Regulation of Yeast RNA Polymerase II Holoenzyme Transcription

by

Christoph J. Hengartner

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Signature of Author:_____
Department of Biology
January 25, 1999

Certified by:_____
Richard A. Young
Professor of Biology
Thesis Supervisor

Accepted by:_____
Terry Orr-Weaver
Professor of Biology
Co-chairperson, Committee for Graduate Students

Dedication

This thesis is dedicated to all my family, far and near, for their love and support. Special thanks to my wife Leticia Vega, my parents Anne and Walter, my grandmother Annemarie Beerli-Rauber, my brothers (Niklaus, Michael, Andreas and Markus) and their respective beloved (Leticia, Béatrice, Linda and Isabelle), and my nephew and nieces (Alex, Andrea, Astride and Sophie).

A special dedication to the memory of Helen Hengartner-Stadelmann and Max Beerli.

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Abstract

RNA polymerase II holoenzyme, a form of eukaryotic RNA polymerase II, had been found to consist of core RNA polymerase II, a subset of general transcription factors and four Srb proteins. The Srb proteins had been identified through a selection for *Saccharomyces cerevisiae* genes involved in transcription initiation and regulation in vivo. Three genes, *SRB7*, *SRB8* and *SRB9*, were isolated and characterized. Along with the prior isolation of 6 other*SRB* genes, this completed the characterization of this genetic selection. All nine gene products were found associated with the RNA polymerase II holoenzyme. A direct interaction between the holoenzyme and a transcriptional activator provided the basis for a model whereby activator recruitment of this apparatus at promoters directs gene expression.

Two yeast cyclin-dependent kinases (CDKs), present in RNA polymerase II holoenzyme, were found to be biochemically indistinguishable in their ability to phosphorylate the RNA polymerase II carboxy-terminal domain (CTD), yet in living cells one acted as a positive regulator and the other as a negative regulator of transcription. This paradox was resolved by the observation that the negative regulator, Srb10, was uniquely capable of phosphorylating the CTD prior to formation of the initiation complex on promoter DNA, inhibiting transcription. In contrast, the TFIIH CDK, Kin28, phosphorylated the CTD only after the transcription apparatus was associated with promoter DNA. Thus, the timing of CTD phosphorylation could account for the positive and negative functions of the two kinases.

Genome-wide expression analysis was performed to identify the genes whose transcription depended on the functions of the two yeast cyclin-dependent CTD kinases. Kin28 function was found to be generally required throughout the genome, while Srb10 functioned negatively at a small subset of genes. Analysis of this subset of genes revealed a novel mechanism for coordinate regulation of specific sets of genes when cells encounter limiting nutrients. A negative role was found for Srb10 in a yeast developmental pathway, suggesting that the ultimate targets of signal transduction pathways could be directly traced to the RNA polymerase II holoenzyme.

Thesis Supervisor: Richard A. Young
Title: Professor of Biology

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Chapter 1

Introduction: RNA polymerase II holoenzyme and transcription initiation

Overview

The genes expressed by a biological organism serve as the dynamic link between its genome and its phenotype. Thus, it comes as no surprise to find that organisms invest a significant degree of complexity and energy in their regulation of gene expression. Protein abundance in a cell is usually related to the abundance of its mRNA, whose expression is commonly controlled at the level of transcription initiation. Transcription is initiated by interactions of the RNA polymerase II holoenzyme and general transcription factors at common core promoter elements, and is regulated by various gene-specific activators and repressors bound to adjacent or distal regulatory elements. Nucleosomes and other chromatin-associated proteins contribute additional layers to the transcriptional regulation process. The communication between the regulatory factors and the components of the transcription machinery is critical for appropriate gene expression.

This chapter aims both to introduce the subject of my studies and to summarize our current knowledge of the yeast RNA polymerase II holoenzyme and transcription initiation.

RNA polymerase II holoenzyme.

RNA polymerase II, the enzyme synthesizing mRNA, exists in several forms. The first form to be isolated was called core RNA polymerase II and is also the simplest form, made up of 10-12 subunits (reviewed in Young, 1991; Woychik and Young, 1994). A much larger form of RNA polymerase II, thought to be the form recruited to promoters in vivo, was discovered twenty years later in *Saccharomyces cerevisiae* and is called a RNA polymerase II holoenzyme (Koleske and Young, 1994; Kim et al., 1994; Thompson and Young, 1995). The RNA polymerase II holoenzyme is not unique to yeast and has been purified

from mammalian cells as well (reviewed in Parvin and Young, 1998). Yeast RNA polymerase II holoenzyme has been reported to contain the hallmark Srb proteins and near stoichiometric levels of most general components involved in transcription initiation with the exception of TFIID and TFIIE. The holoenzyme components can be divided into 3 main groups, which I will review individually: core RNA polymerase II, the general transcription factors (GTFs), and the regulatory factors (Srb proteins and others). Table I lists all yeast RNA polymerase II components identified so far.

RNA polymerase II.

In eukaryotes, three types of DNA-dependent RNA polymerases accomplish the transcription of nuclear genes, each responsible for the transcription of a different class of RNA. RNA polymerase I transcribes the ribosomal RNA-encoding genes, RNA polymerase II transcribes the protein-encoding genes, and RNA polymerase III transcribes the tRNA- and the 5S ribosomal RNA-encoding genes (reviewed in Sentenac, 1985; Roeder, 1996).

Yeast *Saccharomyces cerevisiae* RNA polymerase II is a multisubunit enzyme comprised of 12 core polypeptides encoded by genes designated *RPB1-RPB12*, (reviewed in Young, 1991; Woychik and Young, 1994). All 12 genes are required for normal growth, and all but *RPB4* and *RPB9* are essential for cell viability. Minor differences aside, this enzyme shares many characteristics in subunit composition with all other eukaryotic nuclear RNA polymerases. In yeast, five of the RNA polymerase II subunits (Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12) are also present in RNA polymerases I and III and are referred to as "common" subunits. A human counterpart for each of the 12 genes encoding yeast RNA polymerase II has been isolated (Pati and Weissman, 1989; Pati and Weissman, 1990; Acker et al., 1992; Wintzerith et

Table I. RNA polymerase II Holoenzyme Components in *S. cerevisiae*

Factor	Gene	Subunit	Essential?	Features	References
RNA Polymerase II	*RPB1*	192 kDa	Y	Heptapeptide repeat	Young and Davis, 1983; Ingles et al, 1984; Allison et al., 1985
	RPB2	139 kDa	Y		Sweetser et al., 1987
	RPB3	35 kDa	Y		Kolodziej and Young, 1989
	RPB4	25 kDa	N		Woychik and Young, 1989
	RPB5	25 kDa	Y	Shared with PolI, II, III	Woychik et al., 1990
	RPB6	18 kDa	Y	Shared with PolI, II, III	Woychik et al., 1990
	RPB7	19 kDa	Y		Woychik et al., 1993a
	RPB8	17 kDa	Y	Shared with PolI, II, III	Woychik et al., 1990
	RPB9	14 kDa	N		Woychik et al., 1991
	RPB10	8 kDa	Y	Shared with PolI, II, III	Woychik and Young, 1990
	RPB11	14 kDa	Y		Woychik et al., 1993b
	RPB12	8 kDa	Y	Shared with PolI, II, III	Treich et al., 1992
TFIIH	*TFB1*	73 kDa	Y	Nucleotide excision repair	Feaver et al., 1997
	TFB2	59 kDa	Y	Nucleotide excision repair	Feaver et al., 1997
	TFB3	32 kDa	Y	Nucleotide excision repair	Feaver et al., 1997
	TFB4	37 kDa	Y		Feaver et al., 1997
	RAD3	90 kDa	Y	DNA Helicase	Feaver et al., 1997
	SSL1	52 kDa	Y	Nucleotide excision repair	Feaver et al., 1997
	SSL2/RAD25	95 kDa	Y	DNA Helicase	Feaver et al., 1997
	KIN28	35 kDa	Y	Cyclin dependant CTD kinase	Feaver et al., 1997
	CCL1	45 kDa	Y	Kin28 cyclin pair	Feaver et al., 1997
TFIIE	*TFA1*	55 kDa	Y		Feaver et al., 1994
	TFA2	37 kDa	Y		Feaver et al., 1994
TFIIF	*SSU1/TFG1*	82 kDa	Y		Henry et al, 1994
	TFG2	47 kDa	Y		Henry et al, 1994
	ANC1/TFG3	27 kDa	N	Shared with TFIID and TFIIH	Henry et al, 1994
TFIIB	*SUA7*	38 kDa	Y		Pinto et al., 1992
Srbs	*SRB2*	23 kDa	N		Nonet and Young, 1989; Koleske et al., 1992; Koleske and Young, 1994
	SRB4	78 kDa	Y		Thompson et al, 1993; Koleske and Young, 1994
	SRB5	34 kDa	N		Thompson et al, 1993; Koleske and Young, 1994
	SRB6	14 kDa	Y		Thompson et al, 1993; Koleske and Young, 1994
	SRB7	16 kDa	Y		Hengartner et al., 1995
	SRB8	166 kDa	N		Hengartner et al., 1995
	SRB9	160 kDa	N		Hengartner et al., 1995
	SRB10	63 kDa	N	Cyclin dependant CTD kinase	Liao et al., 1995
	SRB11	38 kDa	N	Srb10 cyclin pair	Liao et al., 1995
Meds	*MED1*	64 kDa	N		Balciunas et al., 1999
	MED2	48 kDa	N		Myers et al., 1998
	MED4	32 kDa	Y		Myers et al., 1998
	MED6	33 kDa	Y		Myers et al., 1998
	MED7	26 kDa	Y		Myers et al., 1998
	MED8	25 kDa	Y		Myers et al., 1998
	MED11	15 kDa	?		Gustafsson et al., 1998
Cse2	*CSE2*	17 kDa	N		Gustafsson et al., 1998
Gal11	*GAL11*	120 kDa	N		Li et al., 1996; Barberis et al., 1996
Nut2	*NUT2*	18 kDa	Y		Gustafsson et al., 1998
Pgd1	*PGD1*	47 kDa	N		Myers et al., 1998
Rox3	*ROX3*	25 kDa	Y		Gustafsson et al., 1997
Rgr1	*RGR1*	123 kDa	Y		Li et al., 1996
Sin4	*SIN4*	111 kDa	N		Li et al., 1996
(Nut1)*	*NUT1*	129 kDa	N		Gustafsson et al., 1998
(Xtc1)*	*XTC1*	27 kDa	N		Emili et al., 1998
Fcp1	*FCP1*	83 kDa	Y		Archambault et al., 1997
SWI/SNF	*SWI1*	148 kDa	N		Cairns et al., 1994; Cote et al., 1995
	SWI2/SNF2	194 kDa	N	DNA dependant ATPase	Cairns et al., 1994; Cote et al., 1995
	SWI3	93 kDa	N		Cairns et al., 1994; Cote et al., 1995
	SNF5	103 kDa	N		Cairns et al., 1994; Cote et al., 1995
	SNF6	38 kDa	N		Cairns et al., 1994; Cote et al., 1995
	SNF11	19 kDa	N		Treich et al., 1995
	ARP9	53 kDa	Y / N	Actin related	Cairns et al., 1994; Cote et al., 1995; Cairns et al., 1998; Peterson et al., 1998
	ARP7	54 kDa	Y / N	Actin related	Cairns et al., 1994; Cote et al., 1995; Cairns et al., 1998; Peterson et al., 1998
	SNF12	64 kDa	N		Cairns et al., 1996A
	SWp82p	~ 82 kDa	?		Cairns et al., 1994; Cote et al., 1995
	ANC1/TFG3	27 kDa	N	Shared with TFIID and TFIIH	Cairns et al., 1996B

* These proteins have not yet been formally demonstrated to be stoichiometric subunits of purified RNA polymerase II holoenzyme.

al., 1992; Acker et al., 1993; Acker et al., 1994; Pati, 1994; Khazak et al., 1995; McKune et al., 1995; Shpakovski et al., 1995; Khazak et al., 1998). They share a sequence similarity with their yeast orthologs that is accompanied by a remarkable conservation of function as six out of seven human RNA polymerase II subunits tested are able to functionally substitute for their yeast counterparts (McKune and Woychik, 1994; Khazak et al., 1995; McKune et al., 1995; Shpakovski et al., 1995).

There is a profound similarity between yeast RNA polymerase II and the bacterial RNA polymerases as well. The bacterial RNA polymerases are composed of a specificity factor (σ), and of a three-component bacterial core enzyme, structured as a $\beta\beta'\alpha_2$ tetramer. The two largest subunits, Rpb1 and Rpb2, are homologous to the β' and β bacterial core subunits, respectively. Rpb3 and Rpb11 share a weaker homology with the α bacterial core subunit and can form a Rpb3-Rpb11 heterodimer in vitro , consistent with the hypothesis that this heterodimer is the ortholog of the α_2 homodimer (Larkin and Guilfoyle, 1997). As expected, the yeast orthologs of the bacterial core enzyme, Rp1, Rpb2 and Rpb3, are found to be largely responsible for the enzymatic RNA catalysis (reviewed in Young, 1991; Woychik and Young, 1994).

Rpb4 and Rpb7 form a dissociable subcomplex that has been implicated in the stress response and in the initiation of transcription (Dezelee et al., 1976; Woychik and Young, 1989; Edwards et al., 1991; Choder and Young, 1993). While Rpb4 and Rpb7 are present only in substoichiometric amounts during the exponential phase of yeast growth, they are found as stoichiometric subunits of core RNA polymerase II under suboptimal conditions such as the stationary phase of cell growth (Choder and Young, 1993).

CTD. Rpb1 contains an unusual carboxy-terminal domain CTD) that consists of tandem repeats of the consensus sequence YSPTSPS (reviewed in Chao and Young, 1991; Corden, 1990). This repeat is highly conserved among eukaryotes and is unique to the largest subunit of RNA polymerase II, suggestive of a role in mRNA biogenesis. The CTD has been found to contain as few as 26 heptapeptide repeats in yeast, or as many as 52 repeats in mouse (Allison et al., 1985; Corden et al., 1985; Allison et al., 1988). Deletion studies have demonstrated that the CTD is essential for cell viability in yeast, Drosophila, and mammalian cells (Nonet et al., 1987; Allison et al., 1988; Bartolomei et al., 1988; Zehring et al., 1988).

Partial CTD truncation studies were the first to suggest the domain's role in transcription initiation. CTD partial truncation mutations cause growth defects in yeast (Nonet et al., 1987) and impair inducible gene expression at a subset of promoters in both yeast and mammalian cells (Allison and Ingles, 1989; Scafe et al., 1990; Gerber et al., 1995). The defects in gene expression can be mapped in vivo and in vitro to the UAS of the genes affected, and can be shown to occur at the level of transcription initiation (Allison and Ingles, 1989; Scafe et al., 1990; Liao et al., 1991). The CTD provided the focal point for the genetic analysis that led to the discovery of the Srb proteins and the RNA polymerase II holoenzyme.

The general transcription factors.

While purified RNA polymerase II is sufficient and necessary for template-dependent RNA synthesis, additional factors are required for accurate and selective initiation at promoters in vitro (Matsui et al., 1980). These general transcription factors (GTFs) were originally defined through fractionation and in vitro transcription reconstitution experiments and include TFIIA, TFIIB,

TFIID, TFIIE, TFIIF and TFIIH (reviewed in Zawel and Reinberg, 1993; Orphanides et al., 1996; Roeder, 1996). As seen in many aspects of gene transcription, the general transcription factors show a high degree of functional and structural homology between eukaryotes. The yeast genes coding for the various GTF subunits have now all been cloned (reviewed in Hampsey, 1998). With the exception of a number of TBP-associated factors ($TAF_{II}s$), the genes are all essential (reviewed in Hampsey, 1998).

Step-wise assembly model of PIC fomation. A decade ago, DNA footprinting, gel electrophoretic mobility shift assays and kinetic assays had revealed that the GTFs and core RNA polymerase II could be assembled in a orderly and step-wise fashion on promoters in vitro to form a pre-initiation complex (PIC) (reviewed in Conaway and Conaway, 1993; Zawel and Reinberg, 1993; Buratowski, 1994). In short, core promoter elements were recognized by TFIID or TBP, followed by the sequential assembly of TFIIA, TFIIB, RNA polymerase II/TFIIF, TFIIE and TFIIH. The discovery of a form of RNA polymerase II that already contained a number GTFs suggested that in vivo, ordered GTF asssembly may be bypassed in favor of direct recruitment of a preassembled complex consisting of RNA polymerase, GTFs, and regulatory proteins (Srb, Med, etc.) to TFIID-bound promoters (Koleske and Young, 1995). Before undertaking a review of the main properties and function of each GTF, I will introduce the main structural elements found in the promoters of protein-encoding genes.

Promoter structure.

Promoters of genes transcribed by eukaryotic RNA polymerase II (class II genes) contain core elements and regulatory elements (reviewed in Struhl, 1995). The core elements encompass the site for assembly of the transcription

preinitiation complex (PIC) and include the TATA box and the initiator sequence (Inr). The regulatory elements, located upstream of the core promoter, are gene-specific sequences that serve to control the rate of transcription initiation and include upstream activation sequences (UAS), upstream repression sequences (URS) and poly(dA-dT) tracts.

Core promoter elements: TATA box core element. Most yeast promoters contain a TATA elements (consensus TATAAA) that is important for transcriptional initiation (Breathnach and Chambon, 1981; Struhl, 1995). The TATA-binding protein TBP), a pivotal component of the general transcription factor TFIID, binds the TATA element and serves to nucleate the assembly of the PIC. TBP is also required for transcription from promoters lacking a canonical TATA box (TATA-less promoters), where it is presumed to function in concert with other components of the TFIID complex in recognizing a promoter structure other than the TATA to nucleate PIC assembly (Brandl et al., 1992; Cormack and Struhl, 1992).

Inr core element. In the budding yeast, *S. cerevisiae*, initiator (Inr) elements are defined as the DNA sequences surrounding the transcription start sites where RNA polymerase II initiates mRNA synthesis. The yeast Inr element is typically located at a variable 40 to 120 bp downstream of the TATA element, in marked contrast to most eukaryotes where RNA polymerase II initiates trasncription at a fixed 25-30 bp downstream of the TATA element (reviewed in Struhl, 1989). This fixed distance between the TATA element and the transcription start site suggested that, in most eukaryotes, the TATA element would be sufficient to specify the location of transcription initiation. Conversely, the variable distance between TATA and Inr elements in yeast, suggested that specific sequences would exist to direct transcription initiation. Such Inr sequences have been described (Chen and Struhl, 1985; Hahn et al.,

1985; Nagawa and Fink, 1985; Healy et al., 1987; Rudolph and Hinnen, 1987). As opposed to the TATA and the regulatory promoter elements, yeast Inr lack a strong sequence consensus and their contributions to the overall level of transcription are relatively modest.

In higher eukaryotes, the Inr element is defined differently from the yeast Inr: a core promoter element near the transcription start site, that could nucleate PIC assembly at TATA-less promoters (Smale and Baltimore, 1989). Besides functional PIC assembly, metazoan TATA and Inr elements share additional functional similarities: each can, independently or in concert, direct accurate transcription initiation and activator responsive transcription (reviewed in Smale, 1997). A number of factors have been demonstrated or inferred to recognize the Inr element, such as the TBP-associated factor (TAF_{II}) subunits of TFIID, RNA polymerase II and novel Inr-binding proteins (reviewed in Weis and Reinberg, 1992) ; (Smale, 1997). The favored model is that TFIID, either through direct TAF_{II}s-Inr element recognition or with the help of other factors, nucleates PIC assembly. Core promoter structures can contain both TATA and Inr elements (composite promoters), either element alone (TATA- or Inr-directed promoter) or none (null promoter) (reviewed in Novina and Roy, 1996). The composite promoters are found primarily in viral genes (reviewed (Roeder, 1991) while most cellular class II genes contain TATA-directed promoters and, to a lesser extent, Inr-directed promoters. The null promoters seem to contain no strong Inr element and usually have multiple transcription start sites, suggestive of imprecise initiation (Geng and Johnson, 1993; Lu et al., 1994; Ince and Scotto, 1995). Their frequency is unknown but they have been proposed to function at "cellular housekeeping" genes. Several null promoters share an intragenic sequence element called MED-1 (Ince and Scotto, 1995).

In *Saccharomyces cerevisiae*, it is still not known whether Inr elements are able to nucleate PIC assembly as well, or if there are specific Inr-recognition factors. One study so far, has shown that the Inr element of *GAL80* was capable of directing transcription initiation in the absence of a canonical TATA element (Sakurai et al., 1994).

DPE and BRE core elements. Studies in *Drosophila* have described a conserved downstream promoter element (DPE) located 30 bp downstream of the transcription start site in many TATA-less promoters (Burke and Kadonaga, 1996). The DPE is necessary for efficient transcription in vitro and can contact $TAF_{II}40$ and $TAF_{II}60$, working in concert with the Inr element to recruit TFIID to the promoter (Burke and Kadonaga, 1996; Burke and Kadonaga, 1997). A new core promoter element that modulates the binding of the general transcription factor TFIIB, the TFIIB recognition element (BRE), has just recently been described (Lagrange et al., 1998). While no conserved yeast DPE element has been described yet, the strong conservation of the TFIIB protein among eukaryotes leads to expect that BRE elements will be generally conserved as well.

Regulatory promoter elements: UAS and URS. While the core promoter elements serve to direct the accurate transcription initiation of a gene, positive transcription factors (activators) and negative transcription factors (repressors) regulate the level of transcription through upstream activation sequences (UAS) and upstream repressor sequences (URS), respectively (reviewed in Mitchell and Tjian, 1989; Struhl, 1995). The UAS and URS elements are typically located within a few hundred bp upstream of the initiation start site and are specifically and directly bound by activators and repressors.

In metazoans, sequences directing transcriptional activation can be divided into promoter-proximal elements, binding glutamine-rich activators such as Sp1, Oct-1 and Oct-2, and enhancer elements, located many kilobases from the core promoter in either 5′ or 3′ direction (Seipel et al., 1992). Yeast UAS elements are analogous to enhancers in that they function in either orientation and at variable distances upstream from the core promoter. However, in marked contrast to enhancers, UAS elements fail to activate transcription when positioned downstream of the core promoter (Guarente and Hoar, 1984; Struhl, 1984). UAS-bound activators have been found to facilitate or stabilize PIC formation through direct or indirect contacts between their transcriptional activation domain and the transcription initiation machinery. This activation domain is functionally distinct, and usually physically separate, from the activator's DNA-binding domain Ptashne, 1988; Ptashne and Gann, 1990). URS-bound repressors inhibit transcription by a variety of mechanisms, such as activator-UAS binding interference; activation domain function interference; or by direct inhibitory contacts with the transcription initiation machinery (reviewed in Johnson, 1995).

URS and co-represssor complexes. URS-bound proteins can also mediate repression indirectly by recruiting the Tup1/Ssn6 co-repressor complex. This general transcription co-repressor complex is recruited to different promoters via pathway-specific DNA-binding proteins and has been proposed to inhibit transcription by targeting the transcription machinery and/or core nucleosomal histones (reviewed in Roth, 1995). Chromatin-packaged genes present problems of promoter DNA accessibility to general and regulatory transcription factors. Modulating changes in nucleosomal structure at promoters is thought to allow or inhibit transcription. What role, if any, the Tup1/Ssn6 complex plays in regulating nucleosomal structure is

unknown. However, two classes of factors that facilitate nucleosomal structure changes have come to the foreground recently: ATP-dependent chromatin remodelling complexes and enzymatic complexes which control histone acetylation and deacetylation (reviewed in Grunstein, 1997; Tsukiyama and Wu, 1997; Armstrong and Emerson, 1998; Ashraf and Ip, 1998; Davie, 1998; Workman and Kingston, 1998). A general in vivo correlation between core histone acetylation and gene expression has long been recognized. Conversely, histone deacetylation has been thought to lead to transcriptional repression. In yeast, recruitment of the Sin3-Rpd3 histone deacetylase co-repressor complex by the URS-bound Ume6 repressor, leads to inhibition of transcription (Kadosh and Struhl, 1997). The Sin3-Rpd3 co-repressor complex and function are found to be conserved in metazaons, while the identification of Tup1/Ssn6 homologs also suggest evolutionary conservation of this co-repressor complex (Grbavec et al., 1999; Pazin and Kadonaga, 1997).

Poly(dA-dT) tracts regulatory element. As opposed to the protein-mediated effects of UAS and URS elements, the regulatory properties of yeast promoters can also be determined by poly(dA-dT) tracts. The Poly(dA-dT) type of upstream element functions not by interacting with a specific DNA-binding protein, but by virtue of it's intrinsic DNA structure (Iyer and Struhl, 1995). Poly(dA-dT) elements have distinct structural characteristics that impair nucleosome assembly and stability (reviewed in Iyer and Struhl, 1995). The analysis of the complete yeast genome sequence has revealed that these elements are found frequently one nucleosome DNA length upstream and downstream of open-reading frames (ORFs), suggestive of a modulatory role in nucleosome positioning (Raghavan et al., 1997).

TFIID and TBP-associated factors.

TBP and TFIID. The TATA-binding protein TBP) is a universal transcription factor that is used for transcription by all three nuclear RNA polymerases (reviewed in Sharp, 1992; Hernandez, 1993). As befits a protein central to so many processes, TBP displays an uncommonly strong evolutionary sequence conservation among eukaryotes. Yeast TBP is encoded by *SPT15* (Hahn et al., 1989). *SPT15* was isolated as a suppressor of a transcription defect that originated from transposon element insertion into the core promoter region of a class II gene (Eisenmann et al., 1989). As a RNA polymerase II GTF, the TBP protein is found associates with approximately 10 polypeptides, called TBP-associated factors (TAF_{II}s), to form a large complex referred to as TFIID (reviewed in Burley and Roeder, 1996). While TBP functions to specifically recognize the TATA elements present in most eukaryotic core promoters, the TAF_{II}s subunits can interact with initiator and downstream elements (reviewed in Burley and Roeder, 1996; Verrijzer and Tjian, 1996; Burke and Kadonaga, 1997). Together, TBP and TAF_{II}s direct promoter recognition and the bound TFIID-DNA complex in turn recruits RNA polymerase II holoenzyme and other factors required for PIC nucleation.

Crystallography studies of TBP, alone or complexed to promoter DNA and GTFs, have been instrumental in resolving a number of the protein's functions at a high level of details (reviewed in Burley, 1996). TBP was found to resemble a symmetrical molecular saddle (Nikolov et al., 1992; Chasman et al., 1993). When bound to a promoter, this saddle structure sits atop the DNA, contacting the minor groove of the TATA element and inducing a sharp DNA bend accompanied by a partial unwinding of base pairs (Kim et al., 1993; Kim et al., 1993). Small surfaces on opposite sides of the TBP

"saddle" are involved in contacting the general transcription factors TFIIA and TFIIB (Geiger et al., 1996; Nikolov et al., 1995). The TBP-TFIIB interaction is thought to be the primary connection between TFIID and the RNA polymerase II holoenzyme although interactions between TBP and the Srb2 holoenzyme component have also been described (Koleske et al., 1992). While TFIID and TBP have not been found to be *bona fide* subunits of highly purified yeast RNA polymerase II holoenzyme, substoichiometric amounts of TBP have been reported, consistent with a weak interaction between TFIID and holoenzyme (Thompson et al., 1993; Koleske and Young, 1994). TBP crystals first suggested that the protein could form homodimers: two TBP molecules interacting with each other through their DNA binding surfaces (Nikolov and Burley, 1994). In vitro and in vivo crosslinking studies show that TBP has the ability to form homodimers in solution, even when part of the TFIID complex (Coleman et al., 1995; Taggart and Pugh, 1996). It is unknown what effect, if any, this homodimerization has on RNA polymerase II transcription regulation.

TAF_{II}s. TFIID complexes have been purified from *Drosophila* (Dynlacht et al., 1991), human (Pugh and Tjian, 1991; Tanese et al., 1991; Takada et al., 1992; Zhou et al., 1992) and yeast (Reese et al., 1994; Poon et al., 1995) sources, and the genes encoding the major TAF_{II}s have all been cloned. The *Drosophila* TFIID complex (TBP and eight TAF_{II}s) could be entirely reconstituted from recombinant proteins (Chen et al., 1994). A majority of yeast TAF_{II}s are essential for viability (Table II) and a homologous counterpart for each yeast TAF_{II} ,except $TAF_{II}47$ and $TAF_{II}30$, has been found in higher eukaryotes (reviewed in Burley and Roeder, 1996; Tansey and Herr, 1997).

Table II. TBP-associated regulators in *S. cerevisiae*

Factor	Gene	Subunit	Essential?	Features	References
TBP	*TBP1/SPT15*	27 kDa	Y		Eisenmann et al., 1989
TAF_Is	*RRN6*	102 kDa	Y		Keys, et. al., 1994
	RRN7	60 kDa	Y		Keys, et. al., 1994
	RRN11	59 kDa	Y		Lalo, et. al., 1996; Lin, et. al., 1996
$TAF_{II}s$	*TAF150/TSM1*	161 kDa	Y	Cell cycle progression role	Verrijzer, et. al., 1994; Poon, et. al., 1995
	TAF145/TAF130	121 kDa	Y	Histone acetyltransferase	Reese, et. al., 1994; Poon, et. al., 1995
	TAF90	89 kDa	Y	Cell cycle progression role, shared with TFIID, SAGA	Reese, et. al., 1994; Poon, et. al., 1995
	TAF67	67 kDa	Y		Moqtaderi, et. al., 1996
	TAF68/TAF61	61 kDa	Y	Similar to histone H2B, shared with TFIID, SAGA	Moqtaderi, et. al., 1996; Walker, et. al., 1996
	TAF60	58 kDa	Y	Similar to histone H4, shared with TFIID, SAGA	Poon, et. al., 1995
	TAF47	40 kDa	Y		Walker, et. al., 1997
	TAF40	41 kDa	Y		Moqtaderi, et. al., 1996; Klebanow, et. al., 1997
	TAF30/ANC1/TFG3	27 kDa	N	Component of TFIIF and SWI/SNF	Henry, et. al., 1994
	TAF25/TAF23	23 kDa	Y		Moqtaderi, et. al., 1996; Klebanow, et. al., 1996
	TAF19/FUN81	19 kDa	Y		Moqtaderi, et. al., 1996
	TAF17/TAF20	17 kDa	Y	Similar to histone H3, shared with TFIID, SAGA	Moqtaderi, et. al., 1996
$TAF_{III}s$	*BRF1/TDS4/PCF4*	67 kDa	Y	Homologous to TFIIB	Buratowski & Zhou, 1992; Colbert & Hahn, 1992; Lopez-de-Leon, et. al., 1992
	TFC5/TFC7	68 kDa	Y		Kassavetis, et. al., 1995; Ruth, et. al., 1996
SAGA	*SPT3*	38 kDa	N		Eisenmann, et. al., 1992
	SPT7	153 kDa	N		Gansheroff, et. al., 1995
	SPT8	66 kDa	N		Eisenmann, et. al., 1994
	SPT20/ADA5	68 kDa	N		Marcus, et. al., 1996; Roberts, et. al., 1996
	ADA2/SWI8	51 kDa	N		Berger, et. al., 1992
	ADA3/NGG1	79 kDa	N		Brandl, et. al., 1993; Pina, et. al., 1993
	GCN5/ADA4/SWI9	51 kDa	N	Histone acetyltransferase	Georgakopoulos & Thireos, 1992; Brownell, et. al., 1996; Grant, et. al., 1997
	TAF90	89 kDa	Y	Cell cycle progression role, shared with TFIID, SAGA	Grant et al., 1998
	TAF61/68	61 kDa	Y	Similar to histone H2B, shared with TFIID, SAGA	Grant et al., 1998
	TAF60	58 kDa	Y	Similar to histone H4, shared with TFIID, SAGA	Grant et al., 1998
	TAF25/TAF23	23 kDa	Y		Grant et al., 1998
	TAF17/TAF20	17 kDa	Y	Similar to histone H3, shared with TFIID, SAGA	Grant et al., 1998
Mot1	*MOT1/BUR3/ADI*	210 kDa	Y	Member of SNF2 family of ATPases	Davis, et. al., 1992; Piatti, et. al., 1992; Auble, et. al., 1994
NC2	*NCB1/BUR6*	16 kDa	Y	Histone fold motif	Goppelt & Meisterernst, 1996; Gadbois, et. al., 1997; Prelich, 1997
	NCB2/YDR1	17 kDa	Y	Histone fold motif	Goppelt & Meisterernst, 1996; Gadbois, et. al., 1997; Kim, et. al., 1997
Nots	*NOT1/CDC39*	240 kDa	Y		Reed, et. al., 1980; Collart & Struhl, 1993
	NOT2/CDC36	22 kDa	N		Reed, et. al., 1980; Collart & Struhl, 1994
	NOT3	94 kDa	N		Collart & Struhl, 1994
	NOT4/MOT2/SIG1	65 kDa	N	Zinc finger protein	Cade, et. al., 1994; Collart & Struhl, 1994; Irie, et. al., 1994; Leberer, et. al., 1994
	NOT5	66 kDa	?	Regions of homology with NOT3	Oberholzer & Collart, 1998

Histone-like TAF_{II}s. Sequence analysis has revealed that $TAF_{II}61/68$, $TAF_{II}60$ and $TAF_{II}17/20$ share a similarity to nucleosomal histones H2B, H3 and H4, respectively (reviewed in Burley and Roeder, 1996). The significance of the sequence alignments is underscored by the crystal structure solution of *Drosophila* $TAF_{II}60$ and $TAF_{II}40$ fragments, homologs of yeast $TAF_{II}60$ and $TAF_{II}17/20$, showing that they interact with each other through a "histone fold" motif (Xie et al., 1996). This type of protein interaction is also seen in the H3/H4 heterotetramer structure. Furthermore, the human homologs of the 3 histone-like TAF_{II}s can associate together in a pattern similar to the one found in core nucleosomes (Hoffmann et al., 1996). Yeast genetic analysises strongly support a direct physical interaction between $TAF_{II}61/68$, $TAF_{II}60$ and $TAF_{II}17/20$ (Michel et al., 1998). It has been speculated that the nucleosome-like assembly of these TAF_{II}s might be indicative of a particular DNA-interacting function for TFIID. The histone-like TAF_{II}s, along with $TAF_{II}90$ and $TAF_{II}25/23$ are also found as subunits of another TBP-interacting complex, called the SAGA complex (Grant et al., 1998). The TFIID and SAGA complexes both possess histone acetyltransferase activity (Mizzen et al., 1996; Grant et al., 1997), raising the possibility that they play similar, or even redundant, chromatin modifying functions at promoters.

TBP and TAF_{II}s in transcriptional activation. In some *Drosophila* (Hoey et al., 1990; Pugh and Tjian, 1990; Dynlacht et al., 1991) and human (Smale et al., 1990) reconstituted in vitro transcription systems, TFIID but not TBP, was required for a transcriptional response to activators. These findings inspired many biochemical studies in higher eukaryotes to probe the TAF_{II}s' function in activation. A large number of direct and specific contacts between various TAF_{II}s and transcriptional activators were identified as a result of this effort (Hoey et al., 1993; Chen et al., 1994; Gill et al., 1994; Jacq et al., 1994;

Chiang and Roeder, 1995; Klemm et al., 1995; Lu and Levine, 1995; Thut et al., 1995; Sauer et al., 1995; Uesugi et al., 1997). The pattern of some of these activator-TAF_{II} contacts was also found to be consistent with the requirement for a given TAF_{II} in activated transcription in vitro. A model emerged from these experiments, where activators would generally increase the rate of transcription by directly targeting TBP and the TAF_{II}s, thus enhancing the recruitment of TFIID to the core promoter.

The first yeast in vivo studies looking at TBP seemed to agree with this model. In vivo recruitment of TBP to a previously untranscribed promoter was shown to be a rate-limiting step enhanced by transcriptional activators (Klein and Struhl, 1994). Furthermore, enhanced recruitment of TBP by artificial tethering to a promoter-bound factor could activate transcription in vivo (Klages and Strubin, 1995; Chatterjee and Struhl, 1995; Xiao et al., 1995). Again, this implied that TBP-binding could be a limiting step in PIC formation. A similar experiment in human cells showed that artificial recruitment of TBP activated transcription from TATA-containing but not from TATA-less promoters (Majello et al., 1998). The strength of acidic activator-TBP interaction in vitro, was also found to correlate the magnitude of activation seen both in vitro and in yeast cells (Wu et al., 1996). As predicted, hybrid fusion proteins tethering yeast TAF_{II}s to promoter DNA in vivo activated transcription as well (Apone et al., 1996; Gonzalez-Couto et al., 1997).

However, when these studies where extended further to the in vivo analysis of TAF_{II}s function, a different picture emerged. Since most of the TAF_{II}s are essential genes, loss of TAF_{II} function in yeast cells can only be achieved by using temperature sensitive TAF_{II} mutants (Moqtaderi et al., 1996; Walker et al., 1996; Apone et al., 1996) or by TAF_{II} depletion (Moqtaderi

et al., 1996; Walker et al., 1996; Apone et al., 1996). Against expectations, loss of $TAF_{II}150$/TSM1, $TAF_{II}145/130$, $TAF_{II}90$ and $TAF_{II}30$ function in vivo showed no global defects in activated transcription, but rather, impaired the transcription of a small number of genes (Moqtaderi et al., 1996; Walker et al., 1996; Apone et al., 1996; Walker et al., 1997; Holstege et al., 1998). Furtermore, cells defective for $TAF_{II}150$/TSM1 and $TAF_{II}90$ showed a G2/M cell cycle arrest (Apone et al., 1996; Walker et al., 1996), while cells defective for $TAF_{II}145/130$ showed a G1/S cell cycle arrest (Walker et al., 1996). A more extensive analysis of a $TAF_{II}145/130$ showed that it was required for the transcription of several G1 cyclin genes (Walker et al., 1997), a requirement conferred by core promoter elements (Shen and Green, 1997). A genome-wide gene expression analysis further demonstrated that a subset of genes involved in DNA repair, DNA synthesis and cell cycle progression all had a direct requirement for $TAF_{II}145/130$ function (Holstege et al., 1998). These yeast results echoed earlier findings in ts13 human cells containing a temperature sensitive mutation in $TAF_{II}250$, the homolog of yeast $TAF_{II}145/130$. At the restrictive temperature, these cells arrested at the G1/S stage of the cell cycle as well (Wang and Tjian, 1994). This was also correlated with transcription defects in cell cycle genes (Wang and Tjian, 1994; Suzuki-Yagawa et al., 1997). The evidence suggests that some of these TAF_{II}s do not play the role of general coactivator but rather, function as core promoter selectivity factors required at a subset of genes involved in cell cycle progression and other functions.

Loss of the histone-like TAF_{II}s' ($TAF_{II}17/20$, $TAF_{II}60$, $TAF_{II}61/68$) function shows they are broadly required for transcription in vivo (Michel et al., 1998; Natarajan et al., 1998). Different groups disagree on the universality of the requirement for $TAF_{II}17$, reflecting differences in methodology or

mutant severity (Apone et al., 1998; Michel et al., 1998; Moqtaderi et al., 1998). In marked contrast to the core promoter dependence seen for $TAF_{II}145/TAF_{II}130$, the gene-dependence on $TAF_{II}17$ was mapped to the regulatory UAS elements (Apone et al., 1998). The interpretation of these results has been complicated by the fact that these histone-like $TAF_{II}s$ also exist in the SAGA complex (Grant et al., 1998). Whether $TAF_{II}17$ function reflects the importance of TFIID, SAGA or a combination of both complexes is unknown.

Recent results in vitro and in vivo suggest that $TAF_{II}s$ in higher eukaryotes might not be as generally required for coactivators function as previously thought. Mammalian TAF_{II}-independent transcription activation in vitro has been reported recently. In one case the coactivator function for VP16 or CTF1 activation in nuclear extracts was provided not by TFIID but by RNA polymerase II holoenzyme (Oelgeschlager et al., 1998). In a highly purified reconstituted transcription system, TBP could support VP16 activated transcription in the presence of the coactivator PC4 (Wu et al., 1998). Mutations in two *Drosophila* $TAF_{II}s$, $TAF_{II}110$ and $TAF_{II}60/62$, were recently shown to perturb Dorsal activator-dependent transcription in vivo, but their effect was limited to only a small subset of embryonic cells (Zhou et al., 1998). It is unknown whether this $TAF_{II}110$ and $TAF_{II}60/62$ requirement mapped to the Dorsal binding site or the core promoter region.

TFIID autoinhibition. Experiments with recombinant $TAF_{II}s$ (reviewed in Liu et al., 1998) or reconstituted complexes (Verrijzer et al., 1995; Guermah et al., 1998), showed that *Drosophila* and human $TAF_{II}250$, can interfere with the formation of a stable TBP-TATA bound complex in vitro. In the same assay, the yeast homolog $TAF_{II}145/130$ has proven to be inhibitory as well (Kokubo et al., 1998; Kotani et al., 1998). NMR analyses

determined that the *Drosophila* $TAF_{II}250$ inhibitory domain binds to the DNA binding surface of TBP and mimicks the minor groove DNA surface recognized by TBP (Liu et al., 1998). This incredible protein mimickry of the TATA element surface clearly establishes the specificity of the negative regulation of TBP-TATA interactions by $TAF_{II}250$. These data suggest that activators also work by antagonizing TFIID autoinhibition. If so, part of the TFIID coactivator function observed in vitro really is the result of activator-dependent relief of TFIID autoinhibition (Burley and Roeder, 1998).

