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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:

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(January 14, 1998)

Biochemistry

ANALYSIS OF TAF_{II} FUNCTION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

A dissertation Presented

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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 ($TAF_{II}s$). Results from numerous *in vitro* experiments indicate that $TAF_{II}s$ 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 $TAF_{II}s$, their proposed targets.

Results from the genetic disruption of a number of $TAF_{II}s$ 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 TAF_{II}s by either of these two methods displayed no defect in global transcription activation. Inactivation of $yTAF_{II}17$, however, resulted in a promoter specific defect. In addition, inactivation of $yTAF_{II}145$, $yTAF_{II}90$, or TSM1, resulted in an inability of cells to progress through the cell-cycle.

In an attempt to identify genes whose expression required $yTAF_{II}90$, 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 $yTAF_{II}90$.

These results indicate that TAF_{II}s are not the obligatory targets of activators, and that other factors must provide this role *in vivo*. Furthermore, that many of TAF_{II}s 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 it's 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.

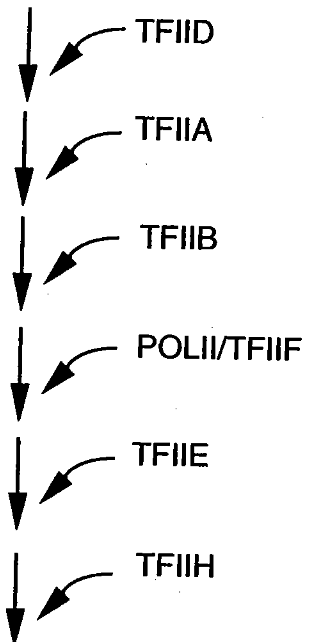
Comparison of their amino acid sequences show that they are highly conserved among eukaryotes (reviewed in Orphanides *et al.* 1996; Nikolov and Burley 1997). In addition, many of the GTFs have regions that are similar to regions of the bacterial σ factors.

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.

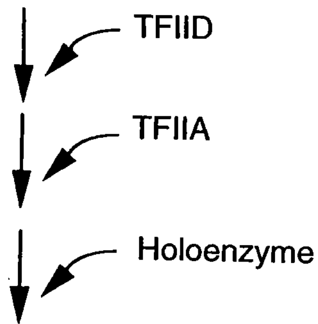
Basal transcription reaction

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

Stepwise assembly



Holoenzyme recruitment



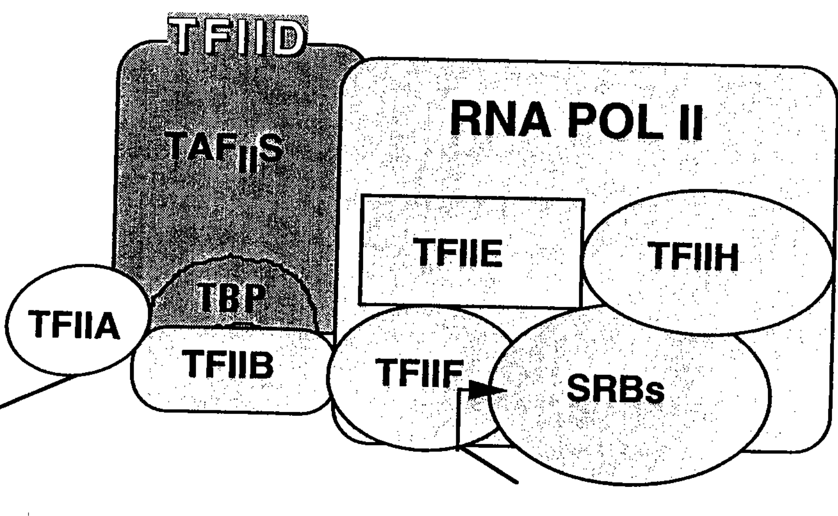


Figure 1-1. Alternative pathways to PIC formation.

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 it's 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 (TAF_{II}s) (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 TAF_{II}s 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 *hsp*70 promoter destabilized the TFIID/DNA complex, but had no effect on TBP binding (Verrijzer *et al.* 1995).

Drosophila $TAF_{II}150$ ($dTAF_{II}150$) is the primary subunit of TFIID believed responsible for the extended DNA contacts. Although no human homologue for $dTAF_{II}150$ 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 $dTAF_{II}150$ binds specifically to an area which includes the initiator and downstream sequences. (Verrijzer *et al.* 1994). In addition, the DNaseI footprint of $dTAF_{II}150$ on DNA resembles that of the TFIID complex. Human $TAF_{II}250$

Yeast	Human	Drosophila
$TAF_{II}130/145$	$TAF_{II}250$	$TAF_{II}230$
TSM1	?	$TAF_{II}150$
None	$TAF_{II}135$	$TAF_{II}110$
$TAF_{II}90$	$TAF_{II}100$	$TAF_{II}80$
$TAF_{II}68/61$	$TAF_{II}20/15$	$TAF_{II}30\alpha$
$TAF_{II}67$	$TAF_{II}55$	?
$TAF_{II}60$	$TAF_{II}70$	$TAF_{II}60/62$
$TAF_{II}40$	$TAF_{II}28$	$TAF_{II}30\beta$
$TAF_{II}30$/TFG3/ANC1	AF-9/ENL	?
TBP	TBP	TBP
$TAF_{II}25/23$	$TAF_{II}30$	?
$TAF_{II}19$/FUN81	$TAF_{II}18$	?
$TAF_{II}17$	$TAF_{II}32$	$TAF_{II}40/42$

Table 1-1: TFIID subunits isolated from the yeast *Saccharomyces cerevisiae* have homologues in both human and *Drosophila*

($hTAF_{II}250$) has also been shown to contact DNA (Verrijzer *et al.* 1995). Furthermore, reconstitution experiments have shown that both $dTAF_{II}150$ and $hTAF_{II}250$ 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 $TAF_{II}s$ 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 $yTAF_{II}145$, the homologue of Drosophila $dTAF_{II}250$, is required for transcription from promoters containing non-consensus TATA boxes (Moqtaderi *et al.* 1996a). These authors found that upon depletion of $yTAF_{II}145$ (also called $yTAF_{II}130$), 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 $yTAF_{II}145$/$yTAF_{II}130$ may be required for the transcription of genes containing weak TATA boxes. However, while $yTAF_{II}145$ may be required for transcription of some genes whose promoters contain non-consensus TATA boxes, a weak TATA box is not the only determinant. $yTAF_{II}145$ 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 $yTAF_{II}145$ dependent *RPS5* promoter, with a consensus TATA box, does not make it $yTAF_{II}145$ 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 cyc1 phenotype associated with an aberrant ATG codon in the yeast *CYC1* 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 TFIIB. Like the addition of TFIIB, 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 *SRB*s 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 constituitively 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 TAF_{II}s (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 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_{II}s). In addition to the TAF_{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 where 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_{II}s (Sauer *et al.* 1995a,b). Despite the plethora of *in vitro* data, clear *in vivo* data supporting a role for TAF_{II}s as coactivators is lacking. This deficiency compelled our group and others to analyze the function of TAF_{II}s *in vivo*. The data generated thus far both supports and contradicts the role of TAF_{II}s as coactivators *in vivo*.

Tjian and colleagues have presented data supporting a coactivator function for TAF_{II}s during Drosophila eye development. These authors found that the presence of transdominant alleles of $dTAF_{II}60$ and $dTAF_{II}110$ 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_{II}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_{II}s did not lead to a global transcriptional defect or to developmental defects in the Drosophila embryo. In contrast, genetic disruption of *Sp1*, an activator believed to function via an interaction with $dTAF_{II}110$, caused severe developmental defects in mice. Embryos in which *Sp1* had been inactivated died at around day eleven of gestation (Marin *et al.* 1997).

In mammalian cells, overexpression of $hTAF_{II}28$ enhanced transcription activation directed by the viral activator Tax (Caron *et al.* 1997). $hTAF_{II}28$ overexpression also partially alleviated the squelching observed when Tax was overexpressed. $hTAF_{II}28$ 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.

$hTAF_{II}28$ 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). $hTAF_{II}28$ 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 $hTAF_{II}28$ and a nuclear receptor could be detected. Transcription activation did, however, depend on the ability of $hTAF_{II}28$ to interact with TBP.

Overexpression of $hTAF_{II}135$ was also shown to potentiate ligand induced transcription activation by a subset of nuclear receptors *in vivo* (Mengus *et al.* 1997). Unlike $hTAF_{II}28$, $hTAF_{II}135$ enhanced activation of mainly the class II nuclear receptors. $hTAF_{II}135$ 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 $hTAF_{II}135$ was overexpressed in these cells, ligand induced transcription activation was obtained in the presence of the fusion proteins. The ability of $hTAF_{II}135$ to enhance transcription was specific for the class II nuclear receptors. Enhancement was not obtained when $hTAF_{II}135$ was overexpressed in the presence of fusion proteins containing a number of unrelated transcription activation domains. These results demonstrate that $hTAF_{II}135$ enhances transcription activation of only a subset of activators, although an interaction between $hTAF_{II}135$ and an activator could not be detected. There is no known homologue for $hTAF_{II}135$ in yeast, however, a yeast homologue has been identified for $hTAF_{II}28$. It will be interesting to determine if this $yTAF_{II}$ is required for activation by class I nuclear receptors in yeast.

While the majority of data generated in higher eukaryotes supports a role for $TAF_{II}s$ as bridging factors or coactivtors, recent results in yeast have challenged this belief. Yeast cells lacking the function of any one of a number of $TAF_{II}s$ fail to display defects in global

transcription activation (Apone *et al.* 1996; Walker *et al.* 1996; Moqtaderi *et al.* 1996a). Even when the largest subunit of the yeast TFIID complex, $yTAF_{II}145$, is conditionally depleted, no global transcriptional defects are observed (Walker *et al.* 1996; Moqtaderi *et al.* 1996a). This result is surprising since $yTAF_{II}145$ 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_{II}s results in an inability to progress through the cell cycle. The loss of $yTAF_{II}145$ results in a G1 arrest (Walker *et al.* 1996), while the loss of $yTAF_{II}90$ 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 $yTAF_{II}90$ and TSM1 mutant cells has yet to be determined. In the $yTAF_{II}145$ 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_{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 $yTAF_{II}145$-dependent gene, with that of *ADH1*, a $yTAF_{II}145$-independent gene, did not convert *CLN2* to a $yTAF_{II}145$-independent gene (Shen and Green 1997). These results indicate that the requirement for $yTAF_{II}145$ was not due to the upstream activator, but rather

the core promoter. $yTAF_{II}145$ is also required for the expression of ribosomal genes in yeast (Shen and Green 1997). The $yTAF_{II}145$-dependent region of these genes also mapped to the core promoter. These results further substantiate the idea that $yTAF_{II}145$ functions not as a coactivator, but rather to facilitate core promoter activity.

Mammalian cells containing a temperature-sensitive allele of $hTAF_{II}250$ 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 $yTAF_{II}145$. 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 $hTAF_{II}250$-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 $TAF_{II}s$ act as bridging factors by interacting with specific classes of activators was obtained from recent studies of $yTAF_{II}17$. $yTAF_{II}17$ is the homologue of human $hTAF_{II}32$ and Drosophila $dTAF_{II}40$. Both the Drosophila and human $TAF_{II}s$

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 TAF_{II} could interact with a transcriptionally inactive form of p53. $dTAF_{II}40$ 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-TAF_{II} interaction appears to be significant: the addition of anti-$dTAF_{II}40$ antibodies to an *in vitro* transcription reaction abolished transcription activation by the activation domain of VP16. Although $hTAF_{II}32/dTAF_{II}40$ 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 $hTAF_{II}32/dTAF_{II}40$ as a coactivator required for transcription activation by acidic activators. Since yeast are believed to possess only acidic activators, the yeast homologue of $hTAF_{II}32/dTAF_{II}40$ 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 TAF_{II}. Interestingly, these are not the results obtained when yeast cells were examined for their ability to activate transcription in the absence of functional $yTAF_{II}17$. Yeast

cells containing a temperature-sensitive allele of $yTAF_{II}17$ 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 $yTAF_{II}17$ 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_{II}/one activator theory.

