal proteins are imported into the nucleus to obtain the rRNA essential for protein synthesis by the ribosome. The ribosomal protein is then exported to the cytoplasm and in the absence of rap, TBP remains within the nucleus. However, in the presence of rap, the FRB tag on TBP binds to the FKBP12 tag on the ribosomal protein with nanomolar affinities (Chen et al., 1995b). The TBP-ribosomal complex is then exported from the nucleus and becomes anchored in the cytoplasm. To adapt this procedure for TBP-promoter dynamic studies, exogenous untagged TBP expressed from the natural TBP-promoter, was introduced to maintain cellular viability upon the loss of the rap-dependent TBP molecules. By fluorescent microscopy we confirmed rapid removal of tagged TBP from the nucleus upon rap addition (FIGURE 5.2).

A core-promoter mutation does not alter TBP occupancy or TBP-promoter dynamics. We examined the gene expression levels during galactose induction from the TATA and CATA reporter genes (FIGURE 5.3 A and B). These results were consistent with our previous work (Stewart and Stargell, 2001), which showed that TATA and CATA have similar induction kinetics, but the overall transcription output is reduced near 100-fold for CATA. Thus, by introducing a single point mutation, the CATA strain is compromised for maximum output.

To investigate if the reduced transcription output was a result of decreased TBP occupancy at the CATA promoter, we performed ChIP assays testing total TBP occupancy in the two strains.

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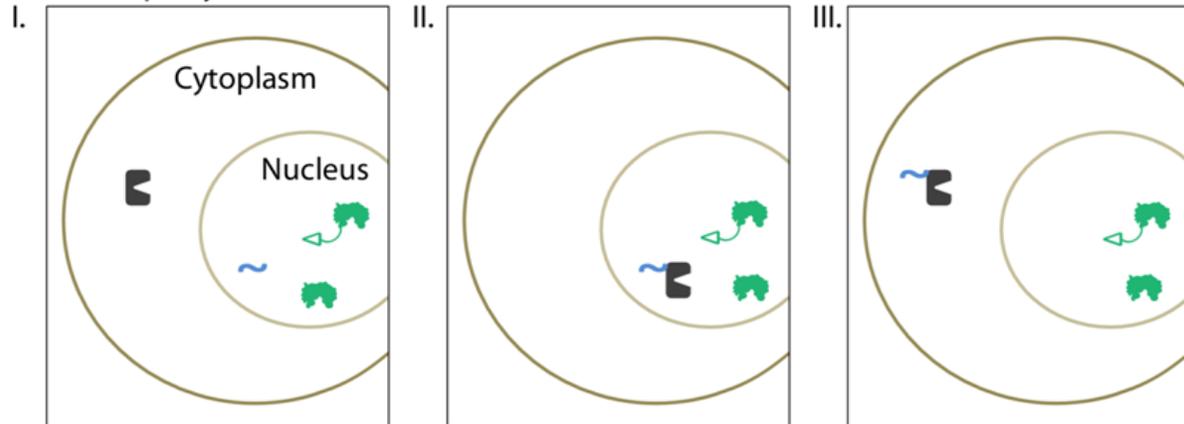
FIGURE 5.1. A schematic showing the anchor-away system for examining TBP dynamics *in vivo*. Prior to rapamycin addition, the tagged TBP molecule remains in the nucleus with the untagged TBP. Upon addition of rapamycin, the tagged TBP molecule is removed from the nucleus via interaction with the tagged ribosomal protein. See text for details.

