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Analysis of TAF II Function in the Yeast Saccharomyces Cerevisiae

Lynne Marie Apone
University of Massachusetts Medical School

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Analysis of TAF_{II} function in the yeast *Saccharomyces cerevisiae*

A dissertation Presented

By

Lynne Marie Apone

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY

(January 14, 1998)

Biochemistry
ANALYSIS OF TAF$_{II}$ FUNCTION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

A dissertation Presented

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Lynne Marie Apone

Approved as to style and content by:

Craig L. Peterson, Ph.D., Chair of Committee

Martha T. Fedor, Ph.D., Member of Committee

Stephen J. Doxsey, Ph.D., Member of Committee

Peter M. Pryciak, Ph.D., Member of Committee

Richard A. Young, Ph.D., Member of Committee

Michael R. Green, Ph.D., Dissertation Mentor

Thomas B. Miller, Ph.D., Dean of the Graduate School of Biomedical Sciences

Department of Biochemistry
Program of Molecular Medicine

January 14, 1998
DEDICATION

In loving memory of my father

and with love and appreciation to my husband Paul for his
love, support and encouragement.
ABSTRACT

Transcription by RNA polymerase II is a highly regulated process requiring a number of general and promoter specific transcription factors. Although many of the factors involved in the transcription reaction are known, exactly how they function to stimulate or repress transcription is not well understood. Central to understanding gene regulation is understanding the mechanism by which promoter specific transcription activators (activators) stimulate transcription.

A group of factors called coactivators have been shown to be required for activator function in vitro. The best characterized coactivators to date are members of the TFIID complex. TFIID is a multisubunit complex composed of the TATA box binding protein (TBP) and 8-12 TBP associated factors (TAF\textsubscript{IIS}). Results from numerous in vitro experiments indicate that TAF\textsubscript{IIS} function by binding to activators and forming a bridge between the activator and the basal transcription machinery. In order to gain insight into the mechanism by which activators stimulate transcription, we chose to analyze the in vivo function of TAF\textsubscript{IIS}, their proposed targets.

Results from the genetic disruption of a number of TAF\textsubscript{IIS} in the yeast *Saccharomyces cerevisiae* showed that most are
encoded by essential genes. In order to study their function, temperature-sensitive and conditional alleles were constructed. Cells depleted of individual TAFrrs by either of these two methods displayed no defect in global transcription activation. Inactivation of yTAF117, however, resulted in a promoter specific defect. In addition, inactivation of yTAF1145, yTAF90, or TSM1, resulted in an inability of cells to progress through the cell-cycle.

In an attempt to identify genes whose expression required yTAF90, we performed subtractive hybridization on strains containing wild-type and temperature-sensitive alleles. Although this technique successfully identified genes differentially expressed in the two strains, it failed to identify genes whose expression required yTAF90.

These results indicate that TAFrrs are not the obligatory targets of activators, and that other factors must provide this role in vivo. Furthermore, that many of TAFrrs are required for cell-cycle progression.
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CHAPTER I

INTRODUCTION
Overview

Transcription activation is a highly regulated process required throughout the life of an organism. It is essential for the development and maintenance of an organism, as well as for its ability to respond to environmental stimuli. Although there is a general understanding of how genes are regulated, the specifics are not completely understood. What has become clear, however, is that the loss of regulation can have catastrophic consequences. The importance of regulation, and the detrimental effects which can result from its loss, make it imperative that we understand the exact mechanism by which genes are regulated.

There are many steps involved in the accurate transcription of a gene. To begin with, a preinitiation complex (PIC) must be formed at the appropriate site on the promoter. The double stranded DNA must then be separated to expose the template for RNA synthesis. This is followed by formation of the first phosphodiester bond, detachment of the RNA synthesis machinery from the promoter area, elongation of the transcript, and termination. The whole process must then be reinitiated to begin a new transcription cycle. Each of these steps involve the function of many proteins, any one of which could serve as a target for regulation. In vivo, the
Repressive effects of chromatin create an additional level of complexity, and additional targets for regulation.

In bacteria, transcription is carried out by a single RNA polymerase. Transcription in eukaryotes, however, is carried out by three distinct RNA Polymerases, each required for the transcription of a different subset of genes (reviewed in Sentenac 1985). RNA polymerase II, which is the focus of this thesis, is responsible for transcribing all of the protein-encoding genes, as well as some small nuclear RNAs.

The formation of a PIC onto the core promoter is thought to constitute one of the most highly regulated steps in the transcription reaction. Core promoters of both prokaryotic and eukaryotic genes contain distinct elements which are required for accurate transcription initiation.

In E. coli, most core promoters contain a -35 and a -10 hexamer sequence (reviewed in Busby and Ebright 1994). The -35 hexamer is located approximately 35 bases upstream of the start site of transcription with the consensus sequence TTGACA. The -10 sequence is located approximately 10 base pairs upstream of the start site and has the consensus sequence TATAAT.

Core promoters of genes transcribed by eukaryotic RNA polymerase II also contain two distinct elements, the TATA box and the initiator. The presence of either one of these
elements is sufficient for accurate transcription initiation, and together they can greatly enhance promoter strength. The TATA box is an A/T rich sequence with the consensus TATAa/tAa/t. In higher eukaryotes it is located 25-30, (Breathnach and Chambon 1981) and in yeast between 40 and 90, base pairs upstream of the start site of transcription (reviewed in Struhl 1987). The second element, the initiator, is a pyrimidine rich sequence which encompasses the start site.

Both bacterial RNA polymerase and eukaryotic RNA polymerase II require auxiliary factors to accurately position RNA polymerase onto the promoter (Weil et al. 1979; reviewed in Young 1991). In bacteria a family of proteins called sigma (σ) factors carry out this function (Lewin 1997). Each σ factor recognizes a different class of promoter. Multiple σ factors allow the polymerase to recognize different promoters in response to different environmental stimuli. While only a single σ factor is required to accurately position the bacterial enzyme, a group of six factors are required in eukaryotes (Matsui et al. 1980). These factors, TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, are commonly referred to as the general transcription factors (GTFs). In the presence of the GTFs and RNA polymerase II a low level (basal) of transcription can be obtained. Many of the GTFs are composed of multiple subunits.
Comparison of their amino acid sequences show that they are highly conserved among eukaryotes (reviewed in Orphanides et al. 1996; Nikolov and Burley 1997). In addition, many of the GTFs have regions that are similar to regions of the bacterial σ factors.

The exact mechanism by which the GTFs assemble onto the promoter, and the function of each of the polypeptides, is under intense investigation. Although the GTFs were originally shown to assemble onto the promoter in a stepwise fashion in vitro, (Buratowski et al. 1989) recent evidence indicates that this may not be the case in vivo. RNA polymerase II can be isolated in association with many of the GTFs in a complex that has been termed the holoenzyme (Koleske and Young 1994; Kim et al. 1994; Chao et al. 1996; Maldonado et al. 1996). If this holoenzyme complex exists in vivo, then it is possible that there are multiple pathways leading to the formation of a PIC. On some promoters the GTFs may be recruited individually, and on others, in a single step much like that of the bacterial core RNA polymerase (Figure 1-1). The GTFs and the formation of a PIC will be discussed in the following section.

**Basal transcription reaction**

**TBP.** In eukaryotes, the formation of a preinitiation complex begins with the binding of the TBP (TATA box binding
Stepwise assembly

TFIID
TFIIB
POLII/TFIIF
TFIIE
TFIIF

Holoenzyme recruitment

TFIID
TFIIB
Holoenzyme

RNA POL II
TFIIE
TFIIF
SRBs
TFIID
TAF15
Figure 1-1. Alternative pathways to PIC formation.

The GTFs may assemble into the PIC individually, in a stepwise fashion, or many may enter as members of a preformed complex called the holoenzyme. Potential components of the holoenzyme are represented in light gray, and those of the TFIID complex, in dark gray. The solid line represents the promoter which is distorted upon TFIID binding, and the bent arrow, the start site of transcription. Complexes are not drawn to scale.
protein) subunit of TFIID to the TATA box. TBP has been highly conserved throughout evolution (reviewed in Hernandez 1993). Most TBPs examined display greater than 80% identity in their C-terminal 180 amino acids. In addition, this domain is sufficient for viability in yeast (Gill and Tjian 1991; Poon et al. 1991; Reddy and Hahn 1991; Zhou et al. 1991). Unlike the C-terminus, the N-terminus of TBP is highly divergent and its function is not known. The high degree of conservation between yeast and human TBP is evidenced by the ability to interchange the two proteins in vitro (Buratowski et al. 1988; Cavallini et al. 1988) and in vivo (Cormack et al. 1994) for transcription from TATA box-containing promoters.

Crystallographic studies of TBP show that it folds up into a unique saddle shape (Nikolov et al. 1992; Chasman et al. 1993). The underside of the saddle straddles the DNA and induces a 90°C bend (Kim et al. 1993a,b). It is this bent structure that is recognized by the remaining factors which enter into the complex.

It has been believed for some time that most organisms, yeast, flies, and humans included, contain a single functional species of TBP. Although this is still the case for yeast, Drosophila appear to contain at least two highly homologous species, one which is ubiquitous and one which is cell type specific (Crowley et al. 1993). The cell type
specific TBP, called TRF (TBP-related factor), is capable of binding to the TATA sequence and of directing RNA polymerase II transcription (Hansen et al. 1997).

TBP is a unique basal factor in that it is required for transcription by all three RNA polymerases. The ability of TBP to recognize and function on promoters transcribed by RNA polymerase I, II and III, stems from the various factors with which it associates. TBP is a component of SL1, TFIID, and TFIIIB, complexes required for transcription by RNA polymerase I, II and III, respectively (Hernandez 1993).

**TFIID.** TFIID is a multisubunit complex composed of TBP and 8-12 TBP associated factors (TAF11s) (Dynlacht et al. 1991; Tanese et al. 1991). The analysis of TFIID has focused almost exclusively on its role in activated transcription. This is not surprising since in vitro the TAF11s are dispensable for basal transcription from TATA box containing promoters (Hoey et al. 1990; Hoffman et al. 1990; Peterson et al. 1990; Pugh and Tjian 1990; Smale et al. 1990). TFIID does, however, play a role in basal transcription from many promoters and appears to function mainly through the initiator element. TFIID is required for transcription from TATA-less promoters, TBP alone is not sufficient (Pugh and Tjian 1990; Smale et al. 1990; Pugh and Tjian 1991). In addition, it is required for enhanced basal transcription from core promoters containing multiple elements, such as a
TATA box and an initiator (Verrijzer et al. 1995). The requirement for TFIID on these promoters has been explained by the ability of TFIID, but not TBP, to make extensive contacts with core promoter elements in addition to the TATA box. On the adenovirus major late (ADML) promoter, DNaseI footprinting assays have shown that TBP protects an area of approximately twenty base pairs surrounding the TATA box. TFIID, however, footprints over the TATA box, as well as the start site and downstream sequences. (Sawadogo and Roeder 1985; Nakajima et al. 1988; Zhou et al. 1992). The extended contacts made by TFIID are thought to stabilize the TFIID/DNA complex. This theory is supported by experiments in which removal of the initiator and downstream elements from the hsp70 promoter destabilized the TFIID/DNA complex, but had no effect on TBP binding (Verrijzer et al. 1995).

Drosophila TAF₅₁₅₀ (dTAF₅₁₁₅₀) is the primary subunit of TFIID believed responsible for the extended DNA contacts. Although no human homologue for dTAF₅₁₁₅₀ has been found (see Table 1-1), TSM₁ has been identified as the yeast homologue (Verrijzer et al. 1994). Numerous in vitro experiments have shown that dTAF₅₁₁₅₀ binds specifically to an area which includes the initiator and downstream sequences. (Verrijzer et al. 1994). In addition, the DNaseI footprint of dTAF₅₁₁₅₀ on DNA resembles that of the TFIID complex. Human TAF₅₂₅₀
<table>
<thead>
<tr>
<th>Yeast</th>
<th>Human</th>
<th>Drosophila</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAF II,130/145</td>
<td>TAF II,250</td>
<td>TAF II,230</td>
</tr>
<tr>
<td>TSM1</td>
<td>?</td>
<td>TAF II,150</td>
</tr>
<tr>
<td>None</td>
<td>TAF II,135</td>
<td>TAF II,110</td>
</tr>
<tr>
<td>TAF II,90</td>
<td>TAF II,100</td>
<td>TAF II,80</td>
</tr>
<tr>
<td>TAF II,68/61</td>
<td>TAF II,20/15</td>
<td>TAF II,30α</td>
</tr>
<tr>
<td>TAF II,67</td>
<td>TAF II,55</td>
<td>?</td>
</tr>
<tr>
<td>TAF II,60</td>
<td>TAF II,70</td>
<td>TAF II,60/62</td>
</tr>
<tr>
<td>TAF II,40</td>
<td>TAF II,28</td>
<td>TAF II,30β</td>
</tr>
<tr>
<td>TAF II,30/TFG3/ANC1</td>
<td>AF-9/ENL</td>
<td>?</td>
</tr>
<tr>
<td>TBP</td>
<td>TBP</td>
<td>TBP</td>
</tr>
<tr>
<td>TAF II,25/23</td>
<td>TAF II,30</td>
<td>?</td>
</tr>
<tr>
<td>TAF II,19/FUN81</td>
<td>TAF II,18</td>
<td>?</td>
</tr>
<tr>
<td>TAF II,17</td>
<td>TAF II,32</td>
<td>TAF II,40/42</td>
</tr>
</tbody>
</table>

Table 1-1: TFIID subunits isolated from the yeast *Saccharomyces cerevisiae* have homologues in both human and *Drosophila*
(hTAF_{150}, 250) has also been shown to contact DNA (Verrijzer et al. 1995). Furthermore, reconstitution experiments have shown that both dTAF_{150} and hTAF_{250} are required for discrimination between promoters containing or lacking initiator and downstream sequences (Verrijzer et al. 1995).

The experiments described above were performed in vitro. Recent experiments performed in vivo also indicate that TAF_{150}s function, at least in part, through the core promoter. The exact promoter element is, however, under debate. Experiments performed in the Struhl laboratory indicate that yeast yTAF_{145}, the homologue of Drosophila dTAF_{250}, is required for transcription from promoters containing non-consensus TATA boxes (Moqtaderi et al. 1996a). These authors found that upon depletion of yTAF_{145} (also called yTAF_{130}), yeast cells were incapable of transcription from the TRP3 and HIS3'1 promoters. These cells were, however, capable of global transcription activation. Since the TRP3 and HIS3'1 promoters contain non-consensus TATA boxes, the Struhl laboratory hypothesized that yTAF_{145}/yTAF_{130} may be required for the transcription of genes containing weak TATA boxes. However, while yTAF_{145} may be required for transcription of some genes whose promoters contain non-consensus TATA boxes, a weak TATA box is not the only determinant. yTAF_{145} is required for the transcription of genes, including CLN2, whose promoters contain consensus TATA boxes (Walker et al.
1997). In addition, replacing the non-consensus TATA box from the yTAF_{145} dependent RPS5 promoter, with a consensus TATA box, does not make it yTAF_{145} independent (Shen and Green 1997).

Once TFIID is bound to the promoter, the remaining GTFs are capable of entering into the complex. These factors will be described as though they assembled individually into the PIC. However, one should keep in mind that in vivo, pathways may exist in which a PIC is formed by the recruitment of a preassembled complex to the promoter (Figure 1-1).

**TFIIB.** TFIIB enters into the PIC by recognizing the unique structure of the TBP/DNA complex (Nikolov et al. 1995). Upon entering the complex it binds TBP and DNA both upstream and downstream of the TATA box. Through these interactions it acts as a clamp to stabilize the TBP/DNA complex. Genetic and biochemical data identified an additional role for TFIIB, that of marking the start site of transcription. TFIIB was originally isolated as a suppressor of the cycl phenotype associated with an aberrant ATG codon in the yeast CYCl gene (Pinto et al. 1992). Suppression resulted from the utilization of a rarely used start site downstream of the aberrant ATG. In vitro, TBP, TFIIB and RNA polymerase II are sufficient for accurate transcription initiation on supercoiled templates (Parvin and Sharp 1993).
The TFIIB gene has been cloned from a number of species including human (Ha et al. 1991) and yeast (Pinto et al. 1992) and found to encode a small protein of approximately 34kD in humans and 38kD in yeast. Both proteins are highly homologous sharing 52% similarity over their entirety, with higher degrees of similarity found within various conserved motifs.

**TFIIA.** TFIIA can enter into the complex either before or after TFIIB. Upon entering the complex it stabilizes the TFIID/DNA and TFIIB/TFIID/DNA complexes (Orphanides et al. 1996). TFIIA is not always required in transcription reactions in vitro: it is required in reactions using purified TFIID but not in those using recombinant TBP (Cortes et al. 1992). The variable requirement for TFIIA is thought to be due to its ability to remove inhibitors associated with TBP (Merino et al. 1993; Auble et al. 1994; Ma et al. 1996). When the inhibitors are present, such as in the purified TFIID complex, TFIIA is required, when they are not, which is the case with recombinant TBP, TFIIA is dispensable for basal transcription.

