

MOLECULAR GENETIC ANALYSIS OF A  
YEAST RNA POLYMERASE II HOLOENZYME

by

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## **Dedication**

To my parents, David and Virginia Thompson.

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# MOLECULAR GENETIC ANALYSIS OF A YEAST RNA POLYMERASE II HOLOENZYME

by

CRAIG MARSHALL THOMPSON

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## Abstract

The carboxy-terminal domain (CTD) of RNA polymerase II plays a role in transcription initiation. The isolation and characterization of extragenic suppressors of *Saccharomyces cerevisiae* RNA polymerase II CTD truncation mutations led to the identification of ten genes involved in CTD function in vivo. Seven of these genes, *SRB4*, *SRB5*, *SRB6*, *SRB7*, *SRB8*, *SRB9*, and *RPB2*, were cloned and sequenced. An eighth gene, *SRB2*, was previously identified as a suppressor of CTD truncation mutations. The sequences revealed that *SRB4*, *SRB5*, *SRB6*, *SRB7*, *SRB8*, and *SRB9* are novel proteins while *RPB2* is the second largest subunit of RNA polymerase II. Genetic analysis suggests that *SRB8* and *SRB9* function to negatively regulate CTD function while *SRB2* and *SRB5* positively regulate CTD function. The CTD, therefore, is influenced by positively and negatively acting SRB factors.

Antibodies generated against recombinant SRB proteins were produced and used to monitor the SRBs during purification using column chromatography. Most of the SRB protein in cells was found to be tightly associated with a large multisubunit complex containing RNA polymerase II, the general transcription factors  $\gamma$ TFIIB,  $\gamma$ TFIIF, and  $\gamma$ TFIIH, and substoichiometric amounts of TATA-binding protein (TBP). This RNA polymerase II holoenzyme is capable of site-specific initiation when supplemented with purified  $\gamma$ TFIIE and recombinant TBP and is responsive to activators. In vitro transcription and template commitment assays confirm that *SRB2* and *SRB5* are components of a functional preinitiation complex and are required for efficient transcription initiation.

Although the holoenzyme contains most of the SRB protein in a cell, it contains only a small fraction of RNA polymerase II. The fraction of genes that employ the RNA polymerase II holoenzyme in vivo was investigated by studying the effects of a temperature-sensitive mutation in the *SRB4* gene on transcription of mRNA. Upon transfer to the restrictive temperature, there is a rapid and general shutdown of mRNA synthesis. These findings suggest a general requirement for *SRB4* and the RNA polymerase II holoenzyme in transcription.

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## **Chapter 1**

### **Introduction: The RNA Polymerase II Initiation Complex**

## Overview

The first step in gene expression, and a major point of gene control, is the initiation of messenger RNA (mRNA) synthesis. The synthesis of mRNA begins with the assembly of RNA polymerase II and general transcription factors onto promoter DNA (Conaway and Conaway, 1993; Zawel and Reinberg, 1993). RNA polymerase II requires these general transcription factors for selective promoter recognition and accurate transcription initiation. Additional transactivating factors regulate the establishment and activity of the transcription initiation complex. The assembly of the transcription initiation complex and the factors involved is the focus of this introduction. A portion of the introduction is also devoted to the carboxy-terminal domain (CTD) of RNA polymerase II. Later chapters in this thesis describe how analysis of the CTD in yeast led to the identification of a novel set of proteins required for transcription initiation complex formation and how these studies led to the identification of the RNA polymerase II holoenzyme, the form of the enzyme recruited to most promoters in the cell.

## RNA Polymerase II

Eukaryotic cells contain three nuclear DNA-dependent RNA polymerases: RNA polymerase I (A), RNA polymerase II (B), and RNA polymerase III (C) (Chambon, 1975; Roeder, 1976; Sentenec, 1985). RNA Polymerase I transcribes ribosomal RNA (rRNA) genes, RNA polymerase II transcribes protein-coding genes, and RNA polymerase III transcribes the genes encoding the 5S rRNA and tRNA. These three enzymes were first purified from sea urchin and rat liver and resolved on the basis of chromatographic and enzymatic properties (Roeder and Rutter, 1969). They can be separated by DEAE-Sephadex chromatography and were named I, II, and III according to their order of elution by increasing concentrations of ammonium sulfate. Each of the three enzymes display different salt optima for activity, different divalent cation and template preferences, and differential sensitivity to inhibition by  $\alpha$ -amanitin.

RNA polymerase II is a multisubunit enzyme approximately 500 kd in size (Young, 1991). In the yeast *Saccharomyces cerevisiae* RNA polymerase II is composed of 12 protein subunits and the genes encoding each of these subunits have been cloned and sequenced (Young, 1991; Treich et al., 1992; McKune et al., 1993; Woychick et al., 1993). All of the genes encoding RNA

polymerase II subunits are essential for wild-type growth rates, and all but two of these genes are essential for cell viability.

The three largest subunits of RNA polymerase II appear to be homologues of the three core subunits of *E. coli* RNA polymerase (Young, 1991). *E. coli* core RNA polymerase is composed of three different subunits,  $\beta$ ,  $\beta'$ , and  $\alpha$  (McClure, 1985). The core enzyme  $\beta\beta'\alpha_2$  is responsible for the synthesis of the RNA transcript. The core enzyme plus a  $\sigma$  subunit is called a holoenzyme and it is this form of the enzyme that specifically recognizes and binds to promoter sites and initiates RNA synthesis. RPB1 and RPB2, the largest and second largest subunits of yeast RNA polymerase II, share extensive sequence similarities with  $\beta'$  and  $\beta$ , respectively (Allison et al., 1985; Sweetser et al., 1987). Features of the third largest subunit of yeast RNA polymerase II, RPB3, show some similarities to the *E. coli*  $\alpha$  subunit. Both proteins are similar in size and play similar roles during the assembly of their respective RNA polymerases (Yura and Ishihama, 1979; Kolodziej and Young, 1989). Furthermore, both RPB3 and  $\alpha$  are present in two copies per RNA polymerase molecule and they share limited spans of amino acid identity (Kolodziej and Young, 1989; Kolodziej et al., 1990; Martindale, 1990). It is not known, though, if the three largest subunits of RNA polymerase II are sufficient for core RNA polymerase activity.

Even though RNA polymerase II is a complex, multisubunit enzyme, purified preparations of polymerase are unable to selectively initiate transcription (Lewis and Burgess, 1982). Just as *E. coli* core RNA polymerase requires the addition of a  $\sigma$  subunit for selective promoter recognition (McClure, 1985), RNA polymerase II requires additional factors for selective transcription initiation. The situation for eukaryotes, however, is far more complex.

### **General Transcription Factors**

Purified RNA polymerase II is unable to properly initiate transcription in the absence of additional factors. A first step towards defining these factors was the demonstration that selective and accurate transcription initiation is possible in a cell-free system consisting of purified RNA polymerase II, template DNA with the adenovirus major late (AdML) promoter, and human KB cell extract (Weil et al., 1979). A system dependent only on whole-cell extract derived from human HeLa cells and template DNA was soon available (Manley et al., 1980). Investigators soon began fractionating human cell extracts and demonstrated a

requirement for multiple components distinct from RNA polymerase II for accurate transcription from the AdML promoter (Matsui, et al., 1980; Samuels et al., 1982; Dignam et al., 1983). These fractions were designated TFIIA (Transcription Factor IIA), TFIIIB, TFIIIC, TFIIID, and TFIIIE. TFIIIC was later shown to be poly (ADP-ribose) polymerase and not required in more purified systems (Slattery et al., 1983). The enzyme suppresses random initiation by binding to nicks in the DNA template.

Continued fractionation and purification has increased the number of general transcription factors required for RNA polymerase II to selectively initiate transcription in vitro (Conaway and Conaway, 1993; Zavel and Reinberg, 1993). Many of these factors (TFIIA, TFIIIB, TFIIID, TFIIIE, TFIIIF, TFIIIH, and TFIIJ) have been purified to homogeneity, characterized in some detail, and their genes cloned. General transcription factors from different species show a high degree of structural and functional conservation. TFIIID is the only one of these general factors capable of site-specific DNA binding. The other general transcription factors and RNA polymerase II assemble into a preinitiation complex following binding of TFIIID to promoter DNA.

**TRANSCRIPTION FACTOR IID** Using DNase I footprinting techniques and gel shift assays TFIIID has been shown to specifically bind the TATA element of the promoter (Sawadogo and Roeder, 1985; Nakajima et al., 1988; Buratowski et al., 1989). The TATA box is the core element of a eukaryotic promoter and is located 25 to 120 bases upstream of the start site of transcription (Struhl, 1989). TFIIID is a multisubunit complex composed of the TATA-binding protein (TBP) and the tightly associated TBP-associated factors (TAFs) (Gill and Tjian, 1992; Pugh and Tjian, 1992). The TAFs appear to mediate interactions with transcriptional regulatory proteins and are discussed in more detail below. TBP was initially purified from yeast because yeast TBP can substitute for human TFIIID in a reconstituted system and bind to the TATA element (Buratowski et al., 1988; Cavallini et al., 1988). The gene encoding TBP in yeast is the *SPT15* gene, originally identified as a suppressor of Ty element insertions (Fassler and Winston, 1988; Eisenmann et al., 1989; Hahn et al., 1989). cDNAs encoding TBP from a large number of other species have now been cloned (Hernandez, 1993). The carboxy-terminal core domains are highly conserved (generally >75% identical to human TBP) while the amino-terminal domain is not conserved and is variable in size. The structure of Arabidopsis and yeast TBP

has been determined (Nikolov et al., 1992; Chasman et al., 1993). Co-crystals of TPB and the TATA element have also recently been described (J.L. Kim et al., 1993; Y. Kim et al., 1993). TBP has a symmetrical saddle shape structure with the inner surface of the saddle interacting with the minor groove of the TATA element and the outer surface of the protein exposed to allow contact with other proteins.

**TRANSCRIPTION FACTOR IIA** TFIIA stabilizes the interaction between TFIID and the TATA element (Davison et al., 1983; Fire et al., 1984; Buratowski et al., 1989). DNA encoding the two subunits of TFIIA from yeast (yTFIIA) has been cloned (Ranish et al., 1992). Recombinant yTFIIA binds to a TBP-DNA complex in a gel shift assay and will substitute for purified yeast and mammalian TFIIA in *in vitro* transcription assays. However, a requirement for TFIIA in reconstituted systems is quite variable (Conaway and Conaway, 1993; Zawel and Reinberg, 1993). The influence of TFIIA on transcription *in vitro* seems to depend a great deal on the purity of the system. TFIIA is not required in highly purified systems derived from yeast and rat (Sayre et al., 1992a; Conaway et al., 1990). This is consistent with the observation that TFIIA is required for *in vitro* transcription in the human system when TFIID is used but not when TFIID is substituted with recombinant TBP (Cortes et al., 1992). This suggests that TFIIA is required to counteract inhibitory activities in TFIID and is likely to play an essential role *in vivo* in preinitiation complex assembly. The issue of TFIIA function should become easier to resolve with the recent cloning of cDNAs encoding some of the subunits of human and *Drosophila* TFIIA (Dejong and Roeder, 1993; Ma et al., 1993; Yokomori et al., 1993a).

**TRANSCRIPTION FACTOR IIB** TFIIIB activity resides in a single polypeptide and cDNAs encoding this polypeptide have been cloned from a number of species (Conaway and Conaway, 1993; Zawel and Reinberg, 1993). TFIIIB appears to play a role in start site selection and is absolutely required for transcription *in vitro*. The *SUA7* gene in yeast encodes yTFIIIB and mutations in this gene alter start site selection *in vivo* (Pinto et al., 1992). Mutations in the largest subunit of yeast RNA polymerase II (*RPB1/SUA8*) display similar start site selection defects (Berroteran et al., 1994). A variety of genetic interactions between *sua7* and *sua8* mutants suggests close interactions between these two proteins. This is supported by additional physical and functional data. First,

both yeast and *Drosophila* TFIIB can directly interact with RNA polymerase II *in vitro* in the absence of DNA (Tschochner et al., 1992; Wampler and Kadonaga, 1992). Second, when components of reconstituted systems were exchanged, TFIIB and RNA polymerase II were found to be solely responsible for the differences in start site selection between the yeasts *S. pombe* and *S. cerevisiae* (Li et al, 1994). The role of TFIIB in mediating interactions with transcriptional regulatory proteins is discussed below.

**TRANSCRIPTION FACTOR IIF** TFIIF is composed of two polypeptides known as RAP30 (RNA polymerase II-associated protein) and RAP74 and binds tightly to RNA polymerase II in solution (Flores et al., 1989; Price et al., 1989; Greenblatt, 1991). RAP30 and RAP74 have been purified to homogeneity and cDNAs isolated (Aso et al., 1992; Finkelstein et al., 1992; Sopta et al., 1989). Curiously, there is a 70 amino acid stretch in RAP30 with limited sequence similarity to conserved regions of bacterial and bacteriophage  $\sigma$  factors. These regions of  $\sigma$  are thought to contact core RNA polymerase. This similarity is supported by the observation that TFIIF can bind to *E. coli* RNA polymerase and be displaced by  $\sigma^{70}$  (McCracken and Greenblatt, 1991). TFIIF, like  $\sigma$ , appears to act as a selectivity factor for RNA polymerase II. Formation of non-specific RNA polymerase II-DNA complexes is reduced by the presence of TFIIF and purified TFIIF will disrupt preformed polymerase-DNA complexes (Conaway and Conaway, 1990; Killeen and Greenblatt, 1992). In addition, TFIIF is required for selective binding of RNA polymerase II to the promoter complex (Buratowski et al., 1991; Conaway et al., 1991b; Flores et al., 1991).