The picture that appears to be emerging from these studies is that TAF_{II}s are not required for global transcription activation *in vivo*, but rather that specific TAF_{II}s are required for the transcription of subsets of genes. It is possible that some TAF_{II}s function as coactivators and other to facilitate core promoter activity. The identification of $hTAF_{II}250$ 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. $hTAF_{II}250$ 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 $hTAF_{II}31/hTAF_{II}32$, $hTAF_{II}80$, $hTAF_{II}15/hTAF_{II}20$ 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 Sawadago 1985) indicate that DNA topology may play an important role in TAF_{II} function. In any case, the isolation of TAF_{II}s 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 TAF_{II}s.

Although TAF_{II}s 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 TAF_{II}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 TAF_{II}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 TAF_{II}s. 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 nucleosomal 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 *tsm1*. I am also responsible for the cell-cycle analysis of strains containing

temperature-sensitive alleles of *tsm1*. Scott Walker is responsible for the transcription and cell-cycle analysis of strains containing conditional alleles of *yTAF$_{II}$145* and Joseph Reese for the analysis of transcription in cells containing conditional alleles of *yTAF$_{II}$68*, *yTAF$_{II}$47*, and in strains in which *yTAF$_{II}$30* has been deleted.

In chapter four, I analyze the *in vivo* function of yTAF$_{II}$17. I am responsible for the cloning, and genetic disruption of *yTAF$_{II}$17*. I am also responsible for the analysis of transcription in cells containing a temperature-sensitive allele of *yTAF$_{II}$17*. Under my guidance, Jing Wang and Lijian Chiang are responsible for the isolation of temperature-sensitive alleles of *yTAF$_{II}$17*.

In chapter five I am responsible for the generation of *yTAF$_{II}$90* temperature-sensitive strains containing temperature-sensitive alleles of *CDC28* and those in which *RAD9* has been genetically deleted. In addition, I am responsible for all work presented in appendix A: namely the subtractive hybridization performed on strains containing wild-type and temperature-sensitive alleles of *yTAF$_{II}$90*.

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

CHAPTER II

Yeast $TAF_{II}90$ 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_{II}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_{II}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_{II}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.

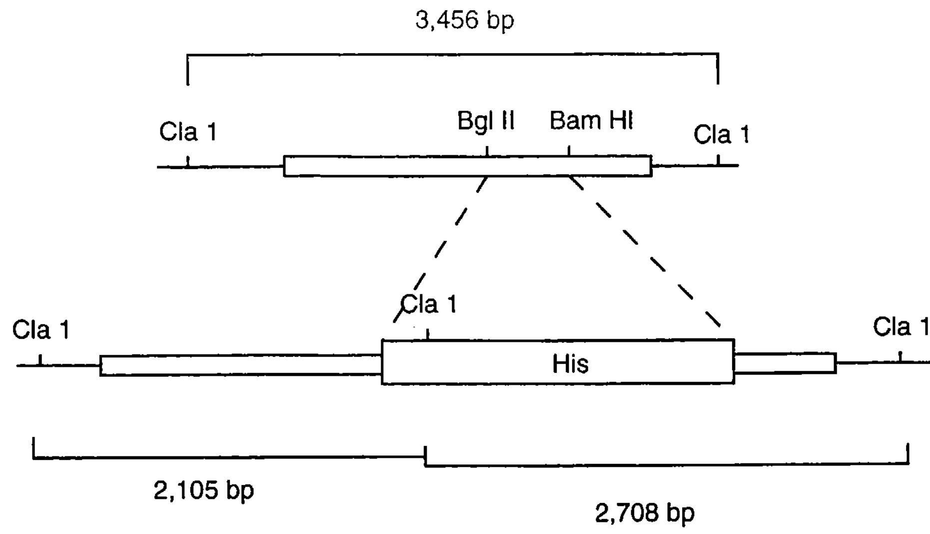
The first $yTAF_{II}$ identified by our group was $yTAF_{II}90$. $yTAF_{II}90$ has been highly conserved throughout evolution and is the homologue of $hTAF_{II}100$ and $dTAF_{II}80$ (Burley and Roeder 1996). In this chapter we describe the *in vivo* analysis of $yTAF_{II}90$. Interestingly, and unexpectedly, $yTAF_{II}90$ 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, $yTAF_{II}90$ 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 $yTAF_{II}90$ 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 $yTAF_{II}90$ temperature-sensitive strains grew at 25°C but not 37°C. Figure 2-2B shows that in liquid culture

A



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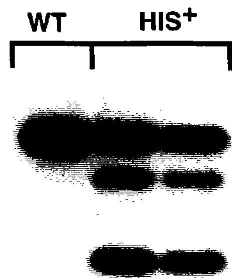

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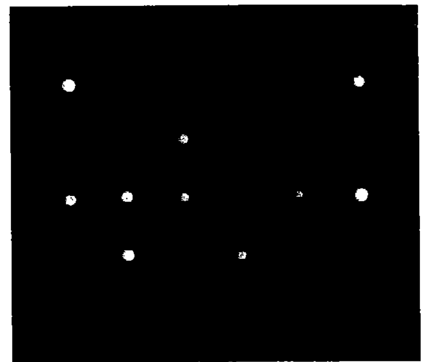


Figure 2-1. $yTAF_{II}90$ is essential for viability.

(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

37°C

WT

ts3-1

ts2-1

WT

ts3-1

ts2-1

B

wild type

ts2-1

ts3-1

OD_{660}

Shift

TIME (hrs)

0 2.5 5 7.5 10 12.5

0 5 10 15

C

```
                                   ts2-1     ts3-1
                                     D        ES
yTAF_II90   678   WDIGTGKRLKQMRGHGKNAIYSLSYSKEGNVLISGGADHTVRVWD   721
dTAF_II80   604   WDLSNGSLVTTLLRHT.STVTTITFSRDGTVLAAAGLDNNLTLWD   646

            722   LKKATTEPSAEPDEPFIGYLGDVTASINQDIKEYGRRRTVIPTSD   767
            647   FHKVT........EDYIS..NHITVSHHQDEND........EDVY   673
                                     ts2-1
                                       R
            768   LVASFYTKKTPVFKVKFSRSNLALAGGAFRP   798
            674   LMRTFPSKNSPFVSLHFTRRNLLMCVGLFKS   704
```

Figure 2-2. Temperature-sensitive alleles of $yTAF_{II}90$.

(A) Growth of strains containing wild-type (LY3) and temperature-sensitive alleles of *yTAF90* (ts2-1 and ts3-1) at 25°C and 37°C.
(B) Growth curve of wild-type and $yTAF_{II}90$ temperature-sensitive strains at 37°C.
(C) Sequence of $ytaf_{II}90$ temperature-sensitive alleles. The segment of the $yTAF_{II}90$ WD40 domain containing the temperature-sensitive mutations is compared with the corresponding sequence in the Drosophila homologue $dTAF_{II}80$. Mutated residues are marked with a dot. The amino acid changes and the strains containing the mutations are indicated above.

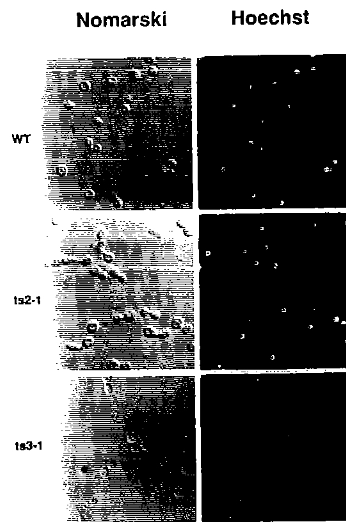
these strains displayed a rapid growth-arrest upon transfer from the permissive to the non-permissive temperature. Even at the permissive temperature the $yTAF_{II}90$ mutant strains grew more slowly than wild-type (data not shown).

Sequencing of the mutants identified double-point substitutions in each of the two alleles. $yTAF_{II}90^{ts2-1}$ (strain LY20) contains an asparagine substituted for a serine at amino acid 703 and an arginine substituted for a glycine at position 793; $yTAF_{II}90^{ts3-1}$ (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

A



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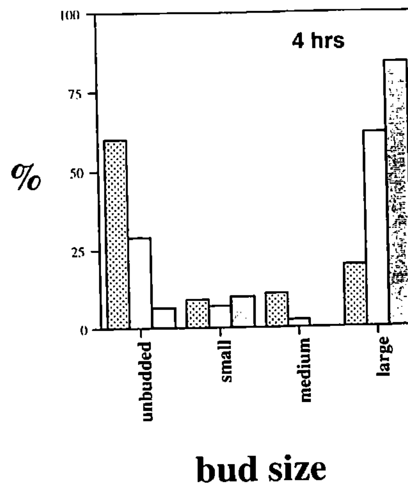

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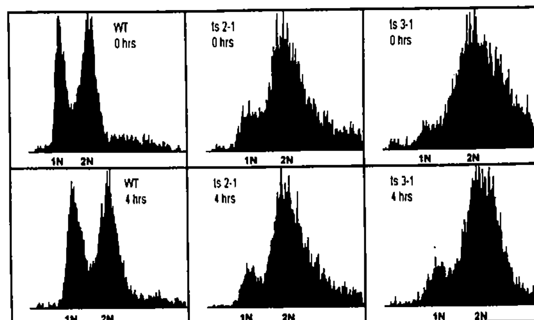


Figure 2-3. Cell cycle phenotype of strains bearing temperature-sensitive alleles of $yTAF_{II}90$.

(A) Nomarski optics or Hoechst staining was used to visualize the morphology and DNA, respectively, of wild-type and strains harboring temperature-sensitive $yTAF_{II}90$ 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 $yTAF_{II}90$ 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 1*N* content of DNA, while the majority of ts2-1 cells and virtually all of the ts3-1 cells contained a 2*N* 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.

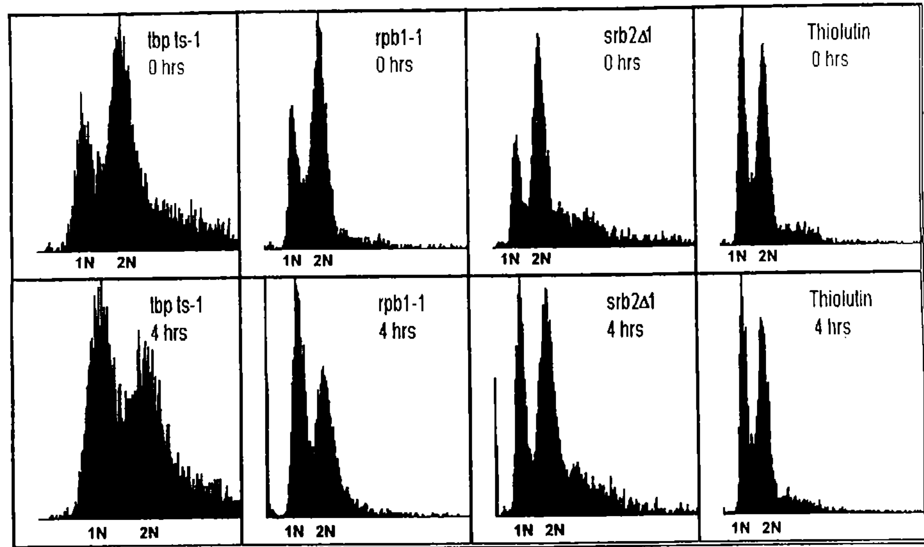
Even at the permissive temperature it was evident that a much greater than normal percentage of mutant cells had a 2*N* DNA content. The inability of these cells to efficiently progress through G2 helps explain their slow growth phenotype and in addition indicates that $yTAF_{II}90$ function is impaired even under permissive conditions. Lack of viability at higher temperatures could be explained either by a complete loss of $yTAF_{II}90$ 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 yTAF$_{II}$90 mutants is also not readily explained by

A



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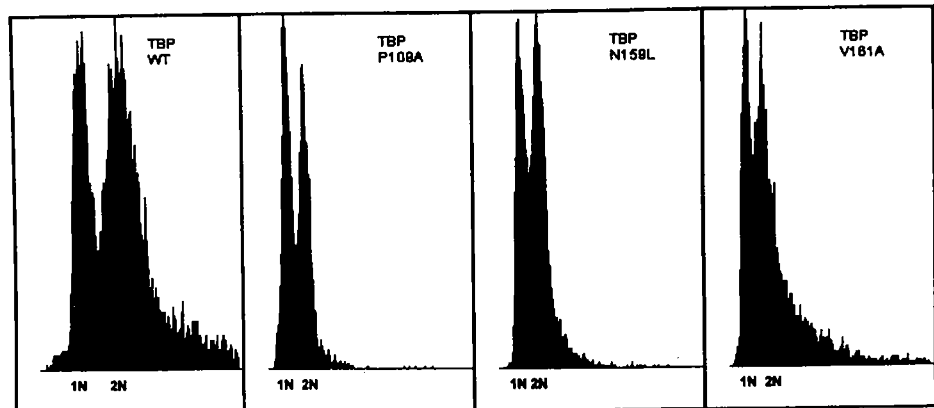


Figure 2-4. Inhibition of RNA polymerase II-directed transcription does not lead to a cell cycle phenotype.