| | DAPI (DNA) | TBP-GFP | Overlay |
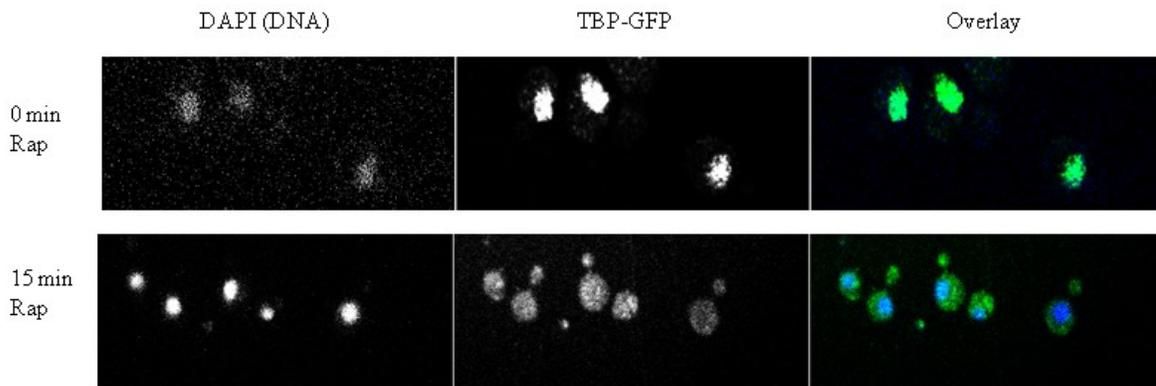
FIGURE 5.2. TBP is rapidly depleted from the nucleus upon addition of rapamycin. The left column shows DAPI staining for DNA visualization, the middle column shows GFP to visualize TBP and the right column shows the merge of the first two columns to show localization. The top panel was not treated with rapamycin (rap), while the bottom column was incubated in rapamycin for 15 before imaging. After 15 minutes rapamycin treatment TBP is removed from the nucleus and relocalized to the cytoplasm.
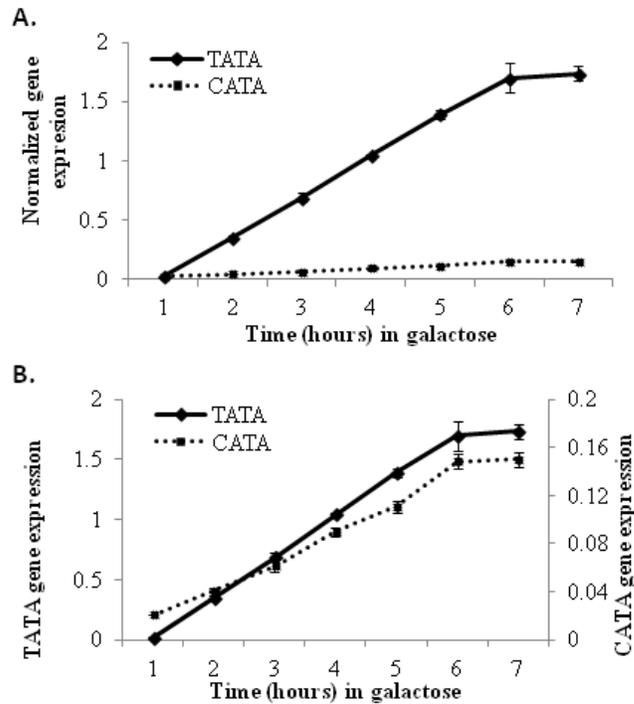
FIGURE 5.3. A single point mutation in the TATA box dramatically reduces gene expression. (**A**) Gene expression of TATA (solid line) and CATA (dotted line) shown through lacZ assays. Introduction of the point mutation into the TATA box (CATA) results in a sever defect in expression. (**B**) The same result as shown above, but CATA is set on a secondary y-axis (right) to show that kinetics of induction are conserved between the two core-promoters. For both analysis, three biological replicates were averaged and the error bars represent biological variation.

This analysis revealed TBP occupancy to be comparable between the two core-promoters (FIGURE 5.4 A). Therefore, the compromised transcription output from the CATA strain was not a result of reduced TBP recruitment. To examine the impact of the SNP on TBP-promoter dynamics, we monitored the loss of tagged TBP-promoter occupancy following removal of tagged TBP from the nucleus. We did not observe any difference in exchange between these two promoters, which suggests that the promoter mutation does not impact TBP-promoter dynamics (FIGURE 5.4 B).

5.3 DISCUSSION

By incorporating a single mutation into the core-promoter region we observed significant loss of transcription output. We found that the reduced transcript levels were not due to a decrease in TBP occupancy or a change in TBP-promoter dynamics. Of course, it is always possible that the differences in TBP-promoter dynamics are too subtle for the anchor-away exchange system to detect. However, a system using a comparable time-scale was capable of observing differences in TBP-promoter dynamics (van Werven et al., 2009). These studies found TATA-containing genes to be enriched for higher turnover of TBP, but these comparisons were between completely different genes. Our approach was a very controlled system, which is best suited to test if core-promoter mutation impacts TBP-promoter dynamics to alter transcriptional output.

