DISSERTATION

Mechanisms of RNA Polymerase II-Mediated Transcription

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY AARON GLENN LOUIS FLETCHER ENTITLED UNDERSTANDING THE MECHANISM OF RNA POLYMERASE II POST-PIC-RECRUITMENT TRANSCRIPTION REGULATION IN YEAST SACCHAROMYCES CEREVISIAE BE ACCEPTED AS FULLFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

Mechanisms of RNA Polymerase II-Mediated Transcription

Transcription by RNA Polymerase II (RNAPII) is a critical step in controlling biological events such as cell growth, cell differentiation, response to environmental change, homeostasis, and disease. The regulation of transcription initiation of some genes is controlled at the level of TBP and RNAPII recruitment to the promoter. At such genes, the binding of TBP/RNAPII is the rate-limiting step for gene expression. Other genes already have TBP/RNAPII occupying the promoter before induction of gene expression, and the rate limiting step is no longer recruitment of TBP/RNAPII. These genes are collectively known as post-recruitment regulated genes. The yeast *CYC1* gene is a post-recruitment regulated gene and serves as an excellent model for understanding the mechanism behind post-recruitment regulation. A TBP recruitment bypass screen was developed to investigate the mechanism of post-recruitment regulation. The results of the bypass screen revealed that SAGA and Mediator play important roles in post-recruitment regulation. Further analysis of SAGA uncovered a new function: that suggests SAGA is important in recruitment of Mediator to post-recruitment regulated genes. In addition to RNAPII and TBP, the *CYC1* gene was found to have TFIIH, capping enzyme and serine 5 phosphorylation of the RNAPII C-terminal domain occupying the promoter in the uninduced condition. These results indicate that much of the Pre-Initiation

Complex (PIC) occupies the *CYC1* promoter in the uninduced state. In addition to PIC occupancy at *CYC1*, a conserved and essential protein, Spn1, is found to occupy the promoter during uninduced conditions. To further understand the role of this essential protein, genome localization studies and transcription profiling were performed. These studies suggest that in addition to playing an important role in post-recruitment regulation of gene expression, Spn1 may be involved in the transcription of ribosomal proteins. Taken together, this body of work contributes significantly to understanding the regulatory mechanisms of post-recruitment regulation.

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Regulation of Eukaryotic RNA Polymerase II transcription of post-PIC-recruitment genes

1.1 Significance of RNA polymerase II mediated transcription

Each organism must develop from germination to adulthood carefully regulating cell growth throughout the process. In addition, preserving a normal and healthy life is a continual balance of homeostasis within each individual organism. Homeostasis is the maintenance of the internal environment within tolerable limits and a key regulation area is that of protein level. RNA polymerase II transcription is the first echelon of protein level regulation. Improper RNA polymerase II transcription regulation leads to a wide variety of diseases. A greater understanding of RNA polymerase II transcription regulation will arm the medical and scientific world with the right tools needed to combat the thousands of diseases that detract from our quality and longevity of life.

1.1a The essential role of Eukaryotic RNA Polymerase II transcription regulation

The internal environment of a living organism is highly complex as it carries out vital functions such as cell growth, cell tissue differentiation, proper response to environmental change, and disease management. In each of these areas of cellular regulation, the molecular mechanism of transcription regulation

influences the ability to properly maintain homeostasis (Maniatis et al., 1987; Mitchell and Tjian, 1989). In addition, the molecular mechanism of transcription regulation is fundamentally similar in all cell types and environmental situations (McKnight and Yamamoto Eds., 1992). Protein levels throughout different cell types or different environmental situations are first controlled at the level of gene expression (Levine and Tjian, 2003). Therefore, regulation of gene expression is of utmost importance in cellular survival. The RNA polymerase II enzyme is most responsible for the transcription of protein-encoding genes in the nucleus of the cell. The transcribed product creates pre-mRNA, which undergoes extensive processing before being properly targeted to the cytoplasm. In the cytoplasm the mRNA is translated at the ribosome into protein. This process offers numerous opportunities for the cell to properly maintain homeostatic protein levels. The different areas for protein level regulation can be sub-classed as follows: a) chromatin structure b) RNA polymerase II transcription initiation c) RNA polymerase II transcription elongation and termination d) RNA processing e) RNA transport to the nucleus f) mRNA stability g) translation of mRNA h) protein stability i) protein compartmentalizing j) protein activity (Maniatis et al., 1987; Sauer and Tjian, 1997). Due to the coupling of RNA processing and exportation with RNA polymerase II transcription, an important step of protein homeostasis is at the level of transcription regulation (Bentley, 1999; Orphanides and Reinberg, 2002).

1.1b *Saccharomyces cerevisiae* as a eukaryotic model system

The molecular mechanism of RNA polymerase II transcription is fundamentally similar throughout a wide spectrum of organisms ranging from yeast to worms to humans (McKnight and Yamamoto Eds., 1992). In addition, the completion of numerous genome projects have shown that this spectrum of species have conserved genomes. With both fundamentally similar mechanisms and genome similarity of different eukaryotic species, scientists can choose "model" systems. Each model system is used to study the regulation of different functions within the cell and apply the knowledge obtained to *Homo sapiens.* Therefore, model systems provide an avenue by which the scientific community can study the cause and determine potential cures for human diseases without having to undermine ethical principals in doing so. These model systems are easier to manipulate, often have high reproduction rates, and are more cost effictive than human systems.

The yeast *Saccharomyces cerevisiae* provides an unique opportunity for studying the regulation of eukaryotic transcription by RNA polymerase II *in vivo*. The first eukaryotic organism to have its full genome sequenced was that of *S. cerevisiae* in 1996. The sequenced genome can be easily obtained for scientific research at (http://www.yeastgenome.org). In addition to the genome sequence, the yeast genome database (YGD) has collated copious amounts of information from scientists who have used *S. cerevisiae* as a model system in the past. This gives the scientific community a powerful tool to efficiently study eukaryotic fundamental mechanisms.

S. cerevisiae have short generation times for eukaryotes (90 minutes) and are capable of haploid propagation. The ability to propagate in the haploid state makes phenotypic analysis very simple. *S. cerevisiae* also have unique genetic tools for conducting research; one such tool is the *S. cerevisiae* deletion collection. The deletion collection consists of ~4,600 strains (approximately 73% of the *S. cerevisiae* genome), each having one non-essential open reading frame replaced with a kanamycin resistance-conferring (kan^R) cassette (Giaever et al., 2002). The kan^R allows selection for the deletion strain by growing on medium containing the drug G418. The deletion collection of *S. cerevisiae* can be used to see what changes occur in the homeostasis of the cell due to the loss of that one gene and corresponding product. The deletion collection also provides a way to create unique genetic studies that are not possible in mammalian systems. These genetic assays are significantly more cost efficient in *S. cerevisiae* model systems than mammalian systems.

1.2 Fundamentals of transcription activation of RNA polymerase II

During the last half century the combined effort of scientists in the fields of genetics, biochemistry, molecular biology, and biophysics, have deeply broadened our understanding of RNA polymerase II mediated transcription. Vast spectrums of genes, promoters, proteins, organisms and experimental assays have been carried out to provide us with a "big picture" view of transcription. From this work it is known that trans-acting transcription factors occupy cis-acting elements of the DNA sequence. In addition, chromatin structure helps to

determine which genes are exposed for transcription activation (Hampsey, 1998; Lee and Young, 2000; Orphanides and Reinberg, 2002; Sims et al., 2004; Svejstrup, 2004). The information obtained over the last half century is astounding and enormous. I will focus primarily on the key aspects of RNA polymerase II mediated transcription of *S. cerevisiae* that pertain to my doctoral studies.

1.2a Protein-encoding gene promoter architecture.

The DNA of protein-encoding genes can be divided into several sections: the core promoter, the initiator element (Inr), upstream activating sequences (UAS), open reading frame (ORF) and the terminator region (Blackwood and Kadonaga, 1998; Struhl, 1989; Struhl, 1995) (Figure 1.1).

The UAS is occupied by gene-specific regulatory elements that bind upstream (to the 5' end) of the core promoter. The UAS is defined as a 10-30 base pair (bp) promoter-specific sequence that is typically located 50-500 bp upstream of the +1 site (Guarente and Hoar, 1984). The UAS is bound by sequence specific activator proteins that help to enhance transcription and often help to stimulate pre-initiation complex (PIC) assembly (Blackwood and Kadonaga, 1998; Buratowski, 2000). The UAS sequence can conversely be occupied by repressors which help to repress transcription (Huang et al., 1997; Pazin and Kadonaga, 1997). The core promoter is where the TATA element is located (Chen and Struhl, 1988; Wobbe and Struhl, 1990). The TATA element is an AT-rich region that is located 40-120 bp upstream of the +1 site (Singer et al.,

1990) and it is estimated that 20% of genes contain the canonical TATA-element TATAA (Basehoar et al., 2004). TATA-binding protein binds to the TATA element of canonical sequences, and at the core promoter of non-canonical sequences via protein-protein interactions (Burke and Kadonaga, 1997). The core promoter defines the region in which the pre-initiation complex (PIC) assembles and the binding of TBP nucleates this process. The Initiator element (Inr) is the sequence that surrounds the transcription start site (tis) or +1. The location of the Inr determines where transcription will begin on the gene and is also functional in the assembly of the PIC, particularly in TATA-less (non-canonical) elements (Burke and Kadonaga, 1997; Struhl, 1995; Weis and Reinberg, 1992). The open reading frame is where the protein coding portion of the gene is found. Directly following the ORF is the termination region. The termination region is the sequence of the DNA that signals polyadenylation and cleavage of the nascent RNA and triggers the end of the transcription process (Kerppola and Kane, 1991; Kim et al., 2004b). Additional factors involved in the regulation of transcription include silencers, enhancers, and insulators (Lee and Young, 2000).

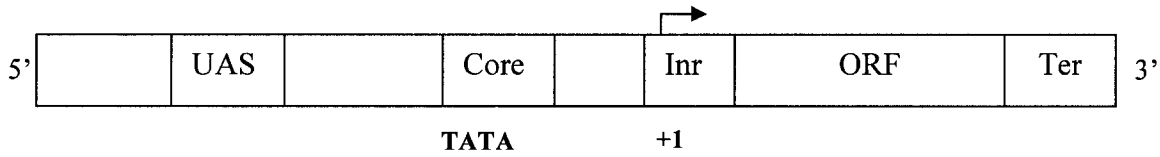


Figure 1.1: Simplified view of a typical Eukaroytic RNA polymerase II promoter broken down into: Upstream Activating Sequences (UAS), Core promoter (Core), Initiator element (Inr), Open Reading Frame (ORF), and the Terminator element (Ter). Each of these sections are required to properly regulate the transcription process.

1.2b The role of chromatin structure in RNA polymerase II transcription

1.2b.1 The organization of chromatin

In vivo, the DNA is neatly packaged within the nucleus. The nucleosome is the basic organization unit to package the DNA by wrapping 146 bp 1.65 times around a core histone octomer. The histone octomer consists of two copies of four different proteins: H2A, H2B, H3, and H4 (Kornberg, 1974; Luger et al., 1997). The histones of the nucleosome interact with the DNA phosphate backbone and the amino-terminal tails of the histones extend beyond the nucleosome to interact with other adjacent nucleosomes (Luger et al., 1997). The linker histone H1 helps to further compact the DNA by bringing the nucleosomes together in higher order structure (Ramakrishnan, 1997; Schwarz et al., 1996). Not all DNA in the genome is packaged equally. Areas of the genome that are highly compacted are called heterochromatin. In contrast, areas of the genome that are more accessible to transcription factors are less dense and called euchromatin (Babu and Verma, 1987; Kimura and Horikoshi, 2004).

1.2b.2 Factors involved in the modification of chromatin

DNA must be available at appropriate times to the transcription machinery to maintain proper homeostasis. The way in which chromatin is assembled allows the DNA to be accessed by disruption of the DNA-histone contacts within the nucleosome. There are two predominant strategies the cells deploy to activate genes embedded within the nucleosome. The first is that of covalent

modifications of the histones and the second is ATP-dependent chromatin remodeling (Kornberg and Lorch, 1999; Lee and Young, 2000).

Histone modification can be broken down into four different classes based on enzymatic activity and include: histone acetyltransferases (HATs) (Hampsey, 1997b; Struhl, 1998), histone deacetylases (HDACs) (De Rubertis et al., 1996; DeRubertis et al., 1996; Rundlett et al., 1996), histone methyltransferases (Ng et al., 2003), and histone kinases (Lee and Young, 2000). HATs are the most understood of the enzymatic classes and are most relevant to my thesis work. Acetylation of histones by HATs occurs at lysine residues located on the N-terminal tails of histones and this modification helps to mark activation of gene expression. Acetylation of histone N-terminal tails results in neutralization of positively charged lysines. This neutralized histone now has less affinity for the negatively charged phosphate backbone of DNA and the DNA is made available for transcription machinery nucleation and expression of the gene (Hampsey, 1997b; Kim et al., 2002; Struhl, 1998). In *S. cerevisiae* a number of protein complexes have been implicated in HAT activity including SAGA, ADA, HAT1, NuA3/4, and Elongator.

ATP-dependent chromatin remodeling complexes release the DNA from nucleosomes by altering the chromatin conformation. Mobilization of nucleosomes occurs by breaking and reforming DNA-histone contacts. Each ATP-dependent remodeling complex contains an ATPase subunit that is essential for hydrolyzing ATP to provide energy for the remodeling activity (Kingston and Narlikar, 1999). Complexes involved in ATP-dependent chromatin

remodeling include Swi/Snf, RSC complexes, INO80, ISW1/2, and CHD1 (Vignali et al., 2000). Swi/Snf is an ATP-dependent remodeling complex relevant to my doctoral studies. Swi/Snf is a 2 MDa molecular weight protein complex composed of 11 subunits (Peterson and Tamkun, 1995; Smith et al., 2003; Sudarsanam and Winston, 2000). The ATPase enzymatic protein of Swi/Snf is Snf2 (Peterson and Herskowitz, 1992). Snf5 is essential in maintaining Swi/Snf complex assembly (Geng et al., 2001). In addition, proper function of Snf2 requires both the Snf5 and Snf6 subunits (Cairns et al., 1994; Geng et al., 2001; Laurent and Carlson, 1992). The Swi/Snf complex has been shown to remodel nucleosomes by either sliding the histone octomer on the same DNA template or by transferring the histone octomer to another adjacent DNA template (Lorch et al., 1999; Sudarsanam and Winston, 2000).

1.2c The transcription cycle of RNA polymerase II

The transcription cycle of RNA polymerase II can be broken down into five separate events: assembly of the PIC, initiation, promoter clearance, elongation, and termination (Figure 1.2).

1.2c.1 The assembly of the pre-initiation complex

After chromatin modifiers have made the DNA sequence available the recruitment of the PIC can begin (Lee and Young, 2000). UAS sequences and their respective activator proteins have also been shown to be fundamental in helping set up the PIC (Guarente and Hoar, 1984). Biochemical analysis has shown that the formation of

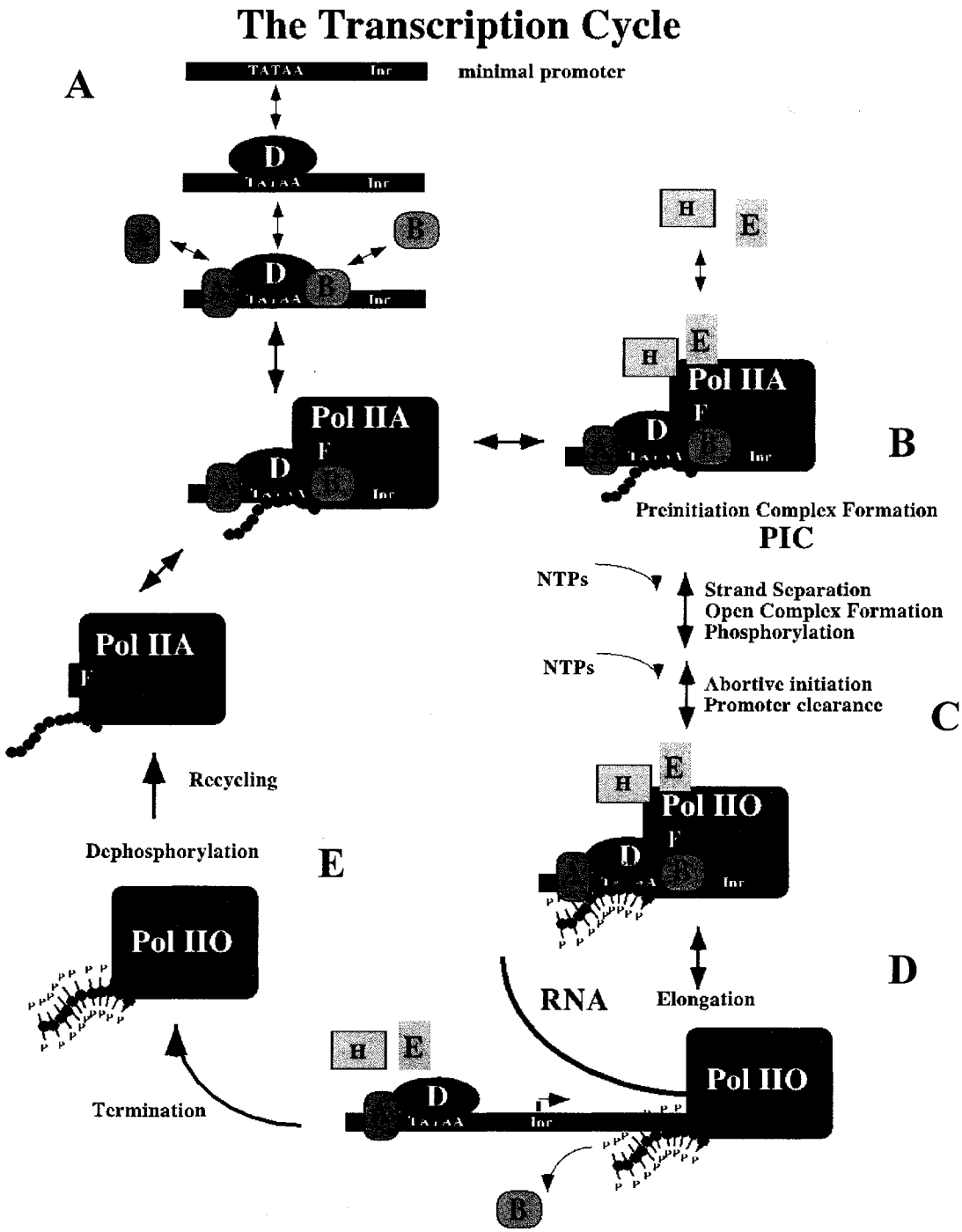


Figure 1.2: The transcription cycle.

- **A)** TBP/TFIID binds to the core promoter and begins the nucleation of the PIC.
- **B)** Once the PIC has formed the helicase activity of TFIIH melts the DNA and forms the transcription bubble, and phosphorylation of RNA Pol II CTD occurs.
- **C)** The transcription machinery is now ready to clear the promoter.
- **D)** Elongation begins with TFIID remaining at the promoter to allow for reinitiation.
- **E)** Termination of the transcript leads to the recycling of the GTFs for the next initiation process.

a functional initiation complex can be dependent on the TATA element, and a number of general transcription factors (GTFs). A number of genes are TFIID dependent while others are SAGA dependent (Chapter 1.4). The dependence on TFIID or SAGA is mutually exclusive (Huisinga and Pugh, 2004). TFIID, a multi-subunit general transcription factor consisting of the TATA binding protein (TBP) and at least a dozen TBP associated factors (TAFs), that is recruited to the core promoter (Hernandez, 1993; Struhl, 1994). These factors, along with a number of GTFs: TFIIA, TFIIB, TFIIF, RNA polymerase II, TFIIE, and TFIIH (Hampsey, 1998; Kornberg, 2001; Kuras and Struhl, 1999; Lee and Young, 2000) nucleate to form the pre-initiation complex which is now ready for transcription (Figure 1.2a). In addition to GTFs, coactivators, such as Mediator, are often recruited to the promoter to aid in the initiation of gene expression (Fan et al., 2006; Kornberg, 2005). Mediator is a large multi-subunit complex that is thought to relay information between activators and RNA Pol II (Chapter 1.3). In the majority of genes, initiation of transcription is regulated by the recruitment of TBP and RNA Pol II to the promoter DNA; in such cases, the level of TBP occupancy is positively correlated with the level of transcription activity (Kim and Iyer, 2004).

1.2c.2 Transcription Initiation

TFIIH is the last general transcription factor to join the preinitiation complex (Cortes et al., 1992) and has two important enzymatic functions, helicase and kinase, for initiation of transcription (Feaver et al., 1991; Lu et al.,

1992; Schaeffer et al., 1993; Serizawa et al., 1992; Serizawa et al., 1993b). RAD3 is the ATP-dependent subunit of *S. cerevisiae* TFIIH with helicase enzymatic activity (Goodrich and Tjian, 1994; Kim and Iyer, 2004; Kim et al., 2000). The helicase activity of TFIIH melts the double stranded DNA to form the transcription bubble. Once the pre-initiation complex is assembled, transcription is ready to initiate. The RNA polymerase II now reads the single stranded DNA and the first phosphodiester bond of nacent RNA is formed when two nucleotide triphosphates (NTPs) are added to the nascent RNA (Figure 1.2b). These NTPs are dictated by the DNA sequence. RNAPII will synthesize and release short RNA transcripts ~2 to 9 nucleotides in length and is known as abortive intiation.

1.2c.3 Promoter Clearance

Now that the nascent RNA synthesis has begun, the RNA polymerase II enzyme must clear the promoter to transcribe the ORF (Dvir et al., 1996). During promoter clearance, the PIC disassembles and RNA polymerase II and TFIIF are able to escape the promoter and begin the initiation process (Yudkovsky et al., 2000; Zawel et al., 1995). A subset of GTFs are left behind on the core promoter to serve as a scaffold for future PIC formation during reinitiation of transcription. The beginning of promoter clearance is hallmarked by phosphorylation of the C-terminal domain (CTD) of the RNA polymerase II (Figure 1.2c). Yeast CTD consists of 26 heptad repeats of the sequence YSPTSPS in which serine 2 and 5 are targets for phosphorylation (Dahmus, 1996). Typically, serine 5 is phosphorylated by the TFIIH subunit *KIN28* during promoter clearance

(Hengartner et al., 1998) and can be enhanced by the Mediator complex (Akoulitchev et al., 2000; Drapkin et al., 1994). Serine phosphorylation of the CTD has also been shown to enhance interactions with numerous transcription factors: Ceg1, yeast 5' capping enzyme (Kim et al., 2004a; Proudfoot et al., 2002; Rodriguez et al., 2000; Yue et al., 1997); pre-mRNA processing factors (Proudfoot et al., 2002); and RNA export factors (Luo and Reed, 1999). Now that the first round of promoter clearance has occurred, the next round of transcription is reinitiated and is responsible for the greater part of transcription in the cell (Jiang and Gralla, 1993; Orphanides, 2000; Ranish et al., 1999). If promoter clearance is not accomplished then transcription is aborted.

1.2c.4 Transcriptional Elongation

The forth step in transcription is that of elongation, which involves the passage of RNAPII through the protein coding region. A number of elongation factors have been identified. In *S. cerevisiae* some well-characterized elongation factors include: TFIIF(Tan et al., 1995), TFIIS (Jeon and Agarwal, 1996), Elongins (Elmendorf et al., 2001), Elongator (Krogan and Greenblatt, 2001), FCP1 (Cho et al., 2001), DSIF (Wada et al., 1998), Spt6 (Bortvin and Winston, 1996; Neigeborn et al., 1986), and Paf1 (Shi et al., 1996; Wade et al., 1996). While transcribing, RNA polymerase II encounters pausing and arresting sites within the DNA sequence. The elongation factors contribute to the overall efficiency of transcription by helping the polymerase to overcome these pause sites and to stimulate the rate of transcription elongation (Reines et al., 1999).

TFIIF is part of the PIC that cleared the promoter with RNA polymerase II. It serves an elongation factor and by helping to reduce the time RNAPII remains paused (Tan et al., 1994) (Figure 1.2d). The general transcription factor TFIIF subunit, FCP1, has phosphatase enzymatic activity that targets the RNAPII CTD and provides additional stimulation to elongation in vitro (Chambers et al., 1995; Cho et al., 1999; Kamada et al., 2003). TFIIS, a general transcription factor, promotes RNA polymerase II to read through arrest sites found throughout the DNA sequence (Awrey et al., 1997; Kulish and Struhl, 2001).

The ORF also has nucleosomes that must be navigated through by the polymerase during transcriptional elongation. To help ensure efficient elongation, the nucleosomes are transferred by a number of chromatin remodeling factors. Many of these factors play a larger role at a certain genes compared to others but their roles are to ensure proper elongation through the protein-coding region of the gene. Many of the factors mentioned earlier in transcription initiation also play a chromatin remodeling function during elongation, such as SAGA, Swi/Snf, Spt4/5 (DSIF), Spt6, and FACT. At a genome level, elongation is often coupled with the level of H3 acetylation (De Rubertis et al., 1996). Swi/Snf seems to traverse along with elongating RNAPII to remodel chromatin during elongation (O'Brien and Lis, 1991; Ossipow et al., 1999; Wilson et al., 1996). In vitro, FACT has been shown to be required for RNAPII to escape the promoter on chromatin template (Bortvin and Winston, 1996; Formosa et al., 2001), and FACT, along with Spt6, also helps to remove nucleosomes in the path of RNAPII and re-deposit them after RNAPII has

passed (Belotserkovskaya et al., 2003; Kaplan et al., 2003; Saunders et al., 2003). SAGA has also been implicated in having a role on elongation efficiency of *GAL1* by the HAT activity of the Gcn5 subunit (Govind et al., 2007).

1.2c.5 Termination of transcription.

Now that the polymerase has effectively transcribed the coding region of the gene, it is ready to terminate the transcription process (Figure 1.2e). During this state of transcription the mRNA is cleaved, polyadenylated, and transported to the cytoplasm. In the cytoplasm the mRNA undergoes the translation process of synthesizing protein from the RNA template at the ribosome (Proudfoot et al., 2002). The termination of RNAPII transcription is thought to be coupled with the maturation of 3' mRNA ends at the poly (A) site (Proudfoot, 2004). Studies have supported this idea by illustrating the consensus sequence of poly (A) is essential in triggering termination (Logan et al., 1987; Zaret and Sherman, 1982). Recent studies have also shown that RNAPII is knocked off the DNA template by exonucleases to elicit transcriptional termination (Kim et al., 2004b; West et al., 2004) . When the nascent RNA is cleaved at the poly (A) site, the exonuclease is recruited by RNA 3'-end processing factors. In this model of termination these 3'-end processing factors are associated with the CTD of RNAPII. Once the 3'-end processing factors have recruited exonuclease to the RNA 3' end, it begins to rapidly degrade RNA until it reaches the transcribing RNAPII and terminates transcription. As a whole, the termination process is not precise and the polymerase usually terminates at multiple positions located hundreds or

thousands of bases downstream of the poly (A) site, even after releasing the nascent RNA transcript (Sims et al., 2004).

1.3 The role of Mediator in RNA polymerase II transcription

1.3a Mediator function.

Coactivator proteins play an important role in transcription regulation of RNA polymerase II. Of particular interest to my doctoral studies is that of the coactivator Mediator. Mediator was first discovered in *S. cerevisiae* when it was observed that over-expression of one activator protein led to interference in activation of RNAPII by another activator, known as squelching (Gill and Ptashne, 1988). The reason for the squelching observation was due to the activators competing for a common target. This target was initially thought to be a direct interaction between the activator and the PIC and was supported by evidence that activators can bind with TPB, TFIIH, and RNA Pol II (Brandl and Struhl, 1989; Stringer et al., 1990; Truant et al., 1993). It was then shown that this direct mechanism may not be all that occurs via analysis of squelching in a crude yeast RNAPII transcription system in vitro (Kelleher et al., 1990). It was shown that the addition of excess amounts of GTFs or RNAPII did not relieve squelching and argued against these proteins being the direct targets of activators. However, if crude yeast protein fraction was added to the in vitro system, squelching was relieved. This yeast protein fraction was called Mediator. Mediator was later purified to homogeneity (Kim et al., 1994) from a complete fractionated yeast

RNAPII transcription system (Flanagan et al., 1991). In this purification Mediator was isolated as two independent forms, Mediator alone or in a complex with RNA polymerase II. Mediator was shown to help in the response of transcriptional activators and to stimulate basal transcription in the absence of an activator (Kim et al., 1994).

1.3b The composition of Mediator.

Mediator complex consists of 25 subunits that create a total mass greater than one million Daltons. Fifteen of the 25 subunits are nonessential proteins; therefore deletion of a single specific gene does not reduce cell viability. Due to the vast size of the protein a high resolution X-ray crystal structure has not yet been solved. Mapping of protein-protein interactions as well as biochemical and EM analyses have provided low resolution structures of Mediator (Asturias et al., 1999; Dotson et al., 2000; Guglielmi et al., 2004; Sato et al., 2003). Mediator can be subcomplexed into four distinct regions: Head, Middle, Tail, and Cdk8 (Guglielmi et al., 2004) (Figure 1.3). The Head region of mediator has been shown to interact with RNAPII (Ranish et al., 1999); the Middle region also contributes to Mediator RNAPII interactions. The Tail region of Mediator interacts with a variety of activator proteins, and the Cdk8 region has a kinase enzymatic domain that is thought to phosphorylate the CTD of RNAPII (Kim et al., 1994).

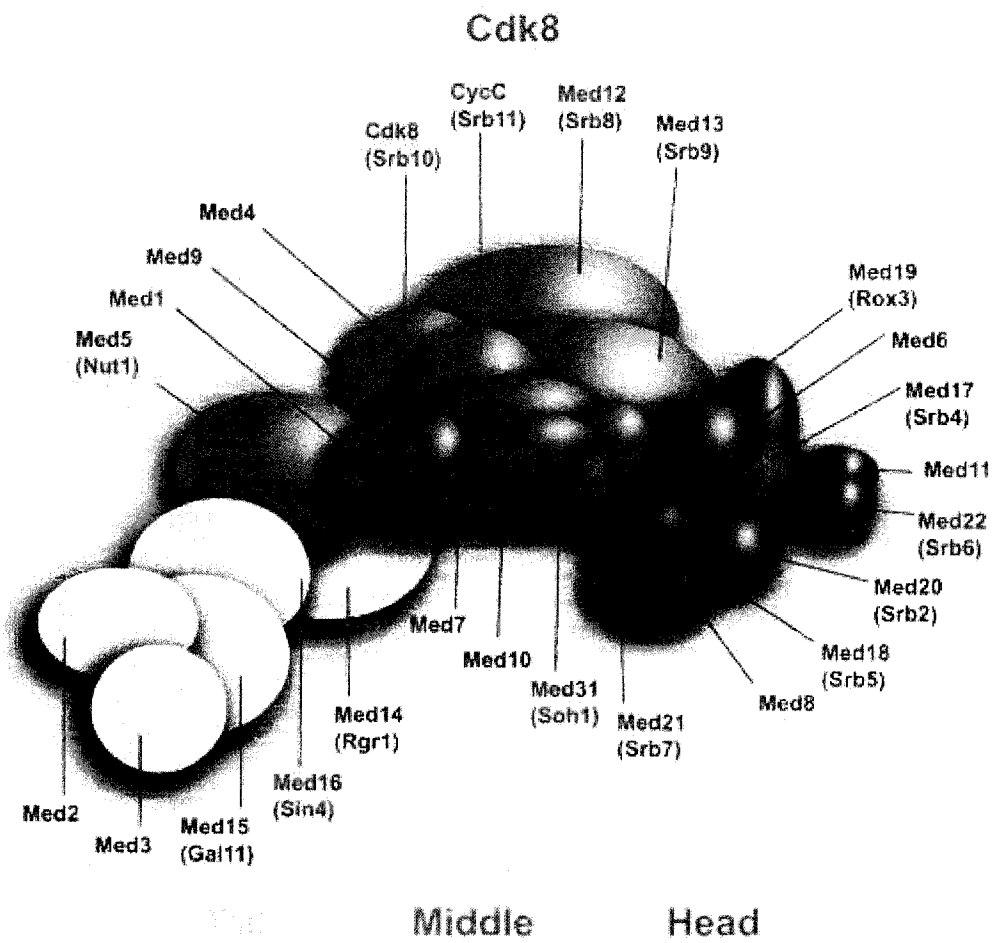


Figure 1.3: The Mediator complex consists of 25 subunits with a molecular weight of 1MDa. This multi-protein complex consists of three groups: Head, Middle, Tail, and Cdk8. the and Middle modules interact with RNA Pol II, while the Tail module interacts with activator proteins. The Cdk8 module has kinase enzymatic activity. Of the 25 subunits 15 are essential for cell viability. (Guglielmi et al., 2004)

1.3c Mediator function in transcription activation and eukaryotic conservation.