Catalytic activities in TFIID. Two catalytic activities can be found in the largest subunit of human and *Drosophila* TFIID, $TAF_{II}250$: a serine/threonine kinase activity and a histone acetyltransferase activity. A bipartite (C-terminal and N-terminal) kinase domain is able to autophosphorylate $TAF_{II}250$ and transphosphorylate TFIIF in vitro (Dikstein et al., 1996). Mutations in the N-terminal kinase domain establish a correlation between kinase activity and the capacity of a transfected $TAF_{II}250$ to rescue growth defects in a ts13 cell line ($TAF_{II}250$ ts) (O'Brien and Tjian, 1998). The yeast homolog of $hTAF_{II}250$, $TAF_{II}145/130$, lacks both kinase domains (Dikstein et al., 1996). Human and *Drosophila* $TAF_{II}250$ were also found to possess histone acetyltransferase activity, an activity found to be conserved in yeast $TAF_{II}145/130$ (Mizzen et al., 1996). The TFIID histone-modifying activity would enable itself and the rest of transcription initiation machinery to access nucleosome-bound promoter sequences.

Multiple TFIID complexes. Several studies have shown that multiple species of TAF-specific TFIID can be purified from metazoan cells (Timmers and Sharp, 1991; Brou et al., 1993; Jacq et al., 1994; Bertolotti et al., 1996). This has been followed with the discovery of a tissue-specific TFIID, isolated from differentiated B cells, containing a cell type-specific TAF_{II} subunit replacing a

more general subunit (Dikstein et al., 1996). Two TBP like factors have also been reported, the TBP-related factor (TRF) in Drosophila (Crowley et al., 1993) and a TBP-like factor (TLF) in humans (Wieczorek et al., 1998). TRF exhibits neural-specific expression and can substitute for TBP to support transcription (Hansen et al., 1997). TRF is also found associated with other proteins, called nTAFs, in 500 kD complex (Hansen et al., 1997). In another striking study, a TBP-free TAF_{II}-containing complex (TFTC) was purified from human cells and shown to be able to replace TFIID on both TATA-containing and TATA-less promoters in transcription assays in vitro (Wieczorek et al., 1998). While many TAF_{II}s were shared between TFTC and TFIID, TFTC was also associated with a number of non-TAF protein species. While TFTC was found to be devoid of the TBP-like factor, TLF, the presence of another factor functionally similar to TBP cannot be excluded.

Other TBP-containing complexes. TBP is found as part of a number of other complexes as well. In a manner analogous to TAF_{II}s, TAF_I and TAF_{III} class proteins also associate with TBP to form promoter selectivity complexes for RNA polymerase I and III transcription (reviewed in Geiduschek and Kassavetis, 1995). PTF/SNAPc is a mammalian promoter selectivity factor made up of TBP and associated proteins. It functions at the promoters of small nuclear RNA genes, subsets of which are transcribed by either RNA polymerase II or III (reviewed in Geiduschek and Kassavetis, 1995). No homologs of the PTF/SNAPc subunits have been found in the yeast genome.

Mot1. TBP-Mot1 complexes have been purified from yeast, human and Drosophila extract (Timmers et al., 1992; Auble et al., 1994; Poon et al., 1994; van der Knaap et al., 1997). Genetic evidence from yeast suggests that Mot1 serves to influence RNA polymerase II promoter selection by TBP in vivo

(Collart, 1996; Madison and Winston, 1997). Yeast Mot1 protein can repress TBP binding to DNA in an ATP-dependent manner to repress trancription in vitro (Auble and Hahn, 1993; Auble et al., 1994; Wade and Jaehning, 1996).

TBP-interacting complexes.

Genetic studies in *Saccharomyces cerevisiae* have suggested the existence of a functional interaction between TBP and three complexes involved in regulating the transcription of protein-coding genes: NC2, NOT and SAGA (reviewed in Lee and Young, 1998). Additionally, a physical interaction between mammalian topoisomerase I and TBP has been characterized in vitro. How can one protein, like TBP, be an integral or associated component of so many complexes ? It turns out that yeast TBP is found to be at least ten times more abundantly in the cell than its associated regulatory proteins ($TAF_{II}s$, SAGA, Mot1, NC2 and NOT proteins) (Lee et al., 1998). Thus a large pool of TBP interacts with smaller pools of diverse regulators in vivo.

NC2. NC2 and it's mammalian homolog (Dr1/DRAP1) is a heterotetramer composed of two NC2α/β dimers that can bind directly to TBP (Goppelt and Meisterernst, 1996; Kim et al., 1996; Gadbois et al., 1997). It acts as a general negative regulator of transcription by RNA polymerase II and III (Meisterernst and Roeder, 1991; Inostroza et al., 1992); ; (Goppelt and Meisterernst, 1996; Mermelstein et al., 1996; Gadbois et al., 1997; Kim et al., 1997; Prelich, 1997). NC2 can bind directly to TBP on promoter DNA and can prevent RNA polymerase II holoenzyme, or its TFIIB subunit, from assembling into a PIC (Meisterernst and Roeder, 1991; Inostroza et al., 1992; Kim et al., 1995; Goppelt et al., 1996; Mermelstein et al., 1996; Gadbois et al., 1997).

Not complex. *NOT* genes were identified through no less than seven different yeast genetic screens looking for negative regulators of transcription (reviewed in Lee and Young, 1998). The 5 *NOT* encoded proteins, Not1, Not2, Not3, Not4 and Not5, are found complexed together (Collart and Struhl, 1994) and can associate with Ccr4 and Caf1 (Liu et al., 1998). While their mechanism of action is not yet solved, biochemical and genetic evidence has clearly linked these proteins, functionally and physically, to TBP (Collart and Struhl, 1993; Collart and Struhl, 1994; Lee et al., 1998). Yeast mutant suppression analysis has also shown them to have a negative function on RNA polymerase II holoenzyme transcription in vivo (Lee et al., 1998). Coding sequences for 5 *NOT* homologs can be found in mammalian genome databases (T. Lee and R.A. Young, unpubl.).

SAGA complex. The yeast SAGA histone acetyltransferase complex and it's human counterpart, the PCAF complex, are made up of around 20 different proteins (Grant et al., 1997; Grant et al., 1998; Martinez et al., 1998; Ogryzko et al., 1998). These include the catalytic component Gcn5, as well as Spt, Ada and even TAF_{II} subunits. The SAGA complex has been reported to stimulate in vitro transcription from preassembled nucleosomal template, but not from naked DNA (Grant et al., 1998); Steger et al., submitted). While the SAGA complex does not copurify withTBP, purified SAGA can bind directly to a TBP-affinity column and SAGA immunoprecipitation in crude extracts retains TBP (Barlev et al., 1995; Roberts and Winston, 1997; Saleh et al., 1997; Sterner et al., 1999). This interaction requires Spt3 and to a lesser extent Spt8, a finding buttressed by genetic evidence of a direct functional interaction between these two proteins and TBP (Eisenmann et al., 1992; Eisenmann et al., 1994; Sterner et al., 1999). Another potential relationship between SAGA and TBP comes from the recent discovery that 5 of the yeast

TAF_{II}s present in TFIID, including the histone-like TAF_{II}s, are also present in the SAGA complex (Grant et al., 1998). The TBP interaction with the SAGA complex has been postulated to facilitate nucleosome disruption at the subset of promoters requiring SAGA function in vivo. Most recently, Tra1, a protein similar to TRAPP, has been reported to be a subunit of yeast SAGA as well (Grant et al., 1998). Human TRAPP protein is a member of the ATM/phosphatidylinositol-3 kinase family and can associate with the c-Myc and E2F-1 trasncription factors (McMahon et al., 1998).

Topoisomerase I. In mammalian systems, topoisomerase I has been found to physically interact with TBP to depress basal (i.e. without activator proteins) transcription and stimulate activated transcription (Merino et al., 1993; Kretzschmar et al., 1993; Shykind et al., 1997). The DNA relaxation activity of topoisomerase I is dispensable for repression of basal transcription (Merino et al., 1993) and stimulation of activated transcription (Shykind et al., 1997). The latter activity is also dependent on the presence of TAF_{II}s (Shykind et al., 1997). No genetic evidence of topoisomerase I involvement in transcription has been found yet.

TFIIB.

TFIIB is an essential protein that can bind core RNA polymerase II (Pinto et al., 1992; Tschochner et al., 1992; Bushnell et al., 1996), an interaction supported by genetic interactions between the genes coding for TFIIB and Rpb9, a core RNA polymerase II subunit (Sun et al., 1996). Not surprisingly, TFIIB can be found as a component in some yeast RNA polymerase II holoenzyme preparations (Koleske and Young, 1994; Koleske et al., 1996; Wilson et al., 1996). Besides TBP and core RNA polymerase II, TFIIB binds TFIIF as well. In humans, TFIIB interacts biochemically with the human

homologs of the Tfg1 (Fang and Burton, 1996) and Tfg2 (Ha et al., 1993) subunits of TFIIF, while in yeast, *SUA7* (TFIIB) interacts genetically with *TFG1* (Sun and Hampsey, 1995) in transcriptional start site selection. Drosophila TFIIB has also been reported to bind $dTAF_{II}40$, homologue of the yeast $TAF_{II}17$ (Goodrich et al., 1993). TFIIB possesses a limited DNA binding ability (Lagrange et al., 1998) that could partly explain its role in start site selection (Pinto et al., 1992; Pinto et al., 1994; Berroteran et al., 1994).

In the sequential assembly model of PIC formation, TFIIB enters the PIC after TFIID and as a prerequisite for the recruitment of the TFIIF bound RNA polymerase II (reviewed in Conaway and Conaway, 1993; Zawel and Reinberg, 1993; Buratowski, 1994). TFIIB entry into PIC was considered to be another possible rate-limiting step that could be modulated by transcriptional activators. Again, a number of biochemical studies have supported this view. TFIIB binds to diverse classes of activation domains and its recruitment to the TBP-TATA complex is enhanced by the acidic activator VP16 and the proline-rich activator CTF1 (Kim and Roeder, 1994; Lin and Green, 1991). The use of VP16 and GAL4 acidic activation domain mutants shows that their TFIIB binding correlates with the strength of their activation domain Lin et al., 1991; Wu et al., 1996). Conversely, TFIIB point mutants failing to interact with the activation domain of VP16 support basal but not activated transcription (Roberts et al., 1993; Roberts et al., 1995). Aside from enhanced TFIIB recruitment into PIC, acidic activators have also been shown to induce a conformational change in TFIIB (Roberts and Green, 1994). However, a yeast in vivo experiment has cast some doubts on the model of direct TFIIB recruitment by activators. A number of TFIIB mutants had been characterized in vitro and shown to be unable to form a stable TFIIB-TBP-TATA complex and/or to bind the VP16 activation domain Chou and Struhl,

1997). Contrary to the prediction, introduction of these mutants into yeast cells did not result in any loss of transcriptional activation in response to VP16 in vivo (Chou and Struhl, 1997). Additionally, TBP mutants defective in direct TFIIB interaction in vitro show no defect in transcriptional activation in vivo either (Lee and Struhl, 1997). These data do not preclude the more complex model where TFIIB, as a holoenzyme component, still provides a vital link to the TFIID-promoter DNA complex in an activator-regulated step in vivo. Consistent with this view, recruitment of TFIIB by artificial tethering to a promoter-bound factor is sufficient to activate transcription in yeast cells (Gonzalez-Couto et al., 1997).

TFIIF.

While mammalian TFIIF is composed of two subunits, called RAP74 and RAP30 (Flores et al., 1990), yeast TFIIF contains three subunits (Henry et al., 1992). Tfg1 and Tfg2 subunits are respectively homologs of RAP74 and RAP30 (Henry et al., 1994), while Tfg3 is a weakly associated component. Tfg3 is unique to yeast and is a subunit also found in TFIID and Swi/Snf complexes (Henry et al., 1994; Cairns et al., 1996). This shared component could be symptomatic of a larger complex tying together the three different subcomplexes. The mammalian TFIIF subunits were first identified as RNA polymerase II associated proteins (RAP) and both yeast and human TFIIF have been found tightly associated with RNA polymerase II (Sopta et al., 1985; Flores et al., 1989; Henry et al., 1992). Not surprisingly, TFIIF is a component of all yeast (Koleske and Young, 1994; Kim et al., 1994; Koleske et al., 1996) and mammalian (reviewed in Parvin and Young, 1998) RNA polymerase II holoenzymes isolated to date.

In higher eukaryotes TFIIF appears to exist as $\alpha_2\beta_2$ hetertetramer (Conaway and Conaway, 1989; Flores et al., 1990) and binds to TFIIB (Ha et al., 1993; Fang and Burton, 1996), TFIID (Ruppert and Tjian, 1995; Dubrovskaya et al., 1996; Tang et al., 1996) and TFIIE (Maxon et al., 1994). As described in the TFIIB section, yeast genetic evidence supports a TFIIF-TFIIB interaction. TFIIF shows a high degree of structural and functional similarity to the prokaryotic sigma (σ) factors (Sopta et al., 1989; Conaway and Conaway, 1990; McCracken and Greenblatt, 1991; Garrett et al., 1992; Killeen et al., 1992; Tan et al., 1994). TFIIF can suppress nonspecific RNA polymerase II DNA binding and can also stabilize PIC formation (Conaway and Conaway, 1993; Greenblatt, 1991). In addition to its role in transcription initiation, TFIIF also stimulates the rate of RNA polymerase II RNA chain elongation ((Flores et al., 1989; Bengal et al., 1991). An intriguing TFIIF function has emerged recently from in vitro DNA-protein crosslinking studies with purified GTFs. TFIIF has been shown to be responsible for the isomerization of a promoter DNA-TBP-TFIIB-core RNA polymerase II-TFIIF-TFIIE complex (Robert et al., 1998). This isomerization is accompanied by tight wrapping of the promoter DNA around the complex for almost a full turn (Robert et al., 1998). TFIIF subunits have also been suggested to be a direct target for activators, such as the Serum Response Factor (Joliot et al., 1995) and Fos/Jun (Martin et al., 1996). Yeast and human TFIIF have also been shown to interact with Fcp1, a component of a TFIIF associated CTD phosphatase activity (Chambers and Kane, 1996; Archambault et al., 1997; Archambault et al., 1998). Fcp1 has been detected in crude yeast and human RNA polymerase II holoenzyme fractions (Archambault et al., 1997; Archambault et al., 1998). Little else is known of Fcp1's function except that it carries out an essential function in yeast (Archambault et al., 1997).

TFIIH.

With nine subunits, TFIIH is a complex factor possessing three enzymatic subunits with either ATP-dependent DNA helicases (Schaeffer et al., 1993; Serizawa et al., 1993) or CTD kinase (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992) activities. TFIIH purified from yeast (Feaver et al., 1991), rat liver (Conaway and Conaway, 1989) and human (Flores et al., 1992; Gerard et al., 1991) cells share conserved polypeptides and common subunit activities (reviewed in Svejstrup et al., 1996; Feaver et al., 1997). TFIIH has also been found as a component in some RNA polymerase II holoenzyme preparations (Koleske and Young, 1994; Koleske et al., 1996) but not in others (Kim et al., 1994).

Human TFIIH has been shown to perform critical roles at early and late stages of transcription initiation. The transcription initiation process can be thought of as three broad steps: PIC formation, open complex formation and promoter clearance (reviewed in Gralla, 1996). Open complex formation is a required intermediate in which 12-15 p of promoter DNA are in the form of a single-stranded bubble. Initiation is then completed by a series of reactions coverting the open complex to an activately elongating complex, a transition called promoter clearance. Open promoter complex formation by RNA polymerase II requires the ATP-dependent DNA helicase activity of TFIIH (Wang et al., 1992; Jiang et al., 1994). TFIIE, TFIIH and ATP hydrolysis are dispensable for in vitro transcription initiation from supercoiled (Parvin and Sharp, 1993; Parvin et al., 1994; Tyree et al., 1993) or pre-melted promoter DNA (Holstege et al., 1995; Pan and Greenblatt, 1994; Tantin and Carey, 1994). Additional detailed biochemical analysis strongly support a role for TFIIE, TFIIH and ATP hydrolysis in open complex formation (Dvir et al., 1996;

Holstege et al., 1996; Holstege et al., 1997) and promoter clearance (Holstege et al., 1997; Shilatifard, 1998; Kumar et al., 1998).

Yeast (Feaver et al., 1991) and mammalian TFIIH (Lu et al., 1992) can both phosphorylate the CTD in vitro, an activity stimulated by TFIIE (Lu et al., 1992; Ohkuma and Roeder, 1994). The TFIIH CTD kinase activity has also been suggested to regulate promoter clearance by phosphorylating the CTD of core RNA polymerase II. A role for TFIIH in transcriptional elongation has been suggested by anti-TFIIH antibody microinjection experiments in Xenopus oocytes (Yankulov et al., 1996) and in vitro transcription experiments (Dvir et al., 1996; Dvir et al., 1997). Mammalian TFIIH can also phosphorylate TBP and both TFIIE subunits (Ohkuma and Roeder, 1994).

Yeast TFIIH is a holoenzyme that can be dissociated into three subcomplexes: a core TFIIH complex, the Ssl2 protein and the TFIIK subcomplex (Svejstrup et al., 1995). The core TFIIH complex contains six subunits: Tfb1, Tfb2, Tfb3, Tfb4, Rad3 and Ssl1 (Svejstrup et al., 1995; Wang et al., 1995; Feaver et al., 1997). Core TFIIH subcomplex exists in multiple forms, functioning as a fundamental RNA polymerase II GTF in one form (TFIIH) and as an essential component in nucleotide excision repair (NER) in the other (repairosome) (Feaver et al., 1991; Wang et al., 1994; Svejstrup et al., 1995). While both complexes share the same core TFIIH subcomplex and the Ssl2 protein, the repairosome contains the repair proteins Rad1, Rad2, Rad4, Rad10, and Rad14, instead of the TFIIK subcomplex found in the TFIIH GTF. The dual role of core TFIIH subcomplex in DNA repair and transcription could account for previous observations of a link between gene transcription and preferential repair (Bohr et al., 1985; Mellon and Hanawalt, 1989). The TFIIK subcomplex contains a cyclin homolog, Ccl1 (Svejstrup and Feaver, 1996), and an associated kinase, Kin28 (Feaver et al., 1994). Kin28 is a cyclin-

dependent kinase (CDK) homologous to Cdk7/MO15, the catalytic subunit in human TFIIH (Roy et al., 1994). Biochemical studies have shown that the human TFIIK kinase, Cdk7, plays a dual role; it serves as a CTD kinase to control transcription, and as a CDK activating kinase to control cell cycle progression (Fesquet et al., 1993; Poon et al., 1994); ; (Solomon et al., 1993; Fisher and Morgan, 1994; Makela et al., 1994; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). In yeast however, Kin28 only exhibits CTD kinase activity and its in vivo role seems restricted to transcription (Cismowski et al., 1995; Valay et al., 1995). Kin28 does not phosphorylate Cdc28, the CDK involved in yeast cell cycle progression. Yeast cells with a temperature sensitive *KIN28* mutant show no cell cycle progression defects, but rather, suffer a rapid loss of class II gene transcription (Cismowski et al., 1995; Valay et al., 1995). The yeast CDK activating kinase function is found to be executed by a monomeric kinase with no involvement in transcription: Cak1/Civ1 (Espinoza et al., 1996; Kaldis et al., 1996; Thuret et al., 1996). Thus yeast seems to have evolved two protein kinases, Kin28 and Cak1, to carry out the same functions as Cdk7 in vertebrate cells.

As with other transcription factors, TFIIH has also been reported to interact with activators, such as VP16 and p53 (Xiao et al., 1994). Certain activators stimulate transcriptional elongation (Yankulov et al., 1996) and the strength of these activators correlates with their abilities to bind TFIIH (Blau et al., 1996). Most significantly, the transcriptional activator HIV Tat can stimulate transcriptional elongation and CTD phosphorylation by human TFIIH in vitro (Parada and Roeder, 1996; Garcia-Martinez et al., 1997). The use of a specific TFIIH kinase inhibitor shows that CTD phosphorylation by TFIIH is necessary for stimulation of elongation by Tat (Cujec et al., 1997).

TFIIE.

TFIIE contains 2 subunits which have been conserved from yeast to humans (Feaver et al., 1994; Ohkuma et al., 1991; Peterson et al., 1991; Sumimoto et al., 1991). TFIIE is physically (Bushnell et al., 1996) and functionally (Li et al., 1994) linked to TFIIH, stimulating CTD phosphorylation by TFIIH (Lu et al., 1992; Ohkuma and Roeder, 1994). TFIIE can also interact with the TFIIF and the unphosphorylated form of RNA polymerase II (Flores et al., 1989; Maxon et al., 1994). TFIIE can bind to single-stranded DNA (Kuldell and Buratowski, 1997), a result consistent with its requirement in open complex formation. This GTF is not present in yeast RNA polymerase II holoenzymes (Koleske and Young, 1994; Kim et al., 1994; Koleske et al., 1996). In *Drosophila*, TFIIE has been implicated as the direct target of homeodomain class of activators (Zhu and Kuziora, 1996) and the Krüppel repressor (Sauer et al., 1995).

The largest TFIIE subunit, Tfa1, contains a zinc-binding motif of the C_2C_2 class. The TFIIE zinc-binding motif was necessary for reconstituted in vitro transcription of the Ad2 MLP template in yeast and human systems (Tijerina and Sayre, 1998; Maxon and Tjian, 1994), but not for TFIIE's ability to bind single-stranded DNA (Kuldell and Buratowski, 1997). Mutants in this motif were shown to cause thermosensitive growth in yeast (Kuldell and Buratowski, 1997; Tijerina and Sayre, 1998). At the restrictive temperature, bulk mRNA was found to decrease substantially (Kuldell and Buratowski, 1997), yet a subset of genes showed no defects when assayed individually (Tijerina and Sayre, 1998). A tight TFIIE ts mutant in a region other than the zinc-binding motif was also used to probe the GTF's function (Sakurai and Fukasawa, 1997). Such a mutant failed to show a strict and obligatory requirement for TFIIE at all promoters (Sakurai and Fukasawa, 1997; Holstege et al., 1998). This promoter variability in TFIIE requirement matches in vitro

findings that the dependence of promoters on human TFIIE is determined by a DNA region around the transcription start site (Tijerina and Sayre, 1998; Holstege et al., 1995).

TFIIA.

Yeast TFIIA is not a component of the RNA polymerase II holoenzyme (Koleske and Young, 1994; Kim et al., 1994; Koleske et al., 1996) and its activity copurifies with two polypeptides encoded by *TOA1* and *TOA2* (Ranish and Hahn, 1991; Ranish et al., 1992). Mammalian TFIIA is encoded by two genes homologous to *TOA1* and *TOA2*(De Jong and Roeder, 1993; Ma et al., 1993). The human *TOA1* product is modified post-translationally to yield two different polypeptides, which, when assembled with the human *TOA2* product, yield a TFIIA factor with 3 subunits (Reinberg et al., 1987).

Mammalian TFIIA was originally identified as a general transcription factor, i.e. required for promoter-specific in vitro transcription in a reconstituted system (Reinberg et al., 1987). Since then, TFIIA has been found to be dispensable for this role, acting rather to stimulate transcription in TFIID-directed systems at TATA-containing and TATA-less promoters (Cortes et al., 1992; De Jong et al., 1995; Ozer et al., 1994; Sun et al., 1994; Martinez et al., 1998; Emami et al., 1997). TFIIA joins the PIC through interactions with TBP (Buratowski et al., 1989) and stabilizes the promoter-TBP interaction (Imbalzano et al., 1994). TFIIA acts as a transcriptional coactivator or anti-repressor (Kang et al., 1995; Ma et al., 1996), presumably by antagonizing TBP-interacting repressors such as NC2 (Inostroza et al., 1992), topoisomerase I (Meisterernst et al., 1991; Merino et al., 1993), HMG1 (Ge and Roeder, 1994), Mot1 (Auble et al., 1994) and even TAF_{II}s themselves (Ozer et al., 1998). Drosophila or human TFIIA also interact with activators such as Zta, Sp1,

VP16 and NTF-1 (Ozer et al., 1994; Yokomori et al., 1994), with a TAF_{II} subunit (Yokomori et al., 1993), with coactivators PC4 and HMG2 (Ge and Roeder, 1994; Shykind et al., 1995) and with the repressor RBP (Olave et al., 1998).

Yeast TFIIA stimulates basal and activated in vitro transcription in a nuclear extract but is dispensable for these same functions when purified general transcription factors are used (Sayre et al., 1992; Kang et al., 1995). These results are consistent with an anti-repressor function for TFIIA. In vivo, yeast mutants of TBP or TFIIA defective in TBP-TFIIA interaction fail to respond to a number of transcriptional activators and show transcriptional defects at a subset of promoters (Stargell and Struhl, 1995; Ozer et al., 1998). In one study, the defects associated with a TBP defective for TFIIA binding was rescued by fusing the smallest TFIIA subunit (Toa2) to TBP (Stargell and Struhl, 1995). This argues that an efficient TBP-TFIIA interaction is required for transcriptional activation at a subset of promoters.

The crystal structure of a yeast promoter DNA-TBP-TFIIA crystal has been solved and confirms a number of functions assigned to TFIIA: TBP/TFIIA has extended contacts with DNA and may explain the stabilizing effect on TBP binding (Geiger et al., 1996; Tan et al., 1996). TFIIA offers a wide surface for activator contacts and requires only a limited contact surface with TBP, relying upon relative spatial constraints to achieve specificity. This suggests that limited contacts between multiple factors may be sufficient to assemble a large number of proteins into the PIC complex (Jacobson and Tjian, 1996).

Suppressors of RNA polymerase B (II) (*SRBs*).

Genetic selection. The CTD of yeast core RNA polymerase II consists of 26 repeats of a seven amino acid consensus sequence and is involved in transcription initiation and the response to activators (See core RNA polymerase II). Yeast cells containing RNA polymerase II mutants that are progressively more truncated for their CTD, exhibit increasingly more severe phenotypes (Nonet et al., 1987): with a CTD from 26 (full length) to 13 repeats, cells grow normally; from 12 to 10 repeats, cells show conditional growth defects; with 9 repeats or less, cells are inviable. The conditional growth defects include cold sensitivity (Nonet et al., 1987), temperature sensitivity (Nonet et al., 1987), inositol auxotrophy (Nonet and Young, 1989) and an inability to use pyruvate as a sole carbon source (Thompson et al., 1993). This set of pleiotropic phenotypes is virtually identical to the set seen when RNA polymerase II is underproduced, consistent with a strong transcription defect for CTD truncations (Archambault et al., 1992).

A genetic selection for factors likely to be involved in transcription initiation and regulation was initiated by looking for suppressor mutations that allowed CTD truncation mutant (*rpb1Δ104*) cells to grow under restrictive conditions (Nonet and Young, 1989; Thompson et al., 1993). Both intragenic (in *rpb1Δ104*) and extragenic (in a gene other than *rpb1Δ104*) suppressors were found (Nonet and Young, 1989). Intragenic suppressors fell in either of two classes: 1) CTD heptapeptide repeat duplications that increased the number of repeats from to 13 or more ; 2) a specific valine to phenylalanine amino acid substitution in the homology box H region of *RPB1* (Nonet and Young, 1989). This suggests that the H box region, a region outside of the CTD that is conserved among RNA polymerases II, could function with the CTD in transcription initiation. The extragenic suppressor

mutants, called *SRB*s (suppressors of RNA polymerase B or II), were classified as dominant and recessive on the basis of their ability to suppress the CTD truncation mutants in the presence of the wild-type suppressor gene (Nonet and Young, 1989; Thompson et al., 1993; Hengartner et al., 1995). The dominant *SRB*s were postulated to be positive (necessary) transcription factors that had gained or strengthened a function, while the recessive suppressors were postulated to be negative (inhibitory) transcription factors that would have lost their repressive function. Both types of mutants, when combined with a partly defective RNA polymerase II, would partially restore transcription function. Since the positive factors were deemed more interesting, initial cloning efforts focused on the dominant *SRB*s.

In a first small scale *SRB* genetic selection, two different extragenic suppressors were analyzed and classified as *SRB2-1* and *srb3-1*, respectively (Nonet and Young, 1989). Note that the *SRB1/KEX2* protease gene is unrelated to all the other *SRB* designates and was found as a suppressor of *rpb1-1*, a tight RNA polymerase II ts mutant different from the CTD truncation mutants (Nonet et al., 1987; Martin and Young, 1989). *srb3-1* was found to be a weak recessive suppressor and was not characterized further. The dominant suppressor *SRB2-1* was isolated (Nonet and Young, 1989) and sequenced (Koleske et al., 1992). A larger genetic selection for suppressors yielded the isolates from which all other Srb genes were cloned (Thompson et al., 1993): the dominant suppressor genes *SRB4*, *SRB5* and *SRB6* first (Thompson et al., 1993),the recessive suppressor genes *SRB7* (Hengartner et al., 1995), *SRB8* (Hengartner et al., 1995), *SRB9* (Hengartner et al., 1995), *SRB10* (Liao et al., 1995), and *SRB11* (Liao et al., 1995) later. Mutant alleles of *RPB2*, the second largest subunit of core RNA polymerase II, were also isolated as recessive suppressors of CTD truncation mutants (Hengartner et al., 1995).

This demonstrated that this genetic selection could find factors physically linked to Rpb1. All 9 *SRB* genes encoded for novel proteins and, with the exception of *SRB10* and *SRB11* , showed no homology to previously characterized proteins.

Genetic analysis. *SRB* deletion analysis showed *SRB4, SRB6* and *SRB7* to be essential genes, with all other *SRB*s showing cold sensitive (cs) and temperature sensitive (ts) phenotypes (Nonet and Young, 1989; Thompson et al., 1993; Hengartner et al., 1995; Liao et al., 1995). These phenotypes are characteristic of CTD truncation, and *srb2Δ* even caused inositol auxotrophy (Nonet and Young, 1989). The deletions of *SRB8, SRB9, SRB10* or *SRB11* were also shown to suppress some of the CTD defects such as the inability to use pyruvate as sole carbon source, arguing that the recessive suppressor alleles were indeed loss-of-function mutants (Hengartner et al., 1995; Liao et al., 1995). Further genetic characterization of the *SRB*s clearly showed that they were functionally interacting with the RNA polymerase II CTD.

First, the original suppressor alleles of all nine *SRB* genes suppressed only the conditional growth defects associated with CTD truncation mutants. Similar conditional phenotypes by six or seven other *RPB1* mutations unrelated to the CTD, were not rescued (Nonet and Young, 1989; Thompson et al., 1993; Hengartner et al., 1995; Liao et al., 1995). One exception of unknown significance was noted: the cs phenotype of *rpb1-14* could be suppressed by the dominant suppressor alleles (*SRB2-1, SRB4-1, SRB5-1* and *SRB6-1*) (Nonet and Young, 1989; Thompson et al., 1993).

Second, the genetic interaction of the CTD with *SRB2* (Koleske et al., 1992)and *SRB10* (Hengartner et al., 1998) alleles came to examplify how dominant and recessive suppressors were indeed acting positively and negatively on transcription in vivo. Yeast cells containing a spectrum of

different length of CTD truncation mutants, and a mutant or wild type *SRB* allele were assayed for growth. It was shown that the dominant *SRB2-1* allele restored full viability to conditionally growing cells and even allowed cells with only 9 heptapeptide repeats to grow conditionally. On the other hand, a *SRB2Δ* allele acted to render most CTD truncations lethals, and even full length CTD remained only conditionally viable. This is consistent with *SRB2* encoding a positive transcription factor and the dominant suppressor being a gain-of-function mutant. In sharp contrast, recessive *srb10-1* and *srb10Δ* alleles had the same effect as *SRB2-1*. That is, they all rescued conditionally viable cells and both allowed conditional growth of cells with only 9 heptapeptide repeat CTD. Thus, the loss of *SRB10* function mimicks the loss of a repressor protein.

Third, the *SRB*s were also linked to the CTD using a different genetic approach. In vivo, substitution of the second heptapeptide Ser ($Y_1S_2P_3T_4S_5P_6S_7$) for Ala or Glu, was lethal when enough repeats where altered (Yuryev and Corden, 1996). *SRB9* loss-of-function mutations as well as the dominant *SRB* alleles tested (*SRB2-1*, *SRB4-1*, *SRB5-1*, *SRB6-1*) suppressed these mutants.

Yeast RNA polymerase II holoenzyme discovery.

The attempt to purify the dominant Srb2, Srb4, Srb5 and Srb6 proteins by conventional chromatography led to the discovery of the RNA polymerase II holoenzyme (Thompson et al., 1993; Koleske and Young, 1994), a large protein complex containing a subset of the GTFs, core RNA polymerase II, the dominant Srb proteins and other regulatory proteins (Koleske and Young, 1994; Kim et al., 1994). The other Srb proteins were later found to be holoenzyme components as well (Liao et al., 1995; Hengartner et al., 1995).

Incubating the holoenzyme with anti-CTD antibodies could break apart the holoenzyme into core RNA polymerase II, general transcription factors and a CTD-associated subcomplex (Kim et al., 1994; Hengartner et al., 1995). This CTD-associated subcomplex, called the Srb/mediator complex, was found to contain the Srb and other holoenzyme-associated regulatory proteins (Kim et al., 1994; Hengartner et al., 1995).

When supplemented with the missing GTFs, the RNA polymerase II holoenzyme was able to initiate transcription and respond to an activator in vitro (Koleske and Young, 1994; Kim et al., 1994). This response to activator could also be reproduced by adding the Srb/mediator complex to the purified GTFs and core RNA polymerase II (Kim et al., 1994). The transcriptional activation witnessed was remarkable because, as opposed to crude yeast extracts, transcription reconstituted with purified yeast GTFs and core RNA polymerase II had been found to be unresponsive to activators (Sayre et al., 1992; Sayre and Kornberg, 1993; Flanagan et al., 1991). Furthermore, neither TFIIA nor TAF_{II}s were required as coactivators. Analysis of temperature-sensitive mutants of two of the Srb components, Srb4 and Srb6, demonstrated that they were globally required for transcription in yeast cells (Thompson and Young, 1995). Since these Srb proteins had been found to be tightly and exclusively associated with the RNA polymerase II holoenzyme, it was argued that this is likely to be the form of RNA polymerase II recruited to promoters in vivo.

Heterogeneity in holoenzyme subunit composition.

A protein is considered to be a holoenzyme subunit, if it is found present in stoichiometric amounts in highly pure holoenzyme preparations (Koleske and Young, 1994). This is usually determined by quantitative Western

blotting of purified RNA polymerase II holoenzyme alongside recombinant protein standards. The amount of query protein found is compared to the amounts of a known holoenzyme subunit such as Srb5 or Rpb1 (Koleske and Young, 1994; Rubin et al., 1996; Gadbois et al., 1997). In one case, the erroneous report of Sug1 as a holoenzyme component was corrected through the application of this stringent standard (Rubin et al., 1996).

Even when this standard is applied, the exact polypeptide composition of yeast and especially mammalian RNA polymerase II holoenzymes is found to vary from study to study. This is a direct consequence of the inherent difficulties in purifying intact megadalton-sized complexes (Parvin and Young, 1998). In yeast, these variations can be traced to a small number of possible causes (strain background, growth conditions, extract and purification procedures) combined with a relative holoenzyme instability. From the very start, quite different yeast strains have been used as holoenzyme sources: the protease-deficient BJ926 *Saccharomyces cerevisiae* laboratory strain S288C derived), (used in Koleske and Young, 1994; Hengartner et al., 1995; Li et al., 1996) ; (Koleske et al., 1996; Wilson et al., 1996); the closely related laboratory strains (S288C derived) for studying mutant holoenzymes (used in Liao et al., 1995; Li et al., 1995; Koleske et al., 1996; Hengartner et al., 1998; Myers et al., 1999); and finally the less defined commercial yeast (Fleischmann or Red Star brand) (used in Kim et al., 1994; Hengartner et al., 1995; Koleske et al., 1996; Myers et al., 1998). As a rule of thumb, all Srb/mediator complex preparations (Kim et al., 1994; Hengartner et al., 1995; Myers et al., 1998) originate from commercial yeast, while all RNA polymerase II holoenzyme preparations, with a few exceptions described in Kim et al., 1994; Li et al., 1995; Koleske et al., 1996; Wilson et al., 1996; Myers et al., 1999), originate from S288C derived strains. The second difference is growth condition and time of cell collection:

laboratory strains have been grown under aerobic conditions in rich (YPD) or minimal (YNB) media (Sherman, 1991) and collected at mid-log or late logarythmic growth phase while commercial baking yeast strains are grown in fermentors and collected when cell density nears saturation. The point on the growth curve at which cells are harvested is known to dramatically affect the composition of at least two holoenzyme subcomplexes: the Rpb4/Rpb7 core RNA polymerase II subcomplex is only found as a stoichiometric subunit near stationary phase (Choder and Young, 1993) while the amount of the catalytic component of the Srb10 CDK subcomplex is found to decrease several fold upon diauxic shift (Hengartner et al., 1998). The extract preparation and chromatographic purification are also likely to cause partial fragmentation of a larger RNA polymerase II holoenzyme into different isoforms lacking one or more subcomplexes. Indeed, the differences between the smallest (Li et al., 1996) and largest (Koleske and Young, 1994) yeast holoenzyme preparations described, involve a discrete and small number of genuine subcomplexes. Furthermore, a holoenzyme that seems to be an intermediate between these two forms has also been reported (Koleske et al., 1996). Importantly, no single purification attempt has ever resulted in the purification of two different holoenzyme preparations from the same cells. The existence of a radically different form of yeast RNA polymerase II holoenzyme, devoid of Srb proteins, has also been postulated but not demonstrated (Shi et al., 1997). The physiological importance of this so-called holoenzyme is questionable, since in vivo disruptions of the function of two purported components, Paf1 and Cdc73, showed little if any effect on gene transcription (Shi et al., 1996; Shi et al., 1997).

Despite all the differences in purification procedures, the yeast RNA polymerase II holoenzymes described show discrepancies in subunit

composition limited to TFIIH, TFIIB, Srb10 CDK subcomplex and Swi-Snf subcomplex. In this review, it will be assumed that the more complex RNA polymerase II holoenzyme is closer to the physiological form of holoenzyme. The combination of yeast genetic and biochemical criteria is of great help in deducing the nature of the holoenzyme components, and seems to support this assumption. Thus, the regulatory proteins found in the RNA polymerase II holoenzyme can be divided into three subcomplexes, the multifunctional core SRB-mediator complex, and the less tightly associated Srb10 CDK and Swi-Snf chromatin-remodeling subcomplexes.

The core SRB-mediator complex

In vitro transcription reactions reconstituted with highly pure core RNA polymerase II and GTFs are not responsive to activators (Sayre et al., 1992; Sayre and Kornberg, 1993). This formed the basis for the purification of factors that could act as a "mediator of activation" (Flanagan et al., 1991). It was recognized that the CTD-associated subcomplex of regulatory proteins associated with the RNA polymerase II holoenzyme was just such a mediator (Kim et al., 1994; Hengartner et al., 1995). By far the largest of all holoenzyme subcomplexes, it has now been fractionated to high purity from commercial yeast using only conventional chromatography and thus bypassing the need for a CTD affinity column step (Myers et al., 1998). This yields a simpler "core" Srb/mediator complex composed of around 20 polypeptides: Srb2, Srb4, Srb5, Srb6, Srb7, Med1, Med2, Med4, Med6, Med7, Med8, Med11, Cse2, Gal11, Pgd1, Rgr1, Rox3, Sin4, Nut2.

In addition to supporting activated transcription in vitro, the Srb/mediator complex stimulates basal transcription and CTD phosphorylation by TFIIH in vitro (Kim et al., 1994). As predicted by the *SRB*

genetic analysis, all three in vitro effects required the RNA polymerase II CTD (Myers et al., 1998). The Srb/mediator complex was found to bind tightly and specifically to recombinant CTD (Myers et al., 1998). The stimulation of basal transcription is probably a direct consequence of the stable association of GTFs in a RNA polymerase II holoenzyme. This stable GTF association is also supported by a role for Srb proteins in holoenzyme stability (Farrell et al., 1996). Analysis of DNA-bound and free forms of RNA polymerase II in crude cell extracts suggests that the Srb/mediator complex is not associated with elongating RNA polymerase II, but rather, is limited to the initiating form of RNA polymerase II (Svejstrup et al., 1997). On the basis of their genetic and biochemical properties, the Srb/mediator complex factors can be classified into a minimum of 4 functional categories: Dominant Srbs subcomplex, Srb7, Med proteins, and Cse2, Gal11, Pgd1, Rgr1, Rox3, Sin4, Nut2.

Dominant Srbs subcomplex (Srb2, Srb4, Srb5, Srb6, Med6 and Rox3 subcomplex). The dominant group of *SRB* genes code for Srb2, Srb4, Srb5 and Srb6. As suppressors of RNA polymerase II CTD truncation mutants, they are functionally linked to the CTD. While CTD truncation does not seem to alter core RNA polymerase II stability (Nonet et al., 1987), it reduces the ability of the holoenzyme to respond to activators (Allison and Ingles, 1989; Scafe et al., 1990; Liao et al., 1991). The *SRB* gain-of-function mutations compensate for CTD truncation by affecting the ability of activators to interact with the holoenzyme, such as with *SRB4-1* (Koh et al., 1998), or by stabilizing the holoenzyme, such as *SRB2-1* (Farrell et al., 1996).

The holoenzyme stabilizing role of Srb2 is also consistent with in vitro assays showing that Srb2 and Srb5 were required for transcription and assembly of a stable PIC in nuclear extracts (Koleske et al., 1992; Thompson et al., 1993). Srb2 and Srb5 are likely to be both involved in the same

holoenzyme stabilization function as they are biochemically and genetically similar. A strain deleted for *SRB5* shows severely reduced levels of Srb2, and the rescue of the in vitro defects of a *srb5Δ* nuclear extract requires addition of both Srb2 and Srb5 proteins (Thompson et al., 1993). Both proteins are encoded by non-essential genes whose deletions have nearly identical phenotypes (Koleske et al., 1992; Thompson et al., 1993).