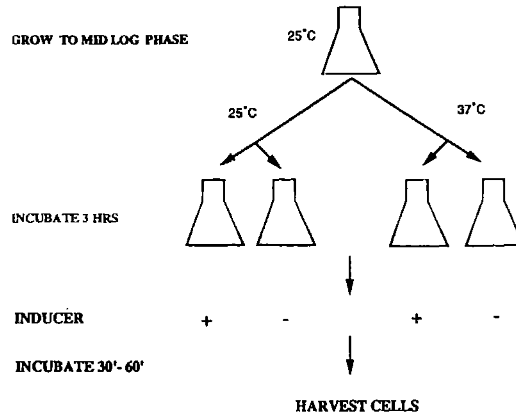
(A) FACS analysis was performed on strains before and 4 hrs after transfer from 25°C to 37°C, or 4 hrs after addition of thiolutin to wild-type strain CY245.
(B) FACS analysis was performed on strains harboring activation-defective TBP mutants grown to mid-log phase at 30°C.

an activation-specific defect in RNA polymerase II-directed transcription.

Numerous experiments have shown that *in vitro* TAF_{II}s are required for activated, but not basal, transcription (Burley and Roeder 1996). We therefore sought to determine whether cells bearing the $yTAF_{II}90$ 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°C). Under non-permissive conditions (37°C), the wild-type (LY3), but not the RNA polymerase II mutant strain (rpb1-1), supported *GAL10* transcription. Significantly, in both $yTAF_{II}90$ temperature-sensitive strains (ts2-1 and ts3-1) *GAL10* was transcriptionally activated at 37°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 $yTAF_{II}90$ was required for the function of another, unrelated activator. The acidic

A



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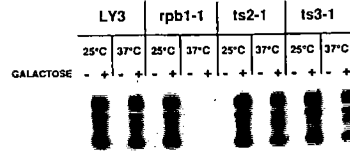

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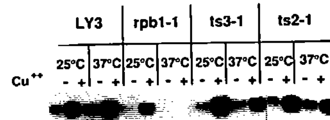

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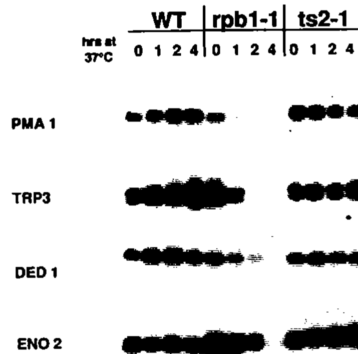


Figure 2-5. Transcription in strains bearing ytaf$_{II}$90 temperature-sensitive mutants.

(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 100um 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.

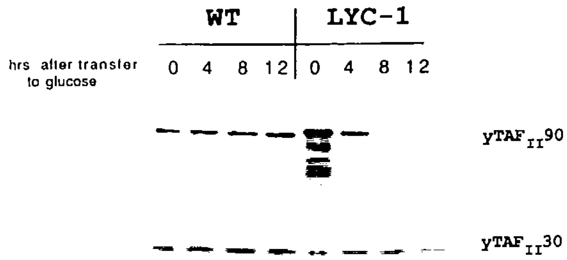
Figure 2-5D analyzes four other endogenous genes whose transcription is controlled by a diverse set of activators: *PMA1* is regulated by the activators MCM1 and RAP1 (Capieaux et al. 1989; Kuo & Grayhack 1994); *TRP3* is regulated by the activator GCN4 (Aebi *et al* 1984; Hope and Struhl 1985; Arndt and Fink 1986); *DED1* is under the control of the activator ABF1 (Buchman and Kornberg 1990); and *EN02* is glucose-regulatable (Johnston and Carlson 1992). Once again, transcription of these genes was unaffected following inactivation of $yTAF_{II}90$. We conclude that in vivo $yTAF_{II}90$ is dispensable for normal transcription of many yeast genes.

Immunoblot analysis of strains bearing the $yTAF_{II}90$ temperature-sensitive mutants revealed that at the non-permissive temperature these strains contained significant levels of $yTAF_{II}90$ as well as several other $yTAF_{II}s$ (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 transription 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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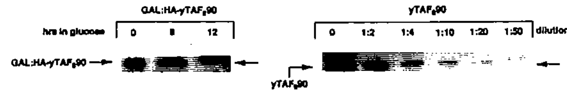

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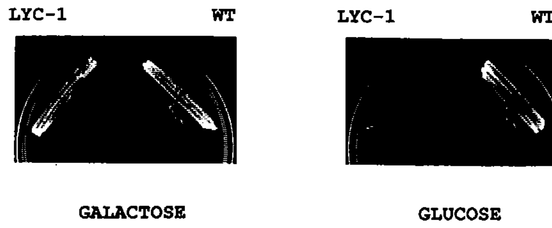

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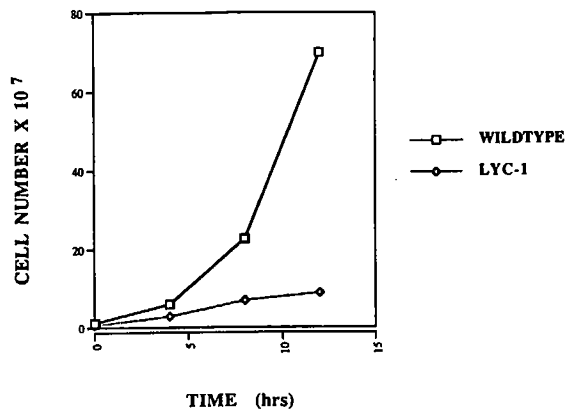
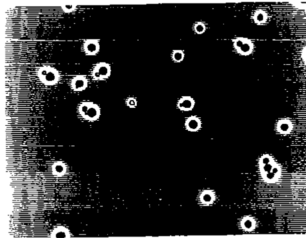


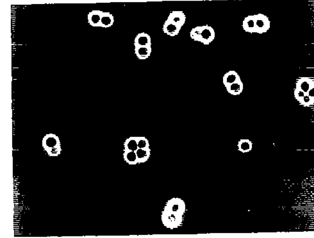
Figure 2-6. Characterization of strains conditionally expressing $yTAF_{II}90$.

(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.

A

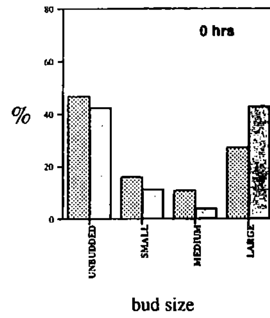


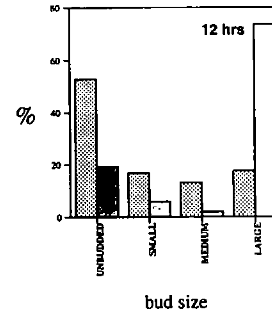
WT



LYC1

B





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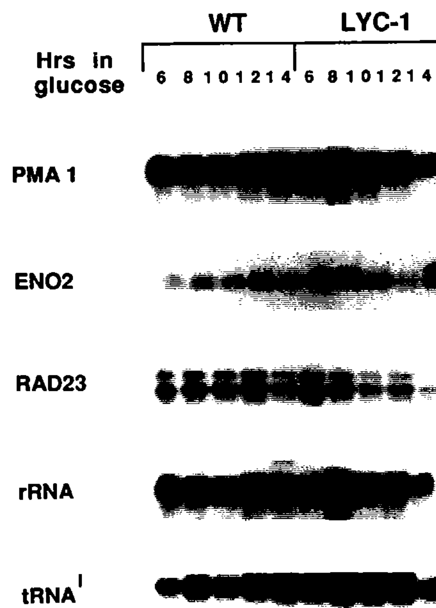


Figure 2-7. Cell cycle and transcriptional properties of strains conditionally expressing $yTAF_{II}90$.

(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 $yTAF_{II}90$ 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 $yTAF_{II}90$ (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 $yTAF_{II}90$ 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 yTAF$_{II}$90 mutant strains described here (Stoler et al. 1995). The Northern blot of Figure 2-8 shows that transcription of *CLB2* and *CSE4*, and as a control, *CLN3*, which encodes a constitutively expressed G1 cyclin (Nash et al. 1988), was unaffected following temperature-sensitive inactivation of yTAF$_{II}$90. Thus, failure to transcribe the *CLB2* and *CSE4* genes cannot explain the cell cycle arrest phenotype of strains harboring yTAF$_{II}$90 mutants.

A possible explanation for the lack of a transcriptional effect in the above experiments was that yTAF$_{II}$90 was not a component of an RNA polymerase II PIC. To address this possibility we performed both biochemical and *in vivo* transcription experiments. First, we asked whether yTAF$_{II}$90 was associated with TBP and other TAF$_{II}$s. The immunoprecipitation experiment of Figure 2-9A shows that an α-yTAF$_{II}$90 antiserum immunoprecipitated TBP and yTAF$_{II}$145, the yTAF$_{II}$ that directly contacts TBP (Reese et al. 1994). Likewise, an α-yTAF$_{II}$145 antiserum immunoprecipitated both