Our results suggest that something other than TBP occupancy and TBP-promoter dynamics is responsible for the reduced transcriptional output caused by the introduction of a SNP into the core-promoter. Examination of TBP-promoter structures in solution has shown DNA sequence can cause dramatic changes in TBP-induced DNA bend (Starr et al., 1995; Wu et al., 2001). TBP bound to a canonical TATA sequence results in a ~80° bend angle, while single point mutations

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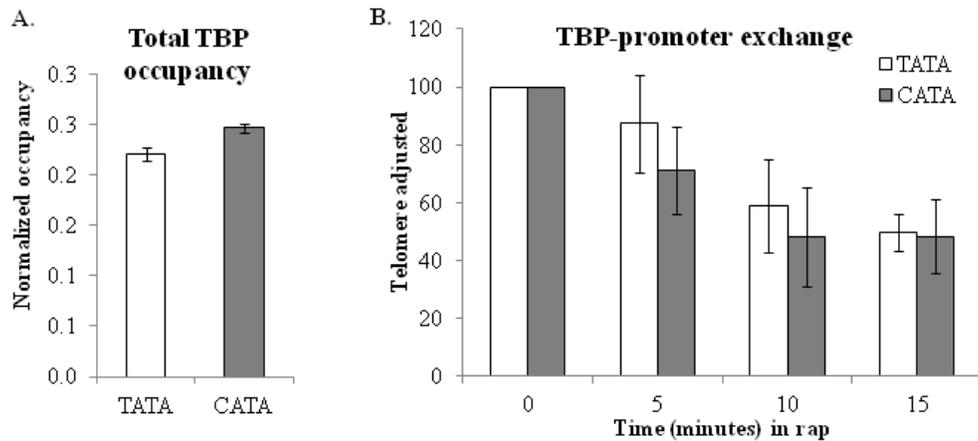
FIGURE 5.4. The reduced transcription output caused by the CATA point mutation is not a result of altered TBP occupancy or TBP-promoter dynamics. (A) TBP occupancy at TATA (white bar) and CATA (grey bar) at the induced core-promoter regions. (B) The occupancy of tagged TBP before and at the indicated times after rapamycin (rap) treatment Shown is the average of three independent biological replicates. The error bars represents biological variation.

can reduce that angle to ~30°. *In vitro*, transcription output from these variant core-promoters correlates strongly with DNA bend angles. For these studies, it has been speculated that the bend angle impacts the orientation of related transcription components to alter the activity of RNAPII. For instance, TFIIA contacts the DNA directly flanking the downstream TBP binding region while TFIIB makes contact with both upstream and downstream flanking regions (Coulombe et al., 1994; Lagrange et al., 1998; Lagrange et al., 1996; Lee and Hahn, 1995; Reinberg et al., 1998). It's clear that alteration of the bend angle would impact the orientation of the two factors. *In vivo* though, the results aren't so clear. For instance, identical sequence mutations in two separate genes have a different impact on the degree of transcription dysregulation (Savinkova et al., 2009). It's important to continue the *in vivo* investigations since numerous genetic diseases have been linked to TATA box mutations in humans (Savinkova et al., 2009). Our results suggest that examining the impact of TATA box mutations on transcription factor occupancy, such as TFIIA and TFIIB, should be studied to look for a link between core-promoter mutations and transcription output.

CHAPTER 6. PERSPECTIVES AND FUTURE DIRECTION

Here we described a stable TBP-promoter association at the poised *CYC1* gene, which persisted

for hours before exchanging in the uninduced condition (FIGURE 3.5). This stable conformation

is found in complex with RNAPII in a low activity state, despite the presence of coactivators and

other related transcription machinery (Lee et al., 2010; Martens et al., 2001; Zhang et al., 2008).

Upon induction, RNAPII takes on a highly active conformation (Lee et al., 2013), which is

associated with dynamic TBP-promoter exchange (FIGURE 3.5). Rapid exchange during activated

transcription was also observed at other genes, including recruitment regulated gene promoters

as well as constitutively active gene promoters. For a recruitment-regulated galactose inducible

promoter, rapid TBP exchange was observed even when the TBP binding site was mutated. This

mutation resulted in dramatically reduced transcription output, but did not alter the rapid TBP-

promoter exchange. Thus, we did not observe a correlation between the level of transcription

output and TBP-promoter dynamics. We showed rapid TBP-promoter exchange to be associated

with active transcription. Interestingly, dynamics have been associated with activation in other

work as well. Specifically, the ERα protein functions as a stronger activator when dynamically

associated with the target gene than when the binding event is prolonged (Metivier et al., 2003;

Reid et al., 2003). From our findings we propose a model where frequently clearing the promoter

offers a functional advantage to support activated transcription.