Biochemical and genetic assays convincingly show that Srb4 is the direct physiological target of the Gal4 activator (Koh et al., 1998). This activator interacting role of Srb4 is likely to be shared by Srb6 as well, since they are both encoded by essential genes that affect transcription of virtually all protein-coding genes in vivo (Thompson et al., 1993; Thompson and Young, 1995; Holstege et al., 1998). A temperature-sensitive *SRB4* mutant that produced a rapid and general shutdown of mRNA synthesis (Thompson and Young, 1995; Holstege et al., 1998), could be suppressed by a dominant (gain-of-function) mutation in *SRB6* or*MED6* (Lee et al., 1998). Conversely, a *med6-ts* mutation can be suppressed by a dominant allele of *SRB4* (Lee and Kim, 1998). In this case, suppression of the *med6-ts* growth defects is correlated with a partial rescue of the transcriptional defects in vivo (Lee and Kim, 1998). All these genetic suppression interactions have been shown to occur in an allele specific fashion, strongly suggesting that direct physical contacts link Srb4 to Srb6 and Med6.

Med6 has been shown to contribute to the mediation of activation function of the Srb/mediator subcomplex. It is found required for the full induction of a subset of genes in vivo (Lee et al., 1997), and a genome-wide analysis of genes requiring Med6 function showed that it plays an essential role in at least 10% of all yeast promoters (Holstege et al., 1998). Furthermore, in vitro trancription experiments with RNA polymerase II holoenzymes

impaired for Med6 function showed that Med6 was required for activated transcription by the VP16 acidic activator, a requirement limited to the initiation of transcription step (Lee et al., 1997).

As predicted, recombinant Srb2, Srb4, Srb5, Srb6 and Med6 can form a stable complex in vitro, where Srb2 associates with Srb5, and Srb4 associates with Srb6 and Med6 (Koh et al., 1998; Lee et al., 1998). A bridging interaction between Srb2 and Srb4 brings them together in a single complex. A purified yeast Srb/mediator subcomplex can also be disrupted into two small stable subcomplexes after denaturing urea treatment (Lee and Kim, 1998). The first subcomplex has been reported to contain Srb2, Srb4, Srb5, Srb6 Med6 and Rox3. The other was reported to contain Srb7, Med1, Med4, Med7, Med8, Med9, Gal11, Pgd1, Rgr1 and Sin4. While no mammalian homologs of dominant Srbs have been found yet, Med6 homologs have been described in humans and *C. elegans* (Lee and Kim, 1998) ;(Sun et al., 1998).

Srb7. Srb7 has characteristics that straddle those of both dominant and recessive Srb proteins. The gene for Srb7 was first isolated as a recessive suppressor of CTD truncation, yet like *SRB4* and *SRB6*, it is essential for viability (Hengartner et al., 1995). Unlike the other recessive Srbs (Srb8, Srb9, Srb10 and Srb11), it is tightly associated with the core Srb/mediator complex (Myers et al., 1998). A possible explanation for these phenotypes is that Srb7 is a Srb4- and Srb6-like protein that anchors the recessive Srb subcomplex (Srb10 CDK subcomplex). A mutation impairing the interaction with the Srb10 CDK subcomplex would appear as a recessive suppressor, while the other functions of Srb7 would be more akin to Srb4 and Srb6. This predicts that conditional mutants in Srb7 will show that it is generally required for transcription in vivo. The human homolog of *SRB7* has been cloned and shown to function in yeast when in the form of a human-yeast chimera (Chao et al., 1996).

Med proteins. The Med proteins represent the set of polypeptides found in the core Srb/mediator subcomplex that had not previously been identified through genetic analysis: Med1, Med2, Med4, Med6, Med7, Med8, Med11 (Myers et al., 1998; Gustafsson et al., 1998). Med4 and Med5 turned out to be a single protein migrating as a doublet (Myers et al., 1998) and the corresponding gene has been named *MED4*. Med3, Med9 and Med10 were found to be encoded by *PGD1*, *CSE2* and *NUT2*, respectively, and will be discussed in the next section (Myers et al., 1998; Gustafsson et al., 1998).

Aside from Med2 and Med6, the function of most Med proteins is still unknown. A mouse and human homolog of *MED7* have been described recently (Jiang et al., 1998; Myers et al., 1998).

Genetics of *CSE2*, *GAL11*, *PGD1*, *RGR1*, *ROX3*, *SIN4* and *NUT2*. With the exception of *CSE2* and *PGD1*, the genes in this category were identified in a variety of genetic selections and screens as a result of their positive or negative influence on the transcription of genes of interest (*CYC7*, *HO*, *IME1*, *GAL1*, *GAL10*, *SUC2*, etc.) (reviewed in Carlson, 1997; Tabtiang and Herskowitz, 1998; Piruat et al., 1997). Depending on the promoter studied, many of these genes show signs of positive or negative regulation. The elucidation of each protein's role is complicated by the fact that the bulk of our knowledge about them derives from genetic observations. It is hard to determine whether these factors truly possess both positive and negative functions in the trancription of different genes, or whether the various effects observed are the indirect consequence of a simpler function. This apparent dual role in transcription in vivo has also been seen for some Med proteins. Yeast whole genome analysis of transcription in a *med2Δ* strain has also failed to clarify its role in vivo: of the 6000 genes analyzed, approximately 200

increased two-fold or more while a similar number decreased two-fold or more (Myers et al., 1999).

Gal11, Pgd1, Rgr1, Sin4 and Med2 subcomplex. A careful analysis of phenotype similarities and genetic suppression analysises suggest a functional link between Gal11, Pgd1, Rgr1 and Sin4 (discussed in Li et al., 1995; Carlson, 1997; Piruat et al., 1997). A direct physical interaction between Gal11, Pgd1, Rgr1, Sin4 and Med2 has been inferred from the biochemical analysis of RNA polymerase II holoenzymes purified from strains deleted for *MED2*, *PGD1* and *SIN4* or partially deleted for *RGR1* (Li et al., 1995; Myers et al., 1999). The first analysis to be conducted concerned the subunit composition of these purified holoenzymes (Li et al., 1995; Myers et al., 1999). From the pattern of the subunits missing after the deletion of a given gene, one can model an ordered arrangement of subunits that tether each other to the RNA polymerase II holoenzyme complex. This modeling predicts a Med2/Pgd1 subcomplex and Gal11 bound to Sin4, and Sin4 in turn anchored to RNA polymerase II holoenzyme by Rgr1. To attribute a function to these proteins, these same holoenzymes were assayed in vitro for basal transcription, activated transcription by VP16 and Gcn4, and TFIIH CTD phosphorylation. Stimulation of basal transcription and TFIIH-dependent CTD phosphorylation was relatively constant, while activated transcription by VP16 required the Med2/Pgd1 (/Gal11 ?) subcomplex and GCN4 activation required Sin4 (/Gal11 ?).

Cse2, Nut1 and Nut2. Protein sequencing of polypeptides found in the purified Srb/mediator complex, recently identified Cse2 and Nut2 as holoenzyme subunits (Gustafsson et al., 1998). *CSE2* deletion causes pleiotropic defects, and the gene was originally isolated for its role in chromosome segregation (Xiao et al., 1993). *NUT2* was isolated in a selection

for factors responsible for the transcription inhibition of the *HO* gene by URS2 (Tabtiang and Herskowitz, 1998). This same screen also found inhibitory holoenzyme components (*SIN4, ROX3, SRB8, SRB9, SRB10* and *SRB11*), a negative TBP-associated factor (*CCR4*) and a novel factor (*NUT1*). Microsequencing of polypeptides around the expected size of Nut1 yielded matching peptide sequences (Gustafsson et al., 1998). Combined with the genetic similarity to Nut2, this data makes a strong case for Nut1 being a Srb/mediator complex subunit. However, the stoichiometry and stable association of Nut1 within RNA polymerase II holoenzyme remain to be shown. Mouse and *C. elegans* homologs of *RGR1* (Jiang et al., 1998), as well as human and *C. elegans* homologs of *NUT2* (Tabtiang and Herskowitz, 1998; Gustafsson et al., 1998) have been reported.

Srb10 CDK subcomplex.

The recessive *SRB* genes (*SRB8, SRB9, SRB10* and *SRB11*) behave in a manner opposite to the dominant *SRB* genes (*SRB2, SRB4, SRB5* and *SRB6*), suggesting they encode negative transcription factors (Koleske et al., 1992; Hengartner et al., 1998). Loss of function in these genes suppresses CTD truncation mutations, suggesting that they negatively affect the stability or the activity of the RNA polymerase II holoenzyme (Hengartner et al., 1995; Liao et al., 1995). Indeed, a large number of genetic screens and selections have found that *SRB8, SRB9, SRB10* and *SRB11* were all required for complete repression of transcription at a variety of genes, consistent with their role as inhibitors of transcription ((Kuchin et al., 1995; Song et al., 1996; Balciunas and Ronne, 1995; Hengartner et al., 1995; Liao et al., 1995; Wahi and Johnson, 1995; Tabtiang and Herskowitz, 1998; Cooper et al., 1997); reviewed in Carlson, 1997). In all of these genetic tests, all four genes behave the same.

Furthermore, cells mutated for any of these four *SRB*s share the same pleiotropic phenotypes of flocculence, cold sensitivity and temperature sensitivity (Hengartner et al., 1995; Liao et al., 1995).

These genetic data make a strong case for a common function for these gene products. A direct biochemical evidence for a Srb8-Srb9-Srb10-Srb11 subcomplex is lacking and reconstitution assays with recombinant proteins have failed to get a complex larger than the Srb10/Srb11 heterodimer (Hengartner et al., 1998); S.S. Koh and R.A. Young, unpublished observations). On the other hand, the yeast RNA polymerase II holoenzymes described, either contain all four or lack all four of these Srb proteins, suggesting that the tight genetic association is also indicative of their tight physical association (Kim et al., 1994; Myers et al., 1998; Koleske and Young, 1994; Liao et al., 1995; Hengartner et al., 1995). RNA polymerase II holoenzyme purified from a *srb8Δ* strain is also shown to lack Srb10 and Srb11 (Myer and Young, 1998).

Srb10/Srb11: a cyclin-dependent kinase pair. Sequencing of *SRB10* and *SRB11* revealed that they were homologous to cyclin-dependent kinase (CDK) and cyclin C genes, respectively. As opposed to cyclins involved in cell cycle control and progression, Srb11 mRNA and protein levels do not vary with the cell cycle (Cooper et al., 1997). Instead Srb11 protein levels have been found to decrease at the onset of meosis and possibly, after heat shock. Srb10 protein levels can also be found to vary, depending on the yeast cell growth state (Hengartner et al., 1998). No cell cycle arrest phenotypes have been found in *SRB10* or *SRB11* mutants. In this respect, Srb10 and Srb11 are similar to Kin28 CDK and Ccl1 cyclin, a yeast CDK-cyclin pair found in TFIIH that appears also to function exclusively in transcription. In higher eukaryotes, Cdk8 and cyclin C share sequence and functional homology with

Srb10 and Srb11, respectively. Furthermore, Cdk8-cyclin C are almost always found in purified preparations of mammalian holoenzymes (reviewed in Parvin and Young, 1998). Both Srb10 and Kin28 kinases possess the ability to phosphorylate the CTD of RNA polymerase II in vitro (Feaver et al., 1994; Liao et al., 1995; Hengartner et al., 1995).

A negative function for Srb10 kinase in vivo. A point mutation in a highly conserved catalytic domain of Srb10, can abolish the proteins' kinase activity (Liao et al., 1995; Hengartner et al., 1995). This mutant allele (*srb10-3*) can suppress the in vivo defects associated with CTD truncation mutants as well as a deletion (srb10Δ) or a recessive suppressor allele (srb10-1) (Hengartner et al., 1998). Cells with *srb10-3* show similar, if not more severe, pleiotropic phenotypes to those found in cells deleted for *SRB8, SRB9, SRB10* or *SRB11* (Liao et al., 1995); Liao et al., unpublished observation). The Srb10-3 mutant protein is still incorporated in the RNA polymerase II holoenzyme in vivo, since holoenzymes purified from *SRB10* and *srb10-3* strains contain similar anounts of Srb10 and other proteins (Liao et al., 1995; Hengartner et al., 1998). This suggests that the non-kinase functions of Srb10, if any, remain unperturbed in the Srb10-3 mutant. It follows then, that the inhibitory effect of *SRB10* on transcription in vivo must be due to its' kinase activity. Taking this logic further, this also implies that Srb8, Srb9 and Srb11 function either as direct kinase substrates or as positive contributors to Srb10's kinase activity.

CTD phosphorylation and transcription. The RNA polymerase II CTD, consisting of multiple repeats of the heptapeptide YSPTSPS, contains an uncommonly high density of potential phosphorylation sites (reviewed in Dahmus, 1995; Dahmus, 1996). In vitro transcription experiments have led to the hypothesis that the CTD is reversibly phosphorylated with each transcription cycle: RNA polymerase II with hypophosphorylated CTD

assembles into preinitiation complexes and, as RNA polymerase II shifts to productive mRNA elongation, the CTD becomes hyperphosphorylated.

Consistent with its role in transcription initiation, yeast RNA polymerase holoenzymes contains the unphosphorylated form of CTD (Koleske and Young, 1994; Kim et al., 1994). When reconstituted with purified GTFs, core RNA polymerase II with the unphosphorylated form of CTD preferentially assembles into a PIC (Laybourn and Dahmus, 1990; Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994). Since the phosphorylated CTD has a role in recruiting the mRNA capping enzyme to the nascent transcript, and mRNA capping occurs soon after promoter clearance (Coppola et al., 1983; Cho et al., 1997; McCracken et al., 1997a; McCracken et al., 1997b; Yue et al., 1997), CTD phosphorylation most likely occurs during the transition from transcription initiation to elongation. Pol II molecules in the midst of elongation contain CTDs which are highly phosphorylated (Bartholomew et al., 1986; Cadena and Dahmus, 1987; Weeks et al., 1993; O'Brien et al., 1994). The CTD has also been found to be required for 3′ mRNA processing in vitro (Hirose and Manley, 1998).

The TFIIH kinase is apparently responsible for CTD phosphorylation subsequent to PIC formation (Laybourn and Dahmus, 1990; Ohkuma and Roeder, 1994; Akoulitchev et al., 1995; reviewed in Dahmus, 1996). Loss-of-function mutations in the yeast TFIIH kinase subunit also cause a general defect on class II transcription in vivo (Cismowski et al., 1995; Valay et al., 1995; (Holstege et al., 1998), confirming the positive role in transcription inferred from in vitro studies.

A role for CTD phosphorylation in transcription elongation is reinforced by evidence that the HIV-1 transcriptional activator Tat, enhances transcription elongation by interacting with two CTD kinases, TFIIH and P-

TEFb, to stimulate or direct CTD phosphorylation (reviewed in Jones, 1997; Yankulov and Bentley, 1998). P-TEFb (positive transcription elongation factor b), first identified as a *Drosophila* factor that could stimulate transcription elongation in vitro (Marshall and Price, 1992), contains a CDK/cyclin pair, named Cdk9 and cyclin T (Zhu et al., 1997; Peng et al., 1998). Human cyclin T has been shown to directly interact with the Tat activator (Wei et al., 1998) in species-specific manner (Gerber et al., 1998). HumanTFIIH has also been reported to bind the activation domain of Tat, although the reports differ on which subunits make contact (Parada and Roeder, 1996; Cujec et al., 1997; Garcia-Martinez et al., 1997). Tat stimulates the CTD phosphorylation activity of TFIIH. Both TFIIH and P-TEFb CTD kinases seem to be required for the Tat-dependent activation in vitro (Parada and Roeder, 1996; Cujec et al., 1997; Garcia-Martinez et al., 1997; Mancebo et al., 1997; Zhu et al., 1997). Whether the two kinases act sequentially or simultaneously to phosphorylate the CTD is unknown.

A large number of kinases have been found to be able to phosphorylate the CTD in vitro (reviewed in Trigon et al., 1998). However of all these kinases, only the TFIIH subunit Kin28, and Srb10 have been found closely associated or complexed with the yeast transcription initiation machinery (Feaver et al., 1994; Liao et al., 1995). Another yeast CDK, Ctk1, associates with a cyclin, Ctk2, and is also able to phosphorylate the CTD in vitro (Sterner et al., 1995). While, to date, there is no evidence that Ctk1 and Ctk2 play any role in transcription, they have been suggested as possible yeast homologs of Cdk9 and cyclin T.

Recombinant Srb10 /Srb11, and recombinant Kin28/Ccl1 can form a CDK/cyclin pair that is able to specifically phosphorylate the serines in the heptapeptide repeats found in the CTD (Hengartner et al., 1998). Both

CDK/cyclin pairs preferentially phosphorylate the same residues, possessing virtually indistinguishable CTD kinase activities (Hengartner et al., 1998). This is rather surprising, because these two kinases seem to have opposite function in vivo, yet phosphorylate the CTD the same way.

A negative function for Srb10 kinase in vitro. The function of the Srb10 kinase can be assayed in vitro by purifying yeast RNA polymerase II holoenzyme from wild-type and *srb10-3* cells. An Srb10-3 holoenzyme is severely deficient for CTD phosphorylation (Liao et al., 1995). The Srb10-dependent CTD phosphorylation shows that the CTD can be efficiently and extensively phosphorylated by Srb10 in the holoenzyme context. In vitro transcription assays showed that the Srb10 kinase played an inhibitory role in transcription (Hengartner et al., 1998). As opposed to the TFIIH kinase (Kin28), Srb10 is uniquely capable of phosphorylating the CTD prior to template binding to inhibit holoenzyme transcription. CTD phosphorylation was accompanied by an inhibition of transcription. If the phosphorylation of holoenzyme CTD by Srb10 is left to ocur after PIC formation, transcription is unaffected. This suggests that the RNA polymerase II holoenzyme can be regulated positively and negatively, with the appropriate timing of CTD phosphorylation. In this model, Srb10/Srb11 CTD kinase activity occurs before competent PIC formation to inhibit transcription activity or PIC assembly, while Kin28/Ccl1 CTD kinase activity occurs after stable PIC formation to establish the elongating form of polymerase (Hengartner et al., 1998).

Swi-Snf subcomplex

The convergence of two yeast genetic screens led to the discovery of the Swi-Snf complex: *SWI* genes were isolated thanks to a screen for mating type

switching defects (Stern et al., 1984), whereas *SNF* genes where identified through a screen for defects in sucrose utilization (Neigeborn and Carlson, 1984). The *SWI/SNF* genes were genetically found to be acting opposite to the *SPT/SIN* genes, previously found to encode either histone or chromatin related factors. (Winston and Carlson, 1992). This lead to a model wherein the Spt-Sin factors repressed transcription by promoting a transcriptionally inactive form of chromatin, while the Swi-Snf factors would function to help transcription overcome chromatin-mediated repression (Winston and Carlson, 1992; Peterson and Tamkun, 1995). A Swi-Snf complex has been purified from yeast and found to consist of 11 subunits (Cairns et al., 1994; Cote et al., 1994; Treich et al., 1995). The complex exhibited a DNA-dependent ATPase activity that could result in nucleosome destabilization, an activity catalyzed by the Swi2/Snf2 subunit.(Cote et al., 1994). The Swi-Snf complex has been found to be an accessory subcomplex of the RNA polymerase II holoenzyme (Wilson et al., 1996). This is thought to allow direct targeting of nucleosome disruption activity to the appropriate promoter regions, facilitating PIC assembly.

A Human SWI/SNF-like complex has been isolated and shown to possess proteins homlogous to yeast Swi-Snf, as well as nucleosome remodeling activity (Imbalzano et al., 1994; Kwon et al., 1994; Wang et al., 1996; Wang et al., 1996).

RSC complex. A Swi-Snf related complex has been purified from yeast and shown to possess chromatin remodeling activity as well (Cairns et al., 1996). This 15 subunit complex, called RSC complex, shares common (Cairns et al., 1998) and related (Cairns et al., 1996) subunits with the Swi-Snf complex. So far, no role in transcription has been found for RSC, instead the complex is favored to play a role in cell cycle progression (Du et al., 1998; Cao et al., 1997).

Yeast holoenzyme component candidates.

In yeast and humans, Fcp1, a presumptive subunit of a CTD phosphatase activity, was detected in partially purified preparations of RNA polymerase II holoenzyme (Chambers and Kane, 1996; Archambault et al., 1997; Archambault et al., 1998). Fcp1 was shown to bind TFIIF but exhibited no CTD phosphatase activity on its own.

A recent study has identified a novel yeast protein, Xtc1, that could interact physically with the cell-cycle regulated mammalian transcriptional activator E2F-1 (Emili et al., 1998). Yeast cells deleted for *XTC1* exhibit temperature sensitivity, galactose auxotrophy and a heightened activator response to GAL4. While Xtc1 can be detected in partially purified RNA polymerase II holoenzyme, it has not yet been shown to be a stoichiometric component of a highly purified holoenzyme fraction.

RNA polymerase II holoenzyme in higher eukaryotes

As mentioned earlier, the detailed make-up of human RNA polymerase II holoenzymes in different reports varies greatly. Nonetheless, mammalian RNA polymerase II holoenzyme and related subcomplexes have been purified and consistently found to contain hSrb7 and hSrb10/hSrb11 (Cdk8/Cyclin C), in addition to GTFs (reviewed in Parvin and Young, 1998)

Subcomplexes of RNA polymerase II holoenzyme, reminiscent of those found in yeast, have also been described (Sun et al., 1998; Jiang et al., 1998). A purified mouse "Srb/mediator" complex, containing mMed7, mMed6, mRgr1, mRing-3 (nuclear kinase), mTrf-proximal and a dozen more 13 polypeptides, has been shown to bind to the RNA polymerase II CTD and stimulate CTD phosphorylation (Jiang et al., 1998). However, no stimulation of activated transcription was reported. A human "Srb10 CDK" subcomplex

that repressed activated transcription in vitro was also purified and included hRGR1, hSrb7, hMed6 and hSrb11 (Sun et al., 1998). This complex, called NAT for negative regulator of activated transcription, was able to inhibit activated transcription in a system reconstituted with RNA polymerase II and GTFs. NAT could also interact directly with RNA polymerase II. NAT's RNA polymerase II interaction and repression were independent of the CTD, yet precluded by phosphorylated CTD. NAT could not inhibit transcription after stable preinitiation complex formation. Because the study always included ATP along with NAT in the transcription reactions, it is unknown if this inhibitory activity required ATP.

Perspective.

Regardless of the technical difficulties associated with yeast RNA polymerase II holoenzyme purification, it is clear that the task of identifying its components has reached near completion. With all the initiation components in hand, we are now in a better position to look at the biochemical steps after initiation that lead to a fully elongated and processed mRNA, and at how activators might regulate them. Some studies have already also suggested that there are different RNA polymerase II holoenzymes for different stages of transcription besides initiation (mRNA elongation, mRNA capping, mRNA polyadenylation, mRNA splicing, etc.). It will be interesting to see how all these functions are coordinated. It is increasingly clear that the holoenzyme has the capacity to integrate and respond to intra- and extracellular signals leading to activation or repression of gene expression. Since some holoenzyme components can be shown to have an activity at just a subset of promoters, how is this gene-specificity achieved ? Another relatively unexplored area is the biochemical analysis of

the role of posttranslational modifications of RNA polymerase II holoenzyme components in the coordinated control of gene expression. The use of genome-wide analysis of gene expression to dissect how the different regulatory pathways feed into the transcription initiation complex, should also produce interesting results.

Personal contributions to these projects

Genetic analysis and cloning of *SRB* genes. When I joined the laboratory of Dr. Richard Young in 1991, Mike Nonet and Craig Thompson had already identified a set of novel transcription factors through a selection for genes involved in transcription initiation by RNA polymerase II in vivo: Srb2, Srb4, Srb5 and Srb6 (Nonet and Young, 1989; Thompson et al., 1993). This selection had yielded a large collection of suppressors of CTD truncation mutation. I perfomed a genetic analysis on the remainder of these suppressors to define five complementation groups among the recessive suppressors, suggestive of the existence of at least five more *SRB* genes (*SRB7*, *SRB8*, *SRB9*, *SRB10*, and *SRB11*). While I proceeded to clone and characterize two of these genes, *SRB7* and *SRB9*, Sha-Mei Liao and Jiang-Hua Zhang focused on the remaining three genes (Liao et al., 1995; Hengartner et al., 1995).

Characterization of SRB proteins. In 1994, biochemical analysis of the first *SRB* genes cloned by Tony Koleske and Craig Thompson led to the discovery of the RNA polymerase II holoenzyme that consisted of RNA polymerase II, a subset of general transcription factors and the Srb2, Srb4, Srb5 and Srb6 proteins (Thompson et al., 1993; Koleske and Young, 1994). Young-Joon Kim from the lab of Roger Kornberg subsequently confirmed Tony Koleske's holoenzyme finding, and showed that a subcomplex, named the mediator of activation, could be dissociated from the holoenzyme (Kim et al., 1994). This mediator contained Srb2, Srb4, Srb5 and Srb6. The clones of the remainder of the *SRB* genes allowed us (Sha-Mei Liao, Jiang-Hua Zhang and I) to purify recombinant protein and to produce antibodies. These antibodies were then use to demonstrate that all nine Srb proteins found in our genetic screen were found as components of the RNA polymerase II holoenzyme

(Liao et al., 1995; Hengartner et al., 1995). I extended this finding further by purifying the mediator of activation subcomplex according to (Kim et al., 1994), and showing that all nine Srb proteins are present (Hengartner et al., 1995).

Association of an activator with the RNA polymerase II holoenzyme. Tony Koleske had shown that the RNA polymerase II holoenzyme was capable of responding to a transcriptional activator (Koleske and Young, 1994), suggesting a model in which activators function, in part, through direct interactions with the holoenzyme. I validated this model by first showing a biochemical interaction between the potent trans-activator VP16 and the RNA polymerase II holoenzyme. I then proceeded to show a specific and direct interaction of this activator with the mediator subcomplex. These results provided the first evidence that this form of the transcription apparatus may be recruited to promoters via direct interactions with activators (Hengartner et al., 1995).

Temporal model of cyclin-dependent kinases (CDKs) in the RNA polymerase II holoenzyme. Two cyclin-dependent kinases (CDK) had been identified in yeast and mammalian RNA polymerase II transcription initiation complexes: the yeast kinase Kin28, a subunit of the general transcription factor TFIIH, and the yeast kinase Srb10, a subunit of the RNA polymerase II holoenzyme. While characterizing *SRB10*, Sha-Mei Liao had accumulated genetic evidence that this kinase was a negative regulator of transcription in vivo while other investigators had provided evidence that the kinase Kin28 was a positive regulator of transcription in vivo (Cismowski et al., 1995; Valay et al., 1995). The CTD of RNA polymerase II has been proposed to be a substrate of both kinases. In order to find the biochemical basis for their different activities in vivo, I purified epitope-tagged

recombinants of these two CDKs, with the help of Sang Seok Koh. My first objective was to compare and contrast the kinase activity of Kin28 and Srb10: I demonstrated that the two yeast kinases were biochemically indistinguishable in their ability to phosphorylate the RNA polymerase II CTD. The paradox of two CDKs with opposite in vivo function but similar kinase activity in vitro was resolved with the help of Vic Myer. Vic Myer had set up a reconstituted in vitro transcription assay using purified RNA polymerase II holoenzyme and general transcription factors. We observed that the negative regulator, Srb10, was uniquely capable of phosphorylating the CTD prior to formation of the initiation complex on promoter DNA, with consequent inhibition of transcription. In contrast, the TFIIH kinase phosphorylated the CTD only after the transcription apparatus was associated with promoter DNA. These results revealed that the timing of CTD phosphorylation was able to account for the positive and negative functions of the two kinases and provided a model for Srb10-dependent repression of genes involved in cell type specificity, meiosis, and sugar utilization (Hengartner et al., 1998).

Genome-wide survey of genes regulated by transcriptional cyclin-dependent kinases *SRB10* and *KIN28*. In 1997, Frank Holstege and John Wyrick embarked on an ambitious project to analyze the genome-wide of requirements for key components of the general transcription factors for yeast gene expression. Using technology that had only recently become available, they compared the mRNA profile of strains that were impaired in the function of components of the RNA polymerase II holoenzyme, the general transcription factor TFIID, and the SAGA chromatin modification complex. I helped analyze the results obtained with strains defective in Srb10 and Kin28. The collection of genes affected by both CDKs not only buttressed the conclusions of my earlier studies (Hengartner et al., 1998), but also provided

new insights in Srb10 function and regulation. I tested these new predictions and found the following. Diminution of Srb10p levels correlated with the loss of negative control of a coordinated set of genes during the diauxic shift. Furthermore, Srb10 negatively regulates the yeast developmental pathway of pseudohyphal growth, a new important function heretofore unknown.

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Chapter 2

Association of an activator with an RNA polymerase II holoenzyme

Summary

RNA polymerase II holoenzymes have been described that consist of RNA polymerase II, a subset of general transcription factors and four SRB proteins. The SRB proteins, which were identified through a selection for genes involved in transcription initiation by RNA polymerase II in vivo, are a hallmark of the holoenzyme. We report here the isolation and characterization of additional *SRB* genes. We show that the products of all nine *SRB* genes identified thus far are components of the RNA polymerase II holoenzyme and are associated with a holoenzyme subcomplex termed the mediator of activation. The holoenzyme is capable of responding to a transcriptional activator, suggesting a model in which activators function, in part, through direct interactions with the holoenzyme. Indeed, immunoprecipitation experiments with anti-Srb5 antibodies demonstrate that the acidic activating domain of VP16 specifically binds to the holoenzyme. Furthermore, the holoenzyme, and the mediator subcomplex, bind to a VP16 affinity column. These results provide a more complete description of the RNA polymerase II holoenzyme and suggest that this form of the transcription apparatus can be recruited to promoters via direct interactions with activators.

Introduction

Large multisubunit complexes containing RNA polymerase II, a subset of the general transcription factors, and additional factors implicated in regulation of transcription initiation in vivo, can assemble independently of promoter DNA (Koleske and Young, 1994; Kim et al., 1994). These complexes, termed RNA polymerase II holoenzymes, have been purified from *Saccharomyces cerevisiae*. The larger form of holoenzyme contains RNA polymerase II, TFIIB, TFIIF, TFIIH, and SRB proteins (Koleske and Young, 1994). Another form of holoenzyme has been described which contains RNA polymerase II, TFIIF, and SRB proteins but lacks TFIIB and TFIIH (Kim et al., 1994). The two holoenzyme forms may exist simultaneously in vivo or the isolation of the smaller complex may be a consequence of the instability of the RNA polymerase II holoenzyme during purification.

Selective transcription initiation in vitro by the twelve subunit core RNA polymerase II was previously shown to require the action of at least five general initiation factors: TATA-binding protein (TBP), TFIIB, TFIIE, TFIIF, and TFIIH (reviewed in Conaway and Conaway, 1993; Zawel and Reinberg, 1993). Consistent with these data, selective transcription initiation in vitro with the larger form of RNA polymerase II holoenzyme required TBP and TFIIE (Koleske and Young, 1994), and initiation with the smaller form required TBP, TFIIB, TFIIE and TFIIH (Kim et al., 1994).

The holoenzymes were discovered by virtue of their association with SRB proteins. *SRB* (Suppressor of RNA Polymerase B) genes were obtained through a genetic selection designed to identify genes involved in RNA polymerase II carboxy-terminal domain (CTD) function (Nonet and Young, 1989; Thompson et al., 1993). The CTD had been implicated in the response to

various transcriptional regulatory signals (Allison and Ingles, 1989; Scafe et al., 1990; Liao et al., 1991; Peterson et al., 1991), and the *SRB* alleles were isolated by virtue of their ability to suppress the cold-sensitive phenotype of cells containing CTD truncation mutations. Purification of the products of the *SRB2*, *SRB4*, *SRB5* and *SRB6* genes led to the observation that the vast majority of these SRB proteins in cell lysates are tightly associated with a portion of the RNA polymerase II and general factor molecules (Thompson et al., 1993; Koleske and Young, 1994). These results, and evidence that the SRB proteins have essential roles in transcription in vivo, suggested that the holoenzyme may be the form of RNA polymerase II that initiates transcription at promoters in vivo. The results also suggested that the isolation of more *SRB* genes might lead to the identification of additional components of the holoenzyme, and we show here that this is indeed the case.

The RNA polymerase II holoenzymes are responsive to activators (Koleske and Young, 1994; Kim et al., 1994), a feature not observed with purified RNA polymerase II and general transcription factors alone (Flanagan et al., 1991; Flanagan et al., 1992). Thus, these holoenzymes contain components necessary and sufficient for some level of response to transcriptional activators. A subcomplex, called the mediator of activation, can be dissociated from the RNA polymerase II holoenzyme by using monoclonal anti-CTD antibodies (Kim et al., 1994). The purified mediator contains SRB2, SRB4, SRB5, SRB6, SUG1, GAL11, TFIIF, and as yet unidentified polypeptides, and is capable of reconstituting the ability of purified RNA polymerase II and general factors to respond to transcriptional activators. Genetic studies previously suggested that GAL11, SUG1 and the SRB proteins are involved in transcriptional regulation (Suzuki et al., 1988;

Fassler and Winston, 1989; Himmelfarb et al., 1990; Nishizawa et al., 1990; Vallier and Carlson, 1991; Swaffield et al., 1992; Yu and Fassler, 1993).

The mechanisms involved in transcriptional activation are not yet clear. Transcriptional activators generally contain separable DNA binding and activation domains (Ptashne, 1988; Mitchell and Tjian, 1989). It is believed that transcriptional activators function in part by binding to promoter elements and to components of the transcription initiation apparatus, thereby contributing to their stable binding to the promoter (reviewed in Struhl, 1989; Ptashne and Gahn, 1990; Roeder, 1991; Drapkin et al., 1993). Some of the most compelling evidence for interactions between transcriptional activators and general factors comes from the analysis of TFIID, where physical and functional interactions have been reconstituted with recombinant TBP and TAFs (Dynlacht et al., 1991; Tanese et al., 1991; Gill and Tjian, 1992; Goodrich et al., 1993; Hoey et al., 1993; Chen et al., 1994; Gill et al., 1994; Jacq et al., 1994).

Transcriptional activators have been reported to interact with additional general factors. For instance, the herpes simplex virus transactivator VP16 (Triezenberg et al., 1988) has been shown to interact with TFIIB (Lin et al., 1991; Goodrich et al., 1993) and TFIIH (Xiao et al., 1994). This raises the possibility that some transcriptional activators can interact simultaneously or sequentially with multiple components of the transcription initiation apparatus. These results have generally been interpreted in the context of a model in which each of the general transcription factors and RNA polymerase II assemble onto the promoter in a stepwise fashion (Buratowski et al., 1989; Flores et al., 1991; Conaway and Conaway, 1993; Zawel and Reinberg, 1993; Buratowski, 1994).

The identification of RNA polymerase II holoenzymes that contain a subset of the general factors and are capable of responding to activators suggests a different model for transcriptional activation. In this model, transcriptional activator proteins first contribute to the establishment of a promoter-bound TFIID complex. The activators then interact with the holoenzyme to facilitate its association with the TFIID/promoter complex.

In this report, we describe the isolation and characterization of additional *SRB* genes and show that the products of all nine *SRB* genes identified thus far are components of the RNA polymerase II holoenzyme and the mediator of activation subcomplex. We also show that the transcriptional activator VP16 binds to the holoenzyme and the mediator subcomplex in vitro, thus providing evidence consistent with the model that transcriptional activators interact with the holoenzyme.

Results

Extragenic suppressors of RNA polymerase II CTD truncation mutations

Extragenic suppressors of a *S. cerevisiae* RNA polymerase II CTD truncation mutant were isolated to identify components of the transcription apparatus involved in initiation. The cold-sensitive phenotype of *rpb1Δ104* cells containing RNA polymerase II CTDs with 11 intact heptapeptide repeats was used to obtain 75 independent suppressing isolates. Genetic analysis revealed that mutations in a total of nine *SRB* genes suppress growth defects of cells containing a truncated CTD (Table I). We previously described the isolation and characterization of *SRB2, SRB4, SRB5, SRB6, SRB10* and *SRB11* (Koleske et al., 1992; Thompson et al., 1993; Liao et al., 1995). Thorough genetic analysis of the 75 independent suppressing isolates led to the identification of three additional genes: *SRB7, SRB8,* and *SRB9.*

The suppressing alleles of *SRB2, SRB4. SRB5, SRB6, SRB10* and *SRB11* were found to suppress the conditional phenotypes associated with the CTD truncation mutation *rpb1Δ104,* but did not suppress similar conditional phenotypes caused by other mutations in *RPB1* (Koleske et al., 1992; Thompson et al., 1993; Liao et al., 1995). Similarly, the mutations *srb7-1, srb8-1,* and *srb9-1* were also specific in suppressing conditional phenotypes due to CTD truncation mutations (not shown). This specificity of suppression suggests that the *SRB* gene products and the CTD are involved in the same process in transcription initiation.

Genomic DNA clones containing *SRB7, SRB8,* and *SRB9* were isolated by genetic complementation. Sequence analysis revealed that *SRB7, SRB8,* and *SRB9* are all newly identified genes. The predicted SRB7 protein is 140 amino acids long and has a molecular mass of 16 kd (Figure 1). Physical

Table 1. *SRB* Gene Summary

Gene	Dominant alleles	Recessive alleles	Deletion viability	Chromosomal location	Protein mass (kDa)	SDS-PAGE mobility (kDa)	pI	References*
SRB2	3	0	conditional	VIII	23	27	5.2	1, 2, 3, 4
SRB4	14	7	inviable	V	78	98	5.1	3, 4
SRB5	7	0	conditional	VII	34	38	4.7	3, 4
SRB6	4	2	inviable	II	14	15	4.6	3, 4
SRB7	0	3	inviable	IV	16	19	4.8	5
SRB8	0	4	conditional	III	144	160	5.7	5
SRB9	0	26	conditional	IV	160	180	5.5	5
SRB10	0	4	conditional	XVI	61	68	9.6	6
SRB11	0	1	conditional	XIV	38	33	7.0	6

* 1) Nonet and Young 1989, 2) Koleske et al. 1992, 3) Thompson et al. 1993, 4) Koleske and Young 1994, 5) this publication, 6) Liao et al. 1995

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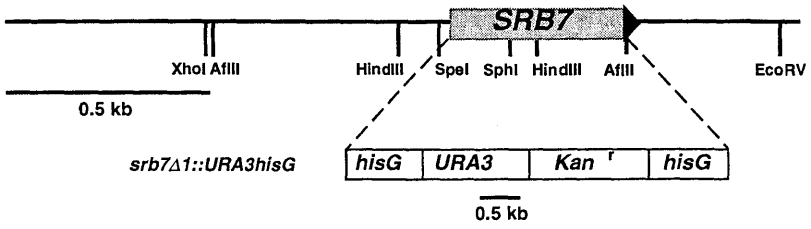

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-423  AATAACTAGAGCTAACAATATTATTTCTTCTGCTTTAGTTACAAAACAAGGACATTCATTTAACTTGGCGTTATC
-348  CCATACATTCGTTTATTATATCTTCTTTTAAAACACAATTTCTTTTACAGTTAAACTTTTCTGATTTATTATATA
-273  TTACTTAAGATTGTTCATATAACTAACATTTATATGCTTATATGCGTGAAGTGCGCTTTTGTAGAACATGTGGCT
-198  GTTTCTGTAGAAGCCTTGTCTTTCTCTGTAATCCTTTAAAGGCCAACCGTACGTGCTTAATTACAAGCTTTGTTC
-123  GCATTGCAAGAAAGTTAGAAAAAAAAATCAATTCTGAAAGATAATTATAATTCAAACGGTAAACCATTGGTTAAAA
 -48  GAGGGACATAACATTTCACTAGTTCAATACATTATATGCTCTTTAACAATGACAGATAGATTAACACAATTGCAG
                                                    M  T  D  R  L  T  Q  L  Q
                                        A(srb7-1)
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 103  TTGACCGTAAATGAACCTCAGATGTCCGATAAGCATGCCACAGTAGTACCTCCTGAGGAATTTTCTAACACGATA
       L  T  V  N  E  P  Q  M  S  D  K  H  A  T  V  V  P  P  E  E  F  S  N  T  I

 178  GATGAGCTATCCACGGACATTATACTTAAAACAAGACAGATAAACAAGCTTATTGACTCGTTACCTGGTGTTGAC
       D  E  L  S  T  D  I  I  L  K  T  R  Q  I  N  K  L  I  D  S  L  P  G  V  D

 253  GTTTCAGCTGAAGAGCAATTAAGGAAGATTGATATGTTGCAGAAAAAGCTAGTTGAAGTGGAAGACGAAAAAATT
       V  S  A  E  E  Q  L  R  K  I  D  M  L  Q  K  K  L  V  E  V  E  D  E  K  I

 328  GAGGCCATCAAAAAGAAGGAGAAACTTTTAAGGCACGTTGATTCTTTAATTGAAGATTTTGTAGATGGCATTGCA
       E  A  I  K  K  K  E  K  L  L  R  H  V  D  S  L  I  E  D  F  V  D  G  I  A

 403  AACTCAAAAAAGAGCACATAAACTTAAGTTTTACAAAGAAATTTGCGAACAGAGGACAGAAAATGTACTATAGTT
       N  S  K  K  S  T  *

 478  ATATGGCAGAGTTAAGCGTATGTATGTTATTCTTATAAATAATTGTGCTACTCTATTTGTACCGGAGAATTATTG
 553  AAGCAATGGGAGAAAAATCATAATGGAGAAAATCTTTTCTACGAGTTACTTTGCAAGGCAATCTAACGATTCTAAA
 628  AGACACAATACACTAAAGAAAAAACTTTGGAAGTACAGTTTTTTCCCCAAGTTGAAGTGTGGACTCATTGTGAAG
 703  ATGTAAAAATGTAAAAACCAACCGACAATGCACTCCCAGCCAAATTCATTGTAGACCTCCCATTTGATAGAAAAG
 778  GAAGGTTCAGCAGTTGTCCACGGATTCCAAGATATCATTCTCTTACATTGCACGCACATGAAAATGATC  846
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Figure 1. Map and sequence of the *SRB7* gene.