Mediator is most well characterized in *S. cerevisiae* (Borggrefe et al., 2002; Kang et al., 2001; Myers and Kornberg, 2000); however Mediator is conserved throughout eukaryotes. Mediator genes have also been investigated in *Schizosaccharomyces pombe, Caenorhabditis elegans,* and *Drosophila melanogaster* (Boube et al., 2000; Gu et al., 2002; Rachez and Freedman, 2001; Spahr et al., 2003). Human cells have homologs to nearly all *S. cerevisiae* Mediator subunits (Boube et al., 2002; Bourbon et al., 2004; Sato et al., 2004) and biophysical studies have shown that these homologous subunits are similar in size, shape and internal structure (Davis et al., 2002; Dotson et al., 2000).

Mediator has been shown to interact with both activators and RNAPII (Naar et al., 1998) and, therefore, is thought to assist in gene expression by relaying information between activator proteins and the PIC. Mediator has been shown to have activator-dependent recruitment to the promoter (Bhoite et al., 2001; Bryant and Ptashne, 2003; Cosma et al., 2001; Cosma et al., 1999; Kuras et al., 2003). Though Mediator and RNAPII have been shown to form a complex in solution (Kim et al., 1994; Myers and Kornberg, 2000; Taatjes and Tjian, 2004) promoter occupancy of Mediator is not strictly correlated with that of RNAPII (Cosma et al., 2001; Fan et al., 2006; Park et al., 2001). Mediator has been shown to stimulate basal transcription from core promoters even when RNAPII is not limiting (Kim et al., 1994). In humans Mediator is essential for proper core promoter activity (Baek et al., 2002; Mittler et al., 2001; Wu et al., 2003b). The

general transcription factor TFIIH has a cordial relationship with Mediator, which can stimulate the kinase activity of the TFIIH subunit Kin28 (Giot et al., 2003; Sakurai and Fukasawa, 2000). Mediator has also been implicated to have a regulatory role in the activation of post-recruitment in *Drosophila* heat shock proteins as well. The activator to *Drosophila HSP70* (Heat Shock Factor) and mediator are not recruited to the promoter until activation. The activator-mediator complex is recruited in a RNAPII free form and is mechanistically separable from the assembly of the preinitiation complex (Park et al., 2001).

1.4 The role of SAGA in RNA polymerase II transcription

1.4a SAGA function

As previously mentioned, chromatin plays an important role in properly maintaining a homeostatic environment within the cell. The packaging of DNA into chromatin is generally regarded as repressive to activation of transcription and in general can affect each step of RNAPII transcription because efficient transcription requires access to the DNA template (Kornberg and Lorch, 1992; Kornberg and Lorch, 2002; Svejstrup, 2004). Therefore, chromatin modifying proteins play an important role in transcription regulation of RNA polymerase II. Of particular interest to my doctoral studies is the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex.

Spt proteins are a group of transcription factors that are highly conserved throughout evolution and have a diverse presence in the different steps of the

transcription cycle, as well as a variety of other biological processes (Winston, 1992; Yamaguchi et al., 2001). Fred Winston and co-workers developed a genetic screen to identify suppressors that could restore transcriptionally deficient mutants caused by the insertion of the yeast transposon, Ty, in the upstream region of *HIS4* or *LYS2* gene. Selection for *spt* mutants in these strains was carried out by looking for His+ or Lys+ revertants and this screen has led to the identification of over two dozen SPT genes (Winston et al., 1984a; Winston et al., 1987; Winston et al., 1984b; Yamaguchi et al., 2001).

The yeast SAGA complex is a multiple subunit complex comprised of TAFs, Ada proteins, and Spt proteins. This large multi-protein complex is required for normal transcription in ~10% of yeast genes (Lee et al., 2000). The mammalian SPT3-TAF_{II}31-$GCN5_L$ acetylase (STAGA) transcription coactivator complex is the human homolog of yeast SAGA complex (Martinez et al., 1998; Martinez et al., 2001). The role of SAGA has been extensively studied at the *S. cerevisiae* gene *GAL1* (Bhaumik and Green, 2001; Bhaumik and Green, 2002; Bhaumik et al., 2004; Dudley et al., 1999; Larschan and Winston, 2001; Roberts and Winston, 1997; Sterner et al., 1999). The activator for the *GAL1* promoter, Gal4, recruits SAGA to the upstream activating sequence (UAS). Gal4/SAGA at the UAS facilitates recruitment of TBP to the core promoter, thereby stimulating formation of preinitiation complex (PIC) and hence transcription (Bhaumik and Green, 2001; Bhaumik et al., 2004; Larschan and Winston, 2001). One of the SAGA subunits, Gcn5, contains a histone acetyltransferase that acelylates the N-terminal tail of histone H3 (Grant et al., 1999; Kuo et al., 2000). Taken together

the SAGA complex to date has been shown to help regulate the recruitment of TBP (Belotserkovskaya et al., 2000; Bhaumik and Green, 2001; Dudley et al., 1999; Larschan and Winston, 2001; Qiu et al., 2005) and acetylate chromosomal histones (Brownell et al., 1996; Grant et al., 1997; Grant et al., 1998b; Utley et al., 1998)

1.4b The composition of SAGA

SAGA is a large multi-protein complex that contains Ada1, Ada2, Ada3, Ada5/Spt20, Spt3, Spt7, Spt8, Gcn5, Tra1, TAF5, TAF6, TAF9, TAF10, TAF12, Ubp8, Chd1 and Sgf11 (Brown et al., 2000; Daniel et al., 2004; Grant et al., 1998a; Hampsey, 1997b; Henry et al., 2003; Ingvarsdottir et al., 2005; Lee et al., 2005; Powell et al., 2004; Pray-Grant et al., 2005; Sanders et al., 2002), and has a combined molecular weight of 1.8 MDa (Figure 1.4a). Recently, Sgf73 has been biochemically implicated as a new component of SAGA (McMahon et al., 2005; Shukla et al., 2006). Studies have shown that Spt7, Spt20 and Ada1, together with several TAFs, are the core subunits of the SAGA complex and deletion of any of these subunits disrupts the structural integrity of the SAGA complex (Dudley et al., 1999; Grant et al., 1998a; Horiuchi et al., 1997; Wu et al., 2004; Wu and Winston, 2002). Gcn5 is the HAT subunit of the SAGA complex and its function is modulated by two other subunits, Ada2 and Ada3 (Balasubramanian et al., 2002; Candau et al., 1997; Grant et al., 1997; Horiuchi et al., 1995; Ruiz-Garcia et al., 1997). The structure of the SAGA complex has not been solved at the atomic level. However, a 3D structure of SAGA has been

determined by electron microscopy (Wu et al., 2004). The SAGA complex has five modular domains that have a high degree of structural conservation with the human homolog of SAGA (Figure 1.4b).

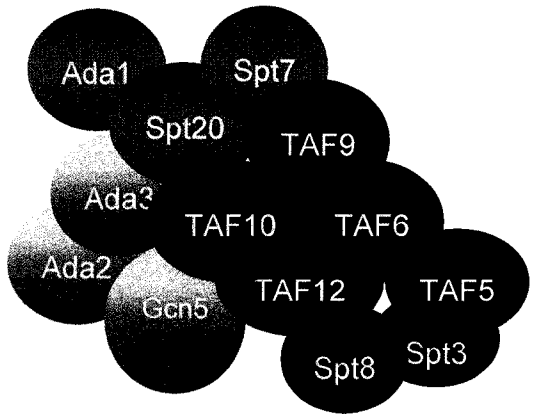
1.5 Defining recruitment and post-recruitment regulated gene activation.

1.5a Recruitment regulated gene activation.

Transcription initiation is the level where most eukaryotic gene expression is regulated (Iyer and Struhl, 1996; Klein and Struhl, 1994; Kuras and Struhl, 1999). RNAPII gene expression can be activated by the binding of a single activator protein to the UAS, while other activators (AP-1, ATF/CREB, Swi5/Ace2) require the binding of multiple proteins (McNabb et al., 1995; Primig et al., 1992). After the activator(s) have bound to the UAS they stimulate gene expression by a transcription activation domain that is functionally distinct, and usually physically separable, from the DNA-binding domain (Ptashne and Gann, 1997).

A

- SAGA integrity: Spt7, Spt20 and Ada1
- HAT activity: Gcn5
- HAT-related: Ada2 and Ada3
- TBP interaction: Spt3 and Spt8



1.8 MDa complex

B

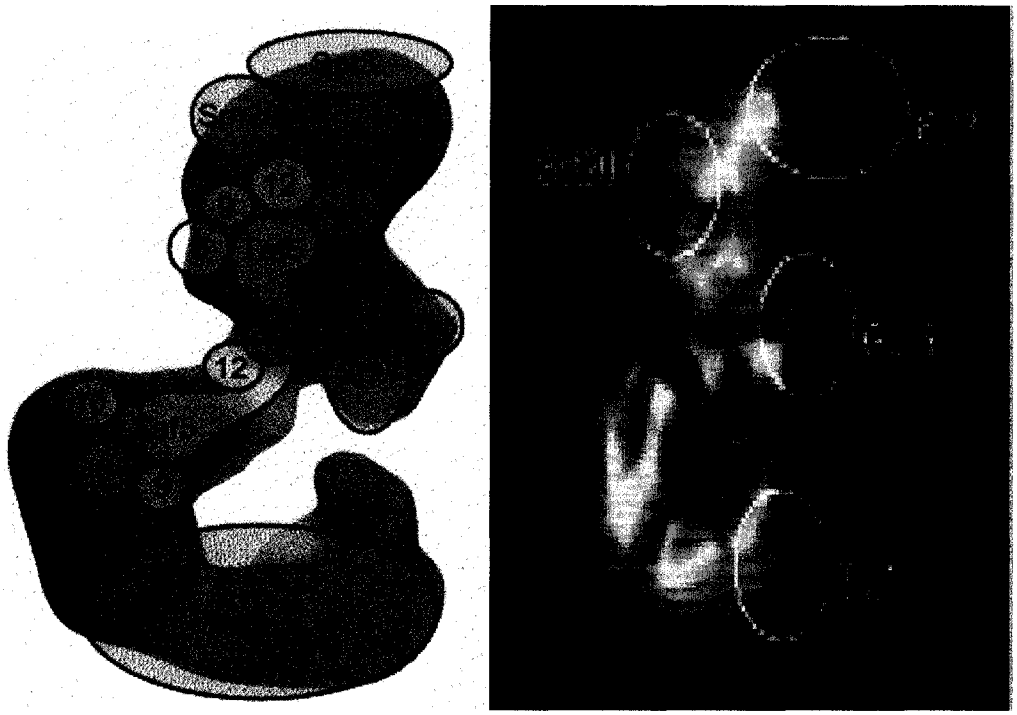


Figure 1.4a: A schematic drawing of the 1.8MDa multi-subunit SAGA complex. Subunits are grouped according to similar function and not spatial representation. Subunits important in SAGA integrity are shown in purple and TBP associated factors present in the SAGA complex and shown in green. The two functions to date of SAGA are shown in gold and red.
1.4b: Spatial representation of the SAGA complex. Immunoblotting of Tra1, Gcn5, Spt20, Spt3 in combination of a number of mutants and electron microscopy led to solving the 3D representation of SAGA (Wu et al., 2004).

The activator proteins serve to recruit the PIC on the promoter DNA. This has been shown both in vitro (Lin and Green, 1991) and in vivo (Kuras and Struhl, 1999). In the majority of genes, initiation of transcription is regulated by the recruitment of TBP and RNAPII to the promoter DNA. In such cases, the level of TBP occupancy is positively correlated with the level of transcription activity (Kim and Iyer, 2004; Kuras and Struhl, 1999; Li et al., 1999). At such genes, the rate-limiting step of transcription is the ability to recruit TBP to the promoter and initiate nucleation of the PIC. Artificial recruitment experiments have shown that fusing a subunit of the general transcription machinery to a DNA-binding domain can allow for the assembly of the PIC on promoter DNA (Ptashne and Gann, 1997). Therefore, functions of these factors can be defined as "recruitment-functions" due to their ability to recruit TBP/TFIID to the promoter and nucleate the formation of the PIC. Throughout my thesis functions that promote the assembly of the PIC will be referred to as recruitment-functions. Genes in which the rate limiting step for gene expression is after the recruitment of TBP/RNAPII are referred to as post-recruitment regulated genes.

1.5b Post-recruitment regulated gene activation.

1.5b.1 Post-recruitment regulation definition

In the case of recruitment regulated genes, initiation of transcription is regulated by the recruitment of TBP and the PIC to the promoter DNA. In such cases, the level of TBP occupancy is positively correlated with the level of transcription activity (Kim and Iyer, 2004; Kuras and Struhl, 1999; Li et al., 1999).

However, a growing number of genes have a high level of TBP and RNAPII bound without a high level of transcription activity (Kuras and Struhl, 1999; Martens et al., 2001) and are therefore post-recruitment regulated (Figure 1.5). In post-recruitment regulation, TBP/RNAPII recruitment is no longer the rate limiting step to initiate transcription. Post-recruitment regulated genes must undergo a step after the recruitment of TBP and RNAPII and that step serves to be the rate limiting step. Many of the genes that are post-recruitment regulated are important in a variety of environmental responses that are needed for homeostasis. It is therefore imperative to understand the transcriptional regulatory mechanisms of post-recruitment regulated genes. The promoter of a post-recruitment regulated gene is constrained and ready to be activated upon proper stimulation. My thesis is focused on obtaining a better understanding of the mechanism and factors involved in the transition of the constrained state to the transcriptionally activated state at this class of genes.

1.5b.1 The prevalence of post-PIC-recruitment regulated genes.

In eukaryotes, the occurrence of post-recruitment regulation of transcription is not unusual (Uptain et al., 1997). For example, the *Drosophila* heat shock genes, *HSP70* and *HSP26,* and the mammalian c-myc, have promoter proximal pausing of the polymerase during elongation of transcription (Andrulis et al., 2000; Krumm et al., 1995; Krumm et al., 1992; Rasmussen and Lis, 1993; Rasmussen and Lis, 1995; Wu et al., 2003a). During the uninduced state, such promoters have TBP and RNAPII bound and initiated, but paused

after ~20 nucleotides. Upon proper stimulation (i.e. heat shock) transcription is fully activated. By definition these promoters are post-recruitment regulated due to the fact that they have high levels of TBP and Pol II occupancy but low levels of transcription expression.

Another example of post-recruitment regulation important to higher eukaryotes, especially humans, is the transcription of the integrated human immunodeficiency virus type 1 (HIV-1) provirus by RNAPII. In the case of HIV-1, the 5' LTR of the virus serves as the promoter for RNAPII. In the absence of the viral Tat protein, the RNAPII initiates transcription from the 5' LTR but is defective in elongation (Liou et al., 2004). As such, understanding the mechanism and factors involved at post-recruitment regulated gene as it transitions from the uniduced to the induced state of transcription is of great interest.

1.5b.2 *CYC1* a post-recruitment regulated gene

The *S. cerevisiae* gene *CYC1* is a gene that is post-recruitment regulated and serves as an excellent model for the study of factors involved in this type of regulation. The *CYC1* gene encodes iso-1-cytochrome c, which is involved in the electron transport chain in the mitochondria (Sherman et al., 1966). In the presence of a fermentable carbon source (such as glucose), the *CYC1* gene has extremely low levels of transcription and is referred to as partially repressed. On a non-fermentable carbon source (such as lactate or ethanol), *CYC1* transcription expression is ten-fold greater (Guarente et al., 1984; Guarente and Mason, 1983). During the partially repressed condition of *CYC1,* TBP and

RNAPII are present on the *CYC1* promoter (Kuras and Struhl, 1999; Martens et al., 2001). TBP and RNAPII occupancy increases marginally (less than two fold) at the *CYC1* promoter during activation of gene expression. Therefore the *CYC1* promoter is in a constrained state during partial repression where full occupancy of TBP and RNAPII does not lead to complete activation of the gene.

In addition to TBP and RNAPII being present at the *CYC1* promoter during partial repression, Dr. Lei Zhang of Dr. Stargell's lab has shown that Spn1 is also present. Upon activation, Dr. Zhang has found that *SPN1, SPT6,* and Swi/Snf are present (Figure 1.6); however, it was not known what other factors occupy the promoter along with TBP and RNAPII. Understanding what factors are present during the constrained state of *CYC1* will shed light on the mechanism of post-recruitment regulated genes in eukaryotes. By knowing which factors are present we know what factors are either required to maintain dormancy or acted upon to fully activate a post-recruitment regulated gene.

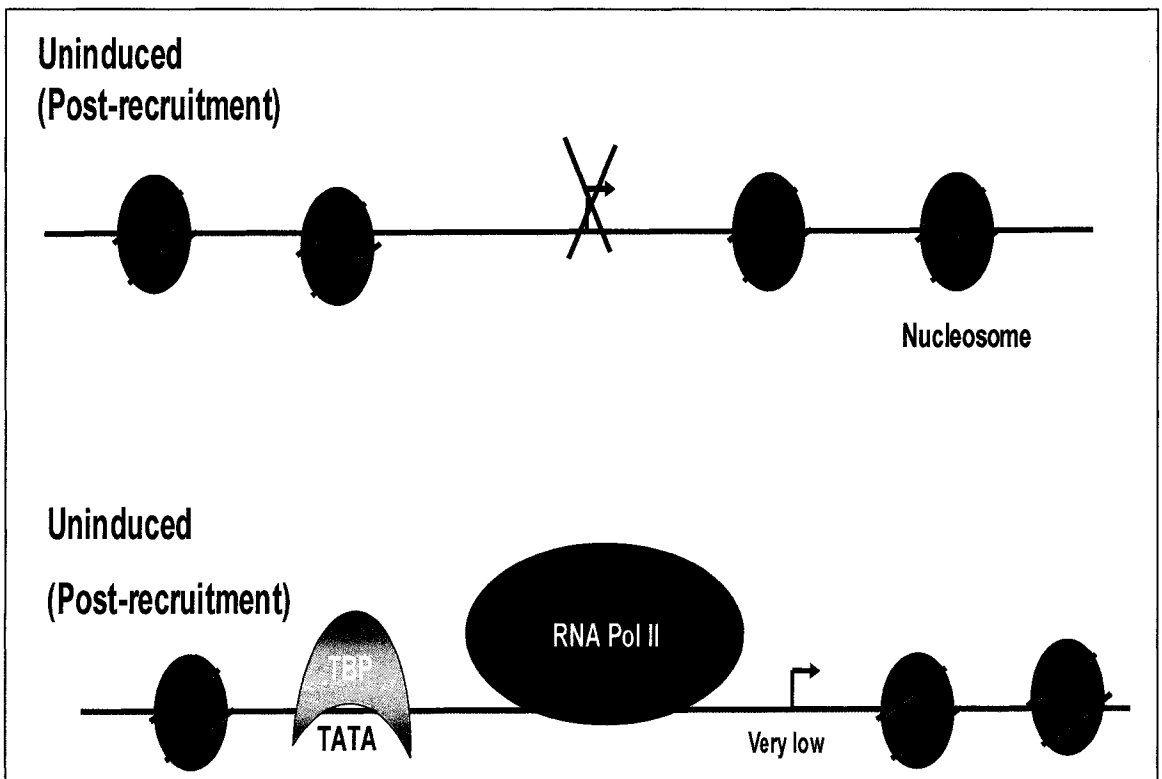


Figure 1.5: : Schematic view of a recruitment vs. post-recruitment gene in the inactive state. In a recruitment regulated gene TBP and RNAPII are not bound. In a post-recruitment regulated gene TBP and RNAPII are bound but transcription is not initiated. Before my thesis work it was unknown whether other transcription factors (such as SAGA, mediators, kinases, capping enzymes, elongation factors etc.) were present during the inactive state of post-recruitment regulated genes.

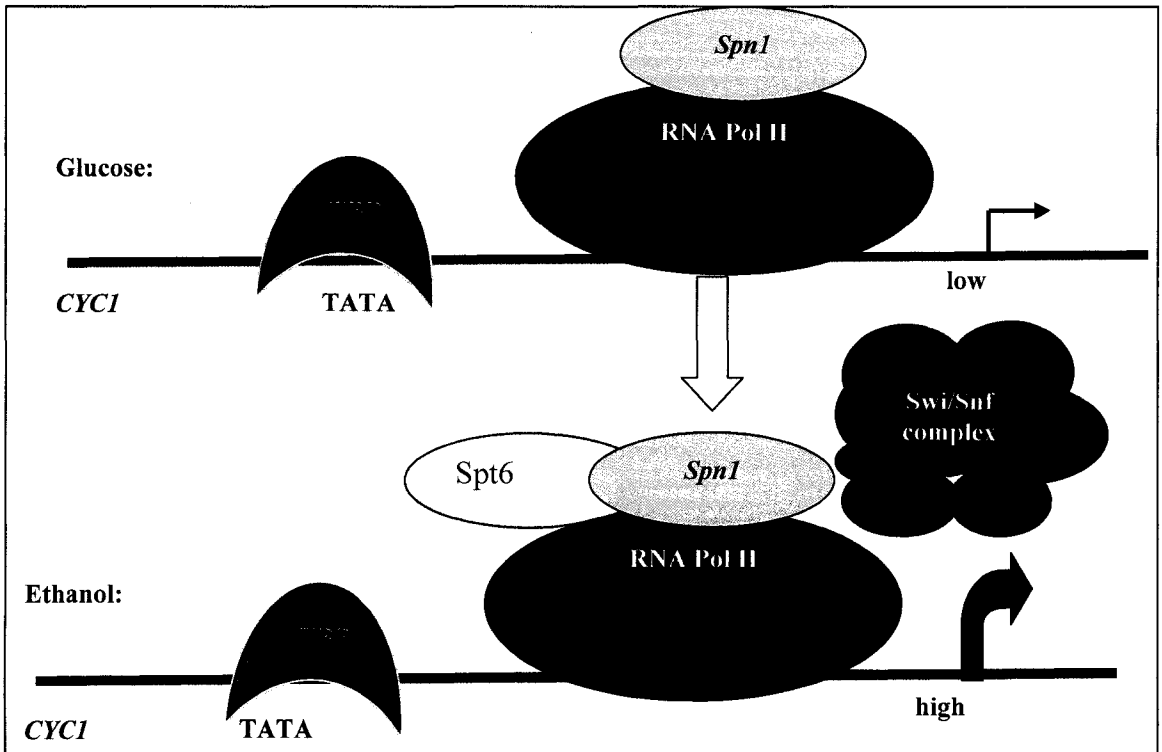


Figure 1.4: *CYC1* promoter is regulated in a post-recruitment fashion. *CYC1* is partially repressed in media containing glucose and TBP, RNAPII, & Spn1 are bound but transcription is inactive. During the transition to fully activated state of transcription there is a recruitment of Spt6 and Swi/Snf.

1.5b.3 *SPN1* a post-recruitment regulating factor.

SPN1 (Suppresses Post-recruitment functions gene Number 1) is an essential gene in *S. cerevisiae* and is conserved throughout evolution. There is a wide range of eukaryotic organisms with *SPN1* homologs, including humans, mice, flies, and worms (Altschul et al., 1997). The central region of Spn1, ~113 amino acids, is highly conserved throughout eukaryotes and is essential for *SPN1* function, while the N- and C- termini are required for proper function *in vivo* (Fischbeck et al., 2002). The *SPN1* gene product (Spn1) is 410 amino acids in length, has a molecular weight of approximately 46 KDa, and a pI of 7.81.

SPN1 was initially identified through a genetic screen using a TBP mutant allele (Fischbeck et al., 2002). The TBP mutant consists of a single point mutation of a phenylalanine at residue 237 to an aspartic acid, TBP^{F237D}. This mutant allele is capable of binding to the DNA, but transcription is not initiated (Stargell and Struhl, 1996). Thus, TBP^{F237D} binds to the DNA but has a post-recruitment constraint. A temperature sensitive *SPN1* allele, $spn1^{K192N}$, is capable of allowing transcription in the presence of TBP^{F237D}. Because the mutant phenotype of $spn1^{K192N}$ allows transcription to occur in a post-recruitment regulated strain, the wild type function of *SPN1* is maintaining the constrained state of a promoter regulated via post-recruitment.

If one looks at an endogenous gene one would expect $spn1^{K192N}$ to release the constrained state of a promoter, i.e. release a post-recruitment regulated gene such as *CYC1*. In a $spn1^{K192N}$ cell, *CYC1* transcription is de-repressed on a fermentable carbon source (which represses *CYC1* in a wild-type cell) and is

super-activated on a non-fermentable carbon source. The effect of spn1^{K192N} on *CYC1* again indicates the role *SPN1* has in maintaining the constrained state of a post-recruitment regulated gene.

1.6 Significance and thesis layout

Transcription initiation is a precisely regulated event that enables a cell to respond promptly to environmental cues. The "dogma" of transcription is that the level of TBP and RNAPII occupancy is correlated with the level of transcription activity. However, the regulation of genes like *CYC1,* provide evidence that certain promoters are regulated in an alternative fashion. *CYC1* has TBP and RNAPII already at the promoter, and presumably recruits these factors in a similar manner as other promoters that are not post-recruitment regulated via general transcription factors, activators, and accessory proteins to provide chromatin accessibility. *CYC1*, however, does not move on to transcription until properly cued and *CYC1* transcription activity does not correlate to TBP occupancy.

Throughout my thesis, I seek to broaden the understanding of post-recruitment regulation. The partially repressed state of *CYC1* is intriguing and serves as my model for the study of post-recruitment regulation. I have investigated the mechanism behind post-recruitment regulation.

To determine if other transcription factors have a regulatory role in post-TBP/RNAPII-recruitment, a genetic assay was developed to screen non-essential genes. I used this genetic assay to screen over 100 non-essential transcription

factors. I also screened over 50 non-essential genes of unknown function to uncover potentially new gene products involved in post-recruitment regulation (Chapter 2). I found that the SAGA complex has a role in post-recruitment regulation through the genetic screen, and this was further characterized (Chapter 3). My research determined that the role of SAGA in post-recruitment is not a carry over of its previously known functions but suggests a new function for SAGA.

The general transcription factor TFIIH is the last factor to come in and complete the nucleation of the PIC. TFIIH contains two (helicase and kinase) very important enzymatic subunits (Rad3 and Kin28 respectively) for transcription initiation and more importantly promoter clearance. To help determine the state of the *CYC1* promoter I determined the kinetics of TFIIH occupancy to the promoter. In addition, the phosphorylated state of the RNAPII was determined and occupancy of capping enzyme was shown in the uninduced condition (Chapter 4).

I also had the pleasure to collaborate with Dr. Lei Zhang, a former graduate student of Dr. Laurie Stargell's lab, to greater understand the role of Spn1 in coordinating the chromatin remodeling factors Spt6 and Swi/Snf on the *CYC1* gene (Appendix I). I summarize a screen I developed in the search for a dominant suppressor of Spn1 (Appendix II).

To determine the role of Spn1 function on a broader scale a collaboration with Dr. Vishy Iyer of University of Texas Austin was initiated. A genome wide

study was conducted to determine the requirement for Spn1 and its genomic localization (Chapter 5).

A summary of my conclusions drawn from my studies and their significance are provided in Chapter 6. In addition, Chapter 6 provides a direction for future studies of post-recruitment regulation and the function of Spn1. Taken together my thesis further explores the mechanism of post-recruitment regulation and broadens our knowledge of the essential and conserved protein Spn1.

Chapter 2

Transcription machinery factors with post-recruitment functions.

To understand the mechanism behind the transition from a partially repressed to a fully activated post-recruitment regulated gene one must determine what factors are involved in such a mechanism. Due to the large number of known subunits involved in the transcription initiation process, it is appropriate to hypothesize that members of the transcription machinery may also contain post-recruitment functions. Testing this hypothesis is described in Chapter 2.

Transcription machinery factors with post-recruitment functions

Aaron Fletcher and Laurie Stargell

2.1 Abstract

To define which, if any, of the transcription machinery members also possess post-recruitment functions, a plasmid-based reporter system and a corresponding genetic screen were created. Through this TBP recruitment bypass screen the deletion collection of *S. cerevisiae* was used to examine non-essential transcription components. The idea was to fuse TBP to a DNA binding protein whose binding site is just upstream of the TATA element. This will bind TBP near the TATA element and bypass the regulation of recruiting TBP to the promoter. Through this bypass screen we showed SAGA as being involved in post-recruitment regulation and also found Mediator to have a high percentage of subunits with post-recruitment function. A large number of genes with unknown function were also tested and a select few may have important functions in post-recruitment regulation.

2.2 Introduction

Initiation of transcription by RNA polymerase II (RNAPII) is a major regulation site of eukaryotic gene expression. The formation of a functional initiation complex is often dependent on the TATA element, and a number of general transcription factors (GTFs). TFIID is a multi-subunit general transcription factor, consisting of the TATA binding protein (TBP) and at least a dozen TBP associated factors. These factors, along with other GTFs, nucleate to form the pre-initiation complex. In the majority of genes, initiation of transcription is regulated by the recruitment of TBP to the promoter DNA; in such cases, the level of TBP occupancy is positively correlated with the level of transcription activity (Kim and Iyer, 2004). However, a growing number of genes have a high level of TBP and RNAPII bound without a high level of transcription activity, (Kuras and Struhl, 1999; Martens et al., 2001) and are therefore post-recruitment regulated. In such an environment, the promoter is constrained and ready to be activated upon proper stimulation. To understand the mechanism and factors involved in the transition of the constrained state to the transcriptionally activated state a genetic assay was constructed. Over 100 genes whose products are important in the transcription machinery in a genetic assay that probes post-recruitment functions were tested.

Spn1, a concerved and essential protein, is a repressor found to play an important role in regulation of the post-recruitment regulated gene *CYC1*. Spn1 was shown to be involved in post-recruitment regulation when a mutant form of Spn1 was able to intiatate transcription in a mutant strain of TBP, TBP^{F237D}, that

binds to DNA but does not intiate transcription (Fischbeck et al., 2002). Here TBP^{F237D} was further used to create a genetic screen to determine potential factors involved in post-recruitment regulation via repression.

2.3 Materials and Methods

2.3a Yeast strains, media and DNA

The deletion mutant strains and its parental strain (BY4741, MATa his3Δ1 ura3Δ0 leu2Δ01 met15Δ01) used in this study were purchased from Research Genetics Inc. The names of the genes deleted are provided in Figure 3.2. The yeast complete and synthetic complete (SC) media was made according to literature (Hampsey, 1997a). The 3- aminotriazol (3-AT) containing plates were made by supplementing the SC-ULH medium with different concentrations of 3-AT. To create the HIS3 reporter plasmid LexAopHIS3, *HIS3* gene in SK1 strain (Fischbeck et al., 2002) that has its Gcn4 binding site replaced by LexA operator was amplified by PCR and sub-cloned into YCp111 plasmid (*LEU2*, CEN). LexA and LexA fused TBP derivatives cloned into pRS316 (*URA3*) plasmids were obtained from previous studies (Stargell and Struhl, 1996). All LexA derivatives had HA module in front of LexA-protein fusion sequence for testing the expression levels of the fusion proteins.