We also showed the stably poised TBP complex to be association with RNAPII containing serine

5 phosphorylation marks consistent with initiation (FIGURE 3.1). In addition, previous work from

our lab showing the presence of TFIIH including subunit responsible for DNA unwinding

capability, suggests that *CYC1* has melted and initiated transcription at the uninduced promoter

(Lee et al., 2010; Zhang et al., 2008). In yeast however, there is no evidence for transcripts that

have initiated and paused using a whole genome approach (Mayer et al., 2010; Pelechano et al.). This might be due to the strain or cell culturing conditions, since we found both influence *CYC1* transcript levels (CHAPTER 3 and 4). To examine pausing directly, in our strain and culturing conditions, we can use native elongating transcript sequencing (NET-seq) assays (Churchman and Weissman, 2011). In this approach, RNAPII is immunoprecipitated and the associated nascent RNA is sequenced. The 3' end of the RNA sequence maps to the location of RNAPII. If RNAPII has initiated and paused, a higher ratio of transcripts will be present at the 5' end of the gene than the 3' end of the gene in the uninduced state. Through this whole genome approach we can also determine if pausing occurs at other genes besides *CYC1*.

Key factors known to be required for pausing in metazoans that have not been found in yeast and thus, identifying a paused RNAPII at *CYC1* would open up many avenues for investigation into the missing pieces [reviewed in (Adelman and Lis, 2012)]. Conversely, it is possible *CYC1* is under a distinct form of post-recruitment regulation where RNAPII is not paused. It is intriguing that occupancy of SAGA (Lebedeva et al., 2005), a requirement for Mediator (Meyer et al., 2010; Park et al., 2001; Wang et al., 2005), a dependency on Spt6 (Ardehali et al., 2009), the involvement of Spn1 (Yoh et al., 2008) and the chromatin architecture (Gilchrist et al., 2010), play critical roles in pausing and/or post-recruitment transcriptional events in metazoan cells. Taken together, these results suggest that there are universal requirements for the activities of multiple complexes in the transition of RNAPII from an inactive to the actively elongating form. Whether the poised RNAPII in yeast is an evolutionary precursor to the more sophisticated version of paused RNAPII in metazoans is an open question, but investigating the status of the RNAPII enzyme will help to address this.

By mapping the chromatin at the poised gene we indentified some unique characteristics including, pre-acetylation of H3K9 and K14 and high histone occupancy in the both the uninduced and induced conditions (FIGURE 3.7). To determine if the chromosomal context is important for establishing the poised complex, we created a plasmid for *CYC1* expression in yeast, which included the upstream and downstream flanking sequences. We found the expression of exogenous *CYC1* to be regulated similar to the chromosomal *CYC1* (C. Radebaugh, unpublished results). It will be interesting to determine if TBP and RNAPII occupy the exogenous *CYC1* gene promoter in the uninduced condition. Also, to investigate whether the promoter remains nucleosome depleted and contain histone acetylation marks prior to activation. If true, this would suggest that the chromosomal context of *CYC1* is not required for preloading the promoter or for the response to environmental stress.

Several important questions remain. For example, what creates the poised polymerase in the first place? We have found that the occupancy of RNAPII at *CYC1* is an incredibly robust phenomenon: single deletion of dozens of different transcription factors and co-activator complex subunits has not resulted in RNAPII occupancy defects (APPENDIX I) (Yearling et al., 2011). It could be that RNAPII preloading is an intrinsic property of the *CYC1* promoter and/or its nuclear and chromosomal context. This can be tested using the construct described above. In contrast to the resiliency of RNAPII occupancy, the transition to an actively elongating form is an incredibly delicate phenomenon, requiring the efforts of several prodigious and powerful transcription complexes: SAGA, Mediator and Swi/Snf. Intriguingly, these complexes and their functions appear to work independently at *CYC1*. As shown previously (Lee et al., 2010), Mediator and SAGA occupancy are not dependent on each other, and mutations that result in constitutive occupancy of Swi/Snf do not bypass the need for SAGA or Mediator for activation

of the poised promoter (FIGURE 1.2) (Yearling et al., 2011). As such, three distinct pathways are required to shift the polymerase into its active form. Further studies are needed to elucidate how each complex directly contributes to the transition from the poised to the active form, but it is clear that in accord with Newton's first law (a body at rest tends to stay at rest), these large macromolecular assemblies must provide the essential outside forces to initiate the process.