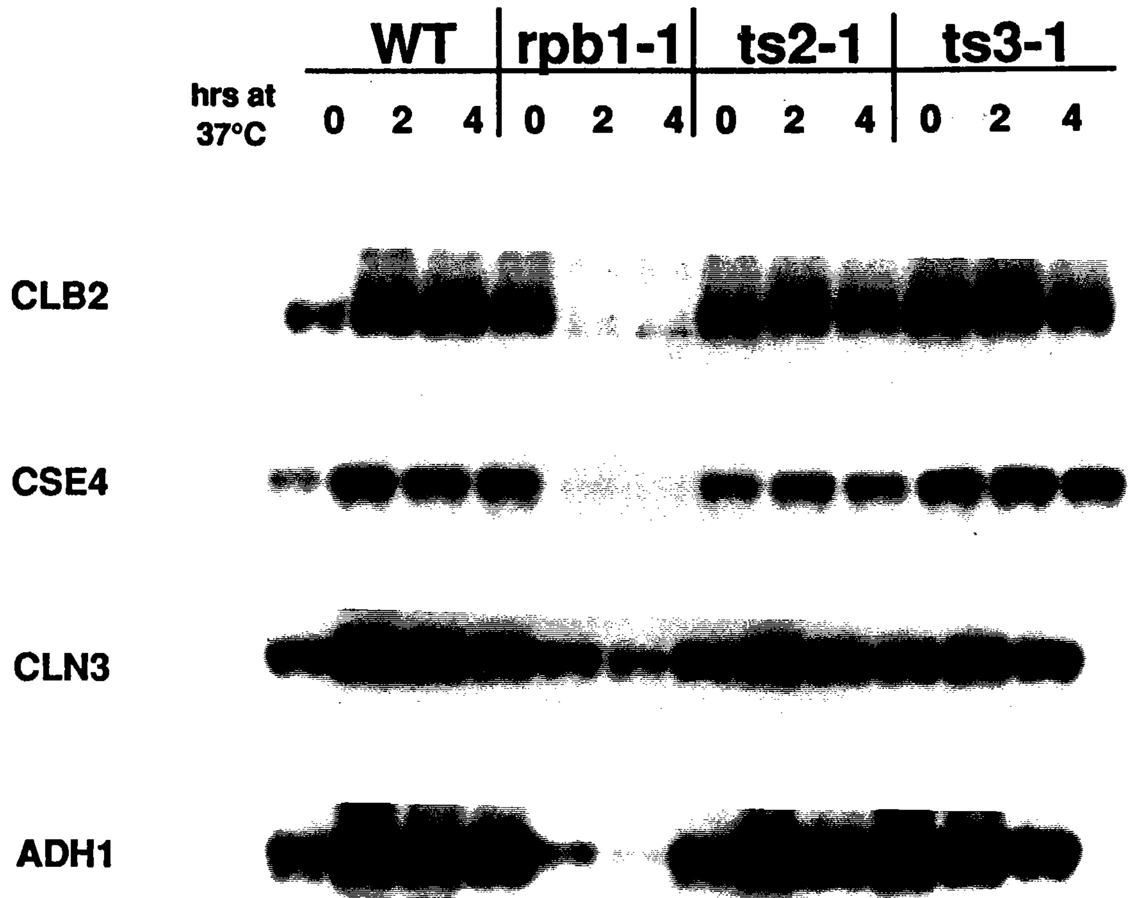
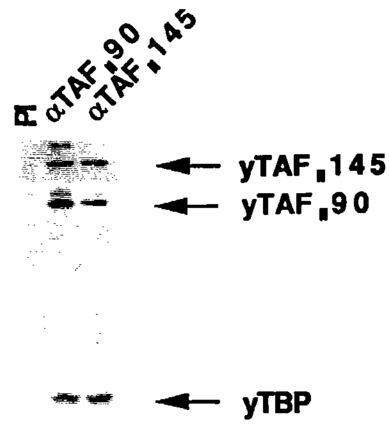

WT
rpb1-1
ts2-1
ts3-1
hrs at 37°C
0 2 4 0 2 4 0 2 4 0 2 4
CLB2
CSE4
CLN3
ADH1

Figure 2-8. Transcription of genes required for progression through G2/M following $yTAF_{II}90$ inactivation. Transcription of the indicated genes was measured by Northern blot analysis.

A



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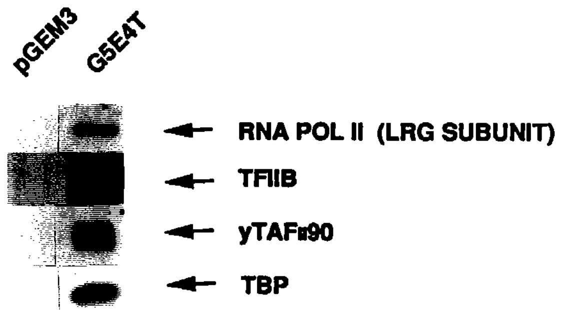

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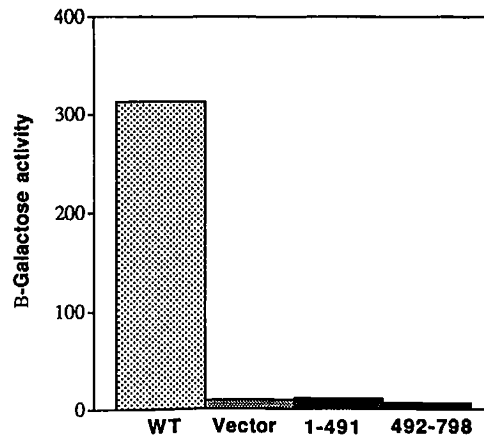

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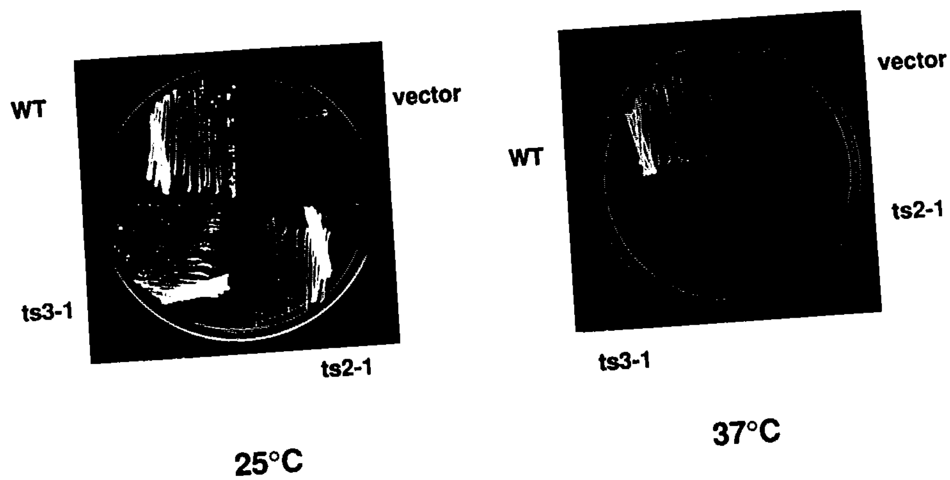

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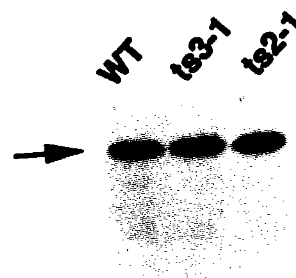


Figure 2-9. $yTAF_{II}90$ is a component of the PIC and can activate transcription as a LexA fusion protein.

(A) Immunoprecipitation analysis. Immunoprecipitates formed with α-$yTAF_{II}90$ or α-$yTAF_{II}145$ antisera were analyzed by immunoblotting for the presence of TBP, $yTAF_{II}90$, and $yTAF_{II}145$.
(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-$yTAF_{II}90$ 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 $yTAF_{II}90$ or amino-terminal (1-491) or carboxy-terminal (492-798) portions of $yTAF_{II}90$ were analyzed for β-galactosidase activity.
(D) LexA-$yTAF_{II}90$ fusion proteins containing wild-type or temperature-sensitive alleles of $yTAF_{II}90$ 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-$yTAF_{II}90$ 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 irrelevanat 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 $yTAF_{II}90$ 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-$yTAF_{II}90$ fusion-protein activated transcription, whereas the LexA DNA binding domain alone (vector) did not.

It was important to rule out the possibility that $yTAF_{II}90$ 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 $yTAF_{II}90$. Significantly, neither of these $yTAF_{II}90$ fragments had $yTAF_{II}90$ function (data not shown). Figure 2-9C shows that whereas the LexA fusion of full-length $yTAF_{II}90$ activated transcription, the N- and C-terminal derivatives did not. These results strongly suggest that activated transcription by LexA-$yTAF_{II}90$ was not due to a cryptic activation domain in $yTAF_{II}90$.

The results of Figure 2-9D provide additional support for this conclusion. LexA-$yTAF_{II}90^{ts2-1}$ and LexA-$yTAF_{II}90^{ts3-1}$ 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 $yTAF_{II}90$ 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-$yTAF_{II}90$ fusion-proteins described above were expressed at comparable levels. Taken together, these results indicate that *in vivo* $yTAF_{II}90$ is a component of a PIC and further suggest that the molecular defect of the $yTAF_{II}90$ 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 $TAF_{II}s$ 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 TAF_{II}, $yTAF_{II}90$, 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 $yTAF_{II}90$, which was

decreased greater than 98% by glucose-depletion (Figure 2-6B and data not shown). The maximal 120 copies of $yTAF_{II}90$ 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 $yTAF_{II}90$ 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 $TAF_{II}90$ can support transcription directed by several types of activators (Chen *et al.* 1994b; Sauer *el al.* 1995a,b).

Our results are also not inconsistent with the limited *in vivo* analysis of other $TAF_{II}s$ in higher eukaryotes. For example, mammalian cell lines harboring a temperature-sensitive $TAF_{II}250$ 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 $TAF_{II}250$ 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 $yTAF_{II}90$ at a specific stage of the cell cycle. Significantly, an identical cell cycle phenotype was observed when $yTAF_{II}90$ was inactivated by two independent strategies:

temperature-sensitive inactivation or conditional depletion. We emphasize that the cell cycle phenotype resulting from $yTAF_{II}90$ 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-deficive 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 $yTAF_{II}s$ 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 transcripion 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 soley 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 TAF_{II}s

In vitro, the TFIID complex is required for activator function. Results presented in the previous chapter, however, indicate that not all TAF_{II}s are required for transcription activation *in vivo*.

The TFIID complex is composed of 8-12 TAF_{II}s. Therefore, while $yTAF_{II}90$ did not possess coactivator activity *in vivo*, it is possible that one or more of the remaining TAF_{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_{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_{II}s, including $TAF_{II}145$, which is believed to be a structurally important component of the complex. However, while the cells were competent to activate transcription, inactivation of $TAF_{II}145$ or TSM1 resulted in an inability to progress through the cell cycle.

Results

The TBP-associated factor $yTAF_{II}145$ is the homologue of higher eukaryotic $TAF_{II}250$, and is the only $yTAF_{II}$ known to directly contact TBP (Reese *et al.* 1994). For these and other reasons, $yTAF_{II}145/TAF_{II}250$ 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

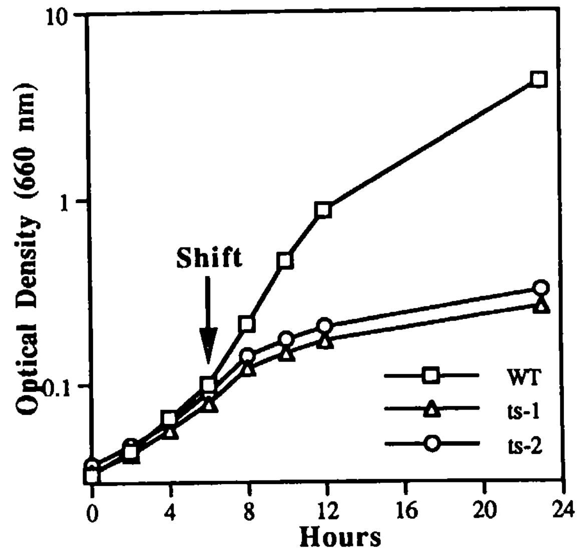
$yTAF_{II}145$, which is encoded by an essential gene (Reese *et al.* 1994; Poon *et al.* 1995). Cells harboring these $yTAF_{II}145$ mutant alleles did not grow at 37°C and displayed a rapid cessation of cell division upon shifting from 23°C to the non-permissive temperature (Figure 3-1A). Immunoblot analysis (Fig. 3-1B) shows that when shift to 37°C, the level of $yTAF_{II}145$ rapidly decreased and by four hours was undetectable in both temperature-sensitive strains. The levels of several other $yTAF_{II}s$, and to a lesser extent yTBP, also decreased. In contrast, components that are not part of the $yTAF_{II}$ complex, such as Sua7 (yTFIIB), were unaffected by the temperature shift. These data suggest that loss of $yTAF_{II}145$ disrupts the $yTAF_{II}$ complex, resulting in the degradation of other $yTAF_{II}s$.

To investigate the consequences of $yTAF_{II}$ inactivation on transcription, we initially examined strains bearing temperature-sensitive mutations in $yTAF_{II}145$ or TSM1, which is encoded by an essential gene and is the homologue of the higher eukaryotic $TAF_{II}150$ (Verrijzer *et al.* 1994), or strains in which $yTAF_{II}30$ have been deleted. The gene encoding $yTAF_{II}30$, *ANC1*, is not essential, but cells lacking $yTAF_{II}30$ grow slowly and are inviable at 37°C (Welch *et al.* 1993).