In addition to its role in transcription initiation, TFIIF also stimulates elongation of RNA polymerase II (Flores et al., 1989; Bengal, et al., 1991). Recently, a set of experiments using recombinant RAP30 and recombinant RAP74 sought to individually determine their roles in transcription initiation and elongation (Chang et al., 1993). Sarkosyl eliminates reinitiation by RNA polymerase II. A sarkosyl resistant complex is formed following the formation of the first few phosphodiester bonds of the transcript. RAP30 must be added prior to the addition of sarkosyl, while RAP74 can be added to the transcription reaction following the addition of sarkosyl. This data is consistent with RAP30 playing a role in bringing RNA polymerase II to the promoter and/or tethering RAP74 to polymerase. RAP74 appears to act subsequent to initiation, possibly in assisting RNA polymerase II in clearing the promoter and/or elongation.

These results are consistent with a prior set of experiments demonstrating that recombinant RAP30 is sufficient for recruitment of RNA polymerase II to the promoter (Flores et al., 1991). RAP30, though, may still play a role subsequent to initiation.

**TRANSCRIPTION FACTOR IIH** Probably the least understood and perhaps the most complicated general factor is TFIID. This factor is a multisubunit complex whose composition has not yet been clearly defined. Human (Drapkin et al., 1994a) and rat TFIID (Conaway et al., 1992a) are composed of at least 8 polypeptides while yeast is composed of at least 5 polypeptides (Feaver et al., 1993). TFIID from all three species is closely associated with a protein kinase that will specifically phosphorylate the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992). Phosphorylation of the CTD has been proposed to serve as a switch that regulates the transition from initiation to elongation (Corden, 1990; Chao and Young, 1991; Peterson and Tjian, 1992). The CTD is discussed in more detail below.

In addition to its kinase activity, ATPase and DNA helicase activities are also associated with TFIID (Feaver et al., 1991; Serizawa et al., 1992; Schaefer et al., 1993; Serizawa et al., 1993b; Roy et al., 1994). A variety of evidence suggests that the ATPase and helicase are part of the same activity and this activity is distinct from the CTD kinase activity. The ATPase and helicase activities display similar nucleotide substrate specificities. In contrast, the ATPase and CTD kinase differ in their nucleotide specificities, responses to DNA, and sensitivities to kinase inhibitors and sarkosyl (Serizawa et al., 1993b; Roy et al., 1994). In addition, two of the subunits of human TFIID, ERCC2 and ERCC3, display ATPase and helicase activities when purified from *E. coli* or yeast (Sung et al., 1993; Drapkin et al., 1994a; Roy et al., 1994). Although TFIID contains CTD kinase activity a subunit with kinase homologies has not yet been identified.

In addition to a role in transcription initiation, TFIID is also involved in DNA repair (Drapkin et al., 1994b). The largest subunit of human TFIID is the excision repair protein ERCC3, the homologue of yeast RAD25/SSL2 (Schaeffer et al., 1993). RAD25, however, does not appear to be a component of yTFIID but rather a protein that only interacts with yTFIID (Feaver et al., 1993; Bardwell et al., 1994). Two of the subunits of yTFIID are encoded by the DNA



repair genes *RAD3* and *SSL1* (Feaver et al., 1993). The human homologues of *RAD3* and *SSL1* are *ERCC2* and *p44*, respectively, both subunits of *TFIIH* (Drapkin et al., 1994a; Humbert et al., 1994). *RAD3* and *RAD25* are both essential for transcription by RNA polymerase II *in vivo* and *in vitro* (Qiu et al., 1993; Guzder et al., 1994), and extracts from *rad3* mutants are deficient in nucleotide-excision repair; a defect that can be complemented by purified  $\gamma$ *TFIIH* (Wang et al., 1994). Furthermore, cell extracts deficient in nucleotide-excision repair due to mutations in *ERCC2* or *ERCC3* can be complemented by human *TFIIH* (Drapkin et al., 1994a). It is not yet clear if individual components of *TFIIH* function in either transcription initiation or DNA repair, respectively, or if various components serve dual roles in both transcription and repair.

**TRANSCRIPTION FACTOR IIE** *TFIIE* has been purified to homogeneity from HeLa cells (Ohkuma et al, 1990; Inostroza et al, 1990), rat liver (Conaway et al., 1991a) and yeast (Sayre et al, 1992b) and it is composed of two different polypeptides. cDNA clones for both of the human *TFIIE* subunits have been isolated (Ohkuma et al., 1991; Peterson et al., 1991; Sumimoto et al., 1991). *TFIIE* and *TFIIH* are not always essential for transcription *in vitro*. A recent report showed that *TFIIE* and *TFIIH* were not required for transcription from the immunoglobulin heavy chain (IgH) promoter while transcription from the AdML promoter was absolutely dependent upon *TFIIE* and *TFIIH* (Parvin et al., 1992). Transcription in the absence of *TFIIE* and *TFIIH* from the IgH promoter is dependent upon the template being negatively supercoiled (Parvin and Sharp, 1993).

The precise role of *TFIIE* in transcription is not yet clear. Recent experiments, however, suggest that at least one thing *TFIIE* does is regulate *TFIIH*. The CTD-kinase and ATPase activities of *TFIIH* are both stimulated by the presence of *TFIIE* (Lu et al., 1992; Ohkuma and Roeder, 1994). Helicase activity, in contrast, is inhibited by *TFIIE* (Drapkin et al., 1994a). Stimulation of ATPase activity and inhibition of helicase activity by *TFIIE* appears to be inconsistent with the hypothesis that these two activities of *TFIIH* are tightly linked (Serizawa et al., 1993b; Roy et al., 1994). It is possible that the ATPase activity of *TFIIH* is provided by one subunit while the helicase activity is provided by another subunit and they are differentially regulated by *TFIIE*. Recombinant *ERCC2* and *ERCC3*, subunits of *TFIIH*, both have helicase activity, however only the helicase activity of *ERCC3* is inhibited by *TFIIE* (Drapkin et al., 1994a).

TFIIH-associated helicase activity may reside with ERCC3 while ATPase activity may reside with ERCC2 or another subunit of TFIIH. Differences in reagents and protocols offer a more trivial explanation for these discrepancies.

In vitro transcription experiments suggest that TFIIE and TFIIH play a role in the transition of an initiation complex to an elongation complex (Goodrich and Tjian, 1994). The production of a functional initiation complex, assayed by the formation of short abortive transcripts, was independent of supercoiling of the template or the presence of TFIIE and TFIIH. The transition to elongation (promoter clearance), however, was found to depend upon either TFIIE, TFIIH, and ATP hydrolysis or a supercoiled template. This indicates that the TFIIH-associated ATPase/helicase activity plays a role in promoter clearance. These experiments do not address the issue of phosphorylation of the CTD. If TFIIH contains the physiological CTD kinase then phosphorylation of the CTD may also occur during promoter clearance.

**TRANSCRIPTION FACTOR IIJ** TFIIJ appears to be an elongation factor (Zawel and Reinberg, pers. comm.). Synthesis of short transcripts do not require the action of TFIIJ. Efficient synthesis of RNA greater than 100 nucleotides in length, however, does require TFIIJ activity. TFIIJ has only been defined as a separate transcription factor in humans (Cortes et al., 1992). Curiously, TFIIJ is only required when TBP is used in lieu of TFIID, suggesting that TFIIJ may be a component of TFIID or a contaminate of the TFIID fraction.

### **Assembly of the Preinitiation Complex**

The formation of a functional preinitiation complex has been proposed to occur through the ordered assembly of the general transcription factors and RNA polymerase II onto promoter DNA (Conaway and Conaway, 1993; Zawel and Reinberg, 1993). This model of initiation complex formation is based upon DNase I footprinting techniques and gel shift assays using purified components. The first step in the formation of the preinitiation complex is the binding of TFIID or TBP to the TATA element of the promoter (Davison et al., 1983; Fire et al., 1984; Buratowski et al., 1989). Next, TFIIA binds and stabilizes TFIID binding to the promoter (Davison et al., 1983; Fire et al., 1984; Buratowski et al., 1989). As discussed above, however, TFIIA is not an essential factor in a minimal in vitro transcription system and is not required for formation of a preinitiation complex.

Gel shift assays indicate that TFIIB recognizes and binds to the TBP-DNA complex and then recruits an RNA polymerase II-TFIIF complex (Buratowski et al., 1989; Buratowski et al., 1991; Flores et al., 1991). Template challenge assays, though, performed under conditions required for transcription *in vitro*, indicate that TFIIB does not stably associate with the promoter in the absence of RNA polymerase II and TFIIF (Conaway et al., 1991b). Furthermore, TFIIB will stably associate with RNA polymerase II in the absence of a TBP-DNA complex (Tschochner et al., 1992; Wampler and Kadonaga, 1992). This suggests that a TFIIB-RNA polymerase II-TFIIF complex is recruited to promoters subsequent to TBP/TFIID binding.

Following the formation of the DNA-TBP-TFIIB-pol II-TFIIF complex, TFIIE, TFIIH, and TFIIF join (Cortes, et al., 1992; Flores et al., 1992). Gel shift assays indicate that TFIIE incorporation is necessary for subsequent recruitment of TFIIH, followed by incorporation of TFIIF. When TFIIE and TFIIH are incubated together much less TFIIE is required for the formation of a preinitiation complex than when TFIIE is incubated in the absence of TFIIH, suggesting that association of TFIIE and TFIIH with the preinitiation complex is cooperative. Consistent with this result is the observation of a direct interaction between the largest subunit of TFIIE and the ERCC3 subunit of TFIIH (Drapkin et al., 1994a; Maxon et al., 1994). TFIIE also appears to directly contact RNA polymerase II in the absence of DNA as determined by glycerol gradient sedimentation analysis (Flores et al., 1989). The interaction of TFIIE with polymerase, though, does not appear to be as strong as the interaction between TFIIF and polymerase.

While RNA polymerase II and general transcription factors can be readily separated, purified and then assembled onto promoter DNA in a sequential manner, much of the data described above suggests that RNA polymerase II and some of the general factors may associate with one another before assembling at the promoter. Evidence for such a complex was recently reported (Serizawa et al., 1994). In the absence of DNA, a transcriptionally active assembly of purified RNA polymerase II, TFIIB, TFIIE, TFIIF, and TFIIH can be immunoprecipitated with an antibody directed against the RNA polymerase II CTD. This suggests that there may be multiple ways to assemble a preinitiation complex *in vitro* and that the situation *in vivo* may be equally complex.

## Activation of Transcription

Selective transcription initiation by RNA polymerase II, under most conditions *in vitro*, requires the general transcription factors TBP, TFIIB, TFIIE, TFIIIF, and TFIIH (Conaway and Conaway, 1993; Zawel and Reinberg, 1993). Highly purified preparations of RNA polymerase II and general factors, however, are not responsive to regulatory signals. These regulatory signals come from sequence-specific DNA binding proteins (Mitchell and Tjian, 1989). An individual gene may have nearby binding sites, or enhancers, for many different activators. A typical transcriptional activator has a sequence-specific DNA binding domain and an activation domain. Additional factors, broadly defined as coactivators, are required for the response to these regulatory signals and are thought to serve as a functional and physical link to the general transcription apparatus. These include TBP-associated factors (TAFs) (Gill and Tjian, 1992; Pugh and Tjian, 1992), mediators (Flanagan et al., 1991), and adaptors (Berger et al., 1992; Pina et al., 1993).

The mediator is a partially purified fraction isolated from yeast that is required for stimulation of transcription in a reconstituted system (Flanagan et al., 1991). In the absence of activator, the mediator has no effect on transcription. It is not clear, though, what the mediator is since its components have not been identified or purified to homogeneity. Adaptors, on the other hand, were identified genetically in yeast (Berger et al., 1992). Mutations in *ADA2* and *ADA3* relieve the toxicity due to overexpression of the potent acidic activator GAL4-VP16. In the absence of *ADA2* (Berger et al., 1992) or *ADA3* (Pina et al., 1993) the ability to respond to some activators, but not others, is compromised both *in vivo* and *in vitro* suggesting that different activators may use different classes of adaptor molecules. Recent immunoprecipitation experiments identify *ADA2* as a direct target of VP16, a feature expected of coactivators (N. Silverman and L. Guarente, pers. comm.). In addition, mutations in *ADA2* can be suppressed by mutations in the gene encoding TFIIB, providing a functional link to the general transcription machinery (R. Knaus and L. Guarente, pers. comm.).