2.3b Phenotypic studies

For phenotypic studies, 10-fold serial dilutions of strains were applied as spots to plates containing synthetic complete medium lacking either leucine, uracil, histidine, or a combination of the three and incubated at 30°C. The

majority of strains were phenotypically screen via streaks on selectable medium and medium containing 3-aminotriazol. Plates were spotted or streaked with cells within 36 hours of pouring.

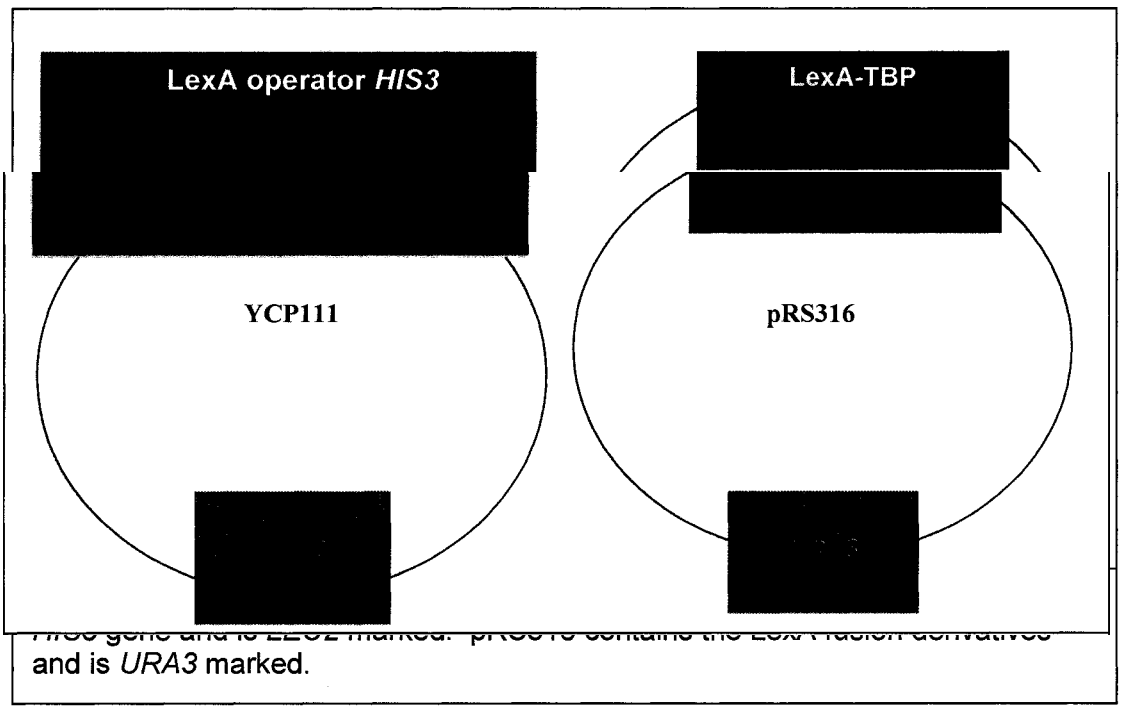
2.3c Transformations

Reporter plasmids and DNA-binding derivative plasmids were transformed into each deletion strain tested. Deletion strains were grown to log phase and cells were harvested and washed in LiAc/TE (0.1M LiAc, 10mM Tris pH 8, 1mM EDTA). Each transformation was performed in 50mL of log phase cells, 5 µl of ssDNA, 1 µl plasmid DNA, and 300 µl LiAc PEG (40% PEG 3350) mixed thoroughly and incubated at 30 degrees Celsius for 30 minutes. Cells were then heat shocked for 20 minutes and plated to selectable media. After 48-72 hours colonies were purified to ensure single colonies and proper selection.

2.4 Results

2.4a Development of a TBP recruitment bypass screen to test for post-recruitment functions

My TBP recruitment bypass genetic screen (bypass screen) consisted of two different plasmids (Figure 2.1): a plasmid based reporter system and a plasmid with TBP fused to a DNA binding protein. The plasmid based reporter system consisted of a DNA binding site, the LexA operator, with a TATA-element downstream and the *HIS3* reporter gene and is *LEU2* marked. This plasmid enabled selection for proper transformation of the reporter gene by growing in medium lacking leucine (Stargell and Struhl, 1996).



HIS3 gene and is *LEU2* marked. pRS316 contains the LexA fusion derivatives and is *URA3* marked.

The LexA operator and its corresponding DNA binding protein, LexA, are found in *E. coli*, thereby making an artificial system in which regulation of these genes in yeast should be unaffected. A second plasmid, pRS316, that is *URA3*-marked contains different LexA derivatives: LexA alone, LexA-TBP, and LexA-TBP^{F237D}. This mutant gene is capable of binding to the DNA, but transcription is not initiated (Stargell and Struhl, 1996). Thus, TBP^{F237D} binds to the DNA but has a post-recruitment constraint. With the addition of this second plasmid, via transformation, I can select for cells that contain both the reporter gene and the LexA derivative. A flow chart of the genetic screen is provided in Figure 2.2.

The fusion construct of LexA-TBP results in the unregulated binding of TBP to the TATA element, nucleation of the pre-initiation complex, and activation of *HIS3* transcription (Chatterjee and Struhl, 1995). The transformed strains were grown on medium containing 3-aminotriazole (AT), a competitive inhibitor of the *HIS3* gene product. Growth of wild-type cells containing LexA-TBP on medium containing AT was robust, and in wild-type cells containing LexA-TBP^{F237D} growth on AT was limited or non-existent (Figure 2.3). Cells containing LexA alone, which serves as a negative control, also show little to no growth in medium containing AT. The plasmid based reporter system is transformed into cells containing a deletion of one non-essential transcription machinery gene. These cells are grown on varying concentrations of AT and post-recruitment phenotypes are looked for, i.e. no growth of LexA-TBP in the deleted strain background.

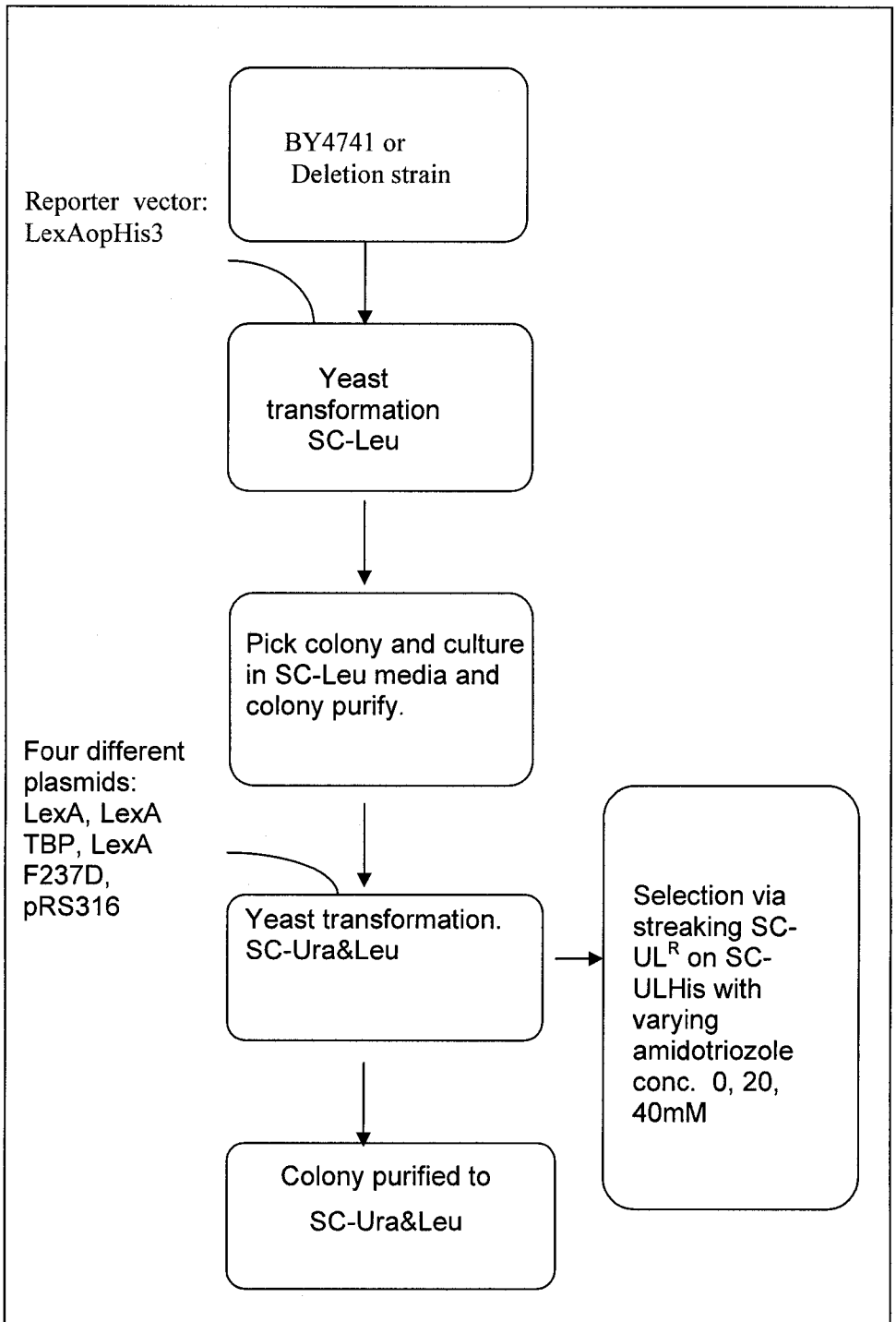


Figure 2.2: A flow chart of the genetic screen. BY4741 is the parental strain with no deleted open reading frames. Synthetic complete (SC) medium lacking either leucine (L), uracil (U), histidine (H), or a combination of the three was used throughout the genetic screen as a selectable marker. 3-amidotriozole (AT) was used to determine promoter strength in the deletion background.

Parental Strain Growth

Figure 2.3: Sequential dilutions of wild type stains with different DNA binding (DB) derivatives grown on medium containing 3-aminotriazole (AT). Cultures at OD600 between 0.6 and 0.8 are diluted to 0.1 and 10-fold serial dilutions are prepared and spotted on media. Wild type DB-TBP grows robustly on medium containing AT, while DB alone and DB- TBP^{F237D} grow poorly on medium containing AT.

2.4b Mediator and SAGA found to have post-recruitment functions

Deletion strains that exhibit no growth in the LexA-TBP strain may have post-recruitment functions. This is due to the fact that TBP is already bound and transcription should occur, yet if a gene is deleted and transcription is unable to occur, they must be affecting something required after the recruitment of TBP. LexA- TBP^{F237D} is the strain in which *SPN1* was found. If growth occurs on LexA- TBP^{F237D}, in the background of a deleted gene, that gene product may also be involved in maintaining the constrained state of a post-recruitment regulated promoter. One hundred and seven different non-essential transcription machinery deletion strains have been assayed through this bypass screen (Figure 2.4) and a couple of complexes suggested post-recruitment phenotypes.

Thus, there is a collection of known gene products whose function may also include post-recruitment regulation. Nearly 40% of the mediator subunits sent through the bypass screen indicated a post-recruitment phenotype, and 30% of the 10 SAGA subunits have a post-recruitment function. Interestingly 83 gene products from other transcription complexes (activators, NuA, Swi/Snf, holoenzyme, general suppressors, elongation factors, casein kinases, and HDAC) showed 5% or less a post-recruitment phenotype.

An important characteristic of this bypass screen is that it provides an unbiased approach to define gene products with a potential role in post-recruitment functions. It is also significant to note that not all transcription complexes were involved in post-recruitment regulation, which indicates a high stringency of the bypass screen.

Parental	LexA	LexA-TBP	LexA-F237D
BY4741	+	+++	+++
Mediators	LexA	LexA-TBP	LexA-F2372
GAL 11	+/-	+++	+/-
ANC1	+	++	+
ROX 3	-	-	-
NUT1	+	+++	+
SIN4	-	-	-
SRB2	-	+++	-
SRB5	++	+++	++
SRB8	-	+/-	-
SRB9	++	+++	+++
SSN3	+/-	+++	+/-
SSN8	+/-	++	+
PGD1	+	+++	+
CSE2	-	+	-
Activators	LexA	LexA-TBP	LexA-F237D
MBF1	-	+++	-
GAL80	+	+++	++
GAL4	+	+++	+
HPC2	+	+++	-
GCN4	-	-	-
STB5	-	-	-
SFL1	-	+++	+
MOT3	-	+++	-
ASC1	+/-	+++	-
NHP6A	-	+++	-
BAS1	-	+++	-
RLR1	-	+++	+/-
MET18	-	+++	+/-
PHO23	+/-	+++	-
TBS1	+/-	+++	+/-

General Suppressors	LexA	LexA-TBP	LexA-F237D
CCR4	+/-	+++	+/-
SIG1	-	++	-
CAF16	-	+++	++
NOT3	-	+++	+
CAF17	-	+++	-
CAF4	+	+++	+
TUP1	-	+++	-
POP2	-	+++	-
SUT1	+/-	+++	+/-
NOT5	-	+++	+/-
Med1	-	++	-
SSN6	-	+++	-
SAGA/ADA	LexA	LexA-TBP	LexA-F237D
SPT8	-	+++	-
AHC1	+	+++	+
HFI1	+	+++	+/-
SPT3	-	+++	-
NGG1	-	+	-
NIF3	++	+++	+
SPT7	+	+	+/-
ADA2	-	++	-
SPT20	-	+	-
GCN5	+/-	++	+/-
SGF11	+/-	++	+/-
NuA HAT	LexA	LexA-TBP	LexA-F237D
YNG1	-	+++	-
HAT2	+	+++	++
HAT1	-	++	-
EAF3	++	+++	+

Holoenzyme components	LexA	LexA-TBP	LexA-F237D
CDC73	+	+++	-
CTK2	-	+++	-
CTK1	+/-	+++	+/-
MHR1	+	+++	+
CTK3	+/-	++	+
RPB9	-	+++	-
PAF1	-	+++	+/-
RPB4	-	+++	+
Elongation Factors			
THP1	+/-	+++	+/-
ELP4	++	+++	+++
ELP2	++	+	+/-
THP2	-	+++	-
IKI3	+	+++	+
RTF1	-	+++	-
ELP3	+	+++	+
ELC1	+	+++	+
ELA1	+/-	+++	+
DST1	+/-	+++	+/-
ELP6	+	++	+
SPT4	+	++	+
HDACs	LexA	LexA-TBP	LexA-F237D
RPD3	-	+++	-
SIN3	-	+++	+/-
HST1	+	+++	+
HOS3	+	+++	++
HOS2	-	+++	+
HOS1	-	+++	+
HDA1	+/-	+++	++
SAP30	+/-	+++	++

SWI/SNF	LexA	LexA-TBP	LexA-F237D
SPT21	-	+++	-
SNF2	-	+++	-
SNF11	+	+++	+
SWI5	+	+++	+
SWI6	+	++	+/-
SPT23	+	+++	+
SWI3	+	+++	+
SNF6	-	++	-
SPT10	-	++	+/-
SNF5	-	+++	+
SPT2	++	+++	+
SNF1	+/-	+++	-
Other Chormatin Remodeling Factors			
	LexA	LexA-TBP	LexA-F237D
ISW2	-	+++	++
ISW1	+	+++	+
MGA2	-	+	+++
MCM22	-	++	-
RAD26	+	+++	++
NRG2	+	+++	++
ITC1	+	++	++
CHD1	-	+++	+/-
Casein Kinase II			
	LexA	LexA-TBP	LexA-F237D
CKA2	+/-	+++	++
CKB2	-	+++	-
CKB1	-	+++	-
CKA1	+	+++	+

YJR Unknowns	LexA	LexA-TBP	LexA-F237D
YJR087W	+	+++	+
YJR097W	+/-	+++	-
YJR056C	+	+++	+
YJR100C	+	+++	-
YJR106W	+/-	+++	+
YJR116W	+/-	+++	+/-
YJR118C	+	+++	+/-
YJR058C	-	+++	+/-
YJR120W	+	+++	-
YJR124C	+/-	+++	+/-
YJR126C	+	+++	+/-
YJR142W	+	+++	-
YJR150C	+/-	+++	+
YJR003C	+	+++	+/-
YJR010C-A	-	+++	+
YJR011C	+	+++	+
YJR015W	+	+++	+/-
YJR018W	-	+/-	-
YJR020W	-	+++	+/-
YJR021C	-	+++	+
YJR024C	+/-	+++	+
YJR030C	+	+++	+/-
YJR033C	+	+++	+
YJR054W	+	+++	+
YJR079W	+/-	+++	+/-
YJR082C	+/-	+++	+/-
YJR083C	+/-	+++	+/-
YJR088C	+/-	+++	+/-
YJR102C	+/-	+++	+/-
YJR108W	+/-	+++	+/-

YJR Unknowns	LexA	LexA-TBP	LexA-F237D
YJR115W	+/-	+++	+/-
YJR055W	+	+++	+
YJR034W	+	+++	+
YJR039W	+/-	+++	+/-
YJR044C	-	+++	+
YJR055W	-	+++	+/-
YJR038C	-	+++	+
YJR024C	+/-	+++	+
YJR030C	+	+++	+/-
YJR033C	+	+++	+
YJR054C	+	+++	+
YJR055C	+/-	+++	+/-
YJR056C	-	+++	+
YJR058C	-	+++	+/-
YJR003C	+	+++	+/-
YJR055W	+	+++	+
YJR034W	+	+++	+
YJR039W	+/-	+++	+/-
YJR044C	-	+++	+
YJR055W	-	+++	+/-
YJR080C	+/-	+++	+
YJR038C	-	+++	+
YJR084W	-	+++	-
YJR128W	+/-	+++	+/-
YJR146W	+/-	+++	+/-
YJR147W	-	+++	+++
YJR149W	+/-	+++	-
YJR154W	-	+++	+
YJR069C	+/-	+++	+/-
YJR111C	+/-	+++	+/-

YJL206C	-	++	-
YJL169W	-	-	-
YJL171C	-	-	-
YJL170C	-	+++	-
YJL154C	-	-	-
YJL151C	-	+++	-
YJL147C	-	+++	-
YJL146W	-	+++	-
YJL144W	-	+++	-
YJL131C	-	+++	-
YJL123C	-	+++	-
YJL122W	-	+++	-
YJL116C	-	+++	-
YJL107C	+	+	+
YJL082W	-	+++	-

Table 2.1: Different transcriptional machinery components and their corresponding scores on medium containing 3-aminotriazole. Growth, defined as individual colony size, was scored on a scale of (-) for no growth to (+++) for robust growth. Listed strains contain a deletion of the indicated non-essential genes. Unannotated ORFs are designated by their SGD systematic name. Stains highlighted in yellow are stains with post-recruitment phenotypes.

2.4c Unknown gene products found to have post-recruitment functions

In addition to transcription factors, a set of randomly selected strains containing deletions in genes with unknown functions were assayed in the screen. The entire right arm of chromosome 10 (YJR) was selected to screen unknown non-essential gene products for post-recruitment regulation function. In addition, the majority of the left arm of chromosome 10 was also screened (YJL) for a total of 81 unknown gene products. The results indicated very few, approximately 5% of the 81 tested, obtained a phenotype indicative of post-recruitment regulation (Table 2.1).

Six of the unknown gene products were found to potentially have post-recruitment functions: *YJL171C, YJL169W, YJL154C, YJL107C, YJR018W,* and *YJR147W.* Four gene products may have a role in post-recruitment regulation, although one of them is an unverified gene. *YJL169W* is an unverified gene that has not been studied and may be of interest for potential follow-up as a new gene product for post-recruitment function. *YJL107C* may play a role in post-recruitment regulation and no evidence to date would suggest that it is not localized to the nucleus. *YJL107C* also came out of a screen that has shown it to be activated by the Hog1 mitogen-activated pathway (Brachat et al., 2003; Harris et al., 2001; O'Rourke and Herskowitz, 2004; Sychrova et al., 2000). *YJL107C* as been shown to interact with an RNA-binding protein Jsn1 through yeast-two hybrids (Ito et al., 2001).

YJR147W, also known as HMS2, protein product is similar to that of heat shock transcription factors and has been found to genetically interact with Mep1

mutants (Huh et al., 2003; Lorenz and Heitman, 1998; Sahara et al., 2002; Yu and Gerstein, 2006). *Drosophila HSP70* is perhaps the most well characterized post-recruitment regulated gene (Andrulis et al., 2000; Krumm et al., 1995; Krumm et al., 1992; Rasmussen and Lis, 1993; Rasmussen and Lis, 1995; Wu et al., 2003a). The potential similarity of *YJR147W* with other heat shock transcription factors makes it a good candidate for further study in post-recruitment regulation. *YJR018W* is another unverified gene, but has been shown to have growth defects on non-fermentable carbon sources. The most well characterized *Saccharomyces cerevisiae* post-recruitment regulated gene, *CYC1*, as well as *Cox5a* have both been shown to have growth defects on non-fermentable carbon sources (Capaldi et al., 1995; Cumsky et al., 1985; Guarente et al., 1984; Guarente and Mason, 1983; Hodge et al., 1989). Though YJR018W is unverified, its similarity in having a growth defect on non-fermentable carbon sources and its presence in my stringent post-recruitment screen also makes it a potential candidate for future studies as a player in post-recruitment regulation.

Upon extensive literature and computational research, two of the six genes do not seem to be good candidates for potential post-recruitment functions. *YJL171C* is a verified gene whose product has recently been found to be bound to the cell wall (Duttagupta et al., 2005; Hamada et al., 1999; Reinders et al., 2006; Sickmann et al., 2003), and has not been shown to occupy the nucleus and may not be the best product to study. *YJL154C* is a verified gene that is also known as GRD9/VPT7 that is involved in protein transport (Carlton et al., 2005; Dechamps et al., 2005; Hancock et al., 2006; Iwaki et al., 2006; Kama

et al., 2007; Oliviusson et al., 2006; Voronkova et al., 2006) and does not seem to have a role in the nucleus.

2.5 Discussion

The TBP recruitment bypass screen has allowed successful study of over one hundred and fifty different gene products to determine if they have a function important in post-recruitment regulation. These studies strongly suggest that the SAGA complex and the Mediator complex play an important role in post-recruitment regulation (further studied in Chapter 3).

The design of the bypass screen allows efficient study of a large number of factors in a relatively short time span. SAGA, described in detail in chapter three, was found to have a post-recruitment phenotype in our genetic screen. Additionally, Mediator was suggested to be important in post-recruitment regulation. The bypass screen has allowed investigation of a large number of transcription factors and accurately identifed two complexes that look to play an important role in the mechanism of post-recruitment regulation.

In addition to determining what transcription factors are important in post-recruitment regulation a number of unknown gene products were tested for post-recruitment function. The stringency of the screen was again seen when ~5% of the genes tested showed a potential for post-recruitment regulation. After comprehensive computational and literature research, four of the six factors suggested to have post-recruitment fuction appear to be potential candidates. Though these four gene products may be of interest in post-recruitment

regulation they were not further studied. Understanding the mechanism of post-recruitment regulation is a very large undertaking and the role that SAGA and Mediator play in that role are of interest to pursue. In addition it is important to understand the general set up of a post-recruitment regulated gene and to determine the commonality of this type of regulation on a genome wide scale.

Chapter 3

A new role for SAGA in transcriptional activation by RNA polymerase II

The SPT gene family has long been known to play important roles in RNA Pol II transcription, and has been implicated in various processes such as maintaining chromatin structure, initiation, elongation and RNA processing . Chapter 3 focuses on determining which SPT genes have post-recruitment functions. This study was performed in collaboration with three additional members of the Stargell lab: Lei Zhang, Julie Fischbeck, and Sarah Lee. I initiated the entire project, created figures, and wrote the findings into a manuscript, which will be submitted to the scientific journal Molecular Cell Biology.

A new role for SAGA in transcriptional activation by RNA polymerase II

Aaron Fletcher, Lei Zhang, Julie Fischbeck, Sarah Lee, and Laurie Stargell

3.1 Abstract

A growing number of genes appear to have TBP and/or RNA polymerase II (TBP/RNAPII) occupied at the promoter prior to transcriptional activation of the gene. As such, these promoters must depend on events which are post-recruitment of TBP/RNAPII for regulated expression, although little is known about the mechanism of activation at these promoters. Here we employ a TBP recruitment bypass assay, and transcription assays on a bona fide post-recruitment regulated gene, to determine the role of SPT genes in this process. Interestingly two subunits of the SPT genes tested are members of the SAGA complex (Spt7 and Spt20) demonstrated post-recruitment functions. Expansion of the study to include the remainder of the members of the SAGA complex revealed that Ada1 was also important in post-recruitment regulation. Interestingly, the histone acetyl transferase activity of SAGA is not required. Likewise (and as expected), the TBP-delivery functions of SAGA are not involved in these post-recruitment functions. Occupancy of Mediator is diminished in the activated state when Spt20 is deleted from the cell. These studies reveal an additional and essential function of the SAGA complex in the mechanism of activation of genes with rate-limiting steps after recruitment of TBP and RNA Pol II.

3.2 Introduction

Initiation of transcription by RNA polymerase II (RNAPII) is a major regulation site of eukaryotic gene expression. The formation of a functional initiation complex can be dependent on the TATA element and on a number of general transcription factors (GTFs). TFIID is a multi-subunit general transcription factor, consisting of the TATA binding protein (TBP) and at least a dozen TBP associated factors. A number of genes are TFIID dependent while others are SAGA dependent. The SAGA complex consists of Spt3, Spt7, Spt8, Spt20 and to date has been shown to help regulate the recruitment of TBP (Belotserkovskaya et al., 2000; Bhaumik and Green, 2001; Dudley et al., 1999; Larschan and Winston, 2001; Qiu et al., 2005) and acetylate chromosomal histones (Brownell et al., 1996; Grant et al., 1997; Grant et al., 1998b; Utley et al., 1998). The dependence on TFIID or SAGA is mutually exclusive (Huisinga and Pugh, 2004). Mediator is a large multi-subunit complex that is thought to relay information between activators and RNAPII (Kim et al., 1994). These factors, along with other GTFs, nucleate to form the pre-initiation complex. In many genes, initiation of transcription is regulated by the recruitment of TBP/RNAPII to the promoter DNA; in such cases, the level of TBP occupancy is positively correlated with the level of transcription activity (Kim and Iyer, 2004). However, a growing number of genes have a high level of TBP and Pol II bound without a high level of transcription activity (Kuras and Struhl, 1999; Martens et al., 2001), and are therefore post-PIC-recruitment regulated. In such a situation,

the promoter is constrained and ready to be activated upon proper stimulation and the rate limiting step is no longer PIC occupancy to the promoter.

The *S. cerevisiae* gene *CYC1* is post-recruitment regulated and serves as an excellent model for the study of factors involved in post-recruitment regulation. The *CYC1* gene encodes iso-1-cytochrome c, which is involved in the electron transport chain in the mitochondria (Sherman et al., 1966). In the presence of a fermentable carbon source (such as glucose) the transcription level of the *CYC1* gene is low even though TBP and Pol II occupy the *CYC1* promoter (Kuras and Struhl, 1999; Martens et al., 2001). The gene is fully activated when the cell is grown on a non-fermentable carbon source (such as lactate or ethanol). During full activation *CYC1* transcription levels are ten-fold greater (Guarente et al., 1984; Guarente and Mason, 1983). However, TBP and RNAPII occupancy increases marginally (less than two fold) and therefore has PIC occupancy during the un-induced condition.

SPT15 encodes TBP (Eisenmann et al., 1989), and products of the yeast *SPT* (Suppressor of Ty) gene family represent over 20 transcription factors that have been implicated in various processes such as maintaining chromatin structure, initiation, elongation and RNA processing (Fassler and Winston, 1988; Winston et al., 1984a; Winston et al., 1987; Yamaguchi et al., 2001). Functions of many Spt proteins are related to TBP function including Spt3, Spt7, Spt8, and Spt20 (Dudley et al., 1999; Eisenmann et al., 1992; Lee et al., 2000; Roberts and Winston, 1996; Sterner et al., 1999). *SPT11* and *SPT12* encode histone H2A and H2B, respectively (Grunstein, 1992; Hereford et al., 1979). Spt1, Spt10 and

Spt21 are the regulatory factors that control the expression levels of histone genes (Dollard et al., 1994; Hess et al., 2004; Natsoulis et al., 1994; Sherwood et al., 1993). Many key regulators of transcription and/or chromatin structure, such as DSIF, SAGA, and FACT complexes, all contain *SPT* gene product components. Spt4/Spt5, the yeast homolog of human DSIF regulates transcription elongation through chromatin (Hartzog et al., 1998; Swanson and Winston, 1992).

To define which, if any, of the *SPT* gene family members also possess post-recruitment functions, a genetic assay using a plasmid-based reporter system was developed. Through this TBP recruitment bypass screen (bypass screen) the deletion collection of *S. cerevisiae* was utilized to examine non-essential components of the *SPT* gene family. We find that multiple subunits of SAGA have a role in post-recruitment regulation. Further analysis of SAGA on the *CYC1* gene showed that SAGA is required for proper regulation of *CYC1.* Furthermore, the required function of SAGA at the *CYC1* gene is not acetyl-transferase activity or TBP recruitment activity. Taken together, these results indicate a new role for SAGA in post-recruitment regulated genes.

3.3 Material and methods

3.3a Yeast strains, media and DNA

The deletion mutant strains and its parental strain (BY4741, MATa his3Δ1 ura3Δ0 leu2Δ01 met15Δ01) used in this study were purchased from Research Genetics Inc. The names of the genes deleted are *SPT1, 2, 3, 4, 7, 8, 10, 13, 20,*

21, 23, GCN5, ADA1, 2, and *3.* The yeast complete and synthetic complete (SC) media were made according to literature (Hampsey, 1997a). The 3- aminotriazol (3-AT) containing plates were made by supplementing the SC-ULH medium with different concentrations of 3-AT. To create the HIS3 reporter plasmid LexAopHIS3, *HIS3* gene in SK1 strain (Fischbeck et al., 2002) that has its Gcn4 binding site replaced by LexA operator was amplified by PCR and subcloned into YCp111 plasmid (*LEU2*, CEN). LexA and LexA fused TBP derivatives cloned into pRS316 (*URA3*) plasmids were obtained from previous studies (Stargell and Struhl, 1996). All LexA derivatives had HA module in front of LexA-protein fusion sequence for testing the expression levels of the fusion proteins. Protein tagging with either HA or Myc epitopes was carried out according to literature (Longtine et al., 1998). Proteins tagged were Spt7, Spt8, Spt20, Gcn5, Ada1, and Srb8.

3.3 b Plasmid based TBP recruitment bypass screen

Yeast cells of both parental and deletion strains were transformed with the plasmids using standard procedures (Beggs, 1978). The plasmid based reporter system consists of a DNA binding domain, the LexA operator, with a TATA-element downstream encoded into YCP111, which contains a *HIS3* reporter gene and is *LEU2* marked. A second plasmid, pRS316, that is *URA3*-marked carries different LexA derivatives: LexA alone and LexA-TBP. With the addition of this second plasmid, via transformation, we can select for cells that contain both the reporter gene and the LexA derivative. Briefly, yeast cells were first transformed with the LexAopHIS3 plasmid DNA, then the *LEU2*+ yeast cells were

further transformed with LexA and LexA-TBP fusion constructs in *URA3* marked plasmids; finally the *LEU2*+ *URA3*+ yeast cells were streaked onto SC-UL medium and SC-ULH medium containing 3-AT. Cell growth was scored as ranging from "-" to "++++". "-" indicating little or no growth. "++++" indicating robust growth.