Yeast strains containing these TAF_{II} mutants or, as controls, a temperature-sensitive GTF (TBP or RNA polymerase

Figure 3-1

A



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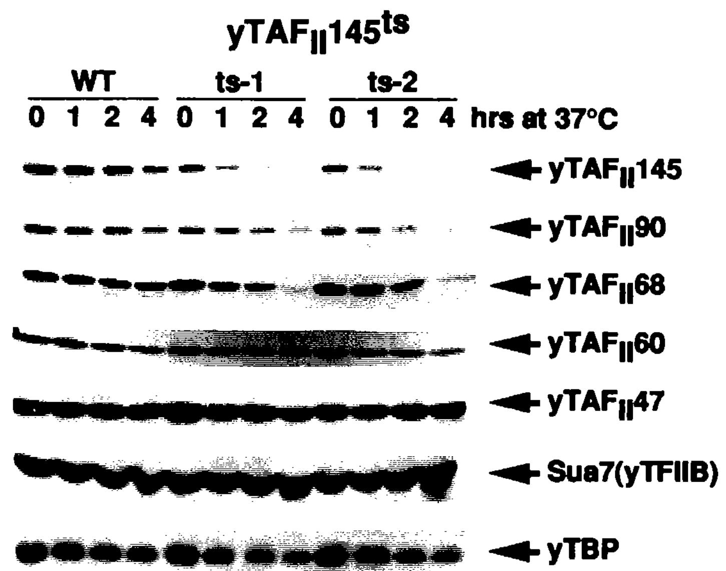


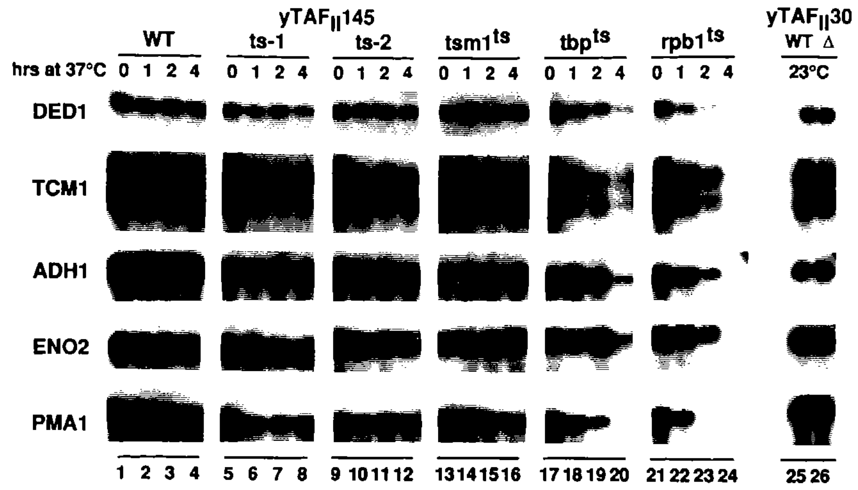
Figure 3-1. Temperature-sensitive $yTAF_{II}145$ mutants.

(A) Rapid cessation of cell growth follows shifting yeast strains containing temperature sensitive alleles of $yTAF_{II}145$ from 23°C to 37°C.
(B) Immunoblot of extracts from wild-type and $yTAF_{II}145$ 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 $yTAF_{II}145$ (lanes 5-12), TSM1 (lanes 13-16) or in the strain deleted of $yTAF_{II}30$ (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 $yTAF_{II}s$ might be required for the initiation, but not maintenance, of a transcriptionally active state. To examine this possibility we tested whether $yTAF_{II}$ inactivation affected the inducible transcription of the yeast copper metallothionein gene, *CUP1*. Transcriptional induction of *CUP1* is extremely rapid and involves the direct

A



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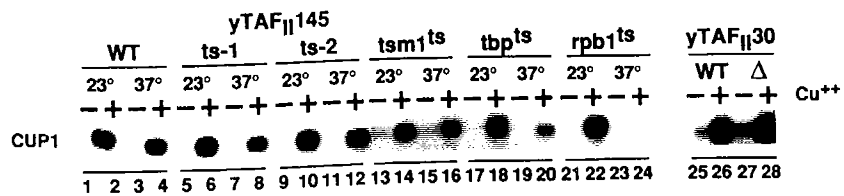


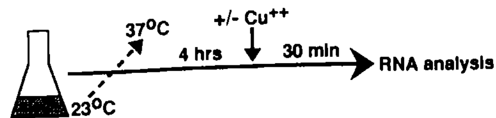
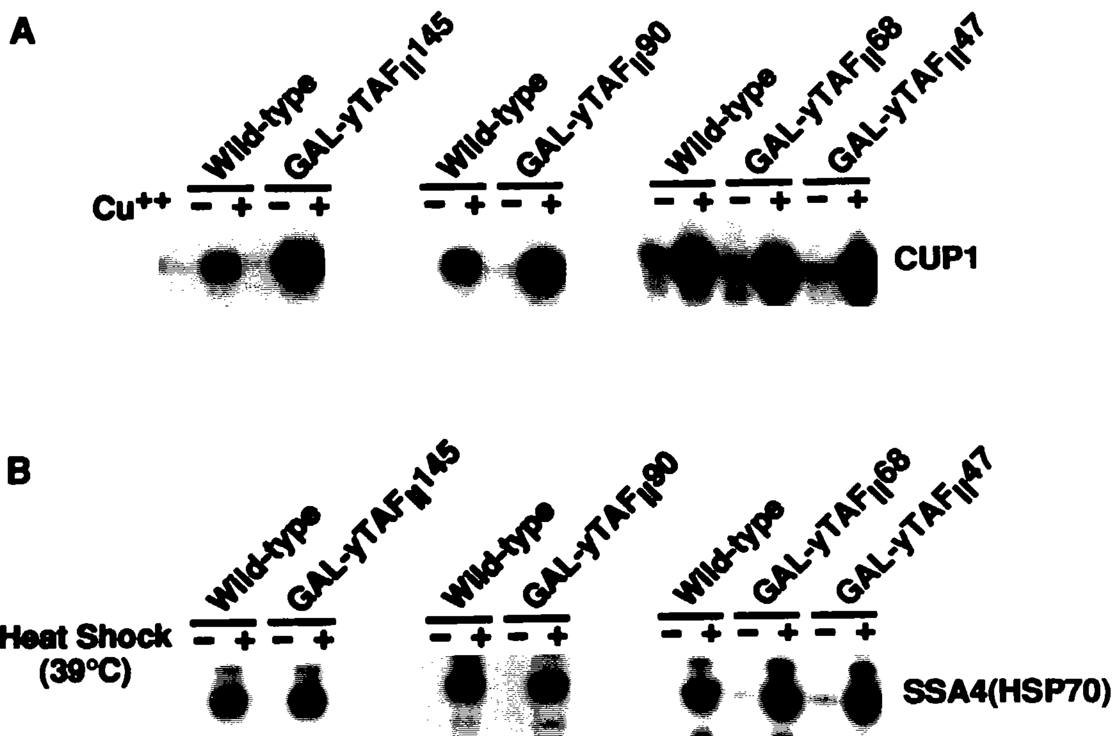


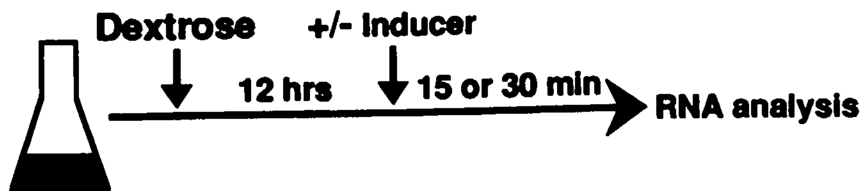
Figure 3-2. Unabated transcription after $yTAF_{II}$ inactivation.

(A) Transcription of several endogenous, chromosomal genes. For the $yTAF_{II}$, 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 $yTAF_{II}30$ 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 $yTAF_{II}$ mutant strains. The experimental scheme is shown below the autoradiogram. Growth and induction of CUP1 transcription in $ytaf_{II}30$ deletion and wild-type strains was performed at 23°C.

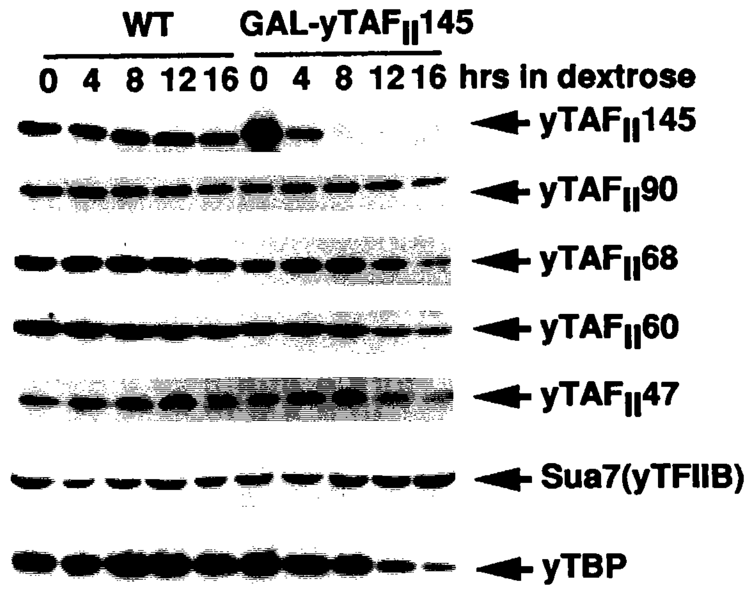
binding of Cu^{++} to the acidic activator ACE1, converting it to an active form (Butt *et al.* 1984; Furst *et al.* 1988). Cultures of cells containing temperature-sensitive mutations in $yTAF_{II}145$, TSM1, TBP, or RNA polymerase II were shifted to the non-permissive temperature for four hours, exposed 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 $yTAF_{II}$ mutations ($ytaf_{II}145^{ts}$ 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 $ytaf_{II}30\Delta$ strain (lanes 25-28).

To confirm and extend the results with the temperature-sensitive $yTAF_{II}$ mutants, we depleted individually four $yTAF_{II}s$ ($yTAF_{II}145$, $yTAF_{II}90$, $yTAF_{II}68$, and $yTAF_{II}47$) by using another experimental strategy. Of these, $yTAF_{II}145$ and $yTAF_{II}90$ have been previously described (Reese *et al.* 1994), but $yTAF_{II}47$ and $yTAF_{II}68$ are new $yTAF_{II}s$, cloned on the basis of microsequence analysis of the corresponding subunits of

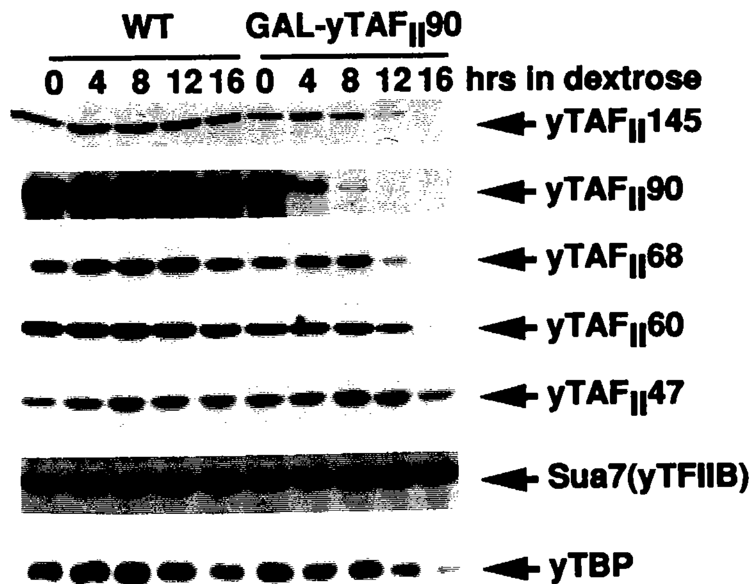

A
Wild-type
GAL-yTAFII145
Wild-type
GAL-yTAFII90
Wild-type
GAL-yTAFII68
GAL-yTAFII47
Cu++
− + − + − + − + − + − + − +
CUP1
B
Wild-type
GAL-yTAFII145
Wild-type
GAL-yTAFII90
Wild-type
GAL-yTAFII68
GAL-yTAFII47
Heat Shock
(39°C)
− + − + − + − + − + − + − +
SSA4(HSP70)