(A) Restriction map of a 2.0 kb DNA fragment from pCH7 containing the *SRB7* gene. The entire coding region of *SRB7* was replaced with a 5.5 kb DNA fragment containing the *URA3* and kanamycin genes flanked by direct repeats of *Salmonella hisG* DNA to create the deletion allele *srb7Δ1::URA3hisG*. (B) Sequence of the *SRB7* gene and adjacent DNA. The predicted 140 aa sequence of the SRB7 protein is shown below the sequence of the gene. Positive numbering of the nucleotides begins with the predicted start site of translation. The *srb7-1* mutation is a G to A transition (nt 61) that changes aa 21 from Ala to Thr.

mapping showed that *SRB7* is located on the right arm of chromosome IV, approximately 45 kb distal to *GCN2* (λ clone 6118). Partial sequence analysis of the *SRB8* coding sequence revealed that it is identical to ORF YCR81W on chromosome III (Oliver et al., 1992). The SRB8 protein is predicted to be 1226 amino acids in length with a molecular mass of 144 kd (Figure 2). The predicted SRB9 protein is 1420 amino acids long and has a molecular mass of 160 kd (Figure 3). *SRB9* maps to the right arm of chromosome IV, approximately 35 kb centromere distal to *ADE8* (λ clone 5513). A search of the sequence data banks revealed that SRB7, SRB8, and SRB9 do not have significant sequence similarity to previously identified proteins.

To determine whether the *SRB* genes are essential for cell viability, most or all of the coding regions were deleted, producing *srb7Δ1* (Figure 1A), *srb8Δ1* (Figure 2A), and *srb9Δ1* (Figure 3A). Heterozygous diploid cells containing these deletion alleles were sporulated and tetrad analysis was carried out. The results revealed that *SRB7* is essential for cell viability. In contrast, cells lacking either *SRB8* or *SRB9* are viable, but flocculate and exhibit mild cold-sensitive and temperature-sensitive phenotypes.

All nine SRBs are components of an RNA polymerase II holoenzyme

An RNA polymerase II holoenzyme has been purified and shown to consist of RNA polymerase II, the general transcription factors TFIIB, TFIIF and TFIIH, and the SRB proteins SRB2, SRB4, SRB5, SRB6, SRB10 and SRB11 (Nonet and Young, 1989; Thompson et al., 1993; Koleske and Young, 1994; Liao et al., 1995). We investigated whether SRB7, SRB8, and SRB9 are also components of this holoenzyme. Rabbit polyclonal antibodies were generated against recombinant SRB7, SRB8, and SRB9. Column fractions from the final purification step of the RNA polymerase II holoenzyme were tested in

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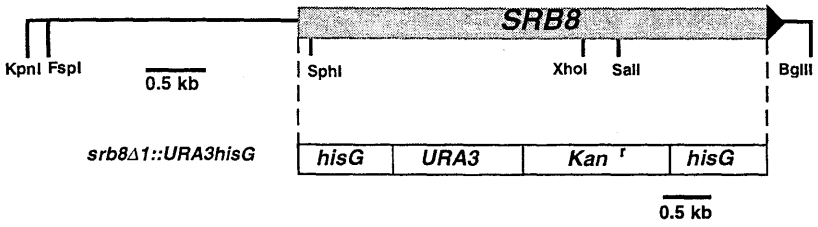

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 151  TCACTAAGATTGATCAATAAGGAAATCTTTCATCATTGGCTTGTAGAGTTTATAAATAAGATGGAAAACTTCGAA
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 976  GAGGATTTGCACGAATTCCAGATAGAAGATGCCATTTGGTCATTGGTTTTCCAATTAGCCAAAAATTTTTCGGCC
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        V  T  S  F  M  P  F  W  K  F  F  M  K  N  F  P  F  V  L  K  V  D  N  D  L

1801  AGGATTGAGTTACAATCTGTTTACAATGATGAGAAATTGAAAACTGAGAAGCTGAAGAATGATAAATCAGAAGTC
        R  I  E  L  Q  S  V  Y  N  D  E  K  L  K  T  E  K  L  K  N  D  K  S  E  V

1876  TTGAAGGTGTATTCCATGATCAATAATTCAAACCAAGCTGTTGGACAGACTTGGAATTTTCCCGAGGTGTTTCAA
        L  K  V  Y  S  M  I  N  N  S  N  Q  A  V  G  Q  T  W  N  F  P  E  V  F  Q

1951  GTAAACATCAGGTTTCTACTACACAACTCCGAGATCATTGATACAAATACAAGCAAACAGTTCCAGAAAGCACGA
        V  N  I  R  F  L  L  H  N  S  E  I  I  D  T  N  T  S  K  Q  F  Q  K  A  R

2026  AACAATGTCATGCTTTTGATTGCCACTAACTTGAAGGAGTACAATAAATTTATGTCCATTTTCTTGAAAAGGAAA
        N  N  V  M  L  L  I  A  T  N  L  K  E  Y  N  K  F  M  S  I  F  L  K  R  K

2101  GACTTTACTAACAAAAATTTAATTCAATTGATCTCTCTAAAACTTCTAACTTTTGAAGTGACGCAGAATGTGTTG
        D  F  T  N  K  N  L  I  Q  L  I  S  L  K  L  L  T  F  E  V  T  Q  N  V  L

2176  GGGCTCGAGTATATTATTCGATTATTACCAATAAACTTGGAAAATAATGACGGCTCATATGGTCTGTTTTTGAAG
        G  L  E  Y  I  I  R  L  L  P  I  N  L  E  N  N  D  G  S  Y  G  L  F  L  K

2251  TATCATAAAGAACAATTCATAAAGTCAAATTTTGAGAAAATTTTACTTACATGTTATGAATTAGAAAAAAAATAT
        Y  H  K  E  Q  F  I  K  S  N  F  E  K  I  L  L  T  C  Y  E  L  E  K  K  Y

2326  CATGGCAACGAATGTGAAATAAATTATTATGAGATCCTATTGAAAATTTTAATAACTTATGGGTCATCTCCCAAA
        H  G  N  E  C  E  I  N  Y  Y  E  I  L  L  K  I  L  I  T  Y  G  S  S  P  K

2401  TTACTTGCAACATCTACAAAAATCATTATGTTGTTATTGAATGATAGCGTGGAAAACTCATCTAATATTTTGGAG
        L  L  A  T  S  T  K  I  I  M  L  L  L  N  D  S  V  E  N  S  S  N  I  L  E

2476  GATATTTTGTACTACTCAACTTGTCCGTCGGAAACCGATCTTAACGATATTCCATTGGGTAGTGGACAACCAGAC
        D  I  L  Y  Y  S  T  C  P  S  E  T  D  L  N  D  I  P  L  G  S  G  Q  P  D

2551  AATGACACTGTTGTAACCAACGATGATAAAAGTGACGATGATGATCACACAGTCGACGAAATTGATCATGTAGAA
        N  D  T  V  V  T  N  D  D  K  S  D  D  D  D  H  T  V  D  E  I  D  H  V  E

2626  TATTACGTTATGATGGACTTTGCCAATCTTTGGGTTTTCCAAGCGTTTACCTGTTTCTGCATCAAAAAAATCATG
        Y  Y  V  M  M  D  F  A  N  L  W  V  F  Q  A  F  T  C  F  C  I  K  K  I  M

2701  GAGAATAATGAGCCAGCAATGGCAATGGAAGACTTGAAGAACTTCATATTCCAAATTATCGAAATAACTAATTCT
        E  N  N  E  P  A  M  A  M  E  D  L  K  N  F  I  F  Q  I  I  E  I  T  N  S

2776  AATGATTTATGTTCACAAATATTTGACCAACTGAAGGATATGCAGACCATTGAGATGATAACCCAAATAGTGGAG
        N  D  L  C  S  Q  I  F  D  Q  L  K  D  M  Q  T  I  E  M  I  T  Q  I  V  E

2851  AAAGATTTCTGCACTTCTTGTTTGCAAAACAACAACCAAAAGATAGATGATAATTACATCGTTGTGGTGATCGAG
        K  D  F  C  T  S  C  L  Q  N  N  N  Q  K  I  D  D  N  Y  I  V  V  V  I  E

2926  ATTATAACGTCATTATCGATGAGGTTTCAAAGAGAAACTTCTGGTATGATAGTTATTTCCATGGAGAACTATCAT
        I  I  T  S  L  S  M  R  F  Q  R  E  T  S  G  M  I  V  I  S  M  E  N  Y  H

3001  TTACTAATAAAGATCATAAGACAATTAAGTGAACTGAACGAAGGAAATTTATCTAAGAGAGAAATCCAAATAGAT
        L  L  I  K  I  I  R  Q  L  S  E  L  N  E  G  N  L  S  K  R  E  I  Q  I  D

3076  GCCGTCTTGAAAATTTTTAGCTTTCATCAGGATTCCATTTTCCAACGCATCATCGCTGATTTATCAGCTGATAAA
        A  V  L  K  I  F  S  F  H  Q  D  S  I  F  Q  R  I  I  A  D  L  S  A  D  K

3151  CCCACAAGTCCATTCATTGATAGCATATGCAAGCTGTTTGATAAAATATCATTTAATTTAAGATTGAAGCTGTTC
        P  T  S  P  F  I  D  S  I  C  K  L  F  D  K  I  S  F  N  L  R  L  K  L  F

3226  TTGTACGAAATTTTGTCTTCATTGAAATCATTCGCCATCTATTCATCCACAATTGATGCCCCAGCATTCCACACA
        L  Y  E  I  L  S  S  L  K  S  F  A  I  Y  S  S  T  I  D  A  P  A  F  H  T

3301  AGCGGTAAGGTCGAACTACCGAAGAAATTGCTGAACTTACCACCATTCCAAGTGTCCTCTTTCGTTAAGGAAACA
        S  G  K  V  E  L  P  K  K  L  L  N  L  P  P  F  Q  V  S  S  F  V  K  E  T

3376  AAACTTCATAGTGGCGACTACGGGGAAGAAGAAGATGCAGACCAAGAAGAATCGTTTAGTTTAAATTTAGGAATC
        K  L  H  S  G  D  Y  G  E  E  E  D  A  D  Q  E  E  S  F  S  L  N  L  G  I

3451  GGCATAGTTGAAATAGCGCACGAAAACGAACAGAAATGGCTCATTTATGACAAGAAAGATCATAAATATGTCTGC
        G  I  V  E  I  A  H  E  N  E  Q  K  W  L  I  Y  D  K  K  D  H  K  Y  V  C

3526  ACATTTTCCATGGAGCCGTACCACTTCATCTCCAACTATAATACCAAGTACACAGATGACATGGCTACAGGCAGT
        T  F  S  M  E  P  Y  H  F  I  S  N  Y  N  T  K  Y  T  D  D  M  A  T  G  S

3601  AATGATACGACTGCGTTTAACGATTCCTGTGTAAACCTGAGTCTTTTGATGCTCGGTTTGAGAGGAAAAATCCA
        N  D  T  T  A  F  N  D  S  C  V  N  L  S  L  F  D  A  R  F  E  R  K  N  P

3676  CATTGATCTCAGAATATATCCAAATGGATAAATTATAAATTTACCAATAACAGTAATTATGTGTCAGTTTTAATA
        H  *

3751  CCCAACCAATTGATTCCTCTAAATGATACCGTACCAAATGACATGTTTGCCACCGATTATAAAACTGGGGTTTAT
3826  GAGCGATGGTTAAGGAGGATTAAGACAAAGTGCTCATCCGCTTACAAGATCAGTACTAGCGTGTACCAGTCTATT
3901  TAAACTGGAATGATCACCCCAAAGGGAACGCACGATGCTGTGGCCAAGTTTCAAAAAACTGACCTGCATCAAGAT
3976  CTCGAT  3971
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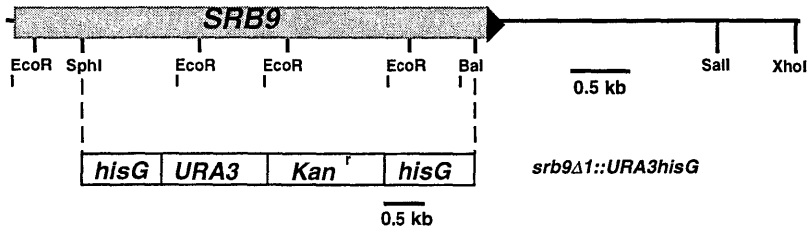
Figure 2. Map and sequence of the *SRB8* gene.

(A) Restriction map of a 6.0 kb DNA fragment from pSL311 containing the *SRB8* gene. The entire coding region of *SRB8* was replaced with a 5.5 kb DNA fragment containing the *URA3* and kanamycin genes flanked by direct repeats of *Salmonella hisG* DNA to create the deletion allele *srb8Δ1::URA3hisG*. (B) Sequence of the *SRB8* DNA and adjacent DNA. The predicted 1226 aa sequence of the SRB8 protein is shown below the sequence of the gene. Positive numbering of the nucleotides begins with the predicted start site of translation. The *SRB8* coding sequence is identical to ORF YCR81W (Oliver et al., 1992).

A



B

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-147  GATCAAGTAGTGTAGTATTTATTGTAGTACACTCTTACAACAACCCTTTAAGACGAATGGTGTGAAATCGGAAAT
 -72  TACTTTGTTGAAGTAAGGTGTAACTATATTTTAAGAACGTTTAAGCTGGATATCAAGATCTGAGGAGGTAGTATG
                                                                              M
   4  AGTTCTGACGCTTCCACGTACAGACTTGAGGATGTTTTATCCAGCTTCTATAGAGTGGAGAAAATCAAAAAGATC
        S  S  D  A  S  T  Y  R  L  E  D  V  L  S  S  F  Y  R  V  E  K  I  K  K  I

  79  AACTATCATCAGTACATTTCTAAAGCCCAAAACGATCAATGGTCTATCCAAATGGAATTCATGCTACGGAAGCAG
        N  Y  H  Q  Y  I  S  K  A  Q  N  D  Q  W  S  I  Q  M  E  F  M  L  R  K  Q

 154  GATCCAAAGACTCTAGTTGCGCTGCTTTCAAGGGATTTATGGTGTTTCAGTATAAATGATGATCCGGTACCGACA
        D  P  K  T  L  V  A  L  L  S  R  D  L  W  C  F  S  I  N  D  D  P  V  P  T

 229  CCTCCTGCGATAGAACATAAACCAGTGAGCCCAGATAAAATCGGAACTTTCACTGCCGATTATTCAAAGCCAAAC
        P  P  A  I  E  H  K  P  V  S  P  D  K  I  G  T  F  T  A  D  Y  S  K  P  N

 304  TTACCGCCACACTATGCTCTTTTTTTAAAAGCTTTAAGAAGGAAAATTTACATTAATTTGGCATTAGGTTCACAC
        L  P  P  H  Y  A  L  F  L  K  A  L  R  R  K  I  Y  I  N  L  A  L  G  S  H

 379  AATAAGCTAATACAATTTGGGAATGCCTGCATATCATTAAGCGGAGTGCCAAATTATCTCGTACAGCTAGAACCA
        N  K  L  I  Q  F  G  N  A  C  I  S  L  S  G  V  P  N  Y  L  V  Q  L  E  P

 454  CACCTTTTTGTAAACGGAGATCTCACAGTGTCGTTATGTGCCAAGAACATGGGATTAGTACCAATGAAGGAGGAA
        H  L  F  V  N  G  D  L  T  V  S  L  C  A  K  N  M  G  L  V  P  M  K  E  E

 529  AATTTGGAAGAATCTTTCCTTTCAAAGCATGCGCTTTATTTAGCACCATCTGGAATAAGGATGCATTTGGCCCCT
        N  L  E  E  S  F  L  S  K  H  A  L  Y  L  A  P  S  G  I  R  M  H  L  A  P

 604  GCTTCCAAGCAAGGATACTTGATAACGCCACCAAAACATACAGAACTTCTCTTGACGACGTTAAGTGTATCTCAT
        A  S  K  Q  G  Y  L  I  T  P  P  K  H  T  E  L  L  L  T  T  L  S  V  S  H

 679  GGTATAAACTTACAAAATAAAAAAAATTTGAAATGGGTTGCTGTTGTTCCTGACTTAGGACATCTCAACGGCCAC
        G  I  N  L  Q  N  K  K  N  L  K  W  V  A  V  V  P  D  L  G  H  L  N  G  H

 754  ACACCTACTATAGCTTCGTATTTAACTCCCTTACTTGAAGCAAAGAAGCTAGTATGGCCGCTGCATTTAATCTTC
        T  P  T  I  A  S  Y  L  T  P  L  L  E  A  K  K  L  V  W  P  L  H  L  I  F

 829  GCCCAACCAGTAGCTGATATAGAAAATTCTACTTCCGGAGATCCATCAGAATTTCACTGTTTGCAAGATGCTCTG
        A  Q  P  V  A  D  I  E  N  S  T  S  G  D  P  S  E  F  H  C  L  Q  D  A  L

 904  GATGCCATTGATGATTTCATACAATTAAAGCAAACAGCTGCCTATAGGACTCCAGGAAGTTCCGGCGTATTGAGC
        D  A  I  D  D  F  I  Q  L  K  Q  T  A  A  Y  R  T  P  G  S  S  G  V  L  S

 979  AGTAATATTGCTGGTACAAATCCCTTAAGCTCAGATGGAGCATATACAGAACAGTTTCAACATTATAAGAACAAC
        S  N  I  A  G  T  N  P  L  S  S  D  G  A  Y  T  E  Q  F  Q  H  Y  K  N  N

1054  TCAATTAGTTCTCAACCCGCTTCTTATCATTCTGTCCAAGAAACTAATAAGATATCTCCGAAAGATTTCTCCCCT
        S  I  S  S  Q  P  A  S  Y  H  S  V  Q  E  T  N  K  I  S  P  K  D  F  S  P

1129  AATTTCACAGGCATTGATAAATTAATGCTGTCGCCCAGCGATCAATTTGCTCCAGCTTTCTTAAATACCCCTAAT
        N  F  T  G  I  D  K  L  M  L  S  P  S  D  Q  F  A  P  A  F  L  N  T  P  N

1204  AATAACATCAATGAGAATGAATTATTTAATGATAGGAAACAAACTACAGTATCAAATGACTTAGAGAACAGCCCA
        N  N  I  N  E  N  E  L  F  N  D  R  K  Q  T  T  V  S  N  D  L  E  N  S  P

1279  CTGAAAACGGAACTGGAGGCAAATGGTAGATCACTCGAAAAGGTAAATAATTCCGTGAGCAAGACAGGAAGCGTA
        L  K  T  E  L  E  A  N  G  R  S  L  E  K  V  N  N  S  V  S  K  T  G  S  V

1354  GACACACTTCATAATAAAGAGGGAACACTGGAACAACGAGAACAGAACGAAAATCTGCCAAGTGATAAAAGTGAC
        D  T  L  H  N  K  E  G  T  L  E  Q  R  E  Q  N  E  N  L  P  S  D  K  S  D

1429  TCTATGGTAGACAAGGAATTGTTTGGTGAGGATGAGGATGAGGATTTATTTGGCGATAGCAATAAATCGAATTCT
        S  M  V  D  K  E  L  F  G  E  D  E  D  E  D  L  F  G  D  S  N  K  S  N  S

1504  ACAAACGAATCGAACAAAAGTATATCGGACGAAATTACCGAGGATATGTTCGAAATGTCTGATGAAGAAGAAAAT
        T  N  E  S  N  K  S  I  S  D  E  I  T  E  D  M  F  E  M  S  D  E  E  E  N

1579  AATAACAATAAAAGCATTAATAAAAATAACAAGGAAATGCATACTGATCTTGGTAAAGATATTCCATTTTTTCCC
        N  N  N  K  S  I  N  K  N  N  K  E  M  H  T  D  L  G  K  D  I  P  F  F  P
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B (Continued 1/2)

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1654  TCATCTGAAAAACCGAATATCCGTACGATGAGCGGAACTACAAAAAGATTAAATGGAAAGAGGAAATATTTGGAT
        S  S  E  K  P  N  I  R  T  M  S  G  T  T  K  R  L  N  G  K  R  K  Y  L  D

1729  ATTCCGATAGATGAAATGACCTTGCCAACGAGTCCATTATATATGGACCCAGGTGCGCCACTCCCTGTGGAAACA
        I  P  I  D  E  M  T  L  P  T  S  P  L  Y  M  D  P  G  A  P  L  P  V  E  T

1804  CCCCGCGATAGACGCAAAAGTGTGTTCGCTCCACTGAATTTTAACCCCATAATAGAAAACAATGTTGATAACAAA
        P  R  D  R  R  K  S  V  F  A  P  L  N  F  N  P  I  I  E  N  N  V  D  N  K

1879  TACAAATCTGGAGGGAAATTTTCCTTCAGTCCGTTGCAAAAGGAGGAAGCATTAAACTTTGATATTTCTATGGCG
        Y  K  S  G  G  K  F  S  F  S  P  L  Q  K  E  E  A  L  N  F  D  I  S  M  A

1954  GATCTTTCTAGCTCTGAAGAGGAAGAGGATGAAGAAGAGAACGGTAGCAGCGATGAGGATCTAAAGTCATTGAAC
        D  L  S  S  S  E  E  E  E  D  E  E  E  N  G  S  S  D  E  D  L  K  S  L  N

2029  GTACGCGACGACATGAAACCTTCTGATAACATCAGTACTAATACTAATATTCATGAGCCTCAATACATAAATTAC
        V  R  D  D  M  K  P  S  D  N  I  S  T  N  T  N  I  H  E  P  Q  Y  I  N  Y

2104  TCTTCGATCCCAAGTCTACAAGACTCTATTATAAAGCAAGAAAATTTCAATTCAGTAAACGATGCTAATATCACT
        S  S  I  P  S  L  Q  D  S  I  I  K  Q  E  N  F  N  S  V  N  D  A  N  I  T

2179  AGCAATAAGGAAGGCTTCAACTCTATTTGGAAAATTCCTCAAAATGATATACCACAGACCGAGTCACCACTGAAG
        S  N  K  E  G  F  N  S  I  W  K  I  P  Q  N  D  I  P  Q  T  E  S  P  L  K

2254  ACCGTTGATTCATCTATTCAACCCATAGAATCCAATATAAAGATGACCTTGGAAGATAATAATGTTACCAGTAAT
        T  V  D  S  S  I  Q  P  I  E  S  N  I  K  M  T  L  E  D  N  N  V  T  S  N

2329  CCGTCCGAATTTACGCCGAATATGGTAAATTCTGAAATTTCTAACCTACCAAAGGACAAGAGTGGTATCCCCGAA
        P  S  E  F  T  P  N  M  V  N  S  E  I  S  N  L  P  K  D  K  S  G  I  P  E

2404  TTCACACCGGCGGACCCCAATTTATCTTTTGAATCATCAAGTAGTCTACCGTTTCTATTGAGACACATGCCGCTA
        F  T  P  A  D  P  N  L  S  F  E  S  S  S  S  L  P  F  L  L  R  H  M  P  L

2479  GCATCTATACCGGACATTTTCATCACGCCTACTCCCGTTGTTACAATTTCAGAAAAAGAACAAGACATCTTAGAT
        A  S  I  P  D  I  F  I  T  P  T  P  V  V  T  I  S  E  K  E  Q  D  I  L  D

2554  TTAATTGCAGAACAAGTCGTCACTGATTATAATATCTTAGGAAACCTCGGTATTCCAAAGATCGCCTATAGGGGA
        L  I  A  E  Q  V  V  T  D  Y  N  I  L  G  N  L  G  I  P  K  I  A  Y  R  G

2629  GTTAAAGATTGCCAAGAAGGTTTAATAACAACCACAATGTTACAGTTATTTTCCACTTCGGATAGATTAAATGGC
        V  K  D  C  Q  E  G  L  I  T  T  T  M  L  Q  L  F  S  T  F  D  R  L  N  G

2704  AATGATACGATCTCCAAATTCTATAACATGAAGCAGCCGTACGTTTTTGTAAAGAAACATCACGAACTAATCAAA
        N  D  T  I  S  K  F  Y  N  M  K  Q  P  Y  V  F  V  K  K  H  H  E  L  I  K

2779  GTCAAACACGACTCTCAGCCATTTATTAAGTTCCTCAATTTTCGCCCTCCAAATGGAATAAAAAACTTCAAATCC
        V  K  H  D  S  Q  P  F  I  K  F  L  N  F  R  P  P  N  G  I  K  N  F  K  S

2854  TTATTATTAAGTTCATCTTTCAAAGAAGATTGTCTGTCATTTGCGCCAACTCTATCTCAAACATATATTAATCAA
        L  L  L  S  S  S  F  K  E  D  C  L  S  F  A  P  T  L  S  Q  T  Y  I  N  Q

2929  GAGTTAGGGTTTTGTGAGTTGCTTAAACTAACTAATGAAGACCCGCCCGGACTGATGTACTTGAAGGCATTTGAT
        E  L  G  F  C  E  L  L  K  L  T  N  E  D  P  P  G  L  M  Y  L  K  A  F  D

3004  AAAAACAAGTTACTGTTGTTAGCTGCGCAGATTGTTTCATACTGTTCTAATAATAAGAACTCCATCAAAAACGTG
        K  N  K  L  L  L  L  A  A  Q  I  V  S  Y  C  S  N  N  K  N  S  I  K  N  V

3079  CCACCAATATTAATAATTTTACCCTTGGATAATGCAACTCTGACTGAATTAGTAGACAAGGCGAATATTTTTCAG
        P  P  I  L  I  I  L  P  L  D  N  A  T  L  T  E  L  V  D  K  A  N  I  F  Q

3154  GTGATCAAGAACGAAGTTTGTGCCAAGATGCCTAACATTGAACTATATTTGAAAGTTATTCCTATGGATTTCATT
        V  I  K  N  E  V  C  A  K  M  P  N  I  E  L  Y  L  K  V  I  P  M  D  F  I

3229  AGAAACGTACTGGTGACAGTGGATCAGTACGTCAACGTAGCAATTTCTATATATAACATGCTGCCGCCAAAATCT
        R  N  V  L  V  T  V  D  Q  Y  V  N  V  A  I  S  I  Y  N  M  L  P  P  K  S

3304  GTAAAGTTCACCCACATTGCGCATACGCTGCCGGAGAAAGTGAATTTCAGAACCATGCAGCAACAGCAAATGCAA
        V  K  F  T  H  I  A  H  T  L  P  E  K  V  N  F  R  T  M  Q  Q  Q  Q  M  Q

3379  CAGCAACAGCAACAGCAACAGCAGCAGCAGAATAACAGTACAGGATCATCTTCTATAATATATTATGACTCGTAC
        Q  Q  Q  Q  Q  Q  Q  Q  Q  N  N  S  T  G  S  S  S  I  I  Y  Y  D  S  Y

3454  ATCCACCTGGCATACTCGCGTAGTGTAGATAAAGAGTGGGTTTTTGCAGCTCTTTCAGATAGCTATGGACAAGGC
        I  H  L  A  Y  S  R  S  V  D  K  E  W  V  F  A  A  L  S  D  S  Y  G  Q  G

3529  AGCATGACGAAAACGTGGTACGTCGGGAATTCCAGAGGAAAATTTGACGACGCATGTAATCAAATATGGAATATC
        S  M  T  K  T  W  Y  V  G  N  S  R  G  K  F  D  D  A  C  N  Q  I  W  N  I

3604  GCCCTAAATTTAGCGTCTAAAAAATTCGGAAAAATATGTCTAATTTTAACTAGATTGAATGGCATACTGCCGGAT
        A  L  N  L  A  S  K  K  F  G  K  I  C  L  I  L  T  R  L  N  G  I  L  P  D

3679  GATGAATTGATGAATTGGAGGAGACTTTCTGGTAGGAATATACATCTTGCTGTGGTGTGTGTGGATGACAACTCT
        D  E  L  M  N  W  R  R  L  S  G  R  N  I  H  L  A  V  V  C  V  D  D  N  S

3754  AAAATCTCCTTCATAGATGAGGACAAATTGTACCCTAGTTTCAAGCCGATCTACAAAGACACTAGGTTTGGAGGA
        K  I  S  F  I  D  E  D  K  L  Y  P  S  F  K  P  I  Y  K  D  T  R  F  G  G

3829  CGCATGGATATGACCAGATTATACGACTATGAAATAAGGGATATAGACCAGGACATCCATGGAATAGTATTTCAG
        R  M  D  M  T  R  L  Y  D  Y  E  I  R  D  I  D  Q  D  I  H  G  I  V  F  Q
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B (Continued 2/2)

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3904  CACCCGTTCCCACTGGCACACTCACAGCATCGCTGTGCTATTAGGAGTGGTGCTTTGATCAAATTCAAAAAATGC
       H  P  F  P  L  A  H  S  Q  H  R  C  A  I  R  S  G  A  L  I  K  F  K  K  C

3979  GACGGTGATACGGTTTGGGACAAATTCGCAGTCAACCTTTTAAACTGTCCACATTCTGATAGTACACAATTGCTG
       D  G  D  T  V  W  D  K  F  A  V  N  L  L  N  C  P  H  S  D  S  T  Q  L  L

4054  GAAACCATCTTAGAAGAGTTTCGCAACCTGGCTGCTCTAAACGTGTGGTACGGTCTCTCTGATGGCGAAGATGGC
       E  T  I  L  E  E  F  R  N  L  A  A  L  N  V  W  Y  G  L  S  D  G  E  D  G

4129  CATATTCCATGGCATATCCTAGCCGTGAAAAAAATGATGAACACTCTTGTGCACACCAGAGTAAAAATTGCTAAT
       H  I  P  W  H  I  L  A  V  K  K  M  M  N  T  L  V  H  T  R  V  K  I  A  N

4204  ACTTCCGCCGCTACTGTGCATACCGCTACTTCTTCATCAATTATTCTCTCGGATAAATAAACTTTTTCCGGCAAC
       T  S  A  A  T  V  H  T  A  T  S  S  S  I  I  L  S  D  K

4279  GTTTTCCTGCTCATCTGTAGCCCTATTTACCAGTTTTGGTTTTAGTATTATTCCGGGGGTGTAAACCCAGAAGTCT
4354  ATTTCTCCAGTCGGATTTATAAAAACAAAACCGGAAGCGGGGCGGTACGGCATTTTCACTGGTGATGCACGCCCA
4429  GCGTGTAGTCCGAGACAATTTCCACAGAACGCGAATGAGATTGCGTTTAAGGCTGTATTTTCAAGGCACACGAAG
4504  CGGCCACGTGGGTCTGCGATGGTGTGTTGATGATGTCAAGAATGGTATCATACTCCGTATAAGGTTATGTAATCG
4579  GAAGTCGCGATTCTTTTTCAATTTTTCTTTTTATTTTTTTCCAGTTTTTTCGTCTCTGCGATGGAAAATTGTTG
4654  AAGTTCTCTTGATTAGCAAGTAGTTCTTACATCGCAGGAATCTTATGTT  4702
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Figure 3. Map and sequence of the *SRB9* gene.

(A) Restriction map of a 7.3 kb DNA fragment from pCH47 containing the *SRB9* gene. Most of the coding region of *SRB9* was replaced with a 5.5 kb DNA fragment containing the *URA3* and kanamycin genes flanked by direct repeats of *Salmonella hisG* DNA to create the deletion allele *srb9Δ1::URA3hisG*.

(B) Sequence of the *SRB9* DNA and adjacent DNA. The predicted 1420 aa sequence of the SRB9 protein is shown below the sequence of the gene. Positive numbering of the nucleotides begins with the predicted start site of translation. A polyglutamine repeat, a motif found in several transcription factors (e.g. Sp1, dTAF$_{II}$110, OCT2), is underlined (Courey and Tjian, 1988; Hoey et al., 1993; Clerc et al., 1988). In yeast, a BLAST search (Altschul et al., 1990) reveals that nearly 3% percent of all open-reading frames (ORFs) are found to contain one or more glutamine-rich domains. These include many positive and negative transcription factors such as: Adr6, Caf1, Ccr4, Dat1, Gal11, Hap1, Hap2, Mcm1, Not1, Pho2, Pgd1, Rox1, Snf5, Snf6, Ssn6 and Tup1.

reconstituted transcription reactions and subjected to Western blot analysis with antisera specific to RNA polymerase II and SRB proteins (Figure 4A). SRB7, SRB8 and SRB9 coeluted with the other SRB proteins, RNA polymerase II and transcriptional activity. Thus, all nine SRB genes identified through our genetic selection encode components of the RNA polymerase II holoenzyme.

The most highly purified RNA polymerase II holoenzyme preparation was subjected to SDS-PAGE and silver-stained (Figure 4B). The SRB proteins were assigned to protein bands based on Western blot analysis. RNA polymerase II and general transcription factor subunits were assigned to protein bands based on their mobility and, in some cases, on Western blot analysis. From this analysis, many of the known components of the holoenzyme can be accounted for, although a number of polypeptides remain unidentified. Some of these components may be encoded by genes that have been identified through other genetic screens (Berger et al., 1992; Amakasu et al., 1993; Pina et al., 1993; Fassler et al., 1991).

SRB proteins are components of the mediator of activation

A subcomplex of RNA polymerase II, named the mediator of activation, can be dissociated from a preparation of RNA polymerase II holoenzyme using anti-CTD monoclonal antibodies (Kim et al., 1994). Purified mediator contains SRB2, SRB4, SRB5, SRB6, SUG1, GAL11, the general transcription factor TFIIF, and as yet unidentified subunits (Kim et al., 1994). Since all nine SRB proteins are components of the holoenzyme, and genetic analysis suggests that the SRB proteins are involved in a similar process in transcription, we investigated whether SRB7, SRB8, SRB9, and SRB10 are also part of a mediator preparation.

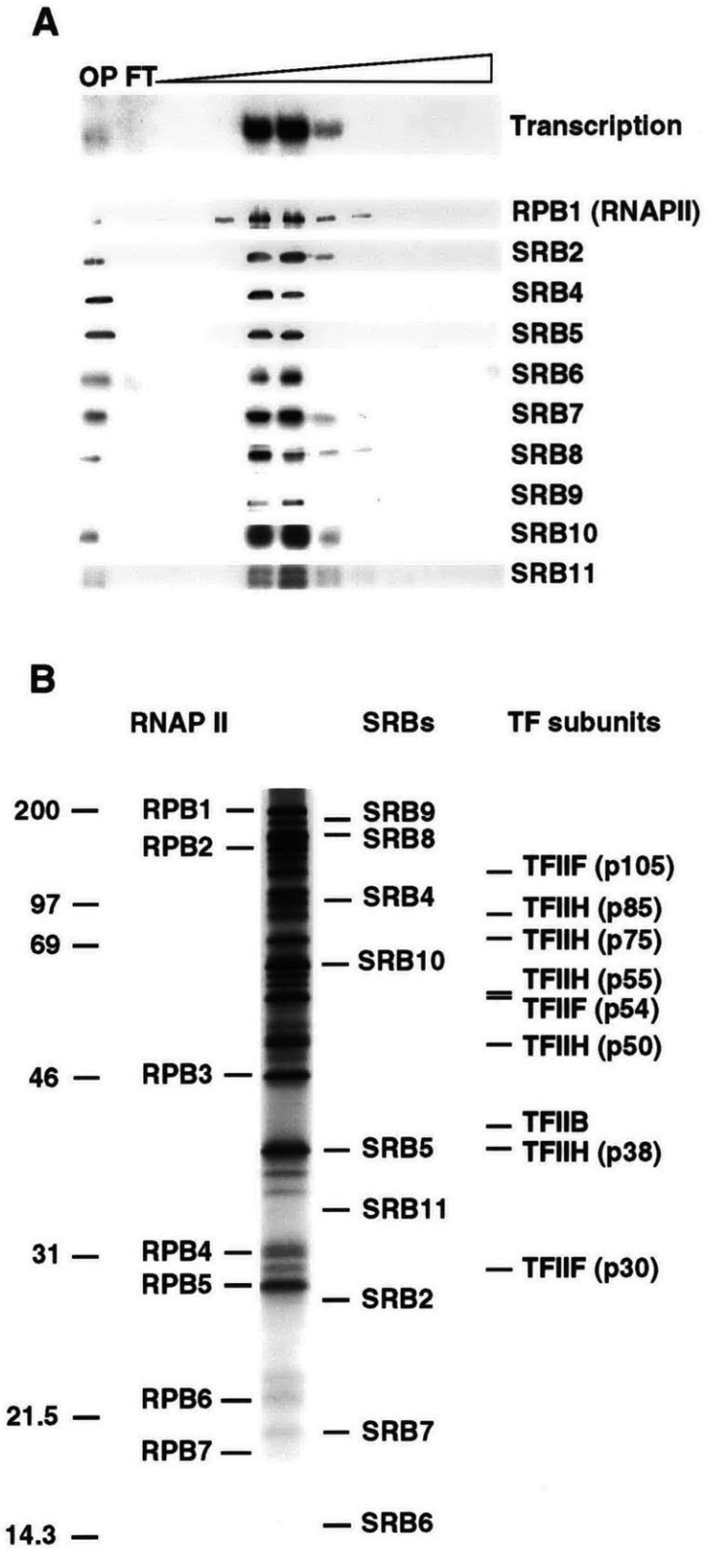

A
OP FT
Transcription
RPB1 (RNAPII)
SRB2
SRB4
SRB5
SRB6
SRB7
SRB8
SRB9
SRB10
SRB11
B
RNAP II
SRBs
TF subunits
200
97
69
46
31
21.5
14.3
RPB1
RPB2
RPB3
RPB4
RPB5
RPB6
RPB7
SRB9
SRB8
SRB4
SRB10
SRB5
SRB11
SRB2
SRB7
SRB6
TFIIF (p105)
TFIIH (p85)
TFIIH (p75)
TFIIH (p55)
TFIIF (p54)
TFIIH (p50)
TFIIB
TFIIH (p38)
TFIIF (p30)

Figure 4. All SRBs are components of an RNA polymerase II holoenzyme.

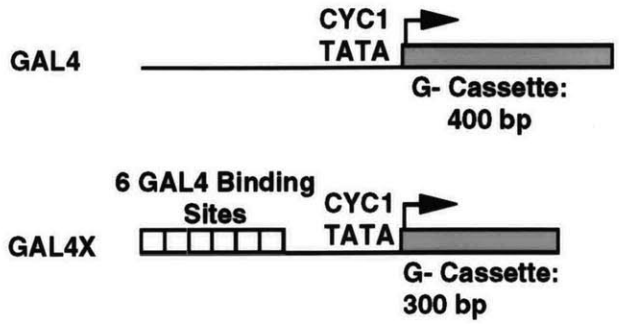
(A) RNA polymerase II holoenzyme was purified as described (Koleske et al., 1994). Holoenzyme loaded onto a Mono S column, the last chromatographic step in the purification procedure, was eluted with a 0.1 - 1.0 M gradient of potassium acetate. The onput (OP) and flow-through (FT) and a portion of every other fraction eluting between 0.1 and 0.9 M potassium acetate were analyzed for holoenzyme activity (top panel). These samples were also analyzed by Western blot for the presence of RNA polymerase II and SRB proteins. The Western blot for SRB11 was done with an RNA polymerase II holoenzyme purified independently from cells with an epitope-tagged SRB11 protein; the purification and transcriptional properties of this holoenzyme were identical to the holoenzyme lacking the epitope tag (Liao et al., 1995). (B) Polypeptide composition of RNA polymerase II holoenzyme. One microgram of purified holoenzyme was subjected to SDS-PAGE and stained with silver. Proteins in the holoenzyme preparation that correspond in size to subunits of RNA polymerase II, SRB proteins, and general transcription factors are indicated. The numbers used for the subunits of transcription factors TFIIF and TFIIH are from previous reports (Henry et al., 1992; Feaver et al., 1993). The sizes of protein molecular weight standards are indicated in kD.

The mediator of activation was purified from commercial baker's yeast as described (Kim et al., 1994). The chromatographic behavior, yield and transcriptional activities of the mediator preparation were similar to those reported. Figure 5 shows the results of transcription and Western blot analysis of fractions from the last chromatographic step in the purification. Fractions containing the mediator stimulated increases in basal and in activated transcription, as previously described (Kim et al., 1994). Western blot analysis with specific antisera against SRB proteins showed that SRB2, SRB4, SRB5, SRB6, SRB7, SRB8, SRB9 and SRB10 proteins all coeluted with the mediator activity. Our lack of anti-SRB11 antibodies prevented us from assaying the presence of SRB11 in the mediator, but evidence that SRB11 is a component of the holoenzyme and binds to directly to SRB10 (Liao et al., 1995) argues that SRB11 is also a component of the mediator. Thus, all nine of the SRB proteins, together with SUG1, GAL11 and TFIIF, can be dissociated as a complex from the RNA polymerase II holoenzyme to produce the mediator of activation.

Acidic activator VP16 coimmunoprecipitates with RNA polymerase II holoenzyme

Two observations suggest that some acidic activators may contact the RNA polymerase II holoenzyme directly. Both forms of RNA polymerase II holoenzyme described thus far are able to respond to activators in reconstituted in vitro transcription systems (Koleske and Young, 1994; Kim et al., 1994). One form of RNA polymerase II holoenzyme contains TFIIB and TFIIH, two factors that have been shown to interact with the VP16 activation domain (Lin et al., 1991; Goodrich et al., 1993; Xiao et al., 1994). To investigate whether the holoenzyme can bind to the acidic activation domain of the

A


GAL4
CYC1
TATA
G- Cassette:
400 bp
6 GAL4 Binding
Sites
GAL4X
CYC1
TATA
G- Cassette:
300 bp

B


GAL4Δ
GAL4X6
Transcription
OP
21
23
25
27
29
Fractions
SRB2
SRB4
SRB5
SRB6
SRB7
SRB8
SRB9
SRB10

Figure 5. SRBs are components of the mediator of activation.