The best characterized coactivators are the proteins tightly associated with TBP. TBP together with the TAFs comprises the general transcription factor TFIID (Gill and Tjian, 1992; Pugh and Tjian, 1992). While recombinant TBP is able to mediate selective transcription by RNA polymerase II in reconstituted human and *Drosophila* systems, the multisubunit TFIID fraction is required for

response to activators (Hoey et al., 1990; Pugh and Tjian, 1990). Addition of TAFs, isolated by treatment of immunopurified TFIID with denaturants, to free TBP restores the ability of activators to stimulate transcription without dramatically influencing basal transcription (Dymlacht et al., 1991; Tanese et al., 1991).

In *Drosophila*, eight TAFs have been identified and cloned (Yokomori et al., 1993b; Hori and Carey, 1994; Verrijzer et al., 1994). Characterization of these proteins has revealed many features expected of factors with coactivating activity. A partial TFIID complex containing recombinant TBP, TAF110, and the majority of TAF250 will support activation by the glutamine-rich activator Sp1 (Weinzierl et al., 1993). Stimulation by Sp1 appears to be mediated, at least in part, via direct interactions with TAF110 (Hoey et al., 1993). Another TAF, TAF40, can be directly contacted by the acidic activation domain of VP16 as well as TFIIB (Goodrich et al., 1993). This result is particularly intriguing because VP16 can also directly contact TFIIB (Lin et al., 1991), suggesting multiple protein-protein interactions involved in activation. These results also indicate that different activators contact different TAFs, suggesting that there may be multiple pathways to leading to activation. Consistent with this is the recent observation that a cell line containing a temperature-sensitive mutation in TAF250 has gene-specific defects in transcription (Wang and Tjian, 1994).

It is important to note that while TAFs appear to be necessary for the optimal response to activators they are not sufficient. A highly purified reconstituted system will not respond to an activator even when native TFIID is used (Kretzschmar et al., 1994)). This indicates that additional components present in partially purified preparations of the other general factors are necessary for the response to activators.

While some of the factors involved in stimulating transcription initiation have been characterized and a few targets of transcriptional activators have been potentially identified, we still do not have a very good picture of the mechanism of activation. An increase in efficiency in any one of a number of steps in the assembly of the preinitiation complex could lead to an increase in transcription initiation. Early mechanistic studies on activation focused on TFIID, the first factor to bind the promoter. Dissociation rate measurements indicate that the gene-specific activator USF increases the binding efficiency of TFIID with the promoter by a cooperative mechanism (Sawadogo and Roeder, 1985). DNase I footprint analysis revealed cooperative interactions between

the ATF and GAL4 activator proteins with TFIID (Horikoshi et al., 1988a; Horikoshi et al., 1988b). Furthermore, a qualitative change in the binding pattern of TFIID was observed. ATF and GAL4 stimulated an increase in protection of the DNA extending beyond the start site of transcription, suggesting activators directly contact TFIID and induce a conformational change. Similar conformational changes in TFIID have recently been reported to be stimulated by the Epstein-Barr virus activator Zta in a TAF-dependent manner (Lieberman and Berk, 1994). This is consistent with the direct interactions that have been detected between activators and TAFs (see above) and a recent report indicating that TAF150 binds specifically to DNA sequences overlapping the start site of transcription (Verrijzer et al., 1994). Affinity chromatography has also detected a direct interaction between the acidic activator VP16 and TBP (Stringer et al., 1990). The significance of this interaction is supported by the observation that mutants in the activation domain of VP16 that are defective in activation are also defective in binding to TBP (Ingles et al., 1991).

TBP is not the only general initiation factor that can interact with activators under the appropriate conditions *in vitro*. TFIIB will selectively bind to the acidic activation domain of VP16 under conditions that do not permit efficient binding of TBP, indicating a stronger interaction between VP16 and TFIIB (Lin et al., 1991). This binding is also dependent upon a functional VP16 activation domain. Functional assays, however, offer the most compelling evidence that TFIIB is a target of transcriptional activators. Using immobilized template DNA and a transcription assay, Lin and Green (1991) were able to isolate complexes at various stages of assembly and determine what stage of preinitiation complex assembly the activator stimulated. They found that association of TFIIB with the DNA-TFIID complex was greatly enhanced by the presence of an activator, while TFIID was able to efficiently assemble on the template in the absence of activator. Recently, mutants in TFIIB have been identified that are unable to bind to VP16 (Roberts et al., 1993). While these TFIIB mutants function normally in basal transcription they are unable to support activated levels of transcription, supporting a functional interaction between TFIIB and acidic activators.

How can all of the observed physical and functional interactions between activators and components of the transcription initiation apparatus be reconciled into a coherent model of activation? A recent report may provide

some of the answers (Choy and Green, 1993). Immunoblot assays on purified transcription complexes were used to determine the influence of activators on the extent of preinitiation complex assembly. While either TFIID or TBP was sufficient for recruitment of TFIIB in the presence of an activator, recruitment of the remaining components (RNA polymerase II, TFII E, TFII F, and TFII H) required TFIID. Activation, therefore, appears to occur in at least two steps. First, TFIIB is recruited to a DNA-TFIID complex. Second, the remaining general transcription factors and RNA polymerase II are recruited through a mechanism requiring the action of TAFs. This second step, if it produces a conformational change in TFIID, would explain the qualitative change observed in DNA protection by TFIID in the presence of activators.

The ability of multiply-bound activators to stimulate transcription synergistically can also be viewed with greater understanding (Ptashne, 1988). Choy and Green (1993) observed that multiple activators did not have a synergistic effect on TFIIB recruitment. They did observe, however, a synergistic effect on complete preinitiation complex formation when TFIID was used. Synergy, therefore, may be, as some investigators have speculated, a result of activators contacting different factors. In support of multiple targets for activators, TFII F (Zhu et al., 1994) and TFII H (H. Xiao and J. Greenblatt, pers. comm.) have recently been reported to interact with some activators as well. While recruitment of TFIIB and the other general factors can be functionally dissected into discrete steps, these steps may, in fact, occur simultaneously. As described above, TFIIB may be associated with RNA polymerase II and other general factors before joining TFIID at the promoter.

Activation *in vivo*, of course, is even more complex than in systems reconstituted *in vitro* with partially purified factors. Transcription factors *in vivo* must compete with nucleosomes for binding to DNA. Activator proteins, however, may enhance the ability of general transcription factors to compete with nucleosomes for DNA binding and thus act as antirepressors (Croston et al., 1992; Adams and Workman, 1993). Similarly, a number of negative regulators have been identified that appear to interact with the general factors, particularly TFIID, preventing initiation complex formation (Drapkin et al., 1993). These effects can also be overcome by activators. Transcriptional activators, therefore, appear to have a hand in a number of events leading up to the formation of a complete preinitiation complex. Activators may not be limited, however, to stimulating preinitiation complex formation. There is no reason to

believe that steps subsequent to preinitiation complex formation can not be stimulated by activators.

### **The CTD and Transcription initiation**

The carboxy-terminal domain (CTD) is a highly conserved feature of the largest subunit of RNA polymerase II (Corden, 1990; Chao and Young, 1991; Young, 1991). The CTD contains 26-52 repeats, depending on the organism, of the consensus heptapeptide sequence sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. This unusual domain is absent from the largest subunit of RNA polymerases I and III, viral, and bacterial RNA polymerases. The CTD is essential for cell viability. Deletion mutants that remove most or all of the CTD are lethal to cells (Nonet et al., 1987; Allison et al., 1988; Bartolomei et al., 1988; Zehring et al., 1988). Genetic and biochemical studies indicate that the CTD plays a role in transcription initiation. CTD partial truncation mutations cause defects in growth and inducible gene expression in vivo (Nonet et al., 1987; Allison and Ingles, 1989; Scafe et al., 1990) and produce substantial defects in transcription initiation in vitro (Liao et al., 1991). This defect in gene expression in vivo has been mapped to upstream activating sequences (Allison and Ingles, 1989; Scafe et al., 1990).

A subset of the RNA polymerase II molecules in yeast and in mammalian cells have highly phosphorylated CTDs (Cadena and Dahmus, 1987; Kolodziej et al., 1990). In vivo, the second and fifth serines are the major sites of phosphorylation while tyrosine and threonine residues are phosphorylated to a lesser degree (Zhang and Corden, 1991; Baskaran et al., 1993). Phosphorylation of the CTD has been proposed to serve as a switch that regulates the transition of RNA polymerase II from initiation to elongation (Corden, 1990; Chao and Young, 1991; Peterson and Tjian, 1992). This model is consistent with the observation that it is the unphosphorylated form of RNA polymerase II that is recruited into the preinitiation complex (Laybourn and Dahmus, 1990; Lu et al., 1991; Kang and Dahmus, 1993). Furthermore, phosphorylation of the CTD is coincident with transcription initiation and it is the phosphorylated form of RNA polymerase II that is actively engaged in elongation (Cadena and Dahmus, 1987; Payne et al., 1989; Laybourn and Dahmus, 1990; Lu et al., 1991; Kang and Dahmus, 1993).

Several kinases have been identified that can phosphorylate the CTD in vitro (Cisek and Corden, 1989; Lee and Greenleaf, 1989; Feaver et al., 1991; Lu



et al., 1992; Serizawa et al., 1992; Baskaran et al., 1993; Dvir et al., 1993). A physiological role, however, for many of these kinases in phosphorylation of the CTD has not been demonstrated. A deletion of *CTK1*, the gene encoding the catalytic subunit of a putative CTD kinase purified from yeast, results in cells that are slow-growing and cold-sensitive (Lee and Greenleaf, 1989; Lee and Greenleaf, 1991). Significantly, phosphorylation of the CTD in these cells is dramatically reduced, suggesting a role for this kinase in CTD phosphorylation in vivo. As discussed above, the general transcription factor TFIID contains a CTD kinase activity (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992). The association of this kinase with TFIID and the apparent role TFIID plays in regulating this activity (Lu et al., 1992; Ohkuma and Roeder, 1994), makes this particular kinase an attractive candidate for an in vivo CTD kinase. Which of these kinases actually phosphorylates the CTD in vivo remains to be established.

A requirement for the CTD in transcription initiation in vitro has been quite variable. Transcription in vitro from the mouse DHFR promoter requires the CTD (Thompson et al., 1989; Buermeyer et al., 1992). In contrast, the CTD is dispensable for transcription in vitro from the AdML and *Drosophila* actin 5C promoters (Zehring et al., 1988; Kim and Dahmus, 1989; Thompson et al., 1989; Buratowski and Sharp, 1990; Buermeyer et al., 1992). In vivo studies indicate that the CTD is partially redundant and that certain promoters are more sensitive to CTD truncations than others (Scafe et al., 1990). It is not clear, though, if some promoters do not require a CTD at all in vivo. It is possible that the use of purified factors used in vitro obviates a requirement for the CTD in transcription. Indeed, yeast RNA polymerase II lacking the CTD is capable of selective-transcription when supplemented with purified transcription factors (Li and Kornberg, 1994). This CTD-less polymerase, however, is unable to restore activity to a crude nuclear extract in which a temperature-sensitive RNA polymerase II has been inactivated, suggesting the CTD is required to overcome some inhibitory activity present in crude extracts.

Phosphorylation of the CTD may serve to regulate interactions between the CTD and inhibitory factors present in crude extracts. A protein kinase inhibitor, H-8, that inhibits CTD phosphorylation abolishes transcription activity in crude yeast extracts (Li and Kornberg, 1994). H-8, however, had no effect on transcription when highly purified factors from yeast or rat were used (Serizawa et al., 1993a; Li and Kornberg, 1994). The requirement for the CTD may be

due, at least in part, to components of the TFIID fraction. Monoclonal antibodies directed against the CTD will specifically inhibit transcription initiation when native TFIID is used but not if recombinant TBP is used in reconstituted reactions (Conaway et al, 1992). The interplay of factors that determine the requirement for the CTD awaits further biochemical analysis. Towards this end Li and Kornberg (1994) have recently been able to reconstitute CTD-dependent transcription in a highly purified system by the addition two fractions. One of these fractions inhibits transcription reconstituted with CTD-less and wild-type polymerase while the other fraction restores activity of the wild-type polymerase but has only a small effect on the CTD-less polymerase.

### **An RNA Polymerase II Holoenzyme**

Suppression analysis of conditional CTD truncation mutations in yeast has been used to identify factors which influence CTD function (Nonet and Young, 1989; Thompson et al., 1993; Chapter 2). This genetic selection identified nine novel factors, SRBs (suppressor of RNA polymerase B), which specifically influence CTD function in vivo. Genetic and biochemical studies showed that the *SRB* genes encode positive and negative regulators of CTD function (Nonet and Young, 1989; Koleske et al., 1992; Thompson et al., 1993; Chapter 2; Chapter 3).