Dextrose
+/- Inducer
12 hrs
15 or 30 min
RNA analysis

C



D



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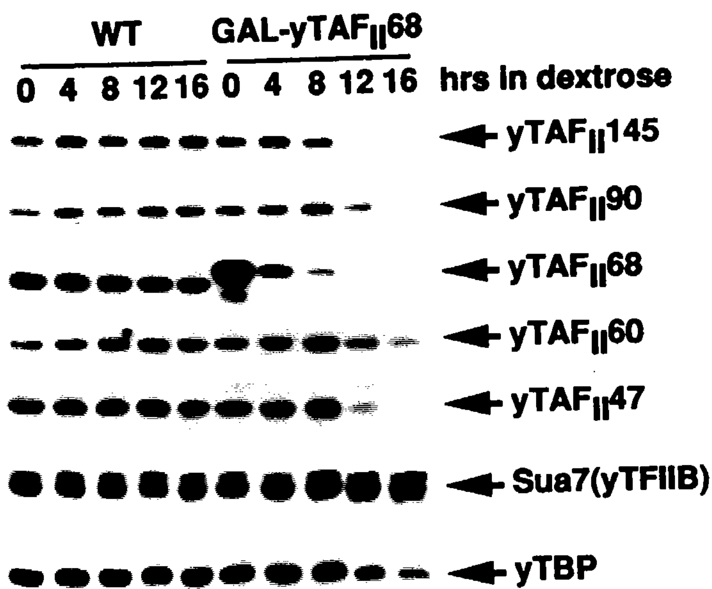

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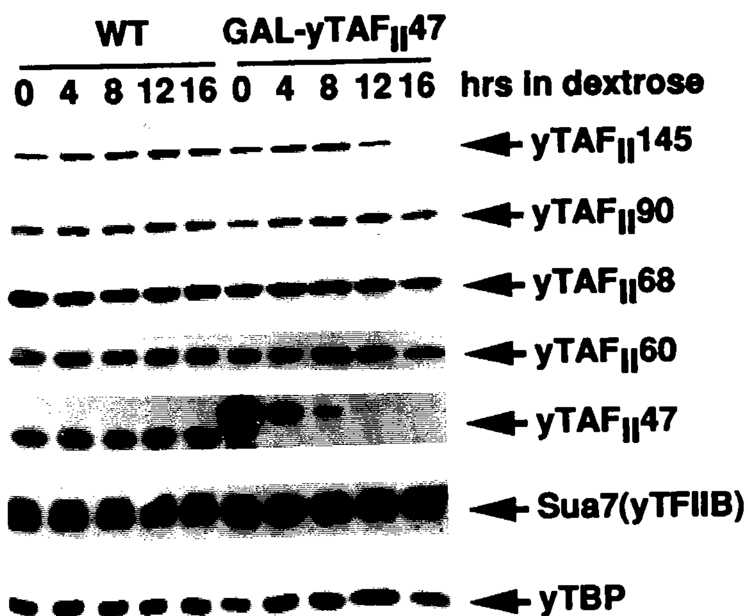


Figure 3-3. Activated transcription in cells depleted of $yTAF_{II}s$.

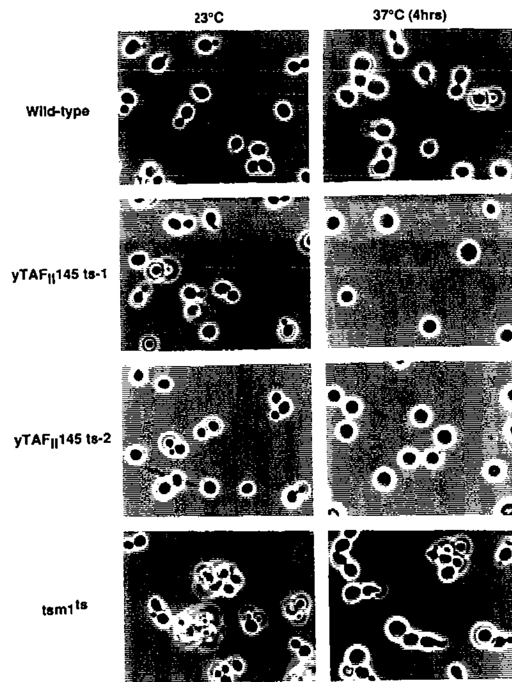
(A) Transcriptional induction of *CUP1* was measured by S1 nuclease protection 30 min. after the addition of 1mM copper sulfate to the medium. $yTAF_{II}s$ were depleted by growing the strains in glucose-containing medium for 12 hrs.
(B) Transcriptional induction of *SSA4*(*HSP70*) was measured by primer extension analysis after shifting the cultures to 39°C for 15 min. $yTAF_{II}s$ were depleted by growing the appropriate strains in glucose-containing medium for 12 hrs.
(C) Immunoblot detection of epitope (HA) tagged $yTAF_{II}s$ in wild-type ($ytaf_{II}145$::LEU2/$pyTAF_{II}145$ [ARS/CEN]), and strain YSW94 ($ytaf_{II}145$::LEU2/trp3::pSW111[GAL1p-HA-$yTAF_{II}145$-CYC_T HIS3]) or
(D) strain LYC-1 ($ytaf_{II}90$::HIS3/Lp16[ARS/CEN LEU2 GAL1p-HA-$yTAF_{II}90$]) or
(E) strain JR18 ($ytaf_{II}68$::hisG-URA-hisG/trp3::pJE20[GAL1p-HA-$yTAF_{II}68$-CYC_T HIS3]) or
(F) strain JR11 ($ytaf_{II}47$::hisG-URA3-hisG/trp3::pJR15[GAL1p-HA-$yTAF_{II}47$-CYC_T 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 $yTAF_{II}$ shut-off (Fig. 3-3A, B, bottom). In cells in which $yTAF_{II}145$, $yTAF_{II}90$, $yTAF_{II}68$ or $yTAF_{II}47$ 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 $yTAF_{II}145$, $yTAF_{II}90$, $yTAF_{II}68$, or $yTAF_{II}47$ were examined for transcriptional induction of the *SSA4* gene (Boorstein and Craig 1990). For each strain, $yTAF_{II}$ depletion did not diminish transcriptional activation (Fig. 3-3B).

Although temperature-sensitive inactivation of $yTAF_{II}145$ 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 $yTAF_{II}s$ enabled us to gain insight into the nature of the growth arrest. When the $ytaf_{II}145^{ts}$ 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 $tsm1^{ts}$ strain arrested as budded cells characteristic of the G2/M phase (Fig. 3-4A). To delineate the cell cycle block

A



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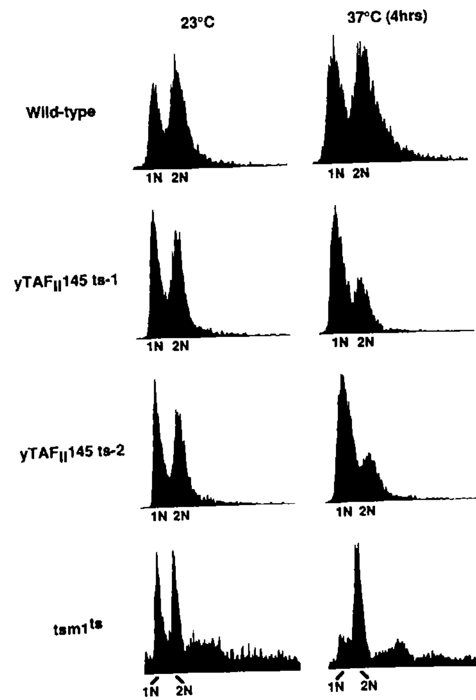


Figure 3-4. Inactivation of $yTAF_{II}145$ and TSM1 leads to distinct cell cycle phenotypes.

(A) Phase-contrast micrographs of wild-type, $ytaf_{II}145$ and tsm1 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, $ytaf_{II}145$ and tsm1 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). $ytaf_{II}145^{ts}$ cells arrested with a 1*N* DNA content (Fig. 3-4B), consistent with a block in G1, and analogous to the results with the mammalian $TAF_{II}250$ homologue (Nishimoto *et al.* 1982); conversely, $tsm1^{ts}$ cells had a 2*N* DNA content, consistent with a block in G2.

Discussion

Taken together, our results indicate that activated transcription can occur in the absence of multiple $yTAF_{II}s$. This conclusion is based upon two independent strategies to functionally inactivate $yTAF_{II}s$: temperature-sensitive mutations and conditional depletion. Moreover, a similar conclusion has been obtained using a third $yTAF_{II}s$ inactivation strategy (Moqtaderi et al. 1996a). Particularly compelling are the results with $yTAF_{II}145$. It is the only $yTAF_{II}$ known to contact TBP directly (Reese *et al.* 1994), and its higher eukaryotic homologue, $TAF_{II}250$, is always required to reconstitute TFIID activity *in vitro* (Chen *et al.* 1994b). Therefore, for those genes tested, $TAF_{II}s$ are not the obligatory targets of activators and so in these instances another transcription component(s), such as TFIIB or TBP (Zawel and Reinberg 1995), must serve this function.

Although inactivation of $yTAF_{II}s$ did not compromise transcription of those genes examined, inactivation of different $yTAF_{II}s$ led to distinct cell cycle phenotypes. These results are consistant 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 $yTAF_{II}s$ 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 $yTAF_{II}17$

In *vitro* TAF_{II}s function as coactivators to facilitate activated transcription. However, our *in vivo* analysis of a number of $yTAF_{II}$s 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 $yTAF_{II}$ with coactivator activity was due to our failure to analyze the TAF_{II} which interacts with acidic activators.

In higher eukaryotes $dTAF_{II}40$ and $hTAF_{II}32$ 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 $dTAF_{II}40$ and $hTAF_{II}32$ would be an ideal candidate to possess coactivator activity in yeast.

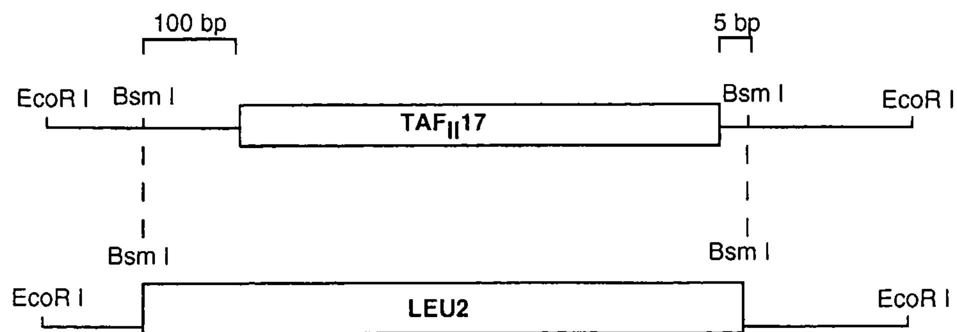
In this chapter we analyze the *in vivo* function of $yTAF_{II}17$, the homologue of $dTAF_{II}40$ and $hTAF_{II}32$. While $yTAF_{II}17$ is required for transcriptional induction of the *GAL1* gene by the activator GAL4, it is not required for global transcription activation. These results indicate that a single TAF_{II} is not the obligatory target of a specific class of activator.

Results

We began our analysis of $yTAF_{II}17$ by creating diploid strains of yeast containing one wild-type and one disrupted copy of the gene. The construct and scheme used to disrupt $yTAF_{II}17$ 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 $yTAF_{II}17$ encodes an essential gene. Furthermore, introduction of a wild-type copy of $yTAF_{II}17$ into the diploid strain resulted in near 100% viability of spores generated from this strain, demonstrating that $yTAF_{II}17$ can rescue the lethal phenotype (data not shown).