Human TFIIA is composed of three subunits α, β, and γ which encode proteins of 37kD, 19kD and 13kD respectively (Orphanides et al. 1996). The α and β subunits are encoded by a single gene whose product is proteolytically processed to produce the two subunits. Yeast contain only two subunits, TOA1 and TOA2, each encoded by a different gene. TOA1 and
TOA2 encode for proteins of 32kD and 13kD, respectively, and each is essential for viability (Ranish et al. 1992). Although varying in subunit composition, TFIIA from yeast and humans are highly conserved. TFIIA from yeast can substitute for human TFIIA in an in vitro transcription reaction (Ranish and Hahn 1992; Ranish et al. 1992).

The crystal structure of yeast TFIIA complexed with TBP and DNA has been solved (Geiger et al. 1996; Tan et al. 1996). In the structure, the two subunits of TFIIA fold up into a unique boot shape which is positioned upstream of TBP, opposite TFIIIB. Like the addition of TFIIIB, the assembly of TFIIA into the complex does not alter the structure of the TBP/DNA complex. The addition of both of these factors does, however, greatly increase the surface area available for interactions with other proteins.

**TFIIF.** TFIIF is a heterodimer consisting of the RAP30 and RAP74 proteins. The genes encoding these subunits have been cloned from many organisms and have been found to be highly conserved throughout evolution (Orphandides et al. 1996). The yeast and human proteins exhibit approximately 50% similarity and 30% identity (Henry et al. 1994). Both RAP30 and RAP74 encode essential genes in yeast (Henry et al. 1994).

The primary role of TFIIF in transcription initiation is to ensure that RNA polymerase II enters into the TFIID/TFIIA/TFIIB complex. This is accomplished by TFIIF
associating with RNA polymerase II (Sopta et al. 1985; Flores et al. 1988) and inhibiting its binding to nonspecific sites (Conaway and Conaway 1990). In addition to its role in transcription initiation, TFIIF has also been shown to stimulate transcription elongation in vitro (Flores et al. 1989; Bengal et al. 1991).

TFIIE/TFIIH. TFIIE is composed of a heterotetramer of 34kD and 56kD subunits (Ohkuma et al. 1990; Inostroza et al. 1991). TFIIH is a complex composed of nine subunits ranging in size from 35-89kD (reviewed in Zawel and Reinberg 1995). Subunits of this complex possess multiple enzymatic activities which include a DNA-dependent helicase, an ATPase, and a kinase which can phosphorylate the carboxy terminal domain (CTD) of the largest subunit of RNA polymerase II. TFIIE and TFIIH are thought to function, at least in part, to separate the double stranded DNA (DNA melting). This process requires ATP hydrolysis (Wang et al. 1992) and TFIIE is capable of stimulating the ATPase activity of TFIIH (Ohkuma and Roeder 1994). Furthermore, neither factor, nor ATP is required when supercoiled templates are used as a substrate (Parvin and Sharp 1993). In addition to its role in transcription, TFIIH also functions in DNA repair (reviewed in Drapkin 1994).

RNA polymerase II. RNA polymerase II is a large multisubunit complex composed of 10-12 subunits (reviewed in Woychik and Young 1990). Some of these subunits are unique to
RNA polymerase II while others are shared among the three RNA polymerases. Like many of the GTFs, the subunits of RNA polymerase II have been highly conserved from yeast to human. Similarly, the two largest subunits of the eukaryotic enzyme (RPB1 and RPB2) share a high degree of conservation with the β' and β subunits of bacterial RNA polymerase. The largest subunit of eukaryotic RNA polymerase II contains a unique structure at its carboxy terminal domain referred to as the CTD. This domain consists of a heptad repeat of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. The number of repeats contained in the CTD correlates with the complexity of the organism: the more complex the organism the greater the number of repeats. Yeast contain 26 or 27 repeats while humans contain 52. The CTD is required for viability (Nonet et al. 1987b; Allison et al. 1988; Zehring et al. 1988; Bartolomei et al. 1988). Cells can survive with partial deletions, but display defects in growth and the response to transcription activators (Nonet et al. 1987b; Bartolomei et al. 1988; Allison and Ingles 1989; Scafe et al. 1990; Peterson et al. 1991). In vivo, the CTD exists in two forms, a nonphosphorylated and a highly phosphorylated form. Conversion between these two forms is believed to result in the transition from initiation to elongation (Payne et al. 1989; Laybourn and Dahmus 1990). The nonphosphorylated form is preferentially associated with the promoter (Laybourn and
Dahmus 1990; Lu et al. 1991), while the phosphorylated form is found associated with elongating polymerases (Cadena and Dahmus 1987; Laybourn and Dahmus 1990; O'Brien et al. 1994). Kinases capable of phosphorylating the CTD in vitro include TFIIH (Feaver et al. 1991; Lu et al. 1992; Serizawa et al. 1992) and SRB10 (Liao et al. 1995), two components of the preinitiation complex. Whether these kinases are responsible for phosphorylation of the CTD in vivo remains to be determined.

**Holoenzyme.** RNA polymerase II has been purified from yeast (Koleske and Young 1994; Kim et al. 1994) and human cells (Chao et al. 1996; Maldonado et al. 1996) as a large multisubunit complex. This complex, called the holoenzyme, is believed to be the form of RNA polymerase II involved in transcription initiation in vivo (Koleske and Young 1994; Thompson and Young 1995; Koleske and Young 1995; Svejstrup et al. 1997). Among the identified components of the holoenzyme are a subset of the basal factors (Koleske and Young 1994; Kim et al. 1994; Chao et al. 1996; Maldonado et al. 1996), and a subcomplex called the mediator (Kim et al. 1994). The mediator will be discussed in a subsequent section of this introduction.

The composition of the holoenzyme varies depending on the procedure and group by which it is isolated. The yeast holoenzyme purified by the Young group contains the general factors TFIIB, TFIIF, TFIIH, and substoichiometric amounts of
TBP (Koleske and Young 1994), while that of the Kornberg group contains only TFIIF (Kim et al. 1994). The human holoenzyme purified by the Young and the Reinberg groups contain substoichiometric amounts of TFIIE and TFIIF (Chao et al. 1996; Maldonado et al. 1996). In addition, the Reinberg group found limiting amounts of TFIIH (Maldonado et al. 1996). Whether different subcomplexes of the holoenzyme exist in vivo, or whether weakly associated subunits are lost during purification remains to be determined. In any case, each complex contains features which have become characteristic of the holoenzyme. Each contains a subset of the GTFs, the SRBs and is capable of responding to transcription activators.

SRBs. The SRBs (suppressor of RNA polymerase B (II)) were originally identified as suppressors of the cold sensitive phenotype associated with truncation of the CTD (Nonet and Young 1989). These proteins have since been shown to exist in a complex associated with the holoenzyme (Thompson et al. 1993; Kim et al. 1994; Koleske and Young 1994; Liao et al. 1995; Hengartner et al. 1995). At this time nine SRBs have been isolated and cloned. SRB4, SRB6 and SRB7 encode essential genes (Thompson et al. 1993; Hengartner et al. 1995; Chao et al. 1996), while cells deleted of SRB2, SRB5, SRB8, SRB9, SRB10 and SRB11 display conditional phenotypes like those observed with CTD truncations (Nonet and Young 1989; Thompson et al. 1993; and Liao et al. 1995;
Hengartner et al. 1995). At least three of the SRBs have been shown to play an important role in basal and activated transcription. Cells expressing temperature-sensitive alleles of SRB4 display global transcriptional defects in RNA polymerase II directed transcription at the non-permissive temperature (Thompson and Young 1995). In addition, both SRB2 and SRB5 are required for basal and activated transcription in vitro (Koleske et al. 1992; Thompson et al. 1993).

While a low level of transcription can be obtained in vitro in the presence of the GTFs and RNA polymerase II, additional transcription factors are required to modulate this level. These factors, which are present in both eukaryotes and prokaryotes, can increase (activators) or decrease (repressors) the level of transcription in response to stimuli. The level of transcription obtained from a particular gene results from the unique combination of activators and repressors bound to its promoter. Transcriptional activators will be discussed in the next section.

Transcription activators

Transcription activators are modular proteins. They contain a DNA binding domain which allows them to bind to sites in their target genes, called enhancers or upstream regulatory sequences (UAS), and an activation domain, which
stimulates transcription (Ptashne 1988; Mitchell and Tjian 1989). Activators can be targeted to different promoters by simply swapping the DNA binding domain of one activator for that of another. Activation domains have generally been classified according to their amino acid composition. Classes of activators found in higher eukaryotes include those rich in acidic, proline, and glutamine residues. Yeast, however, are believed to possess only acidic activators. The mechanism of transcription activation appears to be conserved from yeast to humans. Activators from yeast, including GCN4 and GAL4, function in mammalian cells and activators from mammalian cells, such as Jun and the retinoic acid receptor, function in yeast (Struhl 1989).

How activators function in eukaryotes is not completely understood. Since transcription can proceed in their absence (basal transcription), activators are not believed to be required for promoter function, but rather to overcome a rate limiting step in the reaction. In bacteria, activators have been shown to aid the binding of RNA polymerase to low affinity sites (reviewed in Gralla 1996). They have also been shown to stimulate RNA polymerases which have been bound to their sites in an inactive form. Similar mechanisms are believed to occur in eukaryotes. In eukaryotes, however, the activator has a far greater number of potential targets and possible mechanisms of action.
In eukaryotes, one of the most intensely studied and highly regulated step in the transcription reaction is the formation of a PIC. Activators are thought to function primarily at this step to increase the number of transcriptionally competent PICs. Since the same set of basal factors is required for transcription from all genes, it is believed that activators function by contacting one or more of the basal factors. Interaction between an activator and a basal factor could result in the recruitment of a factor, which is rate limiting, into the PIC. In support of this theory, artificial recruitment of TBP (Chatterjee and Struhl 1995; Klages and Strubin 1995; Xiao et al. 1995), or components of the holoenzyme (Barberis et al. 1995) to the promoter via fusion with a heterologous DNA binding domain overcomes the need for an activator.

Alternatively, the interaction of an activator with a basal factor could result in a conformational change or a covalent modification which facilitates the assembly of additional members into the PIC or which converts the PIC into a more transcriptionally competent complex.

The first indication of an activator-basal factor interaction came in 1985. Sawadogo and Roeder used DNase I and chemical footprinting to show an increase in the stability of TFIID binding to the TATA box of the adenovirus major late promoter in the presence of the activator USF (Sawadogo and Roeder 1985). In these experiments the binding
of the activator was also enhanced. The footprints of the two factors were in close proximity and on the same face of the DNA. These results indicated that the cooperative binding of USF and TFIID was due to a physical interaction. Direct evidence supporting an interaction between TFIID and an activator was obtained in 1990 using affinity chromatography (Stringer et al. 1990). TBP was shown to bind directly to the activation domain of the acidic activator VP16. This interaction was shown to be functionally important in that a single point mutation in the activation domain of VP16 which abolished its ability to activate transcription, also abolished its ability to interact with TBP (Ingles et al. 1991).

Subsequent studies have demonstrated interactions between activators and a number of basal factors (Triezenberg 1995). The significance of many of these interactions remains to be determined. One such interaction which appears to be important is the interaction between TFIIB and VP16 (Lin and Green 1991; Lin et al. 1991). The recruitment of TFIIB into the PIC was shown to be a rate limiting step in PIC formation (Lin and Green 1991). Using an immobilized template assay, Lin and Green (1991) showed that in the presence of an activator, TFIIB was recruited into the PIC. In addition, recruitment of TFIIB facilitated the assembly of the remaining factors into the complex. The significance of this interaction was demonstrated through the use of mutant
proteins. VP16 mutants defective in transcription activation were unable to bind TFIIB. Likewise, TFIIB mutants defective in activated but not basal transcription were unable to bind VP16 (Roberts et al. 1993).

While an important step, recruitment of TFIID and TFIIB is not sufficient for transcription activation from all promoters. First, TFIID is constitutively bound to some promoters in vivo in the absence of transcription activation (Chen et al. 1994a; Weber and Gilmour 1995). Second, recruitment of TFIIB occurs in the presence of TBP or TFIID, however, transcription activation requires the TAFs (Choy and Green 1993). These results may be explained, in part, by the conformational changes observed in the TFIID/DNA complex in the presence of an activator (Sawadogo and Roeder 1985; Horikoshi et al. 1988a,b; Chi and Carey 1996). In the absence of an activator TFIID footprints over a small region surrounding the TATA box of the adenovirus E4 promoter. In the presence of an activator the footprint is extended to include sequences downstream. Furthermore, the extended footprint is associated with increased recruitment of additional factors into the PIC, and to increased transcription activation (Hai et al. 1988; Horikoshi et al. 1988a,b). On the ADML promoter, the extended footprint is observed in the absence of an activator (Sawadago and Roeder 1985), however, it is altered in its presence. It is possible
that this alteration is the result of a conformational change which leads to increased transcription initiation.

TFIIB also appears to undergo a conformational change in the presence of an activator. In the absence of an activator, TFIIB was found to exist in a conformation which precluded the association of TFIIF and RNA polymerase II (Roberts and Green 1994). The presence of an activator altered the TFIIB conformation such that the TFIIF and RNA Polymerase II interacting surface was exposed. Additional factors could now assemble into the complex.

Although activators are believed to function primarily at the step of PIC formation, this is certainly not the only step at which they might function. Activators may function at any step in the transcription reaction. In addition to facilitating PIC formation, activators have been shown to increase the rate of promoter clearance (Narayan et al. 1994). The mechanism by which activators function at this step is not known. It is possible that enhanced promoter clearance is due to the covalent modification of a basal factor. A prime candidate for a role in this process is TFIIH. It contains a kinase which is capable of phosphorylating the CTD of RNA polymerase II (Feaver et al. 1991; Lu et al. 1992), and phosphorylation of the CTD is believed to convert the polymerase from an initiating to an elongating form (Payne et al. 1989; Laybourn and Dahmus 1990).
Activators have also been shown to enhance processivity (Cullen 1993; Yankulov et al. 1994). Again, how this occurs is not known. However, it is possible that the interaction of an activator with a factor capable of stimulating elongation, such as TFIIF, is involved.

The ability of activators to interact with many different basal factors and to affect multiple steps in the transcription reaction seems contradictory at times. However, it may simply indicate that different mechanisms are required for the expression of different genes. In addition, it may explain synergistic activation, the phenomenon by which two or more activators enhance transcription more than the sum of each individually.

In eukaryotes, the addition of transcription activators to the basal reaction is not sufficient to obtain transcription activation. A third class of factors, called coactivators, adapters, or mediators, have been shown to be required for activated but not basal transcription in vitro. These factors are believed to function by forming a bridge between the activator and the basal transcription machinery. The best characterized coactivators to date are components of the TFIID complex (TAF\textsubscript{II}s). In addition to the TAF\textsubscript{II}s, a second group of coactivators have been identified. These factors are not tightly associated with TBP and can be grouped into two categories, general and activator specific. General
Coactivators will be discussed along with TAF$_{II}$s in the following section.

Coactivators

TAF$_{II}$s. The first indication that coactivators were required for activated transcription came with the cloning of the TBP subunit of TFIID. The cloned protein from yeast (Cavallini et al. 1989; Hahn et al. 1989; Horikoshi et al. 1989; Schmidt et al. 1989), human (Hoffman et al. 1990; Kao et al. 1990; Peterson et al. 1990), and Drosophila (Hoey et al. 1990) was found to encode a small polypeptide of 27-38kD. This was a surprising result since TFIID from mammalian and Drosophila cells fractionated as a large complex. (Nakajima et al. 1988; Dynlacht et al. 1991; Tanese et al. 1991). Even more surprising was the finding that the cloned protein could substitute for purified TFIID in a basal transcription reaction in vitro, but could not support activated transcription (Hoey et al. 1990; Hoffman et al. 1990; Peterson et al. 1990; Pugh and Tjian 1990; Smale et al. 1990). Together, these results lead to the hypothesis that there existed coactivators within the TFIID fraction which were required to form a bridge between activators and the basal transcription machinery. TFIID was subsequently purified from human and Drosophila cells and found to consist of TBP and a group of associated factors called TAF$_{II}$s
(Dynlacht et al. 1991; Tanese et al. 1991). The addition of purified TFIID to an in vitro transcription reaction restored transcription activation, indicating that the TAF_{II}s were providing the coactivator activity (Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1992, 1993; Hoey et al. 1993). Since TFIID from yeast fractionated as a single polypeptide, yeast were not believed to possess TAF_{II}s. This notion was proved wrong by the isolation of TAF_{II}s from yeast (Reese et al. 1994; Poon et al. 1995). Not only were the yeast TAF_{II}s found to be highly homologous to TAF_{II}s of higher eukaryotes (Table 1-1; Verrijzer et al. 1994; Reese et al. 1994; Poon et al. 1995; Moqtaderi et al. 1996 a,b), but they were also required for activated transcription in vitro (Reese et al. 1994; Poon et al. 1995). Most of the TAF_{II}s have since been purified and cloned. They range in size from 15kD to 250kD, and most are essential for viability (Burley and Roeder 1996).