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APPENDIX I

SPT4 IS UNIQUELY INVOLVED IN POISED PROMOTER RESPONSE TO OXIDATIVE STRESS

We have found that the occupancy of RNAPII at *CYC1* is an incredibly robust phenomenon: single deletion of dozens of different transcription factors and coactivator complex subunits has not resulted in RNAPII occupancy defects (FIGURE A.1) (Lee et al., 2010). In contrast to the resiliency of RNAPII occupancy, the transition to an actively elongating form is a highly demanding phenomenon, requiring the efforts of several prodigious and powerful transcription complexes: SAGA, Mediator, and Swi/Snf. These complexes, while critical for poised activation, are universal complexes that function at both poised and non-poised genes.

To identify factors that are uniquely involved in activation of poised *CYC1*, the transcriptional response to oxidative stress in deletion strains was assayed comparing the *CYC1* gene to the *GTT2* gene. Both genes respond to oxidative stress induction, but *GTT2* does not have preloaded transcription machinery (Lee et al., 2013). We examined the transcription response of each gene after treatment with H_2O_2 and compared the results of the wildtype strain to each deletion strain. We chose the deletion strains based on their previously characterized involvement in the regulation of transcription after recruitment.

The results of this screen can be classified into three categories: no influence on transcription of either gene, dysregulation of both genes, and a potential poising-specific influence on transcription. Paf1, a component of Paf1 elongation complex is not required for activation of either gene (FIGURE A.2). Leo1, Rtf1, Rpb4 and Rpb7 are required for proper activation of both *CYC1* and *GTT2*, suggesting that the proteins are required for activation in a manner independent of poising (FIGURE A.3 and A.4). Since both Rpb4 and Rpb9 are subunit of RNAPII the

requirement for these proteins at both genes is not too surprising. Leo1 and Rtf1 are components of the Paf1 elongation complex. We previously stated that the Paf1 subunit of the Paf1 complex was not required for activation of either gene. The requirement for different subunits of the Paf1 elongation complex could suggest that this large multi-subunit complex has multiple roles in transcription [reviewed in (Jaehning, 2010)]. Interestingly, the deletion of Spt4 resulted in a differential affect on the poised *CYC1* gene when compared to the non-poised *GTT2* gene (FIGURE A.5). Loss of Spt4 resulted in overactivation of *CYC1*, but had no affect on activation of *GTT2*. This suggests Spt4 has a unique activity at the poised promoter. Consistent with this, Spt4 and its binding partner Spt5 are centrally involved in poising of metazoans [reviewed in (Li and Gilmour, 2011)]. At poised metazoan genes, Spt4-Spt5 heterodimer and the Negative Elongation Factor (NELF) act to maintain the transcription machinery in the uninduced state. Upon induction, Positive Transcription Elongation Factor (P-TEFb) phosphorylates Spt5, among other targets, resulting in the loss of NELF and activated transcription.