In order to study the *in vivo* function of $yTAF_{II}17$ 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

A



B

1 2 3 4 5 6

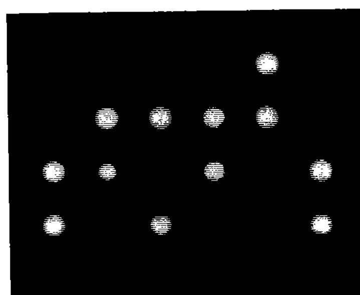
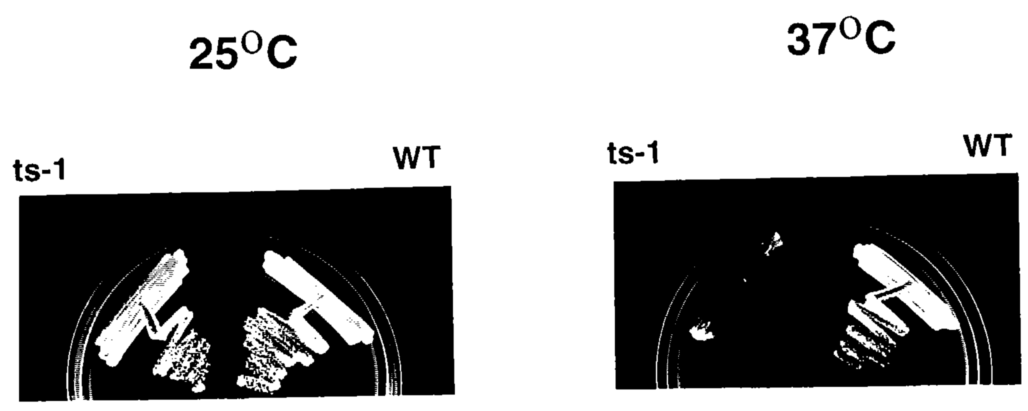


Figure 4-1. *$yTAF_{II}17$* is essential for viability.

(A) Scheme used to delete *$yTAF_{II}17$* from the yeast genome. The entire coding region of *$yTAF_{II}17$* (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 $yTAF_{II}17$ results in two viable spores per tetrad.

A



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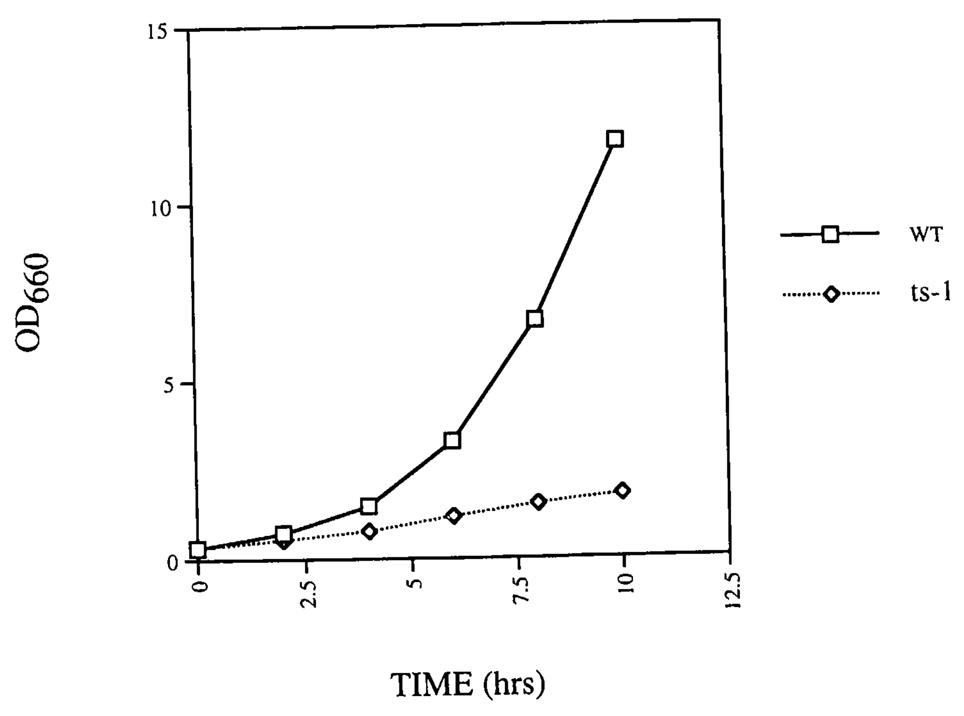


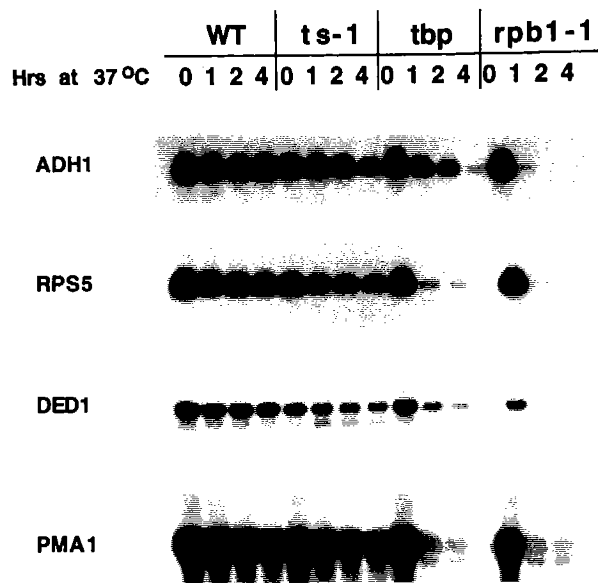
Figure 4-2. Temperature-sensitive allele of $yTAF_{II}17$.

(A) Growth of strains containing wild-type (LY90) and temperature-sensitive alleles, of $yTAF_{II}17$ (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

A



B

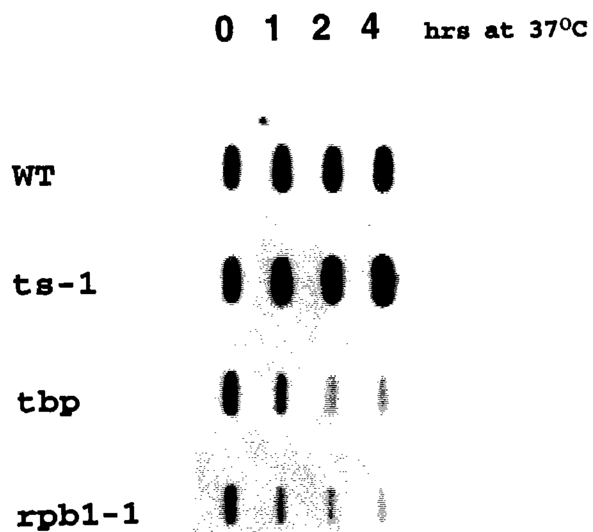


Figure 4-3. Transcription in strains containing wild-type and $yTAF_{II}17$ temperature-sensitive alleles.

(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

A

	WT		ts-1	
	25 °C	37 °C	25 °C	37 °C
GALACTOSE	- +	- +	- +	- +

GAL1

B

	WT		ts-1	
	25 °C	37 °C	25 °C	37 °C
Cu^{++}	- +	- +	- +	- +

CUP1

C

	WT		ts-1	
	25 °C	37 °C	25 °C	37 °C
3AT	- +	- +	- +	- +

HIS3

HIS4

Figure 4-4. Transcription induction in strains bearing wild-type and temperature-sensitive alleles of $yTAF_{II}17$.

(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 $yTAF_{II}17$ 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 $yTAF_{II}17$ 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 $yTAF_{II}17$ 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 $yTAF_{II}17$ 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 $yTAF_{II}17$ is not known. However, it is clear that not all acidic activators require a functional $yTAF_{II}17$ to stimulate transcription. Therefore, activators must interacting with other factors *in vivo* to facilitate transciption acitvation.

CHAPTER VI

Summary

The *in vitro* analysis of TAF_{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_{II}s (Chen *et al.* 1994b). Although numerous TAF_{II}-TAF_{II} and TAF_{II}-activator interactions have been identified, precisely how individual TAF_{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_{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_{II}s (Verrijzer *et al.* 1994; Reese *et al.* 1994; Poon *et al.* 1995; Moqtaderi *et al.* 1996a,b).

Our analysis of individual TAF_{II}s 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 TAF_{II}s, including $yTAF_{II}145$, which is believed to be an essential structural component of the TFIID complex. These results directly contradict those obtained for higher eukaryotic TAF_{II}s *in vitro*. How can these two lines of evidence be reconciled? It is possible that over time TAF_{II}s have evolved different functions in lower and higher eukaryotes. Though possible, data generated *in vivo* indicate that TAF_{II}s of higher and lower eukaryotic cells function similarly. Mammalian and yeast cells containing temperature-sensitive alleles of $hTAF_{II}250$ and $yTAF_{II}145$, 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, TAF_{II}s 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 TAF_{II} function are due to differences between, *in vitro* and *in vivo* systems and not mammalian and yeast TAF_{II}s.

The discrepancies observed may be explained by factors present *in vivo*, but lacking from *in vitro* systems, that are capable of substituting for the TAF_{II}s on most promoters. In yeast, the mediator complex is capable of substituting for the $yTAF_{II}$s 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 TAF_{II}s. However, one must keep in mind that each of the TAF_{II}s examined in this study, with the exception of *$yTAF_{II}30$*, encodes for an essential gene. Therefore, even if redundant factors exist *in vivo*, they cannot substitute for all of the TAF_{II} activities.

An alternative explanation is that TAF_{II}s function differently *in vivo* and *in vitro*. Results obtained from the analysis of strains containing temperature-sensitive alleles of $yTAF_{II}145$ indicate that this may be the case, at least for some promoters. In yeast, $yTAF_{II}145$ 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 $yTAF_{II}145$ 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 it's 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 yTAF$_{II}$145 arrest in the G1 phase of the cell cycle, while cells containing temperature-sensitive alleles of yTAF$_{II}$90 or TSM1 arrest in G2/M. These results led us to speculate that individual TAF$_{II}$s may be required for the transcription of subsets of genes, possibly those required for cell cycle progression. The analysis of strains containing temperature-sensitive alleles of yTAF$_{II}$145 revealed that they were incapable of expressing G1 and S phase cyclins at the non-permissive temperature (Walker et al. 1997). In contrast, strains containing temperature-sensitive alleles of yTAF$_{II}$90 were capable of expressing at least two genes whose expression was required for progression through G2/M, *CLB2* and *CSE4* at the non-permissive temperature. In an attempt to identify genes whose expression required yTAF$_{II}$90 a comprehensive approach was taken. First, cells in which yTAF$_{II}$90 had been inactivated by temperature-sensitive mutation were examined for their ability to transcribe randomly selected genes at the non-permissive temperature. In addition, these cells were subjected to PCR based subtractive hybridization. This technique has the potential to analyze every gene expressed in the yeast genome (see appendix A). To our surprise, neither of these strategies identified a gene that required yTAF$_{II}$90 for expression. It is possible that our inability to identify a yTAF$_{II}$90 dependent gene was due to

limitations in the techniques we employed. Alternatively, $yTAF_{II}90$ 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 $yTAF_{II}90$, 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 $yTAF_{II}90$ are viable (data not shown). These results indicate that the synthetic lethality observed between cdc28-1N and $yTAF_{II}90$ is not simply the result of combining two temperature-sensitive alleles. Furthermore, these results suggest that CDC28 and $yTAF_{II}90$ 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 $yTAF_{II}90$ 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 $yTAF_{II}90$ temperature-sensitive cells in which *RAD9* had been deleted (data not shown). Identifying the *in vivo* function of $yTAF_{II}90$ 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$_{II}$90 function.