(A) In vitro transcription templates used for assaying mediator activity. (B) A mediator preparation was loaded onto a MonoQ column, the last column in the purification procedure, and eluted with a 0.4-0.8 M gradient of potassium acetate. Each column fraction was assayed for mediator activity using a reconstituted transcription system with purified general transcription factors, core RNA polymerase II and recombinant GAL4-VP16. Mediator activity, defined by activator-responsive transcription and increased basal transcription, was found to peak in fraction 25 (top panel). This and nearby fractions were then analyzed by Western blot for the presence of SRB proteins.

activator VP16, we immunoprecipitated the RNA polymerase II holoenzyme from a crude yeast protein preparation in the presence of wild type and mutant VP16 activator proteins. The herpes simplex virus transactivator VP16 has been extensively characterized, and analysis of VP16 mutants suggests the presence of two activation subdomains within the highly acidic C-terminal 78 amino acids (Triezenberg et al., 1988; Regier et al., 1993). The $VP16^{\Delta 456FP442}$ mutant is defective in transcriptional activation; one subdomain of transcriptional activation is deleted (aa 457-490), and the second subdomain (aa 413-456) contains a single amino acid substitution abolishing residual transcriptional activation activity (Phe-442 to Pro-442) (Cress and Triezenberg, 1991; Regier et al., 1993).

RNA polymerase II holoenzyme was immunoprecipitated from a crude yeast protein preparation supplemented with recombinant GST-VP16 or GST-$VP16^{\Delta 456FP442}$ using affinity-purified anti-SRB5 polyclonal antibodies. Western blot analysis of the input, supernatant, wash and pellet show that the pellet contains components of the RNA polymerase II holoenzyme, including the pol II subunit RPB1, SRB proteins, and TFIIB (Figure 6, IP 1 and IP 2). A polyclonal control antibody directed against a human TGFβ receptor did not immunoprecipitate any RNA polymerase II holoenzyme components (Figure 6, IP 3). TBP, which is not a component of purified holoenzymes, could not be detected in any of the pellets, arguing against non-specific aggregation of proteins in the immunoprecipitation (Figure 6, IP 1 and IP 2 pellets). As an additional control, SSA1, a yeast HSP70 protein, also fails to be immunoprecipitated. Thus, holoenzyme components are specifically coprecipitated with SRB5 from crude yeast proteins under these conditions. The results also show that GST-VP16 is coprecipitated with the holoenzyme, whereas GST-$VP16^{\Delta 456FP442}$ is not (Figure 6, IP 1 and IP 2 pellets). We

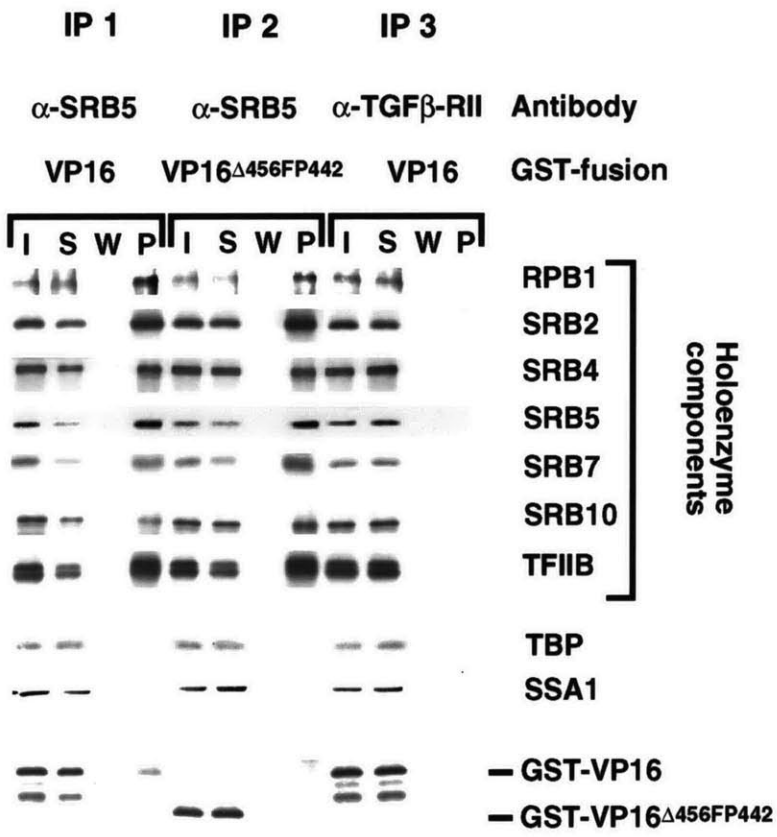

IP 1
IP 2
IP 3
α-SRB5
α-SRB5
α-TGFβ-RII
Antibody
VP16
VP16Δ456FP442
VP16
GST-fusion
I S W P I S W P I S W P
RPB1
SRB2
SRB4
SRB5
SRB7
SRB10
TFIIB
Holoenzyme components
TBP
SSA1
GST-VP16
GST-VP16Δ456FP442

Figure 6. Coimmunoprecipitation of the VP16 activator with the RNA polymerase II holoenzyme.

IP 1, IP 2, and IP 3 represent three separate immunoprecipitation experiments. A yeast whole cell extract was subjected to an ammonium sulfate cut, fractionated by step elution from BioRex 70 and DEAE-Sephacel (Experimental procedures), and immunoprecipitations were carried out with anti-SRB5 antibodies (IP 1 and IP 2), or a control antibody, anti-hTGFβ-RII (IP 3). Recombinant GST-VP16 was added to the input of IP 1 and IP 3, while recombinant GST-VP16$^{\Delta 456FP442}$ was added to the input of IP 2. 1/50 of the input (I) and supernatant (S), and 1/5 of the last wash (W) and pellet (P) were subjected to SDS-PAGE and analyzed by Western blot using specific antibodies.

estimate that the concentration of VP16 proteins was similar to that of holoenzyme components in the crude yeast protein preparation, and that the ratio of GST-VP16 to holoenzyme molecules in the immunoprecipitate was approximately 1:10.

A VP16 column selectively retains the RNA polymerase II holoenzyme together with TBP

To confirm that the holoenzyme interacts specifically with GST-VP16, the same crude protein preparation used in the immunoprecipitation experiments was passed over GST-VP16 or GST-VP16$^{\Delta 456FP442}$ columns. The columns were washed extensively and GST-fusion and associated proteins were eluted with glutathione. Components of the RNA polymerase II holoenzyme bound to GST-VP16 but not to GST-VP16$^{\Delta 456FP442}$ (Figure 7). Consistent with previous reports, TBP also specifically bound to the GST-VP16 (Stringer et al., 1990; Ingles et al., 1991). SSA1 protein did not bind to either column, arguing against non-specific protein aggregation on the GST-VP16 column. Thus, both immunoprecipitation experiments and column chromatography support the conclusion that the RNA polymerase II holoenzyme interacts with the activation domain of VP16.

A VP16 column retains the mediator of activation

We investigated whether the mediator subcomplex interacts with VP16 and, if so, whether the interaction involves both VP16 activation subdomains. A mediator preparation was passed over columns containing GST-VP16, GST-VP16$^{\Delta 456}$, or GST-VP16$^{\Delta 456FP442}$. The columns were washed extensively and GST-fusion and associated proteins were eluted with glutathione. The onput,

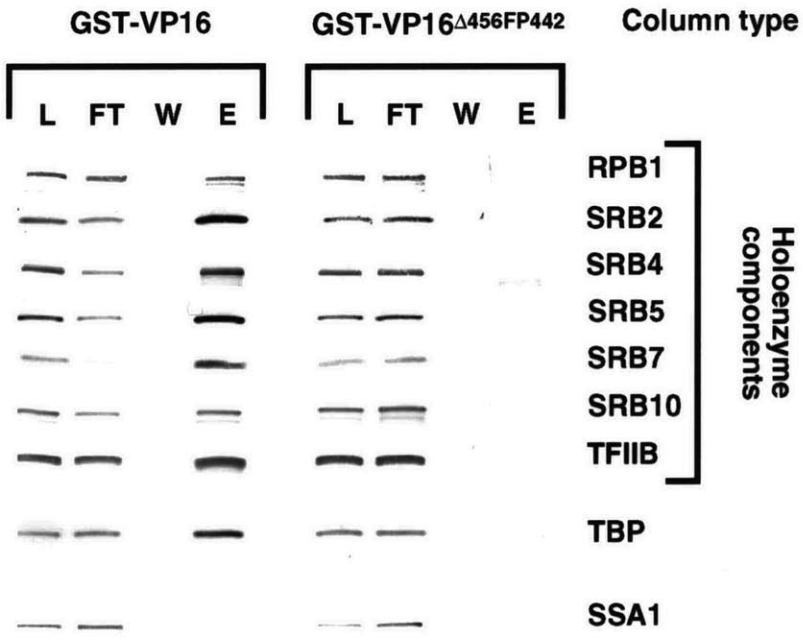
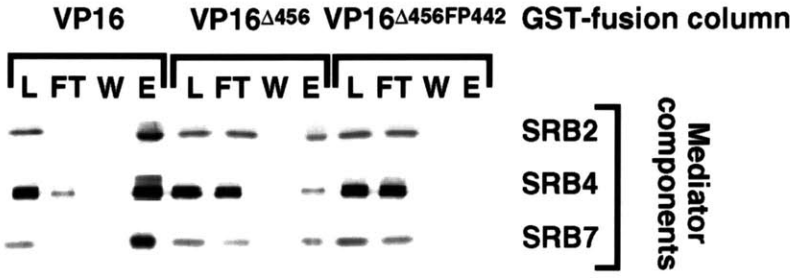

GST-VP16
GST-VP16Δ456FP442
Column type
L FT W E
L FT W E
RPB1
SRB2
SRB4
SRB5
SRB7
SRB10
TFIIB
TBP
SSA1
Holoenzyme components

Figure 7. RNA polymerase II holoenzyme and TBP bind a VP16 activator column.

The DEAE-Sephacel fraction described in Figure 6 was loaded onto GST-VP16 and GST-VP16$^{\Delta 456FP442}$ glutathione-agarose columns. After washing, the columns were eluted with glutathione. 1/200 of the load (L) and the flowthrough (FT), and 1/10 of the last wash (W) and the eluate (E) were subjected to SDS-PAGE and analyzed by Western blot.

flowthrough, wash and eluate fractions from the three columns were subjected to Western blot analysis (Figure 8A). Components of the mediator were bound most effectively by GST-VP16. In contrast, there was limited binding of the mediator to GST-VP16$^{\Delta 456}$, and no apparent binding to GST-VP16$^{\Delta 456FP442}$. Figure 8B shows that the mediator preparation lacks RPB1, confirming that it contains little or no contaminating holoenzyme. These results indicate that the VP16 activator binds to the mediator. Furthermore, both of the activation subdomains of VP16 contribute to maximal binding of the mediator.

A


VP16
VP16Δ456
VP16Δ456FP442
GST-fusion column
L FT W E
L FT W E
L FT W E
SRB2
SRB4
SRB7
Mediator components

B

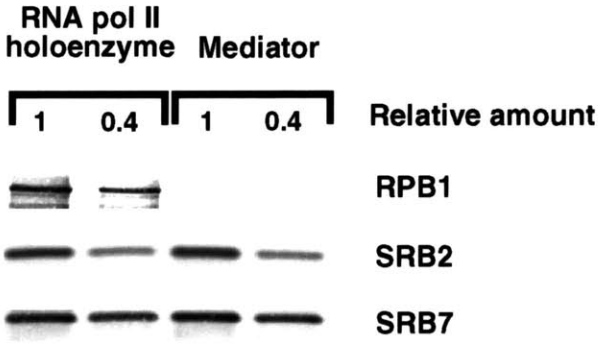

RNA pol II holoenzyme
Mediator
1
0.4
1
0.4
Relative amount
RPB1
SRB2
SRB7

Figure 8. Mediator of activation binds to VP16 activator column.

(A) Mediator of activation (peak fractions from heparin column) was loaded onto GST-VP16, GST-VP$^{\Delta 456}$, and GST-VP16$^{\Delta 456FP442}$ glutathione-agarose columns. After washing, the columns were eluted with glutathione. 1/150 of the load (L) and the flowthrough (FT), and 1/60 of the last wash (W) and the eluate (E) were subjected to SDS-PAGE and analyzed by Western blot.
(B) Mediator of activation (peak fractions from heparin column) and pure RNA polymerase II holoenzyme (Koleske et al., 1994) were analysed by Western blot for the presence of the largest subunit of RNA polymerase II (RPB1) and for SRB2 and SRB7 proteins. The lanes contained 0.8 μg and 0.32 μg of RNA polymerase II holoenzyme, and 2.5 μg and 1 μg of mediator of activation, respectively.

Discussion

The genetic and biochemical analysis reported here provides a more complete description of the RNA polymerase II holoenzyme. The products of all nine *SRB* genes described thus far are components of the holoenzyme. All nine SRB proteins can be dissociated from the holoenzyme in a subcomplex which functions in basal and activated transcription and has been termed the mediator of activation. The ability of holoenzymes to respond to activators suggests a model in which the holoenzyme is recruited to the initiation complex through direct interactions with activator proteins. Indeed, we find that the VP16 activator can bind to the holoenzyme. Since VP16 can bind the mediator, this subcomplex is among the components of the holoenzyme that interact with VP16.

SRB components and the RNA polymerase II holoenzyme

The nine *SRB* genes were identified through a selection for genes whose products are involved in RNA polymerase II transcription in vivo. Suppressing alleles of the *SRB* genes were isolated by their ability to suppress the cold-sensitive phenotype of yeast cells that contain RNA polymerase II molecules with truncated CTDs. The isolation of the *SRB2*, *SRB4*, *SRB5* and *SRB6* genes and the biochemical analysis of their protein products originally led to the discovery of the RNA polymerase II holoenzyme (Koleske and Young, 1994). Two additional *SRB* genes, *SRB10* and *SRB11*, were recently described; they encode kinase and cyclin-like polypeptides that are tightly associated with the holoenzyme and appear to have roles in CTD phosphorylation (Liao et al., 1995). Analysis of all 75 independent suppressor isolates obtained in the genetic selection has led to the identification and

characterization of three additional *SRB* genes. We have summarized the data for the nine *SRB* genes and proteins in Table I.

Biochemical studies have shown that all nine SRB proteins are integral components of the RNA polymerase II holoenzyme. The SRB proteins copurify with RNA polymerase II, TFIIB, TFIIF, and TFIIH in conventional chromatography (Figure 4A; Koleske and Young, 1994). All SRB proteins tested (SRB2, 4, 5, 6, 7, 10 and 11) and other components of the holoenzyme have been shown to co-immunoprecipitate with SRB5 (Figure 6A; Koleske and Young, 1994; Liao et al., 1995). Furthermore, when we purify for any of these SRB proteins, all of the SRB protein detected by Western analysis copurifies with the holoenzyme (Koleske and Young, 1994; Liao et al., 1995; Hengartner and Koleske, unpublished).

A model depicting the RNA polymerase II holoenzyme is shown in Figure 9. This complex consists of RNA polymerase II, the general transcription factors TFIIB and TFIIH, and the mediator of activation. The mediator of activation contains the nine SRB proteins, TFIIF, GAL11 and SUG1 (Figure 5; Kim et al., 1994). In cell lysates, the SRB proteins are only found tightly associated with the holoenzyme; the SRB-containing mediator of activation can be dissociated from the holoenzyme by anti-CTD monoclonal antibodies, indicating that the interaction of these antibodies with the RNA polymerase II CTD disrupts interactions between the CTD and the mediator subcomplex.

Several lines of evidence suggest that the RNA polymerase II holoenzyme is the form of the enzyme involved in transcription initiation in vivo. Genetic analysis has indicated that the CTD and SRB gene products have roles in transcription initiation and are essential for normal yeast cell growth. Using temperature-sensitive mutants, we have found that SRB4 and

RNA Pol II Holoenzyme

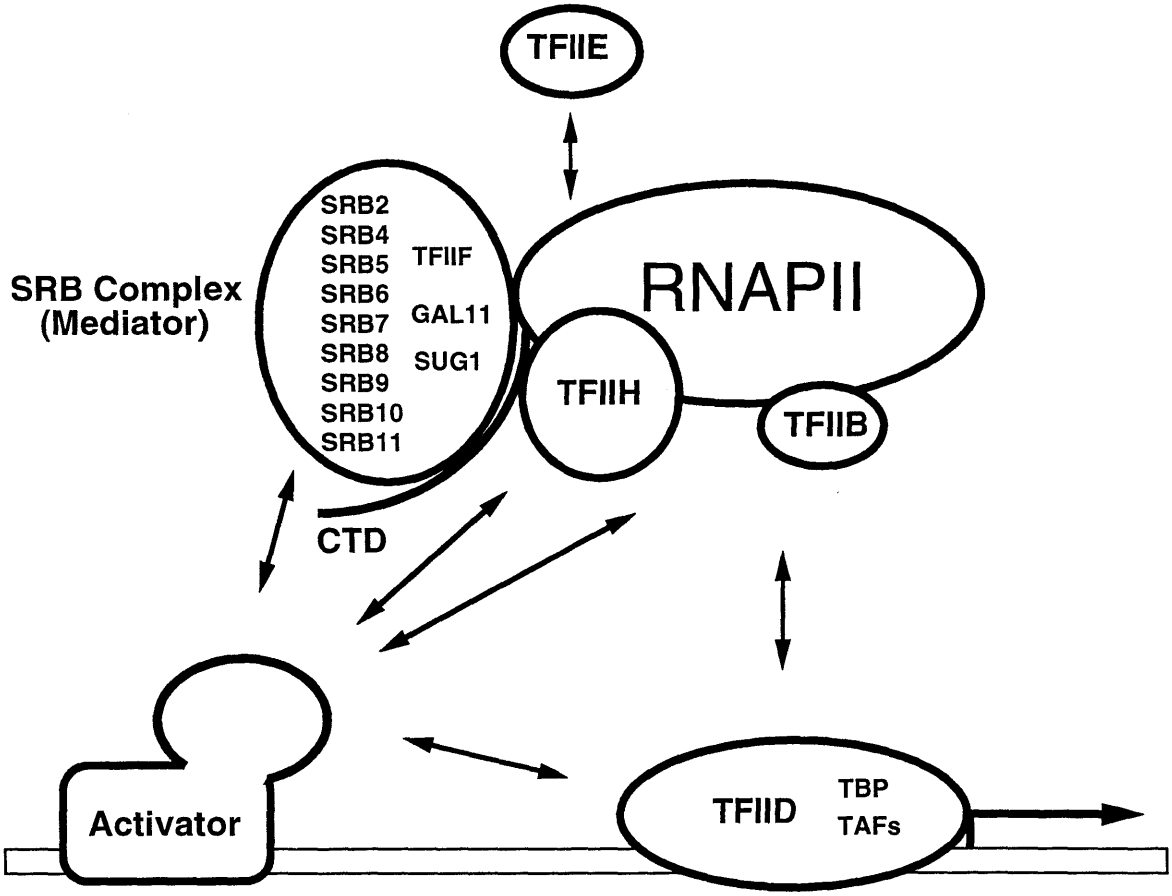


Figure 9. Model for interactions among activators, TFIID, and the RNA polymerase II holoenzyme.

SRB6 are necessary for transcription of most, if not all, genes transcribed by RNA polymerase II in vivo (Thompson and Young, 1995). Since the vast majority of SRB protein in cell extracts is tightly associated with the RNA polymerase II holoenzyme, these results suggest that the holoenzyme is employed at most pol II promoters in vivo.

What roles do the SRB proteins have in the holoenzyme? Some of the SRB proteins are likely to contribute to interactions between RNA polymerase II and general transcription factors. For instance, SRB2 and SRB5 are essential for transcription in unfractionated in vitro systems, where they have roles in stable preinitiation complex formation (Koleske et al., 1992; Thompson et al., 1993). Both SRB2 and SRB5 can bind to TBP in vitro (Koleske et al., 1992; Thompson et al., 1993), raising the possibility that they contribute to the stability of holoenzyme-TBP interactions at the promoter. The mediator subcomplex appears to act as a contact point for at least some transcriptional regulatory proteins and these regulators may bind directly to specific SRB proteins. The SRB-containing subcomplex may also function as a signal processor in the response to transcriptional regulators. SRB10 and SRB11 form a cyclin-dependent kinase that has a role in CTD phosphorylation (Liao et al., 1995). The role of CTD phosphorylation in transcription is not yet clear, but it may serve to disrupt interactions between RNA polymerase II and general factors, which may be necessary for promoter clearance.

Initiation complex formation with the holoenzyme

The identification of a yeast RNA polymerase II holoenzyme suggests a model for initiation complex formation in which the holoenzyme is the form of RNA polymerase recruited to promoters. Activators are believed to

influence the rate of transcription of an adjacent gene in part by interacting with components of the initiation apparatus and affecting stable initiation complex formation. Figure 9 depicts a model that posits interactions between activators and TFIID and between activators and components of the holoenzyme. These interactions could occur sequentially or simultaneously.

The portion of the model that depicts interactions between activators and TFIID has been proposed by others and is supported by several lines of evidence. Activator proteins can stimulate transcription in vitro in reactions directed by the TFIID multisubunit complex but they do not stimulate transcription reactions directed by recombinant TBP alone (Pugh and Tjian, 1990). Reconstitution of activated transcription in vitro can be accomplished by reconstituting TFIID using recombinant TBP and TBP-Associated Factors (TAFs) (Dynlacht et al., 1991; Tanese et al., 1991; Zhou et al., 1992; Chen et al., 1994). These and other results support the idea that activators interact with the TAF subunits of TFIID. A recent report, however, indicates that TFIID may not be sufficient to confer a response to an acidic activator in vitro in highly purified systems (Kretschmar et al., 1994). It is possible that TFIID is necessary but not sufficient for a response to some activators.

The portion of the model in Figure 9 that depicts interactions between activators and the RNA polymerase II holoenzyme is supported by the following lines of evidence. The RNA polymerase II holoenzyme contains components necessary and sufficient for responding to transcriptional activators in a reconstituted system, apparently in the absence of TAFs (Koleske and Young, 1994; Kim et al., 1994). Genetic evidence suggests that GAL11 and SUG1, two components of the mediator, may interact with certain activators (Nishizawa et al., 1990; Swaffield et al., 1992). We have shown that VP16 can bind to the mediator subcomplex of the holoenzyme. Thus, the

activator VP16 has been shown to interact in vitro with three components of the RNA polymerase II holoenzyme: the mediator subcomplex, TFIIB (Lin et al., 1991; Goodrich et al., 1993) and TFIIH (Xiao et al., 1994).

The complexity of the transcription initiation complex suggests that there are numerous molecular mechanisms involved in transcriptional regulation. Precise identification of the interactions that regulatory proteins have with components of the holoenzyme should lead to a better understanding of the mechanisms that regulate transcription initiation.

Experimental Procedures

Genetic manipulations

Yeast strains and plasmids are listed in tables 2 and 3, respectively. Details of strain and plasmid constructions are available upon request. Yeast media was prepared as described (Thompson et al., 1993). Yeast transformations were done using a lithium acetate procedure (Schiestl and Gietz, 1989). Plasmid shuffle techniques were performed as described (Boeke et al., 1987) using 5-fluoro-orotic acid (5-FOA) as a selective agent against *URA3* plasmids. Plasmids were recovered from yeast as described by Hoffman and Winston (1987).

Extragenic suppressors of the cold-sensitive phenotype of Z551 were isolated as previously described (Nonet and Young, 1989, Thompson et al., 1993). Dominant and recessive suppressors were identified by mating to Z26 (Thompson et al., 1993), selecting against the presence of pRP112 (Nonet et al., 1987) using 5-FOA and assaying growth at 12°C on YEPD. Diploids able to grow at 12°C contained a dominant suppressor. Diploids unable to grow at 12°C contained a recessive suppressor.

Yeast strains of the opposite mating type of approximately half of the dominant suppressors and half of the recessive suppressors were generated by inducing a mating type switch by expression of the *HO* gene placed on a plasmid under the control of a galactose inducible promoter. Random spore analysis of the dominantly suppressing mutations was used to determine if two independent isolates were likely to contain mutations in the same gene. Haploids were mated to each other, each containing the CTD truncation mutation *rpb1Δ104* and an independently isolated *SRB* mutation, to form diploids. These diploids were sporulated on plates and a small quantity of spores

scraped off and shaken overnight at 30°C in 0.5 mL 30 mM b-mercaptoethanol and 100 ng/mL Zymolase 100 T (ICN). 0.5 mL of 1.5% NP-40 and 0.4 g glass beads were added and the mixture incubated on ice for 15 min. The suspension was then vortexed 3 min, incubated on ice 5 min, vortexed 2 min, and the glass beads allowed to settle for 10 min at room temperature. The supernatant was removed, spun 2 min, the pellet washed once in water, then resuspended in water and a portion plated onto YEPD. Approximately fifty of the haploid offsprings were assayed for their ability to grow at 12°C. If all haploids were able to grow at 12°C then the two *SRB* isolates were assumed to contain mutations in the same gene. Genetic complementation of the recessive alleles involved mating haploids to each other, each containing the CTD truncation mutation *rpb1Δ104* and an independently isolated *srb* mutation, to form diploids and assessing the ability of these diploids to grow at 12°C. Diploids able to grow at 12°C were assumed to contain *srb* mutations in the same gene. Genomic clones of each complementation group were used to confirm the identity of each member of the complementation group and to identify additional members. Cells containing the CTD truncation mutation *rpb1Δ104* and a recessive *srb* allele were unable to grow at 12°C and on pyruvate media when transformed with the corresponding wild-type *SRB* allele.

Deletions of *SRB7*, *SRB8* and *SRB9* were created by a single step disruption method (Rothstein, 1991). Z558 was transformed with the desired DNA fragment and plated on SC-Ura media (Thompson et al., 1993). Southern analysis was used to confirm that a single copy of the desired *SRB* gene had been deleted. The diploid was sporulated and tetrads dissected (>20) on YEPD plates and scored for nutritional auxotrophies and growth at a variety of temperatures. Z697 was created by transformation with the *srb7Δ1::URA3hisG* fragment from pCH46. Two spores from each tetrad were viable and these spores were uracil

auxotrophs, indicating that *SRB7* is essential. Z698 was created by transformation with the *srb8Δ1::URA3hisG* fragment from pSL315 and Z699 was created by transformation with the *srb9Δ1::URA3hisG* fragment from pCH66. In each case segregants scored 2:2 for uracil prototrophy and all uracil prototrophs exhibited mild cold-sensitive, temperature-sensitive, and slow growth phenotypes, indicating that *SRB8* and *SRB9* deletion strains are conditionally viable. *srb8Δ1* and *srb9Δ1* strains are also flocculent as are the suppressing isolates of *SRB8* and *SRB9*. Strains containing unmarked deletions of *SRB8* and *SRB9* were created by selecting for the excision of the *URA3* gene by growth on 5-FOA (Alani et al., 1987).

DNA methods

DNA manipulations were performed according to Sambrook et al. (1989). Site-directed mutagenesis was performed as described in Kunkel et al. (1987). PCR amplifications to produce pCH45 (*srb7Δ1*), pSL315 (*srb8Δ1*), and pSL307 (*SRB8* in pET-3a) were performed with Taq DNA polymerase (Perkin Elmer) in 100 μL of buffer (provided by the manufacturer) supplemented with 200 mM dNTP for a total of 25 cycles. Primer concentrations were 0.5 mM with 50 ng of DNA and cycling was at 94°C (1.0 min), 50°C (1.0 min) and 72°C (2.5 min).

Cloning and sequence analysis

Genomic clones of *SRB7* (pCH2), *SRB8* (pSL301), and *SRB9* (pCH47) were isolated as previously described (Liao et al., 1995) by transformation and complementation of Z694, Z695, and Z696 respectively. When necessary, the wild-type genes were further localized by subcloning fragments of the genomic inserts and repeating the screen. The clones with the smallest inserts were sequenced. pCH36 was created from pCH7 in vivo by

transforming Z694 with linearized pCH7 lacking *SRB7* coding DNA and isolating the plasmid from a Ura$^+$ transformant which had repaired the plasmid with the mutant *srb7-1* sequences from the chromosome (Rothstein, 1991). *SRB7* and *SRB9* were completely sequenced on each strand using genomic DNA from pCH7 and pCH47, respectively. Unidirectional deletions were constructed using the Erase-a-Base system (Promega) and double stranded sequencing with dideoxynucleotides and Sequenase (US Biochemical) was carried out as described by the manufacturer using T3 and T7 promoter primers. Gaps in the sequence were filled in by sequencing with internal oligonucleotide primers. Sequence comparison analysis was performed at the National Center for Biotechnology Information using the BLAST network service (Altschul et al., 1990).

The *srb7-1* mutant allele obtained by plasmid gap repair in vivo contained a single-point missense mutation changing alanine 21 to threonine (Fig. 1B). Unlike their wild-type counterparts, plasmids containing this mutation did not prevent growth at 12°C when transformed into yeast cells containing the CTD truncation mutation *rpb1Δ104* and *srb7-1*(Z694), indicating that the correct gene was cloned. Similar results were obtained for the *srb8-1* and *srb9-1* mutant alleles obtained by plasmid gap repair in vivo.

The conditional phenotypes associated with CTD truncations are suppressed both by *srb8-1* or by complete deletion of *SRB8* (Z695 vs. Z701). Similarly, the conditional phenotypes associated with CTD truncations are suppressed both by *srb9-1* or by complete deletion of *SRB9* (Z696 vs. Z703). The mutations in *srb8-1* or *srb9-1* are thus likely to have at least partially destroyed gene function, since cells containing either the suppressor or the deletion alleles exhibit very similar phenotypes.

The cloned genes were physically mapped using the prime lambda clone grid filters of the yeast genome (provided by L. Riles and M. Olson, Washington University).

Purification of recombinant proteins

Recombinant SRB proteins were purified for generating polyclonal antibodies in rabbits. SRB7 and a portion of SRB8 (amino acids 868 to 1226) were purified from the bacterial strain BL21(DE3) pLysS (Studier and Moffatt, 1986) carrying the plasmids pCH34 and pSL307, respectively, in the same manner SRB2 was purified (Koleske et al., 1992). A portion of SRB9 (amino acids 45 to 501) was purified as a fusion to glutathione-*S*-transferase from DH5α carrying pCH64 according to the method of Smith and Johnson (1988).

GST-VP16, GST-VP16$^{\Delta 456}$, and GST-VP16$^{\Delta 456FP442}$ recombinant proteins were purified from DH5α carrying pGVP, pGVPΔ456, and pGVPΔ456-FP442 (provided by Michael Green), respectively, as described (Smith and Johnson, 1988).

Mediator purification and assay

Mediator was purified essentially as described (Kim et al., 1994). In vitro transcription assay for mediator activity was performed as described (Sayre et al., 1992) with modifications (Liao et al., 1995). GAL4-VP16, TBP, TFIIB, and TFIIE were prepared as described (Liao et al., 1995). TFIIF and TFIIH were prepared as described (Sayre et al., 1992). RNA polymerase II was prepared as described (Edwards et al., 1990).

Western blot analysis

Western blotting was performed by standard methods (Harlow and Lane, 1989). RPB1 was detected via the CTD with 8WG16 monoclonal antibody ascites fluid (Promega). Polyclonal rabbit anti-SRB2, anti-GST-SRB4, anti-SRB5, anti-GST-SRB6, anti-SRB7, anti-SRB8 (aa 868 to 1226), anti-GST-SRB9 (aa 45 to 501), and anti-SRB10 (aa 1 to 271) antiserum were used to detect the SRBs. TBP, TFIIB, SSA1, and GST were detected using specific rabbit polyclonal antiserum. In all cases, bands were visualized by secondary probing with alkaline phosphatase conjugated secondary antibodies (Promega).

DEAE-400 fraction preparation

2 kg of dry yeast (Red Star) were disrupted essentially as described (Thompson et al., 1993), except that the disruption buffer was 1.2 M ammonium sulfate, 160 mM HEPES-KOH pH 7.6, 4 mM DTT, 40 mM EDTA, 2 mM PMSF, 4 mM benzamidine, 2 μM pepstatin A, 0.6 μM leupeptin, and 2 μg/mL chymostatin. After the initial centrifugation, 1/100 volume of 10% Polymin P pH 7.0 was added to the supernatant. The supernatant was incubated 10 min. at 4°C and then spun for 20 minutes at 5,000 rpm in a Sorvall™ H6000A rotor. Solid ammonium sulfate was added to 35% saturation, and the solution was incubated for 30 minutes. The suspension was centrifuged for 1 hr at 13000 rpm in a GSA rotor. The pellet was resuspended in 9.5 L of Buffer A (20 mM HEPES-KOH pH 7.6, 1 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 0.5 μM pepstatin A, 0.15 μM leupeptin and 1 μg/mL chymostatin) to a conductivity equal to Buffer A-150 mM potassium acetate. The resuspended pellet was centrifuged for 20 minutes at 5,000 rpm in a H6000A rotor. The supernatant (6.14 g) was incubated for 1.5 hours with 500 g of damp BioRex 70 pre-equilibrated in Buffer A-150 mM potassium acetate.

The resin was collected by filtration, washed with 3 L of Buffer A-150 mM potassium acetate, and packed into a column (5 cm width). The column was eluted with Buffer A-600 mM potassium acetate at a flow rate of 8 mL/min. The eluate (0.6 g, 165 mL) was diluted with 500 mL of Buffer B (20 mM Tris-acetate pH 7.8, 1 mM EDTA, 20% glycerol, 0.01% NP-40, 1 mM DTT and the same protease inhibitors as in Buffer A). The diluted eluate was centrifuged for 10 minutes at 10,000 rpm in a GSA rotor. The supernatant was loaded onto a column (5 cm width) containing 100 mL of DEAE-Sephacel pre-equilibrated with Buffer B-200 mM potassium acetate at a flow rate of 8 mL/min. The column was washed with 200 mL of Buffer B-200 mM potassium acetate and eluted with Buffer B-400 mM potassium acetate. The eluate contained 150 mg of protein in 60 mL.

Immunoprecipitation

0.1 mL of the DEAE-400 fraction was dialyzed against Modified Transcription Buffer (50 mM Hepes-KOH pH7.3, 100 mM potassium acetate, 25 mM MgAc, 5 mM EGTA, 1 μM DTT, 10% glycerol, 0.01% NP40, 1 mM PMSF, 2 mM benzamidine, 2 μM pepstatin A, 0.6 μM leupeptin and 2 μg/mL chymostatin) and subsequently diluted to 0.4 mL with Modified Transcription Buffer. 1μg of either affinity purified anti-SRB5 antibodies or purified anti-human TGFβ-RII peptide (aa 30 to 44) IgG, and 1μg of recombinant GST-VP16 or GST-VP16$^{\Delta 456FP442}$ were added to the input. Immunoprecipitation was carried out essentially as described (Harlow and Lane, 1989). 30 μL Anti-rabbit antibody linked to dynabeads (Dynal) were used as the secondary reagent in the immunoprecipitation.

Affinity chromatography

1 mL of the DEAE-400 fraction (for holoenzyme binding experiments) or 1 mL of the mediator preparation (peak fractions from the heparin column) was dialyzed for 2 hours against Modified Transcription Buffer. The GST-VP16, GST-VP16$^{\Delta 456}$, and GST-VP16$^{\Delta 456FP442}$ affinity columns were prepared by immobilizing GST fusion proteins on glutathione-agarose beads as described (Smith and Johnson, 1988). The columns contained 200 µL matrix and contained approximately 300 µg of GST fusion proteins. The columns were equilibrated with 6 mL Modified Transcription Buffer (minus DTT), loaded 3 times over with 0.2 mL of dialyzed DEAE-400 fraction (for holoenzyme binding experiments) or 0.37 ml of the dialyzed mediator preparation (0.5 mg/mL), washed with 6 mL of Modified Transcription Buffer (minus DTT), and eluted with buffer containing 50 mM Tris-Cl pH 7.5, 0.1 mM DTT and 5 mM glutathione, eluting both GST-fusion proteins and interacting proteins.

Figures

The figures were prepared from digital replicas of primary data scanned using a UMAX UC840 Max Vision digital scanner.

Table 2. Yeast Strains

Strain	Alias	Genotype
Z694	S242	*Mata ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb7-1* [pC6 (*LEU2 CEN rpb1Δ104*)]
Z695	S358	*Mata ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb8-1* [pC6 (*LEU2 CEN rpb1Δ104*)]
Z696	S363	*Mata ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb9-1* [pC6 (*LEU2 CEN rpb1Δ104*)]
Z697	CHY102	*Mata /Matα ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb7Δ1::URA3hisG/SRB7*
Z698	SLY35	*Mata /Matα ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb8Δ1::URA3hisG/SRB8*
Z699	CHY105	*Mata /Matα ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb9Δ1::URA3hisG/SRB9*
Z700	SLY61	*Matα ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb8Δ1::hisG* [pRP114 (*LEU2 CEN RPB1*)]
Z701	SLY76	*Matα ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb8Δ1::hisG* [pC6 (*LEU2 CEN rpb1Δ104*)]
Z702	CHY113	*Matα ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb9Δ1::hisG* [pRP114 (*LEU2 CEN RPB1*)]
Z703	CHY116	*Matα ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb9Δ1::hisG* [pC6U (*URA3 CEN rpb1Δ104*)]

Table 3. Plasmids

Plasmid	Description
SRB7	
pCH2	*SRB7* (6.7 kb) *URA3 CEN*
pCH7	*SRB7* (2.0 kb) *URA3 CEN*
pCH36	*srb7-1 URA3 CEN*
pCH34	*SRB7* in pET-3a (Studier and Moffat, 1986)
pCH46	*srb7Δ1::URA3hisG* in pSP72 (Promega)
SRB8	
pSL301	*SRB8* (9.0 kb) *URA3 CEN*
pSL311	*SRB8* (6.0 kb) *URA3 CEN*
pSL307	*SRB8* (encoding aa 868 to 1226) in pET-3a (Studier and Moffat, 1986)
pSL315	*srb8Δ1::URA3hisG* in pBSIISK(+) (Stratagene)
pSL316	*srb8-1 URA3 CEN*
SRB9	
pCH47	*SRB9* (7.3 kb) *URA3 CEN*
pCH64	*SRB9* (encoding aa 45 to 501) in pGEX-1 (Smith and Johnson, 1988)
pCH66	*srb9Δ1::URA3hisG* in pSP72 (Promega)
pJZ995	*srb9-1 URA3 CEN*

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Chapter 3

Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases

Summary

Two cyclin-dependent kinases have been identified in yeast and mammalian RNA polymerase II transcription initiation complexes. We find that the two yeast kinases are indistinguishable in their ability to phosphorylate the RNA polymerase II CTD, yet in living cells one kinase is a positive regulator and the other a negative regulator. This paradox is resolved by the observation that the negative regulator, Srb10, is uniquely capable of phosphorylating the CTD prior to formation of the initiation complex on promoter DNA, with consequent inhibition of transcription. In contrast, the TFIIH kinase phosphorylates the CTD only after the transcription apparatus is associated with promoter DNA. These results reveal that the timing of CTD phosphorylation can account for the positive and negative functions of the two kinases and provide a model for Srb10-dependent repression of genes involved in cell type specificity, meiosis, and sugar utilization.

Introduction

Cyclin-dependent kinases (CDKs), originally described as cell cycle regulators, also have roles in transcription (reviewed in Dynlacht, 1997). Two distinct cyclin-dependent kinases are associated with eukaryotic RNA polymerase II transcription initiation complexes (Liao et al., 1995; Maldonado et al., 1996; Pan et al., 1997; Scully et al., 1997). The yeast kinase Kin28, and it's mammalian homologue Cdk7, are subunits of the general transcription factor TFIIH, which phosphorylates RNA polymerase II subsequent to formation of the pre-initiation complex (PIC) on promoter DNA (Feaver et al., 1994; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). Yeast Srb10, and it's mammalian homolog Cdk8, are subunits of the RNA polymerase II holoenzyme, but their functions are not yet understood (Liao et al., 1995; Maldonado et al., 1996; Pan et al., 1997; Scully et al., 1997).

The C-terminal domain (CTD) of the large subunit of eukaryotic RNA polymerase II (pol II) contains a repeated heptapeptide which is phosphorylated in a portion of pol II molecules in the cell (Cadena and Dahmus, 1987; Kolodziej, et al., 1990; reviewed in Dahmus, 1996). Several lines of evidence indicate that PIC formation involves RNA polymerase II molecules with unphosphorylated CTDs, and that these molecules become phosphorylated during or after the transition to active elongation. The form of pol II found in holoenzymes lacks phosphate on its CTD (Koleske and Young, 1994; Kim et al., 1994). The unphosphorylated form of pol II preferentially assembles into a PIC reconstituted with purified transcription factors (Bartholomew et al., 1986; Laybourn and Dahmus, 1990; Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994). Since the phosphorylated CTD has a role in recruiting the mRNA capping

enzyme to the nascent transcript, and mRNA capping occurs soon after promoter clearance (Coppola et al., 1983; Cho et al., 1997; McCracken et al., 1997a; McCracken et al., 1997b; Yue et al., 1997), CTD phosphorylation most likely occurs during the transition from transcription initiation to elongation. pol II molecules in the midst of elongation contain CTDs which are highly phosphorylated (Bartholomew et al., 1986; Cadena and Dahmus, 1987; Weeks et al., 1993; O'Brien et al., 1994).

The TFIIH kinase is apparently responsible for CTD phosphorylation subsequent to PIC formation (Laybourn and Dahmus, 1990; Ohkuma and Roeder, 1994; Akoulitchev et al., 1995; reviewed in Dahmus, 1996). Consistent with such a role, TFIIH has been found as one of two CTD kinases stimulated by the viral transactivator Tat (Parada and Roeder, 1996; Garcia-Martinez et al., 1997; Cujec et al., 1997). The HIV-1 Tat protein enhances transcription elongation by interacting with either TFIIH or P-TEFb CTD kinases to stimulate CTD phosphorylation (reviewed in Jones, 1997; Yankulov and Bentley, 1998). Loss-of-function mutations in the yeast TFIIH kinase subunit cause a general defect on class II transcription in vivo (Cismowski et al., 1995; Valay et al., 1995), confirming the positive role in transcription inferred from in vitro studies.