3.3c Western blot analysis

10ml of yeast cells grown in SC-UL with an OD600 of ~0.8 were harvested, washed once with sterile water, and resuspended in 200ul lysis buffer (0.5M phosphate buffer, pH 7.5). Whole cell extracts were prepared by vigorous bead beating. Cellular debris was removed by spinning the extracts at 10,000g at 4°C for 10 min. Protein concentrations were determined by Bradford assay (Bio-Rad). Equal amount of whole cell extracts were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The following antibodies were used at the given concentrations: anti-HA (12CA5, from Covance Inc; 1:250), anti-myc (Upstate Inc.), homemade anti-Toa1 (1:10,000). Horseradish peroxidase (HRP) conjugated second antibodies were used at 1:20,000 dilution and protein bands were detected by western blotting detection reagents from Amersham Biosciences.

3.3d Transcription assay

S1 nuclease assays were conducted as described (Iyer and Struhl, 1996). For *CYC1* induction, cultures grown overnight in rich medium containing 2%

glucose were washed three times in medium lacking glucose, diluted into medium containing 3% ethanol, and cultured at 30°C for 6 hr. For un-induced samples, cells were grown in YPD for 6 hours at 30°C till optical density reached 0.8~1.0. Yeast cells were then harvested and total RNA was isolated by hot acid phenol extraction. Hybridization with excess probe was done with 25~30 µg of RNA samples and were hybridized with excess ^{32}P labeled probe overnight at 55°C. S1 nuclease digestion was performed on the hybridized samples for 30~45 min at 37°C. The hybridization signals were visualized by phosphoimager, mRNA loading was normalized to the intensity of the $tRNA^{W}$ band.

3.3e Chromatin immunoprecipitation (ChIP) analysis

Chromatin immunoprecipitations were performed as described (Strahl-Bolsinger et al., 1997), with few a modifications. Cells (50 ml) were grown to OD 600 of 0.8~1.0. Cells were treated with a final concentration of 1% formaldehyde for 15 minutes with occasional swirling of the flasks at intervals of 5 minutes. Glycine was added to a final concentration of 125mM at room temperature for 5 minutes to stop cross-linking. Cells were collected and washed twice in ice cold TBS. Cells were resuspended 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, beuzamidine, pepstatin, leupeptin, and chymostatin). Chromatin was sheared by sonication using a Branson W-350 model of sonifier (10 times at 10 seconds each on continuous pulse at a microtip power setting of 6). 10% of the chromatin material used for the immunoprecipitation was

processed as the input after reversing the cross-links and purifying the DNA. 500 µl of the chromatin material was incubated with approximately 5µl of either homemade anti-TBP, anti-Pol II (8WG16, Covance Inc.), anti-HA (Santa Cruz) or anti-Myc (Upstate) antibodies by rotation overnight at 4°C. 50µl of protein-A sepharose beads (Pharmacia-prepared as slurry as per the manufacturers directions) was further incubated with the chromatin material for 3 hours at 30°C. The beads were spun down and the Antigen-Antibody complexes bound to the beads were recovered and further treated with TE/SDS buffer for 15 minutes at 65°C to elute the complexes. Protein-DNA cross-links were reversed by incubation overnight at 65°C and the DNA was purified by phenol-chloroform extraction and used for the linear PCR analysis or Real Time PCR (RT-PCR) analysis. Linear PCR reactions were carried out in a total volume of 25µl. Each reaction contains 1 ul of 1/100 dilution of ^{32}P labeled-ATP. Different dilutions of each input and immunoprecipitated material were used to determine the linear range of the PCR reaction. The samples were run on 5% native polyacrylamide gels in 0.5XTBE buffer. The gels were dried and exposed to the phosphor-image screen. Image was scanned on STORM and quantified using image quant software analysis to detect the strengths of various signals. No antibody samples were used as controls. Primers for the PCR were designed at the promoter region of the *CYC1* gene and amplified a product of 312 bp (Martens et al., 2001). For analysis of the occupancy of the LexA derivative, primers were designed to encompass the whole engineered HIS3 reporter promoter region and amplified a product of 646 bp. The signal strength ratio between the precipitated

sample and the input, minus background of no antibody control, was used as an indication of the protein occupancy.

SAGA subunits' occupancy, as well as, TBP and Pol II occupancy of ada1Δ were determined via real time PCR (RT-PCR). Standard curves were generated using 10-fold serial dilutions of Input DNA. PCR efficiencies ranged from 90-100%, with a correlation coefficient of 0.990 or greater. Threshold cycle data were quantified relative to the input, as described (Frank et al., 2001). Background levels were calculated as the relative occupancy of untagged strains and these values were subtracted from that of the tagged strains.

3.4 Results

3.4a TBP recruitment bypass assay for *SPT* gene family members with a potential role in post-recruitment regulation

A plasmid-based TBP bypass assay was developed to identify post-TBP/RNAPII-recruitment functions of non-essential *SPT* gene family members. The theory is that a DNA binding domain fused to TBP results in unregulated binding of TBP to a reporter gene activating the reporter gene. The genetic assay consists of two plasmids: a plasmid based reporter system and a plasmid with TBP fused to a DNA binding protein (Materials and Methods). The fusion construct of LexA-TBP results in the unregulated binding of TBP to the TATA element of the reporter gene, nucleation of the pre-initiation complex, and activation of *HIS3* transcription (Chatterjee and Struhl, 1995). Strains are grown

on media containing 3-aminotriazole (AT), a competitive inhibitor of the *HIS3* gene product. In wild-type cells containing LexA-TBP, growth on media containing AT is robust. Cells containing LexA alone, which serve as a negative control, show little to no growth on medium containing AT. The plasmid based reporter system is transformed into cells containing a deletion of one non-essential *SPT* gene family member. These cells were grown on varying concentrations of AT and assayed for growth. If the deletion of a *SPT* gene is defective for functions after TBP recruitment, these defects would not be corrected by the artificial recruitment assay. The *HIS3* gene would not be transcribed to levels of that in a wild-type cell, as such, these strains, though containing LexA-TBP, would grow poorly on AT. If the deletion of a *SPT* gene was defective for TBP recruitment, the artificial recruitment of TBP would correct these defects and the *HIS3* gene would be transcribed similarly to wild-type. Therefore the growth on AT of the deletion strain reflects the potential involvement of the wild-type protein in post-recruitment regulation functions.

Cells containing deletion of *spt1, 2, 3, 4, 8, 10, 13, 21*, or *23* grew similar to that of wild-type cells on medium containing 40 mM AT (Table 3.1) indicating they had no essential function after PIC formation at the reporter gene. Strains containing deletion mutants of *SPT7* and *STP20* grew poorly on medium containing AT (Table 3.1 & Figure 3.1), suggesting that *SPT7* and *SPT20* have a role in post-recruitment function.

The poor growth of strains with *SPT7* and *SPT20* deleted could be due to low expression levels of the LexA-TBP fusion protein leading to a false positive in

our bypass assay. Low levels of LexA-TBP would prevent the formation of the PIC on the reporter gene and result in no growth on 3-aminotriazole. To test this, the protein level of LexA-TBP was assayed in each strain via immuno blot analysis. Expression levels of LexA-TBP were found to be comparable in all strains tested (Figure 3.2A). Since LexA-TBP is expressed normally in *SPT7* and *SPT20* deletion strains, another possibility for the poor growth in our assay is that LexA-TBP fusion protein occupancy to the promoter of the *HIS3* gene requires Spt7 and Spt20. To test this hypothesis, we performed ChIP analysis to determine the occupancy of LexA-TBP on the promoter of *HIS3* reporter gene. We found that in both *SPT7* and *SPT20* deletion strains, LexA-TBP was recruited to the *HIS3* reporter gene promoter indistinguishably from a wild type strain (Figure 3.2B). Taken together, the results of our bypass assay suggest that *SPT7* and *SPT20* are involved in post-recruitment regulation.

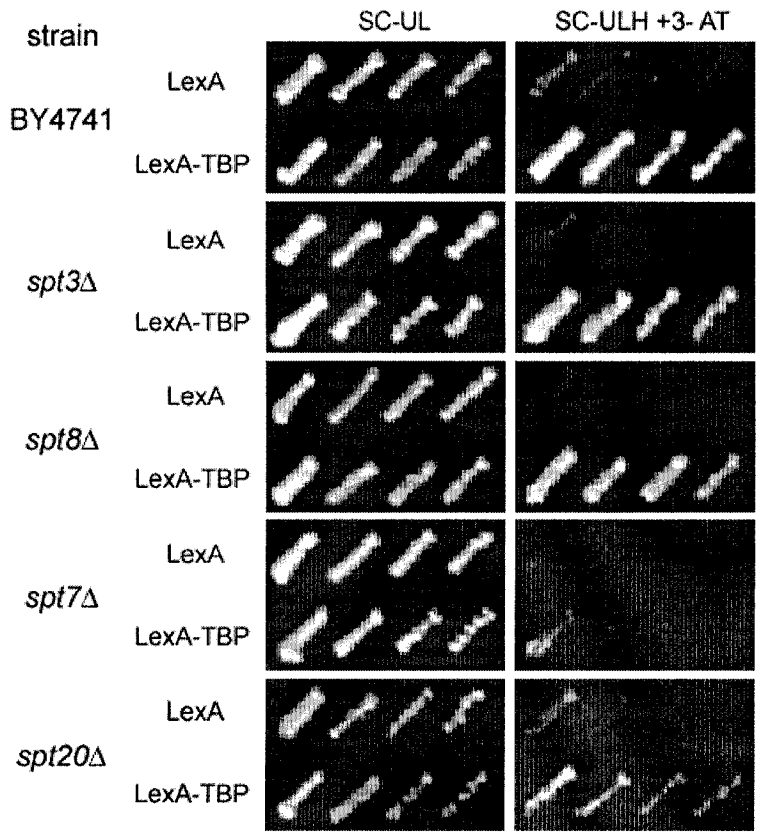


Figure 3.1 Results of plasmid-based artificial recruitment assay. The parental strain (BY4741) and *SPT* gene deleted strains (as indicated) were transformed with reporter plasmid and with either LexA alone or LexA-TBP containing plasmid. Yeast cells for each strain were streaked four times on medium containing 3-aminotriazole and without 3-AT. Pictures were taken after growing for 3 days. Strains transformed with LexA plasmid was used as controls.

Table 3.1

Results of plasmid based artificial recruitment assay *

Strains	LexA	LexA-TBP	[AT] used	Days of growth
BY4741	+	++++	40	3
Δ*spt1*	+	++++	40	3
Δ*spt2*	+	++++	40	3
Δ*spt3*	+	++++	40	3
Δ*spt4*	+	+++	40	3
Δ*spt7*	+/-	+	20	4
Δ*spt8*	+	++++	40	3
Δ*spt10*	+	+++	40	3
Δ*spt13*	+	++++	40	3
Δ*spt20*	+/-	++	20	4
Δ*spt21*	+	++++	40	3
Δ*spt23*	+	++++	40	3

Table 3.1 *: The yeast cells transformed with the plasmid-based artificial recruitment system were grown on SC-UL and SC-ULH 3-aminotriazole (AT) plates at 30 °C. The growth of cells was scored as ranging from "+/-" to "++++". The "+/-" means very weak to no growth, and the "++++" indicates robust growth.

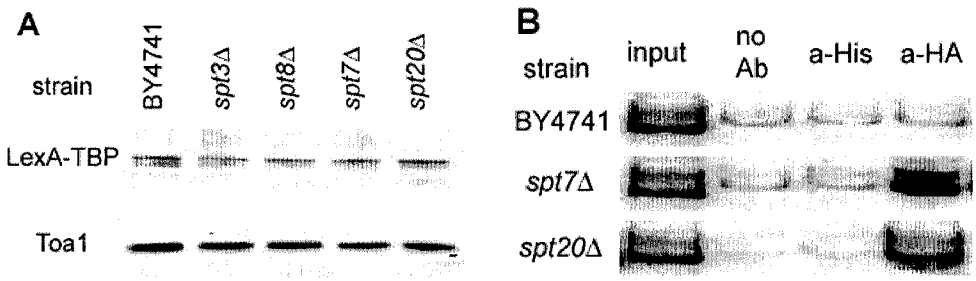


Figure 3.2a Expression levels of LexA-TBP protein were similar in SPT deletion strains Protein extracts from strains transformed with LexA-TBP plasmids were separated on SDS-PAGE and subjected to western blot analysis. Levels of LexA-TBP were detected via anti-HA antibody against HA tag on the N-terminus of the fused protein. Anti-Toa1 antibody was used to detect the Toa1 levels for loading control.

3.2b LexA-TBP is artificially recruited to the reporter *HIS3* gene Strains expressing LexA-TBP were subjected to ChIP analysis of the reporter gene in order to examine the occupancy level of LexA-TBP on the promoter. Anti-HA antibody was used to immunoprecipitate LexA-TBP, anti-His antibody was used as irrelevant antibody control.

3.4b Proper regulation of *CYC1* transcription levels requires the function of *SPT7* and *SPT20.*

To further investigate the role of *SPT7* and *STP20* in post-PIC-recruitment regulation, we tested their requirement on the *CYC1* gene. *CYC1* is a gene regulated by post-recruitment functions (Kuras and Struhl, 1999; Martens et al., 2001) and, regulation of the *CYC1* gene provides an excellent model to study post–recruitment functions. The transcription levels of *CYC1* were measured via S1 nuclease assay. While the *CYC1* activation levels were only slightly decreased in *spt1, 2, 3, 8, 10, 13, 21* and *23* deletion strains, *CYC1* transcription was abolished in *spt7* and *spt20* deletion strains (Figure 3.3). It is significant to note that the transcription results were consistant what was seen in the genetic bypass assay, indicating the high stringency and accuracy of the LexA-TBP post-recruitment functional assay.

3.4c Bypass assay for SAGA members indicates *ADA1* also plays an important role in post-recruitment regulation.

Spt7 and Spt20 are both subunits of the yeast SAGA complex (Grant et al., 1997; Roberts and Winston, 1997). The finding that both Spt7 and Spt20 are involved in post-recruitment functions suggested that other SAGA subunits may also play a role in post-recruitment functions. The yeast SAGA complex is a multiple subunit complex comprised of TAFs, Ada proteins, and Spt proteins. Studies have shown that Spt7, Spt20 and Ada1, together with several TAFs, are the core subunits of the SAGA complex in that deletion of any of these subunits

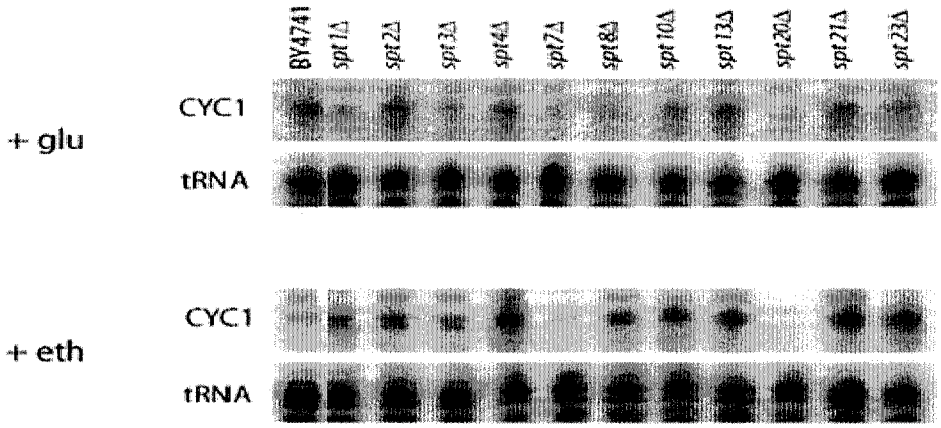


Figure 3.3 Deletion mutants of *SPT7* and *SPT20* are defective for *CYC1* activation. Total RNA from strains as indicated grown under uninduced and induced conditions were analyzed via S1 nuclease assay using ^{32}P labeled *CYC1* and tryptophan tRNA probes. tRNA^{w} signal was used as a load control to normalize signals of *CYC1* transcripts. Strains with *spt4Δ* and *spt10Δ* alleles decrease *CYC1* activation moderately; the *spt7Δ* and *spt20Δ* alleles abolish *CYC1* activation.

disrupts the structural integrity of the SAGA complex (Dudley et al., 1999; Grant et al., 1998a; Horiuchi et al., 1997; Wu et al., 2004; Wu and Winston, 2002). Gcn5 is the HAT subunit of the SAGA complex and its function is modulated by two other subunits Ada2 and Ada3 (Balasubramanian et al., 2002; Candau et al., 1997; Grant et al., 1997; Horiuchi et al., 1995; Ruiz-Garcia et al., 1997).

Cells containing deletion mutants of *ADA2* and *ADA3* showed similar growth as wild-type on medium containing AT. In contrast, the *gcn5* mutant strain showed a moderate effect and *ada1* deletion strain showed very weak growth on AT (Figure 3.4A), suggesting that Ada1 plays an important role after recruitment of the PIC. Interestingly, all three subunits important for structural integrity of SAGA (Spt7, Spt20, & Ada1) were found to have post-recruitment function in our assay. Transcription output of *CYC1* was also measured, and similar to that of *spt7* and spt*20*, *ada1* deletion abolished *CYC1* transcription. Deletion of *GCN5* had a minor effect on *CYC1* transcription, while deletion of *ADA2* or *ADA3* had no effect on *CYC1* transcription output (Figure 3.4B & C).

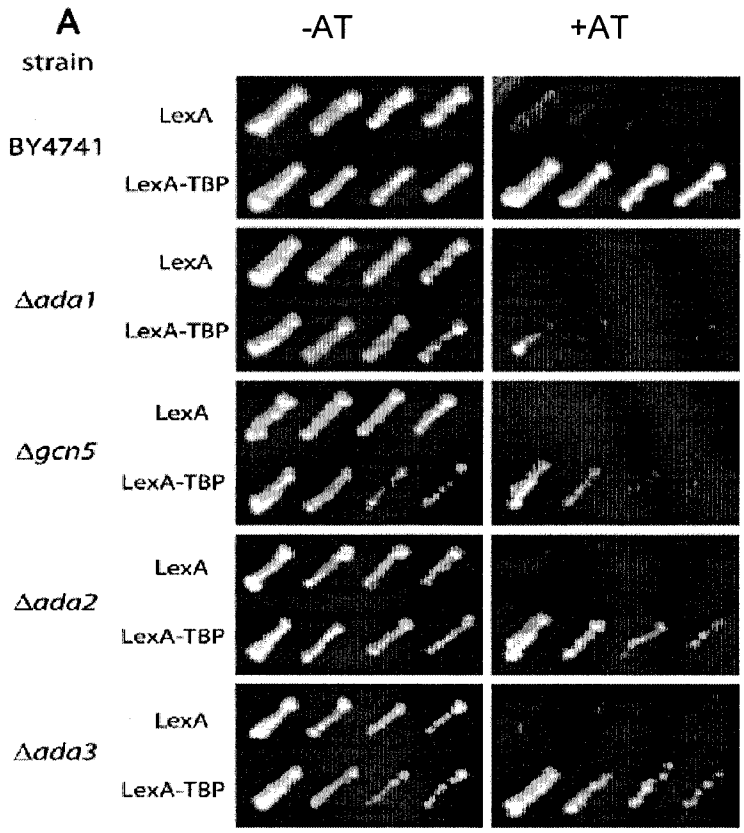


Figure 3.4 Regulation of *CYC1* transcription by non-Spt subunits of SAGA complex

3.4a The parental strain (BY4741) and SAGA gene deleted strains (as indicated) were transformed with reporter plasmid and with either LexA alone or LexA-TBP containing plasmid. Yeast cells for each strain were streaked four times on medium containing 3-aminotriazole and without 3-AT. Pictures were taken after growing for 3 days. Strains transformed with LexA plasmid was used as controls.

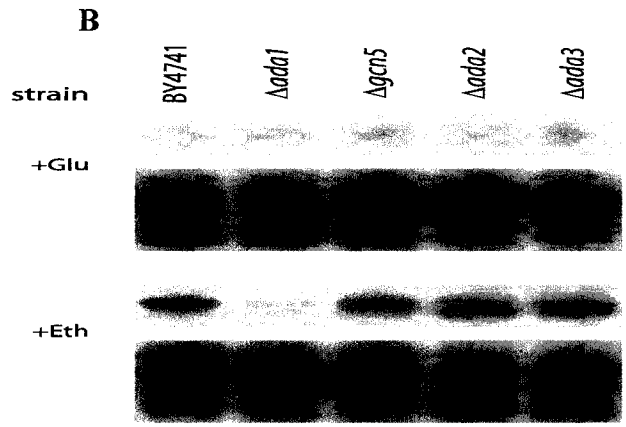


Figure 3.4 Regulation of *CYC1* transcription by non-Spt subunits of SAGA complex

3.4b Total RNA from strains as indicated grown under uninduced and induced conditions were analyzed via S1 nuclease assay using ^{32}P labeled *CYC1* and tryptophan tRNA probes. $tRNA^{W}$ signal was used as a load control to normalize signals of *CYC1* transcripts.

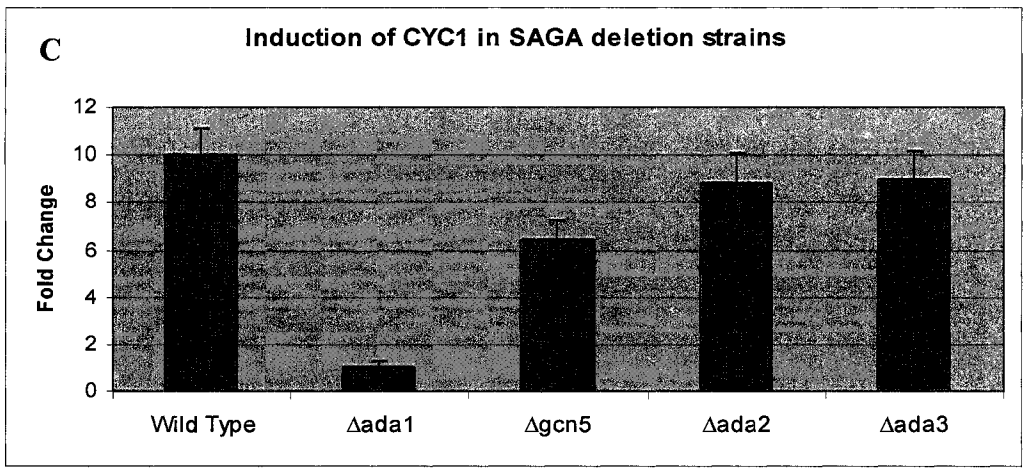


Figure 3.4 Regulation of *CYC1* transcription by non-Spt subunits of SAGA complex

3.4c Bar graphs show fold changes (mean ±SD, $p<0.005$) of *CYC1* levels upon activation versus partial repression from each strain of 3 separate experiments. The induction fold changes were calculated by dividing the signals of *CYC1* transcripts upon activation by those during partial repression.

3.4d The SAGA complex occupies the *CYC1* promoter

SAGA plays an important role in transcription regulation at many promoters; therefore, it is possible that the deletion of SAGA affects *CYC1* indirectly. If SAGA has a direct role in *CYC1* transcription one would hypothesize that SAGA occupies the *CYC1* promoter. To test this hypothesis, chromatin immunoprecipitation (ChIP) assays were performed on a number of SAGA subunits: Spt7, Spt8, Spt20, Gcn5, & Ada1. Each SAGA subunit was epitope tagged with either 3 tandem repeats of hemagglutinin (HA) or 13 tandem repeats of Myc. Deletion of Ada1 disrupts the structural integrity of the SAGA complex (Dudley et al., 1999; Grant et al., 1998a; Horiuchi et al., 1997; Wu et al., 2004; Wu and Winston, 2002). Ada1 occupied the CYC1 promoter in both the uninduced and induced state (Figure 3.5). The presence of the SAGA complex at the *CYC1* promoter indicates that SAGA has a direct role in PIC-post-recruitment regulation of *CYC1*. Deletion of SAGA subunits, important for structural integrity, leads to abolishment of *CYC1* transcription and confirms a direct role for SAGA in post-recruitment regulation.

SPT8 is found only in the SAGA complex and not the SLIK complex (Pray-Grant et al., 2002), additionally SPT7 is truncated at the C-terminal end in the SLIK complex (Pray-Grant et al., 2002; Sterner et al., 2002; Wu and Winston, 2002). To ensure that SAGA had post-recruitment function SPT8 and SPT7 were both epitope tagged at the C-terminus. We found that Spt8 and Spt7, which was myc tagged to the C-terminus, occupy the *CYC1* promoter (data not

shown). Therefore the SAGA complex occupies *CYC1* in both the uninduced and induced conditions.

TFIID and SAGA have been found to share many TAF subunits, however *TAF1* is specific to TFIID (Grant et al., 1998b). In addition, genes whose regulation is dependent on SAGA are largely TFIID-independent (Huisinga and Pugh, 2004). To determine if *CYC1* was TFIID-independent, Taf1 was HA tagged and occupancy of Taf1 was determined at the *CYC1* promoter. An untagged strain was used as a control and immunoprecipiation of Taf1-HA was not greater than background (Figure 3.5). Lack of Taf1 occupancy at *CYC1* suggests *CYC1* is a TFIID-independent gene.

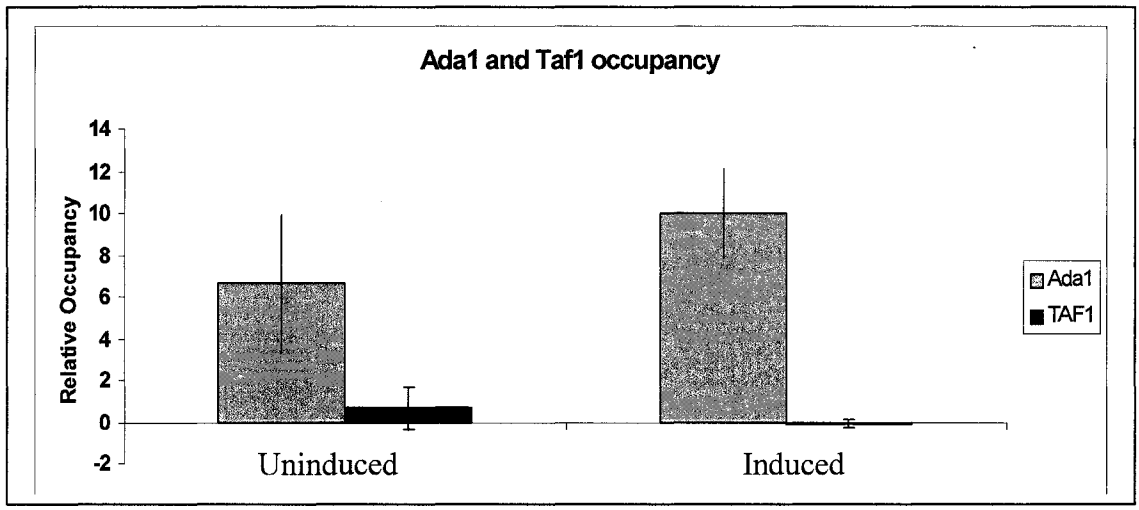
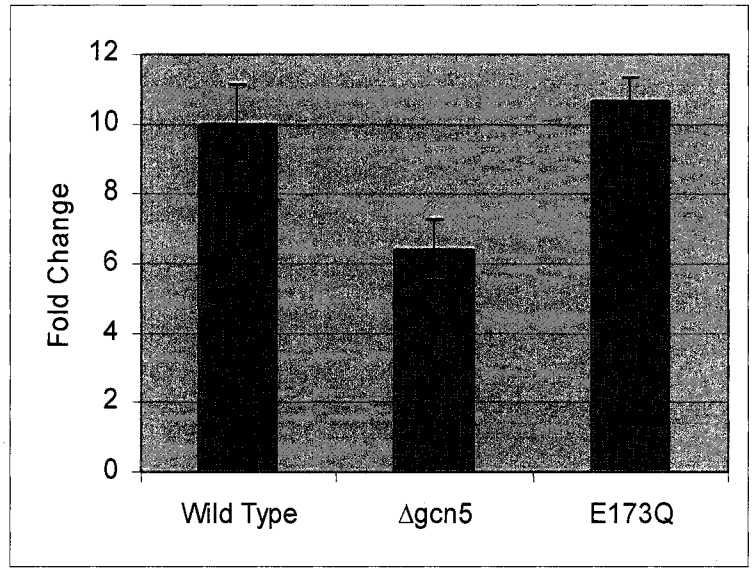


Figure 3.5 SAGA occupancy at the *CYC1* promoter.
Example of SAGA subunit, Ada1, important for SAGA integrity whose occupancy was determined at the *CYC1* promoter. Occupancy of Ada1 showed significant occupancy levels in both the uniduced and induced conditions. Taf1 did not occupy the *CYC1* promoter in either the uninduced or induced conditions suggesting that the *CYC1* promoter is TFIID-independent.

3.4e The histone acetyl-transferase activity of SAGA is not required for proper regulation of *CYC1*

One of the two known functions of SAGA is the enzymatic activity of Gcn5 in transferring acetyl groups to histones (Brownell et al., 1996; Grant et al., 1997; Grant et al., 1998b; Utley et al., 1998). To determine what role the acetyl transferase activity of SAGA had in post-recruitment regulation a number of studies were carried out. First, deletion of the enzymatic subunit of SAGA, Gcn5, does not abolish *CYC1* transcription, and deletion of the two HAT-related SAGA subunits, Ada2 & Ada3, has no effect on transcription (Figure 3.4A & C). Second, S1 nuclease assays were performed using a GCN5 mutant defective for histone acetylation, E173Q (Ricci et al., 2002), and it was shown to have no effect on *CYC1* gene expression (Figure 3.6A). Finally histone occupancy at the *CYC1* promoter was determined. With an antibody targeted to H3, ChIP assays were used to determine histone occupancy at the *CYC1* promoter and ORF. In both the uninduced and induced conditions, the level of histone occupancy was not above background at the *CYC1* promoter, while the coding region had histone occupancy. As a positive control H3 occupancy was measured at the *GAL1* promoter where histones were found in the uninduced condition and diminished upon activation (Figure 3.6B). These results are in accord with previous studies that have shown that the *CYC1* promoter is free from histones while the ORF has some histone occupancy (Martens et al., 2001; Pokholok et al., 2005). Taken together, these observations suggest that the acetyl transferase activity is not important for *CYC1* post-recruitment regulation.

A



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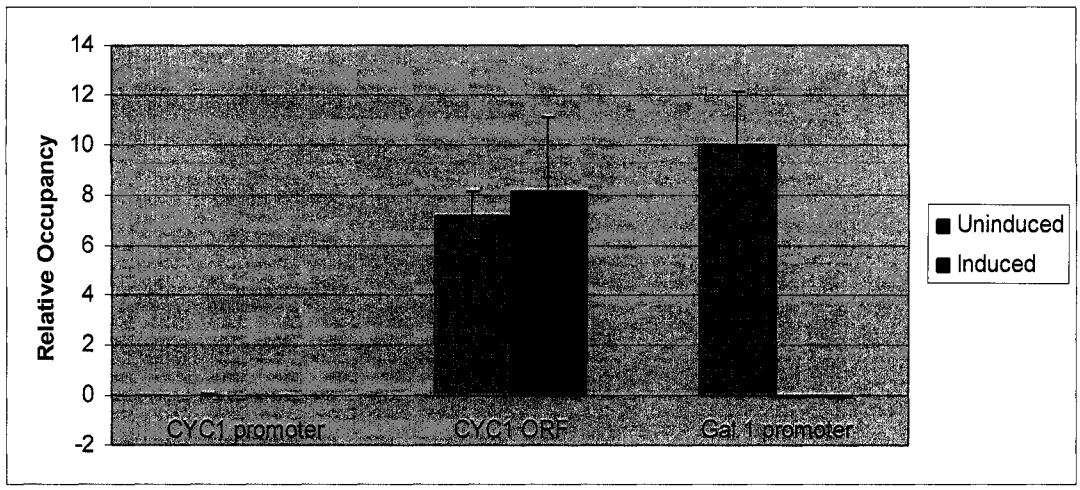


Figure 3.6 HAT activity of SAGA is not required for post-recruitment regulation of *CYC1*

3.6a S1 nuclease assays showed that a *gcn5* mutant defective for HAT activity did not diminish the expression of *CYC1*. In conjunction with the lack of histones at the *CYC1* promoter it is evident that SAGA's function at the *CYC1* promoter is not acetylation of histones.