Purification of the SRB proteins from whole-cell extract led to the identification of a large multisubunit complex containing RNA polymerase II, the SRB proteins, and the general transcription factors yTFIIB, yTFIIF, and yTFIIH (Thompson et al., 1993; Koleske and Young 1994; Chapter 3). This RNA polymerase II holoenzyme is capable of site-specific initiation when supplemented with purified yTFIIE and recombinant TBP and transcription by this holoenzyme is responsive to activator protein. While the majority of SRB protein in cells is contained within the holoenzyme, only a small fraction of RNA polymerase II is found in the holoenzyme. The RNA polymerase II holoenzyme, however, appears to be the form of the enzyme recruited to most promoters in the cell (Chapter 4). Cells containing a temperature-sensitive mutation in the *SRB4* gene rapidly cease mRNA synthesis upon transfer to the restrictive temperature. These findings indicate a general requirement for SRB4 and the RNA polymerase II holoenzyme in transcription.

The holoenzyme probably escaped earlier detection because it only contains a small fraction of the RNA polymerase II in the cell. In addition, the

emphasis over the past fifteen years has been on the separation and purification of factors required for free RNA polymerase II to selectively initiate transcription. This RNA polymerase II holoenzyme is significant for a couple of reasons. First, purified yeast RNA polymerase II is not stimulated by transcriptional activators in the presence of general transcription factors alone (Flanagan et al., 1991; Flanagan et al., 1992). Some component of the holoenzyme, possibly the SRBs, is responsible for the ability to respond to activators. Indeed, an SRB-complex separated from polymerase stimulates basal and activated transcription when added to purified factors and RNA polymerase II (Kim et al., 1994). Second, while others have postulated that RNA polymerase II enters the initiation complex associated with a subset of the general transcription factors (see above), this work provides substantial support for this model as a general mechanism of initiation complex assembly.

The holoenzyme model of preinitiation complex formation is significantly different than the traditional step-wise assembly model that has been proposed. The holoenzyme model of preinitiation complex formation proposes that RNA polymerase II, tightly associated with TFIIB, TFIIF, TFIIH, and additional factors, assembles onto a TFIID containing promoter. TFII E then joins the complex. TFII E can, however, interact with RNA polymerase II and TFIIH in the absence of DNA (Flores et al., 1989; Maxon et al., 1994), suggesting that TFII E may also assemble at the promoter as a component of the holoenzyme. The step-wise assembly model of preinitiation complex formation proposes that TFIIB first recognizes a TFIID containing promoter, then RNA polymerase II, in association with TFIIF, assembles at the promoter, followed by TFII E and TFIIH, in that order. The holoenzyme model of preinitiation complex formation is consistent with much of the earlier data. The general factors TFIIB, TFII E, TFIIF, and TFIIH can all associate with RNA polymerase II in vitro in the absence of DNA (see above). Furthermore, template challenge assays performed under conditions required for transcription in vitro suggest that TFIIB associates with RNA polymerase II and TFIIF before joining TFIID at the promoter (Conaway et al., 1991b). In contrast, gel shift assays indicate that TFIIB alone is able to assemble with TFIID at promoters (Buratowski et al., 1989; Flores et al., 1991). Conditions for gel shift assays, however, may stabilize weak interactions (Fried and Crothers, 1981). Such weak interactions may be very physiologically relevant but may only occur in the context of a greater RNA polymerase II containing preinitiation complex.

The holoenzyme has additional implications for the mechanism of activation. Choy and Green (1993) have recently dissected activation into two steps. First, TFIIB is recruited to a TFIID containing promoter (TBP, however, works equally well in place of TFIID). Second, the remaining general transcription factors and RNA polymerase II are recruited through a mechanism requiring the action of TAFs (TFIID is required for this step). The existence of the holoenzyme suggests that while activation can be functionally dissected into discrete steps, these steps may, in fact, occur simultaneously. Efficient recruitment of the holoenzyme may involve the concerted action of TFIIB and additional regulatory factors, possibly SRBs, in the holoenzyme together with TAFs at the promoter.

### **My Contributions to this Project**

When I joined the Young laboratory in 1990 Tony Koleske was characterizing *SRB2*, the first gene identified as an extragenic suppressor of CTD truncation mutations. In order to identify additional components of the transcription machinery that influence CTD function, I, with the assistance of Sara Okamura, isolated a large collection of suppressors of a CTD truncation mutation. Initial genetic analysis identified additional dominant alleles of *SRB2*, dominant suppressing alleles in *SRB4*, *SRB5*, and *SRB6*, and five complementation groups among the recessive suppressors. At this point I focused my efforts on the further characterization of *SRB4*, *SRB5*, and *SRB6*, while other members of the lab (C. Hengartner, S.-M. Liao, and J. Zhang) eventually began working on the recessive suppressing alleles.

The dominant suppressing alleles in *SRB4*, *SRB5*, and *SRB6* suppress all of the conditional and auxotrophic phenotypes associated with CTD truncation mutations. In addition, these alleles, *SRB4-1*, *SRB5-1*, and *SRB6-1*, generally do not suppress the conditional phenotypes associated with RNA polymerase II mutations outside of the CTD. This is the same type of suppression specificity shown by *SRB2-1*, and this argues that *SRB2*, *SRB4*, *SRB5*, *SRB6*, and the CTD are involved in the same process in transcription initiation. For this reason I cloned and sequenced *SRB4-1*, *SRB5-1*, *SRB6-1*, and their wild-type counterparts. At this time I also constructed a wild-type genomic library which was later used by other members of the laboratory to clone the additional *SRBs* represented by the recessive suppressing alleles.

Clones of *SRB4*, *SRB5*, and *SRB6* permitted the purification of recombinant protein and the production of antibodies. Yeast strains containing complete knockouts of each of these genes were also generated. *SRB4* and *SRB6* are essential for cell viability. *SRB5*, like *SRB2*, is not essential, but cells lacking the gene exhibit the slow growth, cold-sensitive, and temperature-sensitive phenotypes characteristic of CTD truncations.

I took advantage of the fact that *SRB5* is not an essential gene to investigate the activity of SRB5 in vitro. Previous studies by Tony Koleske had revealed that SRB2 is required for efficient basal and activated transcription in vitro and that SRB2 is a component of stable preinitiation complexes. I made nuclear extracts from wild-type and *srb5Δ1* cells and tested their ability to direct synthesis of a specific transcript. Extracts made from cells lacking SRB5 were unable to synthesize significant levels of transcript in both the presence and absence of activator protein. I was able to restore activity to near wild-type levels with the addition of recombinant SRB2 and SRB5; SRB5 alone failed to complement. Western blot analysis revealed that the level of SRB2 protein is greatly reduced in extracts prepared from *srb5Δ1* cells. A template commitment assay was used to show that SRB5 promotes efficient preinitiation complex formation, probably through incorporation into the complex. These results demonstrate that SRB5, like SRB2, is required for efficient basal and activated transcription in vitro and that SRB5 is a component of stable preinitiation complexes.

The fact that SRB2 and SRB5 appeared to be components of the preinitiation complex and that dominant mutations in *SRB2*, *SRB4*, *SRB5*, and *SRB6* all had exactly the same effect on the viability or growth phenotypes of cells that contained CTD truncations led Tony and I to propose that these four proteins may be components of a multisubunit complex. Immunoprecipitation experiments supported this hypothesis. In collaboration with Tony, cells were constructed that produced functional, epitope-tagged SRB4, SRB5, or SRB6 protein, nuclear extracts were prepared, and immunoprecipitations performed. SRB2 coprecipitated in each case.

Using antibodies that Tony and I had generated against SRB2, SRB4, SRB5, and SRB6, Tony followed the elution profile of these proteins through a series of purification steps. Most of the SRB protein in cells was found to be tightly associated with a large multisubunit complex. Additional Western blot analysis and in vitro transcription assays revealed that this complex also

contained RNA polymerase II, the general transcription factors  $\gamma$ TFIIB,  $\gamma$ TFIIF, and  $\gamma$ TFIIH, and substoichiometric amounts of TBP. This RNA polymerase II holoenzyme is capable of site-specific initiation when supplemented with purified  $\gamma$ TFIIE and recombinant TBP and is responsive to activators.

Tony proposed that the RNA polymerase II holoenzyme is a form of the enzyme readily recruited to promoters in the cell. However, because only a small fraction of RNA polymerase II in cells is found in the holoenzyme, it was unclear if the holoenzyme was preferentially recruited to some promoters while free RNA polymerase II and general factors were recruited in a step-wise fashion to others. I reasoned, though, that because the holoenzyme contains most of the SRB protein in the cell, the fraction of genes that employ the RNA polymerase II holoenzyme in vivo could be investigated by studying the effects of a temperature-sensitive mutation in the *SRB4* gene on transcription of mRNA. Upon transfer to the restrictive temperature there is a rapid and general shutdown of mRNA synthesis. These findings suggest a general requirement for SRB4 and the RNA polymerase II holoenzyme in transcription.

## References

- Adams, C.C., and Workman, J.L. (1993). Nucleosome displacement in transcription. *Cell* 72, 305-308.
- Allison, L.A., and Ingles, C.J. (1989). Mutations in RNA polymerase II enhance or suppress mutations in GAL4. *Proc. Natl. Acad. Sci.* 86, 2794-2798.
- Allison, L. A., Moyle, M., Shales, M., and Ingles, C. J. (1985). Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. *Cell* 42, 599-610.
- Allison, L.A., Wong, J.K., Fitzpatrick, V.D., Moyle, M., and Ingles, C.J. (1988). The C-terminal domain of the largest subunit of RNA polymerase II of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and mammals: a conserved structure with an essential function. *Mol. Cell. Biol.* 8, 321-329.
- Aso, T., and Vasavada, H.A., Kawaguchi, T., Germino, F.J., Ganguly, S., Kitajima, S., Weissman, S.M., and Yasukochi, Y. (1992). Characterization of cDNA for the large subunit of the transcription initiation factor TFIIF. *Nature* 355, 461-464.
- Bardwell, L., Bardwell, A.J., Feaver, W.J., Svejstrup, J.Q., Kornberg, R.D., and Friedberg, E.C. (1994). Yeast RAD3 protein binds directly to both SSL2 and SSL1 proteins: Implications for the structure and function of transcription/repair factor b. *Proc. Natl. Acad. Sci.* 91, 3926-3930.
- Bartolomei, M.S., Halden, N.F., Cullen, C.R., and Corden, J.L. (1988). Genetic analysis of the repetitive carboxyl-terminal domain of the largest subunit of RNA polymerase II. *Mol. Cell. Biol.* 8, 330-339.
- Baskaran, R., Dahmus, M., and Wang, J.Y.J. (1993). Tyrosine phosphorylation of mammalian RNA polymerase II carboxyl-terminal domain. *Proc. Natl. Acad. Sci.* 90, 11167-11171.
- Bengal, E., Flores, O., Krauskopf, A., Reinberg, D., and Aloni, J. (1991). Role of the mammalian transcription factors IIF, IIS, and IIX during elongation by RNA polymerase II. *Mol. Cell. Biol.* 11, 1195-1206.
- Berger, S.L., Pina, B., Silverman, N.S., Marcus, G.A., Agapite, J., Regier, J.L., Triezenberg, S.J., and Guarente, L. (1992). Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. *Cell* 70, 251-265.
- Berroteran, R.W., Ware, D.E., and Hampsey, M. (1994). The *sua8* suppressors of *Saccharomyces cerevisiae* encode replacements of conserved residues with the largest subunit of RNA polymerase II and affect transcription start site selection similarly to *sua7* (TFIIB) mutations. *Mol. Cell. Biol.* 14, 226-237.

Buermeyer, A.B., Thompson, N.E., Strasheim, L.A., Burgess, R.R., and Farnham, P.J. (1992). The HIP1 initiator element plays a role in determining the in vitro requirement of the dihydrofolate reductase gene promoter for the C-terminal domain of RNA polymerase II. *Mol. Cell. Biol.* *12*, 2250-2259.

Buratowski, S., Hahn, S., Guarente, L., and Sharp, P. A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* *56*, 549-561.

Buratowski, S., Hahn, S., Sharp, P. A., and Guarente, L. (1988). Function of a yeast TATA element-binding protein in a mammalian transcription system. *Nature* *334*, 37-42.

Buratowski, S., and Sharp, P.A. (1990). Transcription initiation complexes and upstream activation with RNA polymerase II lacking the C-terminal domain of the largest subunit. *Mol. Cell. Biol.* *10*, 5562-5564.

Buratowski, S., Sopta, M., Greenblatt, J., and Sharp, P.A. (1991). RNA polymerase II-associated proteins are required for a DNA conformation change in the transcription initiation complex. *Proc. Natl. Acad. Sci.* *88*, 7509-7513.

Cadena, D.L., and Dahmus, M.E. (1987). Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. *J. Biol. Chem.* *262*, 12468-12474.

Cavallini, B., Huet, J., Plassat, J.-L., Sentenac, A., Egly, J.-M., and Chambon, P. (1988). A yeast activity can substitute for the HeLa cell TATA box factor. *Nature* *334*, 77-80.

Chang, C.-h., Kostrub, C.F., and Burton, Z.F. (1993). RAP30/74 (transcription factor IIF) is required for promoter escape by RNA polymerase II. *J. Biol. Chem.* *268*, 20482-20489.

Chao, D., and Young, R.A. (1991). Tailored tails and transcription initiation: the carboxyl terminal domain of RNA polymerase II. *Gene Expression* *1*, 1-4.