RNA polymerase II holoenzymes have been purified from yeast and mammalian cells which contain a second CDK implicated in CTD phosphorylation. Genes encoding the Srb10 kinase, and its cyclin partner Srb11, were initially identified in a yeast genetic screen designed to reveal factors involved in CTD function; subsequent analysis revealed that their protein products copurify with the other SRB proteins in the RNA polymerase II holoenzyme (Nonet and Young, 1989; Liao et al., 1995). Holoenzymes with a catalytically inactive Srb10 subunit have substantially

reduced CTD kinase activity, suggesting that Srb10 is a CTD kinase (Liao et al., 1995), but this has yet to be directly demonstrated. Mammalian Cdk8 and cyclin C are apparently homologues of Srb10 and Srb11, as they share strong sequence similarity and are both found in mammalian holoenzymes (Maldonado et al., 1996; Pan et al., 1997; Scully et al., 1997; D. Chao and R. Young, unpublished data).

While the function of the TFIIH kinase has been thoroughly studied, the function of Srb10 is poorly understood. It is clear, however, that yeast Kin28 and Srb10 CDKs are not functionally redundant. Substantial genetic and biochemical evidence indicates that Kin28 plays an essential, general and positive role in transcription. In contrast, the evidence suggests that Srb10 is essential for regulation of a subset of genes which are involved in cell type specificity (Wahi and Johnson, 1995), meiosis (Surosky et al., 1994), and sugar utilization (Kuchin et al., 1995). How Srb10 contributes to the regulation of these important genes is not yet clear.

Here we describe evidence that the two holoenzyme CDKs are indistinguishable in their ability to phosphorylate the CTD, yet in living cells Kin28 functions as a positive regulator and Srb10 as a negative regulator. The different regulatory consequences appear to be due to the fact that the Srb10 kinase is able to phosphorylate the CTD prior to holoenzyme binding to promoter DNA, with consequent inhibition of transcription. In contrast, Kin28 is active only after PIC formation, and plays a positive role through CTD phosphorylation. These results support a novel model for transcriptional regulation in which the negative and positive roles of the two kinases, which act on the same substrate, are a consequence of the time at which they are activated. This model describes how Srb10 contributes to

repression of yeast genes involved in cell type specificity, meiosis, and sugar utilization.

Results

Srb10 is a negative regulator of transcription in vivo

The Srb10 gene was identified in a genetic screen designed to reveal genes whose products interact functionally with the RNA polymerase II carboxy-terminal domain (CTD) (Liao et al., 1995). Cells containing a CTD truncation mutation exhibit conditional lethality, and extragenic suppressors (SRBs) were identified which restore the ability of these cells to grow at the nonpermissive temperature (reviewed in Koleske and Young, 1995). We have found that the suppressing phenotype of the *srb10-1* allele, the original recessive suppressor obtained in the selection, can be duplicated by altering a single amino acid residue in Srb10 which is critical for its kinase function (*srb10-3*; Srb10(D290A)) or by deleting the entire *SRB10* gene (*srb10Δ1*) (Figure 1 A). The observation that loss-of-function mutations in *SRB10* can restore viability to yeast cells with a CTD truncation indicates that the Srb10 kinase normally has a negative role in transcription in vivo.

The effect of *SRB10* mutations on yeast cells with a spectrum of CTD truncation mutations (Figure 1B) supports a negative role for Srb10 in transcription. We previously demonstrated that progressive truncation of the RNA polymerase II CTD produced cells with increasingly severe growth phenotypes, and that these phenotypes were due to functional defects rather than reduced stability of the pol II molecules (Nonet et al., 1987; Scafe et al., 1990). The phenotypes exhibited by each of eighteen different strains, which differ only in pol II CTD length, were classified into three categories: nonviable (N) strains which failed to grow under any condition, conditional lethal (C) strains which were cold sensitive, and viable (V) strains which exhibited essentially wild-type growth phenotypes. The wild-type *SRB10* gene

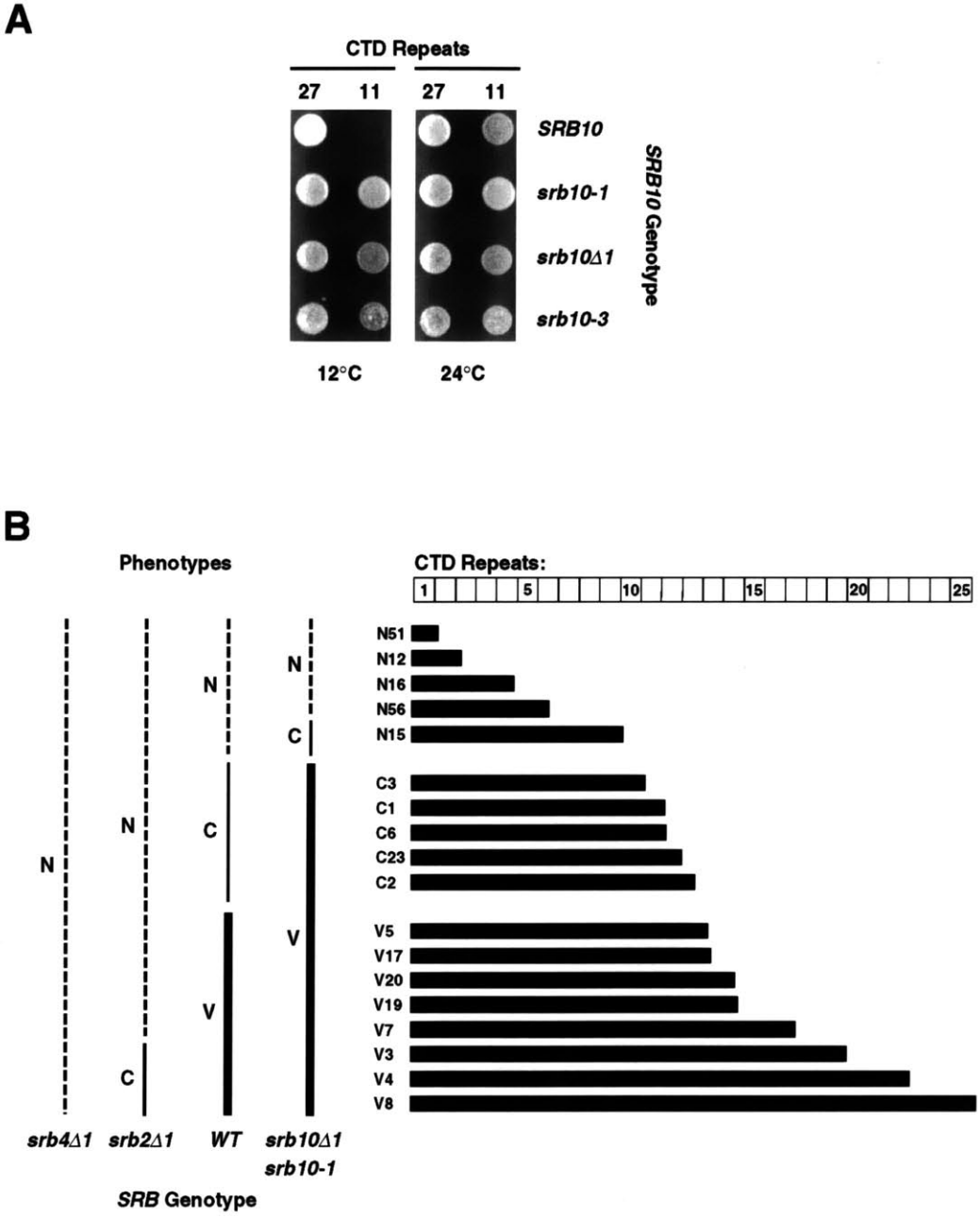

A
CTD Repeats
27
11
27
11
SRB10
srb10-1
srb10Δ1
srb10-3
SRB10 Genotype
12°C
24°C
B
Phenotypes
CTD Repeats:
1
5
10
15
20
25
N51
N12
N16
N56
N15
C3
C1
C6
C23
C2
V5
V17
V20
V19
V7
V3
V4
V8
N
N
C
N
C
V
V
C
srb4Δ1
srb2Δ1
WT
srb10Δ1
srb10-1
SRB Genotype

Figure 1. Srb10 is a negative regulator in vivo.

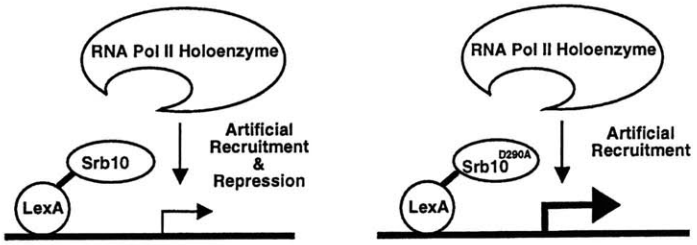
(A) Loss-of-function mutations in *SRB10* rescue the conditional lethality of a CTD truncation mutant. Strains with a truncated CTD (11 heptapeptide repeats) are inviable when grown at 12°C. Three different loss-of-function mutations in the *SRB10* gene restore viability to the CTD truncation strain. *srb10-1*, the original *SRB* suppressor, is a C-terminal truncation of the kinase, *srb10Δ1* is a deletion of the entire Srb10 coding sequence and *srb10-3* is an engineered point mutation (D290A) which renders the kinase catalytically inactive. CTD repeat length is indicated on the left, growth temperature on the right and *SRB10* genotypes across the top.

(B) *SRB10* loss-of-function alleles suppress growth defects across a spectrum of CTD truncation mutations. The effect of loss-of-function mutations in *SRB2*, *SRB4*, and *SRB10* was investigated in strains containing progressively truncated CTDs. The number of CTD repeats is shown on the horizontal axis, and the plasmid carrying each CTD truncation allele is indicated (i.e. pN51). The growth phenotypes exhibited by each CTD truncation mutant in the presence of wild-type *SRB* genes or with mutations in *SRB2*, *SRB4* or *SRB10* is shown. Non-viable (N) cells are indicated by a dashed line, conditional (C) cells that are inviable at 12°C but can grow at 24°C are indicated with a thin line, and viable (V) cells that exhibit wild-type growth characteristics under all conditions tested are indicated by a heavy line. The loss of Srb10 increases the viability of CTD truncation mutants, whereas the loss of Srb2 or Srb4 decreases the viability of the CTD mutants.

was replaced with the *srb10-1* or the *srb10Δ1* allele, and the growth phenotypes of these cells were examined. The results, summarized in Figure 1B, demonstrate that the loss of Srb10 function restores full viability to CTD-mutants which exhibited conditional lethal phenotypes in the presence of wild-type Srb10 kinase activity. In addition, the loss of Srb10 function rescues N15 cells, which were inviable with wild-type Srb10. Thus, the loss of this putative CTD kinase increases the viability of cells whose pol II molecules have shortened CTDs. In contrast, the loss of Srb2 or Srb4, both positive acting transcription factors, decreases the viability of these cells (Figure 1B). These results provide strong evidence that Srb10 is a negative regulator of transcription in vivo.

An artificial holoenzyme recruitment assay (Barberis et al., 1995; Farrell et al., 1996; reviewed in Ptashne and Gann, 1997) provides another in vivo test of the hypothesis that Srb10 is a negative regulator. Tethering of a holoenzyme component (such as Gal11, Srb2, etc.) to a sequence specific DNA-binding domain (LexA) is sufficient to activate transcription from a promoter containing the appropriate upstream element, as the tethered holoenzyme component apparently recruits the remaining transcription apparatus to the promoter. If the kinase activity of Srb10 has a negative function in vivo, a mutation which eliminates kinase activity but does not alter the ability of the protein to interact with the holoenzyme should be a better artificial activator than its wild-type counterpart because it would recruit holoenzyme as efficiently as its wild-type counterpart, but would have no inhibitory effect on transcription (Figure 2A). Strains were constructed which contain the LexA DNA binding domain fused with either the wild-type Srb10 sequence or the Srb10(D290A) sequence. The D290A mutation renders Srb10 protein catalytically inactive but fully capable of being incorporated into the

A


RNA Pol II Holoenzyme
Srb10
LexA
Artificial Recruitment & Repression
RNA Pol II Holoenzyme
Srb10 D290A
LexA
Artificial Recruitment

B

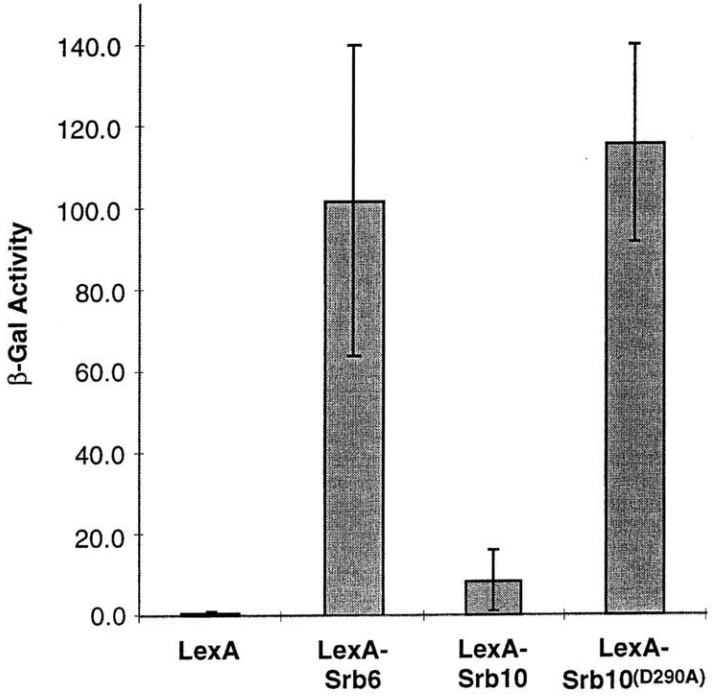

β-Gal Activity
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
LexA
LexA-Srb6
LexA-Srb10
LexA-Srb10(D290A)

Figure 2. Artificial recruitment of holoenzyme with LexA-Srb fusion proteins.

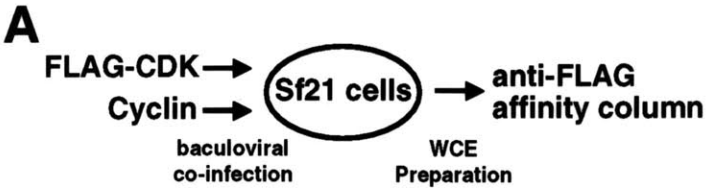
(A) Diagram of the experimental concept. If Srb10 is a negative regulator of the transcription initiation complex, then a LexA-Srb10 fusion protein should recruit the transcription apparatus, yet repress transcription. In contrast, a LexA-Srb10(D290A) fusion protein, in which the kinase is catalyticaly inactive, should recruit the apparatus and produce levels of transcription similar to those observed with Srb proteins which have positive roles in the holoenzyme.

(B) A wild-type strain containing the LexA-lacZ reporter plasmid pSH18-34 was transformed with plasmids expressing LexA alone, or LexA fused to Srb6, Srb10, or Srb10(D290A) as indicated. The specific activity of β-galactosidase is expressed in nmoles of *o*-nitrophenol produced/min/mg of total protein assayed. As predicted by the model in (A), the LexA-Srb10 protein is a very poor artificial activator whereas the LexA-Srb10(D290A) fusion protein is a good activator. The LexA-Srb10(D290A) fusion activates as well as LexA-fusions with Srb proteins which have positive functions; LexA-Srb6 is shown as an example. The Srb10 protein is active in vivo when fused to LexA; the LexA-Srb10 expression plasmid complements all phenotypes associated with a *srb10Δ* strain (data not shown). Western blots of whole cell extracts probed with α-LexA antibodies show that LexA-Srb10, LexA-Srb10(D290A), and LexA-SRB6 are all expressed at similar levels.

holoenzyme (Liao et al., 1995). The results of the experiment (Figure 2B) demonstrate that LexA-Srb10 has substantially less activity in artificial recruitment than LexA-Srb10(D290A). This result supports the model that the Srb10 kinase is a negative regulator of transcription in vivo.

CTD Phosphorylation by Srb10 and Kin28 CDKs

Although two CDKs have been identified in yeast and mammalian holoenzyme preparations (Liao et al., 1995; Maldonado et al., 1996; Pan et al., 1997; Scully et al., 1997), only the TFIIH kinases have been demonstrated to phosphorylate the CTD directly (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992). The Srb10 kinase has been proposed to be a CTD kinase based upon genetic evidence for a interaction with the CTD and evidence that holoenzyme preparations lacking Srb10 activity have substantially reduced CTD kinase activity (Liao et al., 1995). We tested whether purified Srb10 has CTD kinase activity and, if so, how it compares to Kin28 CTD kinase activity. Epitope-tagged recombinant Srb10/Srb11 and Kin28/Ccl1 cyclin-dependent kinases were expressed in a baculovirus expression system and purified in a one-step affinity purification (Figure 3). Catalytically inactive recombinant Srb10(D290A)/Srb11 and Kin28(D147A)/Ccl1 cyclin-dependent kinases were also produced and purified as controls. Both Srb10/Srb11 and Kin28/Ccl1 were found to be capable of phosphorylating recombinant glutathione-S-transferase-CTD (GST-CTD) (Figure 4A). The recombinant kinase-cyclin pairs phosphorylated rGST-CTD and pure yeast RNA polymerase II with similar efficiencies (data not shown). Neither kinase could phosphorylate GST alone, calf thymus histone H1, the other kinase-cyclin pair, or general transcription factors (GTFs) (Figure 4A and data not shown). The activity of Srb10/Srb11 and Kin28/Ccl1 could be directly attributed to the highly purified kinases,


A
FLAG-CDK
Cyclin
Sf21 cells
anti-FLAG
affinity column
baculoviral
co-infection
WCE
Preparation

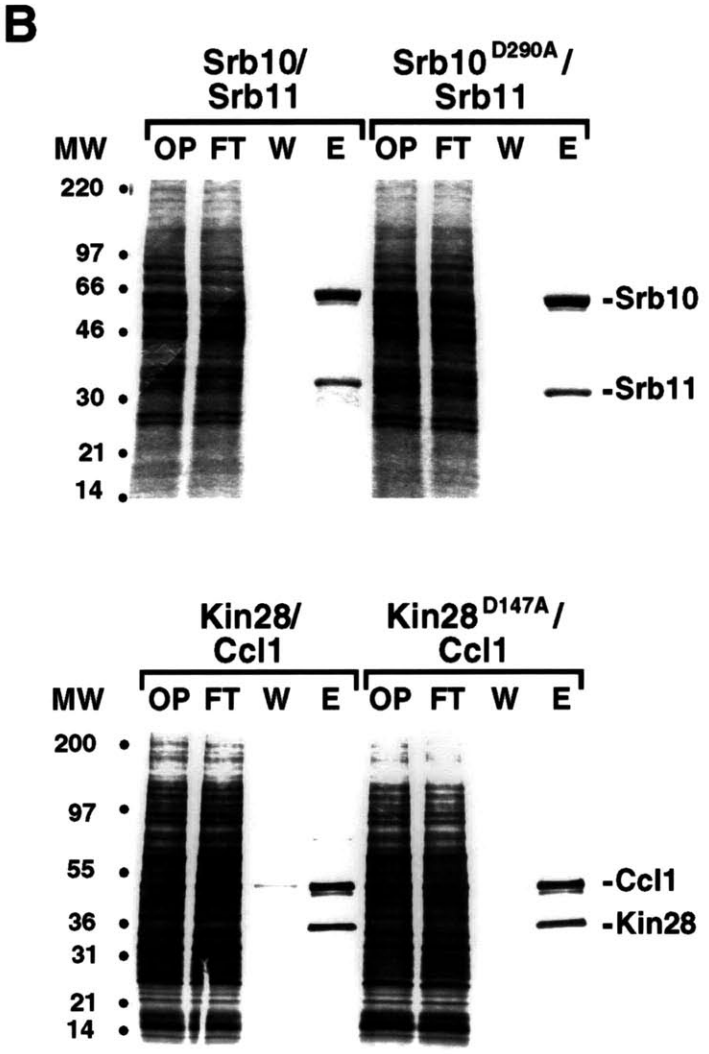
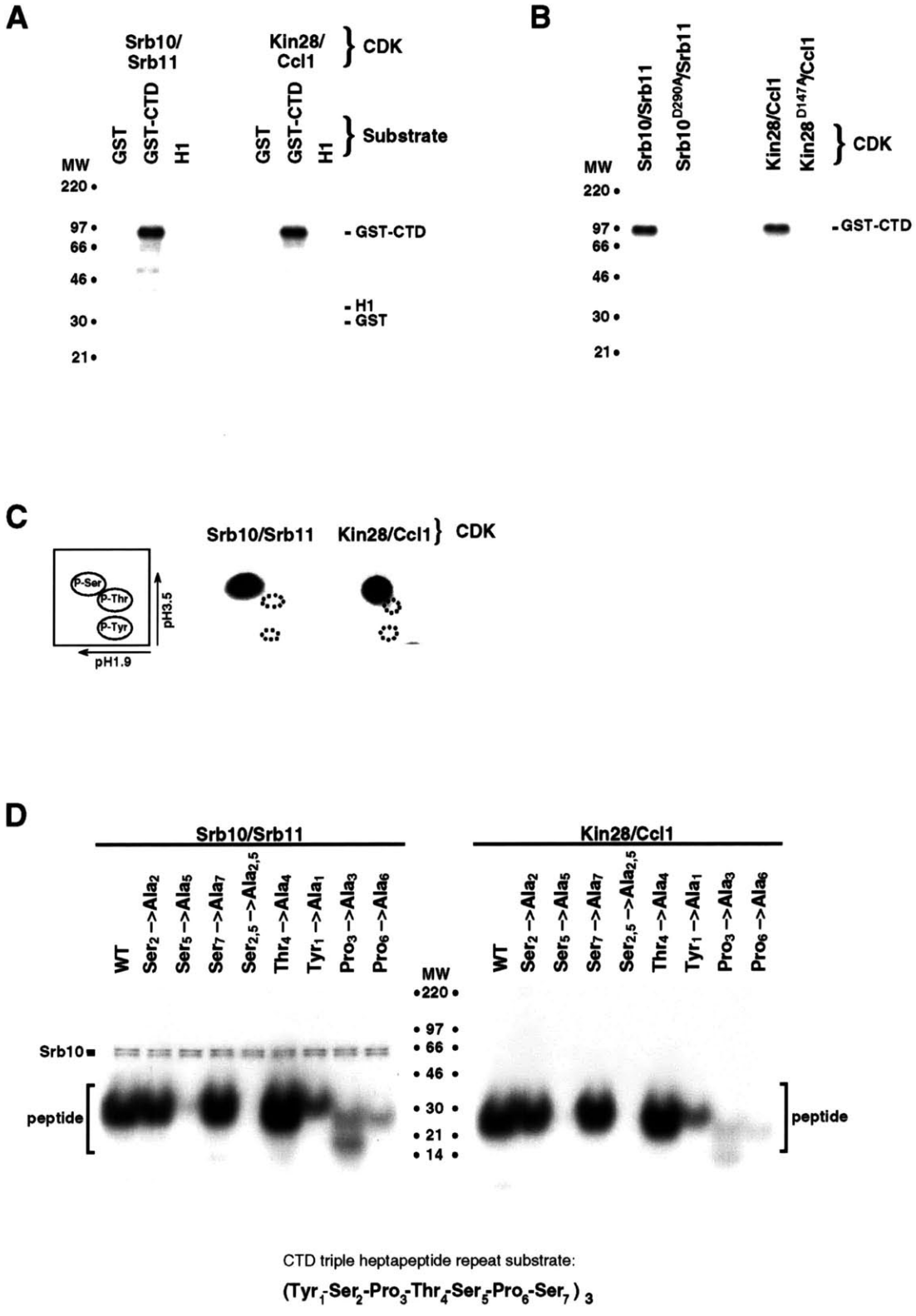

B
Srb10/
Srb11
Srb10 D290A /
Srb11
MW
OP FT W E
OP FT W E
220
97
66
46
30
21
14
-Srb10
-Srb11
Kin28/
Ccl1
Kin28 D147A /
Ccl1
MW
OP FT W E
OP FT W E
200
97
55
36
31
21
14
-Ccl1
-Kin28

Figure 3. Purification of recombinant Srb10/Srb11 and Kin28/Ccl1 cyclin-dependent kinases (CDKs).

(A) Scheme for production and purification of recombinant holoenzyme CDKs from Sf21 cells co-infected with baculovirus encoding a kinase (Srb10 or Kin28) and the corresponding cyclin partner (Srb11 or Ccl1, respectively). FLAG-epitope tagged recombinant Srb10/Srb11 and Kin28/Ccl1 and their inactive mutant derivatives, Srb10^{D290A}/Srb11 and Kin28 ^{D147A}/Ccl1 were purified in a single step from whole cell extracts of baculovirus infected insect cells using an anti-FLAG affinity column.

(B) Purity of recombinant kinase-cyclin pairs. Onput (OP), Flowthrough (FT), Wash (W) and Eluate (E) fractions of the anti-FLAG affinity column were subjected to SDS-PAGE electrophoresis followed by Coomassie (upper panel) or silver staining (lower panel). The identities of the kinase and cyclin subunits and position of the molecular weight markers (MW) in kiloDaltons is shown.



CTD triple heptapeptide repeat substrate:

$(Tyr_1\text{-}Ser_2\text{-}Pro_3\text{-}Thr_4\text{-}Ser_5\text{-}Pro_6\text{-}Ser_7)_3$

Figure 4. Recombinant CDKs are indistinguishable in CTD phosphorylation activity.

(A) Holoenzyme associated CDKs phosphorylate the CTD, but not histone H1 in vitro. Recombinant GST, GST-CTD, or calf thymus H1 substrates were incubated with pure recombinant CDK/cyclin pairs in the presence of γ-^{32}P-ATP, separated by SDS-PAGE and visualized by autoradiography. Label is transferred only to the GST-CTD fusion and not to GST or histone H1, a well studied kinase substrate.

(B) Purified mutant recombinant CDKs exhibit no kinase activity. Wild-type and mutant CDKs were incubated with GST-CTD in the presence of γ-^{32}P-ATP as in panel A. The inactive CDK/cyclin pairs contained a point mutation at a highly conserved aspartate residue critical for catalytic activity. The absence of a labeled product in the mutant CDK/cyclin preparations suggests the observed activity is not due to a contaminating kinase.

(C) Phosphoamino acid analysis of in vitro phosphorylated CTD. Recombinant GST-CTD was incubated with recombinant CDK/cyclin pairs in the presence of γ-^{32}P-ATP. After SDS-PAGE and transfer to a PVDF membrane, the labeled CTD band was cut out and subjected to acid hydrolysis. The phosphoamino acids were separated by two-dimensional thin layer electrophoresis. Amino acid standards were visualized by ninhydrin and their mobilities shown on the left, while the labeled phosphoamino acids were visualized by autoradiography as shown in the middle and right panel. Serine is the primary phosphoacceptor on the GST-CTD substrate for both Srb10/Srb11 and Kin28/Ccl1 kinases.

(D) Holoenzyme CDKs show identical specificity for synthetic CTD peptide substrates. Synthetic peptides consisting of three heptapeptide consensus

repeats, or mutant variations thereof, were used as substrates for recombinant holoenzyme CDKs. The wild-type (WT) heptapeptide consensus sequence as well as the amino acid numbering used in describing various mutants is shown at the bottom of the figure. After SDS-PAGE, the phosphorylated peptides were visualized by autoradiography. The Ser_5 to Ala mutant peptide was unable to serve as a substrate for either CDK, strongly suggesting that Ser_5 is the primary substrate labeled by the CDKs.

since the catalytically inactive CDK mutant kinases were unable to phosphorylate GST-CTD at any level (Figure 4B). These results demonstrate that Srb10 and Kin28 CDKs are both capable of phosphorylating the CTD.

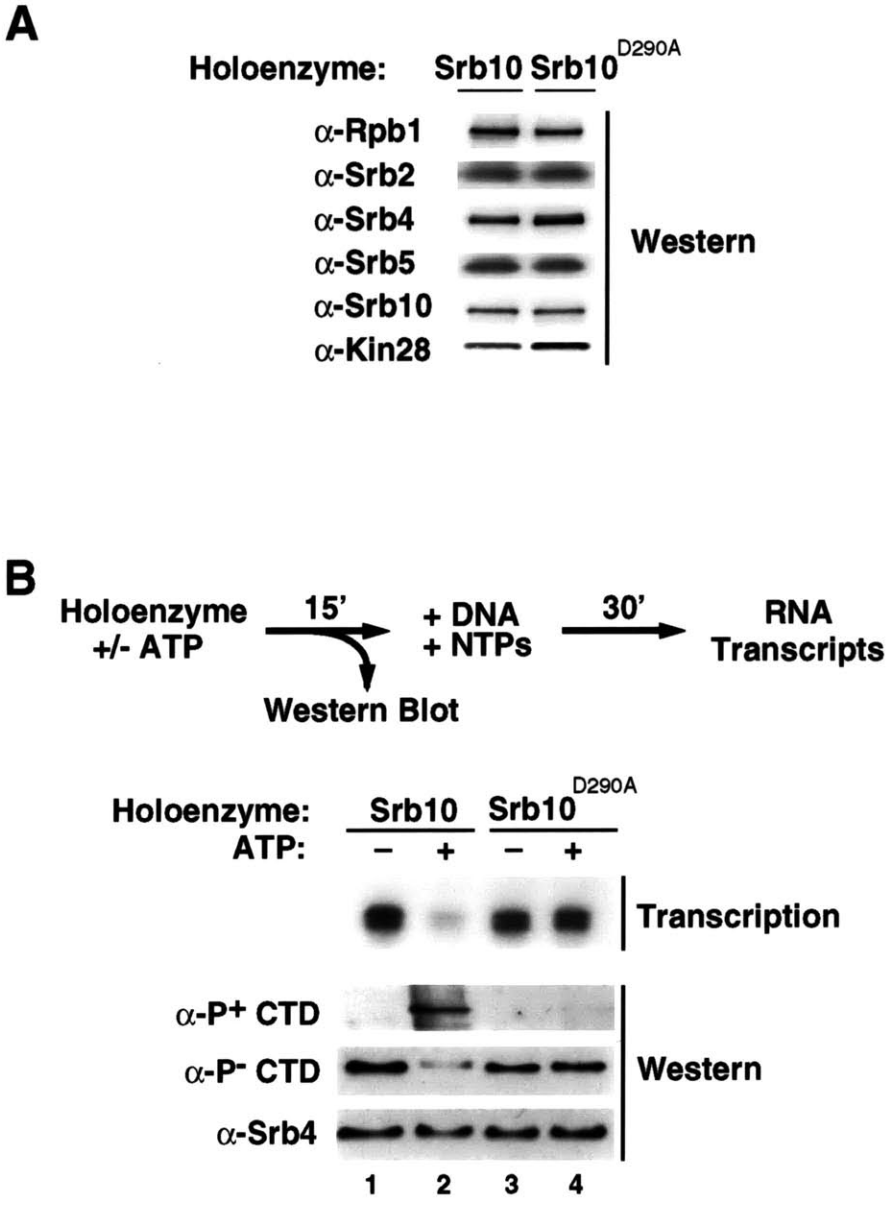
Genetic evidence presented here and elsewhere (Cismowski et al., 1995; Valay et al, 1995) indicate that Kin28 and Srb10 contribute positive and negative functions, respectively, to transcription in vivo. We investigated the possibility that differential phosphorylation of the CTD by the two CDKs might account for their different functions. The amino acid residues of the CTD phosphorylated in vitro by the two CDKs were identified by two dimensional thin layer chromatography. The results demonstrate that both Srb10 and Kin28 phosphorylate serine residues (Figure 4C).

To investigate further the substrate specificity of the two CTD kinases, a battery of synthetic peptides were used as substrates to determine which amino acid residues in the heptapeptide consensus repeat are critical for CTD phosphorylation (Figure 4D). The results show that the activities of Srb10 and Kin28 on these peptide substrates are indistinguishable. Substitution of Ser_2, Thr_4 or Ser_7 with alanine did not significantly affect the ability of the peptide to act as a substrate for either kinase. In contrast, substitution of Ser_5 with alanine led to a dramatic loss in peptide phosphorylation, suggesting that Ser_5 is the principal phosphoacceptor in the heptapeptide repeat. Substitutions of Tyr_1, Pro_3 or Pro_6 reduced phosphorylation of the synthetic peptides, probably due to the effects such alterations have on their structure. These results indicate that Srb10 and Kin28 CDKs are indistinguishable in substrate specificity and activity in these CTD phosphorylation assays.

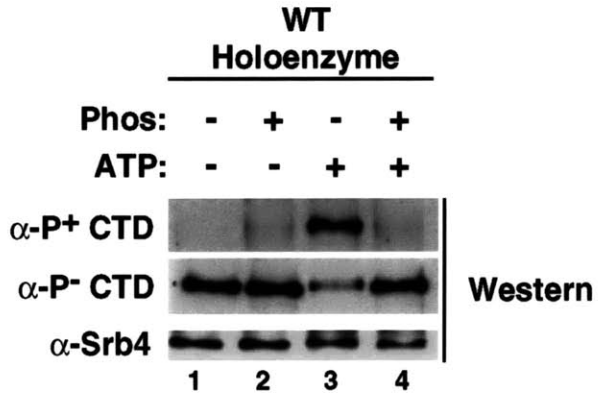
Srb10 Dependent Inhibition of Transcription In Vitro

The existence of two CDKs in the holoenzyme with similar biochemical specificity and activity, yet opposite in vivo function, led us to entertain the possibility that the timing of CTD phosphorylation in the holoenzyme could determine whether the event had a negative or a positive consequence. Although both kinases are capable of CTD phosphorylation as purified, recombinant kinase-cyclin pairs, it is possible that they can function only at certain times when assembled in the holoenzyme. We therefore considered a temporal model for the action of these kinases, in which Srb10 is uniquely capable of CTD phosphorylation prior to initiation complex formation by the holoenzyme, thereby repressing transcription. In contrast, Kin28, when assembled into the holoenzyme, is capable of CTD phosphorylation only after preinitiation complex formation, when such activity would not interfere with transcription.

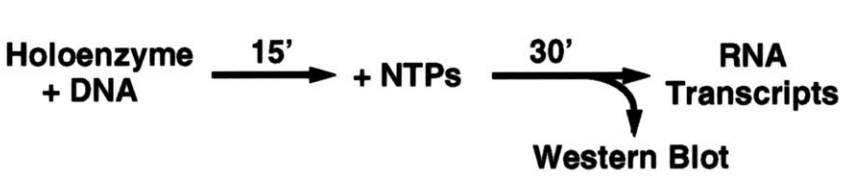
This temporal model predicts that holoenzymes with catalytically active Srb10 should be transcriptionally inhibited when the kinase functions prior to association with template DNA. RNA polymerase II holoenzymes containing Kin28 and either wild-type Srb10 or catalytically inactive Srb10(D290A) were purified in parallel and assayed for kinase and transcriptional activities (Figure 5). The two purified holoenzymes contained comparable amounts of Rpb1, Srb2, Srb4, Srb5, Srb10 and Kin28 (Figure 5A). To determine whether Srb10 kinase activity can inhibit transcription by acting prior to PIC formation, we performed an in vitro transcription experiment in which both wild-type and mutant Srb10 containing holoenzymes were preincubated with ATP prior to addition of template DNA, additional GTFs and nucleoside triphosphates (NTPs) (Figure 5B). Preincubation with ATP produced a significant inhibition of transcription with the wild-type


A
Holoenzyme:
Srb10
Srb10D290A
α-Rpb1
α-Srb2
α-Srb4
α-Srb5
α-Srb10
α-Kin28
Western
B
Holoenzyme
+/- ATP
15'
+ DNA
+ NTPs
30'
RNA
Transcripts
Western Blot
Holoenzyme:
Srb10
Srb10D290A
ATP:
– + – +
Transcription
α-P+ CTD
α-P- CTD
α-Srb4
Western
1 2 3 4

C


WT
Holoenzyme
Phos: - + - +
ATP: - - + +
α-P+ CTD
α-P- CTD
α-Srb4
Western
1 2 3 4

D


Holoenzyme
+ DNA
15'
+ NTPs
30'
RNA
Transcripts
Western Blot

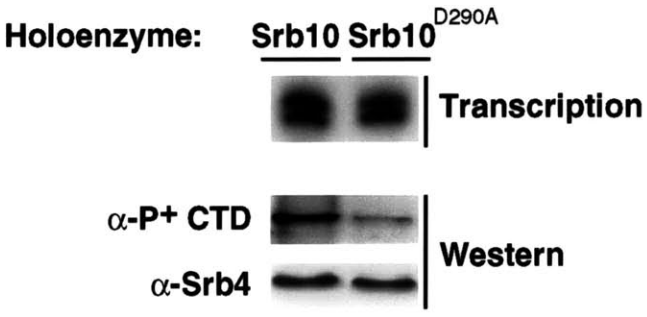

Holoenzyme: Srb10 Srb10D290A
Transcription
α-P+ CTD
α-Srb4
Western

Figure 5. Catalytically active Srb10 can inhibit transcription by RNA polymerase II holoenzyme in vitro.

(A) Purified holoenzymes contain similar amounts of Rpb1, Srb2, Srb4, Srb5 and the kinases Srb10 and Kin28. Wild-type and Srb10 (D290A) mutant holoenzymes were purified in parallel and analyzed by western blot. Monoclonal antibodies specific to unphosphorylated CTD (α-P^- CTD; 8WG16) were used to detect Rpb1.

(B) Holoenzymes containing either wild-type Srb10 kinase (lanes 1-2) or catalytically inactive Srb10(D290A) kinase (lanes 3-4) were preincubated with or without ATP prior to PIC formation and analyzed as diagrammed. Only holoenzymes containing functional Srb10 are inhibited for transcription when kinases are allowed to function before PIC formation (compare lanes 2 and 4). In vitro transcription is performed in the presence of α-^{32}P-UTP resulting in internal labeling of a 400 nucleotide transcript derived from a G-less cassette driven by the *CYC1* promoter. The state of CTD phosphorylation after ATP preincubation was monitored by western analysis using monoclonal antibodies specific to unphosphorylated (α-P^- CTD; 8WG16) or Ser-phosphorylated CTD (α-P^+ CTD; H5). CTD phosphorylation occurs during preincubation only in holoenzymes containing functional Srb10 kinase (compare lanes 2 and 4). Control experiments indicate that the Srb10 CTD kinase activity is largely restricted to the holoenzyme in which it resides (data not shown). Srb4 is probed as a loading control.

(C) Changes in mAb reactivity to RPB1 is due to phosphorylation. Holoenzyme containing functional Srb10 was incubated with ATP (lanes 1-2). The signal obtained when the phosphorylated preparation is probed with mAb 8WG16 (α-P^- CTD) is reduced and the mobility of RPB1 is retarded (lane

3). The same preparation then reacts with the phospho-serine CTD specific H5 mAb (α-P^+ CTD). Subsequent treatment of the sample with protein phosphatase eliminates the H5 reactive band and restores 8WG16 reactivity and mobility to that seen prior to ATP incubation (compare lanes 1 and 4). This indicates the mAbs are accurately probing the phosphorylation state of the CTD.

(D) Srb10 kinase does not affect transcription post PIC formation. Holoenzymes containing either wild-type Srb10 kinase (WT) or catalytically inactive Srb10(D290A) kinase, preincubated with template DNA and GTFs prior to addition of NTPs, exhibit identical transcriptional activity (top panel). The state of CTD phosphorylation after transcription was monitored with the phospho-serine specific H5 mAb (α-P^+ CTD). Both wild-type and srb10(D290A) containing holoenzymes are able to phosphorylate the CTD. Srb4 is probed as a loading control (bottom panel).

holoenzyme, but not with the holoenzyme lacking Srb10 catalytic activity (Figure 5B; compare lanes 2 and 4). These data show that transcription by RNA polymerase II holoenzyme is inhibited when the Srb10 kinase is allowed to function prior to PIC formation.

pol II CTD phosphorylation was monitored in the holoenzymes that were subjected to preincubation with and without ATP (Figure 5B). CTD phosphorylation occurred only in holoenzymes containing catalytically active Srb10 (Figure 5B, compare lanes 2 and 4). Kin28 is apparently not active in the holoenzyme prior to PIC formation, because the Srb10 mutant holoenzyme exhibits essentially no CTD phosphorylation activity during the preincubation. The Srb10-dependent phosphorylation of the CTD was highly efficient; most of the pol II molecules in the wild-type holoenzyme became phosphorylated in this reaction (Figure 5B, compare lanes 1 and 2). A control experiment confirmed the specificity of the antibodies used to detect unphosphorylated and phosphorylated CTDs (Figure 5C). Thus, Srb10-dependent CTD phosphorylation is coincident with repression of transcription, a result consistent with previous evidence that formation of a functional preinitiation complex is impaired if the pol II molecules contain phosphorylated CTDs (Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994).

The temporal model also predicts that RNA polymerase II holoenzymes which are allowed to bind template DNA prior to addition of nucleoside triphosphates should not be transcriptionally inhibited by Srb10 activity. The experiment shown in Figure 5D shows that the wild-type and Srb10 mutant holoenzymes are in fact equally active in transcription under these conditions, confirming the prediction. The state of CTD phosphorylation was also assayed after the transcription reaction, revealing

that CTD phosphorylation occurs in RNA polymerase II molecules from holoenzymes with or without functional Srb10, albeit the levels are three-fold less in holoenzymes lacking catalytically active Srb10 kinase. These results indicate that Srb10-independent CTD phosphorylation occurs during the in vitro transcription reaction, as would be expected from the action of Kin28.