3.6b ChIP assays were conducted using anti-H3 antibodies in three separate trials of un-induced and induced conditions. Anti-myc was used as an irrelevant antibody control and was subtracted from the H3 IP. The *CYC1* promoter showed diminished histone occupancy compared to the *CYC1* ORF or the *GAL1* uninduced promoter.

3.4f TBP recruitment function of SAGA is not required for *CYC1* regulation

The second known function of SAGA is that of TBP delivery (Belotserkovskaya et al., 2000; Bhaumik and Green, 2001; Dudley et al., 1999; Larschan and Winston, 2001; Qiu et al., 2005). TBP is bound directly by way of the SAGA complex (Sterner et al., 1999) and is transferred to the TATA box by SAGA (Sermwittayawong and Tan, 2006). The SAGA complex has been shown to be required for TBP recruitment to the promoter at a number of SAGA dependent genes (Bhaumik and Green, 2002). At such genes, deletion of SAGA components lead to the loss of TBP occupancy, measured via ChIP. It is possible that the dependence of *CYC1* expression on SAGA is connected to the TBP recruitment function of SAGA. If the TBP recruitment function of SAGA is important in regulation of *CYC1*, deletion of SAGA will lead to loss of TBP occupancy to the *CYC1* promoter. To tet the hypothesis that the TBP recruitment function of SAGA is important for *CYC1* regulation we performed ChIP assays in deletion backgrounds, (Δ*spt7,* Δ*spt20,* Δ*gcn5,* and Δ*ada1*) of SAGA. In all four deletion strains TBP occupied the *CYC1* promoter similar to that of wild type in both the uninduced and induced conditions (Figure 3.7A & B). In addition, RNAPII was also found to occupy the *CYC1* promoter in Δ*spt7,* Δ*spt20,* Δ*gcn5,* and Δ*ada1* strains during the uninduced condition. In the Δ*ada1* strain, RNAPII occupancy showed a slight decrease during activation compared to wild type (Figure 3.7A & C). Taken together the TBP recruitment function of SAGA is not required for post-recruitment regulation of *CYC1.*

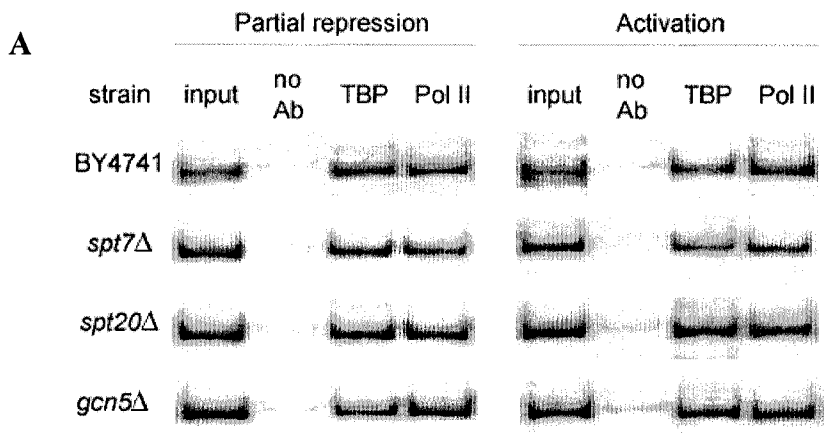
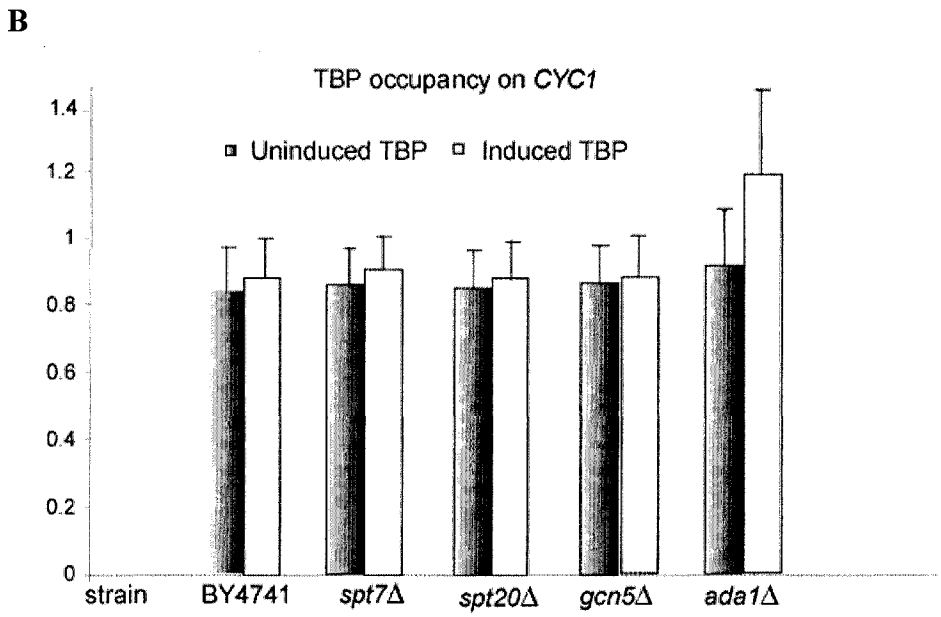
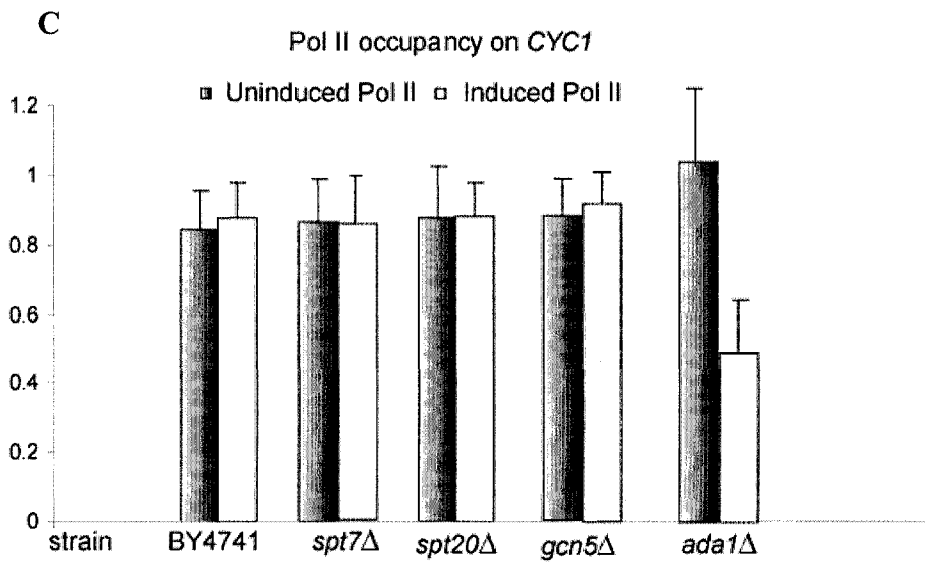


Figure 3.7 Occupancy levels of TBP and Pol II on the *CYC1* gene

3.7a Strains as indicated were grown under partial repressed or activated conditions of *CYC1* gene. ChIP analysis was performed to measure the occupancy levels of TBP and Pol II. Shown here is one representative picture of three independent experiments.



3.7b Bar graph shows the relative occupancy levels of TBP during un-induced and induced conditions of the *CYC1* gene (mean±SD, n=3, $p<0.005$). Protein occupancy was represented by ratio of signal from IP samples versus that of the input minus the signal of no antibody control. TBP occupancy of the *ada1Δ* strain was quantified using Real-Time PCR.



3.7c Bar graph shows the relative occupancy levels of Pol II during un-induced and induced conditions of the *CYC1* gene (mean±SD, n=3, $p<0.005$). Protein occupancy was represented by ratio of signal from IP samples versus that of the input minus the signal of no antibody control. Pol II occupancy of the *ada1Δ* strain was quantified using Real-Time PCR.

3.4g Deletion of SAGA reduces the occupancy of Mediator during activation of *CYC1*

Gene expression is a highly regulated event that encompasses the interaction of a number of complexes to properly initiate transcription. SAGA functions with Mediator to properly regulate transcription initiation (Larschan and Winston, 2005; Qiu et al., 2005). The bypass assay to determine post-recruitment function was applied to non-essential Mediator strains and ~30% of the strains tested showed a post-recruitment phenotype (Table 3.2). Interestingly, the post-recruitment strains were not concentrated to one domain of the mediator complex.

To determine the occupancy of Mediator at *CYC1* during the induced and uninduced conditions, Srb8 was tagged with a HA epitope. Mediator was found to have a four fold increase in occupancy at the *CYC1* promoter upon activation (Figure 3.8b). Mediator occupancy was also measured in a Δ*spt20* mutant strain. This was carried out by HA epitope tagging Srb8 in a strain deleted for *SPT20*. Western blot analysis showed that deletion of Spt20 had no effect on the expression level of Srb8-HA versus wild-type expression (Figure 3.8a). ChIP analysis of Mediator in cells where Spt20 is deleted shows Mediator occupancy fails to increase at the *CYC1* promoter during inducing conditions (Figure 3.8b). This indicates that Spt20 is essential for recruitment of Mediator to *CYC1*.

Table 3.2

Results of TBP recruitment bypass assay with Mediator subunits

Strains	LexA	LexA-TBP	[AT] used	Days of growth
BY4741	+	++++	40	3
Δ*med15*	+	+++	40	3
Δ*anc1*	+	++	40	3
Δ*med19*	+	-	40	3
Δ*med5*	+	+++	40	3
Δ*med16*	+/-	-	40	3
Δ*med20*	+	+++	40	3
Δ*med18*	+	+++	40	3
Δ*med12*	+	+/-	40	3
Δ*med13*	+/-	+++	40	3
Δ*ssn3*	+	+++	40	3
Δ*ssn8*	+	++	40	3
Δ*med3*	+	+++	40	3
Δ*med9*	-	+	40	3

Table 3.2: The yeast cells transformed with the plasmid-based artificial recruitment system were grown on SC-UL and SC-ULH 3-aminotriazole (AT) plates at 30 °C. The growth of cells was scored as ranging from "+/-" to "++++". The "+/-" means very weak to no growth, and the "++++" indicates robust growth.

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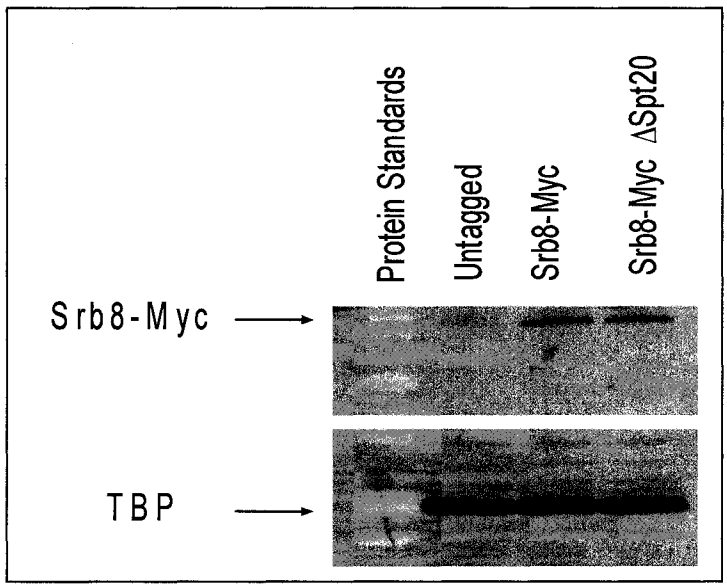

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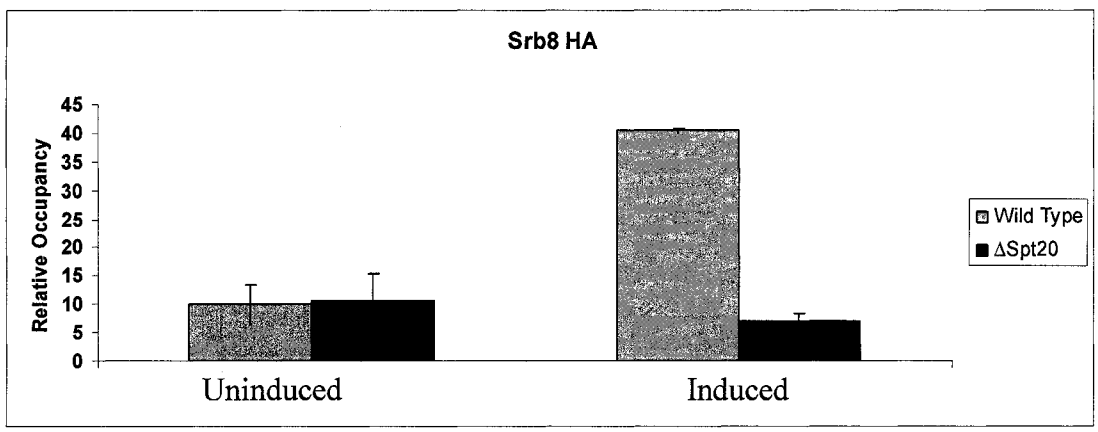


Figure 3.8 Occupancy levels of Mediator on the *CYC1* gene
A) Western blot analysis shows deletion of Spt20 has no effect on expression of Srb8-Myc. TPB was used as a load control and an untagged strain as a negative control.

B) Bar graph shows the relative occupancy levels of Mediator, Srb8, during un-induced and induced conditions of the *CYC1* gene (mean±SD, n=3, $p<0.005$). Wild-type is BY4741 with Srb8 epitope tagged with HA. ΔSpt20 is BY4741 with *SPT20* deleted from the genome and Srb8 epitope tagged with HA. Mediator increases four fold upon activation of *CYC1* in a wild-type background, but does not increase in the absence of SAGA.

3.5 Discussion

To study the effects of the *SPT* gene family on post-recruitment regulation we created a plasmid based reporter system to study non-essential *SPT* genes. Only *SPT* gene products that are part of the SAGA complex were found to have a post-recruitment function in our genetic assay. SAGA was further confirmed to have a post-recruitment function when the Δ*ada1* strain had a post-recruitment function in our bypass assay.

Our results show deletion of SAGA subunits important for SAGA integrity abolishes *CYC1* transcription, and that SAGA occupies the *CYC1* promoter, indicating SAGA has a direct role in post-recruitment regulation. SAGA, however, does not function in TBP recruitment at the *CYC1* promoter; TBP occupancy is not affected by the deletion of *SPT7, SPT20* or *ADA1.* Additionally, the promoter of *CYC1* is nucleosome depressed [(Martens et al., 2001; Pokholok et al., 2005) and herein] and the deletion of the enzymatic subunit of SAGA, *GCN5*, and the HAT-related subunits, *ADA2* and *ADA3*, does not abolish transcription of *CYC1.* In fact, a Gcn5 mutant, E173Q, defective for HAT activity has no effect on *CYC1* transcription. Taken together it is evident that SAGA has a new and unique function in post-recruitment regulation of *CYC1.*

We have shown that SAGA plays an important role in the recruitment of Mediator during activation (Figure 3.8) at post-recruitment regulated gene. Conversely in PIC-recruitment regulated promoters, Srb6 and Gal11, recruitment of SAGA has been shown to be dependent on Mediator (Qiu et al., 2005). In higher eukaryotes mediator plays an important role in promoter-proximal post-

recruitment regulation (Park et al., 2001). Mediator has been shown to have post-recruitment function in transcription initiation (Wang et al., 2005). Mediator has been implicated in promoter proximal regulation of *Drosophila HSP70,* where the Mediator complex is recruited to the promoter upon heat shock in a RNA Pol II free form (Park et al., 2001). Our results are in accord with others that Mediator does play a role in post-recruitment regulation and here we show that SAGA is required for the recruitment of mediator to the post-recruitment regulated gene *CYC1*. At the *CYC1* promoter Mediator occupancy does not increase during activation in strains deleted for Spt20, suggesting that SAGA is important in properly recruiting Mediator to *CYC1* during activation. SAGA function in post-PIC-recruitment regulation may be interacting with Mediator to mediate the trigger which serves as the rate limiting step of post-recruitment regulation.

Lastly it is important to note that the study of post-recruitment genes is not just a study of an important subset of genes, but is also a way for us to take advantage of a natural process to study the range of transcription regulation. Transcription is a complex process that involves a number of factors that are precisely assembled to activate certain genes at specific times according to the cell's needs to properly maintain homeostasis. It has been shown that the cell makes this process as efficient as possible with "full" and "partial-PIC" assembly throughout the genome (Zanton and Pugh, 2006). Post-recruitment regulated genes, such as *CYC1* and *Drosophila HSP70*, are functionally different from that of "PIC-recruitment" regulated genes, like *GAL1*, in which different genes may

possess "Partial or Full-PICs" of distinctly different composition. The study of each of these genes, and their corresponding promoters and "Partial or Full PIC" provides an avenue for the understanding of the continuum of transcription regulation. In studying "PIC-recruitment" regulated genes, i.e. *GAL1*, the rate limiting step is events that lead up to the recruitment of the pre-initiation complex and thereby serves as the point of study. These PIC-recruitment genes are not able to be as well studied in vivo for rate limiting steps after the recruitment and assembly of the PIC. Understanding the mechanism of "post-PIC-recruitment" regulated genes allows the scientific community to continue the study of transcription initiation regulation beyond the recruitment of the PIC. In genes where TBP and RNAPII are already bound to the promoter but transcription is not initiated the point of study is now the events that occur after PIC nucleation but before promoter clearance/elongation. Therefore, the study of "Post-Recruitment" genes provides the opportunity to determine rate-limiting steps of transcription intiation after PIC recruitment in vivo.

Chapter 4

Characterization of the pre-initiation complex at the *CYC1* post-recruitment regulated promoter

Post-recruitment regulation is an important mechanism in higher eukaryotic gene expression. *S. cerevisiae* is a single cell eukaryote that has a variety of unique experimental techniques which allow for effective study of cellular and biochemical mechanisms. Previous studies have shown TBP and RNA polymerase II occupy the *CYC1* promoter. Here we find that the majority of the PIC occupies the *CYC1* promoter. In addition, the C-terminal domain of RNA polymerase II is phosphorylated and capping enzyme is present during the uninduced condition. Also included in Chapter 4 is a list of stains I have created with epitope tagged proteins for future studies.

Characterization of the pre-initiation complex at the *CYC1* post-recruitment regulated promoter

Aaron Fletcher and Laurie Stargell

4.1 Abstract

The *S. cerevisiae CYC1* gene has been shown to have TBP and RNA Polymerase II (RNAPII) bound to the promoter during the uninduced state. Here we characterize the post-recruitment regulated gene and find that most of the members of the PIC are bound to the *CYC1* gene in the uninduced state. In addition to TFIIH being bound to the uninduced *CYC1* gene the C-terminal domain (CTD) of RNAPII is phosphorylated at the serine 5 position of the heptad repeat. The capping enzyme, Ceg1, was also found to occupy the promoter during repressed gene expression. Interestingly, Mediator does not have full occupancy until *CYC1* is fully active. Comparison of *S. cerevisiae* with *Drosophila* post-recruitment regulation indicates that the mechanism of post-recruitment regulation is conserved between eukaryotes.

4.2 Introduction

One can grossly breakdown transcription regulation into two sub-categories: TBP/RNAPII recruitment regulated genes and post-TBP/RNAPII-recruitment regulated genes. In the case of recruitment regulated genes initiation of transcription is regulated by the recruitment of TBP and the corresponding PIC to the promoter DNA; in such cases, the level of TBP occupancy is positively correlated with the level of transcription activity (Kim and Iyer, 2004; Kuras and Struhl, 1999; Li et al., 1999). At such genes the rate-limiting step of transcription is the ability to recruit TBP to the promoter and initiate nucleation of the PIC. However, a growing number of genes have a high level of TBP and RNAPII bound without a high level of transcription activity, (Kuras and Struhl, 1999; Martens et al., 2001) and are therefore post-TBP/RNAPII-recruitment regulated. In post-recruitment regulation TBP/RNAPII recruitment is no longer the rate limiting step of transcription initiation. Many of these genes are important in a variety of environmental responses that are needed for homeostasis. It is therefore important to understand the transcriptional regulatory mechanisms of post-recruitment regulated genes. In such an environment, the promoter is constrained and ready to be activated upon proper stimulation.

The *S. cerevisiae* gene *CYC1* is a gene that is post-recruitment regulated yet little is known about the PIC occupancy at the promoter. The *CYC1* gene encodes iso-1-cytochrome c, which is involved in the electron transport chain in the mitochondria (Sherman et al., 1966). In the presence of a fermentable carbon source (such as glucose) the *CYC1* gene is partially repressed at the

transcriptional level; however, TBP and Pol II are present on the *CYC1* gene (Kuras and Struhl, 1999; Martens et al., 2001). The gene is fully activated when the cell is grown on a non-fermentable carbon source (such as lactate or ethanol) and TBP and RNAPII occupancy increases marginally (less than two fold); however, on a non-fermentable carbon source, *CYC1* transcriptional expression is ten-fold greater (Guarente et al., 1984; Guarente and Mason, 1983). To fully characterize the state of the uninduced *CYC1* promoter, occupancy of the entire PIC was determined.

4.3 Materials and Methods

4.3a Yeast strains, media and DNA

The wild-type strain BY4741: MATa his3Δ1 ura3Δ0 leu2Δ01 met15Δ01 was used throughout this comparative analysis for the *S. cerevisiae* strain. The yeast complete and synthetic complete (SC) media were made as described (Hampsey, 1997a).

4.3b Phenotypic studies

For phenotypic studies, 10-fold serial dilutions of strains were applied 10µl drops on plates. A variety of different plates were used to effectively screen phenotypes and included: YPD (2% Dextrose), YPE (3% ethanol), lacking inositol, containing sorbitol, 0.75-1M NaCl, 30° Celsius, 37° Celsius, 16° Celsius, YPEG (3% ethanol and glycerol), 2% raffinose, 2% galactose, media lacking

histidine as a selectable marker, and media lacking either leucine, or uracil as a negative control. Plates were typically used within 36 hours of pouring.

4.3c Transformations

DNA was isolated from PCR products and transformed into BY4741. BY4741 strains were grown to log phase and cells were harvested and washed in LiAc/TE (0.1M LiAc, 10mM Tris pH 8, 1mM EDTA). Each transformation was performed in 50mL of log phase cells, 5 µl of ssDNA, 1, 5, and 15 µl of purified PCR DNA, and 300 µl LiAc PEG (40% PEG 3350) mixed thoroughly and incubated at 30 degrees Celsius for 30 minutes. Cells were then heat shocked for 20 minutes and plated to selectable medium. After 48 hours, colonies were purified to ensure single colonies and proper selection.

4.3d Western blot analysis

10ml of yeast cells grown in YPD with an OD600 of ~0.8 were harvested, washed once with sterile water, and resuspended in 200ul lysis buffer (0.5M phosphate buffer, pH 7.5). Whole cell extracts were prepared by vigorous bead beating. Cellular debris was removed by spinning the extracts at 10,000g at 4°C for 10 min. Protein concentrations were determined by Bradford assay (Bio-Rad). Equal amount of whole cell extracts were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The following antibodies were used at the given dilutions: anti-HA (sc-7392, from Santa Cruz; 1:500), anti-myc (Upstate Inc.). Horseradish peroxidase (HRP) conjugated second antibodies were used at

1:20,000 dilution and protein bands were detected by western blotting detection reagents from Amersham Biosciences.

4.3e Chromatin immunoprecipitation (ChIP) analysis

Chromatin immunoprecipitations were performed as described (Strahl-Bolsinger et al., 1997), with a few modifications. Cells (50 ml) were grown to OD 600 of 0.8~1.0. Cells were treated with a final concentration of 1% formaldehyde for 15 minutes with occasional swirling of the flasks at intervals of 5 minutes. Glycine was added to a final concentration of 125mM at room temperature for 5 minutes to stop cross-linking. Cells were collected and washed twice in ice cold TBS. Cells were resuspended 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, beuzamidine, pepstatin, leupeptin, and chymostatin). Chromatin was sheared by sonication using a Branson W-350 model of sonifier (10 times at 10 seconds each on continuous pulse at a microtip power setting of 6). 10% of the chromatin material used for the immunoprecipitation was processed as the input after reversing the cross-links and purifying the DNA. 500 µl of the chromatin material was incubated with approximately 10µl of either homemade anti-TBP, anti-Pol II (8WG16, Covance Inc.), anti-HA (Santa Cruz) or anti-Myc (Upstate), anti-Serine 2 CTD phosphorylation (H5, Covance), anti-Serine 5 CTD phosphorylation (8WG16, Covance) antibodies by rotation overnight at 4°C. 50µl of protein-A sepharose beads (Pharmacia-prepared as slurry as per the manufacturers directions) was further incubated with the

chromatin material for 3 hours at 30°C. The beads were spun down and the Antigen-Antibody complexes bound to the beads were recovered and further treated with TE/SDS buffer for 15 minutes at 65°C to elute the complexes. Protein-DNA cross-links were reversed by incubation overnight at 65°C and the DNA was purified by phenol-chloroform extraction and used for Real Time PCR (RT-PCR) analysis. Protein occupancy was determined via real time PCR (RT-PCR). Standard curves were generated using 10-fold serial dilutions of Input DNA. PCR efficiencies ranged from 90-100%, with a correlation coefficient of 0.990 or greater. Threshold cycle data were quantified relative to the input, as described (Frank et al., 2001). Background levels were calculated as the relative occupancy of untagged strains and these values were subtracted from that of the tagged strains.

4.4 Results

4.4a Epitope tagging of proteins for determining occupancy of complexes.

To properly compare *S. cerevisiae* protein complex occupancy levels, each protein of the complex was tagged with epitopes. Epitope tagged proteins provided consistency throughout the experiments during the immunoprecipitation process, and reduced expense by allowing one to immunoprecipitate numerous subunits using the same antibody. Plasmids provided by Mark Longtine were used to insert epitopes onto the subunits of interest (Longtine et al., 1998). Each epitope consists of either three-tandem repeats of the influenza virus hemagglutinin (HA) epitope or 15 tandem repeats of the Myc epitope. Using

these plasmids, we could tag the protein on either the N- or C-terminus. The collection of strains described here each contain protein at the C-terminus, thereby allowing endogenous protein levels by maintaining each gene's natural promoter. The Longtine module consists of the epitope tag and a histidine selectable marker flanked on each side by 60 nucleotide oligos consisting of homologous nucleotides to the C-terminus of the gene of interest and to the Longtine model to allow for specific homologous recombination (Figure 4.1).

Tagging specific proteins with an epitope tag starts by designing oligos to the protein of interest as described in Figure 4.1. The oligos are used to produce a PCR amplicon with the Longtine module consisting of the specific epitope of interest. The DNA is isolated and transformed into BY4741, where homologous recombination incorporates the epitope to the C-terminus of the gene of interest. Western blot analysis ensures that the correct protein was tagged and phenotypic screens determine if the eptitope tag affected the function of the protein (Figure 4.2).

A number of different proteins from numerous protein complexes were tagged (Mediator: Srb8, Rox3; SAGA: Ada1, Spt7, Spt20, Spt8, Gcn5; TFIIH: Rad3, Kin28, Ssl2; Capping Enzyme: Ceg1; RNAPII: Rpb2; TFIID: Taf1). All strains that are tagged and have been screened via Western blot analysis and phenotypic screen have been compiled into Table 4.1. Included in the table are tagged proteins whose data are described in other chapters of my thesis and proteins on which I have not yet performed ChIP assays.

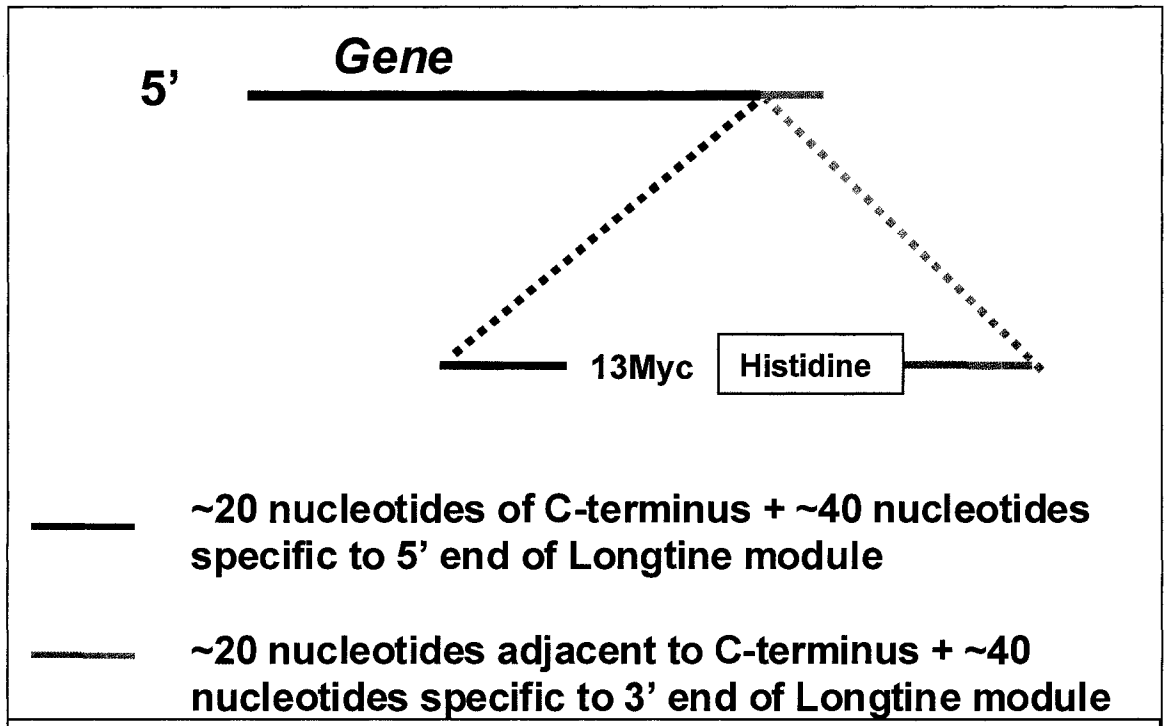


Figure 4.1: Longtine modules allow for epitope tagging of genes via homologous recombination. The PCR amplicon consists of 60 nucleotide flanking regions that incorporate the epitope tag at the C-terminus of the gene of interest. To ensure proper recombination a histidine selectable marker is amplified within the Longtine module.