Chasman, D.I., Flaherty, K.M., Sharp, P.A., and Kornberg, R.D. (1993). Crystal structure of yeast TATA-binding protein and model for interaction with DNA. *Proc. Natl. Acad. Sci.* *90*, 8174-8178.

Chambon, P. (1975). Eukaryotic nuclear RNA polymerases. *Ann. Rev. Biochem.* *44*, 613-638.

Choy, B., and Green, M.R. (1993). Eukaryotic activators function during multiple steps of preinitiation complex assembly. *Nature* *366*, 531-536.

Cisek, L.J., and Corden, J.L. (1989). Phosphorylation of RNA polymerase by the murine homologue of the cell-cycle control protein cdc2. *Nature* *339*, 679-684.



Conaway, J.W., and Conaway, R.C. (1990). An RNA polymerase II transcription factor shares functional properties with *Escherichia coli*  $\sigma^{70}$ . *Science* *248*, 1550-1553.

Conaway, J.W., Bradsher, J.N., and Conaway, R.C. (1992a). Mechanism of assembly of the RNA polymerase II preinitiation complex: transcription factors  $\delta$  and  $\epsilon$  promote stable binding of the transcription apparatus to the initiation element. *J. Biol. Chem.* *267*, 10142-10148.

Conaway, R.C., Bradsher, J.N., and Conaway, J.W. (1992b). Mechanism of assembly of the RNA polymerase II preinitiation complex: evidence for a functional interaction between the carboxyl-terminal domain of the largest subunit of RNA polymerase II and a high molecular mass form of the TATA factor. *J. Biol. Chem.* *267*, 8464-8467.

Conaway, R.C., and Conaway, J.W. (1993). General initiation factors for RNA polymerase II. *Ann Rev. Biochem.* *62*, 161-190.

Conaway, J.W., Hanley, J.P., Garret, K.P., and Conaway, R.C. (1991a). Transcription initiated by RNA polymerase II and transcription factors from liver: structure and function of transcription factors  $\epsilon$  and  $\tau$ . *J. Biol. Chem.* *266*, 7804-7811.

Conaway, R.C., Garrett, K.P., Hanley, J.P., and Conaway, J.W. (1991b). Mechanism of promoter selection by RNA polymerase II: mammalian transcription factors  $\alpha$  and  $\beta\gamma$  promote entry of polymerase into the preinitiation complex. *Proc. Natl. Acad. Sci.* *88*, 6205-6209.

Conaway, J.W., Reines, D., and Conaway, R.C. (1990). Transcription initiation initiated by RNA polymerase II and purified transcription factors from liver. *J. Biol. Chem.* *265*, 7552-7558.

Corden, J. L. (1990). Tails of RNA Polymerase II. *Trends Biochem. Sci.* *15*, 383-387.

Cortes, P. Flores, O., and Reinberg, D. (1992). Factors involved in specific transcription by mammalian RNA polymerase II: Purification and analysis of transcription factor IIA and identification of transcription factor IIJ. *Mol. Cell. Biol.* *12*, 413-421.

Croston, G.E., Laybourn, P.J., Paranjape, S.M., and Kadonaga, J.T. (1992). Mechanism of transcriptional antirepression by GAL4-VP16. *Genes Dev* *6*, 2270-2281.

Davison, B. L., Egly, J. M., Mulvihill, E. R., and Chambon, P. (1983). Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences. *Nature* *301*, 680-686.

Dejong, J., and Roeder, R.G. (1993). A single cDNA, hTFIIA/ $\alpha$ , encodes both the p35 and p19 subunits of human TFIIA. *Genes Dev.* 7, 2220-2234.

Dignam, J.D., Martin, P.L., Shastri, B.S., and Roeder, R.G. (1983). Eukaryotic gene transcription with purified components. *Meth. Enzymol.* 101, 582-598.

Drapkin, R., Merino, A., and Reinberg, D. (1993). Regulation of RNA polymerase II transcription. *Curr. Opin. Cell Biol.* 5, 469-476.

Drapkin, R., Reardon, J.T., Ansari, A., Huang, J.-C., Zawel, L., Ahn, K., Sancar, A., and Reinberg, D. (1994a). Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. *Nature* 368, 769-772.

Drabkin, R., Sancar, A., and Reinberg, D. (1994b). Where transcription meets repair. *Cell* 77, 9-12.

Dvir, A., Stein, L.Y., Calore, B.L., and Dynan, W.S. (1993). Purification and characterization of a template-associated protein kinase that phosphorylates RNA polymerase II. *J. Biol. Chem.* 268, 10440-10447.

Dynlacht, B.D., Hoey, T., and Tjian, R. (1991). Isolation of coactivators associated with the TATA-binding protein that mediates transcriptional activation. *Cell* 66, 563-576.

Eisenmann, D.M., Dollard, C., and Winston, F. (1989). *SPT15*, the gene encoding the yeast TATA binding factor TFIIID, is required for normal transcription initiation in vivo. *Cell* 58, 1183-1191.

Fassler, J.S., and Winston, F. (1988). Isolation and analysis of a novel class of suppressor of Ty insertion mutations in *Saccharomyces cerevisiae*. *Genetics* 118, 203-212.

Feaver, W.J., Gileadi, O., Li, Y., and Kornberg, R.D. (1991). CTD kinase associated with yeast RNA polymerase II initiation factor b. *Cell* 67, 1223-1230.

Feaver, W.J., Svejstrup, J.Q., Bardwell, L., Bardwell, A.J., Buratowski, S., Gulyas, K.D., Donahue, T.F., Friedberg, E.C., and Kornberg, R.D. (1993). Dual roles of a multiprotein complex from *S. cerevisiae* in transcription and DNA repair. *Cell* 75, 1379-1387.

Finkelstein, A., Kostrub, C.F., Li, J., Chavez, D.P., Wang, B.Q. Fang, S.M., Greenblatt, J., and Burton, Z.F. (1992). A cDNA encoding RAP74, a general initiation factor for transcription by RNA polymerase II. *Nature* 355, 464-467.

Fire, A., Samuels, M., and Sharp, P.A. (1984). Interactions between RNA polymerase II, factors, and template leading to accurate transcription. *J. Biol. Chem.* 259, 2509-2516.

Flanagan, P.M., Kelleher, R.J., Sayre, M.H., Tschochner, H., and Kornberg, R.D. (1991). A mediator required for activation of RNA polymerase II transcription in vitro. *Nature* 350, 436-438.

Flanagan, P.M., Kelleher, R.J., Tschochner, H., Sayre, M.H., and Kornberg, R.D. (1992). Simple derivation of TFIID-dependent RNA polymerase II transcription systems from *Schizosaccharomyces pombe* and other organisms, and factors required for transcriptional activation. *Proc. Natl. Acad. Sci.* 89, 7659-7663.

Flores, O., Lu, H., and Reinberg, D. (1992). Factors involved in specific transcription by mammalian RNA polymerase II. *J. Biol. Chem.* 267, 2786-2798.

Flores O., Lu, H., Killeen, M., Greenblatt, J., Burton, Z.F., and Reinberg, D. (1991). The small subunit of transcription factor IIF recruits RNA polymerase II into the preinitiation complex. *Proc. Natl. Acad. Sci.* 88, 9999-10003.

Flores, O., Maldonado, E., and Reinberg, D. (1989). Factors involved in specific transcription by mammalian RNA polymerase II. *J. Biol. Chem.* 264, 8913-8921.

Fried, M., and Crothers, D.M. (1981). Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nuc. Acids Res.* 9, 6505-6525.

Gill, G., and Tjian, R. (1992). Eukaryotic coactivators associated with the TATA box binding protein. *Curr. Opin. Gen. Dev.* 2, 236-242.

Goodrich, J.A., Hoey, T., Thut, C.J., Admon, A., and Tjian, R. (1993). *Drosophila* TAF<sub>II</sub>40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell* 75, 519-530.

Goodrich, J.A., and Tjian, R. (1994). Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. *Cell* 77, 145-156.

Greenblatt, J. (1991). RNA polymerase-associated transcription factors. *Trends Biochem. Sci.* 16, 408-411.

Guzder, S.N., Qui, H., Sommers, C.H., Sung, P., Prakash, L., and Prakash, S. (1994). DNA repair gene *RAD3* of *S. cerevisiae* is essential for transcription by RNA polymerase II. *Nature* 367, 91-94.

Hahn, S., Buratowski, S., Sharp, P.A., and Guarente, L. (1989). Isolation of the gene encoding the yeast TATA binding protein TFIID: a gene identical to the *SPT15* suppressor of Ty element insertions. *Cell* 58, 1173-1181.

Hernandez, N. (1993). TBP, a universal eukaryotic transcription factor? *Genes Dev.* 7, 1291-1308.

Hoey, T., Dynlacht, B.D., Peterson, M.G., Pugh, B.F., and Tjian, R. (1990). Isolation and characterization of the *Drosophila* gene encoding the TATA box binding protein, TFIID. *Cell* 61, 1179-1186.

Hoey, T., Weinzierl, R.O.J., Gill, G., Chen, J.-L., Dynlacht, B.D., and Tjian, R. (1993). Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. *Cell* 72, 247-260.

Hori, R., and Carey, M. (1994). The role of activators in assembly of RNA polymerase II transcription complexes. *Curr. Opin. Gen. Dev.* 4, 236-244.

Horikoshi, M., Carey, M.F., Kadidani, Hitoshi, Roeder, R.G. (1988a). Mechanism of action of a yeast activator: Direct effect of GAL4 derivatives on mammalian TFIID-promoter interaction. *Cell* 54, 665-669.

Horikoshi, M., Hai, T., Lin, Y.-S., Green, M.R., and Roeder, R.G. (1988b). Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. *Cell* 54, 1033-1042.

Humbert, S., Hanneke, V., Lutz, Y., Hoeijmakers, J.H.J., Egly, J.-M., and Moncollin, V. (1994). p44 and p34 subunits of the BTF2/TFIIH transcription factor have homologies with SSL1, a yeast protein involved in DNA repair. *EMBO* 13, 2393-2398.

Ingles, C.J., Shales, M., Cress, W.D., Triezenberg, S.J., and Greenblatt, J. (1991). Reduced binding of TFIID to transcriptionally compromised mutants of VP16. *Nature* 351, 588-590.

Inostroza, J., Flores, O., and Reinberg, D. (1990). Factors involved in specific transcription by mammalian RNA polymerase II: purification and functional analysis of general transcription factor IIE. *J. Biol. Chem.* 266, 9304-9308.

Kang, M.E., and Dahmus, M.E. (1993). RNA polymerase IIA and IIO have distinct roles during transcription from the TATA-less murine dihydrofolate reductase promoter. *J. Biol. Chem.* 268, 25033-25040.

Killeen, M.T., and Greenblatt, J. (1992). The general transcription factor RAP30 binds to RNA polymerase II and prevents it from binding nonspecifically to DNA. *Mol. Cell. Biol.* 12, 30-37.

Kim, J.L., Nikolov, D.B., and Burley, S.K. (1993). Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* 365, 520-527.

Kim, W.-Y., and Dahmus, M.E. (1989). The major late promoter of adenovirus-2 is accurately transcribed by RNA polymerases IIO, IIA, and IIB. *J. Biol. Chem.* 264, 3169-3176.

Kim, Y., Geiger, J.H., Hahn, S., and Sigler, P.B. (1993). Crystal structure of a yeast TBP/TATA-box complex. *Nature* 365, 512-520.

Kim, Y.-J., Bjorklund, S., Li, Y., Sayre, M.H., and Komberg, R.D. (1994). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* 77, 599-608.

- Kolodziej, P.A., Woychik, N., Liao, S.-M., and Young, R.A. (1990). RNA polymerase II subunit composition, stoichiometry, and phosphorylation. *Mol. Cell. Biol.* *10*, 1915-1929.
- Kolodziej, P.A., and Young, R.A. (1989). RNA polymerase II subunit RPB3 is an essential component of the mRNA transcription apparatus. *Mol. Cell. Biol.* *9*, 5387-5394.
- Koleske, A.J., Buratowski, S., Nonet, M., and Young, R.A. (1992). A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. *Cell* *69*, 883-894.
- Koleske, A.J., and Young, R.A. (1994). An RNA polymerase II holoenzyme responsive to activators. *Nature* *368*, 466-469.
- Kretzschmar, M., Stelzer, G., Roeder, R.G., and Meisterernst, M. (1994). RNA polymerase II cofactor PC2 facilitates activation of transcription by GAL4-AH in vitro. *Molec. Cell. Biol.* *14*, 3927-3937.
- Laybourn, P.J., and Dahmus, M.E. (1990). Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promoter and before the initiation of transcription. *J. Biol. Chem.* *265*, 13165-13173.
- Lee, J.M., and Greenleaf, A.L. (1989). A protein kinase that phosphorylates the C-terminal repeat domain of the largest subunit of RNA polymerase II. *Proc. Natl. Acad. Sci.* *86*, 3624-3628.
- Lee, J.M., and Greenleaf, A.L. (1991). CTD kinase large subunit is encoded by CTK1, a gene required for normal growth of *Saccharomyces cerevisiae*. *Gene Expr.* *1*, 149-167.
- Lewis, M. K., and Burgess, R. R. (1982). Eukaryotic RNA polymerases. *The Enzymes* *15*, 109-153.
- Li, Y., Flanagan, P.M., Tschochner, H., and Kornberg, R.D. (1994). RNA polymerase II initiation factor interactions and transcription start site selection. *Science* *263*, 805-807.
- Li, Y., and Kornberg, R.D. (1994). Interplay of positive and negative effectors in function of the C-terminal repeat domain of RNA polymerase II. *Proc. Natl. Acad. Sci.* *91*, 2362-2366.
- Liao, S.-M., Taylor, I.C.A., Kingston, R.E., and Young, R.A. (1991). RNA polymerase II carboxy-terminal domain contributes to the response to multiple acidic activators in vitro. *Genes Dev.* *5*, 2431-2440.
- Lieberman, P.M., and Berk, A.J. (1994). A mechanism for TAFs in transcriptional activation: activation domain enhancement of TFIID-TFIIA-promoter DNA complex formation. *Genes Dev.* *8*, 995-1006.