Interestingly, loss of Spn1 (a preloaded protein at the *CYC1* promoter) is lethal in combination with deletion of Spt4 (Zhang et al., 2008).The Spn1 and Spt4 proteins have been shown to physically interact and the loss of Spn1 results in misregulation of *CYC1* similar to that observed for the loss of Spt4. This evidence suggests that Spn1 and Spt4-Spt5 complex are involved in a parallel pathway. Previous work in our lab has determined that the binding of Spn1 to the preloaded complex blocks the recruitment of chromatin remodelers (the Swi/Snf complex and Spt6) in the uninduced state and also serves as a platform for recruitment during the activated state (Zhang et al., 2008). Taken together it seems likely that Spt4 could play a role in the poised regulation of *CYC1* and makes Spt4 an interesting lead to follow-up on in the future.
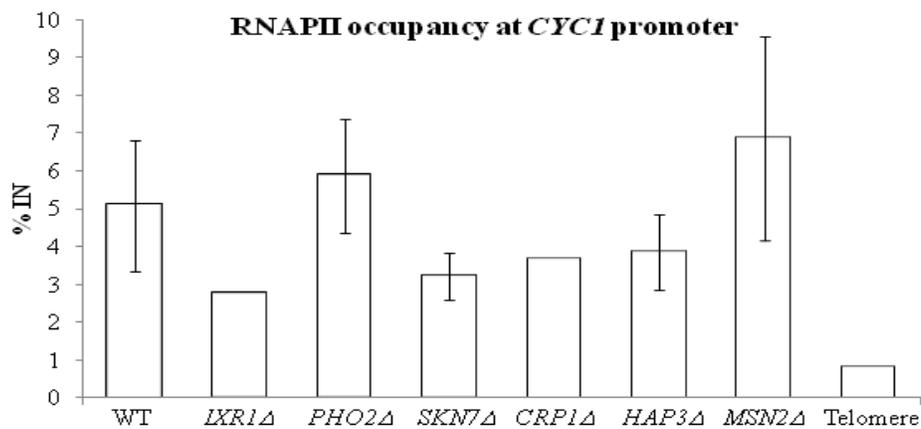
APPENDIX FIGURE A.1. RNAPII occupancy is maintained in multiple transcription factor deletion strains. Poised RNAPII occupancy at the *CYC1* promoter in wildtype and deletion strains (as indicated). Strains with error bars are the average of three independent biological replicates, others are the average of two or less biological replicates. Samples were normalized as the percent of input.