A great deal of in vitro data has been generated supporting the role of TAF_{II}s as coactivators. Individual TAF_{II}s have been shown to interact with specific activators, as well as basal transcription factors (Burley and Roeder 1996). Furthermore, TFIID reconstitution experiments have demonstrated a requirement for particular TAF_{II}s to support activation by specific classes of activators (Chen et al. 1994b). In addition, synergistic activation has been shown to
result from the interaction of multiple activators with multiple TAF\textsubscript{n}S (Sauer et al. 1995a,b). Despite the plethora of in vitro data, clear in vivo data supporting a role for TAF\textsubscript{n}S as coactivators is lacking. This deficiency compelled our group and others to analyze the function of TAF\textsubscript{n}S in vivo. The data generated thus far both supports and contradicts the role of TAF\textsubscript{n}S as coactivators in vivo.

Tjian and colleagues have presented data supporting a coactivator function for TAF\textsubscript{n}S during Drosophila eye development. These authors found that the presence of transdominant alleles of dTAF\textsubscript{n}60 and dTAF\textsubscript{n}110 lead to reduced levels of transcription from genes under the control of the transcription activators bicoid and hunchback (Sauer et al. 1996). The mutant TAF\textsubscript{n}S were incapable of assembling into the TFIID complex, but retained their ability to bind to bicoid and hunchback, respectively. This resulted in the sequestration of the activators into non-productive complexes. Interestingly, the presence of the mutant TAF\textsubscript{n}S did not lead to a global transcriptional defect or to developmental defects in the Drosophila embryo. In contrast, genetic disruption of Spl, an activator believed to function via an interaction with dTAF\textsubscript{n}110, caused severe developmental defects in mice. Embryos in which Spl had been inactivated died at around day eleven of gestation (Marin et al. 1997).
In mammalian cells, overexpression of hTAF\textsubscript{II}28 enhanced transcription activation directed by the viral activator Tax (Caron et al. 1997). hTAF\textsubscript{II}28 overexpression also partially alleviated the squelching observed when Tax was overexpressed. hTAF\textsubscript{II}28 has been shown to interact with Tax as well as TBP. Furthermore, its ability to potentiate transcription activation correlated with the ability of Tax to interact with both of these proteins.

hTAF\textsubscript{II}28 has also been shown to potentiate ligand induced transcription activation by a number of nuclear receptors when overexpressed in cells in which it is believed to be present in limiting quantities (May et al. 1996). hTAF\textsubscript{II}28 functioned mainly with class I nuclear receptors though it was also able to enhance transcription activation by the vitamin D receptor, a class II nuclear receptor. Unlike the case with Tax, no interaction between hTAF\textsubscript{II}28 and a nuclear receptor could be detected. Transcription activation did, however, depend on the ability of hTAF\textsubscript{II}28 to interact with TBP.

Overexpression of hTAF\textsubscript{I}135 was also shown to potentiate ligand induced transcription activation by a subset of nuclear receptors \textit{in vivo} (Mengus et al. 1997). Unlike hTAF\textsubscript{II}28, hTAF\textsubscript{I}135 enhanced activation of mainly the class II nuclear receptors. hTAF\textsubscript{I}135 appeared to function, at least in part, by enhancing the ability of the receptors to recruit
TFIID to the TATA box. Experiments in which cells had been transfected with a reporter construct containing five GAL4 DNA binding sites upstream of a low affinity TATA (TGTA) box, failed to show transcription activation in the presence of a fusion protein containing the GAL4 DNA binding domain fused to various class II nuclear receptors. However, when hTAF\textsubscript{135} was overexpressed in these cells, ligand induced transcription activation was obtained in the presence of the fusion proteins. The ability of hTAF\textsubscript{135} to enhance transcription was specific for the class II nuclear receptors. Enhancement was not obtained when hTAF\textsubscript{135} was overexpressed in the presence of fusion proteins containing a number of unrelated transcription activation domains. These results demonstrate that hTAF\textsubscript{135} enhances transcription activation of only a subset of activators, although an interaction between hTAF\textsubscript{135} and an activator could not be detected. There is no known homologue for hTAF\textsubscript{135} in yeast, however, a yeast homologue has been identified for hTAF\textsubscript{28}. It will be interesting to determine if this yTAF\textsubscript{135} is required for activation by class I nuclear receptors in yeast.

While the majority of data generated in higher eukaryotes supports a role for TAF\textsubscript{135}s as bridging factors or coactivators, recent results in yeast have challenged this belief. Yeast cells lacking the function of any one of a number of TAF\textsubscript{135}s fail to display defects in global
transcription activation (Apone et al. 1996; Walker et al. 1996; Moqtaderi et al. 1996). Even when the largest subunit of the yeast TFIID complex, yTAF\textsubscript{II}145, is conditionally depleted, no global transcriptional defects are observed (Walker et al. 1996; Moqtaderi et al. 1996). This result is surprising since yTAF\textsubscript{II}145 is believed to form a scaffold which holds the TFIID complex together. Although no global transcriptional defects are observed, the inactivation of at least three of the yeast TAF\textsubscript{II}5 results in an inability to progress through the cell cycle. The loss of yTAF\textsubscript{II}145 results in a G1 arrest (Walker et al. 1996), while the loss of yTAF\textsubscript{II}90 and TSM1 result in a G2/M arrest (Apone et al. 1996; Walker et al. 1996). The cause of the cell cycle arrest in the yTAF\textsubscript{II}90 and TSM1 mutant cells has yet to be determined. In the yTAF\textsubscript{II}145 mutant cells, however, the cell-cycle arrest is believed to be due to an inability of the cells to express G1 and S phase cyclins upon TAF\textsubscript{II} inactivation (Walker et al. 1997). Transcription of these cyclin genes is required for progression through the G1 phase of the cell cycle (reviewed in Koch and Nasmyth 1994). Interestingly, replacing the upstream activating sequence of CLN2, a yTAF\textsubscript{II}145-dependent gene, with that of ADH1, a yTAF\textsubscript{II}145-independent gene, did not convert CLN2 to a yTAF\textsubscript{II}145-independent gene (Shen and Green 1997). These results indicate that the requirement for yTAF\textsubscript{II}145 was not due to the upstream activator, but rather
the core promoter. yTAF\textsubscript{145} is also required for the expression of ribosomal genes in yeast (Shen and Green 1997). The yTAF\textsubscript{145}-dependent region of these genes also mapped to the core promoter. These results further substantiate the idea that yTAF\textsubscript{145} functions not as a coactivator, but rather to facilitate core promoter activity.

Mammalian cells containing a temperature-sensitive allele of hTAF\textsubscript{250} also display gene-specific, but not global transcriptional defects at the non-permissive temperature. (Liu et al. 1985; Wang and Tjian 1994). The defects observed are similar to those observed in yeast cells containing a temperature-sensitive allele of yTAF\textsubscript{145}. At the non-permissive temperature they arrest at the G1/S boundary and are incapable of transcribing cyclin genes required for progression through this phase of the cell cycle (Wang and Tjian 1994; Suzuki-Yagawa et al. 1997). Interestingly, experiments designed to identify the elements required for hTAF\textsubscript{250}-dependence point to both the core promoter and the upstream activator (Suzuki-Yagawa et al. 1997; Wang et al. 1997).

Some of the most compelling data challenging the belief that TAF\textsubscript{17}S act as bridging factors by interacting with specific classes of activators was obtained from recent studies of yTAF\textsubscript{17}. yTAF\textsubscript{17} is the homologue of human hTAF\textsubscript{32} and Drosophila dTAF\textsubscript{140}. Both the Drosophila and human TAF\textsubscript{17}S
have been shown to interact with the acidic activator p53 in vitro (Thut et al. 1995). In support of the functional significance of this interaction neither TAFII could interact with a transcriptionally inactive form of p53. dTAFII40 has also been shown to interact with the acidic activator VP16 as well as the basal transcription factor TFIIB in vitro (Goodrich et al. 1993). Likewise, the activator-TAFII interaction appears to be significant: the addition of anti-dTAFII40 antibodies to an in vitro transcription reaction abolished transcription activation by the activation domain of VP16. Although hTAFII32/dTAFII40 is capable of interacting with acidic activators, it is incapable of interacting with the proline rich activation domain of CTF (Goodrich et al. 1993) or of potentiating transcription activation by the glutamine rich activation domain of Sp1 (Thut et al. 1995). Taken together, the data described above supports a role for hTAFII32/dTAFII40 as a coactivator required for transcription activation by acidic activators. Since yeast are believed to possess only acidic activators, the yeast homologue of hTAFII32/dTAFII40 is a prime candidate to possess coactivator activity. In fact, if the theory is correct one would expect to observe global transcriptional defects in the absence of this TAFII. Interestingly, these are not the results obtained when yeast cells were examined for their ability to activate transcription in the absence of functional yTAFII17. Yeast
cells containing a temperature-sensitive allele of \( yTAF_{17} \) displayed no global defects in transcription at the non-permissive temperature. Rather, they displayed a promoter specific defect (this thesis Chapter IV). At the non-permissive temperature cells containing a temperature-sensitive allele of \( yTAF_{17} \) were incapable of inducing transcription of the \( GAL1 \) gene in the presence of the inducer galactose, but were competent to induce the expression of the \( CUP1 \) gene in the presence of the inducer copper sulfate. As would be expected for yeast, the transcription activators required for induction of the \( GAL1 \) (GAL4) and \( CUP1 \) (ACE1) genes are both acidic activators. While this data is preliminary, it contradicts the one TAF\(_i\)/one activator theory.

The picture that appears to be emerging from these studies is that TAF\(_i\)s are not required for global transcription activation in vivo, but rather that specific TAF\(_i\)s are required for the transcription of subsets of genes. It is possible that some TAF\(_i\)s function as coactivators and other to facilitate core promoter activity. The identification of hTAF_{250} as a histone acetyltransferase (Mizzen et al. 1996) opens the possibility that it functions to facilitate TFIID binding to promoters which are inaccessible due to the presence of nucleosomes. hTAF_{250} also contains a kinase domain which is capable of
phosphorylating TFIIF (Dikstein et al. 1996). The phosphorylation of TFIIF may be important for the formation of a transcriptionally competent PIC, or for stimulating elongation, on some promoters. In addition, the ability of hTAF₁₁₀₃₁/hTAF₁₁₀₃₂, hTAF₁₁₀₈₀, hTAF₁₁₀₁₅/hTAF₁₁₀₂₀ and their Drosophila homologues to form a nucleosome like structure (Hoffmann et al. 1996; Xie et al. 1996), and the nucleosome like pattern of DNase I digestion observed on some promoters in the presence of TFIID (Roeder and Sawadogo 1985) indicate that DNA topology may play an important role in TAF₁₁₀ function. In any case, the isolation of TAF₁₁₀s from yeast (Reese et al. 1994; Poon et al. 1995) and the ease with which this organism can be genetically manipulated have created a unique opportunity to determine the physiologically relevant functions of individual TAF₁₁₀s.

Although TAF₁₁₀s are required to achieve activated transcription in some in vitro systems they are dispensable in others. They are dispensable for transcription activation in reactions using TBP, RNA polymerase II, the remaining GTFs and a subcomplex of the holoenzyme called the mediator (Kim et al. 1994).

**Mediator.** Exposure of the holoenzyme to an anti-CTD antibody liberates a complex of approximately 20 polypeptides called the mediator (Kim et al. 1994). Though the identity of many of the peptides remain to be determined, the mediator
was shown to contain SRB2, SRB4, SRB5, SRB6, TFIIF, and a
number of other proteins involved in transcription, but not
TBP or the TAF₁₁'s. In addition to supporting transcription
activation, the mediator is capable of enhancing basal
transcription in the presence of TBP, RNA polymerase II and
the missing basal factors (Kim et al. 1994). While TAF₁₁'s are
not required for activated transcription in the presence of
the mediator, they can substitute for it.

**USA.** The first *in vitro* transcription reactions used
crude fractions isolated from Hela cells which contained many
polypeptides. In an attempt to identify and analyze the role
of individual factors, these fractions were purified to
homogeneity and many of the components cloned.

Transcription reactions reconstituted with highly purified
components, however, were incapable of adequately responding
to transcription activators. Roeder's group used this
observation to purify an activity, called upstream factor
stimulatory activity (USA), which could facilitate high
levels of transcription activation in reactions using highly
purified components (Meisterernst et al. 1991). In the
presence of USA and an activator, basal transcription was
repressed, while activated transcription was stimulated,
resulting in a large net increase in the level of
transcription activation. Partial purification of USA
identified repressive components referred to as NC's and
stimulatory components called PC's. The repressive activity of USA could function with TBP, but the stimulatory activity required TBP and the TAFₙₛ. Analysis of NC1 showed that it functioned by binding to TBP and inhibiting the entry of additional factors into the PIC (Meisterernst et al. 1991). This activity could be inhibited by TFIIA. Interestingly, the positive factors which have been identified encode proteins which are capable of binding to DNA. PC3 encodes topoisomerase I (Kretzschmar et al. 1993), and PC4 is homologous to a mouse single stranded DNA binding protein (Ge and Roeder 1994). How these factors facilitate activated transcription is not known, however, PC4 has been shown to interact with the acidic activator VP16 as well as TFIIA, and may be required to form a bridge between the basal transcription machinery and the activator. The remaining factors must be purified and cloned before a clear picture of how USA functions can be obtained. However, it appears that the presence of USA in an in vitro transcription reaction more closely recapitulates that which is seen in vivo. In vivo, basal transcription is normally repressed, while the presence of an activator induces high levels of transcription. It will be important to understand the mechanism by which USA functions.

Chromatin
In the cell DNA is found associated with histones and nonhistone proteins in a highly compact structure called chromatin (reviewed in Paranjape et al. 1994). While the packaging of DNA into chromatin ensures that the genome fits into the small space allotted to it in the nucleus, it is repressive to transcription (reviewed in Adams and Workman 1993). In order for a gene to be activated, the chromatin must first be altered to allow transcription factors access to the DNA. How this process, known as derepression, is achieved or regulated is not known. However, the recent isolation of a number of complexes which are capable of altering chromatin structure, in an ATP-dependent manner, have shed new light on this process (reviewed in Kingston et al. 1996; Burns and Peterson 1997a). The most extensively studied chromatin remodeling complex to date is the SWI/SNF complex.

**SWI/SNF.** SWI/SNF is a large multisubunit complex composed of 11 subunits which have been highly conserved throughout evolution (reviewed in Peterson and Tamkun 1995; Kingston et al. 1996; Burns and Peterson 1997a). A SWI/SNF complex has been isolated from yeast (Cairns et al. 1994; Cote et al. 1994) and human (Kwon et al. 1994), and factors which are highly homologous to SWI/SNF components have been identified in other chromatin remodeling complexes (Tsukiyama et al. 1995; Cairns et al. 1996; Varga-Weisz et al. 1997). The first indication that SWI/SNF may function by altering
chromatin structure came from genetic studies of swi/snf mutants in Saccharomyces cerevisiae. Cells in which components of the SWI/SNF complex had been deleted were unable to express a number of highly inducible genes including HO and SUC2 (Stern et al. 1984; Neigeborn and Carlson 1994; Peterson and Herskowitz 1992; Kingston et al. 1996). Mutations in genes encoding the chromatin components histone H3, H4, H2A, H2B and the HMG-1 like protein SIN1 were found to alleviate the transcriptional defects in these cells (Kruger and Herskowitz 1991; Hirschhorn et al. 1992; Kruger et al. 1995). Further indications that SWI/SNF functioned by altering chromatin, and a glimpse into its role in transcription activation, was obtained from biochemical studies. Purified SWI/SNF was shown to alter nucleosomonal structure in an ATP-dependent manner (Cote et al. 1994; Imbalzano et al. 1994; Kwon et al. 1994). In addition, purified SWI/SNF was shown to facilitate the binding of an activator (Cote et al. 1994; Kwon et al. 1994), or the GTF TBP (Imbalzano et al. 1994) to their DNA binding sites when these sites were present in nucleosomes. Recent data from the Peterson laboratory indicates that SWI/SNF functions similarly in vivo. SWI/SNF was shown to be required for the activator GAL4 to bind to and activate transcription from two low affinity binding sites when they were encompassed in nucleosomes (Burns and Peterson 1997b). If they were made
nucleosome-free by the insertion of a nucleosome positioning element, however, SWI/SNF was no longer required.

SWI/SNF is not required for transcription of all genes. Exactly how it is targeted to particular promoters is not understood. It is possible that SWI/SNF is targeted via an association with the holoenzyme (Wilson et al. 1996). Previous work has shown that mutations in chromatin components which suppressed defects in SWI/SNF mutants also suppressed defects caused by CTD truncations (Peterson et al. 1991). There is also evidence that SWI/SNF functions in concert with the ADA/GCN5 complex, a known histone acetyltransferase (Pollard and Peterson 1997). Possibly SWI/SNF is targeted via its association with this complex.