- Lin, Y.-S., and Green, M.R. (1991). Mechanism of action of an acidic transcriptional activator in vitro. *Cell* **64**, 971-981.
- Lin, Y.-S., Maldonado, E., Reinberg, D., and Green, M.R. (1991). Binding of general transcription factor TFIIB to an acidic activating region. *Nature* **353**, 569-571.
- Lu, H., Flores, O., Weinmann, R., and Reinberg, D. (1991). The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. *Proc. Natl. Acad. Sci.* **88**, 10004-10008.
- Lu, H., Zawel, L., Fisher, L., Egly, J.-M., and Reinberg, D. (1992). Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. *Nature* **358**, 641-645.
- Ma, D., Watanabe, H., Mermelstein, F., Admon, A., Oguri, K., Sun, X., Wada, T., Imai, T., Shiroya, T., Reinberg, D., and Handa, H. (1993). Isolation of a cDNA encoding the largest subunit of TFIIA reveals functions important for activated transcription. *Genes Dev.* **7**, 2246-2257.
- Manley, J.L., Fire, A., Cano, A., Sharp, P.A., and Gelfand, M.L. (1980). DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. *Proc. Natl. Acad. Sci.* **77**, 3855-3859.
- Martindale, D. (1990). A conjugation specific gene (*cnjC*) from *Tetrahymena* encodes a protein homologous to yeast RNA polymerase subunits (*RPB3*, *RPC40*) and similar to a portion of the prokaryotic RNA polymerase alpha subunit (*rpoA*). *Nucleic Acids Res.* **18**, 2952-2960.
- Matsui, T., Segall, J., Weil, P.A., and Roeder, R.G. (1980). Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. *J. Biol. Chem.* **255**, 11992-11996.
- Maxon, M.E., Goodrich, J.A., and Tjian, R. (1994). Transcription factor IIE binds preferentially to RNA polymerase IIa and recruits TFIIH: a model for promoter clearance. *Genes Dev.* **8**, 515-524.
- McClure, W.R. (1985). Mechanism and control of transcription initiation in prokaryotes. *Ann. Rev. Biochem.* **54**, 171-204.
- McCracken, S., and Greenblatt, J. (1991). Related RNA polymerase-binding region in human RAP30/74 and *Escherichia coli*  $\sigma^{70}$ . *Science* **253**, 900-902.
- McKune, K., Richards, K.L., Edwards, A.M., and Young, R.A. (1993). RPB7, one of two dissociable subunits of yeast RNA polymerase II, is essential for cell viability. *Yeast* **9**, 295-299.
- Mitchell, P.J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371-378.

- Nakajima, N., Horikoshi, M., and Roeder, R.G. (1988). Factors involved in specific transcription by mammalian RNA polymerase II: Purification, genetic specificity, and TATA box-promoter interactions of TFIID. *Mol. Cell. Biol.* **8**, 4028-4040.
- Nikolov, D.B., Hu, S.-H., Lin, J., Gasch, A., Hoffman, A., Horikoshi, M., Chua, N.-H., Roeder, R.G., and Burley, S.K. (1992). Crystal structure of TFIID TATA-box binding protein. *Nature* **360**, 40-46.
- Nonet, M., Sweetser, D., and Young, R.A. (1987). Functional redundancy and structural polymorphism in the largest subunit of RNA polymerase II. *Cell* **50**, 909-915.
- Nonet, M.L., and Young, R.A. (1989). Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. *Genetics* **123**, 715-724.
- Ohkuma, Y., and Roeder, R.G. (1994). Regulation of TFIIH ATPase and kinase activities by TFII E during active initiation complex formation. *Nature* **368**, 160-163.
- Ohkuma, Y., Sumimoto, H., Horikoshi, M., and Roeder, R.G. (1990). Factors involved in specific transcription by mammalian RNA polymerase II: purification and characterization of general transcription factor IIE. *Proc. Natl. Acad. Sci.* **87**, 9163-9167.
- Ohkuma, Y., Sumimoto, H., Hoffman, A., Shimasaki, S., Horikoshi, M., and Roeder, R.G. (1991). Structural motifs and potential s homologies in the large subunit of human general transcription factor TFII E. *Nature* **354**, 398-401.
- Parvin, J.D., and Sharp, P.A. (1993). DNA topology and a minimal set of basal factors for transcription by RNA polymerase II. *Cell* **73**, 533-540.
- Parvin, J.D., Timmers, H.Th.M., and Sharp, P.A. (1992). Promoter specificity of basal transcription factors. *Cell* **68**, 1135-1144.
- Payne, J.M., Laybourn, P.J., and Dahmus, M.E. (1989). The transition of RNA polymerase II from initiation to elongation is associated with phosphorylation of the carboxyl-terminal domain of subunit Ila. *J. Biol. Chem.* **264**, 19621-19629.
- Peterson, M.G., Inostroza, J., Maxon, M.E., Flores, O., Admon, A., Reinberg, D., and Tjian, R. (1991). Structure and functional properties of human general transcription factor IIE. *Nature* **354**, 369-373.
- Peterson, M.G., and Tjian, R. (1992). The tell-tail trigger. *Nature* **358**, 620-621.
- Pina, B., Berger, S., Marcus, G.A., Silverman, N., Agapite, J., and Guarente, L. (1993). *ADA3*: a gene, identified by resistance to GAL4-VP16, with properties similar to and different from those of *ADA2*. *Molec. Cell. Biol.* **13**, 5981-5989.

Pinto, I., Ware, D.E., and Hampsey, M. (1992). The yeast *SUA7* gene encodes a homolog of human transcription factor TFIIB and is required for normal start site selection in vivo. *Cell* 68, 977-988.

Price, D.H., Sluder, A.E., and Greenleaf, A.L. (1989). Dynamic interaction between a *Drosophila* transcription factor and RNA polymerase II. *Mol. Cell Biol.* 9, 1465-1475.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. *Nature* 335, 683-689.

Pugh, B.F., and Tjian, R. (1990). Mechanism of transcriptional activation by Sp1: Evidence for coactivators. *Cell* 61, 1187-1197.

Pugh, B.F., and Tjian, R. (1992). Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. *J. Biol. Chem.* 267, 679-682.

Qui, H., Park, E., Prakash, L., and Prakash, S. (1993). The *Saccharomyces cerevisiae* DNA repair gene *RAD25* is required for transcription by RNA polymerase II. *Genes Dev.* 7, 2161-2171.

Ranish, J.A., Lane, W.S., and Hahn, S. (1992). Isolation of two genes that encode subunits of the yeast transcription factor IIA. *Science* 255, 1127-1129.

Roberts, S.G.E., Ha, I., Maldonado, E., Reinberg, D., and Green, M.R. (1993). Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation. *Nature* 363, 741-744.

Roeder, R. (1976). Eukaryotic nuclear RNA polymerases. In *RNA polymerase*, Losick, R. and Chamberlin, M.J., Eds. (Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York).

Roeder, R. G., and Rutter, W. J. (1969). Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. *Nature* 224, 234-237.

Roy, R., Schaeffer, L., Humbert, S., Vermeulen, W., Weeda, G., and Egly, J.-M. (1994). The DNA-dependent ATPase activity associated with the class II basic transcription factor BTF2/TFIIH. *J. Biol. Chem.* 269, 9826-9832.

Samuels, M., Fire, A., and Sharp, P.A. (1982). Separation and characterization of factors mediating accurate transcription by RNA polymerase II. *J. Biol. Chem.* 257, 14419-14427.

Sawadogo, M., and Roeder, R.G. (1985). Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell* 43, 165-175.

Sayre, M. H., Tschochner, H., and Kornberg, R. D. (1992a). Reconstitution of transcription with five purified initiation factors and RNA polymerase II from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 267, 23376-23382.



- Sayre, M. H., Tschochner, H., and Kornberg, R. D. (1992b). Purification and properties of *Saccharomyces cerevisiae* RNA polymerase II general initiation factor  $\alpha$ . *J. Biol. Chem.* *267*, 23383-23387.
- Scafe, C., Chao, D., Lopes, J., Hirsch, J.P., Henry, S., and Young, R.A. (1990). RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. *Nature* *347*, 491-494.
- Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Verkmeulen, W., Hoeijmakers, J.H.J., Chambon, P., and Egly, J.M. (1993). DNA repair helicase: A component of BTF2 (TFIIH) basic transcription factor. *Science* *260*, 58-63.
- Sentenac, A. (1985). Eukaryotic RNA polymerases. *Crit. Rev. Biochem.* *18*, 31-91.
- Serizawa, H., Conaway, J.W., and Conaway, R.C. (1993a). Phosphorylation of C-terminal domain of RNA polymerase II is not required in basal transcription. *Nature* *363*, 371-374.
- Serizawa, H., Conaway, J.W., and Conaway, R.C. (1994). Transcription initiation by mammalian RNA polymerase II. In *Transcription: Mechanism and Regulation*. (Conaway, R.C., and Conaway, J.W. eds., Raven Press, Ltd., New York).
- Serizawa, H., Conaway, R.C., and Conaway, J.W. (1992). A carboxyl-terminal-domain kinase associated with RNA polymerase II transcription factor  $\delta$  from rat liver. *Proc. Natl. Acad. Sci.* *89*, 7476-7480.
- Serizawa, H., Conaway, R.C., and Conaway, J.W. (1993b). Multifunctional RNA polymerase II initiation factor  $\delta$  from rat liver. *J. Biol. Chem.* *268*, 17300-17308.
- Slattery, E., Dignam, J.D., Matsui, T., and Roeder, R.G. (1983). Purification and analysis of a factor which suppresses nick-induced transcription by RNA polymerase II and its identity with poly (ADP-ribose) polymerase. *J. Biol. Chem.* *258*, 5955-5959.
- Sopta, M., Burton, Z.F., and Greenblatt, J. (1989). Structure and associated DNA-helicase activity of a general transcription initiation factor that binds to RNA polymerase II. *Nature* *341*, 410-414.
- Stringer, K.F., Ingles, J.C., and Greenblatt, J. (1990). Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIIID. *Nature* *345*, 783-786.
- Struhl, K. (1989). Molecular mechanisms of transcriptional regulation in yeast. *Annu. Rev. Biochem.* *58*, 1051-1077.
- Sumimoto, H., Ohkuma, Y., Sinn, E., Kato, H., Shimasaki, S., Horikoshi, M., and Roeder, R.G. (1991). Conserved sequence motifs in the small subunit of human general transcription factor TFIIIE. *Nature* *354*, 401-404.

- Sung, P., Bailly, V., Weber, C., Thompson, L.H., Prakash, L., and Prakash, S. (1993). Human xeroderma pigmentosum group D gene encodes a DNA helicase. *Nature* 365, 852-855.
- Sweetser, D., Nonet, M., and Young, R. A. (1987). Prokaryotic and eukaryotic RNA polymerases have homologous core subunits. *Proc. Natl. Acad. Sci. USA* 84, 1192-1196.
- Tanese, N., Pugh, B.F., and Tjian, R. (1991). Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. *Genes Dev.* 5, 2212-2224.
- Thompson, C.M., Koleske, A.J., Chao, D.M., and Young, R.A. (1993). A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell* 73, 1361-1375.
- Thompson, N.E., Steingerg, T.H., Aronson, D.B., and Burgess, R.R. (1989). Inhibition of in vivo and in vitro transcription by monoclonal antibodies prepared against wheat germ RNA polymerase II that react with the heptapeptide repeat of eukaryotic RNA polymerase II. *J. Biol. Chem.* 264, 11511-11520.
- Treich, I., Carles, C., Riva, M., and Sentenac, A. (1992). *RPC10* encodes a new mini subunit shared by yeast nuclear RNA polymerases. *Gene Expr.* 2, 31-37.
- Tschochner, H., Sayre, M. H., Flanagan, P. M., Feaver, W. J., and Kornberg, R. D. (1992). Yeast RNA polymerase II initiation factor e: isolation and identification as the functional counterpart of human transcription factor IIB. *Proc. Natl. Acad. Sci. USA* 89, 11292-11296.
- Verrijzer, C.P., Yokomori, K., Chen, J.-L., and Tjian, R. (1994). *Drosophila* TAF<sub>II</sub>150: similarity to yeast gene TSM-1 and specific binding to core promoter DNA. *Science* 264, 933-941.
- Wampler, S.L., and Kadonaga, J.T. (1992). Functional analysis of *Drosophila* transcription factor IIB. *Genes Dev.* 6, 1542-1552.
- Wang, Z., Svejstrup, J.Q., Feaver, W.J., Wu, X., Kornberg, R.D., and Friedberg, E.C. (1994). Transcription factor b (TFIIH) is required during nucleotide-excision repair in yeast. *Nature* 368, 74-76.
- Wang, E.H., and Tjian, R. (1994). Promoter-selective transcriptional defect in cell cycle mutant ts13 rescued by hTAF<sub>II</sub>250. *Science* 263, 811-814.
- Weil, P.A., Luse, D.S., Segall, J., and Roeder, R.G. (1979). Selective and accurate initiation of transcription at the Ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. *Cell* 18, 469-484.
- Weinzierl, R.O.J., Dynlacht, B.D., and Tjian, R. (1993). Largest subunit of *Drosophila* transcription factor IID directs assembly of a complex containing TBP and a coactivator. *Nature* 362, 511-517.