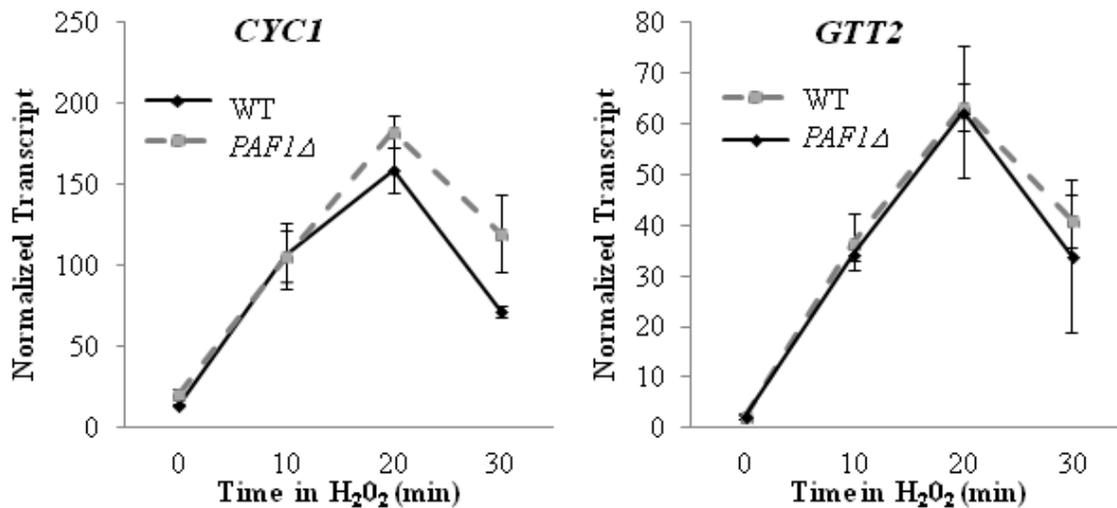
APPENDIX FIGURE A.2. The Paf1 deletion does not impact the transcriptional output of either *CYC1* or *GTT2*. *CYC1* and *GTT2* transcript levels before and at the indicated time after treatment with H_2O_2 using S1 nuclease transcript assays. The wildtype transcript levels are indicated with the solid black line, while the deletion strain is shown as the dotted grey line. Shown is the average of three independent biological replicates normalized to tRNA[W] as an input control.
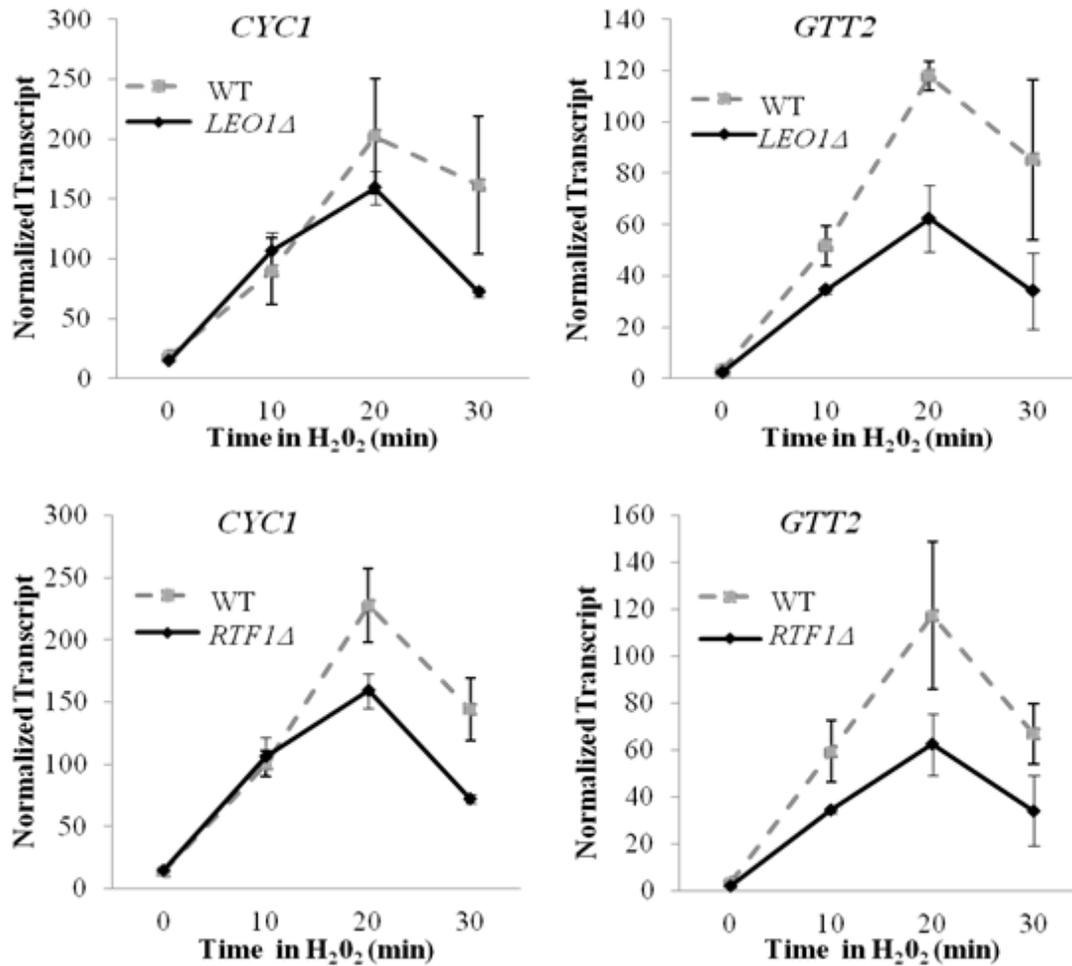
APPENDIX FIGURE A.3. Leo1 and Rtf1 deletions do not differentially impact transcriptional output of *CYC1* and *GTT2*. *CYC1* and *GTT2* transcript levels before and at the indicated time after treatment with H_2O_2 using S1 nuclease transcript assays. The wildtype transcript levels are indicated with the solid black line, while the deletion strains are shown as the dotted grey line. Show n is the average of three independent biological replicates normalized to $tRNA^W$ as an input control.