The intricacies as to how SWI/SNF functions remain to be determined. However, the identification of the SWI/SNF complex as well as a number of related complexes from yeast and higher eukaryotes has opened the door for identifying how the repressive effects of chromatin are overcome to allow gene specific transcription activation. It will be important to keep these advances in mind when reading this thesis.
CONTRIBUTIONS

During my graduate career I was a member of a small group within the laboratory working on yeast TAF$_{II}$s. The work performed by each member of the team was relevant to, and greatly influenced that, of the others. In order to present my data in a way that reflects this interplay, and it's relevance to the field of transcription, I have included some of the work of my collaborators in my thesis. At this time I would like to describe these contributions.

In chapter two, I analyze the in vivo function of yTAF$_{II}$90. I am responsible for the cloning, genetic disruption and generation of conditional alleles, of yTAF$_{II}$90. I am also responsible for the analysis of transcription and cell-cycle progression in strains containing conditional alleles of yTAF$_{II}$90. Joseph Reese is responsible for the immunoprecipitation and immobilized DNA template analysis of yTAF$_{II}$90 and Amy Virbasius for the analysis of LexA-yTAF$_{II}$90 fusion proteins. In addition, Stephen Doxsey is responsible for light micrographs and Rose Tam for immunofluorescence.

In chapter three, the analysis of transcription in cells lacking TAF$_{II}$s is examined. In this chapter I am responsible for the analysis of transcription in strains containing conditional alleles of yTAF$_{II}$90 and tsml. I am also responsible for the cell-cycle analysis of strains containing
temperature-sensitive alleles of tsml. Scott Walker is responsible for the transcription and cell-cycle analysis of strains containing conditional alleles of yTAF1145 and Joseph Reese for the analysis of transcription in cells containing conditional alleles of yTAF68, yTAF47, and in strains in which yTAF30 has been deleted.

In chapter four, I analyze the in vivo function of yTAF17. I am responsible for the cloning, and genetic disruption of yTAF17. I am also responsible for the analysis of transcription in cells containing a temperature-sensitive allele of yTAF17. Under my guidance, Jing Wang and Lijian Chiang are responsible for the isolation of temperature-sensitive alleles of yTAF17.

In chapter five I am responsible for the generation of yTAF90 temperature-sensitive strains containing temperature-sensitive alleles of CDC28 and those in which RAD9 has been genetically deleted. In addition, I am responsible for all work presented in appendix A: namely the subtractive hybridization performed on strains containing wild-type and temperature-sensitive alleles of yTAF90.

I would like to thank each of these individuals for their generous contributions.
CHAPTER II

Yeast TAF₉₀ is required for cell cycle progression through G2/M but not for general transcription activation
In 1994 a TFIID complex composed of TBP and a number of TAF$_{11}$s was isolated from the yeast *Saccharomyces cerevisiae* (Reese et al. 1994). Not only could the yeast TFIID complex support activated transcription *in vitro*, but it also contained homologues of higher eukaryotic TAF$_{11}$s. At this time, a great deal of data had been generated regarding the function of the TFIID complex *in vitro*. The analysis of individual subunits, however, had been hampered by their tight association in the complex, as well as the difficulty of performing genetics in higher eukaryotes.

The isolation of a TFIID complex from yeast created an enormous opportunity to study the function of individual TAF$_{11}$s *in vivo*. In addition, the high degree of conservation between the transcription machinery in yeast and higher eukaryotes (Struhl 1989) indicated that the data generated in the yeast system would be applicable to higher eukaryotes.

The first yTAF$_{11}$ identified by our group was yTAF$_{11}$$^{90}$. yTAF$_{11}$$^{90}$ has been highly conserved throughout evolution and is the homologue of hTAF$_{11}$$^{100}$ and dTAF$_{11}$$^{80}$ (Burley and Roeder 1996). In this chapter we describe the *in vivo* analysis of yTAF$_{11}$$^{90}$. Interestingly, and unexpectedly, yTAF$_{11}$$^{90}$ is not required for global transcription activation. It is, however, required for progression through the G2/M phase of the cell cycle.
Results

The in vitro analysis of TFIID indicates that it plays an essential role in transcription activation. We therefore, wanted to determine if individual subunits of the complex, yTAF\textsubscript{II}90 in particular, were required for cell growth. In order to answer this question we generated a construct containing a disrupted copy of TAF90 (Fig. 2-1A). This construct was then used to create diploid strains of yeast which contained one wild-type and one disrupted copy of the gene. Disruptions were confirmed by Southern blotting (Fig. 2-1B). Tetrads generated by sporulation of the diploid strains were dissected and the resulting spores analyzed for viability. In no case was more than two viable spores obtained from a single tetrad (Fig 2-1C). In addition, no viable spore contained the HIS3 disruption marker (data not shown). These results indicate that yTAF\textsubscript{II}90 is essential for cell viability.

To determine the cellular defects associated with the loss of its function, we constructed and isolated two temperature-sensitive TAF90 alleles. The plasmid shuffle technique (Boeke et al. 1984) was then used to create strains of yeast bearing only a mutant TAF90 allele. Figure 2-2A shows that both yTAF\textsubscript{II}90 temperature-sensitive strains grew at 25°C but not 37°C. Figure 2-2B shows that in liquid culture
Figure 2-1. yTAF1190 is essential for viability.

(A) Scheme used to disrupt yTAF90 in S. cerevisiae. A segment of yTAF90 (BglII-BamHI) was replaced with a larger fragment containing the HIS3 gene.
(B) The disruption of yTAF90 is confirmed by Southern blotting. DNA isolated from wild-type (WT) cells, or cells transformed with the disruption construct (HIS+), were used for Southern blotting. The appearance of two additional bands confirms the proper disruption.
(C) Sporulation and dissection of diploid strains containing one disrupted and one wild-type copy of yTAF90 results in two viable spores per tetrad.
A

25°C

WT

ts2-1

ts3-1

37°C

WT

ts2-1

ts3-1

B

\[ \text{OD}_{600} \]

\[ \text{TIME (hrs)} \]

Shift

C

<table>
<thead>
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<th>ts2-1</th>
<th>ts3-1</th>
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<tr>
<td>yTAF\textsubscript{1}50 678</td>
<td>WSLGKRLKQHRSKSNAIYSLSEYREIYLSKQTVMD</td>
</tr>
<tr>
<td>dTAF\textsubscript{1}50 654</td>
<td>WLSMGSLVTLLRTSTVTITFSGTVLAAAGLDNHMLWD</td>
</tr>
<tr>
<td>\textbf{722} LKARTTEPSARPEPPDGFLQYGTASIMQDGRTVMGSTD</td>
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<tr>
<td>\textbf{768} LVASPTKKTVPVKFSRSNH8ALAGFSP</td>
<td>798</td>
</tr>
<tr>
<td>\textbf{674} LMSTFPPPSPFPYSLHFTRRNLIMCVGLFKS</td>
<td>704</td>
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Figure 2-2. Temperature-sensitive alleles of yTAF\textsubscript{90}.

(A) Growth of strains containing wild-type (LY3) and temperature-sensitive alleles of yTAF\textsubscript{90} (ts2-1 and ts3-1) at 25°C and 37°C.
(B) Growth curve of wild-type and yTAF\textsubscript{90} temperature-sensitive strains at 37°C.
(C) Sequence of ytaf\textsubscript{90} temperature-sensitive alleles. The segment of the yTAF\textsubscript{90} WD40 domain containing the temperature-sensitive mutations is compared with the corresponding sequence in the Drosophila homologue dTAF\textsubscript{80}. Mutated residues are marked with a dot. The amino acid changes and the strains containing the mutations are indicated above.
these strains displayed a rapid growth-arrest upon transfer from the permissive to the non-permissive temperature. Even at the permissive temperature the \( \gamma TAF_{190} \) mutant strains grew more slowly than wild-type (data not shown).

Sequencing of the mutants identified double-point substitutions in each of the two alleles. \( \gamma TAF_{190ts2-1} \) (strain LY20) contains an asparagine substituted for a serine at amino acid 703 and an arginine substituted for a glycine at position 793; \( \gamma TAF_{190ts3-1} \) (strain LY21) contains a glutamic acid and a serine substituted for glycines at positions 713 and 714, respectively (Figure 2-2C). Significantly, all of the mutations are within the highly conserved WD40 domain.

To gain insight into the nature of the temperature-sensitive growth defect, the cells were analyzed for morphological differences at the non-permissive temperature. Within four hours after shifting to 37°C there was a striking difference in the appearance of the wild-type and mutant strains. Cells from both mutant strains were twice as large as wild-type, and consisted of mostly large budded cells (Figure 2-3A). Approximately 62% of ts2-1 and 84% of ts3-1 cells contained large buds, whereas only 20% of the wild-type strain possessed large buds (Figure 2-3B).

Cells arresting with large buds could be blocked in \( S \), G2 or M phase (Pringle and Hartwell 1981). To distinguish
Figure 2-3. Cell cycle phenotype of strains bearing temperature-sensitive alleles of yTAF90.

(A) Nomarski optics or Hoechst staining was used to visualize the morphology and DNA, respectively, of wild-type and strains harboring temperature-sensitive yTAF90 mutants 4 hr after transfer from 25°C to 37°C.
(B) Percentage of unbudded, small, medium and large budded cells after 4 hrs at 37°C. (Light bars) Wild-type; (medium bars) ts2-1; (dark bars) ts3-1.
(C). FACS analysis of strains containing wild-type and temperature-sensitive alleles of yTAF90 immediately before and 4 hrs after transfer from 25°C to 37°C.
between these possibilities, we performed Hoechst staining and FACS analysis. Hoechst staining performed on cells incubated for four hours at 37°C identified a single nucleus in the mutant cells localized near the neck of the bud (Figure 2-3A). The FACS analysis of Figure 2-3C shows that following incubation for four hours at 37°C the majority of wild-type cells contained a 1N content of DNA, while the majority of ts2-1 cells and virtually all of the ts3-1 cells contained a 2N DNA content. On the basis of these combined data we conclude that the mutant cells are capable of replicating their DNA and of nuclear migration to the neck of the bud, consistent with a G2/M block.

Even at the permissive temperature it was evident that a much greater than normal percentage of mutant cells had a 2N DNA content. The inability of these cells to efficiently progress through G2 helps explain their slow growth phenotype and in addition indicates that yTAFII90 function is impaired even under permissive conditions. Lack of viability at higher temperatures could be explained either by a complete loss of yTAFII90 function, or an inability of unhealthy cells to survive additional stress.

In S. cerevisiae there are several phases of the cell cycle at which transcription is required for proper progression (Koch and Nasmyth 1994). Therefore, it seemed unlikely that a general transcription defect would result in
a specific cell cycle phenotype. To verify this notion we inhibited RNA polymerase II-directed transcription by several strategies and analyzed the effect on cell cycle progression. As shown in Figure 2-4A, when transcription was inhibited using RNA polymerase II (Nonet et al. 1987a), TBP (Cormack and Struhl 1992) or SRB2 (Koleske et al. 1992) temperature-sensitive strains, or with the RNA polymerase II chemical inhibitor thiolutin (Parker et al. 1991), the cells did not uniformly arrest at a specific stage of the cell cycle. In several instances there was a modest G1 shift, probably reflecting the elaborate transcriptional program required to traverse G1 (Koleske et al. 1992; Rowley et al. 1993). Thus, a specific cell cycle phenotype cannot be explained by a general defect in RNA polymerase II-directed transcription.

The experiment of Figure 2-4A involved mutant GTFs, which are completely defective in supporting RNA polymerase II-directed transcription. To ask whether interfering specifically with transcription activation affected cell cycle progression, we analyzed several previously described TBP mutants that are unable to support activated transcription of some or all genes (Arndt et al. 1995; Lee and Struhl 1995). The FACS analysis of Figure 2-4B shows that cells harboring three such TBP mutants did not arrest at a specific stage of the cell cycle. Thus, the cell cycle phenotype of yTAFII90 mutants is also not readily explained by
Figure 2-4. Inhibition of RNA polymerase II-directed transcription does not lead to a cell cycle phenotype.

(A) FACS analysis was performed on strains before and 4 hrs after transfer from 25°C to 37°C, or 4 hrs after addition of thiolutin to wild-type strain CY245.
(B) FACS analysis was performed on strains harboring activation-defective TBP mutants grown to mid-log phase at 30°C.
an activation-specific defect in RNA polymerase II-directed transcription.

Numerous experiments have shown that in vitro TAF\textsubscript{II}s are required for activated, but not basal, transcription (Burley and Roeder 1996). We therefore sought to determine whether cells bearing the yTAF\textsubscript{II}90 temperature-sensitive mutants were responsive to activators. We first chose to analyze the well characterized acidic activator GAL4 according to the experimental strategy outlined in Figure 2-5A. As expected, Figure 2-5B shows that transcription of the GAL10 gene was induced in all strains under permissive conditions (25\textdegree C). Under non-permissive conditions (37\textdegree C), the wild-type (LY3), but not the RNA polymerase II mutant strain (rpb1-1), supported GAL10 transcription. Significantly, in both yTAF\textsubscript{II}90 temperature-sensitive strains (ts2-1 and ts3-1) GAL10 was transcriptionally activated at 37\textdegree C. We note that in strain ts3-1 there was an ~50% decrease of GAL10 transcription four hours following the temperature-shift, a time at which the cells had been arrested for at least three hours. This modest effect most likely reflects a general deterioration of all activities in dying cells. In particular, RNA polymerase III-directed transcription also undergoes a comparable decrease at this time point (data not shown).

We next asked whether yTAF\textsubscript{II}90 was required for the function of another, unrelated activator. The acidic
Figure 2-5. Transcription in strains bearing ytafr190 temperature-sensitive mutants.

(A) Experimental design.
(B) Transcriptional activation of GAL10 was measured by primer-extension analysis in cells 60 minutes after induction by 3% galactose.
(C) Transcription activation of CUP1 was measured by S1 nuclease analysis in cells 30 minutes after induction by 100 uM copper sulfate.
(D) Transcription of indicated endogenous genes was measured by S1 nuclease analysis before and at the indicated times after transfer from 25°C to 37°C.
activator ACE1 induces transcription of the CUP1 gene in the presence of copper (Butt et al. 1984; Furst et al. 1988). The experimental design was analogous to the GAL10 experiment except that the cells were grown in glucose and the inducer was 100μm copper sulfate (see Figure 2-5A). The results of Figure 2-5C show that all strains supported copper-inducible transcriptional activation of the CUP1 gene at 25°C. At 37°C, only the rpb1-1 strain failed to support normal CUP1 transcription.

Figure 2-5D analyzes four other endogenous genes whose transcription is controlled by a diverse set of activators: PMA1 is regulated by the activators MCM1 and RAP1 (Capieaux et al. 1989; Kuo & Grayhack 1994); TRP3 is regulated by the activator GCN4 (Aebi et al. 1984; Hope and Struhl 1985; Arndt and Fink 1986); DED1 is under the control of the activator ABF1 (Buchman and Kornberg 1990); and ENO2 is glucose-regulatable (Johnston and Carlson 1992). Once again, transcription of these genes was unaffected following inactivation of yTAFII90. We conclude that in vivo yTAFII90 is dispensable for normal transcription of many yeast genes.

Immunoblot analysis of strains bearing the yTAFII90 temperature-sensitive mutants revealed that at the non-permissive temperature these strains contained significant levels of yTAFII90 as well as several other yTAFIIIs (data not shown). Therefore, a possible explanation for the lack of a
transcriptional defect in the above experiments was that yTAF_{II}90 contained multiple functional domains and the mutations were within a domain not required for transcription of those genes tested. To rule out this possibility, we constructed a strain in which yTAF_{II}90 could be conditionally depleted. In this strain (LYC-1) TAF90 was under the control of the galactose-inducible GAL1 promoter. The immunoblot of Figure 2-6A shows that upon transfer from galactose- to glucose-containing medium, yTAF_{II}90 was rapidly depleted, and by 12 hours was undetectable. The quantitative immunoblot analysis of Figure 2-6B shows that by 12 hours yTAF_{II}90 was below our level of detection, which was 2% of the wild-type level. As expected, LYC-1 cells grew in the presence of galactose but not glucose (Figure 2-6C) and upon transfer to glucose-containing medium, the LYC-1 cells displayed a rapid growth arrest (Figure 2-6D).

We next analyzed the effect of yTAF_{II}90 depletion on cell cycle progression and transcription. Analogous to the results with the temperature-sensitive mutants, cells depleted of yTAF_{II}90 arrested with large buds (Figure 2-7A). After incubation in glucose for 12 hours, 80% of strain LYC-1 consisted of budded cells, 74% of which contained large buds. By comparison, the wild-type strain possessed only 47% budded cells, only 18% of which contained large buds (Figure 2-7B).
A

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<th></th>
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yTAF1190

yTAF130

B

C

LYC-1

WT

LYC-1

WT

GALACTOSE

GLUCOSE

D

CELL NUMBER X 10^7

- - WILDTYPE
- - LYS-1

TIME (hrs)
Figure 2-6. Characterization of strains conditionally expressing yTAF$_{II}$90.

(A) Immunoblot analysis of whole-cell extracts prepared from wild-type (LY3) and LYC-1 (GAL1-HA-yTAF$_{II}$90) at the indicated times following transfer from galactose- to glucose-containing medium.