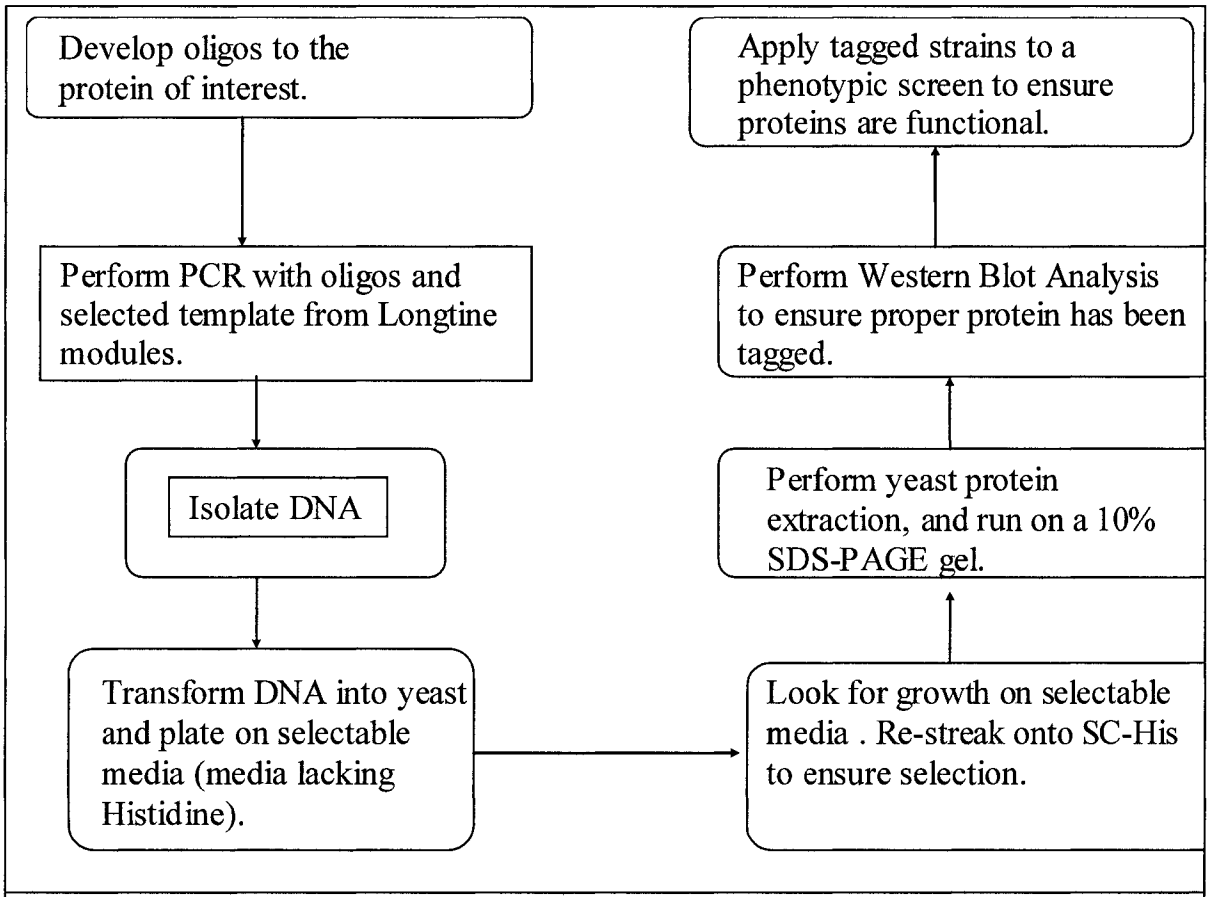


Figure 4.2: Flow chart for epitope tagging proteins. To ensure proper tagging a western is performed to the epitope of interest. In addition, a phenotypic screen is performed on each tagged strain to determine if tagging of the protein affects function in any way.

Table 4.1 Epitope tagged strains in yeast BY4741 background

Epitope Tagged S. cerevisiae strains	Western blot confirmation	Phenotypic Screen confirmation	CYC1 occupancy determined
Rad3-HA	✓	✓	✓
Spt20-HA	✓	✓	✓
Spt8-HA	✓	✓	✓
Rox3-HA	✓	✓	✓
Srb8-HA	✓	✓	✓
Taf1-HA	✓	✓	✓
Hap4-HA	✓	✓	ND
Spt4-HA	✓	✓	ND
Spt5-HA	✓	✓	ND
Ceg1-HA	✓	✓	✓
Rpb2-HA	✓	✓	ND
Srb8-HA Delta Spt20	✓	✓	✓
Kin28-Myc	✓	✓	✓
Rad3-Myc	✓	✓	✓
Spt7-Myc	✓	✓	✓
Ada1-Myc	✓	✓	✓
Gcn5-Myc	✓	✓	✓
Ceg1-Myc	✓	✓	✓
Ssl2-Myc	✓	✓	✓
Ccl1-Myc	✓	✓	✓
Rtf1-Myc	✓	✓	ND
Rox3-Myc	✓	✓	✓
Rox3-Myc Delta Spt20	✓	✓	ND

Table 4.1 List of strains tagged with either a HA or Myc epitope. All strains are in the BY4741 parental strain background and have been confirmed as having the correct protein tagged by Western Blot analysis. Each strain in this table was also found not to disrupt protein function upon eptitope tagging to the C-terminus via phenotypic analysis. ND (Not Done) indicates strains whose tagged product has not been tested for *CYC1* occupancy. Genotype for epitope tagged strains, XXX indicates gene name: BY4741and XXX::13Myc/*HIS3* or BY4741 and XXX::3HA/*HIS3*. For example Rad3-HA strain is BY4741 and *RAD3*::3HA/*HIS3*

4.4b Occupancy of TFIIH to the *CYC1* promoter.

The pre-initiation complex consists of a number of general transcription factors: TFIID, TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH (Hampsey, 1998; Kornberg, 2001; Kuras and Struhl, 1999; Lee and Young, 2000). TFIID is a multi-subunit general transcription factor which consists of TBP and a number of TAFs (Hernandez, 1993; Struhl, 1994) Due to TBP occupying the *CYC1* promoter during partial repression, we determined if the entire pre-initiation complex will be found at the *CYC1* promoter during partial repression of a post-recruitment regulated gene. TFIIH is the last general transcription factor to join the pre-initiation complex (Cortes et al., 1992) and has two important enzymatic functions, helicase and kinase, for initiation of transcription (Feaver et al., 1991; Lu et al., 1992; Schaeffer et al., 1993; Serizawa et al., 1993a; Serizawa et al., 1992).

The helicase activity of TFIIH is an important step in promoter clearance of the polymerase. If TFIIH is found not to occupy the promoter during partial repression, it would imply that the recruitment of TFIIH to the promoter is an important step in the transition to full activation. If TFIIH is indeed occupying the *CYC1* promoter during partial repression one could deduce three possibilities: 1) the polymerase is bound and sitting in the closed conformation because TFIIH has not been cued to enact its helicase activity 2) the polymerase is bound and sitting at the start site in the open conformation and 3) that the polymerase has bound and undergone promoter clearance but is paused in a promoter proximal manner.

The kinase (Kin28) and helicase (Rad3) enzymatic subunits of TFIIH were tagged and *CYC1* promoter occupancy was determined. Interestingly, the helicase subunit was found to occupy the *CYC1* promoter in the uninduced condition (Figure 4.3a). However, the kinase subunit was not detected at the promoter until after induction due to error range and background signal (Figure 4.3b). Kin28 and Rad3 are found to be part of different subcomplexes of TFIIH, TFIIH core and TFIIK (Takagi et al., 2003) and the difference in occupancy at *CYC1* may be do to the recruitment of TFIIK after induction. To test this hypothesis we tagged a second subunit , Ssl2, of TFIIK to see if the entire complex only came in after induction. Interestingly, Ssl2 was at the promoter in both the induced and uniduced conditions (Figure 4.3c). It is possible that due to conformational changes of TFIIH and the transcriptional machinery, Kin28 is not detectable by ChIP analysis until after induction.

4.4c RNAPII phosphorylation state of the CTD at *CYC1.*

To define the PIC composition at the *CYC1* promoter, the phosphorylated state of the RNA Polymerase II CTD was tested. The CTD consists of heptad repeats of the sequence YSPTSPS in which serine 2 and 5 are targets for phosphorylation. Serine 5 is phosphorylated by a number of subunits including the TFIIH subunit *KIN28* (Hengartner et al., 1998), and can be immunoprecipitated with the monoclonal antibody H14 (Bregman et al., 1995; Patturajan et al., 1998). The phosphorylated state of the (CTD) of RNAPII is an indicator for the state of the RNAPII enzyme. In the pre-initiation complex the

RNAPII CTD is thought to be in a hypophosporylated state (Pol IIa) and becomes hyperphosphorylated (Pol IIo) during elongation. Serine 5 phosphorylation is seen only at the promoter proximal region (Cheng and Sharp, 2003; Komarnitsky et al., 2000) and can be immunoprecipitated to determine if RNAPII at the *CYC1* promoter is phosphorylated in a promoter proximal manner.

ChIP assays using H14 antibody showed that the heptad repeat of RNAPII is phosphylated during the uninduced and induced condition of *CYC1* (Figure 4.4). This phosphorylated form of RNAPII further suggests the presence of TFIIH at the *CYC1* promoter and lack of detection by ChIP studies being due to conformational changes.

4.4d Occupancy of mRNA capping enzyme, *CEG1*, at the *CYC1* promoter.

The capping enzyme is present during the uninduced condition of higher eukaryotic post-recruitment regulation (Rasmussen and Lis, 1993). The capping activity of mRNA occurs during early elongation, after the transcript is only 20 to 30 nucleotides in length (Kim et al., 2004a). Occupancy of the Alpha (guanylyltransferase) subunit of the yeast mRNA capping enzyme, *CEG1*, was determined at the *CYC1* promoter (Itoh et al., 1984a; Itoh et al., 1984b; Lima et al., 1999; Shibagaki et al., 1992). During the uninduced condition of *CYC1,* Ceg1 was found to occupy the promoter and upon induction Ceg1 occupancy increased slightly, less than 2 fold (Figure 4.5).

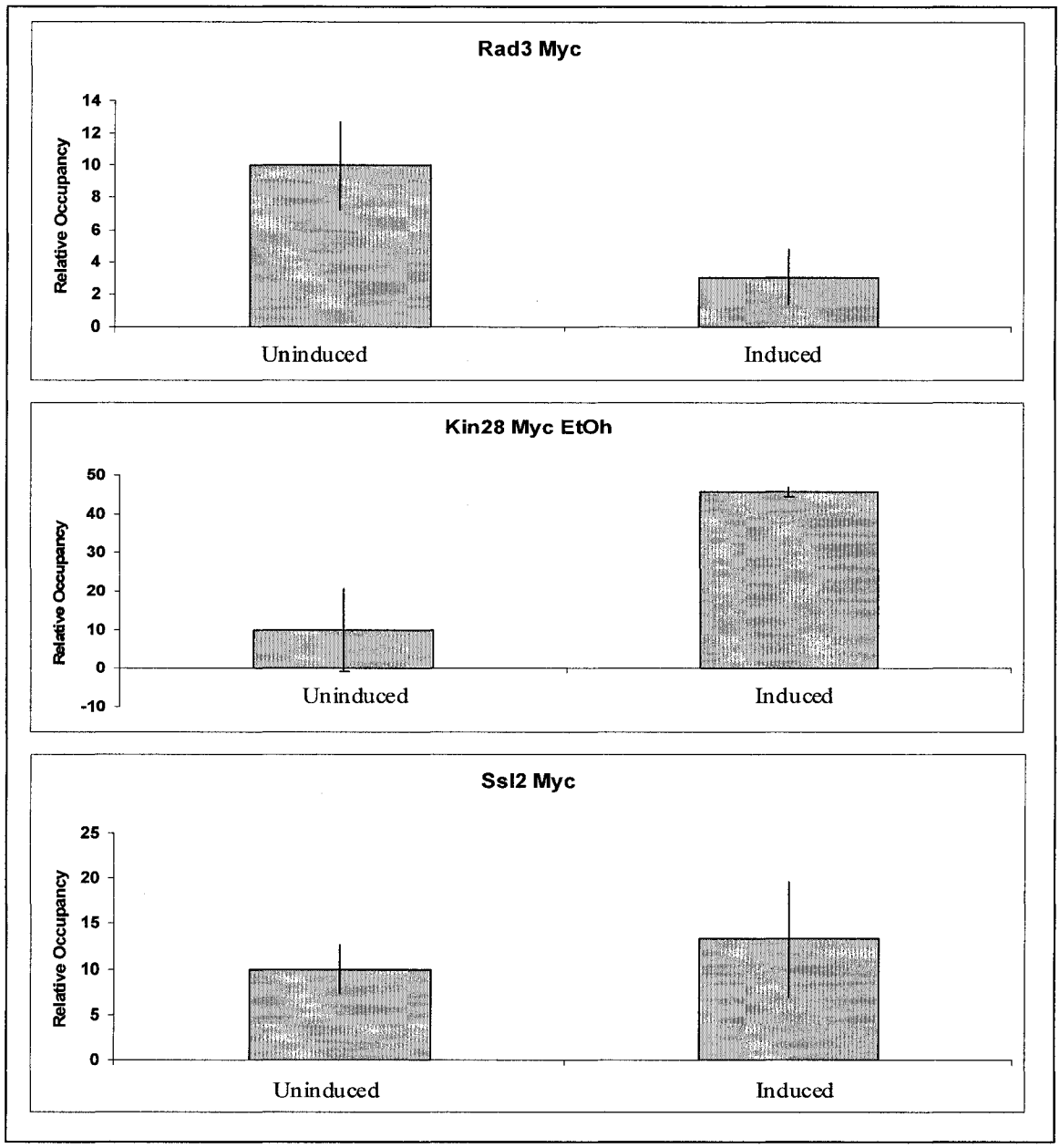


Figure 4.3 ChIP analysis of TFIIH at the *CYC1* promoter (mean±SD, n=3, $p<0.005$).

A) Rad3, the helicase subunit of TFIIH, is at the promoter prior to induction and occupancy decreases upon induction.

B) Kin28, the kinase subunit of TFIIH and part of the TFIIK sub-complex of TFIIH, occupies the promoter after induction but is not seen prior to induction

C) Ssl2, a second subunit of the TFIIK sub-complex of TFIIH, occupies the *CYC1* promoter equally in the induced and uninduced state and suggests the presence of TFIH at the promoter in both the uninduced and induced states in which TFIIH undergoes conformational changes during the transition from uniduced to induced.

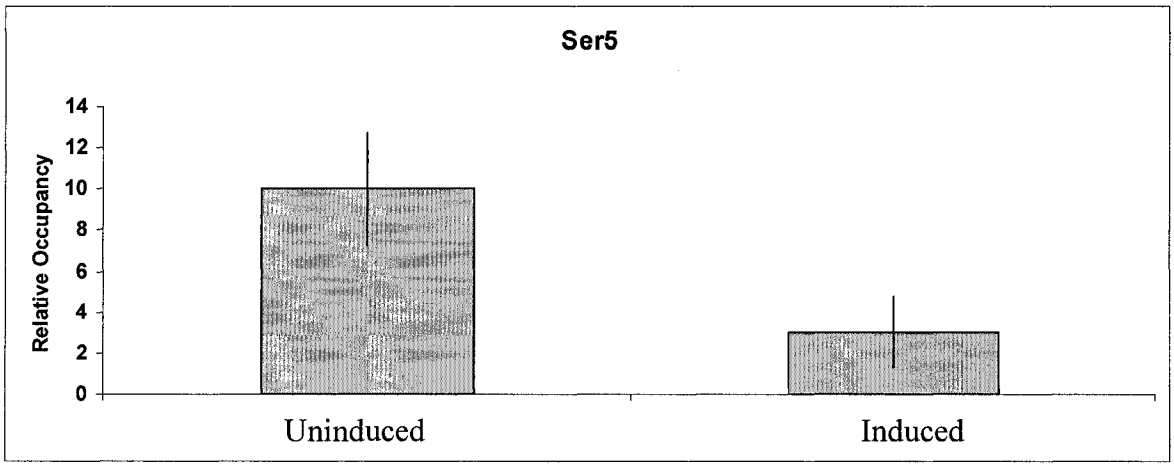


Figure 4.4 ChIP analysis of RNA Pol II CTD phosphorylation of Serine 5 of the heptad repeat at the *CYC1* promoter (mean±SD, n=3, $p<0.005$). During the uninduced state serine 5 of RNA Pol II CTD is phosphorylated at the *CYC1* promoter and serine 5 phosphorylation decreases upon induction.

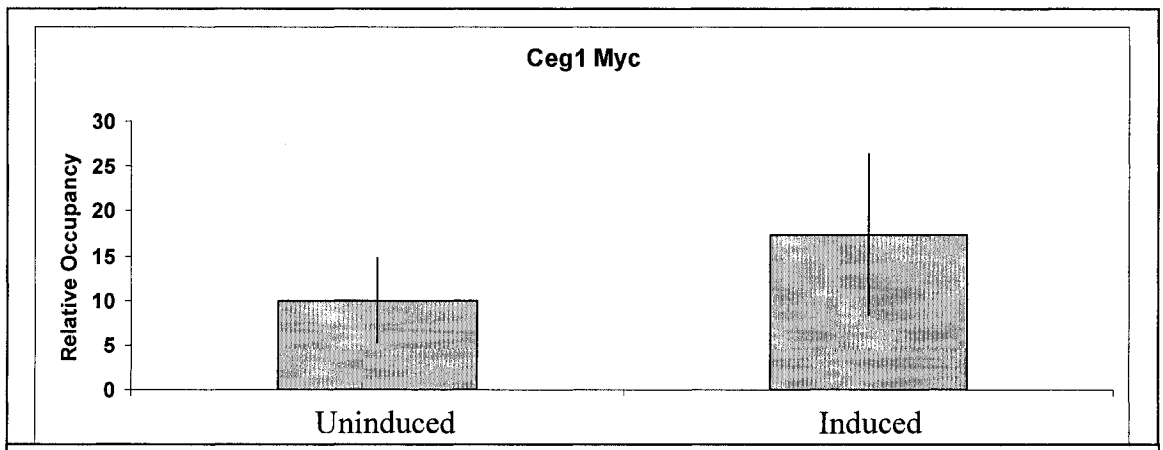
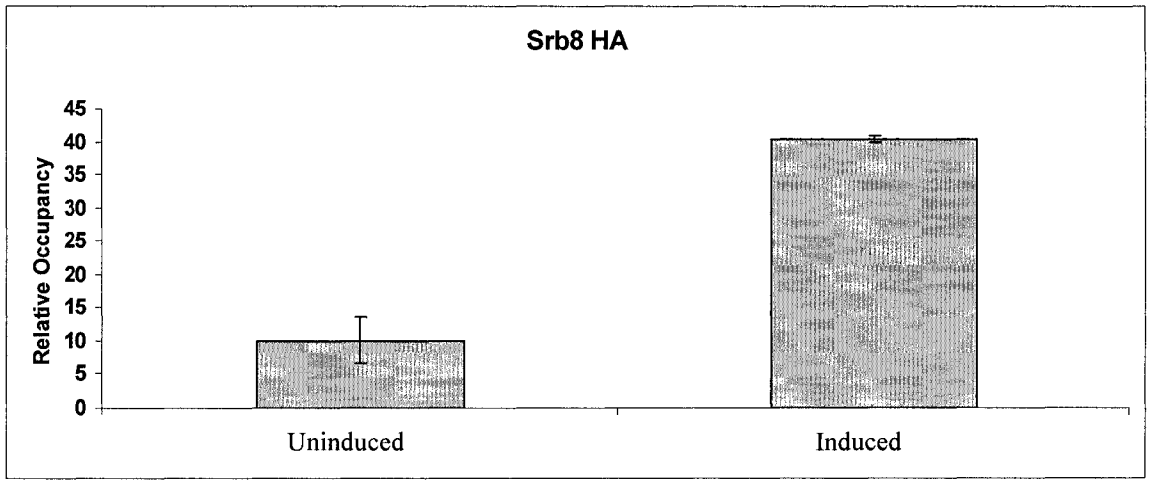


Figure 4.5 ChIP analysis of *S. cerevisiae* capping enzyme Ceg1 at the *CYC1* promoter. During the uninduced and induced state of *CYC1* gene activation Ceg1 occupies the *CYC1* promoter (mean±SD, n=3, $p<0.005$).

4.4e Kinetic analysis of Mediator occupancy at the *CYC1* promoter.

Mediator is found to play a role in post-recruitment regulation in *Drosophila* heat shock proteins and has been shown to be important in post-recruitment regulation of *CYC1* (Chapter 3). The activator-mediator complex is recruited in a Pol II free form and is mechanistically separable from the assembly of the preinitiation complex (Park et al., 2001). Because Mediator was found to have a post-recruitment phenotype through the genetic screen (Chapter 2 and 3), and due to mediator being recruited to the promoter of higher eukaryotes after initiation, timing of Mediator occupancy to the *CYC1* promoter was determined. A constitutive amount of Mediator occupancy is seen in the uninduced condition, when background of an untagged strain is subtracted out; upon induction the *CYC1* promoter is has increased occupancy by Mediator (Figure 4.6a). *CYC1* gene expression is fully activated after 6 hours of growth in a non-fermentable carbon source (Fischbeck et al., 2002). To determine if Mediator occupancy is early or late during activation a time course was performed in one hour intervals. Mediator occupancy occurs late in the induction process (Figure 4.6b).

A



B

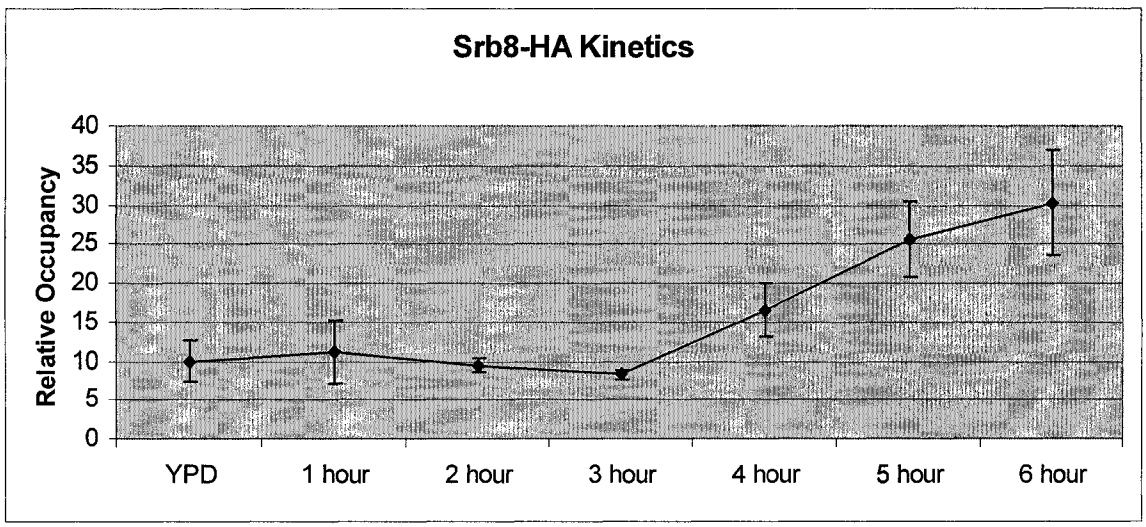


Figure 4.6 ChIP analysis of Mediator, via Srb8-Myc, at the *CYC1* promoter.

A) Cells were grown for 6 hours to log phase in uniduced (2% dextrose) or induced (3% ethanol) media. Cells were cross-linked with formaldehyde and protein-DNA cross-links were analyzed by quantitative PCR. ChIP analysis revealed Mediator has residual occupancy during the uninduced condition and fully occupies the *CYC1* promoter during the induced state of *CYC1* gene expression.

B) Time course of Mediator occupancy on the *CYC1* promoter. Cells were cross-linked at log phase in one hour intervals (mean±SD, n=3, $p<0.005$). Mediator occupancy begins to increase after three hours of induction and is fully occupied by six hours induction.

4.5 Discussion

To determine the state of the PIC at *CYC1* TFIIH occupancy was determined and found that the helicase enzymatic subunit Rad3 occupies the promoter during the uninduced condition. Kin28, the kinase enzymatic subunit, however was not seen until after induction. This discrepancy is most likely due to conformational changes the PIC undergoes during induction that allows Kin28 to be immunoprecipitated during the induced condition. Rad3 and Kin28 are subunits of two subcomplexes of TFIIH, Rad3 in TFIIH core and Kin28 in TFIIK, and it is possible that TFIIH is recruited to the *CYC1* promoter by separate subcomplexes, which would also explain the difference in occupancy in Rad3 and Kin28. This, however, is most likely not the case at *CYC1* as Ssl2, a second subunit of TFIIK, is found at the promoter in both the induced and uninduced condition and further supports the theory of a conformational change to the PIC during the transition from uninduced to induced gene expression of *CYC1.*

Further confirmation of an intact PIC at the *CYC1* promoter during the uninduced condition is the fact that serine 5 of the RNA Pol II CTD heptad repeat is phosphorylated during the uninduced condition. Serine 5 phosphorylation is seen only at the promoter proximal region (Cheng and Sharp, 2003; Komarnitsky et al., 2000). *Drosophila HSP70* has RNA Pol II CTD serine 5 phosphorylated during the uninduced condition (Boehm et al., 2003).

Genetic interactions between the capping enzyme *CEG1* and *KIN28* suggest that Kin28 is responsible for recruitment of the capping enzyme (Rodriguez et al., 2000). To better understand the presence of Kin28 at the

CYC1 promoter and further compare yeast post-recruitment regulation with Drosophila post-recruitment regulation *CEG1* occupancy was determined at *CYC1. HSP70* has the capping enzyme present during the uninduced condition (Rasmussen and Lis, 1993). *CYC1* also was found to have *CEG1,* occupying the promoter during the uninduced condition. *CEG1* further strengthens the theory that Kin28 is at the *CYC1* promoter but unable to be assayed via immunoprecipitation due to PIC conformational changes.

The post-recruitment regulated gene of *S. cerevisiae, CYC1,* was also found to have full Mediator occupancy at the promoter after induction of the gene. Kinetic analysis of Mediator at *CYC1* showed mediator recruitment occurs late in the induction process.

In eukaryotes the concept of post-recruitment regulation of transcription is not uncommon (Uptain et al., 1997). For example, the *Drosophila* heat shock genes, *HSP70* and *HSP26,* and the mammalian c-myc, have been found to have promoter proximal pausing of the polymerase during elongation of transcription (Andrulis et al., 2000; Krumm et al., 1995; Krumm et al., 1992; Rasmussen and Lis, 1993; Rasmussen and Lis, 1995; Wu et al., 2003a). During inactivation, such promoters have TBP and RNAPII bound and initiated, but paused after ~20 nucleotides. Upon proper stimulation (i.e. heat shock) transcription is fully activated. By definition these promoters are post-recruitment regulated due to the fact that they have high levels of TBP and Pol II occupancy but low levels of transcription expression.

To understand the similarities of post-recruitment regulation of *S. cerevisiae* with that of higher eukaryotes, a comparative analysis was performed with *S. cerevisiae CYC1* and *Drosophila HSP70* regulation. *Drosophila* heat shock genes, *HSP70* and *HSP26*, and the mammalian c-myc are regulated in a post-recruitment manner as they have all been found to have promoter proximal pausing of the polymerase during elongation of transcription (Andrulis et al., 2000; Krumm et al., 1995; Krumm et al., 1992; Rasmussen and Lis, 1993; Rasmussen and Lis, 1995; Wu et al., 2003a). During the uninduced condition of *CYC1* gene transcription TBP and RNA Pol II are present on the *CYC1* gene (Kuras and Struhl, 1999; Martens et al., 2001). Post-recruitment regulation of *Drosophila HSP70* has been extensively studied and it has been shown that a complete and functional PIC at the promoter has melted the DNA and transcribed 20-30 nucleotides. In addition, capping enzyme is present in the uninduced condition and Mediator comes in after induction of *HSP70* via heat shock (Park et al., 2001; Rasmussen and Lis, 1993). Mediator does not fully occupy the *Drosophila HSP70* promoter during the uninduced condition (Park et al., 2001). Upon induction of *Drosophila HSP70,* via heat shock, mediator occupies the promoter.

It is evident that post-recruitment regulation of *S. cerevisiae* is mechanistically similar to that of higher eukaryotes. *CYC1* and *HSP70* both have a complete PIC during the uninduced conditions, as seen by the last general transcription factor, TFIIH, being present at the promoter and the RNA Pol II CTD is phosphorylated at the serine 5 position of the heptad repeat. In addition, the

mRNA capping enzyme is also found at the promoter of *CYC1* and *HSP70* during the uninduced condition. Upon induction, Mediator is recruited to the post-recruitment regulated genes of *CYC1* and *HSP70*. The similarities seen between *CYCI* and *HSP70* strongly suggest a highly conserved mechanism for eukaryotic post-recruitment regulation.

Chapter 5

Genome wide analysis of Spn1 function

Spn1 is a highly conserved, essential protein that functions in post-recruitment regulation. To understand the breadth of post recruitment regulation in *S. cerevisiae* and to better understand the role of Spn1, occupancy was assayed at a genome level. Genome-wide localization studies were performed in collaboration with Dr. Vishy Iyer and BumKyu Lee. I spearheaded the project and created the materials needed for genome arrays. BumKyu performed the array studies and we collectively analyzed the data.

Genome wide analysis of Spn1 function

Aaron Fletcher, BumKyu Lee, Vishy Iyer and Laurie Stargell

5.1 Abstract

Promoters with TBP and RNA Polymerase II (RNAPII) already bound in the uninduced state are becoming more and more common. Spn1 has been shown to play an important role in post-TBP/RNAPII-recruitment regulation. Spn1 is encoded by an essential gene that is highly conserved throughout eukaryotes. A mutant form of Spn1, spn1^{K192N}, has been shown to deregulate post-recruitment promoters. To further understand the role of the essential protein Spn1, and to determine what genes are post-recruitment regulated, genome wide localization studies were performed with Spn1 and spn1^{K192N}. Spn1 was found to occupy a number of genes which might be regulated in a post-recruitment manner. In addition Spn1, showed strong occupancy levels at 444 genomic sites. More than 50% of sites occupied by Spn1 were lost in spn1^{K192N} cells. Many of these genes may be recruited by a post-recruitment mechanism. Clustering analysis of Spn1 occupancy showed that ribosomal genes had a high concentration of Spn1 occupancy. The majority of yeast ribosomal genes are spliced. Human Spn1 has been implicated in splicing and the high levels of occupancy of Spn1 at ribosomal genes suggest splicing as an important role for Spn1 function. Mutant Spn1 occupany was completely lost at the promoters of ribosomal protein genes indicating Spn1 plays an important role in the transcription intiation of PIC-recruitment regulated genes.

5.2 Introduction

Transcription is a highly regulated event that involves a concerted effort from a number of different factors collectively referred to as the transcription machinery. The *CYC1* gene in *S. cerevisiae* has TBP and RNA Polymerase II (RNAPII) bound to the promoter in the uninduced state (Kuras and Struhl, 1999; Martens et al., 2001) and is therefore post-recruitment regulated, in that it is regulated after the recruitment of TBP and RNAPII. In the case of recruitment regulated genes, initiation of transcription is regulated by the recruitment of TBP and the corresponding PIC to the promoter DNA. In such cases, the level of TBP occupancy is positively correlated with the level of transcription activity (Kim and Iyer, 2004; Kuras and Struhl, 1999; Li et al., 1999). At such genes the rate-limiting step of transcription is the ability to recruit TBP to the promoter and initiate nucleation of the PIC. In post-recruitment regulation TBP/RNAPII recruitment is no longer the rate limiting step of transcription initiation. Many post-recruitment regulated genes are important in a variety of environmental responses that are needed for homeostasis (Kuras and Struhl, 1999; Martens et al., 2001; Rasmussen and Lis, 1993; Rasmussen and Lis, 1995).

SPN1 (Suppresses Post-recruitment functions gene Number 1) is an essential gene in *S. cerevisiae* and is conserved throughout evolution. A wide range of eukaryotic organisms contain *SPN1* homologs, including humans, mice, flies, and worms (Altschul et al., 1997; Liu et al., 2007). The central region of *SPN1*, ~113 amino acids, is highly conserved throughout eukaryotes and is essential for *SPN1* function, while the N- and C- termini are dispensable for

proper function *in vivo* (Fischbeck et al., 2002). The *SPN1* gene product (Spn1) is 410 amino acids in length, has a molecular weight of approximately 46 KDa, and a pI of 7.81.