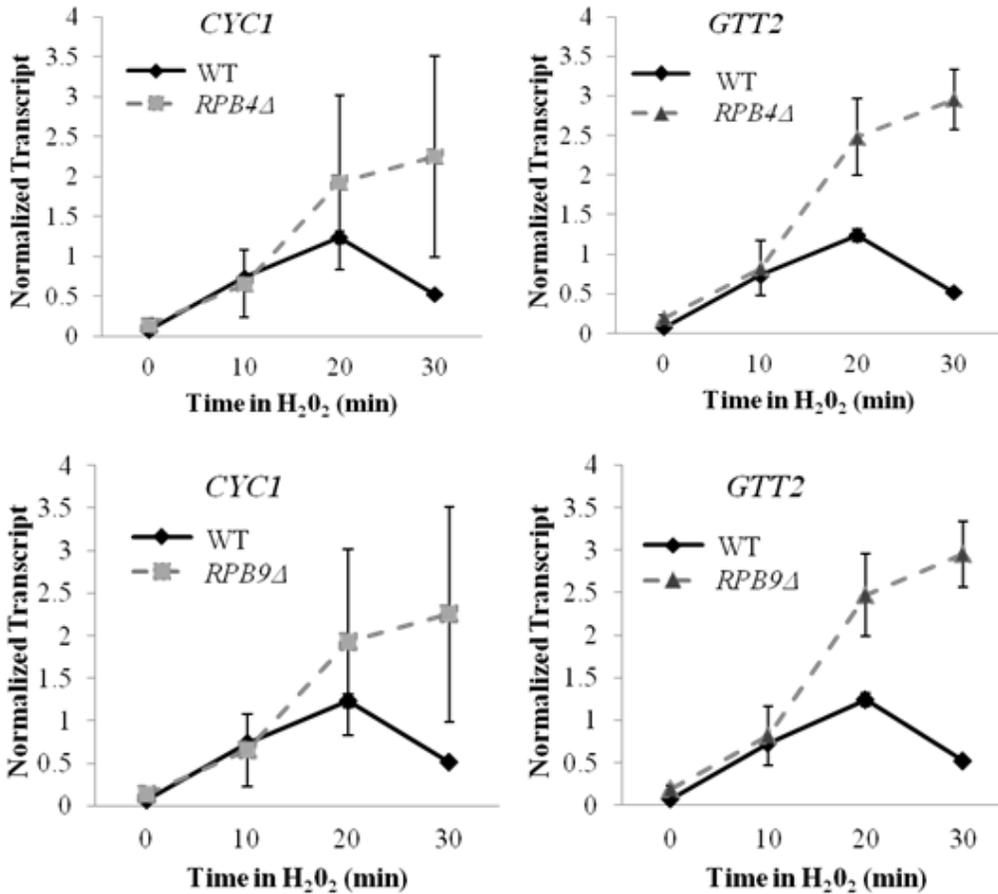
APPENDIX FIGURE A.4. Rpb4 and Rpb9 deletions do not differentially impact transcriptional output of *CYC1* and *GTT2*. *CYC1* and *GTT2* transcript levels before and at the indicated time after treatment with H_2O_2 using S1 nuclease transcript assays. The wildtype transcript levels are indicated with the solid black line, while the deletion strains are shown as the dotted grey line. Shown is the average of three independent biological replicates normalized to tRNA^W as an input control.
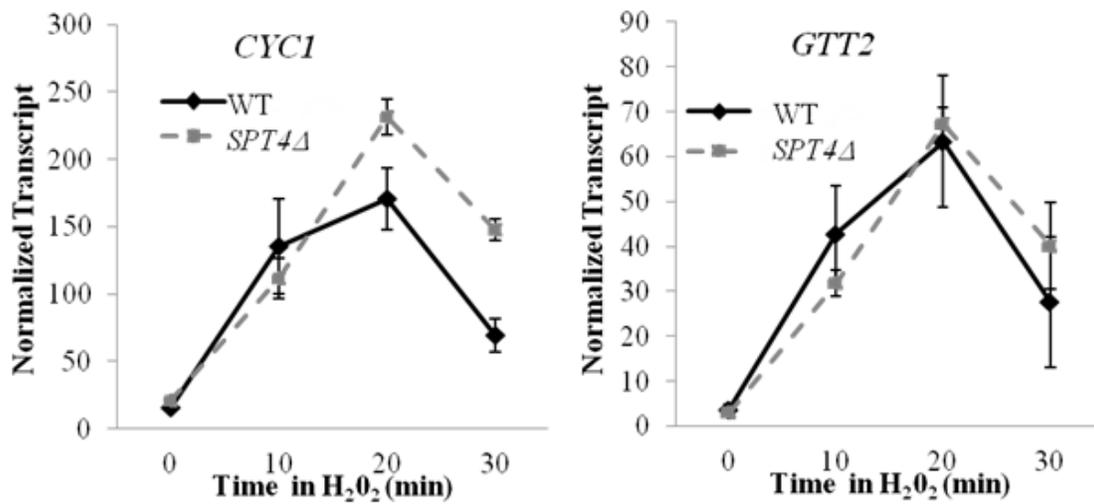
**APPENDIX Figure A.5. Spt4 deletion specifically impacts transcriptional output of *CYC1*
when compared to *GTT2*.** *CYC1* and *GTT2* transcript levels before and at the indicated time
after treatment with H_2O_2 using S1 nuclease transcript assays. The wildtype transcript levels
are indicated with the solid black line, while the deletion strains are shown as the dotted grey
line. Shown is the average of three independent biological replicates normalized to tRNA^W as
an input control.