(B) Quantitative immunoblot analysis. Extracts prepared from the wild-type strain (right) were undiluted (8 and 12 hr) or diluted 1:20 (0hr) following transfer from galactose- to glucose-containing medium. HA-tagged yTAF$_{II}$90, expressed from the GAL1 promoter, was detected by immunoblotting. The proteins are indicated by arrows.

(C) Growth of wild-type and LYC-1 on plates containing galactose or glucose at 30°C.

(D) Growth curve of wild-type and LYC-1 before and after transfer from galactose- to glucose- containing medium.
Figure 2-7. Cell cycle and transcriptional properties of strains conditionally expressing yTAFII90.

(A) Wild-type and LYC-1 cells were visualized by light microscopy 12 hr after transfer from galactose- to glucose-containing medium.
(B) Percentage of unbudded, small, medium, and large budded cells was determined for wild-type (light bars) and LYC-1 (dark bars) immediately before and 12 hrs after transfer from galactose- to glucose-containing medium.
(C) Transcription was measured by S1 nuclease analysis of RNA isolated from wild-type and LYC-1 cells after transfer from galactose- to glucose-containing medium.
Transcription was determined by S1 nuclease analysis of RNA isolated following transfer from galactose- to glucose-containing medium. The half-life of each of these mRNAs is less than thirty minutes (Cormack and Struhl 1992; Thompson and Young 1995; see also Figure 2-5D). As mentioned above, transcription of PMA1 is regulated by the activators MCM1 and RAP1 (Capieaux et al. 1989; Kuo and Grayhack 1994), transcription of ENO2 is glucose-regulatable and involves the activator ABF1 (Johnston and Carlson 1992), whereas transcriptional regulation of RAD23 is not well understood. Figure 2-7C shows that following yTAFII90 depletion, each gene tested was transcribed at wild-type levels (lanes 6-10) even at 12 hours, a time at which there was no detectable yTAFII90 (Figures 2-6A and B). In some cases, a small transcriptional decrease was observed 14 hours after transfer, a time at which the cells had been arrested for approximately six hours. At these late times, RNA polymerase I- and III-directed transcription was also modestly impaired. Thus, this small decrease in transcription reflects a general deterioration of function in dying cells, and not a specific defect in RNA polymerase II-directed transcription.

The results described above raised the possibility that yTAFII90 might be required not for transcription in general but rather for the transcription of a subset of genes involved in progression through the G2/M phase of the cell
cycle. As a first test of this possibility, we analyzed the transcription of two key genes required for progression through G2/M. CLB2 encodes a cyclin involved in G2/M progression and is transcribed only during G2/M (Surana et al. 1991). CSE4 encodes a protein required for proper chromosome segregation and significantly the phenotype of cells bearing CSE4 mutants is identical to that of the yTAFII90 mutant strains described here (Stoler et al. 1995). The Northern blot of Figure 2-8 shows that transcription of CLB2 and CSE4, and as a control, CLN3, which encodes a constitutively expressed G1 cyclin (Nash et al. 1988), was unaffected following temperature-sensitive inactivation of yTAFII90. Thus, failure to transcribe the CLB2 and CSE4 genes cannot explain the cell cycle arrest phenotype of strains harboring yTAFII90 mutants.

A possible explanation for the lack of a transcriptional effect in the above experiments was that yTAFII90 was not a component of an RNA polymerase II PIC. To address this possibility we performed both biochemical and in vivo transcription experiments. First, we asked whether yTAFII90 was associated with TBP and other TAFIIIs. The immunoprecipitation experiment of Figure 2-9A shows that an α-yTAFII90 antiserum immunoprecipitated TBP and yTAFII145, the yTAFII that directly contacts TBP (Reese et al. 1994). Likewise, an α-yTAFII145 antiserum immunoprecipitated both
<table>
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<th>WT</th>
<th>rpb1-1</th>
<th>ts2-1</th>
<th>ts3-1</th>
</tr>
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</table>

- **CLB2**
- **CSE4**
- **CLN3**
- **ADH1**
Figure 2-8. Transcription of genes required for progression through G2/M following \( \gamma \text{TAF}_{190} \) inactivation. Transcription of the indicated genes was measured by Northern blot analysis.
A

RNA POL II (LRG SUBUNIT)

yTAF

yTAF145

yTAF90

yTBP

B

PGEM3

G5CAT

RNA POL II (LRG SUBUNIT)

TFIIB

yTAF190

TBP

C

β-Galactoside activity

WT Vector 1-491 492-798
Figure 2-9. yTAFII90 is a component of the PIC and can activate transcription as a LexA fusion protein.

(A) Immunoprecipitation analysis. Immunoprecipitates formed with α-yTAFII90 or α-yTAFII145 antisera were analyzed by immunoblotting for the presence of TBP, yTAFII90, and yTAFII145.

(B) Immobilized DNA template assay. Promoter (G5E4T) or control (pGEM3) DNA was coupled to Dynal streptavidin beads, and incubated in a yeast whole-cell extract under standard transcription conditions. Factors associated stably were detected by immunoblot analysis.

(C) Transcription activation by LexA-yTAFII90 fusion proteins. Wild-type strains containing an integrated lacZ reporter with two LexA binding sites upstream of the GAL1 TATA box and the DNA binding domain of LexA alone fused to full-length yTAFII90 or amino-terminal (1-491) or carboxy-terminal (492-798) portions of yTAFII90 were analyzed for β-galactosidase activity.

(D) LexA-yTAFII90 fusion proteins containing wild-type or temperature-sensitive alleles of yTAFII90 attached to the DNA binding domain of LexA were transformed into strain EGY48. Strain EGY48 contains a single chromosomal LEU2 gene whose expression is controlled by three LexA-binding sites. Transformants were streaked on selective medium lacking leucine and tested for growth at 25°C to 37°C.

(E) Immunoblot analysis using an α-LexA antibody was performed on extracts prepared from strains harboring the various LexA-yTAFII90 fusion proteins. The proteins are indicated by arrows.
TBP and yTAF_{II}90. Thus, in a yeast whole-cell extract yTAF_{II}90 is associated with TBP and other yTAF_{II}s, as expected for a component of the TFIID complex.

Second, and more importantly, we obtained direct evidence that yTAF_{II}90 was a component of an RNA polymerase II PIC formed on a class II promoter. We have previously described methods for quantitating transcription factors that are stable components of PICs using "immobilized" DNA templates (Choy and Green, 1993). Figure 2-9B uses immunoblotting to analyse the presence of several GTFs, RNA polymerase II, and yTAF_{II}90 in PICs formed under standard transcription conditions in a yeast whole-cell extract. As expected, and consistent with previous results (Choy and Green 1993; Kim et al. 1994), TBP, TFIIB, and RNA polymerase II were stably associated with a DNA fragment containing a class II promoter (G5E4T) but not an irrelevant DNA (pGEM3). Significantly, yTAF_{II}90 was also stably and specifically associated with the promoter, indicating that yTAF_{II}90 is a component of the PIC.

Recent experiments have shown that PIC components, such as TBP and GAL11, can activate transcription when tethered to the promoter via a heterologous DNA binding domain (Chatterjee and Struhl 1995; Klages and Strubin 1995; Xiao et al. 1995; Barberis et al. 1995). We used a similar strategy to determine whether in vivo yTAF_{II}90 entered into a
transcriptionally competent PIC. Fusion-proteins containing the DNA binding domain of LexA attached to yTAFII90 were assayed for their ability to activate transcription from an integrated reporter bearing LexA binding sites upstream of the GAL1 TATA box and the LacZ gene. Figure 2-9C shows that a LexA-yTAFII90 fusion-protein activated transcription, whereas the LexA DNA binding domain alone (vector) did not.

It was important to rule out the possibility that yTAFII90 contained a "cryptic" activation domain, which in yeast are short, acidic sequences (Ma and Ptashne 1987; Ptashne 1988). We therefore constructed LexA fusion-proteins containing an N-terminal (1-491) or C-terminal (492-798) fragment of yTAFII90. Significantly, neither of these yTAFII90 fragments had yTAFII90 function (data not shown). Figure 2-9C shows that whereas the LexA fusion of full-length yTAFII90 activated transcription, the N- and C-terminal derivatives did not. These results strongly suggest that activated transcription by LexA-yTAFII90 was not due to a cryptic activation domain in yTAFII90.

The results of Figure 2-9D provide additional support for this conclusion. LexA-yTAFII90ts2-1 and LexA-yTAFII90ts3-1 fusion-proteins were constructed and tested for their ability to activate transcription from a promoter bearing LexA binding sites upstream of the GAL1 TATA box and LEU2 gene. The ability to support growth on selective media provided a
sensitive assay for transcriptional activation. The results of this experiment demonstrate that when fused to the DNA binding domain of LexA, the temperature-sensitive alleles of TAF90 supported growth on leucine-deficient media at 25°C but not 37°C. Therefore, these yTAFII90 mutants enter into a transcriptionally competent PIC at the permissive but not the non-permissive temperature. The immunoblot analysis of Figure 2-9E indicates that all of the LexA-yTAFII90 fusion-proteins described above were expressed at comparable levels. Taken together, these results indicate that in vivo yTAFII90 is a component of a PIC and further suggest that the molecular defect of the yTAFII90 mutants is failure to engage in a protein-protein interaction(s) at the non-permissive temperature.

Discussion

Although extensive in vitro data has demonstrated a requirement for TAFII's in transcription activation, their function(s) in vivo has not been critically evaluated. Here we report that cells in which a highly conserved and essential TAFII, yTAFII90, was inactivated by either temperature-sensitive mutations or conditional depletion, had no general defect in RNA polymerase II-directed transcription. We estimate from immunoblotting that yeast cells normally contain 3-6,000 copies of yTAFII90, which was
decreased greater than 98% by glucose-depletion (Figure 2-6B and data not shown). The maximal 120 copies of yTAFII90 remaining is far below the ~4,000 actively transcribed genes in a yeast cell (see Lewin 1990). Whereas our experiments do not rule out that yTAFII90 is required for transcription of a particular subset of genes, they do indicate a lack of a general requirement for transcription activation. Consistent with our in vivo results are in vitro experiments demonstrating that partial TFIID complexes lacking TAFII90 can support transcription directed by several types of activators (Chen et al. 1994b; Sauer et al. 1995a, b).

Our results are also not inconsistent with the limited in vivo analysis of other TAFII in higher eukaryotes. For example, mammalian cell lines harboring a temperature-sensitive TAFII250 allele, do not have a global defect in RNA polymerase II-directed transcription under non-permissive conditions (see, for example, Liu et al. 1985). Furthermore, inactivation of TAFII250 did not prevent transcriptional activation of the c-Fos gene, which contains a highly inducible and well characterized promoter (Wang and Tjian, 1994).

The results described in this study point to a role for yTAFII90 at a specific stage of the cell cycle. Significantly, an identical cell cycle phenotype was observed when yTAFII90 was inactivated by two independent strategies:
temperature-sensitive inactivation or conditional depletion. We emphasize that the cell cycle phenotype resulting from $\gamma$TAF$_{II}^{90}$ inactivation is not typical of a transcription defect. Blockage of transcription by exposure to a chemical inhibitor, or through the use of temperature-sensitive alleles of RNA polymerase II, TBP, or SRB2 did not lead to a uniform cell cycle arrest. Most significantly, strains harboring activation-defective TBP mutants also failed to display a cell cycle phenotype. Interestingly, mammalian cell lines expressing a temperature-sensitive allele of TAF$_{II}^{250}$ also displayed a cell cycle phenotype at the non-permissive temperature, although in this instance the arrest was in G1 (Sekiguchi et al. 1988; Sekiguchi et al. 1991; Hisatake et al. 1993; Ruppert et al. 1993). Taken together, these results raise the intriguing possibility that in both yeast and mammalian cells TAF$_{II}$s may have distinct and essential functions in cell cycle progression, and that different TAF$_{II}$s may act during different stages of the cell cycle.

It will be important to determine how TAF$_{II}$s facilitate cell cycle progression. In yeast, cell cycle progression requires the temporally regulated transcription of particular genes (reviewed in Koch and Nasmyth, 1994). An intriguing possibility is that $\gamma$TAF$_{II}$s are required not for transcription in general, but rather for transcription of specific genes involved in cell cycle progression. Although we have analyzed
the ability of yTAF_{II}90 mutant cells to transcribe two important genes required for G2/M progression, our analysis is far from exhaustive. Thus, it still remains possible that yTAF_{II}90 is required for transcription of one or more genes required for progression through G2/M and experiments to test this idea are in progress.

However, it remains possible that yTAF_{II}90 does not act directly in transcription activation. For example, a Drosophila TAF_{II}, dTAF_{II}250, has been recently reported to have a protein kinase activity (Dikstein et al. 1996). This or other biochemical activities could serve to coordinate transcription with cell division or other cellular processes. Thus, yTAF_{II}90 and other TAF_{II}s may function in the cell cycle by a mechanism that does not operate directly through a transcription pathway.

An additional suggestion that yTAF_{II}90 may be performing a function other than as a coactivator is that the phenotype of yeast strains unable to support activated transcription differ substantially from that of the TAF90 mutant strains described here. Activation-defective TBP mutant strains are viable, do not support activated transcription of several genes tested (Kim et al. 1994; Arndt et al. 1995; Lee and Struhl 1995; Stargell and Struhl 1995), and do not have a cell cycle phenotype (Figure 2-4B); conversely, the TAF90 mutant strains are inviable, support activated transcription
of all genes tested, and specifically arrest in G2/M. Collectively, these observations raise the possibility that yTAF_{II}90 may function other than as a general coactivator.

yTAF_{II}90 activates transcription when tethered to the promoter through a LexA DNA binding domain. The portions of yTAF_{II}90 required to support activated transcription in this assay are identical to those required for yTAF_{II}90 function. In particular, substitutions in the WD40 repeats, presumptive protein-protein interaction sites, affected yTAF_{II}90 activity and LexA-yTAF_{II}90 activity comparably. We interpret these results to mean that to activate transcription as a LexA fusion-protein, yTAF_{II}90 engages in the same interactions with other PIC components as it does when it functions as yTAF_{II}90.

Previous studies have shown that both TBP and GAL11, a component of the holoenzyme, can activate transcription in analogous tethering experiments (Chatterjee and Struhl 1995; Klages and Strubin 1995; Xiao et al. 1995; Barberis et al. 1995). Thus, TBP, a GTF; yTAF_{II}90, a component of the TAF_{II} complex; GAL11, a component of the holoenzyme; and TFIIB (our unpublished data) can all activate transcription when fused to a DNA binding domain. Thus, appropriate tethering of diverse PIC components can activate transcription, presumably by nucleating formation of a PIC on the promoter. Based solely on these types of experiments, it is difficult to draw strong
conclusions about the target of an activator or the step(s) an activator affects.
CHAPTER III

Transcription activation in cells lacking TAFs
In vitro, the TFIID complex is required for activator function. Results presented in the previous chapter, however, indicate that not all TAF\textsubscript{II}s are required for transcription activation in vivo.

The TFIID complex is composed of 8-12 TAF\textsubscript{II}s. Therefore, while yTAF\textsubscript{II}90 did not possess coactivator activity in vivo, it is possible that one or more of the remaining TAF\textsubscript{II}s did. This possibility compelled us to analyze the ability of yeast cells to activate transcription in the absence of a number of additional TAF\textsubscript{II}s. In this chapter we present the results of our analysis. Again we were surprised to discover that yeast cells were capable of transcription activation in the absence of any one of a number of TAF\textsubscript{II}s, including TAF\textsubscript{II}145, which is believed to be a structurally important component of the complex. However, while the cells were competent to activate transcription, inactivation of TAF\textsubscript{II}145 or TSM1 resulted in an inability to progress through the cell cycle.

Results

The TBP-associated factor yTAF\textsubscript{II}145 is the homologue of higher eukaryotic TAF\textsubscript{II}250, and is the only yTAF\textsubscript{II} known to directly contact TBP (Reese et al. 1994). For these and other reasons, yTAF\textsubscript{II}145/TAF\textsubscript{II}250 is thought to be the core subunit of the complex (Chen et al. 1994b; Burley and Roeder 1996). We isolated several temperature-sensitive (ts) alleles of
yTAFII145, which is encoded by an essential gene (Reese et al. 1994; Poon et al. 1995). Cells harboring these yTAFII145 mutant alleles did not grow at 37°C and displayed a rapid cessation of cell division upon shifting from 23°C to the non-permissive temperature (Figure 3-1A). Immunoblot analysis (Fig. 3-1B) shows that when shift to 37°C, the level of yTAFII145 rapidly decreased and by four hours was undetectable in both temperature-sensitive strains. The levels of several other yTAFIIIs, and to a lesser extent yTBP, also decreased. In contrast, components that are not part of the yTAFII complex, such as Sua7 (yTFIIB), were unaffected by the temperature shift. These data suggest that loss of yTAFII145 disrupts the yTAFII complex, resulting in the degradation of other yTAFIIIs.

To investigate the consequences of yTAFII inactivation on transcription, we initially examined strains bearing temperature-sensitive mutations in yTAFII145 or TSM1, which is encoded by an essential gene and is the homologue of the higher eukaryotic TAFII150 (Verrijzer et al. 1994), or strains in which yTAFII30 have been deleted. The gene encoding yTAFII30, ANC1, is not essential, but cells lacking yTAFII30 grow slowly and are inviable at 37°C (Welch et al. 1993).