Although transcriptional defects were not observed in the absence of any one of a number of functional TAF$_{II}$s, cells expressing a temperature-sensitive allele of yTAF$_{II}$17 were shown to exhibit a promoter specific defect at the non-permissive temperature. yTAF$_{II}$17 was analyzed specifically because of its homology to higher eukaryotic TAF$_{II}$s shown to interact with acidic activators (Moqtaderi *et al.* 1996b). Since yeast possess only acidic activators, yTAF$_{II}$17 appeared to be the perfect candidate to possess coactivator activity. Interestingly, yTAF$_{II}$17 was required for expression of the *GAL1* gene, but not the *HIS3*, *HIS4* or *CUP1* genes. These results are intriguing because they contradict the long held belief that a single TAF$_{II}$ functions by interacting with a specific class of activator. It is possible, however, that there exists another TAF$_{II}$ 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$_{II}$ which functions as a coactivator for all acidic activators. Furthermore, we can not rule out the possibility that the temperature-sensitive allele of yTAF$_{II}$17

examined in this study retained partial activity at the non-permissive temperature. $yTAF_{II}17$ contains a domain which has similarity to histone H3. In addition, the higher eukaryotic homologues of $yTAF_{II}17$, $hTAF_{II}32$ and $dTAF_{II}40$, have been shown to associate with $hTAF_{II}80$ and $dTAF_{II}60$, respectively, to form a nucleosome like structure. Since the temperature-sensitive allele of $yTAF_{II}17$ 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 $yTAF_{II}17$ 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 $yTAF_{II}17$ dependence on the *GAL1* promoter. If $yTAF_{II}17$ 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_{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_{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_{II} complexes exist in yeast.

The work presented in this thesis has forced us to reevaluate our views on TAF_{II}s and transcription activation. While it was assumed from data generated *in vitro* that the

TAF_{II}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 TAF_{II}s. In contrast, a small number of promoters have been identified which require particular TAF_{II}s for expression. In order to understand how TAF_{II}s are functioning *in vivo* it will be important to determine the elements which confer TAF_{II} dependence on these promoters. In addition, it will be important to determine if there is a common requirement for TAF_{II}-dependence, and what that requirement is. This will require the identification of additional genes that require specific TAF_{II}s for expression and the precise mapping of the elements which confer TAF_{II}-dependence.

In addition, the work presented in this thesis has led to the identification of unexpected roles for three of the TAF_{II}s in cell cycle progression. While $yTAF_{II}145$ is required for the expression of G1 and S phase cyclins (Walker *et al.* 1997), the roles played by $yTAF_{II}90$ and TMS1 are not known. It will be important to determine if these TAF_{II}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 TAF_{II}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 $yTAF_{II}30\Delta$; Welch *et al.* 1993), tsm1 (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 *EcoR1* 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 $ytaf_{II}90^{ts2-1}$ and $ytaf_{II}90^{ts3-1}$, 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-yTAF$_{II}$90, 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-ggcctgaattcacctttaccg and 5-gcggaaagtgctctcaagaagatg. The primers were designed to generate an *EcoR1* restriction site on both ends of the PCR fragment. The *EcoR1* digested PCR fragment was cloned into the *EcoR1* 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 100um 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 yTAF$_{II}$s

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 CGGGATCCGGATCAAGCACTGAGGTAG & CCCTCTTCTCATTCATGCAAG, GCGGATCCGGAAGATCACTCAGTAACGTC & GAAGATCTGCGGAGAGCACTTGTCGAACC, GCGTCTAGATCAGCGAGTTTTCTTGAGGTT & CGGGATCCAATGGCCATATTGAAGGATACC, and ATCGTCTAGACGATTTTTTTCTAAACCGTGGAATATTTCG & CTCGGGATCCGGACGGATTACAACAGGTATTGTCC to generate PCR fragments *CLB2*, *CSE4*, *CLN3* and *ADH1* respectively. The rRNA, $tRNA^{I}$, *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 GGCATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCACTTGG

ENO2 CGGGAGTCGTAGACGGATCTAGCGTAAACTTTAGAGACAGCCTAATAA

PMA1 GCAGGCTTTTCTTGAGTTGGCTGATGAGCTGAAACAGAAGATGCACTTCT

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 Denhardts, 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 ddH_20, 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$_{II}$90 Fusion-Proteins

LexA-yTAF$_{II}$90 fusion-proteins containing full length, N-terminal and C-terminal deletion were transformed into LGY7 which contains an integrated lacZ reporter with 2 LexA operators upstream of the TATA box. β-galactosidase activities were assayed as described (Kaiser *et al.* 1994). Wild-type and LexA-yTAF$_{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 $yTAF_{II}90$ failed to identify promoters which required $yTAF_{II}90$ 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 $yTAF_{II}90$ protein caused derepression, or inappropriate expression, of a subset of genes.

To identify genes whose proper expression required $yTAF_{II}90$, 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 $yTAF_{II}90$ for expression (plus subtraction). The second, to identify genes whose expression increased upon inactivation of $yTAF_{II}90$ (minus subtraction). Each subtraction was performed on mRNA isolated from the wild-type strain LY3 and the $yTAF_{II}90$ 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 $yTAF_{II}90$

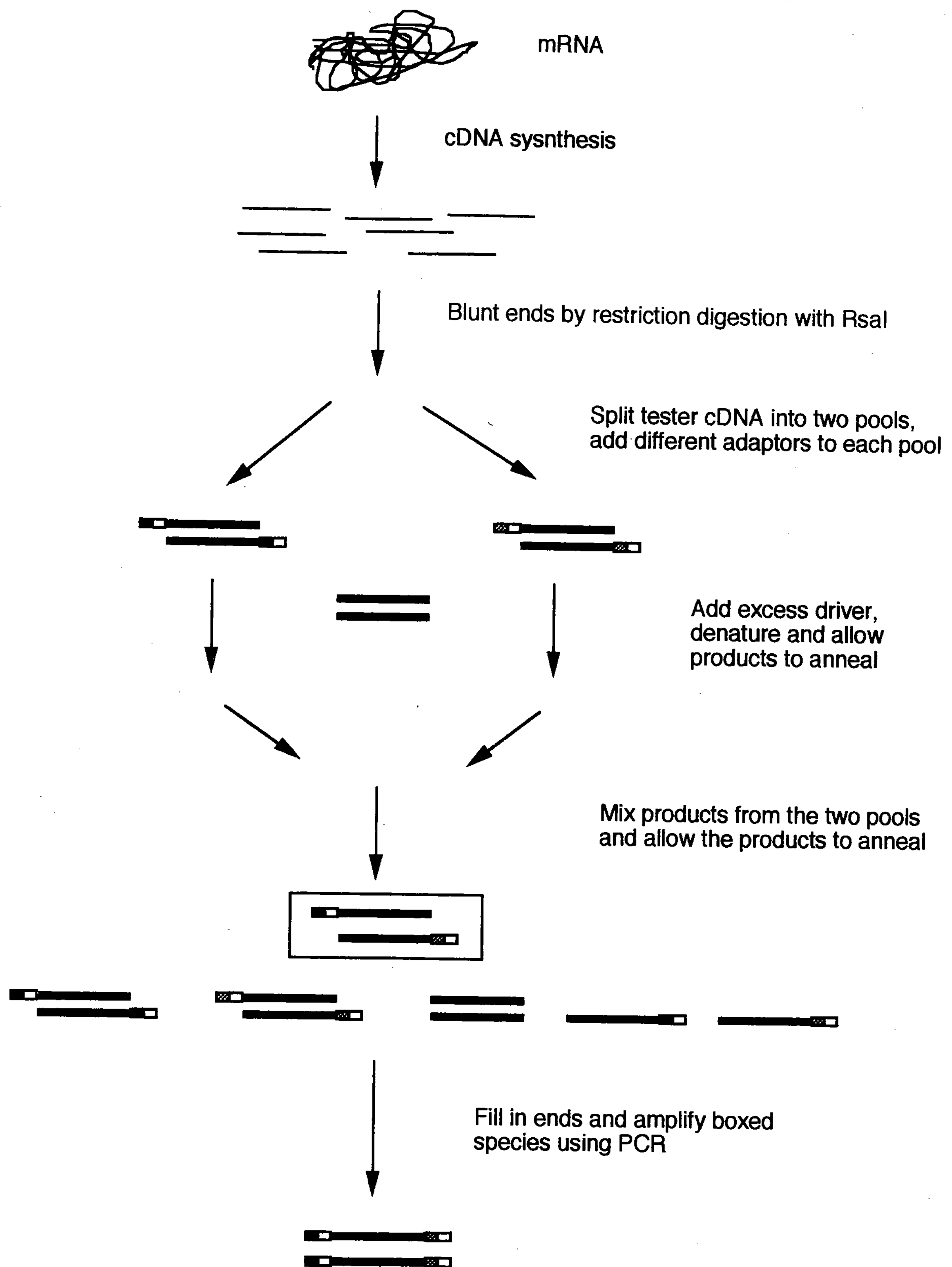
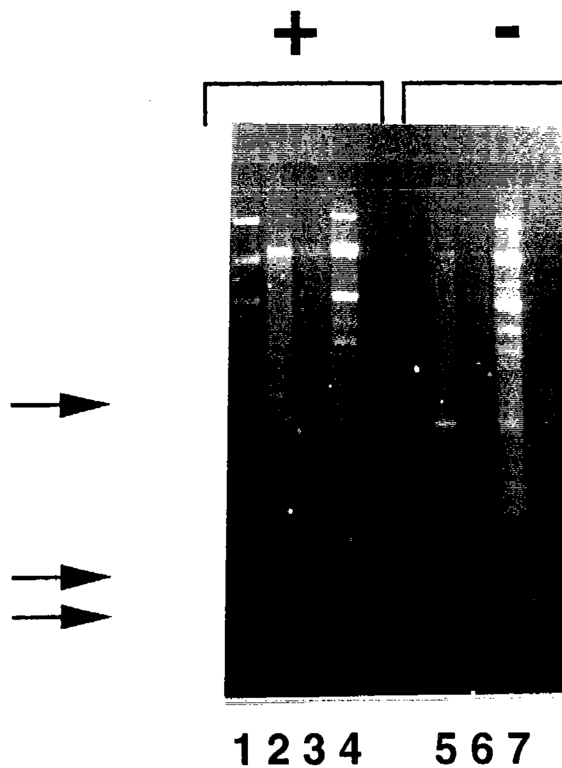


Figure A-1. Subtractive hybridization scheme. Tester cDNAs contain the differentially expressed products.

A



B

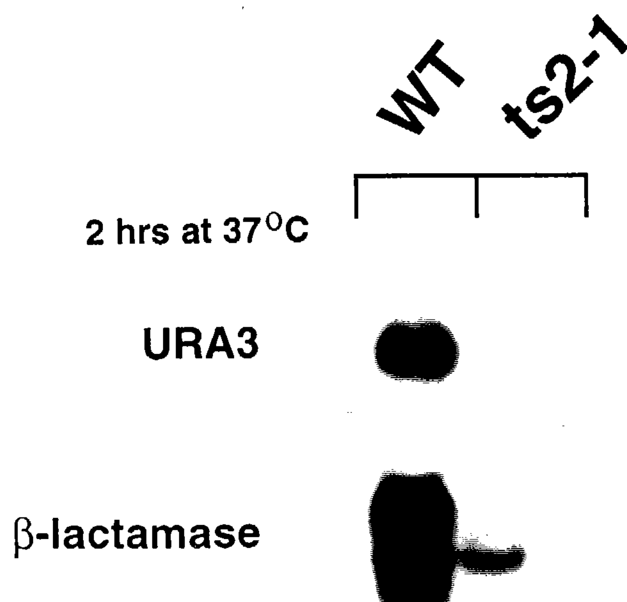


Figure A-2. Differentially expressed genes in wild-type and $yTAF_{II}90$ temperature-sensitive strains.

(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 $yTAF_{II}90$ 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 explaination 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 $yTAF_{II}90$, 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 $yTAF_{II}s$ (data not shown). These results indicate that differential expression of the isolated clones was not due to inactivation of $yTAF_{II}90$, 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 $yTAF_{II}90$ for proper expression because $yTAF_{II}90$ 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 $yTAF_{II}90$ function causes only a small change in the expression of a number of genes. Equally plausible, is that genes requiring $yTAF_{II}90$ for expression, are expressed a 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.