Woychik, N. A., McKune, K., Lane, W. S., and Young, R. A. (1993). Yeast RNA polymerase II subunit RPB11 is related to a subunit shared by RNA polymerase I and III. *Gene Expr.* **3**, 77-82.

Young, R. A. (1991). RNA Polymerase II. *Ann. Rev. Biochem.* **60**, 689-715.

Yokomori, K., Admon, A., Goodrich, J.A., Chen, J.-L., and Tjian, R. (1993a). *Drosophila* TFIIA-L is processed into two subunits that are associated with the TBP/TAF complex. *Genes Dev.* **7**, 2235-2245.

Yokomori, K., Chen, J.-L., Admon, A., Zhou, S., and Tjian, R. (1993b). Molecular cloning and characterization of dTAF<sub>II</sub>30 $\alpha$  and dTAF<sub>II</sub>30 $\beta$ : two small subunits of *Drosophila* TFIIID. *Genes Dev.* **7**, 2587-2597.

Yura, T., and Ishihama, A. (1979). Genetics of bacterial RNA polymerases. *Annu. Rev. Genet.* **13**, 59-97.

Zawel, L., and Reinberg, D. (1993). Initiation of transcription by RNA polymerase II: A multi-step process. *Prog. Nuc. Acids Res.* **44**, 67-108.

Zehring, W.A., Lee, J.M., Weeks, J.R., Jokerst, R.S., and Greenleaf, A.L. (1988). The C-terminal repeat domain of RNA polymerase II largest subunit is essential *in vivo* but is not required for accurate transcription initiation *in vitro*. *Proc. Natl. Acad. Sci.* **85**, 3698-3702.

Zhang, J., and Corden, J.L. (1991). Identification of phosphorylation sites in the repetitive carboxyl-terminal domain of the mouse RNA polymerase II largest subunit. *J. Biol. Chem.* **266**, 2290-2296.

Zhu, H., Joliot, V., and Prywes, R. (1994). Role of transcription factor TFIIIF in serum response factor-activated transcription. *J. Biol. Chem.* **269**, 3489-3497.

## **Chapter 2**

### **Isolation and Characterization of Extragenic Suppressors of *S. cerevisiae* RNA Polymerase II CTD Truncation Mutations**

## Summary

The carboxy-terminal domain (CTD) of RNA polymerase II plays a role in transcription initiation. *SRB2*, identified as a suppressor of CTD truncation mutations, encodes a novel transcription factor that is essential for efficient establishment of the transcription initiation apparatus. We have now isolated a larger set of extragenic suppressors of CTD truncation mutations and identified an additional set of genes, *SRB4*, *SRB5*, *SRB6*, *SRB7*, *SRB8*, *SRB9*, *SRB10*, *SRB11*, and *RPB2*, which influence CTD activity. Seven of these genes, *SRB4*, *SRB5*, *SRB6*, *SRB7*, *SRB8*, *SRB9*, and *RPB2*, were cloned and sequenced. The sequences revealed that *SRB4*, *SRB5*, *SRB6*, *SRB7*, *SRB8*, and *SRB9* are novel proteins while *RPB2* is the second largest subunit of RNA polymerase II. Genetic analysis suggests that *SRB8* and *SRB9* function to negatively regulate CTD function. This is in contrast to *SRB2* which positively regulates CTD function. The CTD, therefore, is influenced by positively and negatively acting SRB factors.

## Introduction

Selective transcription initiation by RNA polymerase II requires the action of at least five general initiation factors: TATA-binding protein (TBP), TFIIB, TFIIE, TFIIF, and TFIIH (reviewed in Sawadogo and Sentenac, 1990; Roeder, 1991; Zawel and Reinberg, 1992; Conaway and Conaway, 1993). Highly purified preparations of RNA polymerase II and general initiation factors, however, are not responsive to regulatory signals. Coactivating factors are thought to contribute to the response to regulatory signals, and these include TBP-associated factors (reviewed in Gill and Tjian, 1992; Pugh and Tjian, 1992), mediators (Flanagan et al., 1991), and adaptors (Berger et al., 1992; Pina et al., 1993). SRB2 identified through functional studies of the carboxy-terminal domain (CTD) of RNA polymerase II, may also contribute to the response to various regulatory signals (Nonet and Young, 1989; Koleske et al., 1992).

The CTD is a highly conserved and apparently unique feature of the largest subunit of RNA polymerase II (reviewed in Corden, 1990; Young, 1991). Depending on the organism, the CTD contains 26 to 52 repeats of the consensus heptapeptide sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. A subset of the RNA polymerase II molecules in yeast and mammalian cells have highly phosphorylated CTDs (Cadena and Dahmus, 1987; Kolodziej et al., 1990). RNA polymerase II molecules lacking phosphorylation on the CTD are preferentially recruited into the initiation complex (Laybourn and Dahmus, 1990; Lu et al., 1991). Deletion mutations that remove most or all of the CTD are lethal to cells (Nonet et al., 1987; Allison et al., 1988; Zehring et al., 1988; Bartolomei et al., 1988). CTD partial truncation mutations, however, cause defects in growth and gene expression in vivo (Nonet et al., 1987b; Bartolomei et al., 1988; Allison and Ingles, 1989; Scafe et al., 1990a; Peterson et al., 1991) and produce substantial defects in transcription initiation at multiple promoters in vitro (Liao et al., 1991).

Suppression analysis of conditional CTD truncation mutations in yeast has been used to identify a factor which influences CTD function. A dominant suppressor in the *SRB2* (*SRB*, suppressor of RNA polymerase B) gene and its product have been characterized (Nonet and Young, 1989; Koleske et al., 1992). Genetic and biochemical studies showed that *SRB2* acts positively to influence transcription initiation (Koleske et al., 1992). Dominant, gain-of-

function mutations in *SRB2* counters the partial loss of function due to CTD truncations while cells containing a deletion of *SRB2* can survive only if the CTD is nearly wild-type in length. In vitro transcription assays demonstrate that *SRB2* has an important role in transcription initiation and promotes efficient establishment of the transcription initiation complex, probably through incorporation into the complex.

We have now studied additional suppressors of CTD truncations in order to further investigate CTD function and identify additional components of the transcription initiation complex. Ten genes were identified with this approach: *SRB2*, *SRB4*, *SRB5*, *SRB6*, *SRB7*, *SRB8*, *SRB9*, *SRB10*, *SRB11*, and *RPB2*. We report here genetic characterization of *SRB4-SRB9* and *RPB2*. *RPB2* encodes the second largest subunit of RNA polymerase II (Sweetser et al., 1987), while *SRB4-SRB9* encode proteins not previously identified. Genetic evidence indicates that two of these genes, *SRB8* and *SRB9*, repress CTD function. Thus, the CTD is influenced by factors which enhance as well as repress its activity.

## Results

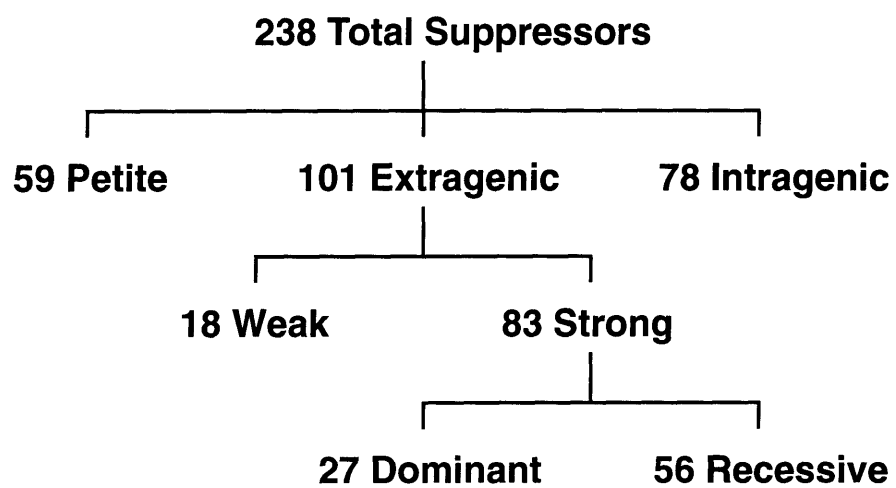
### Suppressors of RNA Polymerase II CTD Truncation Mutations

Extragenic suppressors of a *Saccharomyces cerevisiae* RNA polymerase II CTD truncation mutant were isolated to identify components of the transcription apparatus that affect CTD function. Spontaneous suppressors of the cold-sensitive phenotype of cells containing plasmid-borne RNA polymerase II CTDs with only 11 intact heptapeptide repeats (*rpb1Δ104*) were selected on rich medium at 12°C. Suppressors arose at a frequency of approximately  $10^{-6}$ . 238 suppressing isolates were further characterized (Figure 1). 59 of the suppressors are probably petites, as evidenced by their inability to grow on glycerol/ethanol as a carbon source (YEPG plates), white color, and slow growth rates. These mutants were not further characterized. The plasmid encoding the *rpb1Δ104* mutation was isolated from each of the remaining suppressor strains and reintroduced into a non-suppressed strain background to test if the suppression phenotype was linked to the original *rpb1Δ104* mutation. 78 of the suppressors were linked to the plasmid, indicating that the suppressor mutation was intragenic. 101 of the suppressors were identified as extragenic suppressors and the 83 isolates exhibiting the strongest suppressing phenotype were further characterized. Analysis of diploids heterozygous for the suppressor mutations identified approximately one-third as dominant and two-thirds recessive.

Genetic analysis has revealed that mutations in at least ten genes will suppress growth defects of cells containing a truncated CTD (Figure 2; Appendix A). All of the dominant mutations occurred in four *SRB* genes: *SRB2*, *SRB4*, *SRB5* and *SRB6* (Thompson et al., 1993). We identified recessive suppressing mutations in six additional genes: *SRB7*, *SRB8*, *SRB9*, *SRB10*, *SRB11*, and *RPB2*. Recessive suppressing alleles of *SRB4* and *SRB6* were also identified.

This selection appears to be nearly saturated since, with the exception of *SRB11*, more than one independent isolate of each of the ten genes has been identified. The characterization and cloning of seven of the genes containing suppressing mutations is presented here. *SRB4*, *SRB5*, *SRB6*, *SRB7*, *SRB8*, and *SRB9* are newly identified genes, whereas *RPB2* is the gene encoding the second largest subunit of RNA polymerase II (Sweetser et al., 1987). *SRB2* had





Suppressors arose at a frequency of approximately  $10^{-6}$

Figure 1

**Figure 1. Classification of Suppressors of the CTD Truncation Mutant *rpb1Δ104*.**

238 Suppressors of the conditionally viable CTD truncation mutant *rpb1Δ104* were isolated at the restrictive temperature of 12°C. These suppressors were classified as described in the text.