Yeast strains containing these TAFII mutants or, as controls, a temperature-sensitive GTF (TBP or RNA polymerase
Figure 3-1. Temperature-sensitive yTAF\textsubscript{II}145 mutants.

(A) Rapid cessation of cell growth follows shifting yeast strains containing temperature sensitive alleles of yTAF\textsubscript{II}145 from 23°C to 37°C.
(B) Immunoblot of extracts from wild-type and yTAF\textsubscript{II}145 temperature-sensitive cells, before and after transfer from 23°C to 37°C.
II) were shifted to 37°C and RNA isolated at various times. Transcription was measured by S1 nuclease protection using probes specific for five endogenous yeast genes driven by diverse activators (Hamil et al. 1988; Capieaux et al. 1989; Buchman and Kornberg 1990; Johnston and Carlson 1992; Kuo and Grayhack 1994). For all five genes, as shown in Figure 3-2A, in the tbp (lanes 17-20) and rpb (lanes 21-24) temperature-sensitive strains, shifting to the non-permissive temperature substantially reduced transcription, which by four hours was undetectable. Similar results were obtained with an srb4 temperature-sensitive strain (Thompson and Young 1995 and data not shown). In contrast, even after four hours at the non-permissive temperature, transcription was not significantly affected in the wild-type strain (lanes 1-4), strains bearing temperature-sensitive mutations in yTAFII145 (lanes 5-12), TSM1 (lanes 13-16) or in the strain deleted of yTAFII30 (lanes 25-26).

The genes examined in Figure 3-2A were actively transcribed at the time of the temperature-shift, leaving open the possibility that yTAFIIIs might be required for the initiation, but not maintenance, of a transcriptionally active state. To examine this possibility we tested whether yTAFII inactivation affected the inducible transcription of the yeast copper metallothionein gene, CUP1. Transcriptional induction of CUP1 is extremely rapid and involves the direct
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### Diagram

- **A**
  - Reaction conditions: 4 hrs + 30 min at 37°C.
  - RNA analysis.
- **B**
  - Reaction conditions: 4 hrs + 30 min at 37°C.
  - RNA analysis.
Figure 3-2. Unabated transcription after yTAFII inactivation.

(A) Transcription of several endogenous, chromosomal genes. For the yTAFII, tbp, and rpb, temperature-sensitive strains culture samples were taken for RNA preparation and analysis before (0) and 1, 2, and 4 hr after temperature shift to 37°C. The yTAFII30 deletion (Δ) and parental, wild-type (WT) cells were grown at 23°C before RNA was prepared for analysis. The transcripts detected by S1 nuclease protection in each strain are indicated on the left.

(B) Transcriptional activation of CUP1 in yTAFII mutant strains. The experimental scheme is shown below the autoradiogram. Growth and induction of CUP1 transcription in ytafII30 deletion and wild-type strains was performed at 23°C.
binding of Cu$^{++}$ to the acidic activator ACE1, converting it to an active form (Butt et al. 1984; Furst et al. 1988). Cultures of cells containing temperature-sensitive mutations in yTAFII145, TSM1, TBP, or RNA polymerase II were shifted to the non-permissive temperature for four hours, exposed to copper sulfate (1 mM) for 30 min, and transcription was quantitated (Fig. 3-2B). At the permissive temperature (23°C), activation of CUP1 was similar in all strains (Fig. 3-2B). At the non-permissive temperature (37°C), induction of CUP1 was severely diminished in the tbp temperature-sensitive cells (lanes 19 and 20), and was undetectable in rpb temperature-sensitive cells (lanes 23 and 24). However, cells containing the yTAFII mutations (ytafII145$^{ts}$ and tsm1$^{ts}$) supported transcriptional induction of the CUP1 gene to a level indistinguishable from that of wild-type, even after incubation for four hours at the non-permissive temperature (lanes 7, 8, 11, 12, 15 and 16). CUP1 transcription was also normal in the ytafII30A strain (lanes 25-28).

To confirm and extend the results with the temperature-sensitive yTAFII mutants, we depleted individually four yTAFII$^+$ (yTAFII145, yTAFII90, yTAFII68, and yTAFII47) by using another experimental strategy. Of these, yTAFII145 and yTAFII90 have been previously described (Reese et al. 1994), but yTAFII47 and yTAFII68 are new yTAFII$^+$, cloned on the basis of microsequence analysis of the corresponding subunits of
A

Cup++

Wild-type GAL-YTAF145

Wild-type GAL-YTAF90

Wild-type GAL-YTAF68

Wild-type GAL-YTAF47

CUP1

B

Heat Shock
(39°C)

Wild-type GAL-YTAF145

Wild-type GAL-YTAF90

Wild-type GAL-YTAF68

Wild-type GAL-YTAF47

SSA4(HSP70)

Dextrose +/- Inducer

↓ 12 hrs  ↓ 15 or 30 min  RNA analysis
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Figure 3-3. Activated transcription in cells depleted of yTAF\textsubscript{II}s.

(A) Transcriptional induction of \textit{CUP1} was measured by S1 nuclease protection 30 min. after the addition of 1mM copper sulfate to the medium. yTAF\textsubscript{II}s were depleted by growing the strains in glucose-containing medium for 12 hrs.

(B) Transcriptional induction of \textit{SSA4(HSP70)} was measured by primer extension analysis after shifting the cultures to 39°C for 15 min. yTAF\textsubscript{II}s were depleted by growing the appropriate strains in glucose-containing medium for 12 hrs.

(C) Immunoblot detection of epitope (HA) tagged yTAF\textsubscript{II}s in wild-type (ytaf\textsubscript{II}145::LEU2/pyTAF\textsubscript{II}145 [ARS/CEN]), and strain YSW94 (ytaf\textsubscript{II}145::LEU2/trp3::pSW111[GAL1p-HA-yTAF\textsubscript{II}145-CYC\textsubscript{HIS3}]) or

(D) strain LYC-1 (ytaf\textsubscript{II}90::HIS3/Lp16[ARS/CEN LEU2 GAL1p-HA-yTAF\textsubscript{II}90]) or

(E) strain JR18 (ytaf\textsubscript{II}68::hisG-URA-hisG/trp3::pJE20[GAL1p-HA-yTAF\textsubscript{II}68-CYC\textsubscript{HIS3}]) or

(F) strain JR11 (ytaf\textsubscript{II}47::hisG-URA3-hisG/trp3::pJR15[GAL1p-HA-yTAF\textsubscript{II}47-CYC\textsubscript{HIS3}]) for various times after transfer from galactose- to glucose- containing medium.
Transcriptional induction of CUP1 was examined in these conditional strains 12 hours after glucose-mediated yTAFII shut-off (Fig. 3-3A, B, bottom). In cells in which yTAFII145, yTAFII90, yTAFII68 or yTAFII47 had been depleted, CUP1 transcription was identical to that of the wild-type strain (Fig. 3-3A).

Heat shock is another well characterized inducible transcription response (Bienz and Pelham 1987). Strains containing glucose-regulatable alleles of yTAFII145, yTAFII90, yTAFII68, or yTAFII47 were examined for transcriptional induction of the SSA4 gene (Boorstein and Craig 1990). For each strain, yTAFII depletion did not diminish transcriptional activation (Fig. 3-3B).

Although temperature-sensitive inactivation of yTAFII145 or TSM1 did not result in a general transcription activation defect (Fig. 3-2A, B), a rapid growth arrest was evident (Fig. 3-1A; Mortimer and Schild 1980), and data not shown). The ability to rapidly inactivate these temperature-sensitive yTAFIIIs enabled us to gain insight into the nature of the growth arrest. When the ytafII145ts strain was shifted to 37°C, there was an accumulation of large, unbudded cells (Fig. 3-4A), indicative of a G1-phase cell cycle arrest (Herskowitz 1988). However, at the non-permissive temperature the tsmlts strain arrested as budded cells characteristic of the G2/M phase (Fig. 3-4A). To delineate the cell cycle block
A

Wild-type

yTAF145 ts-1

yTAF145 ts-2

tam1

B

Wild-type

yTAF145 ts-1

yTAF145 ts-2

tam1

23°C

37°C (4hrs)
Figure 3-4. Inactivation of $\gamma$TAF$^{145}$ and TSM1 leads to distinct cell cycle phenotypes.

(A) Phase-contrast micrographs of wild-type, $\gamma$taf$^{145}$ and tsml temperature-sensitive strains at 23°C, and after 4 hrs at 37°C. All micrographs were taken at a magnification of X1000.

(B) Flow cytometric (FACS) analysis, by propidium iodide staining, of the DNA content of wild-type, $\gamma$taf$^{145}$ and tsml temperature-sensitive strains at 23°C and after 4 hr at 37°C.
more precisely, the arrested cells were subjected to fluorescence activated cell sorting (FACS). ytafII145ts cells arrested with a 1N DNA content (Fig. 3-4B), consistent with a block in G1, and analogous to the results with the mammalian TAFII250 homologue (Nishimoto et al. 1982); conversely, tsm1ts cells had a 2N DNA content, consistent with a block in G2.

Discussion

Taken together, our results indicate that activated transcription can occur in the absence of multiple yTAFIIs. This conclusion is based upon two independent strategies to functionally inactivate yTAFIIs: temperature-sensitive mutations and conditional depletion. Moreover, a similar conclusion has been obtained using a third yTAFII inactivation strategy (Moqtaderi et al. 1996a). Particularly compelling are the results with yTAFII145. It is the only yTAFII known to contact TBP directly (Reese et al. 1994), and its higher eukaryotic homologue, TAFII250, is always required to reconstitute TFIIID activity in vitro (Chen et al. 1994b). Therefore, for those genes tested, TAFIIs are not the obligatory targets of activators and so in these instances another transcription component(s), such as TFIIIB or TBP (Zawel and Reinberg 1995), must serve this function.
Although inactivation of yTAFIIIs did not compromise transcription of those genes examined, inactivation of different yTAFIIIs led to distinct cell cycle phenotypes. These results are consistent with those presented in chapter one in which inactivation of yTAFII90 was shown to result in a cell cycle arrest in the absence of a global transcriptional defect. In yeast, cell cycle progression requires the temporally regulated transcription of particular genes (Koch and Naysmith 1994). An intriguing possibility is that yTAFIIIs are not required for transcription in general, but rather for transcription of specific genes, such as those involved in cell cycle progression.
CHAPTER IV

Promoter specific, but not global transcriptional defects in cells lacking functional yTAF_II17
In vitro TAFs function as coactivators to facilitate activated transcription. However, our in vivo analysis of a number of yTAFs had failed to identify one with coactivator activity. As described in the introduction, eukaryotic activators are grouped into classes based on the prevalence of a particular type of amino acid in their activation domain. Although higher eukaryotes possess many classes of activation domains, yeast are believed to possess only the acidic class. Therefore, it is possible that our failure to identify a yTAFII with coactivator activity was due to our failure to analyze the TAFII which interacts with acidic activators.

In higher eukaryotes dTAFII40 and hTAFII32 have been shown to interact with acidic activators in vitro (Goodrich et al. 1993; Thut et al. 1995). With this fact in mind, we reasoned that the yeast homologue of dTAFII40 and hTAFII32 would be an ideal candidate to possess coactivator activity in yeast.

In this chapter we analyze the in vivo function of yTAFII17, the homologue of dTAFII40 and hTAFII32. While yTAFII17 is required for transcriptional induction of the GAL1 gene by the activator GAL4, it is not required for global transcription activation. These results indicate that a single TAFII is not the obligatory target of a specific class of activator.
Results

We began our analysis of yTAF\textsubscript{II}17 by creating diploid strains of yeast containing one wild-type and one disrupted copy of the gene. The construct and scheme used to disrupt yTAF\textsubscript{II}17 is depicted in Figure 4-1A. Sporulation and dissection of the diploid strain resulted in only two viable spores per tetrad (Fig. 4-1B). In no case did the disruption marker segregate with a viable spore (data not shown), indicating that yTAF\textsubscript{II}17 encodes an essential gene. Furthermore, introduction of a wild-type copy of yTAF\textsubscript{II}17 into the diploid strain resulted in near 100% viability of spores generated from this strain, demonstrating that yTAF\textsubscript{II}17 can rescue the lethal phenotype (data not shown).

In order to study the \textit{in vivo} function of yTAF\textsubscript{II}17 we constructed a temperature-sensitive allele. Cells containing the mutant allele grew at 25°C, but not 37°C (Fig. 4-2A) and displayed a rapid cessation of growth when transferred from the permissive to the non-permissive temperature (Fig. 4-2B). Sequencing of the allele revealed that it possessed a single point mutation. This mutation generated a premature stop at amino acid 128 which resulted in the loss of thirty amino acids from the C-terminus. Interestingly, the truncated protein retained the highly conserved histone fold domain
Figure 4-1. \( yTAF_{17} \) is essential for viability.

(A) Scheme used to delete \( yTAF_{17} \) from the yeast genome. The entire coding region of \( yTAF_{17} \) (Bsm 1-Bsm 1) is replaced with the \( LEU2 \) gene.

(B) Sporulation and dissection of diploid strains containing one disrupted and one wild-type copy of \( yTAF_{17} \) results in two viable spores per tetrad.
A

25°C

ts-1  WT  ts-1  WT

37°C

B

TIME (hrs)

OD660

- WT
- ts-1
Figure 4-2. Temperature-sensitive allele of yTAF1717.

(A) Growth of strains containing wild-type (LY90) and temperature-sensitive alleles, of yTAF1717 (ts-1) at 25°C and 37°C.  
(B) Growth curve of wild-type and the temperature-sensitive strain ts-1 immediately after transfer from 25°C to 37°C.
(Burley and Roeder 1996). This domain is believed to be involved in protein-protein interactions. To analyze the effect of $yTAF_{II}17$ inactivation on transcription we used Northern blotting to examine the ability of the temperature-sensitive cells to respond to a number of activators at the non-permissive temperature. Figure 4-3A shows the results of this analysis. Cells containing temperature-sensitive alleles of the basal transcription factor TBP or RNA polymerase II display a rapid decrease in transcription of all genes tested upon transfer from the permissive to the non-permissive temperature. Cells containing a temperature-sensitive allele of $yTAF_{II}17$, however, show only a modest decrease in transcription at the non-permissive temperature. Furthermore, this modest decrease occurs only at the late time points.

Since the loss of a single $yTAF_{II}$ has been shown to disrupt the TFIID complex (Walker et al. 1996), it is possible that the modest decrease in transcription observed in the $yTAF_{II}17$ temperature-sensitive cells is due to degradation of other components of the complex such as TBP, and is not a direct result of a loss of $yTAF_{II}17$ function.

The results from this experiment indicate that $yTAF_{II}17$ is not required for global transcription activation. However, since only five out of the estimated 6,000 genes in the yeast genome were examined, it is difficult to confidently draw this conclusion. An alternative approach to
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Figure 4-3. Transcription in strains containing wild-type and yTAF17 temperature-sensitive alleles.

(A) Transcription of the indicated endogenous genes was measured by Northern blot analysis of RNA collected from strains grown for various times after transfer from the permissive (25°C) to the non-permissive temperature (37°C). (B) mRNA levels were measured in wild-type and temperature-sensitive strains immediately before, and for various times after, transfer to the non-permissive temperature, by slot blot analysis of total RNA with an oligo (dT)20 probe.
analyze the transcriptional competence of cells after yTAF_{II}17 inactivation is to analyze their ability to transcribe bulk mRNA. In this experiment slot blots containing total RNA isolated from cells grown for various times at the non-permissive temperature were probed with an oligonucleotide which hybridizes to the polyA tail of mRNA. Results from this experiment are shown in Figure 4-3B. While mRNA synthesis was rapidly decreased in cells containing temperature-sensitive alleles of TBP or RNA polymerase II, it was unaffected in the yTAF_{II}17 mutant cells.

The analysis described above was designed to determine if transcription could be maintained at the non-permissive temperature in the absence of functional yTAF_{II}17. It is possible that no significant transcriptional defect was observed because yTAF_{II}17 is not required for the maintenance of transcription, but rather for transcription initiation. In order to test this hypothesis the following experiment was performed. Basically, cells were allowed to grow at the permissive temperature to mid-log phase. They were then shifted to the non-permissive temperature for one to two hours. After this time the appropriate inducer was added to the media. Cells were allowed to incubate further at the non-permissive temperature, then collected and analyzed for their ability to respond to various activators. In Figure 4-4A the
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- **ts-1**: 

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### CUP1

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- **ts-1**: 

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### 3AT

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### HIS3

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### HIS4

- **WT**: 
- **ts-1**: 

Figure 4-4. Transcription induction in strains bearing wild-type and temperature-sensitive alleles of yTAF17.

(A) Northern blot analysis was used to measure the induction of the GAL1 gene, in the indicated strains, one hour after the addition of galactose at a final concentration of 3% to the medium.

(B) Induction of the CUP1 gene was measured 30 minutes after the addition of copper sulfate at a final concentration of 100uM to the medium.