We and others have found that Srb10 is critical for regulation of a subset of genes in yeast cells, including those involved in cell type specificity (Wahi and Johnson, 1995), meiosis (Surosky et al., 1994), and sugar utilization (Liao et al., 1995; Kuchin et al., 1995). Srb10 is not a general repressor of transcription, as a variety of genes are expressed normally in Srb10 mutant cells (Surosky et al., 1994, Liao et al., 1995), and the levels of active holoenzyme are similar in wild-type and Srb10 mutant cells (S.S.K. and R.Y., unpublished). The observation that Srb10 is not a general repressor of protein-coding genes suggests that in living cells, where there is abundant ATP, Srb10 activity in holoenzymes must be inhibited in order to prevent constitutive inactivation of the general transcription initiation apparatus. To test this idea, we produced nuclear extracts from wild-type and Srb10(D290A) mutant strains and investigated whether the wild-type extract showed an ATP-dependent inhibition of transcription prior to PIC formation. We previously showed that the transcriptional activity in these extracts is entirely dependent on components of the Srb/mediator complex (Koleske et al., 1992; Thompson et al., 1993), which are tightly associated with pol II holoenzymes (Koleske and Young, 1994). The results, shown in Figure 6, demonstrate that transcription in nuclear extracts is not inhibited by preincubation with ATP, suggesting that these extracts contain an Srb10 inhibitory activity which is lost during holoenzyme purification.

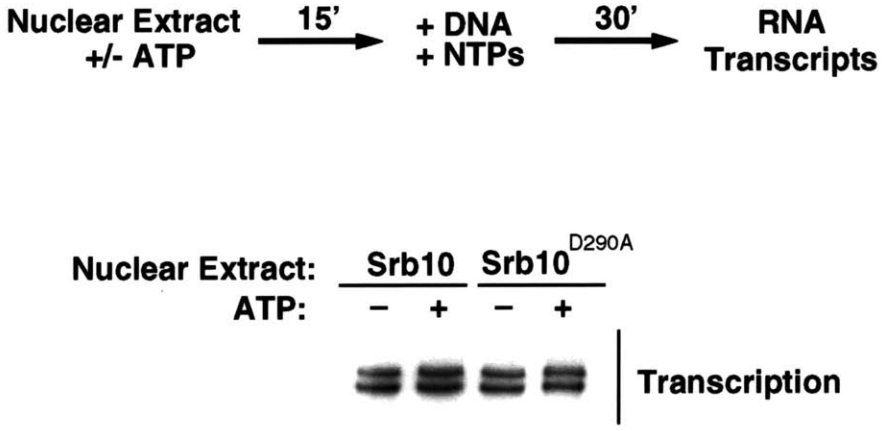

Nuclear Extract
+/- ATP
15'
+ DNA
+ NTPs
30'
RNA
Transcripts
Nuclear Extract:
Srb10
Srb10D290A
ATP:
– + – +
Transcription

Figure 6. Nuclear extracts show no Srb10-dependent inhibition of transcription by RNA polymerase II holoenzyme.

Nuclear extracts from cells containing either wild-type Srb10 kinase or catalytically inactive Srb10(D290A) kinase were preincubated with or without ATP prior to PIC formation, as diagrammed (the experimental design is identical to that in Figure 5B). In vitro transcription is performed in the presence of α-^{32}P-UTP resulting in internal labeling of a 400 nucleotide transcript derived from a G-less cassette driven by the *CYC1* promoter. No inhibition of transcription was observed after ATP preincubation with either extract.

Discussion

Yeast and mammalian RNA polymerase II holoenzymes have been described which contain two cyclin-dependent kinases. Previous studies established that Kin28 is a CTD kinase with a positive role in transcription, that of producing a phosphorylated form of the enzyme which is associated with active elongation. Genetic and biochemical evidence described here reveals that the Srb10 kinase is a CTD kinase with a negative role in transcription. Srb10 is uniquely capable of phosphorylating the CTD in purified holoenzymes prior to template binding, and this phosphorylation inhibits subsequent transcription by the holoenzyme. Srb10 does not appear to inhibit transcription after formation of a stable preinitiation complex. Thus, the transcription initiation apparatus can be regulated positively or negatively via modification of the CTD, depending on the timing of the phosphorylation event (Figure 7).

In arriving at this temporal model, we first examined the two most obvious models which could account for differential regulation by the two CDKs. It was possible that the Srb10 and Kin28 kinases could act on other substrates in the transcription initiation apparatus, but we did not detect phosphorylation of general transcription factors or histones, nor did we find that either kinase could phosphorylate the other. It was also possible that the two kinases phosphorylated different residues on the CTD, but our experiments indicate that they exhibit very similar substrate recognition and modification behaviors. The one clear difference in behavior was the unique ability of Srb10 to phosphorylate the pol II CTD prior to initiation complex formation when a component of the holoenzyme. We conclude that the temporal regulation of transcription by CDKs is an instance where a specific

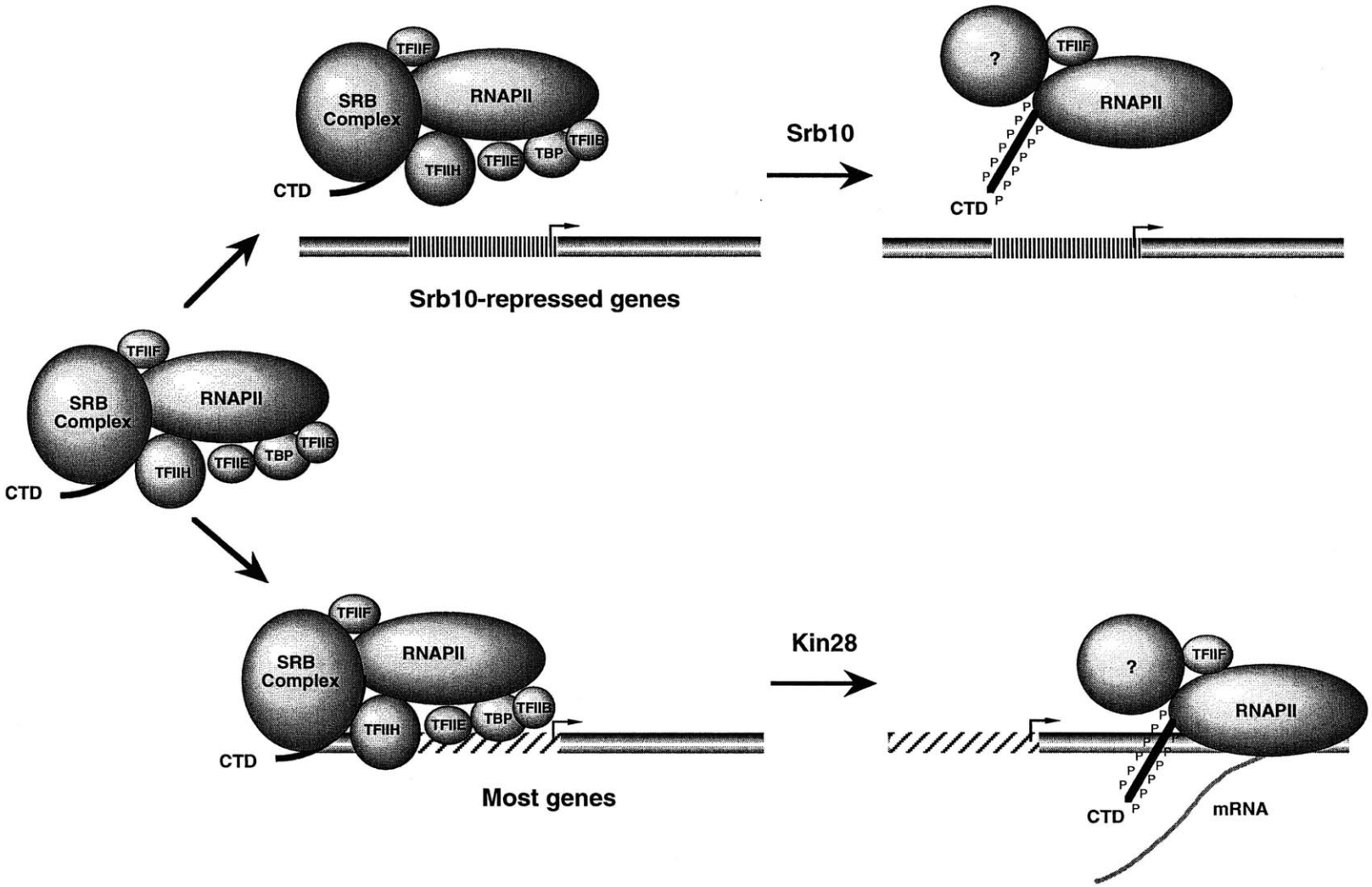

TFIIF
SRB Complex
RNAPII
TFIIB
TBP
TFIIE
TFIIH
CTD
Srb10
?
Srb10-repressed genes
Kin28
Most genes
mRNA

Figure 7. Model for temporal function of holoenzyme CDKs in transcription initiation.

The two holoenzyme cyclin-dependent kinases are CTD kinases which function at different times. Srb10-dependent CTD phosphorylation can occur prior to stable preinitiation complex (PIC) formation at a subset of promoters, presumably activated by factors associated with these promoters, with consequent inhibition of transcription. The Kin28 kinase functions after stable PIC formation at promoters generally, producing the hyperphosphorylated form of pol II associated with productive elongation.

phosphorylation event, carried out at different times, can produce opposite regulatory effects in the cell.

Negative Regulation by Srb10 in vivo

Progressive truncation of the RNA polymerase II CTD produces cells with increasingly severe growth phenotypes (Nonet et al., 1987). The greater the truncation of the CTD, the larger the number of genes affected, accounting for the increasingly severe growth phenotypes (Scafe et al., 1990). The *SRB* genes were originally identified as suppressors of defects due to CTD truncation. A subset of these genes, for example those encoding Srb4 and Srb6, are essential for expression of most protein-coding genes (Thompson and Young, 1995). In contrast, Srb8, Srb9, Srb10 and Srb11 are not essential for expression of protein coding genes generally, but are critical for normal regulation of a subset of genes (Surosky et al., 1994; Kuchin et al., 1995; Liao et al., 1995; Wahi and Johnson, 1995).

Genetic and biochemical evidence indicates that Srb2, Srb4, Srb5 and Srb6 all contribute positively to holoenzyme function (Koleske and Young, 1995; Hengartner et al., 1995). For example, mutations which reduce the function of the Srb2, Srb4, Srb5, Srb6 or Srb7 proteins cause reduced cell viability, and this is exacerbated in cells with CTD truncation mutations (Koleske at al., 1992; Thompson et al., 1993; Hengartner et al., 1995). In contrast, mutations which eliminate Srb10 or Srb11 function actually restore viability to cells with CTD truncation mutations. This, and additional genetic evidence, indicates that Srb10 is a negative regulator of transcription. Highly repressed genes such as *SPO13*, *GAL1*, *SUC2*, *PHO5*, and *MFA2* are derepressed in strains lacking Srb10 activity (Kuchin et al., 1995; Liao et al., 1995; Wahi and Johnson, 1995; S.-M. L. and R. Y., unpublished). Since Srb10

represses only a subset of genes, there must be a mechanism to activate the kinase solely at these genes. We suggest that promoter specific factors repress transcription at these genes by stimulating an otherwise quiescent Srb10 prior to stable association of the holoenzyme with promoter DNA (Figure 7).

Phosphorylation of CTD by Srb10 and Kin28

Previous studies demonstrated that the kinase activity of purified TFIIH could phosphorylate the CTD (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992). Previous reports also indicated that Srb10 is involved in CTD phosphorylation, as the SRB10 gene was identified in genetic selection for suppressors of a CTD truncation defect, and holoenzymes with catalytically inactive Srb10 protein have markedly reduced CTD phosphorylation activity (Liao et al., 1995). The use of highly purified recombinant forms of the two yeast CDKs allowed us to demonstrate that they phosphorylate the CTD, to identify the residues of the heptapeptide which are modified, and to compare and contrast their activities. Srb10/Srb11 and Kin28/Ccl1 phosphorylate the CTD with similar efficiency and are indistinguishable in their specificity towards recombinant full length CTD or synthetic heptapeptide repeats, down to the specific residue they modify, Ser_5. These results suggest that the positive and negative regulatory functions of the two CDKs are not due to differences in substrate specificity.

Temporal Regulation via CTD Phosphorylation

The form of pol II found in RNA polymerase II holoenzyme preparations lacks phosphate on its CTD (Koleske and Young, 1994; Kim et al.. 1994). Several experimental observations led us to postulate that the timing of CTD phosphorylation in the holoenzyme determines whether the event has a

negative or a positive consequence. The two holoenzyme CDKs have very similar biochemical specificity and activity, yet opposite in vivo function. In an assay designed to measure transcriptional activity subsequent to template binding, wild-type and Srb10(D290) mutant holoenzymes are indistinguishable. However, previous studies have shown that the phosphorylation state of the CTD affects PIC formation; formation of such a complex is impaired if the pol II molecules contain phosphorylated CTDs (Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994). If one of the holoenzyme kinases phosphorylates the CTD prior to template association, it could inhibit subsequent transcription.

We carried out an experiment designed to identify an effect of kinase activity in the holoenzyme prior to pre-initiation complex (PIC) formation on template DNA. This experiment revealed that CTD phosphorylation and transcription inhibition does occur when holoenzymes are provided with ATP prior to template association, but only if Srb10 is catalytically active. In contrast, Kin28 kinase activity in these holoenzymes is not evident prior to template association, but is evident later in the transcription reaction. Thus, the positive and negative roles of the two kinases can be attributed to the time at which they act during the process of transcription initiation. In this model, Srb10-dependent CTD phosphorylation prior to stable PIC formation at specific promoters inhibits transcription initiation, accounting for the negative regulatory activity observed for Srb10 in vivo. In contrast, Kin28 phosphorylation of the pol II CTD subsequent to PIC formation has a positive role, that of producing the phosphorylated RNA polymerase II molecule which recruits mRNA capping enzyme and which is associated with efficient elongation of the nascent transcript (Cho et al., 1997; McCracken et al., 1997a; McCracken et al., 1997b; Yue et al., 1997).

Regulation of CDKs

Cyclin-dependent kinases were first described as cell cycle regulators. These kinases are themselves regulated in a temporal fashion through pairing with various cyclins, through phosphorylation events which can have positive or negative effects, and through interactions with CDK inhibitors. The two holoenzyme CDKs are paired with cyclin molecules, but are not typically activated, since holoenzyme preparations contain RNA polymerase II molecules with unphosphorylated CTDs. Furthermore, our experiments suggest that Srb10 kinase activity is inhibited in nuclear extracts. The future identification of this CDK regulator should reveal important new insights into the molecular mechanisms involved in regulation of cell type specificity, meiosis, sugar utilization and other important cellular processes under the control of Srb10.

Experimental Procedures

Genetic Analysis

To examine the ability of various *SRB10* alleles to suppress the conditional phenotypes caused by a truncated CTD (*rpb1Δ104*), yeast strains Z768 and Z769 (Table 1) were transformed with plasmids containing *SRB10* (pRY2973), *srb10-1* (pRY7091), *srb10Δ1* (pRY2966), and *srb10-3* (pRY7096). Growth conditions were assayed as described (Nonet et al., 1987).

Growth phenotype analysis of yeast cells containing CTD truncation mutations was performed as described (Nonet et al., 1987). The various *SRB10* background strains used were N418 (*SRB10*), Z741 (*srb10Δ1*) and Z735 (*srb10-1*) (Table 1). The viability of cells containing CTD truncations in those backgrounds were assayed by plasmid shuffle, and surviving strains were tested for cold sensitivity.

In Vivo Recruitment Assays

β-galactosidase assays were performed as described (Rose and Botstein, 1983). The strains are derivatives of Z719 transformed with the reporter pSH18-34 and the appropriate LexA fusion. To make the LexA fusions, *SRB10*, *srb10-3*, and *SRB6* open reading frames were subcloned into the LexA fusion plasmid pEG202 (Ausubel et al., 1997).

Recombinant CDK/cyclin Production and Purification from Insect Cells

Recombinant CDK/cyclin pairs were produced using a baculovirus expression system. For expression of CDKs, genes for Srb10 and Kin28 were amplified by polymerase chain reactions (PCR) and cloned into baculoviral transfer vectors pSK277 or pSK278 (Koh et al., 1997) to produce recombinants with FLAG

epitope-tag at their N-termini. For expression of cyclins, genes for Srb11 and Ccl1 were amplified by PCR and cloned into baculoviral transfer vectors pBacPAK8 or pBacPAK9 (Clontech). PCRs were performed with Vent DNA polymerase (New England Biolabs). All the PCR clones were verified by DNA sequencing. Mutant CDK clones, Srb10(D290A) and Kin28(D147A), were produced by site-directed in vitro mutagenesis (Kunkel et al., 1987) using oligonucleotides CAAAACCTAAAGCACCAATTTT and CCTTGCTAGACCGAAAGCTGCTACTTTTATCTG, respectively. All mutations were verified by DNA sequencing.

Recombinant baculoviruses were generated from the recombinant transfer plasmids containing CDKs or cyclins by cotransfection of the plasmids with wild-type viral DNA as recommended by the manufacturer (Clontech). *Spodoptera frugiperda* (*Sf21*) cells were coinfected with recombinant baculoviruses expressing CDKs and their cyclin partners at a multiplicity of infection (m.o.i.) of 5-10. The cells from 200 mL of culture (approx. 3×10^8) were collected 60-72 hr post-infection and lysed by sonication. The lysates were then clarified by centrifuging for 3 hr at 100,000 x g yielding 20-40 mg of total protein in 10 mL. CDK/cyclin pairs were purified from the lysates as described (Koh et al., 1997) using 1 mL of anti-FLAG M2 affinity gel and 73 μg/mL of FLAG peptide in the elution buffer. Typical yields were 0.2-0.4 % of total protein from cell lysate.

Kinase Assay

Kinase assays were performed using 1 μg of protein substrate (GST, recombinant GST-CTD, or calf thymus histone H1) or 15 μg of synthetic CTD peptide substrate with 100 ng of pure recombinant CDK/cyclin pairs in 15 μl reaction containing 20 mM Hepes-KOH, pH 7.3, 10% glycerol, 2.5 mM EGTA,

15 mM magnesium acetate, 1 mM DTT, 100 mM potassium acetate, 200 μM ATP, 10 μCi [γ-^{32}P] ATP (NEN, 6000 Ci/mmol, 10 mCi/ml), a mixture of phosphatase inhibitors (1 mM NaN_3, 1 mM NaF, 0.4 mM $NaVO_3$, 0.4 mM Na_3VO_4), a cocktail of protease inhibitors (0.5 mM PMSF, 1 mM benzamidine, 1 μM pepstatin, 0.3 μM leupeptin and 1 μg/ml chymostatin) and 0.5 mg/ml of acetylated BSA. Reactions were assembled on ice and initiated with the addition of ATP. After 60 min at 25°C , the reactions were terminated by adding 15 μl of Stop Buffer (2X SDS-PAGE loading buffer supplemented with 100 mM Tris-HCl, pH 6.8, and 40 mM EDTA) and then resolved by 4-20% acrylamide gradient SDS-PAGE. The dried gels were exposed directly to autoradiographic films.

Kinase substrates GST and GST-CTD were purified from bacteria as described (Thompson et al., 1993) and purified calf thymus histone H1, purchased (Boehringer Mannheim). Triple CTD heptapeptide consensus repeats were synthesized (Research Genetics) and were provided in the form H-YSPTSPSYSPTSPSYSPTSPS-Amide. CTD peptide variants where one or more amino acids of the consensus sequence have been systematically replaced by alanine were also synthesized by Research Genetics.

Phosphoamino Acid Analysis

Using GST-CTD as a substrate, a kinase reaction was performed. After SDS-PAGE, the samples were transferred to a PVDF membrane and the labelled phosphorylated GST-CTD band, localized and cut out after a short film exposure. Two-dimensional electrophoretic analysis of phosphoamino acid content was performed subsequent to acid hydrolysis as described in Coligan et al. (1997). The phospholabelled phosphoamino acids were visualized by autoradiography.

Nuclear Extract Transcription

Nuclear extracts from Z719 and Z690 were prepared according to Lue et al. (1991) with the modifications described by Liao et al. (1991), yielding a final protein concentration of 85 and 75 mg/ml, respectively. In vitro transcription was carried out essentially as described (Liao et al., 1991). Each reaction contained 90 μg of Z719 protein or 120 μg of Z690 protein with 250 ng of template.

Transcription and Western Blot Analysis

Holoenzyme was purified according to Liao et al. (1995). In vitro transcription reactions were performed essentially as described (Gadbois et al., 1997) with the following modifications. Preincubations (19μl total) contained all reaction components except TBP, TFIIE, TFIIB, nucleotides and DNA template; ATP containing reactions were brought to a final ATP concentration of 1mM with 100 mM stock (Pharmacia). After a fifteen minute preincubation at room temperature, GTFs (3 μl) and NTP mix (4 μl; 5 mM ATP, CTP, 0.156 mM UTP, 0.25 mM 3'-O-Me GTP and 10 μCi [α-^{32}P] UTP 3000 Ci/mmole) containing 100ng DNA template (pGALΔ) were added for a final reaction volume of 26 μl. After allowing transcription to proceed for 30 minutes at room temperature, reactions were stopped by addition of 125 μl stop buffer (10 mM Tris-HCl, pH 7.5, 20 mM EDTA, 2 M ammonium acetate, and 10 μg/ml glycogen) and 150 μl isopropanol. Samples were placed on dry ice for 10 min., microcentrifuged at 14K RPM for 10 min., pellets resuspended in 6 μl formamide loading dyes and electrophoresed on a 4% Urea-containing denaturing polyacrylamide gel. Gels were dried and exposed to Kodak X-AR film at -80°C with an intensifying screen.

Samples for western blot analysis were fractionated on 5% SDS-PAGE gels and transferred according to standard procedures. 8WG16 monoclonal antibody (Babco) and Srb4 rabbit anti-serum were used at 1:1000, H5 monoclonal antibody (Babco) was used at 1:250. HRP-conjugated anti-mouse (Pierce) and anti-rabbit (Amersham) secondary antibodies were used at 1:2000. Detection was performed by ECL according to the manufacturers directions (Amersham).

Table 1. Yeast Strains

Strain	Alias	Genotype	Reference
Z768	SLY67	*Mata ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 srb10Δ1::hisG* [L14 (*LEU2 CEN RPB1*)]	This study
Z769	SLY69	*Mata ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 srb10Δ1::hisG* [C6 (*LEU2 CEN rpb1Δ104*)]	This study
N418		*Mata ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 SRB10* [pRP112 (*URA3 CEN RPB1*)]	Nonet et al., 1989
Z741	SLY37	*Mata ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 srb10Δ1::hisG* [pRP112 (*URA3 CEN RPB1*)]	This study
Z735	SLY26	*Mata ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 srb10-1::hisG* [pRP112 (*URA3 CEN RPB1*)]	This study
Z687	SLY7	*Mata ura3-52 his3Δ200 leu2-3,-112 RPB1 srb10Δ1::hisG*	Liao et al., 1995
Z690	SLY96	*Mata ura3-52 his3Δ200 leu2-3,-112 RPB1 srb10-3::hisG*	Liao et al., 1995
Z719	SLY3	*Mata ura3-52 his3Δ200 leu2-3,-112 RPB1 SRB10*	Liao et al., 1995

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Chapter 4

Genome-wide survey of genes regulated by transcriptional cyclin-dependent kinases *SRB10* and *KIN28*

Summary

Eukaryotic transcription of protein encoding genes is initiated by the regulated assembly of an RNA polymerase II preinitiation complex (PIC) at the promoter DNA. This PIC, made up of a variety of protein complexes such as RNA polymerase II holoenzyme, general transcription factors and chromatin modifying complexes, contains two cyclin-dependent kinases (CDKs). Studies using*S. cerevisiae* have led to a model wherein the two CDKs, Srb10 and Kin28, both phosphorylate the RNA polymerase II carboxy-terminal domain (CTD) at different times in the PIC formation to act as a negative and positive regulator of transcription, respectively. While transcription of most protein-coding genes seems to require the function of Kin28, Srb10 has been proposed to negatively regulate only a subset of genes. Genome-wide expression analysis was used to identify the genes whose transcription depends on the functions of the RNA polymerase II core enzyme and of the two cyclin-dependent CTD kinases in yeast. Kin28 function was found to be generally required throughout the genome, while Srb10 was found to function negatively at a small subset of genes. Analysis of this subset of genes revealed a novel mechanism for coordinate regulation of specific sets of genes when cells encounter limiting nutrients. It also allowed us to uncover a negative role for Srb10 in a yeast developmental pathway and suggests that the ultimate targets of signal transduction pathways can be identified within the initiation apparatus.

Introduction

Much of biological regulation occurs at the level of transcription initiation. Genes contain promoter sequences which are bound by transcriptional activators and repressors (Struhl, 1995; Ptashne and Gann, 1997). Activators recruit the transcription initiation machinery, which for protein-coding genes consists of RNA polymerase II and at least 50 additional components (Orphanides et al., 1996; Roeder, 1996; Greenblatt, 1997; Hampsey, 1998; Myer and Young, 1998). The transcription initiation machinery includes factors which bind to DNA, cyclin-dependent kinases which regulate polymerase activity, and acetylases and other enzymes which modify chromatin (Burley and Roeder, 1996; Kingston et al., 1996; Roth and Allis, 1996; Steger and Workman, 1996; Tsukiyama and Wu, 1997; Hengartner et al., 1998; Struhl, 1998).

Our understanding of eukaryotic gene expression remains limited in several ways. The complete set of transcriptional regulators has yet to be identified. How these regulators interact with and regulate components of the transcriptional machinery is not yet clear. The functions of just a fraction of the components of the transcriptional machinery are understood, and then only with respect to a small set of genes. Cells must adjust genome expression to accommodate changes in their environment and in their programs of growth control and development, but precisely how coordinate remodeling of genome expression is accomplished for signal transduction pathways or for the cell cycle clock has yet to be learned.

Genome-wide expression monitoring has recently become feasible with the description of complete genome sequences and through the development of cDNA and high-density oligonucleotide array technology (Lockhart et al,

1996; Chee et al., 1996; DeRisi et al., 1997; Lashkari et al., 1997; Wodicka et al., 1997). Expression profiling has been used to examine differences in gene expression when yeast are grown in various media (Wodicka et al., 1997) and has revealed how yeast genome expression is remodeled during the metabolic shift from fermentation to respiration (DeRisi et al., 1997), during the cell cycle (Cho et al., 1998), and during sporulation (Chu et al., 1998). Expression profiling is also being used to improve our understanding of various aspects of human biology and disease (DeRisi et al., 1996; Schena et al., 1996), and to facilitate drug development (Gray et al., 1998). The data generated with genome-wide expression monitoring technology describes the level of each mRNA species in a population, but this data alone does not always produce significant new biological insights. Our knowledge of genome-wide transcriptional regulation is incomplete, making it difficult to understand how such genome-wide expression signatures transpire.

We are exploring the ability of genome-wide expression analysis to provide insights into the transcriptional regulatory functions of the two yeast cyclin-dependent kinases (CDK) found in the transcription preinitiation complex, Kin28 and Srb10. Since those CDKs have been suggested to play an important role in regulation of gene expression, it becomes important to determine the extent to which each gene in the genome depends on their function. We describe here the mRNA population of yeast cells and the expression requirement of this population on Rpb1, a core RNA polymerase II subunit, and on Srb10 and Kin28 CDKs. While Rpb1 and Kin28 are generally required for protein-coding gene expression, Srb10 negatively regulates a small subset of genes. The insight provided by these results, is followed up with the observation that Srb10 itself is regulated when cells encounter limiting nutrients, and that it is a negative regulator of

pseudohyphal growth, a dimorphic developmental pathway in S. cerevisiae under conditions of nutrients limitation. Our results reveal that the transcriptional CDKs can add an additional level of combinatorial control on eukaryotic gene expression in vivo.

Results

Features of the study

The study described here was designed to assess the requirement for the two cyclin-dependent kinases that are components of the RNA polymerase II transcriptional machinery. This was accomplished by using high density oligonucleotide arrays (HDAs) (Wodicka et al., 1997) to determine the effects of mutations in these components genome-wide. Databases supporting all aspects of this study can be found on the World Wide Web at http://www.wi.mit.edu/young/expression.html.

The yeast transcriptome

Knowledge of the levels of all detectable mRNA species in yeast is useful for evaluating the degree to which these levels depend on any one component of the transcription apparatus. To obtain this information and to assess the reproducibility of the HDA technology, RNA was harvested from two independent wild type cultures and compared using two sets of HDAs on two separate days. The HDAs used here can score mRNA levels for up to 6181 genes. Of the 5460 genes whose mRNA levels were accurately determined and compared in both experiments, 99% of the mRNAs differed no more than 1.7 fold, and only 35 transcripts (0.65%) showed more than a two-fold change (Figure 1). In order to prevent these minimal variations from influencing the results, all experiments were performed in duplicate. The levels determined for the 5460 transcripts in wild type yeast cells and additional information derived from this experiment can be found under "Yeast mRNA population" on the Web site. The SAGE method has previously been used to determine values for 4465 transcripts, the result of

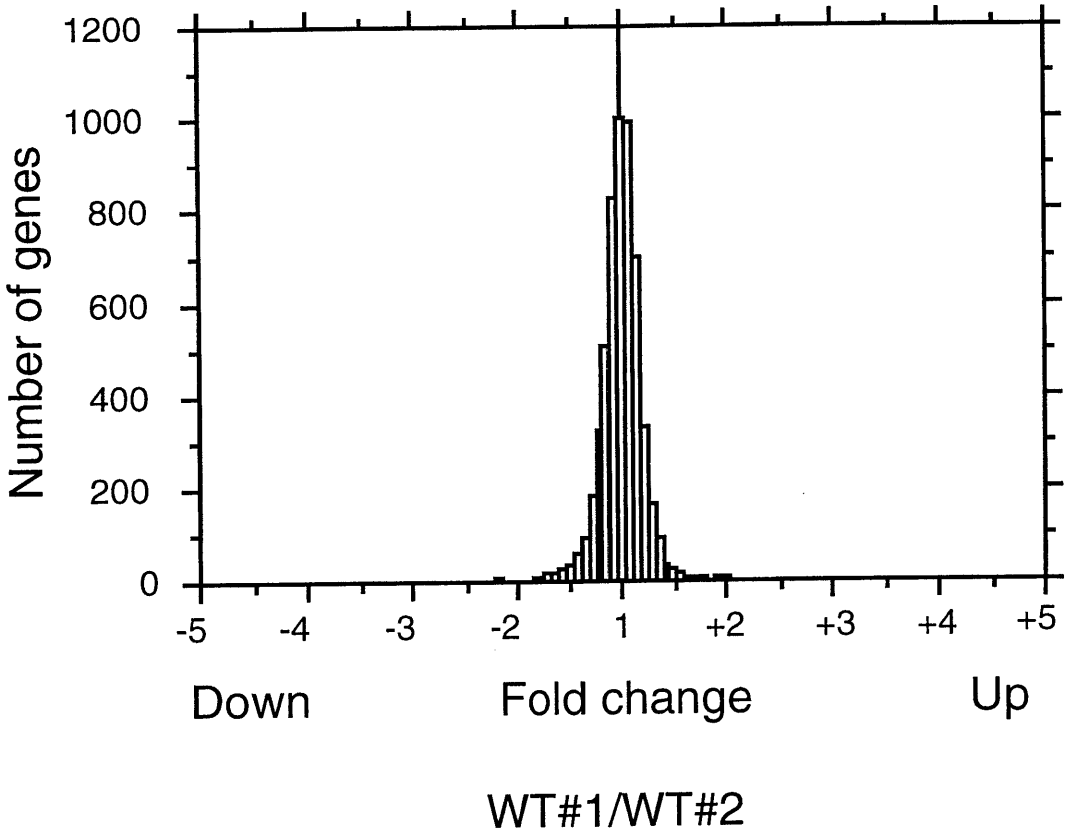

1200
1000
800
600
400
200
0
Number of genes
-5
-4
-3
-2
1
+2
+3
+4
+5
Down
Fold change
Up
WT#1/WT#2

Figure 1. Reproducibility of the HDA technology.

RNA was harvested from two independent wild type colonies and the two RNA preparations were hybridized to two different HDAs on two separate days. The results were plotted on a histogram in which the ratio of levels of each mRNA measured from the two independent experiments (fold change) was plotted against the number of genes whose mRNAs have been counted in that category. For 99% of all genes scored, the expression values produced for each gene in the two experiments were within 1.7-fold. The genes whose expression values scored outside this narrow range in the two experiments tended to be genes whose expression levels are near the lower limits of detection (0.1 mRNA molecules/cell). Thus, the technology generates highly reproducible results.

which has been termed the yeast transcriptome (Velculescu et al., 1996). The sensitivity of the HDA technology permitted a determination of the levels of many additional gene products, and revealed that transcripts from 80% of expressed yeast genes exist at steady state levels of 0.1 to 2 molecules/cell.

Dependence of genome expression on components of the transcriptional machinery

At any one promoter, the transcriptional machinery might include the RNA polymerase II core enzyme, the general transcription factors (GTFs), the core Srb/mediator complex, the Srb10 CDK complex, the Swi/Snf complex, and the SAGA complex, among others (Figure 2). Besides the Srb10 CDK complex, a second cyclin-dependent kinase (CDK), named Kin28, can be found as part of the general transcription factor TFIIH. The kinase subunit of each of the two transcriptional CDK was investigated for its role in genome-wide gene expression through the use of mutations which affect either the function or the physical presence of the subunit (Table 1). As a control, we also analyzed the mutated allele *rpb1-1* (Nonet et al., 1987), a temperature sensitive (ts) mutation in *RPB1*, a critical component of the RNA polymerase II core enzyme. For the essential CDK Kin28, a temperature-sensitive (ts) mutations was valuable because it allowed us to examine the effects on gene expression at any point after inactivating the factor. For the non-essential CDK Srb10, we used a point mutation which knocked out the catalytic kinase function. In each experiment, a mutant cell and its isogenic wild-type counterpart are grown to mid-log phase, the two populations are harvested, RNA is prepared, and hybridization to HDAs is carried out, all in duplicate.

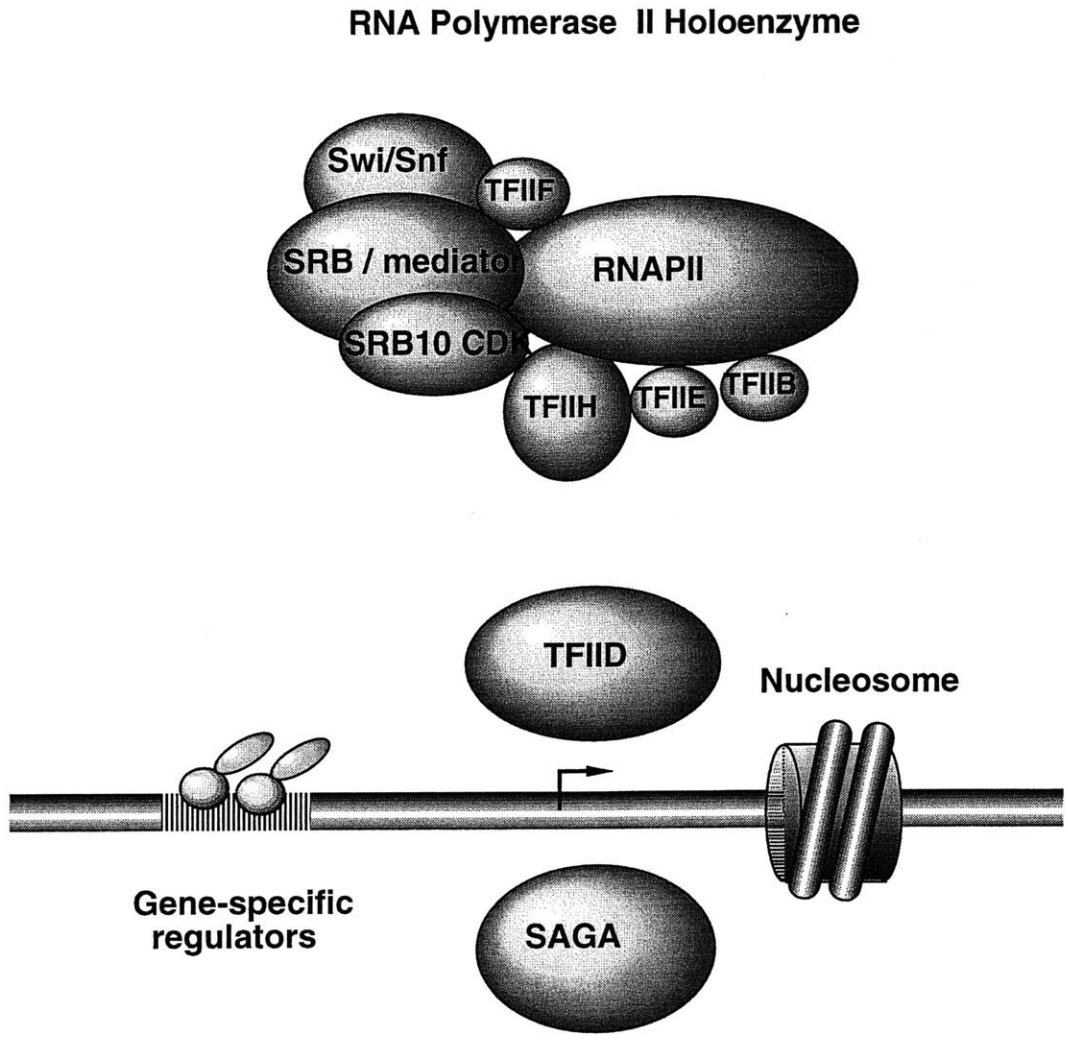

RNA Polymerase II Holoenzyme
Swi/Snf
TFIIF
SRB / mediator
RNAPII
SRB10 CDK
TFIIH
TFIIE
TFIIB
TFIID
Nucleosome
Gene-specific
regulators
SAGA

Figure 2. Model of RNA polymerase II transcription machinery.

The machinery depicted here encompasses over 85 polypeptides in 10 (sub) complexes: core RNA polymerase II (RNAPII) consists of 12 subunits, TFIIH 9 subunits, TFIIE 2 subunits, TFIIF 3 subunits, TFIID 14 subunits, core SRB/mediator more than 16 subunits, Swi/Snf complex 11 subunits, Srb10 kinase complex 4 subunits and SAGA 13 subunits (see Web site for more details). As detailed in Table 1, Rpb1, a subunit of the core RNA polymerase II complex , Srb10, a subunit of the Srb10 CDK complex, and Kin28, a subunit of the TFIIH complex, were choosen for analysis of genome-wide transcription dependence.

le 1. Transcriptional Machinery

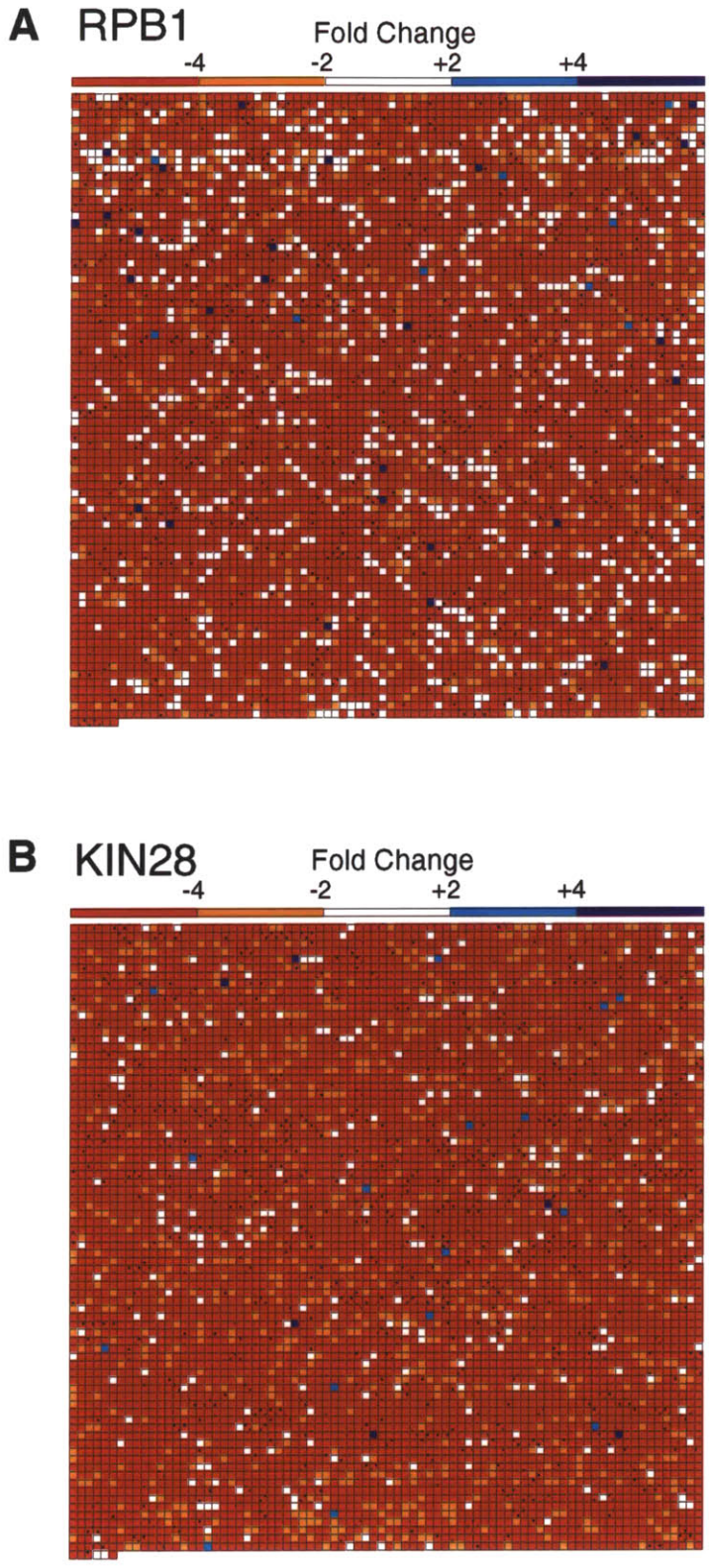
nplex and Subunit	Features	Fraction of genes dependent on subunit function
A Polymerase II		
Rpb1	Largest subunit, mRNA catalysis, contains CTD	100%
ıeral Transcription Factors		
TFIIH (Kin28)	CTD kinase	87%*
CDK complex		
Srb10	CTD kinase, negative regulator	3%

in28 results were essentially identical to Rpb1, but because of the stringency applied by the fit algorithm, a
ıimal estimate is produced.