SPN1 was initially identified through a genetic screen using a TBP mutant allele (Fischbeck et al., 2002). The TBP mutant consists of a single point mutation of a phenylalanine at residue 237 to an aspartic acid, TBP^{F237D}. This mutant allele is capable of binding to the DNA, but transcription is not initiated (Stargell and Struhl, 1996). A temperature sensitive *SPN1* allele, $spn1^{K192N}$, is capable of allowing transcription in the presence of TBP^{F237D}. Because the mutant phenotype of $spn1^{K192N}$ allows transcription to occur in a post-recruitment regulated strain, *SPN1's* wild type function is maintaining the constrained state of a promoter regulated via post-recruitment. Spn1 physically interacts with RNAPII and Spt6 and genetically interacts with TBP, Swi/Snf, and Spt4 (Krogan et al., 2002; Lindstrom et al., 2003).

Due to *SPN1* being essential and having a known role in post-recruitment regulation, understanding 1) the genome wide occupancy of Spn1 2) expressional changes due to $spn1^{K192N}$ will help illuminate the role of Spn 1 on a genome wide scale.

5.3 Materials and Methods

5.3a Yeast strains, media and DNA

The wild-type strain BY4741: MATa his3Δ1 ura3Δ0 leu2Δ01 met15Δ01 was the parental *S. cerevisiae* strain. Spn1 and Spn1^{K192N} were epitope tagged in the BY4741 background. pCR101: BY4741*spn1::LEU2* pRS313-myc-*SPN1* (*HIS3*), pCR102: BY4741*spn1::LEU2* pRS313-myc- *spn1*^{K192N} (*HIS3*). The yeast complete and synthetic complete (SC) media were made according to literature (Hampsey, 1997a).

5.3b Western blot analysis

10ml of yeast cells grown in YPD with an OD600 of ~0.8 were harvested, washed once with sterile water, and resuspended in 200ul lysis buffer (0.5M phosphate buffer, pH 7.5). Whole cell extracts were prepared by vigorous bead beating. Cellular debris was removed by spinning the extracts at 10,000g at 4°C for 10 min. Protein concentrations were determined by Bradford assay (Bio-Rad). Equal amount of whole cell extracts were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The following antibodies were used at the given dilutions: anti-myc (Upstate Inc. 1:500). Horseradish peroxidase (HRP) conjugated second antibodies were used at 1:20,000 dilution and protein bands were detected by western blotting detection reagents from Amersham Biosciences.

5.3c Chromatin immunoprecipitation (ChIP) analysis

Chromatin immunoprecipitations were performed as described (Strahl-Bolsinger et al., 1997), with few modifications. Cells (50 ml) were grown to OD 600 of 0.8~1.0. Cells were treated with a final concentration of 1% formaldehyde for 15 minutes with occasional swirling of the flasks at intervals of 5 minutes. Glycine was added to a final concentration of 125mM at room temperature for 5 minutes to stop cross-linking. Cells were collected and washed twice in ice cold TBS. Cells were resuspended 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, beuzamidine, pepstatin, leupeptin, and chymostatin). Chromatin was sheared by sonication using a Branson W-350 model of sonifier (10 times at 10 seconds each on continuous pulse at a microtip power setting of 6). 10% of the chromatin material used for the immunoprecipitation was processed as the input after reversing the cross-links and purifying the DNA. 500 µl of the chromatin material was incubated with approximately 10µl of anti-Myc (Upstate, Inc) antibodies by rotation overnight at 4°C. 50µl of protein-A sepharose beads (Pharmacia-prepared as slurry as per the manufacturers directions) was further incubated with the chromatin material for 3 hours at 30°C. The beads were spun down and the Antigen-Antibody complexes bound to the beads were recovered and further treated with TE/SDS buffer for 15 minutes at 65°C to elute the complexes. Protein-DNA cross-links were reversed by incubation overnight at 65°C and the DNA was purified by phenol-chloroform extraction and used for Real Time PCR (RT-PCR) analysis. Protein occupancy

was determined via real time PCR (RT-PCR). Standard curves were generated using 10-fold serial dilutions of Input DNA. PCR efficiencies ranged from 90-100%, with a correlation coefficient of 0.990 or greater. Threshold cycle data were quantified relative to the input, as described (Frank et al., 2001). Background levels were calculated as the relative occupancy of untagged strains and these values were subtracted from that of the tagged strains.

5.3d mRNA preparation

Cells where grown to log phase (O.D.600 of 0.7-1.0) and collected via centrifugation at 3,000 x g at room temperature. Each 10ml cell pellet was resuspended in 1ml lysis buffer and stored at -80°C until RNA preparation. Total RNA was prepared by acid phenol lysis as described (Spellman et al., 1998) and quantified spectrophotometrically at 260nm.

5.3e Yeast DNA microarrays and hybridization

Microarrays that include every ORF and intergenic element from the yeast genome were manufactured by Dr. Iyer's lab at the University of Texas, Austin (Iyer et al., 2001). Microarrays were scanned with a GenePix 4000B scanner (Axon Instruments). Fluorescence intensities were quantified using GenePix Pro software (version 4.0), and data were uploaded to a relational database for further analysis (http://www.biomedcentral.com). Data was filtered to exclude spots with obvious defects or a signal intensity below an empirically determined threshold. PCR amplification and fluorescence labeling of immunoprecipitated

DNA and labeling of cDNA was performed by Dr. Iyer's lab at the University of Texas, Austin. The reference hybridization probe used in the experiments was a common pool of wild-type yeast genomic DNA that had been sonicated. Amplification and labeling of the reference was performed by the same protocols used for the ChIP samples.

5.3f Calculation of genomic enrichment and gene-promoter associations.

ChIP enrichment scores (E) for each genomic locus was calculated as follows: E= median $R(Cy5/Cy3)_1$, $R(Cy5/Cy3)_2$,... $R(Cy5/Cy3)_n$, where $R(Cy5/Cy3)_I$ is the percentile rank of Cy5 to Cy3 ratio of the microarray element corresponding to that locus among all genomic loci in the ith independent ChIP experiment. E is expressed as a percentage with values ranging from 0-100 and is a measure of consistent in vivo association of Spn1 with a given genomic locus.

5.4 Results

5.4a Expression change distribution of Spn1 vs. spn1^{K192N} finds 134 genes affected by spn1^{K192N}.

CYC1 gene expression is up-regulated in the presence of spn1^{K192N} (Fischbeck et al., 2002). Therefore, the role of Spn1 at post-recruitment regulated genes is to help maintain a constrained state at the promoter, where the promoter is occupied by the PIC yet transcription is not active. To identify other genes throughout the genome with similar properties in Spn1 function an expression array was performed. Gene expression was compared in cells with wild-type Spn1 vs. cells with spn1^{K192N}. mRNA from cell cultures of spn1^{K192N} were reverse transcribed into cDNA and hybridized to whole genome microarrays together with amplified, Cy3 labeled, Spn1 cDNA from mRNA samples. Each microarray was performed from three independent mRNA samples.

One hundred and thirty-three genes were found to have at least 2 arrays with a 1.5 fold decrease or increase in expression (Figure 5.1), which represents ~2% of the genome tested. These genes are listed in Table 5.1. Over 99% of these genes increased in expression in spn1^{K192N} cells, which further supports a function of Spn1 in repression of transcription expression. As expected, *CYC1* increased in the spn1^{K192N} strain. There is an enrichement of genes that increased in expression in spn1^{K192N} cells that are involved in amino acid biosynthetic pathways, heat shock pathways, and respratory pathways. Many of these genes may be regulated in a post-recruitment manner similar to the respratory gene *CYC1*.

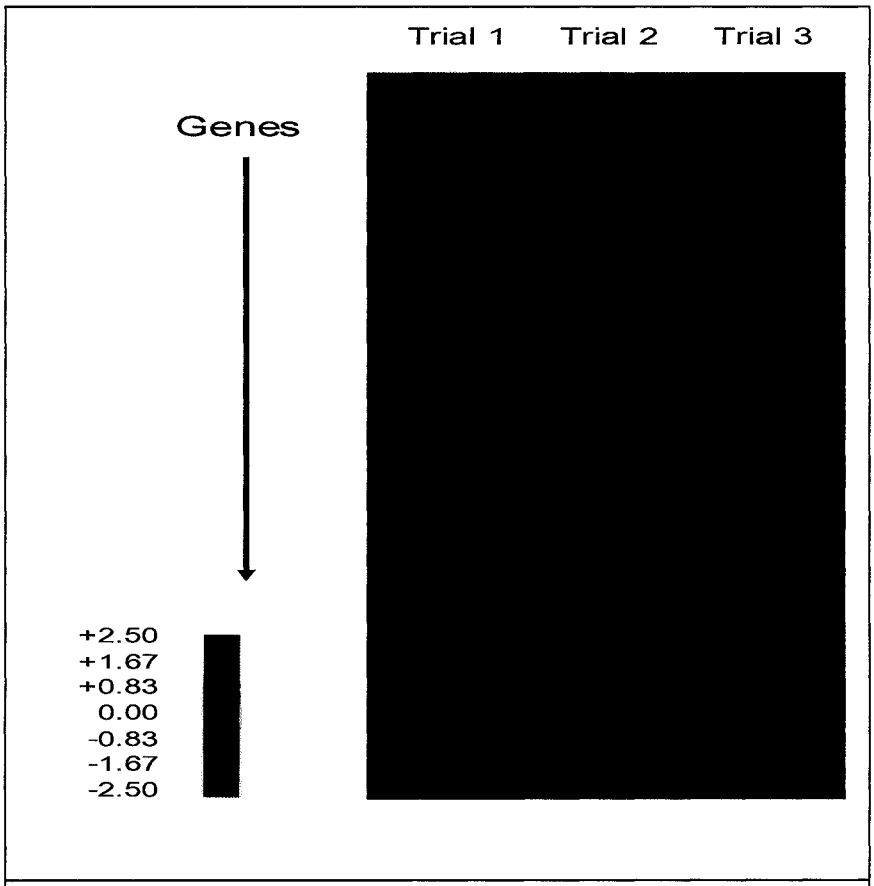


Figure 5.1 Hierarchical clustering of Spn1 expression array. Genome wide analysis of expression change in mutant spn1. Red indicates an increase in expression and green indicates a decrease in expression. One hundred and thirty-three genes showed an expression change with a 1.5-fold increase or decrease in at least 2 arrays.

	Gene name	Common name	Expression Change in spn1^{K192N}		Gene name	Common name	Expression Change in spn1^{K192N}		Gene name	Common name	Expression Change in spn1^{K192N}
1	YHR018C	ARG4	Increase	45	YFL045C	SEC53	Increase	91	YKL103C	LAP4	Increase
2	YOR202W	HIS3	Increase	46	YGL225W	VRG4	Increase	92	YPL111W	CAR1	Increase
3	YER081W	SER3	Increase	47	YDL198C	GGC1	Increase	93	YDR434W	GPI17	Increase
4	CEN6	#N/A	Increase	48	YGR284C	ERV29	Increase	94	YML052W	SUR7	Increase
5	YBR067C	TIP1	Increase	49	YCL030C	HIS4	Increase	95	YDR034W-B	0	Increase
6	YCR021C	HSP30	Increase	50	YBL030C	PET9	Increase	96	YLR110C	CCW12	Increase
7	YNL160W	YGP1	Increase	51	SNR65	SNR65	Decrease	97	YER141W	COX15	Increase
8	YBR296C	PHO89	Decrease	52	YBR162C	TOS1	Increase	98	YDL046W	NPC2	Increase
9	YDR158W	HOM2	Increase	53	YLR356W	0	Increase	99	YIL088C	AVT7	Increase
10	YMR145C	NDE1	Increase	54	YLR350W	ORM2	Increase	100	YLR370C	ARC18	Increase
11	YAR068W	0	Increase	55	YBR104W	YMC2	Increase	101	YDR074W	TPS2	Increase
12	YIR034C	LYS1	Increase	56	YML123C	PHO84	Increase	102	YDL134C	PPH21	Increase
13	YHR051W	COX6	Increase	57	YML028W	TSA1	Increase	103	YBR070C	ALG14	Increase
14	YLR432W	IMD3	Increase	58	YJR048W	CYC1	Increase	104	YDR342C	HXT7	Increase
15	YOR247W	SRL1	Increase	59	YBR004C	GPI18	Increase	105	YLR447C	VMA6	Increase
16	YHR216W	IMD2	Increase	60	YBR096W	0	Increase	106	YDR178W	SDH4	Increase
17	YEL046C	GLY1	Increase	61	YBR085W	AAC3	Increase	107	YBR048W	RPS11B	Increase
18	YKL016C	ATP7	Increase	62	YJL159W	HSP150	Increase	108	YBR014C	0	Increase
19	YHR001W-A	QCR10	Increase	63	YDR298C	ATP5	Increase	109	YBR006W	UGA2	Increase
20	YKR042W	UTH1	Increase	64	YIL124W	AYR1	Increase	110	YJR111C	0	Increase
21	YBR092C	PHO3	Increase	65	YBR256C	RIB5	Increase	111	YPL154C	PEP4	Increase
22	YER055C	HIS1	Increase	66	YKR013W	PRY2	Increase	112	YFR044C	DUG1	Increase
23	YBR078W	ECM33	Increase	67	YBR076W	ECM8	Increase	113	YBL098W	BNA4	Increase
24	YHL048W	COS8	Increase	68	YDR012W	RPL4B	Increase	114	YHR037W	PUT2	Increase

25	YKL141W	SDH3		Increase	69	YDR222W		0	Increase	115	YOR285W		0	Increase
26	YLR120C	YPS1		Increase	70	YML056C	IMD4		Increase	116	YBR139W		0	Increase
27	YHR071W	PCL5		Increase	71	YJR145C	RPS4A		Increase	117	YFR015C	GSY1		Increase
28	YER150W	SPI1		Increase	72	YDR345C	HXT3		Increase	118	YAR028W		0	Increase
29	YDR525W-A	SNA2		Increase	73	YPR016C	TIF6		Increase	119	YCL052C	PBN1		Increase
30	YPL014W		0	Increase	74	YNL055C	POR1		Increase	120	YML110C	COQ5		Increase
31	YLR220W	CCC1		Increase	75	YBR287W		0	Increase	121	YER011W	TIR1		Increase
32	YBR068C	BAP2		Increase	76	YJR016C	ILV3		Increase	122	YMR318C	ADH6		Increase
33	YJL026W	RNR2		Increase	77	YNR061C		0	Increase	123	YCR086W	CSM1		Increase
34	YJL079C	PRY1		Increase	78	YMR305C	SCW10		Increase	124	YOL011W	PLB3		Increase
35	YKL096W-A	CWP2		Increase	79	YBL099W	ATP1		Increase	125	YDR171W	HSP42		Increase
36	YKL163W	PIR3		Increase	80	YDR461W	MFA1		Increase	126	YGR295C	COS6		Increase
37	YER060W	FCY21		Increase	81	YLR390W-A	CCW14		Increase	127	YPL036W	PMA2		Increase
38	YIL116W	HIS5		Increase	82	YCL037C	SRO9		Decrease	128	YKL109W	HAP4		Increase
39	YBR036C	CSG2		Increase	83	YKL207W		0	Increase	129	YPL006W	NCR1		Increase
40	YHL044W		0	Increase	84	YOL092W		0	Increase	130	YPL283C	YRF1-7		Increase
41	YGR279C	SCW4		Increase	85	YBL064C	PRX1		Increase	131	YFL014W	HSP12		Increase
42	YFL026W	STE2		Increase	86	YOR152C		0	Decrease	132	YDR343C	HXT6		Increase
43	YOL086C	ADH1		Increase	87	YER052C	HOM3		Increase	133	iYDR461W		0	Increase
44	YPR069C	SPE3		Increase	88	YGL187C	COX4		Increase	134	iYCR024C		0	Increase
45	YFL045C	SEC53		Increase	89	YKL056C	TMA19		Increase					

Table 5.1 Gene list whose expression profile changes in response to $spn1^{k192N}$

5.4b Spn1 occupancy sites throughout the genome.

To determine if Spn1 occupied promoters that increased in expression in spn1^{k192N} cells, genome wide localization studies were performed. Spn1 was Myc tagged and three independent samples were grown to log phase in medium containing 2% dextrose. ChIP DNA was isolated from each sample and labeled with Cy5. Input DNA or untagged DNA was also isolated independently and fluorescently labeled with Cy3. The Spn1-Myc Cy5 DNA was combined with either input or untagged (Mock) DNA Cy3 and co-hybridized to whole-genome yeast microarrays that contained nearly every ORF as well as every intergenic sequence. ORF sequence encompassed the protein coding region and intergenic sequences were upstream (promoter) sequences to the protein-coding region. A consistent enrichment value for each genomic locus was calculated for each genomic locus across all the independent genome localization experiments. Spn1 was found to occupy 444 sites with a (p)value less than 0.01. Spn1 occupancy was found at both the ORF and the promoter regions. Seventy-six genes had Spn1 occupancy at both the ORF and promoter (Figure 5.2). Three genes that showed an increase of expression in spn1^{K192N} cells also had significant Spn1 occupancy: *HIS3, SER3, and HIS1*. In addition, *HSP150* also showed significant Spn1 occupancy and had an increase in expression when grown in spn1^{K192N} cells.

MIPS clustering analysis of Spn1 occupancy found a significant amount of binding to genes important in ribosome biogenesis and protein synthesis (Table 5.2). Spn1 was found to bind to a number of ribosomal genes at both the ORF

and intergenic regions. Of the 135 ribosomal genes, Spn1 was found to have significant occupancy at 103 of them. The ORF of ribosomal genes were predominantly occupied by Spn1. Eighty of the 114 occupancy sites were positioned at the ORF of ribosomal genes. Thirty-four ribosomal genes had significant Spn1 occupancy to the intergenic region and twenty-three genes had occupancy at both the ORF and intergenic region.

5.4c spn1^{K192N} occupancy sites throughout the genome

To determine the genome wide binding distribution of mutant Spn1, spn1^{K192N}, genome wide localization studies were performed in the same manner as described for the wild type protein. Occupancy of spn1^{K192N} occurred less than wild type occupancy with 189 sites found to have strong occupancy. Approximately half of the Spn1 occupancy sites at the ORF were lost in spn1^{K192N} cells. Seventy percent of Spn1 occupancy at intergenic sites were lost in the spn1^{K192N} cells (Figure 5.3). More than half, (255), of the 444 sites found to have significant Spn1 show no Spn1 occupancy in spn1^{K192N} cells. These are good candidates for post-recruitment regulated genes.

Occupancy of spn1^{K192N} was found at 189 sites. When spn1^{K192N} occupancy was compared to that of wild-type Spn1 occupancy, it was evident that spn1^{K192N} cells did not lead to a number of new binding sites. Eighty percent of spn1^{K192N} binding occurred at sites where Spn1 had previously shown occupancy (Figure 5.4) and suggests a subset of genes where Spn1 may function differently than at *CYC1*. As previously described wild-type Spn1

occupancy was enriched at ribosomal genes. Cells with spn1^{K192N} showed significant binding to ribosomal genes, but to a much lesser extent than wild type genes. Strikingly, spn1^{K192N} occupancy was almost completely absent at the intergenic regions of ribosomal genes. In addition, the ORF regions of ribosomal genes lost ~60% Spn1 occupancy in spn1^{K192N} cells (Table 5.3).

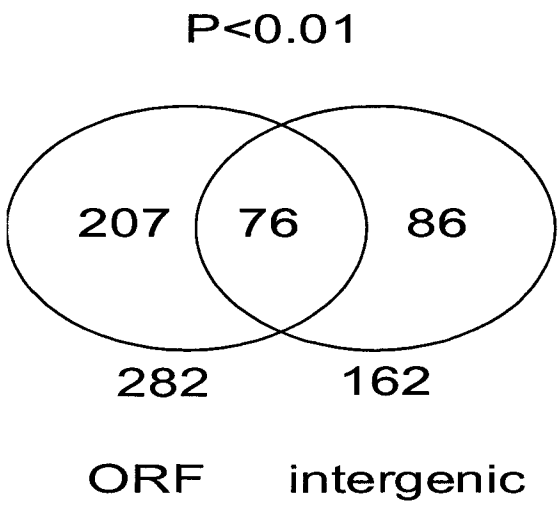


Figure 5.2 Genome wide analysis of Spn1 binding.
ChIP-chip analysis of Spn1 showed significant Spn1 binding at 444 sites. Of the 444 occupancy sites of Spn1 282 of them were to the ORF and 162 were to intergenic sites. There were 76 sites where Spn1 binding was found at both the ORF and the intergenic site.

Table 5.2 Gene clustering analysis of Spn1 occupancy

Category	p-value	Genes with Spn1 occupancy in each category	k	F
RIBOSOME BIOGENESIS	<1e-14	RPL19B RPS8A RPL23A RPL32 RPL4A RPL19A RPS9B RPL21A RPS14A RPS29B RPP1A RPL13A RPL35B RPL4B RPS13 RPL12B RPS18A MRPL28 RML2 RPL34A RPS26B RPL2A RPL29 RPL30 RPL28 RPS2 RPL1B RPL9A RPS25A RPL26B MRP13 RPL11B RPS23A RPS0A RPL14B RPS20 RPL8A RPL27A RPL42B RPS4B RPL2B RPL34B RPL16A RPL40A RPL17B RPS14B RPL43B RPS5 RPS4A RPL14A YKL056C RPL17A RPL8B RPL15A RPS0B RPL22A RPS25B RPP0 RPL26A RPS22B RPS29A RPL6B RPL6A ASC1 RPL20A RPL9B RPL42A RPL18B RPS19B RPP2A RPS15 RPL18A RPS19A RPL25 RPL3 RPL33B RPL21B RPS9A RPS6A RPL5 RPL33A RPL7B RPL1A RPL36B RPL43A	85	215
SUBCELLULAR LOCALISATION	<1e-14	EFB1 CDC19 CDC24 HTB2 HTA2 RRN10 RPL19B RPS8A AAR2 RPL23A RPL32 HHF1 HHT1 IPP1 RPL4A HMT1 BAP2 TAT1 RPL19A MRS5 PHO3 TEF2 MUD1 CBP6 RPS9B RPL21A PGI1 LEU2 PGK1 RPS14A TUP1 RPS29B RPP1A RPL13A RDI1 RPL35B TFP1 RPL4B TPI1 RPS13 HTA1 YRA1 EFT2 RPL12B RPS18A MRPL28 RML2 GCD11 FCY2 RPL34A SHO1 RPS26B STE2 RPL29 PMA1 RPL30 RCS1 MPS2 HNM1 RPL28 RPS2 RPL1B SEC27 RPL9A RPS25A RPL26B ERG25 COX18 MRP13 RPL11B RPS23A NUP57 NSR1 TDH3 RPS0A BGL2 ZUO1 RPL14B RPS20 RPL8A SBP1 RPL27A RPN1 GAR1 RPL42B ENO2 RPS4B RPL2B SYG1 RPL34B RPL16A FLX1 RPL40A RPE1 RPA34 HSP150 RPL17B RPS14B TDH2 RPL43B RPS5 RPS4A RPL14A YKL056C FBA1 VMA5 TEF4 CWP2 RRN3 GPM1 RPL17A FAS1 URA1 TIF1 KTR2 MTD1 SOF1 RPL8B RPL15A RPS0B SHM2 FRS1 YEF3 CTS1 RPS25B RPP0 RPL26A ILV5 RPS22B RPS29A RPL6B RPL6A PHO84 TUB3 MCM1 ASC1 NDE1 RPL20A NIP1 RPL9B RPL42A RPL18B RPS19B PRE6 RPP2A RPS15 ADH1 RPL18A RPS19A HRP1 MDH2 RPL25 RPL3 HIS3 DED1 RPT4 DIG1 RPL21B RPS9A RPS6A RPL5 RPL33A RPL7B RPL1A RPL36B RPL43A	166	2256
PROTEIN SYNTHESIS	1.12E-14	EFB1 RPL19B RPS8A RPL23A RPL32 RPL4A RPL19A TEF2 CBP6 RPS9B RPL21A RRP7 RPS14A RPS29B RPP1A RPL13A RPL35B RPL4B RPS13 EFT2 RPL12B RPS18A MRPL28 RML2 GCD11 RPL34A RPS26B RPL2A RPL29 RPL30 RPL28 RPS2 RPL1B RPL9A RPS25A RPL26B MRP13 RPL11B RPS23A RPS0A RPL14B RPS20 RPL8A RPL27A RPL42B RPS4B RPL2B RPL34B RPL16A RPL40A RPL17B RPS14B RPL43B RPS5 RPS4A RPL14A YKL056C TEF4 RPL17A TIF1 RPL8B RPL15A RPS0B FRS1 RPL22A YEF3 RPS25B RPP0 RPL26A RPS22B RPS29A RPL6B RPL6A ASC1 RPL20A NIP1 RPL9B RPL42A RPL18B RPS19B RPP2A RPS15 RPL18A RPS19A RPL25 RPL3 DED1 RPL33B RPL21B RPS9A RPS6A RPL5 RPL33A RPL7B RPL1A RPL36B RPL43A	97	359

CYTOPLASM	1.58E-14	EFB1 CDC19 RPL19B RPS8A RPL23A RPL32 IPP1 RPL4A RPL19A TEF2 RPS9B RPL21A PGI1 LEU2 PGK1 RPS14A RPS29B RPP1A RPL13A RDI1 RPL35B RPL4B TPI1 RPS13 EFT2 RPL12B RPS18A GCD11 RPL34A RPS26B RPL29 RPL30 RPL28 RPS2 RPL1B RPL9A RPS25A RPL26B RPL11B RPS23A TDH3 RPS0A RPL14B RPS20 RPL8A RPL27A RPL42B ENO2 RPS4B RPL2B RPL34B RPL16A RPL40A RPE1 RPL17B RPS14B TDH2 RPL43B RPS5 RPS4A RPL14A YKL056C FBA1 TEF4 GPM1 RPL17A FAS1 URA1 TIF1 MTD1 RPL8B RPL15A RPS0B SHM2 FRS1 YEF3 RPS25B RPP0 RPL26A RPS22B RPS29A RPL6B RPL6A ASC1 RPL20A NIP1 RPL9B RPL42A RPL18B RPS19B RPP2A RPS15 ADH1 RPL18A RPS19A HRP1 MDH2 RPL25 RPL3 HIS3 DED1 RPL21B RPS9A RPS6A RPL5 RPL33A RPL7B RPL1A RPL36B RPL43A	110	554
GLYCOLYSIS & GLUCONEOGENESIS	7.40E-06	CDC19 PGI1 PGK1 TPI1 TDH3 ENO2 TDH2 FBA1 GPM1	9	35
TRANSLATION	4.46E-05	EFB1 TEF2 RPP1A EFT2 GCD11 TEF4 TIF1 YEF3 ASC1 NIP1 DED1	11	64
PURINE RIBONULEOTIDE METABOLISM	0.00204	ADE1 HPT1 PRS1 MTD1 ADE16 SHM2 ADE17	7	45
ENERGY	0.00305	CDC19 PGI1 PGK1 TPI1 ARO10 PCL10 COX18 TDH3 ENO2 RPE1 GLG2 TDH2 OAR1 FBA1 GPM1 YML125C ASC1 NDE1 ADH1 MDH2	20	252
CHROMOSOME	0.00823	HTB2 HTA2 HHF1 HHT1 HTA1 ZUO1	6	44

Table 5.2 MIPS gene clustering analysis of Spn1 genome wide occupancy. k indicates the number of genes where Spn1 occupancy occurs in a given cluster. f indicates the total number of genes in a given cluster. All Spn1 occupancy had a p value of less than 0.01. Spn1 occupancy occurred at ~40% of ribosome biogenesis genes and ~30% of genes important for protein synthesis.

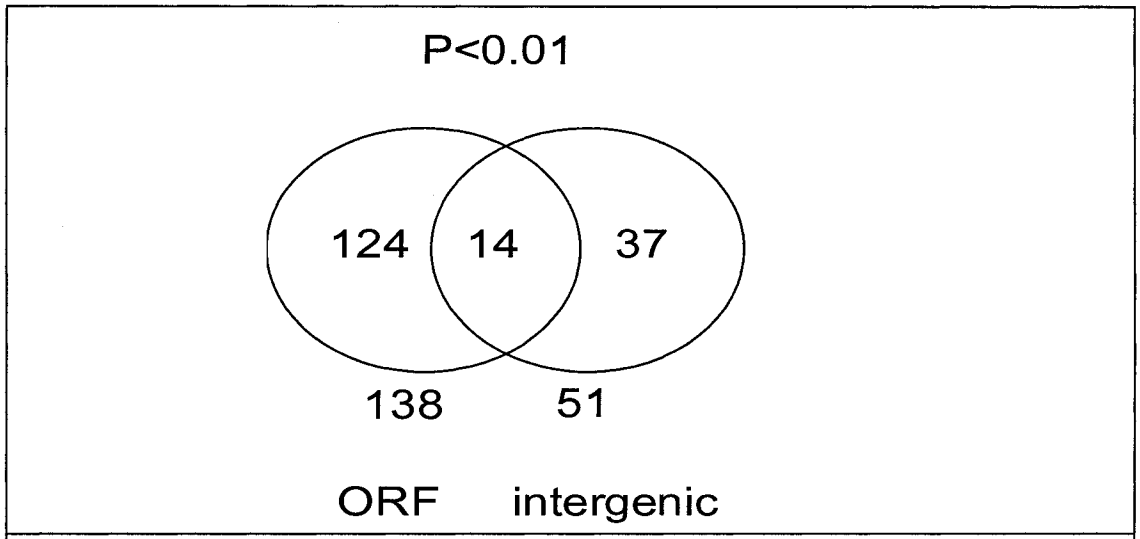


Figure 5.3 Genome wide analysis of spn1^{K192N} binding.
ChIP-chip analysis of spn1^{K192N} showed significant spn1^{K192N} binding at 189 sites. Of the 189 binding sites of Spn1 138 of them were to the ORF and 51 were to intergenic sites. There were 14 sites where Spn1 binding was found at both the ORF and the intergenic site.

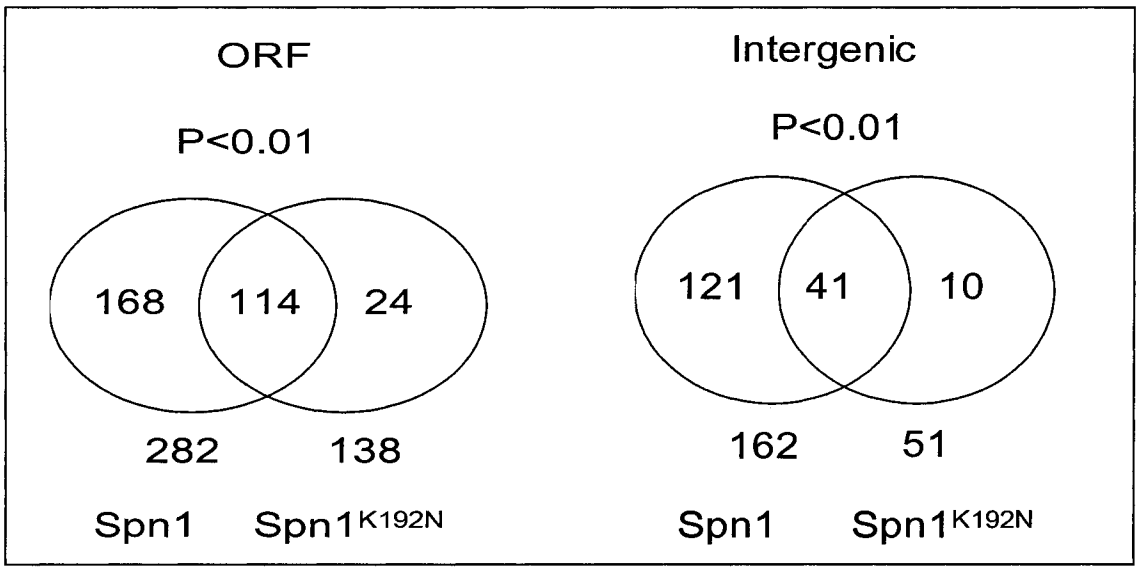


Figure 5.4 Binding of spn1^{K192N} predominately occurs at Spn1 binding sites.
Comparative analysis of ChIP-chip results of spn1^{K192N} and Spn1 binding showed that spn1^{K192N} binding binds to Spn1 sites 80% of the time, at both ORF and intergenic sites.