(C) Induction of the HIS3 and HIS4 genes was measured three hours after the addition of 3-Amino-1-2-4-Triazole at a final concentration of 10mM to the medium.
ability of the cells to respond to the well characterized acidic activator GAL4 upon the addition of galactose to the media is shown. As expected, both wild-type and yTAF$_{II17}$ temperature-sensitive cells were capable of transcription induction of the GAL1 gene at 25°C. However, after incubation at 37°C for two hours only the wild-type cells were capable of inducing the GAL1 gene.

These results were very interesting and compelled us to analyze the ability of the yTAF$_{II17}$ temperature-sensitive cells to induce the transcription of genes under the control of other well characterized activators. We chose to examine the ability of the cells to induce the transcription of the CUP1 gene, by the activator ACE1, in the presence of copper sulfate (Butt et al. 1984; Furst et al. 1988). The results of Figure 4-4B show that both wild-type and yTAF$_{II17}$ temperature-sensitive cells were capable of transcription induction of CUP1 at 25°C and after incubation at 37°C for two hours. These results were not unique to activation by ACE1. Both wild-type and yTAF$_{II17}$ temperature-sensitive cells were capable of transcription induction of the HIS3 and HIS4 genes in the presence of the competitive inhibitor 3-Amino-1-2-4-Triazole (3AT) at 25°C and 37°C (Fig. 4-4C) Transcriptional induction of HIS3 and HIS4 are under the control of the activator GCN4 (Hope and Struhl 1985; Arndt and Fink 1986).
Discussion

The results presented in this chapter show that yTAF_{II}17 is not required for global transcription activation, but rather for the function of at least one specific promoter. These results are consistent with those obtained from the in vivo analysis of another TAF_{II}, hTAF_{II}250. Human and yeast cells containing temperature-sensitive alleles of TAF_{II}250 are incapable of transcription of a number of genes at the non-permissive temperature including the G1 cyclins, but display no global transcriptional defect (Liu et al. 1985; Wang and Tjian 1994; Walker et al. 1996).

These results, however, are not consistent with yTAF_{II}17 being the obligatory target of acidic activators. While yTAF_{II}17 was required for activation by the acidic activator GAL4 it was not required for the acidic activators ACE1 or GCN4. These results may be explained if the yTAF_{II}17 temperature-sensitive alleles retained partial activity. We do not favor this explanation for two reasons. First, the cells show a rapid decrease in cell growth upon transfer from the permissive to the non-permissive temperature, indicating that an essential activity of yTAF_{II}17 has been inactivated. Second, analysis of mRNA synthesis in the mutant cells at the non-permissive temperature failed to identify any defect; indicating that transcriptional defects must be rare.
Exactly what makes a gene dependent on $\gamma$TAF$_{II}$17 is not known. However, it is clear that not all acidic activators require a functional $\gamma$TAF$_{II}$17 to stimulate transcription. Therefore, activators must interacting with other factors in vivo to facilitate transcription activation.
CHAPTER VI

Summary
The *in vitro* analysis of TAF$_{	ext{II}}$s from higher eukaryotes indicates that they are the obligatory targets of activators. They are required for activator function (Hoey et al. 1990; Hoffman et al. 1990; Peterson et al. 1990; Pugh and Tjian 1990; Smale et al. 1990), have been shown to interact with specific activators (Goodrich et al. 1993; Hoey et al. 1993; Jacq et al. 1994; Thut et al. 1995;), and the function of specific activators requires the presence of specific TAF$_{	ext{II}}$s (Chen et al. 1994b). Although numerous TAF$_{	ext{II}}$-TAF$_{	ext{II}}$ and TAF$_{	ext{II}}$-activator interactions have been identified, precisely how individual TAF$_{	ext{II}}$s function in higher eukaryotes has failed to progress far beyond the identification of these protein-protein interactions.

In this thesis we have taken advantage of the isolation of a TFIID complex from the yeast *Saccharomyces cerevisiae* (Reese et al. 1994; Poon et al. 1995) to analyze the function of individual TAF$_{	ext{II}}$s *in vivo*. We are confident that the data generated in this system is applicable to higher eukaryotes for the following reasons. First, there is a high degree of conservation between the transcription machinery of yeast and that of higher eukaryotes (Struhl 1989). Second, TFIID from yeast contains homologues of higher eukaryotic TAF$_{	ext{II}}$s (Verrijzer et al. 1994; Reese et al. 1994; Poon et al. 1995; Moqtaderi et al. 1996a,b).
Our analysis of individual TAF₁₅s in yeast indicate that they are not the obligatory targets of activators. Global transcription activation occurs in the absence of any one of a number of TAF₁₅s, including yTAF₁₅₁₄₅, which is believed to be an essential structural component of the TFIID complex. These results directly contradict those obtained for higher eukaryotic TAF₁₅s in vitro. How can these two lines of evidence be reconciled? It is possible that over time TAF₁₅s have evolved different functions in lower and higher eukaryotes. Though possible, data generated in vivo indicate that TAF₁₅s of higher and lower eukaryotic cells function similarly. Mammalian and yeast cells containing temperature-sensitive alleles of hTAF₂₅₀ and yTAF₁₄₅, respectively, arrest in the G1 phase of the cell cycle and display promoter-specific defects at the non-permissive temperature (Liu et al. 1985; Wang and Tjian 1994; Walker et al. 1996). In addition, TAF₁₅s from higher eukaryotes and yeast function similarly in vitro, each is required for activator function (Hoey et al. 1990; Hoffman et al. 1990; Peterson et al. 1990; Pugh and Tjian 1990; Smale et al. 1990; Reese et al. 1994; Poon et al. 1995). These results indicate that the discrepancies observed in TAF₁₅ function are due to differences between, in vitro and in vivo systems and not mammalian and yeast TAF₁₅s.
The discrepancies observed may be explained by factors present in vivo, but lacking from in vitro systems, that are capable of substituting for the TAFIIIs on most promoters. In yeast, the mediator complex is capable of substituting for the YTAFIIIs in reconstituted transcription reactions (Kim et al. 1994). Therefore, members of the mediator are prime candidates to serve this function. Although a mediator complex has not yet been identified in higher eukaryotic cells, it is possible that components of this complex exist and are capable of substituting for the TAFIIIs. However, one must keep in mind that each of the TAFIIIs examined in this study, with the exception of YTAFII-30, encodes for an essential gene. Therefore, even if redundant factors exist in vivo, they cannot substitute for all of the TAFII activities.

An alternative explanation is that TAFIIIs function differently in vivo and in vitro. Results obtained from the analysis of strains containing temperature-sensitive alleles of YTAFII-145 indicate that this may be the case, at least for some promoters. In yeast, YTAFII-145 is required for the expression of G1 and S phase cyclins (Walker et al. 1997), as well as for the expression of ribosomal genes (Shen and Green 1997). Identification of the element within the CLN2 and RPS5 promoters responsible for YTAFII-145 dependence maps to the core promoter and not the upstream activator site. Interestingly, similar experiments performed in mammalian
cells containing a temperature-sensitive allele of hTAF_{II}250 indicate that both the activator and the core promoter contribute to the dependence of hTAF_{II}250 for expression of the cyclin A promoter (Wang et al. 1997). It is possible that yTAF_{II}145/hTAF_{II}250 is capable of functioning through multiple promoter elements and those used depend on the particular promoter. Alternatively, it is possible that mammalian hTAF_{II}250 functions through multiple promoter elements and yeast yTAF_{II}145 only through the core promoter. This possibility, however, is unlikely since both TAF_{II}s are required for activated transcription in vitro and both perform similar functions in vivo. Interestingly, yTAF_{II}145 and its higher eukaryotic homologues have been shown to possess histone acetyltransferase activity (HAT) that is specific for histones H3 and H4 in vitro. It is possible that on some promoters yTAF_{II}145/hTAF_{II}250 functions to antagonize repression caused by nucleosomes. Alternatively, yTAF_{II}145/hTAF_{II}250 may acetylate factors other than histones, such as the basal transcription factors. Possibly, the acetylation of one or more of these factors results in the formation of a more productive PIC. In order to understand the function of yTAF_{II}145/hTAF_{II}250 it will be important to determine the in vivo targets of the HAT activity.

At least three of the TAF_{II}s appear to play a role in cell cycle progression. At the non-permissive temperature,
cells containing temperature-sensitive alleles of yTAF₁₁₁₄₅ arrest in the G1 phase of the cell cycle, while cells containing temperature-sensitive alleles of yTAF₁₁₁₉₀ or TSM₁ arrest in G2/M. These results led us to speculate that individual TAF₁₁₁s may be required for the transcription of subsets of genes, possibly those required for cell cycle progression. The analysis of strains containing temperature-sensitive alleles of yTAF₁₁₁₄₅ revealed that they were incapable of expressing G1 and S phase cyclins at the non-permissive temperature (Walker et al. 1997). In contrast, strains containing temperature-sensitive alleles of yTAF₁₁₁₉₀ were capable of expressing at least two genes whose expression was required for progression through G2/M, CLB₂ and CSE₄ at the non-permissive temperature. In an attempt to identify genes whose expression required yTAF₁₁₁₉₀ a comprehensive approach was taken. First, cells in which yTAF₁₁₁₉₀ had been inactivated by temperature-sensitive mutation were examined for their ability to transcribe randomly selected genes at the non-permissive temperature. In addition, these cells were subjected to PCR based subtractive hybridization. This technique has the potential to analyze every gene expressed in the yeast genome (see appendix A). To our surprise, neither of these strategies identified a gene that required yTAF₁₁₁₉₀ for expression. It is possible that our inability to identify a yTAF₁₁₁₉₀ dependent gene was due to
limitations in the techniques we employed. Alternatively, yTAF₉₀ may function in activities other than transcription, for example, cell cycle progression. Interestingly, cells containing both a temperature-sensitive allele of CDC28 (cdc28-1N), which arrests in the G2 phase of the cell cycle at the non-permissive temperature, and a temperature-sensitive allele of yTAF₉₀, are inviable. In contrast, cells containing a temperature-sensitive allele of CDC28 (cdc28-4) which arrests in the G1 phase of the cell cycle at the non-permissive temperature and a temperature-sensitive allele of yTAF₉₀ are viable (data not shown). These results indicate that the synthetic lethality observed between cdc28-1N and yTAF₉₀ is not simply the result of combining two temperature-sensitive alleles. Furthermore, these results suggest that CDC28 and yTAF₉₀ function in the same pathway. They may function in a pathway that ensures progression through the cell cycle, or possibly in one that involves check point controls.

If yTAF₉₀ functions in a check point control pathway it does not appear to involve RAD9. No change in the percentage of cells arrested in the G2 phase of the cell cycle was observed in yTAF₉₀ temperature-sensitive cells in which RAD9 had been deleted (data not shown). Identifying the in vivo function of yTAF₉₀ will require the use of highly sensitive procedures to identify differentially expressed genes, as
well as an in depth analysis of the cell cycle defects exhibited by cells lacking yTAF\textsubscript{II}90 function.

Although transcriptional defects were not observed in the absence of any one of a number of functional TAF\textsubscript{II}s, cells expressing a temperature-sensitive allele of yTAF\textsubscript{II}17 were shown to exhibit a promoter specific defect at the non-permissive temperature. yTAF\textsubscript{II}17 was analyzed specifically because of its homology to higher eukaryotic TAF\textsubscript{II}s shown to interact with acidic activators (Moqtaderi et al. 1996b). Since yeast possess only acidic activators, yTAF\textsubscript{II}17 appeared to be the perfect candidate to possess coactivator activity. Interestingly, yTAF\textsubscript{II}17 was required for expression of the GAL1 gene, but not the HIS3, HIS4 or CUP1 genes. These results are intriguing because they contradict the long held belief that a single TAF\textsubscript{II} functions by interacting with a specific class of activator. It is possible, however, that there exists another TAF\textsubscript{II} in yeast which will supply this coactivator activity. It is also possible that our classification of activators is incorrect. Grouping activators into classes based on the preponderance of a particular type of amino acid may be too simplistic. If our classifications are incorrect, then we would not expect to find a single TAF\textsubscript{II} which functions as a coactivator for all acidic activators. Furthermore, we can not rule out the possibility that the temperature-sensitive allele of yTAF\textsubscript{II}17
examined in this study retained partial activity at the non-permissive temperature. \( yTAF_{17} \) contains a domain which has similarity to histone H3. In addition, the higher eukaryotic homologues of \( yTAF_{17} \), \( hTAF_{32} \) and \( dTAF_{40} \), have been shown to associate with \( hTAF_{80} \) and \( dTAF_{60} \), respectively, to form a nucleosome like structure. Since the temperature-sensitive allele of \( yTAF_{17} \) contains a truncation which leaves the H3 homology domain in tact, it is possible that this mutant assembles into the TFIID complex at the non-permissive temperature and is capable of supporting transcription activation of most promoters. If this is true, then the temperature-sensitive defect must result in the rapid loss of transcription of at least one essential gene. It will be important to determine if \( yTAF_{17} \) protein is present in the temperature-sensitive cells at the non-permissive temperature. Furthermore, if protein is detected, if it is capable of assembling into the PIC at the non-permissive temperature. In addition, it will be important to identify the element which confers \( yTAF_{17} \) dependence on the \( GAL1 \) promoter. If \( yTAF_{17} \) is functioning as a coactivator it should map to the activator binding site.

The binding of TFIID to the promoter constitutes the first step in the formation of a PIC. Only after it is bound are the remaining GTFs capable of entering into the PIC. The finding that many of the basal factors may exist in a single
complex, invokes an interesting analogy between TFIID and bacterial σ factors. Possibly TFIID functions like the bacterial σ factor to select a particular promoter to which the holoenzyme is then recruited. In bacteria there are multiple σ factors. Multiple TFIID complexes have recently been found to exist in Drosophila (Hansen et al. 1997). At least one of these complexes has been shown to be capable of associating with the remaining GTFs and of supporting transcription activation by a subset of transcription activators (Hansen et al. 1997). Like the bacterial σ factor, each of these complexes may be required for nucleating the formation of a PIC on different promoters. Though multiple TFIID complexes have not been identified in yeast, it is still possible that TFIID functions as a promoter specificity factor in this organism. The association of TBP with the TAF\textsubscript{II}s is weaker in the TFIID complex isolated from yeast than that from higher eukaryotes. It is possible that while there are multiple TFIID complexes in higher eukaryotes, there are multiple TAF\textsubscript{II} complexes in yeast. Each capable of associating with TBP and selecting a different set of promoters. It will be interesting to determine whether multiple TAF\textsubscript{II} complexes exist in yeast.

The work presented in this thesis has forced us to reevaluate our views on TAF\textsubscript{II}s and transcription activation. While it was assumed from data generated in vitro that the
TAFII's would be required for global transcription activation in vivo, this is not the case. In vivo, global transcription activation is unaffected following inactivation or depletion of numerous TAFII's. In contrast, a small number of promoters have been identified which require particular TAFII's for expression. In order to understand how TAFII's are functioning in vivo it will be important to determine the elements which confer TAFII dependence on these promoters. In addition, it will be important to determine if there is a common requirement for TAFII-dependence, and what that requirement is. This will require the identification of additional genes that require specific TAFII's for expression and the precise mapping of the elements which confer TAFII-dependence.

In addition, the work presented in this thesis has led to the identification of unexpected roles for three of the TAFII's in cell cycle progression. While yTAFII145 is required for the expression of G1 and S phase cyclins (Walker et al. 1997), the roles played by yTAFII90 and TMS1 are not known. It will be important to determine if these TAFII's are required for the transcription of genes required for cell cycle progression or if they play a more direct role in this process. An intriguing possibility is that they function in a check point control pathway, either directly, or indirectly via transcription activation.

The work presented in this thesis has shed new light on the function of TAFII's and the mechanisms of transcription
activation in vivo. In addition, it has generated many interesting question which must be addressed in order to fully understand transcription activation in vivo.
CHAPTER VI

Materials and Methods
Yeast Strains

Strains CY245, LY3, LY20, LY21, and LYC-1, YSW87, YSW90, YSW93, YSW94, JR18, and JR11 are isogenic derivatives of S288C. Strains LY90, LY722, and LY740 are isogenic derivatives of W303. Strains DDY555 (also called yTAFII30Δ; Welch et al. 1993), tsml (Ray et al. 1991), rpb1-1 (Nonet et al. 1987), Srb2Δ1 (Koleske et al. 1992), Egy48 (Gyuris et al. 1993), and strains harboring the TBP mutants, ts-1 (Cormack), P109A (Arndt et al. 1995), N159L and V161A (Lee and Struhl 1995) have been previously described. Mutant taf90, taf145 and taf17 strains were made by the plasmid shuffle technique (Boeke et al. 1984). Basically, strains LY3, YSW85, and LY90, were transformed (Ito et al. 1983), respectively, with various mutant plasmids, followed by screening for cells that had lost the wild-type plasmid on 5-fluoroorotic acid (5-FOA). The TAF17 disruption strain LY80 was constructed by transformation of strain W303 with an EcoRI fragment from plasmid Lp21 containing a disrupted copy of TAF17, followed by selection on leucine-deficient medium. PCR and Southern blotting were used to confirm proper integration of the disruption construct. Strain LY80 was then transformed with plasmid Lp19 to generate strain LY87. LY87 was then sporulated, followed by dissection of the resulting tetrads. Tetrads which grew on selective media lacking uracil and
leucine were selected. One such strain, called LY90, was used to make the wild-type strain LY740 and the temperature-sensitive strain LY722. Strain LY740 and LY722 were made by transformation of LY90 with plasmids Lp20 and Lp34 respectively, followed by plasmid shuffle. Strain LYC-1 which contains a glucose-repressible allele of TAF90 was also made using the plasmid shuffle technique. Strains containing glucose-repressible alleles of TAF145 (YSW94), TAF68 (JR18) and TAF47 (JR11) were made by integration of the appropriate constructs. Strain LGY7 was constructed by digestion of the plasmid Jp167 (J. Pearlberg, unpublished) with ApaI followed by transformation into yeast strain JYY45 (J. Pearlberg, unpublished). Cultures were grown in YEPD unless selection was necessary, in which case all cultures were grown in the appropriate selective (SD) medium.