Dependence on core RNA polymerase II

To determine the genome-wide dependence of gene expression on core RNA polymerase II, RNA was isolated from an *rpb1-1* temperature sensitive (ts) cell and its wild type counterpart 45 minutes after a shift to the nonpermissive temperature and was hybridized to HDAs. Because *rpb1-1* cells shut down transcription of protein-coding genes immediately after a temperature shift, these cells have been used by us and other investigators to determine the half-life of various yeast mRNAs (Nonet et al., 1987; Herrick et al., 1990). The 45 minute time point was used for the analysis of all ts mutants in this study because it is sufficiently long to detect a significant (i.e. a two-fold or more) loss of mRNA levels for 94% of detectable gene products without any loss of rRNA (Nonet et al., 1987). In addition, the 45 minute time point is short enough to minimize the potentially complicating effects of cell cycle arrest and cell death.

The results of genome wide expression analysis of the *rpb1-1* mutant as compared to an isogenic wild type strain are shown in a grid format in Figure 3A. The grid shows the change in mRNA level for each gene, beginning with the left-most gene on chromosome I and proceeding in a linear fashion, left to right, through chromosome I, then II, then III, etc., until the last gene on the right arm of chromosome XVI is reached at the lower right hand corner. 5735 genes were scored in this analysis. The vast majority of mRNAs are reduced more than two-fold in the mutant cells relative to wild type cells, and this reduction provides an apparent half-life for each of the mRNA species (see Yeast mRNA Population on the Web site). The value determined with this approach is an approximation, but is useful for comparative purposes. Comparison of this data with that obtained for the *kin28ts-3* allele identifies the set of genes whose expression is equivalently dependent on RNA


A
RPB1
Fold Change
-4
-2
+2
+4
B
KIN28
Fold Change
-4
-2
+2
+4

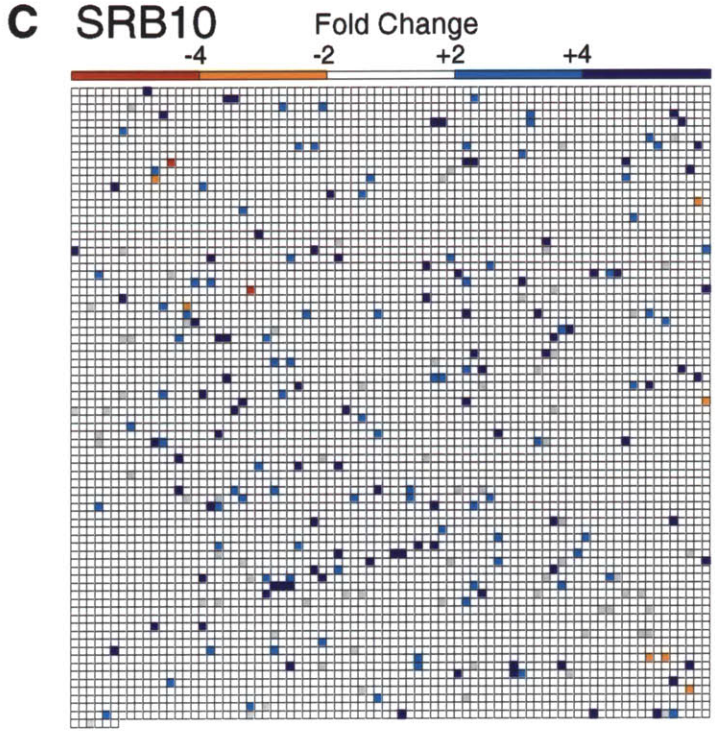

C
SRB10
Fold Change
-4
-2
+2
+4

Figure 3. Genome-wide expression data for *RPB1, KIN28* and *SRB10*.

Data reflecting the change in mRNA levels when a mutant is compared to its isogenic wild type counterpart is presented in a grid format. In the grid, the upper left grid square represents the left-most gene on chromosome I, and the squares to its right represent adjacent genes, proceeding in a linear fashion through chromosome I, then II, then III, etc., until the last gene on the right arm of chromosome XVI is reached at the bottom of the grid. The results are shown for (A) Rpb1, (B) Kin28 and (C) Srb10.

polymerase II and Kin28. There is a set of genes whose mRNAs are not significantly reduced in the mutant cells. These consist of genes that have stable messages as well as genes whose mRNA levels are slightly elevated in the mutant cells relative to wild-type.

In this latter group are many known heat shock or stress response genes (e.g. *SSA4*, *SSA3*, *HSP26*, *HSP30*, *HSP42* and *SSL2*), plus additional ORFs of unknown but perhaps related function. Another group had recently reported that the same temperature-sensitive mutant in Kin28 did not significantly affect activated transcription of the heat shock induced gene, *SSA4*, and the copper induced gene, *CUP1* (Lee and Lis, 1998). At the time, these results were interpreted to suggest that transcription activation at these genes might be independent of the TFIIH kinase. However, subsequent studies could not reproduce these results and found instead that *CUP1* and *SSA4* induction required Kin28 function (H. Causton and R. Young, unpublished). Previous studies in yeast cells had indicated that heat shock responsive genes are maximally transcribed approximately 15 min. after the temperature shift, after which a rapid reduction of heat shock mRNA levels occurs (Werner-Washburne et al., 1989; Miller et al., 1982). Careful time course analysis of *SSA4* induction in vivo has revealed that transcriptional induction required Rpb1 and Kin28, but that the mRNA became highly stable when transcription was inhibited during or immediately after heat shock (Causton and Young, unpublished results). This increased mRNA stability, combined with the measurement of mRNA at a single time point of 45 min after the temperature shift, explains the apparent Rpb1 and Kin28 "independence" seen for heat shock gene transcription.

Dependence on Kin28 CDK

The general transcription factors are necessary to reconstitute promoter-dependent transcription in vitro with core RNA polymerase II. These factors include TFIID, TFIIB, TFIIF, TFIIE and TFIIH. Among these factors, TFIIH is of particular interest because it has been found to be dispensable for transcription in vitro under certain conditions (Parvin et al., 1992; Serizawa et al., 1993; Timmers 1994). Kin28, a CDK subunit of TFIIH, is an RNA polymerase II CTD kinase which is involved in the transition from initiation to elongation (Dahmus, 1996). Analysis of *kin28* ts mutants suggested that most protein coding genes did require the function of *KIN28* in vivo (Cismowski et al., 1995; Valay et al., 1995). Genome-wide expression analysis provides a more rigorous test of the model that expression of all protein-coding genes is dependent on Kin28. The analysis was carried out on a Kin28 ts cell and its isogenic wild type counterpart using the same experimental protocol used for the Rpb1 ts mutant. The reduction in mRNA levels observed in ts mutants soon after a temperature shift (i.e., 45 minutes) is likely a consequence of primary effects due to factor inactivation. A longer time would be required to produce most secondary effects since indirect effects involve a substantial and sequential reduction in both transcription and translation products. Nonetheless, the results obtained in this type of experiment must be regarded as the sum of primary and secondary effects.

To identify the set of genes whose change in expression is most likely a direct consequence of the loss of function of the ts Kin28, we compare data sets from the ts inactivation of Kin28 and Rpb1 mutants and look for transcripts with equivalent decay kinetics (see Experimental procedures and Data Analysis on the Web site for details). The results reveal that Kin28 is generally required for expression of protein coding genes (Figure 3B). Of the 5405 genes whose mRNA expression levels could be compared (i.e. those that

had a greater than two-fold decrease in the experiment with Rpb1 ts and were scored in the Kin28 ts experiment), 87% showed a decrease that closely fit the decrease observed in the Rpb1 ts experiment. While the remaining 13% of the genes did not closely fit the Rpb1 decay rates, they nonetheless consistently decreased in the Kin28 ts experiment, and are thought to require Kin28 as well. The stringent standard applied in the data comparison, the experimental error, and the strain background difference between the Rpb1 ts and Kin28 ts strains, must all have contributed to the difference observed. Our results thus indicate that genome-wide gene expression generally requires Kin28. However, without knowing the exact nature of the Kin28 ts mutation, we can not distinguish between a general requirement for the Kin28 protein, the Kin28 kinase activity or the TFIIH multisubunit complex.

Dependence on Srb10 CDK complex

Srb10 is a cyclin dependent kinase which is part of a holoenzyme subcomplex containing Srb8, 9, 10 and 11 proteins (Liao et al., 1995; Hengartner et al., 1995). Srb10 and its associated proteins have been proposed to form a negative regulatory complex which functions through phosphorylation of the RNA polymerase II CTD (Hengartner et al., 1998). To determine how gene expression depends on Srb10, RNA was isolated from an Srb10 point mutant which lacks catalytic activity and the expression profile was compared to that of its wild type counterpart. The results are shown in a grid format in Figure 3C. Of the 5626 genes which were scored, 173 gene products showed 2-fold or greater increases in mRNA levels in the mutant relative to the wild type. This indicates that Srb10 is normally a negative regulator of these 173 genes (approximately 3% of the genome).

Coordinate negative regulation of nutrient starvation response genes via Srb10

The analysis revealed that Srb10 is a negative regulator of 173 genes. It is notable that nearly half of these genes are derepressed during the nutrient deprivation which occurs during the diauxic shift (DeRisi et al., 1997) (Figure 4). Yeast cells undergo a diauxic shift as nutrients are depleted in culture, and a variety of genes which enable the cell to survive nutrient-limiting conditions are derepressed (Johnston and Carlson, 1992; Yin et al., 1996). These include genes involved in dimorphic morphology (nutrient starved cells alter their morphology to permit foraging for nutrients) and stress responses (starved cells are apparently better able to survive nutrient deprivation when stress proteins are elevated). Srb10 in wild type cells most likely plays a role in repressing this set of genes when cells are in exponential growth on glucose, but no longer performs this function as cells enter the diauxic shift. Coordinate regulation of this set of genes could be accomplished by eliminating the function of Srb10 as cells enter the diauxic shift.

To determine whether Srb10 is physically lost from cells as they enter the diauxic shift, cells containing an epitope-tagged Srb10 protein were grown in YPD media and sampled at various times during the growth curve (Figure 4C). Cell lysates were prepared from each sample and the levels of Srb10 were assayed by Western blot. The data in Figure 4C shows that Srb10 levels become sharply reduced (approx. 4-fold) as cells enter the diauxic phase of growth. This result is consistent with evidence that the levels of Srb11, the cyclin partner of Srb10, are reduced when cells are exposed to the limiting nutrient environment in sporulation media (Cooper et al., 1997). It may also explain why a form of yeast holoenzyme purified from commercially available yeast cells lacks the Srb10/Srb11 kinase/cyclin pair (Li et al., 1995;

Myers et al., 1998), as these cells are typically harvested past mid-log phase (or even near stationary phase). In contrast to what was seen for Srb10, a survey of epitope-tagged Srb5 levels showed no marked decrease as cells encountered the diauxic shift; instead, the protein levels remained relatively constant even as the cells neared stationary phase (data not shown). In addition to Srb5, Srb4 protein levels have also been shown to remain unaffected by the cellular growth state (Walker et al., 1997). These results argue against a general decrease of holoenzyme levels as cells move from logarithmic growth to stationary phase and indicate that the nutrient starvation response is mediated, in part, through the physical loss of the Srb10 CDK from the holoenzyme. This novel mechanism provides an example of how coordinate regulation of gene expression can be accomplished through regulation of components of the general initiation machinery.

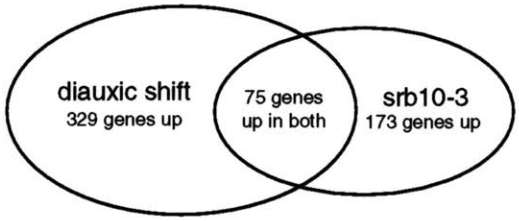
Srb10 is a negative regulator of a yeast developmental program

When nutrients become limiting on solid media, *S. cerevisiae* cells undergo a developmental transition from a single cell form to a filamentous pseudohyphal form, allowing better nutrient foraging. In diploid cells, this dimorphic form is called pseudohyphal growth and can readily be observed as a difference in colony morphology, as filamentous growth extends beyond the perimeter of the colony. Filamentous growth is accompanied by agar penetration, and in haploid cells, the dimorphic growth is referred to as invasive growth. A strain's invasive growth can be observed by washing the top portion of the colony off the plate surface and examining the remaining cells embedded in the agar. *FLO11*, which encodes a cell wall protein which is highly expressed in pseudohyphal cells, is expressed at 15-fold higher levels when Srb10 function is lost (Figure 4A). The dramatic increase in the

A

Gene	Description	Fold Up
FLO1	Flocculence cell wall protein	102
SIP18	Induced by osmotic stress	74
YBR116C	Induced by diauxic shift	61
YMR107W	Induced by diauxic shift	32
ALD3	Induced by diauxic shift	28
HSP26	Induced by osmotic stress, diauxic shift	26
GRE1	Induced by osmotic stress, diauxic shift	25
YER150W	Induced by diauxic shift	24
HSP12	Induced by numerous stresses	18
RCK1	Serine/threonine protein kinase	18
FLO11	Flocculence	15
RTA1	Involved in 7-aminocholesterol resistance	15
YDR070C	Induced by diauxic shift	13
YBR147W	Induced by diauxic shift	10
CTT1	Induced by osmotic stress, diauxic shift	10
YDL204W	Induced by diauxic shift	10
TKL2	Induced by diauxic shift	10
YGR043C	Induced by diauxic shift	9
YNL194C	Induced by diauxic shift	9
SOL4	Induced by diauxic shift	8
CYC7	Induced by numerous stresses, diauxic shift	8
PUT4	Proline permease, nitrogen induced	8
YKL187C	Induced by diauxic shift	8
NCA3	Life-span determination	8
YML128C	Induced by diauxic shift	8
GPH1	Induced by diauxic shift	8
POT1	Induced by diauxic shift	7

B



C

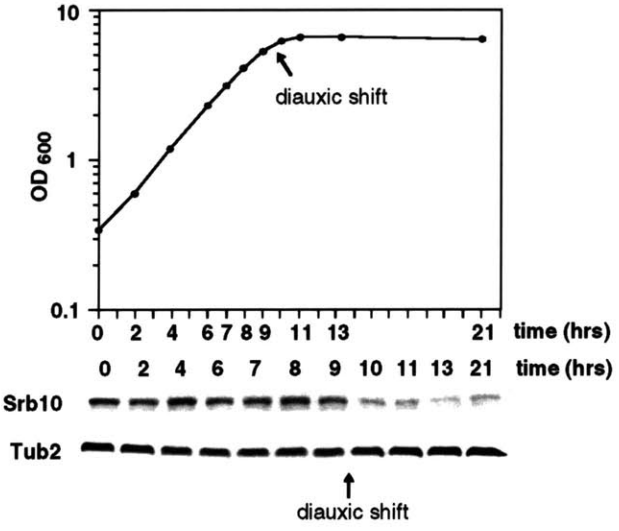


Figure 4. *SRB10* CDK represses genes elevated during response to nutrient starvation.

(A) Subset of 173 genes whose expression is derepressed in cells lacking Srb10 kinase activity.
(B) Venn diagram showing the number of genes which are derepressed during the nutrient deprivation which occurs during the diauxic shift and the fraction of these which are derepressed in cells lacking Srb10 kinase activity.
(C) Srb10 protein is depleted from cells as they enter the diauxic shift. The graph shows the growth curve of a yeast strain allowed to grow near the stationary phase (21 hours). At specified time points, aliquots from a culture were measured for cell density and equal amounts of cells were harvested for Western blot analysis. Western blots against epitope-tagged Srb10p and a control protein Tub2p (β-tubulin) show that Srb10 levels decrease substantially as cells enter the diauxic shift (9 to 10 hours).

expression of *FLO11* and other genes whose products are involved in the dimorphic shift led us to determine whether the absence of Srb10 function would affect two key features of filamentous pseudohyphal growth: filament formation and agar invasion.

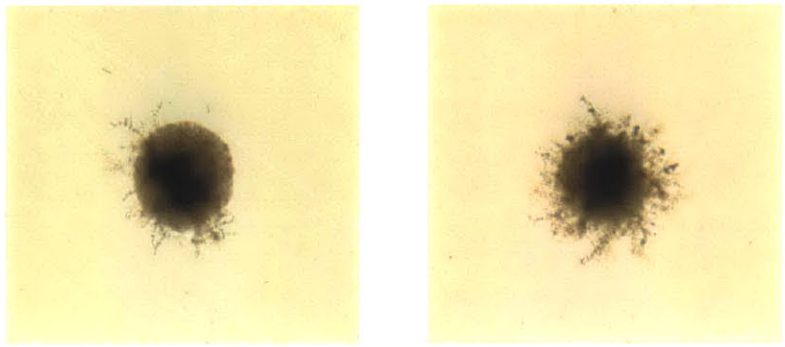
To assay filament formation, both copies of the *SRB10* gene were deleted from a diploid strain which is generally used to assay this phenotype, and colony morphology was examined under the microscope. The results in Figure 5A demonstrate that the complete loss of Srb10 causes cells to grow preferentially in a pseudohyphal form. While Srb10 negatively regulates pseudohyphal growth, other layers of repression must also exist since no pseudohyphal growth was detected on nitrogen rich media in the absence of Srb10 (data not shown).

To assay agar invasion we used haploid cells derived from two different yeast strains: Σ1278b and S288C. Σ1278b, like most feral yeast strains, is able to form pseudohyphae and haploid invasion while the laboratory strain S288C does not, due to a naturally occurring mutation in the *FLO8* gene (Liu et al., 1996). The *SRB10* gene was deleted in both strains and cells that were embedded in the agar could be seen after washing off the surface cells from the yeast plate (Figure 5B). In the S288C strain background, loss of Srb10 now allowed the cells to dig lightly into the agar while the loss of Srb10 in the Σ1278b background promoted an agar superinvasive behavior. Thus, Srb10 negatively regulates two characteristic components of the pseudohyphal developmental pathway. This example demonstrates how expression analysis is useful for predicting unexpected phenotypes. More importantly, specific signal transduction pathways control the dimorphic shift (Madhani

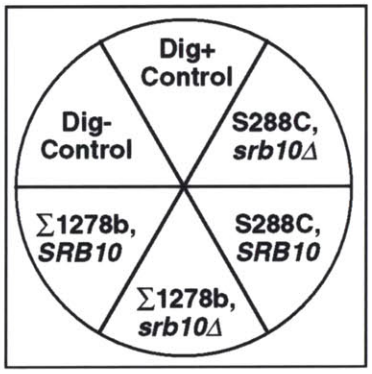
A

SRB10/SRB10

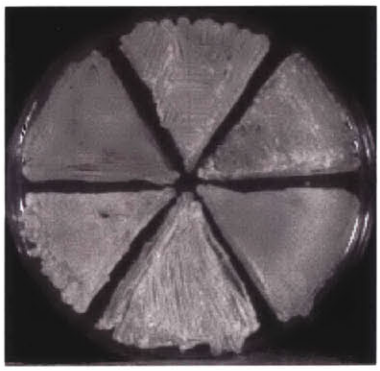
srb10Δ/srb10Δ

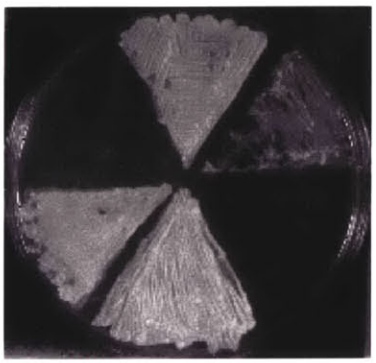


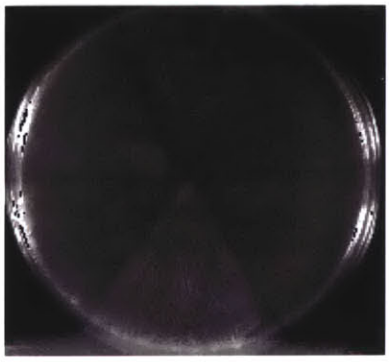
B



Total Cells







Invasive growth

Superinvasive growth

Figure 5. *SRB10* CDK acts negatively on the filamentous pseudohyphal growth developmental pathway in haploid and diploid yeast cells.

(A) Diploid cells lacking Srb10 kinase activity exhibit increased pseudohyphal filament growth upon nitrogen starvation. Diploid strains PHY584 (*SRB10/SRB10*) and CHY134 (*srb10Δ/srb10Δ*) were streaked and grown on SLAD plates at 30°C. Individual colonies were photographed at 5 days.
(B) Haploid cells lacking Srb10 kinase activity exhibit increased invasive growth. Σ1278b derived haploid strains (Dig+ control, 10560-5B), (Dig- control, CSY251), (*SRB10*, CHY131) and (*srb10Δ*, CHY130), as well as S288C derived haploid strains (*SRB10*, Z718) and (*srb10Δ*, Z723) were patched on a YPD plate and incubated at 30°C for 3 days, then at room temperature 3 more days. The cells were first washed off the agar surface with deionized water and later by gently surface rubbing with a gloved finger. The plate was photographed before washing (total growth), after moderate water washing (invasive growth) and after mechanical rubbing of plate surface (superinvasive growth).

and Fink, 1998), and these results suggest that one of the ultimate targets of these pathways is the Srb10 kinase.

Discussion

Using HDA technology, we have characterized the mRNA population of yeast cells and the gene expression requirement for an essential component of the core RNA polymerase II, Rpb1, and two cyclin-dependent kinases (CDK) found in the transcription preinitiation complex, Kin28 and Srb10. The insights obtained from this analysis include the following. While the essential requirement of *RPB1* for genome-wide expression was expected, the general requirement for *KIN28* suggests that TFIIH is generally recruited and functions at promoters of protein-coding genes. The distinct expression signature obtained when the Srb10 kinase is inactivated, reveals the coordinate negative regulation of functionally related genes, as exemplified by the regulation of Srb10p levels under growth limiting amounts of nutrient and the role of this kinase in the various responses to nutrient deprivation. The results obtained here about the effect of these CDKs in genome-wide gene expression has confirmed and united a variety of unlinked original observations about their role in vivo and in vitro.

Transcriptome

We have estimated the number of mRNA molecules present for all genes in a single wild type haploid cell using HDA data (see Yeast mRNA Population on the Web site). This is a more accurate representation of the transcriptome than that previously determined because it is better able to score mRNA species which are expressed at very low levels (5460 genes were scored using HDAs, whereas 4465 genes were scored with SAGE). It is particularly valuable to have information on transcripts from genes expressed at low levels because many of the regulatory components of the cell are expressed at low levels.

Core RNA polymerase II

Because Rpb1 is generally required for expression of protein-coding genes, and it is associated tightly and exclusively with RNA polymerase II (Koleske and Young, 1994; Kim et al., 1994; Myers et al., 1998), we can infer that RNA polymerase II is generally required for transcription. This agrees, of course,with previous studies describing a rapid decrease of the bulk of poly-A^+ RNA levels upon loss of *RPB1* function (Nonet et al., 1987; Cormack and Struhl, 1992; Thompson et al., 1995).

Kin28 CDK and the TFIIH complex

Assuming that the function of Kin28 is restricted to TFIIH, the data obtained with the Kin28 mutant demonstrates that TFIIH is a generally required factor. This requirement for Kin28 in transcription is consistent with prior observations of a rapid decrease of overall mRNA levels upon a shift to restrictive temperatuer with the *kin28ts-3* mutant allele used here (Valay et al., 1995) or an unrelated *kin28* ts allele (Cismowski et al., 1995). The use of a mutant of that specifically knocked out the kinase function would allow us to narrow down the Kin28 function required for transcription.

In contrast to our results, a recent report has suggested that the same Kin28 mutant does not affect activated transcription of the heat genes such as *SSA4* and *CUP1* (Lee and Lis, 1998). However, a number of problems tarnish these results. First, the temperature used to inactivate Kin28 was lower than suggested (Valay et al., 1995), raising the distinct possibility of an incomplete inactivation of Kin28 function. Second, levels of mRNA were assayed at a single time point after Kin28 inactivation, a source of possible error as explained in the Results section. Indeed, when a time course experiment was

performed at the proper restrictive temperature, a clear requirement for Kin28 was found for both *SSA4* and *CUP1* induction (H. Causton and R. Young, unpublished).

Srb10 CDK complex

The function of the Srb10 CDK complex can be defined by the kinase itself, since loss-of-function mutations in any of the four components of this complex produce identical phenotypes (Hengartner et al., 1995; Carlson, 1998). The Srb10 kinase is a negative regulator of a substantial fraction of genes which are repressed when cells grow vegetatively in rich media and are induced as cells experience nutrient deprivation. These results complete and confirm the description of the negative function of Srb10 inferred from amassed genetic and biochemical evidence (Hengartner et al., 1998). The genes regulated by Srb10 include those which are critical for the morphological change which permits foraging for nutrients and stress responses. Since Srb10p levels are reduced as cells enter the diauxic shift, a simple mechanism for derepression of this set of genes can be proposed: Srb10 in wild type cells is responsible for repressing this set of genes when cells are in exponential growth on glucose, but due to reduced protein levels, no longer can perform this function as cells enter the diauxic shift. The idea that Srb10 is part of a nutrient sensing pathway is also consistent with the finding that it negatively regulates *SPO13*, *GAL1* and *SUC2*. The first is a gene activated upon yeast sporulation, one of the possible responses to nutrient limitation, the other two genes are sugar utilization genes that are subject to glucose-repression (Surosky et al., 1994; Liao and Young, unpublished; Kuchin et al., 1995). What mechanism restricts the negative activity of Srb10 to only a subset of genes is as yet not known, but we have suggested that

promoter specific factors at these genes stimulate an otherwise quiescent Srb10 to hinder formation of pre-initiation complex through early CTD phosphorylation (Hengartner et al., 1998). The identification of such factors and of the promoter elements that recruit them is currently under way.

The results described here reveal that a layer of regulation is available to the cell in addition to that provided by gene-specific regulators: the expression of specific sets of genes can be regulated by affecting the availability or function of a specific component of the RNA polymerase II holoenzyme, TFIID and other general transcription initiation factors. A good example of such a mechanism of regulation involves the modification of core RNA polymerase II structural composition by alteration of the incorporation of subunits in response to stress signals. Rpb4 and Rpb7 form a dissociable subcomplex that has been implicated in the stress response and in the initiation of transcription (Dezelee et al., 1976; Woychik and Young, 1989; Edwards et al., 1991; Choder and Young, 1993). While Rpb4 and Rpb7 are present only in substoichiometric amounts during the exponential phase of yeast growth, they are found as stoichiometric subunits of core RNA polymerase II under suboptimal conditions such as the stationary phase of cell growth (Choder and Young, 1993). Another example of regulation of core RNA polymerase II by post-translational modifications of it's subunits is illustrated by the reversible phosphorylation of the carboxy-terminal domain (CTD) of Rpb1 (reviewed in Dahmus, 1996). While core RNA polymerase II with unphosphorylated CTD are needed for efficient assembly onto a promoter to initiate transcription, mRNA elongation efficiency and processing is correlated with a heavily phosphorylated CTD. Future surveys might determine whether the regulation of other RNA polymerase II

holoenzyme components is critical to the coordination of genome-wide gene expression regulation in response to extracellular and intracellular signals.

Distinct requirements for other components of transcriptional machinery

A more extended genome-wide analysis has been performed by F.C. Holstege et al. (1998), that included not only the three components described here (Rpb1, Kin28 and Srb10) but also components of the Srb/mediator complex (Srb4, Srb5 and Med6), Swi/Snf complex (Swi2), SAGA complex (Gcn5 and $TAF_{II}17$), and the two general transcription factors TFIID ($TAF_{II}145$ and $TAF_{II}17$) and TFIIE (Tfa1) (refer to the following world wide web site for additional details: http://www.wi.mit.edu/young/expression.html). In addition to the Rpb1 subunit of core RNA polymerase II and the Kin28 subunit of the general transcription factor TFIIH, the Srb4 subunit of the Srb/mediator complex was also generally required for transcription of protein coding genes. In contrast, Tfa1, a subunit of TFIIE, and Taf17, a subunit of both the SAGA complex and TFIID, were found to be required for more than half, but not all genes. Most other components investigated were necessary for transcription of less than a fifth of the genome (Srb5, Med6, Srb10, Swi2, Taf145, Gcn5). In this latter group, the evidence indicates that Srb5, Med6, and Taf145 have predominantly positive roles, Srb10 has an almost exclusively negative role, and Swi2 and Gcn5 can have either a positive or a negative role in gene expression. These data suggest that coordinate regulation of large sets of genes could be accomplished by affecting the function of specific components of the transcriptional machinery. If this is the case, then it would be expected that functional relationships exist among some genes within these sets, as has been observed with $TAF_{II}145$ (Holstege et al., 1998) and Srb10. It also raises the possibility that the ultimate targets of certain

signal transduction pathways could be identified by comparing genome expression signatures from these experiments and those which modify the cellular environment.

Development of a genome control map

The results described here support the feasibility of dissecting the regulatory circuitry of the yeast genome by using genome-wide expression analysis on cells with mutations in the transcription apparatus. The set of yeast genes whose expression depends on the functions of key components of the transcriptional machinery has been identified (Holstege et al., 1998). The genome-wide expression signatures produced by lesions in specific components of the transcription apparatus are quite distinct, making it possible to envision a genome control map. Such a map would identify all the components of the transcriptional machinery which have roles at any particular promoter and the contribution which specific components make to coordinate regulation of genes. The map will facilitate modeling of the molecular mechanisms which regulate gene expression and implicate components of the transcription apparatus in functional interactions with gene-specific regulators.

Experimental Procedures

High-density oligonuleotide array (HDA) technology

Affymetrix GeneChip high-density oligonucleotide arrays (HDAs) were used in this study. The yeast genome HDAs are described in detail in Wodicka et al. (1997). The arrays can detect as few as 0.1 mRNA molecules/cell; the dynamic range over which detection is accurate is approximately 0.1 - 100 mRNA molecules/cell.

With the Genechip arrays, the entire yeast genome is covered by four HDAs. In total, 6181 ORFs are present within this set. Each gene is represented on the HDA by 20 25-mer oligos that match the sequence of the message (perfect match oligos) and 20 oligos that are identical but differ by one base (mismatch oligos). mRNA levels are calculated by subtracting the signal of a mismatch from its perfect match partner and averaging the difference for each oligo pair representing a given gene. Based on various criteria that assay the consistency of behavior of each perfect match and mismatched oligomer pair, a Present or Absent call is also returned for each mRNA.

Genome-wide survey

Two independent experiments were performed for each wild type versus mutant comparison. For any experiment involving a comparison between a mutant and its isogenic wild type counterpart, the HDAs used were from the same lot number.

Expression profiles were determined by growing yeast cultures to mid-log phase, isolating total RNA, spiking in known amounts of control RNA for normalization and isolating poly A^+ RNA. This was used to generate double stranded complementary DNA (dscDNA) that in turn was used to generate

biotin labeled copy RNA (cRNA; the oligo used for dscDNA synthesis contains a T7 RNA polymerase promoter). 1-3 μg of poly A^+ RNA thus resulted in synthesis of approximately 60 μg cRNA. This was fragmented and hybridized to the oligonucleotide arrays, arrays were washed, stained, washed and scanned. All steps subsequent to poly A^+ RNA purification were carried out exactly as described in the Affymetrix product information supplied with the Genechips (the leaflets are part numbers 700187 Rev 1 and 700163 Rev 1 5/98).

HDA result analysis

Individual mRNA levels were scored if the computer algorithm analyzing the scanned results (Wodicka et al., 1997) returned a "Present" call in both the two wild type and the two mutant expression profiles for that gene or if the expression levels of that gene changed in the same direction and the amplitude of the change was greater than background levels in both wild type and mutant comparisons.

For all genes scored, the fold change was calculated by dividing the mutant value by the wild type value. The reported fold changes are the average of the two independent experiments. For the grid representation comparing the change in mRNA levels between mutant and wild type strains (Figure 3), a 2-fold change was called if it satisfied the following two conditions: a given mRNA level had to change more than two-fold in both comparisons, and the change in the values was above background values in both comparisons.

For comparing the dependence of gene expression on Kin28 and Rpb1, the fold change in genes scored in the *rpb1-1* and the *kin28ts-3* experiments were compared. Transcripts whose levels dropped less than 2-fold in the *rpb1-1* experiment were not included in the analysis because the decay rates were not sufficient to provide a meaningful comparison. A gene was

determined to be equivalently dependent on Rpb1 and Kin28 if it satisfied two conditions: the gene's mRNA level dropped more than two-fold in the *kin28ts-3* experiment, and the fold decrease in the *kin28ts-3* experiment was two-fold within that seen in the *rpb1-1* experiment.

Cell Harvesting

Freshly streaked colonies were used to inoculate a pair of 10 ml YPD culture grown overnight at 30°C. After dilution to an $O.D._{600}$ of 0.05 in 200 ml pre-warmed YPD culture, the cells were grown an additional 4-8 hours to an $O.D._{600}$ of 0.5.

For temperature shift experiments, the culture were diluted 1:2 with pre-warmed YPD so that the final temperature of the mix would be correct (e.g., to shift 200 ml of culture from 30°C to a final temperature of 37°C, 200 ml of YPD pre-warmed to 44°C were added). Cultures were then incubated at the restrictive temperature for exactly 45 minutes.

The cells were harvested as quickly as possible by centrifugation for 3 min at 3500 rpm in a GSA rotor, resuspended in 6 ml ice-cold ddH_20 and respun 2.5 min at 2600 rpm in a Sorvall H1000B rotor. The resulting pellet was frozen in liquid nitrogen for at least 2 minutes and stored at -80°C. The steps from the harvesting to the frozen pellet took approximately 15 min.

The wild type and mutant strains used for the *RPB1*, *KIN28* and *SRB10* gene analysis were , Z579 and Z460, GF1047 and GF2092, and Z579 and Z690, respectively. The *rpb1-1* ts, the *kin28ts-3* and the *srb10-3* mutants have all been described (Nonet et al., 1987; Valay et al., 1995; Liao et al., 1995). Z460, Z579 and Z690 are all S288C derived. It is unclear whether GF1047 and GF2092 were also S288C derived.

RNA preparation

Total RNA was prepared using a hot acidic phenol RNA preparation procedure (Ausubel et al., 1997). Total RNA was resuspended in DEPC-treated water to a final concentration of 15 mg/ml, snap-frozen and stored at -80°C. We verified that the yield of total RNA obtained from wild type and mutant cells was very similar. Equivalent amounts of total RNA from each strain were used isolate mRNA since stable RNAs such as rRNA and tRNA account for approximately 96% of total RNA. mRNA was prepared using an Oligotex^{TM} resin (Qiagen Inc.) and was resuspended to a final concentration of 0.5 mg/ml. For strains where no general loss of mRNA was expected, 1 mg of total RNA was used. Otherwise 2 or 3 mg of total RNA were used. For each mg of total RNA, 5 µl of poly A$^+$ control RNA (see below) was added.

Poly A$^+$ control RNA

PolyA tagged lys, phe, thr, trp and dap T7/T3 IVT expression constructs were obtained from the ATCC (#'s87482, 87483, 87484, 87485, 87486, respectively). The polyA tagged RNA was generated with NotI digested template DNA and Ambion's Megascript T3 IVT kit according to their instructions. Transcripts were purified using the Qiagen RNeasy kit. An undiluted stock of these controls was prepared by mixing the various transcripts: 133 µg lys, 58.5 µg phe, 24.5 µg dap, 8.8 µg thr, 3.3 µg trp in 225 µl DEPC water. This was aliquoted in 22.5 µl amounts. A diluted stock was made by adding 477.5 µl DEPC water to the undiluted stock and this was again aliquoted. The diluted stock was spiked into the total RNA, 5 µl per mg total RNA. The final amounts of the controls in the total RNA were then: 4 trp, 13.3 thr, 40 dap, 133.3 phe, 400 lys (pmol/mg). By using theses same controls in every experiment all experiments can be normalized to equivalent amounts of total RNA.

Srb10 epitope surveying

An *SRB10* epitope-tagged culture (CHY159) grown at 30°C overnight in YPD was diluted to an $O.D._{600}$ of 0.2 in YPD and grown further for 2-3 hours. 50 $O.D._{600}$ of cells were harvested every 1-2 hours for whole cell extract (WCE) preparation, washed twice with 1 ml ddH_20, flash frozen and stored at -70°C until needed. Frozen cells were resuspend in 350 µl of lysis buffer (50 mM Hepes-KOH pH7.6, 5 mM EDTA, 5 mM EGTA, 50 mM of neutralized ammonium sulfate, 10% glycerol, 1x Protease inhibitors) and added to 400 µl of glass beads. The cells were lysed using a combination of bead beating and freeze thawing: frozen in liquid nitrogen for 1 min, thawed at 37°C for another and vortexed for 30 sec. This procedure was repeated 4 times. The supernatant was collected, the remaining beads were washed with 100 µl of lysis buffer (+ 5 µl of 100 mM DTT), and the wash was combined with the supernatant. After spinning in a microfuge at 14,000 rpm for 20 min at 4°C, the supernatant (WCE) was flash frozen and stored at -70°C until needed. Yields ranged from 3.7 to 2.3 mg/ml of total protein. WCE (30 µg) samples for western blot analysis were fractionated on 4-20% SDS-PAGE gradient gels and transferred according to standard procedures. Tub2 rabbit anti-serum was used at 1:3500, anti-HA mouse ascites fluid was used at 1:1000. HRP-conjugated anti-mouse (Pierce) and anti-rabbit (Amersham) secondary antibodies were used at 1:2000. Detection was performed by ECL according to the manufacturers directions (Amersham).

CHY159 was obtained by PCR epitope-tagging the genomic copy of *SRB10* in Z22 to introduce a N-ternimal His_6-HA_3 tag (Schneider et al., 1995).

Pseudohyphal and invasive growth assay

Pseudohyphal growth of the diploid strains was assayed as described (Gimeno et al., 1992) using synthetic low ammonia dextrose media (SLAD). CHY134 was created by deletion of *SRB10* using a single step disruption method. In short, L5978 was transformed with a 5.1 kb *srb10Δ::HIS3* DNA fragment (SalI/NotI double digestion of pRY2969) and plated on SC-His media. Southern analysis was used to confirm that a single copy of *SRB10* had been deleted. The resulting heterozygous diploid (CHY126) was sporulated and tetrads dissected on YEPD plates and scored for nutritional auxotrophies and colony morphology. Mating of 2 *srb10Δ::HIS3* haploids (CHY129 and CHY130) produced CHY134, a strain homozygous for the *srb10Δ*. All strains described as derived from Σ1278b are congenic to each other (Gimeno et al., 1992).

Haploid invasive growth was assayed as described (Roberts and Fink, 1994). Haploid strains CHY131 (Σ1278b, *SRB10*) and CHY130 (Σ1278b, *srb10Δ*) were both obtained from tetrad dissection of *SRB10/srb10Δ* heterozygote, CHY126.

Figures

The figures were prepared from digital replicas of primary data scanned using a UMAX UC840 Max Vision digital scanner.

Additional information

A variety of additional detailed information on experimental procedures, genetic reagents, HDA technology and data analysis can be found on the world wide web at http://www.wi.mit.edu/young/expression.html in the section titled Study Design.

Table 2. Yeast Strains

Strain	source	Genotype
CHY126	this study	*Mata/Matα ura3-52/ura3-52 LEU2/leu2::hisG his3::hisG/his3::hisG TRP1/trp1 SRB10/srb10Δ::HIS3*
CHY129	this study	*Mata ura3-52 his3::hisG trp1 srb10Δ::HIS3*
CHY130	this study	*Matα ura3-52 leu2::hisG his3::hisG srb10Δ::HIS3*
CHY131	this study	*Matα ura3-52 leu2::hisG his3::hisG*
CHY134	this study	*Mata/Matα ura3-52/ura3-52 LEU2/leu2::hisG his3::hisG/his3::hisG TRP1/trp1 srb10Δ::HIS3/srb10Δ::HIS3*
CHY159	this study	*Matα ura3-52 leu2-3,112 his3Δ200 His_6-HA_3-SRB10*
CSY251	Fink lab	*Mat α ura3-52 leu2::hisG TRP1 his3::hisG inv1::LEU2+lacZ* (dig- strain)
GF1047	Faye lab	*Mata ura3 leu2 trp1 lys2*
GF2092	Faye lab	*Mata ura3 leu2 trp1 lys2 kin28-ts3*
L5978	Fink lab	*Mata/Matα ura3-52/ura3-52 LEU2/leu2::hisG his3::hisG/his3::hisG TRP1/trp1*
PHY584	Fink lab	*Mata/Matα ura3-52/ura3-52 LEU2/leu2::hisG HIS3/his3::hisG TRP1/trp1 srb10Δ::HIS3/srb10Δ::HIS3*
Z22	Young lab	*Matα ura3-52 leu2-3,112 his3Δ200*
Z460	Young lab	*Mata ura3-52 leu2-3,112 his3Δ200 his4-912 lys2-128 rpb1Δ187::HIS3* [pRP1-1U (*URA3 CEN rpb1-1*)]
Z579	Young lab	*Mata ura3-52 leu2-3,112 his3Δ200 srb4Δ2::HIS3* [RY2884 (*LEU2 CEN SRB4*)]
Z690	Young lab	*Mata ura3-52 leu2-3,112 his3Δ200 srb10-3::hisG*
Z718	Young lab	*Matα ura3-52 leu2-3,112 his3Δ200*
Z723	Young lab	*Matα ura3-52 leu2-3,112 his3Δ200 srb10Δ1::HIS3*
10560-5B	Fink lab	*Matα ura3-52 leu2::hisG trp1::hisG* (dig+ strain)

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