Table5.3 A significant majority of ribosomal protein genes are bound by Spn1 and spn1^{K192N}

Chip	P value	ORF	Intergenic	Overlap	Total ribosomal protein genes in yeast
Spn1	P<0.01	80	34	23	137
K192N	P<0.01	45	1	1	

5.5 Discussion

A genome wide approach was taken to obtain a better understanding of Spn1 function. Genome wide expression mircoarrays provided us with genes that are potentially regulated in a similar manner to *CYC1*, a post-recruitment regulated gene. To determine genes where Spn1 was important in the regulation process expression levels in a mutant form of Spn1 was compared with wild-type expression. This recessive mutant, spn1^{K192N}, no longer represses gene expression at *CYC1* during the uninduced state and exacerbates expression of *CYC1* during the induced state (Fischbeck et al., 2002). Microarray analysis revealed 133 genes where expression changed more than 1.5 fold when wild type Spn1 was compared with spn1^{K192N}. Ninety-nine percent of these genes increased expression in the presence of spn1^{K192N}, and are potential candidates for post-recruitment regulation. *CYC1* was one of the 133 genes to show an increase in expression and further validates the microarray analysis.

Genome wide localization analysis was performed to obtain an understanding of Spn1 function at a genome wide scale. Spn1 and spn1^{K192N} were both myc tagged and DNA where each protein binds throughout the genome was isolated by chromatin immunoprecipitation. A number of interesting observations are seen from the localization results of Spn1 and spn1^{K192N}. Spn1 showed significant occupancy to 444 different sites. Approximately 45% of these sites were located only in the ORF of genes, while 20% of Spn1 binding sites were only at the promoter of genes. The remaining 35% of sites had Spn1 bound to both the promoter and ORF of the gene. The binding of Spn1 to only

the ORF and both the ORF and promoter suggest Spn1 plays a role in elongation, as has been seen at the *ADH1* gene (Krogan et al., 2002). Spn1 also has a role in regulation of transcription initiation, as seen at *CYC1.* A number of genes also suggest Spn1 is important in transcription initiation, as 36% of the significant Spn1 occupancy sites were to the promoter region. Microarray analysis showed that *CYC1* expression increased in spn1^{K192N} cells (Table 5.1). Oddly, the *CYC1* gene did not show Spn1 occupancy in the genome wide localization assays (Table 5.2). Spn1 has previously been shown to occupy the *CYC1* promoter in the uninduced state, while spn1^{K192N} no longer occupies the *CYC1* promoter (Fischbeck et al., 2002). To confirm that Spn1 occupied the *CYC1* promoter and to ensure immunoprecipitated DNA was of high quality for genome wide localization studies ChIP analysis was carried out on *CYC1.* Spn1-Myc was immunoprecipitated and occupancy was determined at *CYC1.* ChIP analysis confirmed previous results that wild-type Spn1 occupies *CYC1* in the uninduced condition while mutant spn1^{K192N} does not. This suggests that the whole genome approach is more stringent in occupancy detection compared to the examination of a single gene using real-time PCR methods. As such, it is likely that additional genes in the genome are occupied by Spn1.

Genome wide localization analysis of spn1^{K192N} revealed that ~60% of Spn1 binding is lost in spn1^{K192N} cells, which is similar to what is seen at *CYC1.* The other 40% of Spn1 binding sites did not lose spn1^{K192N} binding and suggests that Spn1 may have a different function at these genes. Interaction with Spt6 is lost in spn1^{K192N} cells. It is possible that sites where spn1^{K192N} binds to the DNA

are genes where the Spt6 and Spn1 interaction are not important for Spn1 function. To test this hypothesis Spt6 occupancy can be determined at these genes in both wild-type Spn1 cells and spn1^{K192N} cells.

Clustering analysis of Spn1 binding showed a majority of ribosomal genes had Spn1 occupancy. Yeast ribosomal protein (RP) genes are highly conserved with mammalian RP genes (Wool et al., 1995). The ribosome of yeast and mammals is also highly conserved with the yeast ribosome consisting of 78 RPs while the mammalian ribosome is composed of 79 RPs (Warner, 1999; Warner et al., 2001). Most yeast RPs are encoded by two genes producing nearly identical proteins, with the 78 proteins being encoded by 137 RP genes (Wolfe and Shields, 1997). RP genes represent only 2% of the yeast genome but RP genes contain 101 of the yeast genome's 234 introns (Spingola et al., 1999). Due to the vast abundance of rRNA transcription and high ratio of introns in rRNA, nearly 40% of yeast mRNAs are spliced with 90% of all mRNA splicing occurring at RP transcripts (Warner, 1999; Warner et al., 2001). A number of other yeast genes with introns were also found to have significant Spn1 occupancy: *SNR18, OM14, UBC33, YRA1, MM2, SEC27, SNR87, TEF4, TUB3, ASC1, VPS75, NOG2, SCS22, EMC33, VMA9, HAC1, MOB2, ACT1,* and *MOB1.* Spn1 has recently been implicated in human splicing. RNAi knockdown of hSpn1 results in mRNA processing defects and nuclear retention of poly(A) RNA (Yoh et al., 2007). In addition to having a role in post-recruitment regulation, the high Spn1 occupancy found at yeast ribosomal genes and the prevalence of introns throughout ribosomal genes further suggests Spn1 may play a role in the regulation of

splicing. Though Spn1 may be involved in the regulation of splicing, it is very evident that Spn1 is important in the transcription intiation of ribosomal protein genes. Nearly 100% of ribosomal protein genes lost Spn1 occupany in spn1^{K192N} cells. In addition, RPL4B, RPS11B, and RPS4A all showed an increase in transcription expression during the genome wide expression assays (Table 5.1). Although RPS11B and RPS4A both have introns, RPL4B does not and further suggests an important role in transcription intiation of ribosomal protein genes. Due to the increase in expression Spn1 most likely serves in the capacity of a repressor to transcription intiation.

Chapter 6

Perspectives and future directions

The focus of my doctoral work was to better understand the mechanism of post-TBP/RNAPII-recruitment regulation. Previous studies had shown that Spn1, a highly conserved and essential protein, is important in post-recruitment regulation and serves as a repressor. These studies also showed that a mutant form of Spn1, spn1^{K192N}, no longer regulates the post-recruitment gene *CYC1* properly. As initiation of transcription is further studied, it has become evident that post-recruitment regulated genes are an important subset of the genome. For example, two genes important to higher eukaryotic health and regulated in a post-recruitment fashion are the proto-oncogene c-myc and the HIV-1 gene. In addition, post-recruitment regulated genes may be more likely to be induced by environmental change or stress as seen by *Drosophila* heat shock genes and *Saccharomyces cerevisiae FLR1, CYC1,* and *COX5A*. Taken together these studies sparked my interest to understand the mechanism of post-TBP/RNAPII-recruitment in greater detail.

Examining the mechanism of post-recruitment regulation first involved determining what additional factors are involved in post-recruitment regulation. To initiate this study I designed a TBP recruitment bypass screen to examine non-essential genes with a role in post-recruitment regulation.

I initially focused on genes encoding proteins with a known role in transcription to determine what factors also had post-recruitment regulation functions. Harnessing the power of yeast genetics, I was able to screen hundreds of non-essential gene products from a number of different transcription machinery complexes. The genetic screen proved to be quite successful in providing efficient, as well as, stringent information. Only two complexes, SAGA and Mediator, showed a high occurrence of post-recruitment function.

Follow up studies of SAGA and Mediator on the post-recruitment regulated gene *CYC1* confirmed important roles for SAGA and Mediator in post-recruitment regulation. Analysis of SAGA at the *CYC1* promoter suggests a new role for SAGA directly involved in post-recruitment function. The TBP recruitment function, as well as the histone acetyl transferase activities of SAGA, were not required to properly regulate *CYC1* in a post-recruitment manner.

Interestingly, dependence between SAGA and Mediator is seen at the *CYC1* promoter. A time course of Mediator shows its occupancy correlates closely with *CYC1* induction. When subunits of SAGA are deleted you lose Mediator occupancy to the *CYC1* promoter and expression is lost. This suggests that SAGA helps recruit Mediator to the promoter and facilitate transcription initiation. Non-essential genes of SAGA and Mediator proved to be essential when growth on non-fermentable carbon sources was required. This dependence on specific non-essential factors during cell growth on non-optimal conditions redefines the meaning of non-essential.

The composition of the *CYC1* promoter was further analyzed to determine how complete the PIC was during partial repression. Similar to that of higher eukaryotes the majority of the PIC was found at the promoter duning uninduced conditions. TFIIH, the last factor of the PIC, occupied *CYC1* during partial repression and did not have a substantial increase in occupancy upon induction. In addition, serine 5 of the RNAPII CTD heptad repeat is phosphoryalted during uninduced conditions. The capping enzyme Ceg1 has been shown to associate with the RNAPII phosphorylated CTD. To further understand the mechanism of post-recruitment regulation Ceg1 occupancy was determined. Interestingly, not only is the PIC at the promoter during uninduced conditions, but so is the capping enzyme.

To better understand the functions of the essential protein Spn1, and to determine genes potentially regulated in a post-PIC-recruitment manner, genome wide localization studies were performed. This resulted in a fruitful collaboration with BumKyu Lee and Dr. Vishy Iyer from the University of Texas, Austin. This collaboration provided us with an abundance of data that will lead to a number of future endeavors. Over 100 genes showed an increase in expression in the $spn1^{K192N}$ allele strains, which can be further analyzed as potential genes where Spn1 suppresses post recruitment function. Genes that are induced by environmental stress will be first analyzed for TBP and RNAPII occupancy. Approximately one-third of the genes where Spn1 bound did not lose occupancy in $spn1^{K192N}$ cells and may be further characterized for additional Spn1 function. Finally, Spn1 was found to significantly bind to a number of ribosomal genes.

Within the yeast genome more than half of all genes with introns are ribosomal genes. Human Spn1 has also been shown to be important for splicing. Yeast Spn1 may serve as an excellent model for further investigating the mechanism of splicing and mRNA export.

A model for post-recruitment regulation of *CYC1* can be made from these results. In the uninduced state the *CYC1* promoter is occupied by the PIC, Spn1, serine 5 phosphorylated RNAPII CTD, SAGA, and Ceg1 (Figure 6.1). Upon induction Dr. Lei Zhang showed that Spt6 is rapidly recruited (Figure 6.2) followed by Mediator and Swi/Snf later in the induction process (Figure 6.3).

These findings establish a strong correlation between yeast post-recruitment regulation and higher eukaryotes. Understanding the mechanisms of post-recruitment regulation is important for the development of treatments in diseases where expression of proteins i.e. c-myc, c-jun, and HIV-1 is regulated in a post-recruitment manner. In higher eukaryotes post-recruitment regulation has been established via promoter-proximal pausing. However, to date, post-recruitment regulation in a promoter-proximal pausing manner has not been seen in *S. cerevisiae*. With the strong correlation of similar complexes and composition in yeast and higher eukaryotic post-recruitment regulation, future work is aimed at identifying promoter-proximal pausing in yeast. *FLR1*, a gene studied by Dr. David Goldstrohm, and *CYC1* are both prime targets for promoter-proximal pausing. Specifically, these studies will involve potassium permanganate footprinting to determine the architecture of the yeast promoter.

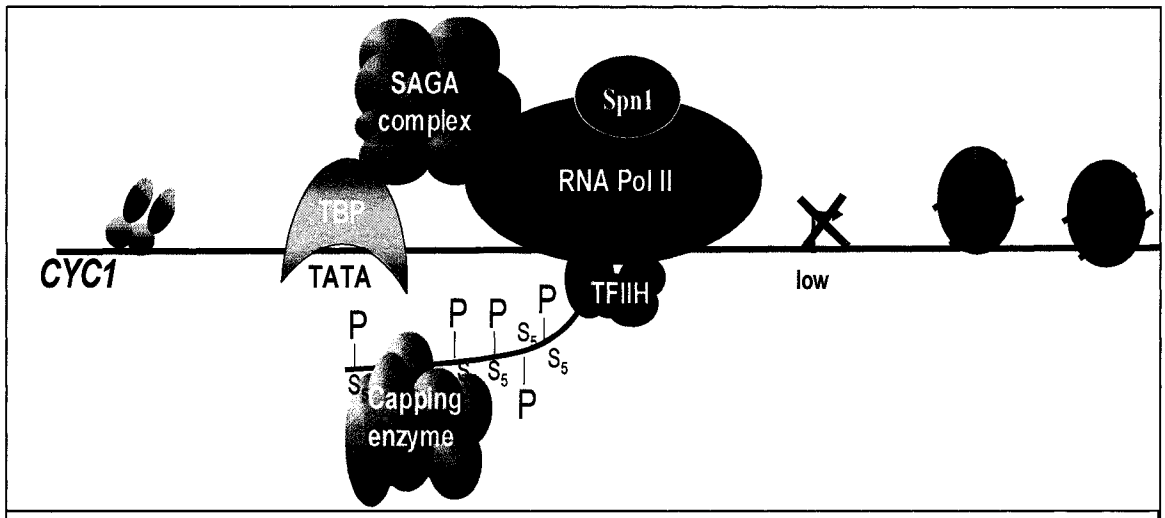


Figure 6.1 Model of Post-Recruitment Regulation during the uninduced condition: *CYC1* is a post-recruitment regulated gene. In the uninduced state TBP, RNA Pol II with serine 5 of the CTD phosphorylated, Spn1, SAGA, and Capping Enzyme all occupy the promoter.

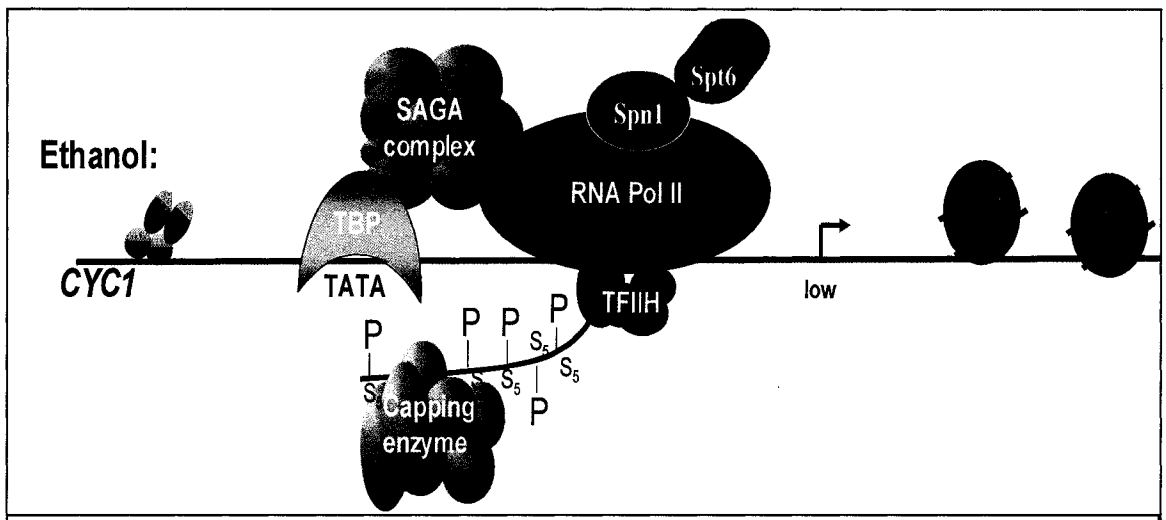


Figure 6.2 Model of Post-Recruitment Regulation after 1 hour activation: *CYC1* is a post-recruitment regulated gene. Immediately following activation of *CYC1* Spt6 is recruited to the promoter and interacts with Spn1. During activation TBP and RNA Pol II do not increase in occupancy.

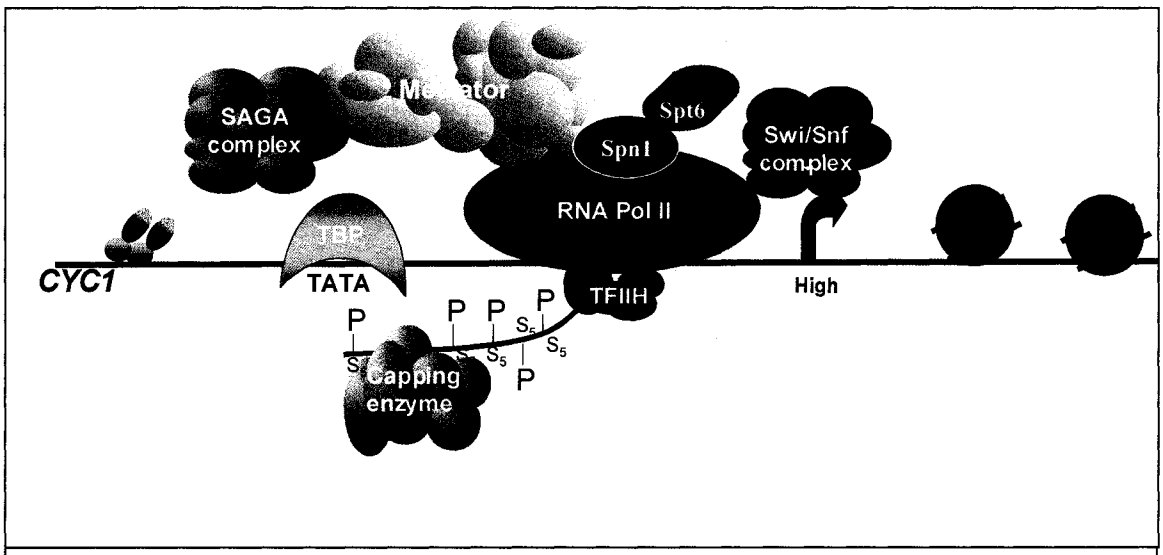


Figure 6.3 Model of Post-Recruitment Regulation at full activation: *CYC1* is a post-recruitment regulated gene. At full induction, 6 hours in non-fermentable carbon media, the *CYC1* promoter has recruited Mediator and Swi/Snf. During full activation TBP and RNA Pol II marginally increase in occupancy while expression increases 10 fold.

These footprinting studies should reveal if the promoter is melted prior to induction. In addition nuclear run-on assays can be carried out to determine if initiation of transcription by RNAPII occurs before gene activation.

Continued examination of the *CYC1* promoter will not only enhance our understanding of post-recruitment regulation, but it will also provide insight into the overall regulation of transcription initiation. The study of transcription initiation where recruitment of TBP and the PIC are rate limiting steps allows one to understand what events must occur to overcome this rate limiting step (Figure 6.4). Study of *CYC1* where recruitment of TBP and the PIC are not the rate limiting steps allows one to understand what events must occur in transcription initiation after the PIC has been formed (Figure 6.5). Therefore, the examination of post-recruitment regulation is the study of a continuum of transcription initiation regulation.

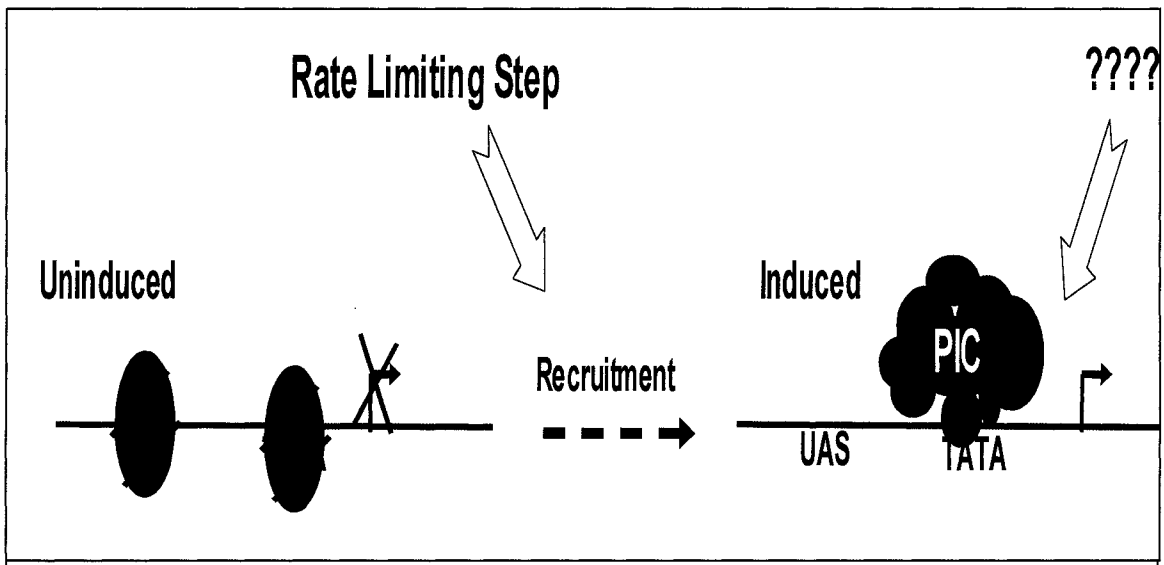


Figure 6.4 Study of Recruitment promoters: Studying the mechanism of transcription initiation is an intense process that has been studied for many years. Promoters where TBP and RNA Pol II are rate limiting steps allow one to study events that lead up to the recruitment of these rate limiting steps. Events that take place after the rate limiting step are not conducive to scientific analysis.

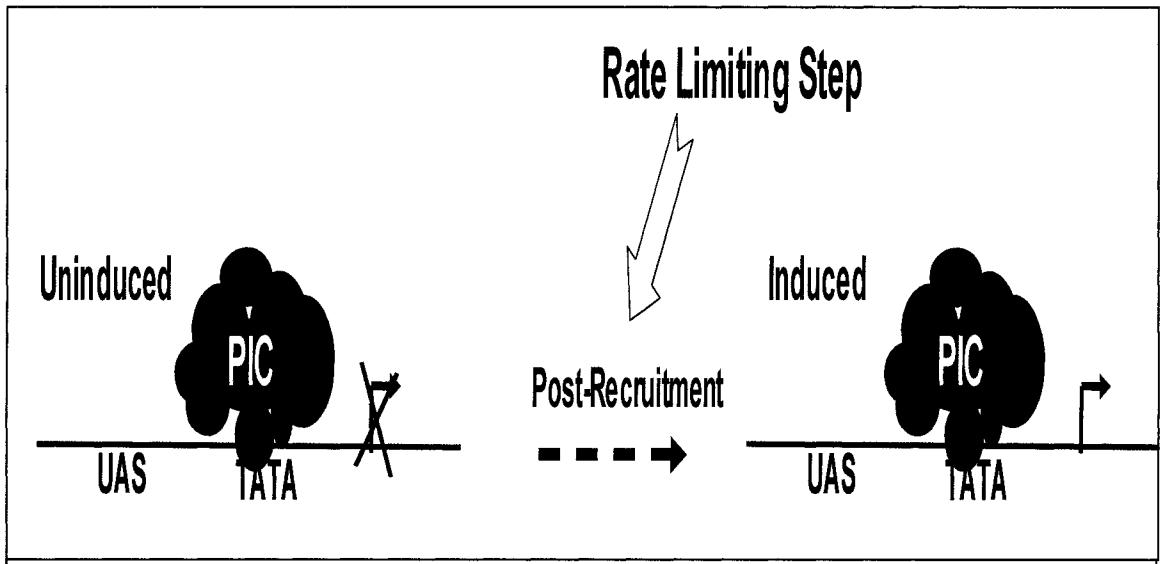


Figure 6.5 Study of Post-Recruitment promoters: The study of post-recruitment promoters allows one to investigate processes after TBP and Pol II are bound to the promoter but before elongation has begun. Therefore, the study of post-recruitment regulation allows one to take advantage of a natural process to study the continuum of transcription initiation.

Appendix I

Spn1 coordinates the recruitment of the Swi/Snf complex and Spt6 during RNA polymerase II transcription.

Dr. Lei Zhang, a former graduate student of Dr. Laurie Stargell, directed much of his work to understanding the functional relationship between Spn1 and Spt6. In his work he found that Spn1 played an important role of helping coordinate the chromatin remodeling factors Spt6 and Swi/Snf. I examined histone occupancy on the *CYC1* promoter to help better understand the role of Spt6, Swi/Snf, and Spn1 in transcription initiation of *CYC1.* In addition, I performed Co-IP studies of Spn1 and RNA Pol II CTD phosphorylation and found that Spn1 co-immunoprecipitated with both serine 2 and 5 phosphoryated derivatives of RNA Pol II. This collaboration with Dr. Zhang resulted in a manuscript currently being prepared for Molecular Cell submission. My contributions to this work included the following: Figure 2B, Figure 6, data analysis and construction of Figure 1C, Figure 4C, helping compose our findings into manuscript format. Here I have attached the abstract for the manuscript entitled: Spn1 coordinates the recruitment of the Swi/Snf complex and Spt6 during RNA polymerase II transcription.

Spn1 coordinates the recruitment of the Swi/Snf complex and Spt6 during RNA polymerase II transcription.

A.1 Abstract

Through a targeted genetic screen, strong and specific counteracting interactions of *SPN1* with three SWI/SNF genes are observed. The mechanistic functions of Spn1, Spt6, and the Swi/Snf complex are investigated on the yeast *CYC1* gene, which is an excellent model system to study regulatory mechanisms after TATA-binding protein (TBP) and Polymerase II (Pol II) recruitment. Spn1 is constitutively recruited to the *CYC1* promoter, through its association with Pol II under partial repression and full activation; however, Spt6 and Swi/Snf appear at *CYC1* only after activation. Spt6 significantly precedes Swi/Snf occupancy at the promoter. A Spn1 mutant defective for interacting with Pol II is no longer recruited to the *CYC1* gene. In this mutant *SPN1* strain, Spt6 is absent from the *CYC1* promoter, and Swi/Snf is now constitutively recruited. These observations support a model whereby Spn1 negatively regulates recruitment of Swi/Snf to the *CYC1* promoter, and this inhibition is abrogated by the Spn1-Spt6 interaction.

Appendix II

Attempt to suppress SNF5 deletion mutant by dominant mutant alleles of SPN1

Malignant Rhabdoid Tumor (MRT) is a fatal form of cancer typically found in infants or children. Those diagnosed with MRT have a short life expectancy, generally six months to 2 years, due to the lack of treatment available for MRT. Deletions or truncations in the human SNF5 gene are associated strongly with MRT. SNF5 is a member of the ATP dependent chromatin remodeling complex Swi/Snf, which is conserved from humans to yeast. In yeast, deletion of the SNF5 gene (ΔSNF5) results in the inability of cells to grow on media lacking inositol. Dr. Lei Zhang had found genetic interactions between the Swi/Snf complex and the Spn1 protein (Appendix I). Due to this interaction it was hypothesized that one might find a SPN1 mutant that could dominantly repress the mutant phenotype of a cell harboring ΔSNF5. Using polymerase chain reaction (PCR) SPN1 was randomly mutated via misincorporation as described previously (Michel et al., 1998). A selection was developed to screen through thousands of colonies to find a dominant mutant SPN1 that could allow growth on inositol-lacking media in a ΔSNF5 cell. I screened over 250,000 colonies and was unsuccessful in finding a dominant Spn1 mutant that was capable of suppressing ΔSNF5 phenotypes. The following is a flowchart for the genetic screen developed to find a dominant Spn1 mutant that could suppress ΔSNF5 phenotypes (Figure AII.1).

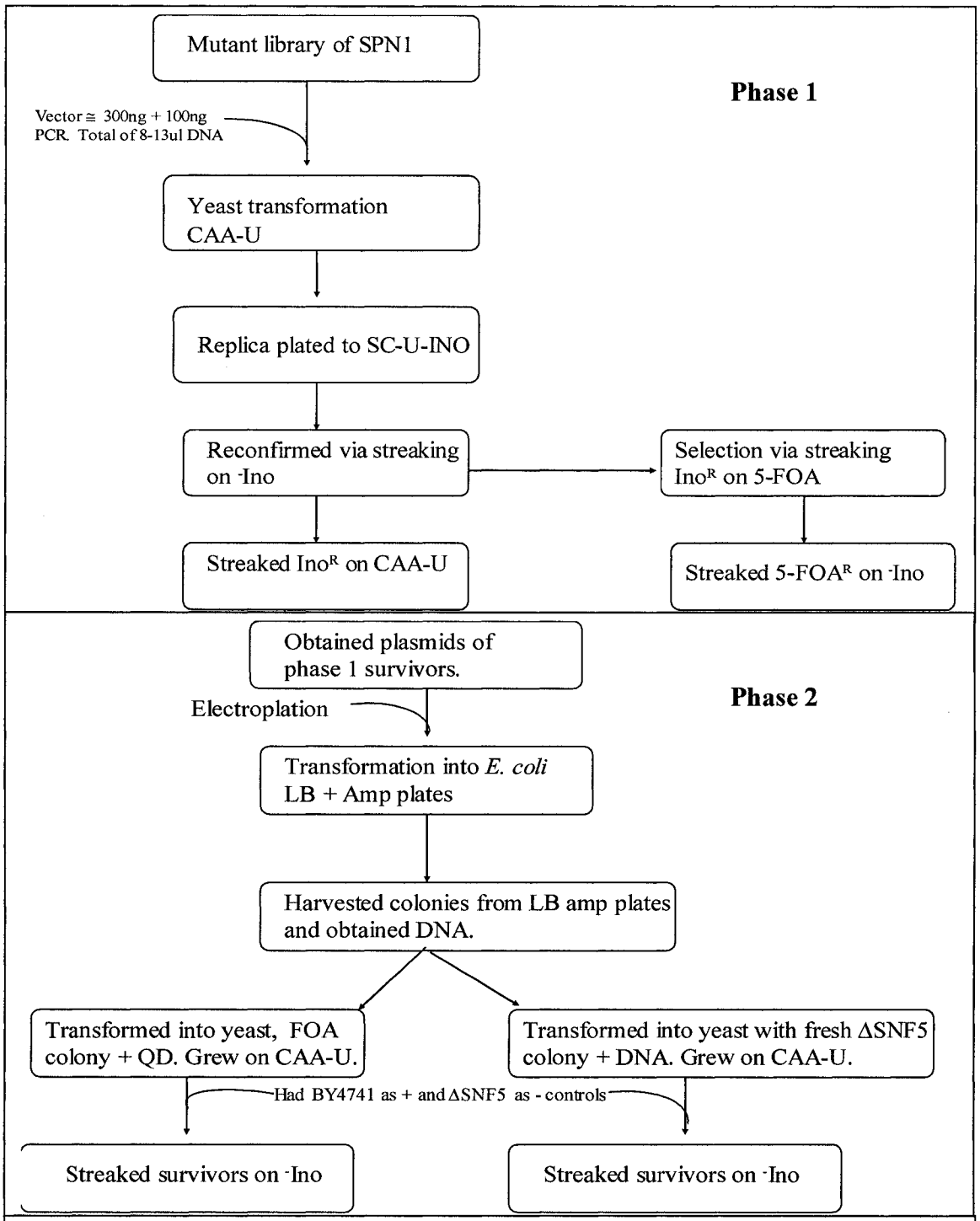


Figure AII.1 Genetic Screen for dominant Spn1 suppressors of Δsnf5 phenotypes. In phase 1 Spn1 was randomly mutated via PCR and transformed into Δsnf5 cells with a URA3 marker. Cells that could grow on medium lacking inositol (-INO) was further taken through the screen. Reconfirmation of a dominant Spn1 mutant that suppresses Δsnf5 phenotypes was carried out in phase 2. None of the over 250,000 colonies screened were able to suppress Δsnf5 phenotype.

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