Plasmid Constructions

The TAF90 plasmid Lp7 that was mutagenized was constructed by cloning the HindIII fragment of plasmid Lp6 (Reese et al. 1994), which contains the entire coding region of TAF90, into the HindIII site of pGAD (Chien et al. 1991). Mutants generated in plasmid Lp7, namely ytaf1190ts2-1 and ytaf1190ts3-1, were liberated from the plasmid by HindIII digestion and cloned into the HindIII site of plasmid Lp5, to generate plasmids Lp12 and Lp11, respectively. Lp5 is a
derivative of the LEU2 marked CEN plasmid pRS415 (Sikorski and Hieter 1989), which contains the ADH1 promoter and terminator. Plasmid Lp16, in which TAF90 is under control of the GAL1 promoter, was constructed by ligating the HindIII fragment from plasmid Lp7 into the HindIII site of plasmid Lp15. Lp15 is also a derivative of pRS415 with the addition of the GAL1-GAL10 UAS. LexA-TAF90 fusions containing wild-type and temperature-sensitive alleles were constructed by cloning the XbaI/XhoI fragment of plasmid Lp1, which contains the entire coding region of TAF90 in-frame with LexA(1-87). LexA-yTAF1190, N-terminal (Δ) and C-terminal (Δ) deletion mutants were constructed by cloning the XbaI/BglII fragment and the BglII/XhoI fragment of the wild-type TAF90 coding region respectively, in frame with LexA(1-87). Expression of all LexA-TAF90 constructs are under control of the ADH1 promoter cloned into plasmid pRS423 (Sikorski and Hieter 1989). To construct TAF17-containing plasmids a fragment containing wild-type TAF17 was generated by PCR using genomic DNA and the primers 5-ggcctgaattcctttttaccg and 5-gcggaagtgtctctcaagaagatg. The primers were designed to generate an EcoRI restriction site on both ends of the PCR fragment. The EcoRI digested PCR fragment was cloned into the EcoRI site of the HIS3-marked, single copy number plasmid pRS413 (Sikorski and Hieter 1989) to generate plasmid Lp20, into the URA3 marked single copy number plasmid pRS416
(Sikorski and Hieter 1989), to generate Lp19, and into the plasmid Bluescript (Stratagene) to generate plasmid Lp17. Lp17 was then digested with BsmI, and the ends made blunt with T4 DNA polymerase. A DNA fragment containing the LEU2 gene was isolated from plasmid pJJ282 (Jones and Prakash 1990) by digestion with SstI, and cloned into the blunted Lp17 plasmid to generate the TAF17 disruption construct Lp21. The plasmid pSW104 used as a substrate for mutagenesis of TAF145 was constructed by inserting a DNA fragment containing the promoter and coding sequence of TAF145 into the multicloning site of pRS313 (HIS3, ARS/CEN) (Sikorski and Hieter 1989).

**Isolation of Temperature-Sensitive Alleles**

Plasmids containing the coding regions of TAF145 (pSW104), TAF90 (LP7) and TAF17 (LP20) were treated with 0.5M hydroxylamine for 1-2 hours at 70°C. The mutagenized DNA was then transformed into the yeast strains YSW85, LY3, and LY90, respectively, and grown at room temperature on selective plates. Transformants were then patched to selective plates, grown at room temperature, and replica plated to two plates containing 5-FOA to select for cells which have lost the wild-type plasmid. One plate was incubated at room temperature and the other at 37°C. Colonies which grew at room temperature but not 37°C were restreaked and rescreened
for growth at the two temperatures. Mutagenized plasmids from colonies that grew at room temperature but not at 37°C were isolated, subcloned into fresh vectors, and rescreened for the temperature-sensitive phenotype.

**Temperature-Shift Experiments**

Unless otherwise stated, cells were grown at room temperature to log phase followed by transfer to 37°C either directly, or after the addition of an equal volume of the appropriate media which had been warmed to 37°C. Similar results were obtained by both methods. For galactose induction cells were grown at room temperature in 3% raffinose to mid log phase. Each culture was then divided into four flasks, two of the flasks were incubated at room temperature and the other two at 37°C. After two or three hours one of the two flasks at each temperature received galactose to a final concentration of 3% and the other remained as the uninduced control. The cells were incubated for an additional 30 minutes to 1 hour then harvested at 4°C, frozen on dry ice, and stored at -80°C. Copper, and histidine induction was performed identically to the galactose induction with the following exceptions. For copper induction the cells were grown in the presence of 3% glucose, the inducer was 100μm copper sulfate, and the cells were harvested 30 minutes after induction. For histidine induction cells were grown in complete medium containing 3%
glucose. Immediately before temperature shift cells were collected, washed once in medium lacking histidine, and resuspended in the same medium if cultures were to be induced and complete medium for cultures not induced. After incubation at the appropriate temperature for one hour, 3-Amino-1-2-4-Triazole (3AT) was added to a final concentration of 10mm to cultures grown in medium lacking histidine. Cells were then incubated for an addition three hours before harvesting.

**Conditional Depletion of yTAFⅢⅢs**

Cultures were grown to log phase at 30°C in the presence of 3% galactose and 0.25% sucrose. The cells were then harvested, washed two times with sterile water, resuspended in selective media containing 3% glucose, and incubated for an additional 16 hours. Aliquots of cells were taken throughout this time period, frozen on dry ice, and stored at -80°C to be used for protein and RNA analysis.

**RNA Analysis**

Total RNA was isolated as previously described (Peterson et al. 1991) and quantitated by absorbence at 260 nM and visualization on an agarose gel. mRNA was isolated using the PolyATtract mRNA Isolation system from Promega (Madison, WI). S1 nuclease analysis, primer extension analysis and Northern
blotting were performed as previously described (McKnight and Kingsbury 1982; Cormack and Struhl 1992). In each case, 10-20 ug of total RNA was used. Probes for Northern blot analysis were generated by using the primer pairs

CGGGATCCGATCAAGCAGTGGTAG & CCGGTCTCAGTCAGTAACGTC

GAAGATCCGAGACTCTGCGAACC,

GGATCTAGATCCGAGCAGTGGTAG

CGGGATCCGAGCCATATTGAAGATACC, &

ATCGTCTAGACGATTTTTGGAATATTTCG

CTCGGGATCCGAGATTACAACAGGTATTGTCC to generate PCR fragments CLB2, CSE4, CLN3 and ADH1 respectively. The rRNA, tRNA1, DED1, RAD23, TRP3 and GAL10 probes have been reported previously (Cormack and Struhl 1992; Peterson and Herskowitz 1992; Thompson and Young 1995). The sequence of the remaining probes are listed below.

CUP1 GGCACTGCTGACCTTACATTTGAATATTGAAGTCTGG

ENO2 CGGGATCTGAGACGATCTAGCTGGAAACATTAGAGACAGCCTAATAAA

PMA1 GCAGGGTTTCTTGAGGGCTGAGCTGAGCTGAAACAGGATGCTTCT

**Slot Blots**

Analysis of polyA RNA was performed as follows. 5ug of total RNA was transferred to GeneScreen (NEN Research Products, Boston Ma.) as recommended by the manufacturers (Hoefer
Scientific Instruments, San Francisco Ca.). RNA was fixed to the filter by UV crosslinking, and the filter hybridized over night in prehybridization buffer consisting of 5X SSC, 5X Denhardt's, 0.5% SDS and 0.2 mg/ml salmon sperm DNA. The filter was then hybridized over night (at least 18 hours) in fresh prehybridization buffer containing 5 X 10^5 cpm/ml of end-labeled oligo dT_{20} probe. The filter was then washed once for fifteen minutes at 37°C in prehybridization buffer, followed by three room temperature washes for twenty minutes each in 1 X SSC, 0.1% SDS.

**Whole-Cell Extracts and Immunoblot Analysis**

Cultures were grown to mid-log phase and aliquots harvested by centrifugation for 5 minutes at 3.4K. The cells were then washed with cold ddH2O, transferred to a microfuge tube, and frozen at -80°C until all aliquots were taken. The cells were resuspended in 50ul of extraction buffer (0.1M Tris pH 8.0, 0.5M NaCl, 5mM EDTA, 0.01% NP40 and 15% Glycerol) with freshly added protease inhibitors (10mM DTT, 10ug/ml leupeptin, 10ug/ml aprotinin, 2mM benzamidine-HCL, 1.0mM PMSF and 2ug/ml pepstatin), followed by vortexing six times for 10 seconds with one minute of cooling on ice in the presence of 50-75ul of glass beads. The extracts were then clarified twice for ten minutes at 4°C and protein concentration
determined by the Bradford Assay (Bradford 1976). Proteins were visualized by the ECL detection system from Amersham Life Science.

**Immunoprecipitation Analysis**

Yeast whole cell extracts (Reese et al. 1994) were diluted to 10mg/ml protein and adjusted to 0.25M KOAc buffer T plus 0.01% NP40. The extracts were clarified and 100ul was incubated with 2ul of rabbit polyclonal antisera for 4 hours on ice. Immune complexes were recovered by the addition of 3ul protein-A agarose beads followed by a 4 hour incubation at 4°C with rotation. The beads were recovered and washed 4 times in ice-cold 0.75M KOAc Buffer T plus 0.01% NP40. Proteins were solubilized with SDS-PAGE loading dye.

**Immobilized DNA Template Assays**

Yeast whole cell extracts (600ug protein in 100ul) was incubated under transcription conditions (Reese et al. 1994) with a fragment of biotinylated pGEM3 or G5E4T (0.15pmol) complexed to Dynal strepavadin beads prepared as described (Choy and Green 1993). 200ng of PolydI-dC was added. The reactions with G5E4T contained 200ng of GAL4-VP16 to stimulate the formation of the Preinitiation complex. After a 40 minute incubation at 23°C, the beads were collected and washed 4 times with wash buffer (20 mM HEPES-KOH pH 7.5, 10mM
MgOac, 2.5 mM EGTA, 100 mM KOAc, 1mM DTT, 0.003% NP40 and 10% glycerol). The proteins were eluted from the beads by digestion with 5U of micrococcal nuclease for 15 minutes and 23°C, and analyzed by immunoblotting.

**Analysis of LexA-yTAF\textsubscript{II}90 Fusion-Proteins**

LexA-yTAF\textsubscript{II}90 fusion-proteins containing full length, N-terminal and C-terminal deletion were transformed into LGY7 which contains an integrated lacZ reporter with 2 LexA operators upstream of the TATA box. β-galactosidase activities were assayed as described (Kaiser et al. 1994). Wild-type and LexA-yTAF\textsubscript{II}90 temperature-sensitive hybrids were transformed into strain EGY48 (Gyuris et al. 1993). Transformants were restreaked on minimal medium without leucine and tested for the ability to grow at 25°C and 30°C.

**Flow Cytometry**

Samples were prepared for FACS analysis as previously described (Lew et al. 1992), and FACS analysis performed by the Flow Cytometry Facility at the University of Massachusetts Medical Center.
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APPENDIX
As detailed in the previous pages, the analysis of transcription activation in cells lacking functional yTAF_H90 failed to identify promoters which required yTAF_H90 for expression. However, since it has been estimated that there are between 4,000 and 6,000 actively transcribed genes in a yeast cell and we had examined only a small number of genes, we could not rule out the possibility that such promoters existed. Furthermore, we could not rule out the possibility that the loss of functional yTAF_H90 protein caused derepression, or inappropriate expression, of a subset of genes.

To identify genes whose proper expression required yTAF_H90, we performed two subtractive hybridizations using the CLONTECH PCR-select cDNA subtraction kit (outlined in Figure A-1). This technique has the potential to identify a small population of differentially expressed genes among a large population of genes not differentially expressed. The first subtraction was designed to identify genes that required yTAF_H90 for expression (plus subtraction). The second, to identify genes whose expression increased upon inactivation of yTAF_H90 (minus subtraction). Each subtraction was performed on mRNA isolated from the wild-type strain LY3 and the yTAF_H90 temperature-sensitive strain ts2-1 (LY20), one hour after transfer from the permissive to the non-permissive temperature. Results of the minus subtraction failed to identify genes up regulated by the loss of yTAF_H90
mRNA

cDNA synthesis

Blunt ends by restriction digestion with Rsal

Split tester cDNA into two pools, add different adaptors to each pool

Add excess driver, denature and allow products to anneal

Mix products from the two pools and allow the products to anneal

Fill in ends and amplify boxed species using PCR

Figure A-1. Subtractive hybridization scheme. Tester cDNAs contain the differentially expressed products.
A

B

2 hrs at 37°C

URA3

β-lactamase
Figure A-2. Differentially expressed genes in wild-type and yTAF₁₁₀ temperature-sensitive strains.

(A) Products from the plus (lanes 2-4) and minus (lanes 5-7) subtractions were run on an agarose gel. Candidates of differentially expressed genes are designated by arrows. Lane 1 contains a control subtraction supplied by CLONTECH, lanes 4 and 7 contain unsubtracted controls from the plus and minus subtraction respectively. Lanes 2 and 3 contain two dilutions of the plus subtracted products, and lanes 5 and 6 two dilutions of the minus subtracted products. 

(B) Bands from the above gel, designated by arrows, were cloned and used to probe Northern blots made from RNA isolated from wild-type and yTAF₁₁₀ temperature-sensitive strains two hours after temperature shift. Northern blots for two of the cDNAs are shown above.
function (Figure A-2a, lanes 5-7). A single cDNA present above background was visible among the subtracted products, however, it was also present in the unsubtracted control (Figure A-2a ,lanes 5-7), indicating that it represented a highly abundant cDNA that was amplified during the PCR reaction. In contrast, at least three differentially expressed cDNAs were present in the subtracted, but not the unsubtracted controls of the plus subtraction (Figure A-2a, lanes 2-4). In addition, two bands were present in the subtracted and unsubtracted controls. Again, these cDNAs probably represent highly abundant cDNAs amplified during the PCR reaction.

In order to determine if these three cDNAs represented differentially expressed genes they were cloned and used as probes in Northern blot analysis. To ensure that we had not isolated genes whose expression was decreased transiently, we probed Northern blots made with RNA isolated from wild-type and temperature-sensitive strains two hours after temperature-shift. Results from two such Northern blots are shown in Figure A-2b. Both of the cDNAs used as probes were expressed at high levels in the wild-type but not the temperature-sensitive strains two hours after temperature shift. Sequencing of the clones identified two as URA3 and the other as β-lactamase. The differential expression of both of these genes was easily explained by differences in the
strains used in the subtraction. Although these results had a trivial explanation they indicated that the technique worked well and compelled us to isolate additional clones from the background smear. Approximately forty cDNAs were cloned and used to probe Northern blots. Seven of these clones were found to be differentially expressed in the two strains two hours after transfer to the non-permissive temperature (data not shown). However, analysis of these clones in a variety of strains conditionally arrested in the G2/M phase of the cell-cycle, independent of yTAF$_{11}$90, showed that they were differentially expressed in these strains as well (data not shown). In addition, many of them were also differentially expressed in cells containing temperature-sensitive alleles of a variety of yTAF$_{11}$s (data not shown). These results indicate that differential expression of the isolated clones was not due to inactivation of yTAF$_{11}$90, but rather to the cell cycle arrest exhibited by these cells, or to a general defect in the ability of dying cells to express these genes.

It is possible that we failed to identify genes that required yTAF$_{11}$90 for proper expression because yTAF$_{11}$90 is not required for gene expression. It is also possible that our failure was due to limitations in our technique. Our ability to identify URA3 which is highly expressed in the wild-type strain and absent from the temperature-sensitive strain, implies that this technique is capable of identifying highly expressed genes, that are expressed at very different levels
in the two strains. This technique may not be capable of identifying low abundance genes, or genes which are expressed at only slightly different levels in the two strains. If this is the case, then it is possible that the loss of $\gamma$TAF$_{I1}$90 function causes only a small change in the expression of a number of genes. Equally plausible, is that genes requiring $\gamma$TAF$_{II}$90 for expression, are expressed at levels below that which could be detected by this technique. In any case, there are a number of additional techniques, such as differential display, which should be attempted and which may yield more fruitful results.