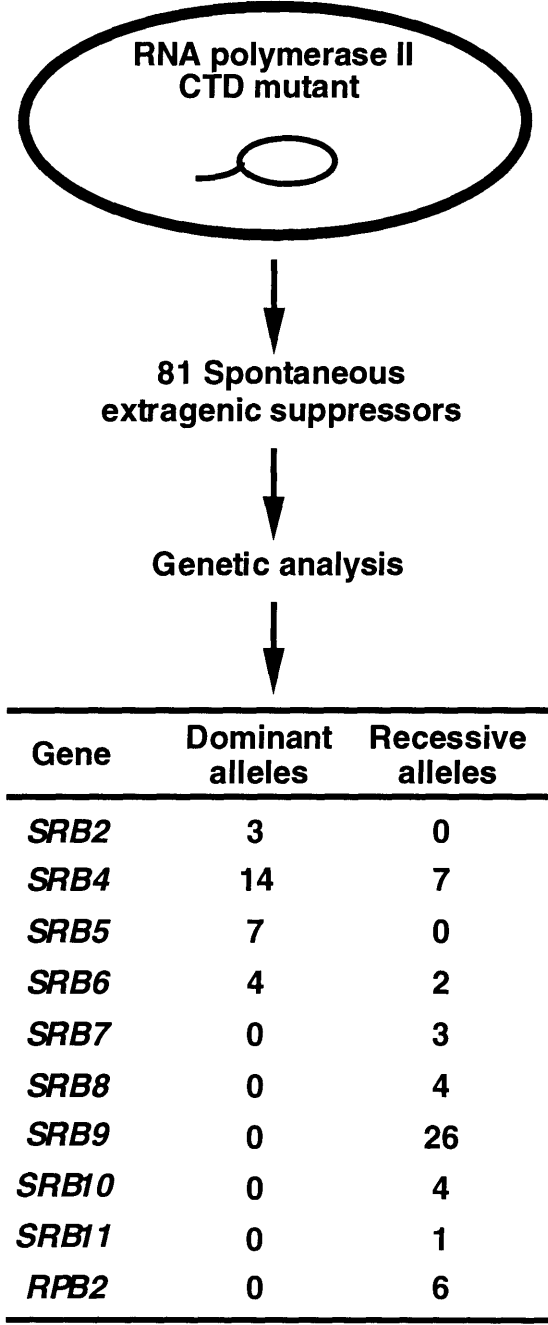


Figure 2

previously been isolated in a similar genetic selection (Nonet and Young, 1989).

### **Genetic Analysis of *SRB4*, *SRB5*, and *SRB6***

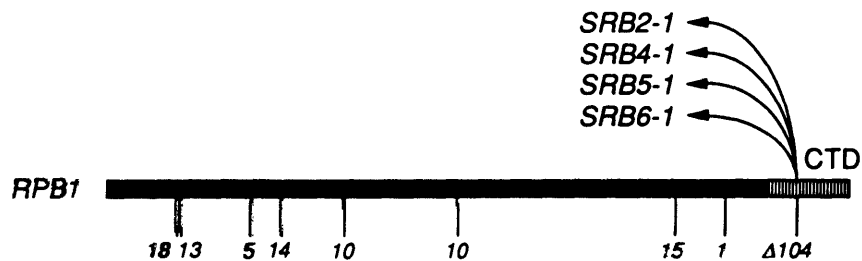
Two genetic assays were performed to obtain support for a functional relationship between the SRB gene products and the CTD (Figure 3). The ability of the suppressing alleles of *SRB4*, *SRB5* and *SRB6* to suppress all of the conditional and auxotrophic phenotypes associated with the CTD truncation mutation *rpb1Δ104* was investigated. These phenotypes include cold-sensitive and temperature-sensitive growth, inositol auxotrophy and the inability to utilize pyruvate as a sole carbon source. Cells containing either *SRB4-1*, *SRB5-1* or *SRB6-1* suppress all of these defective phenotypes (Figure 3B), as does *SRB2-1* (Nonet et al., 1989).

To assess whether the suppressing activities of *SRB4-1*, *SRB5-1* and *SRB6-1* are specific to CTD mutations, the ability of the *SRB* alleles to suppress the conditional phenotypes associated with mutations elsewhere in RNA polymerase II was investigated (Figure 3C). *SRB4-1*, *SRB5-1* and *SRB6-1* generally do not suppress the conditional and auxotrophic phenotypes associated with *RPB1* point mutations. *SRB4-1*, *SRB5-1* and *SRB6-1* do suppress the cold-sensitive phenotype of the *rpb1-14* mutation. This is the same type of suppression specificity shown by *SRB2-1* and argues that *SRB2*, *SRB4*, *SRB5*, *SRB6* and the CTD are involved in the same process in transcription initiation.

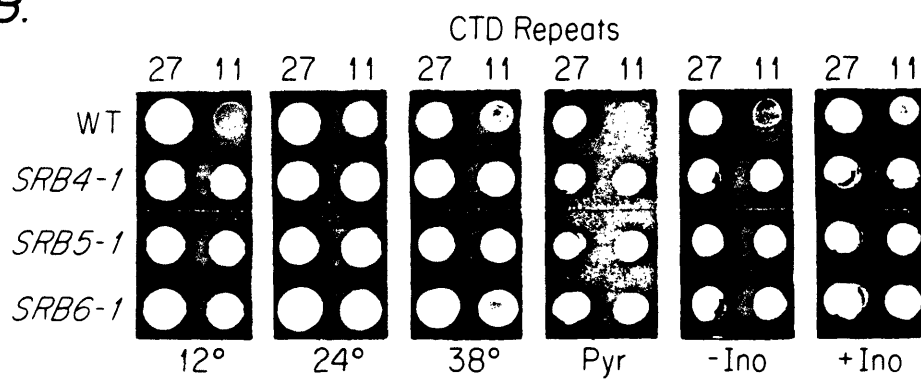
### **Cloning and Sequence Analysis of *SRB4*, *SRB5* and *SRB6***

Genomic DNA clones containing *SRB4-1*, *SRB5-1* and *SRB6-1* were isolated by taking advantage of their ability to dominantly suppress the cold-sensitive phenotype of a cell containing the CTD truncation mutation *rpb1Δ104*. Genomic DNA was isolated from strains containing the dominant suppressing alleles of *SRB4*, *SRB5* and *SRB6*. Libraries were constructed in a yeast centromeric plasmid containing the *URA3* gene as a selectable marker. These libraries were transformed into yeast cells containing the cold-sensitive CTD truncation mutation and genomic clones were isolated from Ura<sup>+</sup> transformants able to grow at 12<sup>o</sup>. The mutant genes were further localized by constructing subgenomic libraries with fragments of the *SRB4-1*, *SRB5-1* and *SRB6-1*

A.



B.



C.

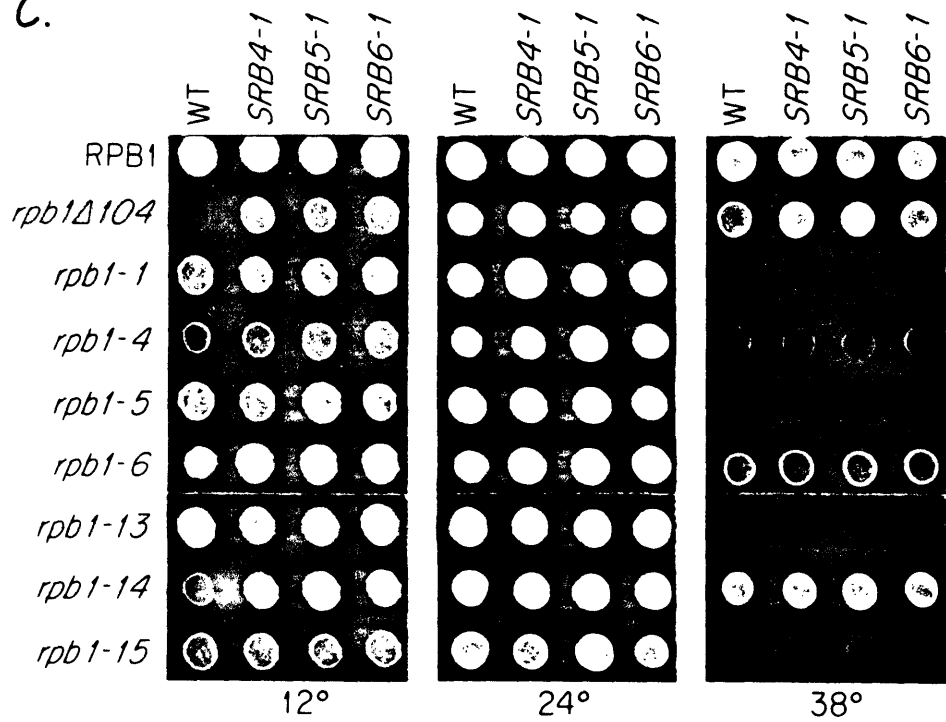


Figure 3. Genetic Characterization of *SRB4-1*, *SRB5-1* and *SRB6-1*.

(A) Diagram of *RPB1* conditional mutations used to isolate and characterize suppressors of *rpb1 $\Delta$ 104* mutations. The positions of the conditional mutations utilized in this study are indicated, except for *rpb1-4*, *6* and *12*, which have not been determined.

(B) Growth phenotypes of cells containing an *RPB1* CTD truncation mutation and *SRB4-1*, *SRB5-1* and *SRB6-1*. Cells were spotted on YEPD medium and incubated at 12°C, 24°C and 38°C (first three panels), on SC medium containing pyruvate as a sole carbon source (fourth panel) and on minimal medium with or without inositol (fifth and sixth panels). Isogenic wild-type, *SRB4-1*, *SRB5-1* and *SRB6-1* backgrounds contained either wild-type *RPB1* (27 repeat CTD) or *rpb1 $\Delta$ 104* (11 repeat CTD).

(C) Influence of *SRB4-1*, *SRB5-1* and *SRB6-1* on the growth phenotypes of cells containing various conditional *RPB1* mutations. Isogenic wild-type, *SRB4-1*, *SRB5-1* and *SRB6-1* backgrounds containing *RPB1* alleles indicated on the left were assayed for growth by spotting on YEPD medium and incubating at 12°C, 24°C and 38°C. Similar experiments revealed that *SRB4-1*, *SRB5-1* and *SRB6-1* do not suppress the growth defects of cells containing *rpb1-10*, *rpb1-12* or *rpb1-18*.

genomic inserts and again selecting for Ura<sup>+</sup> transformants able to grow at 12<sup>o</sup>. Genomic clones with the smallest inserts were identified and sequenced.

The wild-type allele of *SRB4* was cloned from a wild-type genomic DNA library. Wild-type *SRB5* and *SRB6* alleles were obtained by plasmid gap repair in vivo (Rothstein, 1991). Plasmids containing the wild type *SRB4*, *SRB5* and *SRB6* genes do not suppress the cold-sensitive phenotype of CTD truncation mutants, confirming that in each case the correct locus was cloned. *SRB4*, *SRB5* and *SRB6* were physically mapped using the prime  $\lambda$  clone grid filters of the yeast genome (provided by L. Riles and M. Olson, Washington University). *SRB4* maps to the right arm of chromosome V approximately 40 kb from the centromere ( $\lambda$  clones 5961 and 6224). *SRB5* maps to the right arm of chromosome VII approximately 30 kb centromere proximal to *SPT6* ( $\lambda$  clones 5146 and 4624). *SRB6* maps to the right arm of chromosome II approximately 75 kb centromere distal to *CDC28* ( $\lambda$  clone 4796).

DNA fragments containing *SRB4*, *SRB5* and *SRB6* were sequenced and the open reading frames were established by unidirectional deletion analysis and identification of the suppressing mutations. The predicted *SRB4* protein is 687 aa long and has a molecular mass of 78 kd (Figure 4). *SRB5* is predicted to be 307 aa in length with a molecular mass of 34 kd (Figure 5). The predicted *SRB6* protein is 121 aa long and has a molecular mass of 14 kd (Figure 6). A search of sequence data banks revealed that *SRB4*, *SRB5* and *SRB6* have no significant sequence similarity to previously identified proteins. One notable feature of the *SRB* proteins is their acidic content. The predicted pKa of *SRB2*, *SRB4*, *SRB5* and *SRB6* is 5.2, 5.1, 4.7 and 4.6 respectively.

The suppressing mutations in all three genes were identified by comparing the complete sequences of the cloned wild type and suppressing alleles of *SRB4*, *SRB5* and *SRB6*. In each case, the alterations were single point missense mutations. The mutation in *SRB4-1* changes glycine 353 to cysteine. The *SRB5-1* mutation changes threonine 22 to isoleucine and the *SRB6-1* mutation changes asparagine 86 to lysine.

To determine whether the *SRB* genes are essential for cell viability, the entire coding region of each of the *SRB* genes was deleted to produce *srb4 $\Delta$ 2*, *srb5 $\Delta$ 1* and *srb6 $\Delta$ 1* (Figures 4A, 5A and 6A). *SRB4* and *SRB6* are essential. *SRB5*, like *SRB2*, is not essential but cells lacking the gene exhibit the slow-

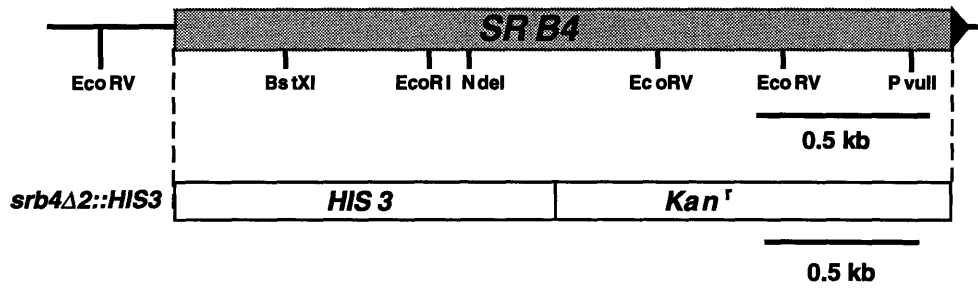


Figure 4A



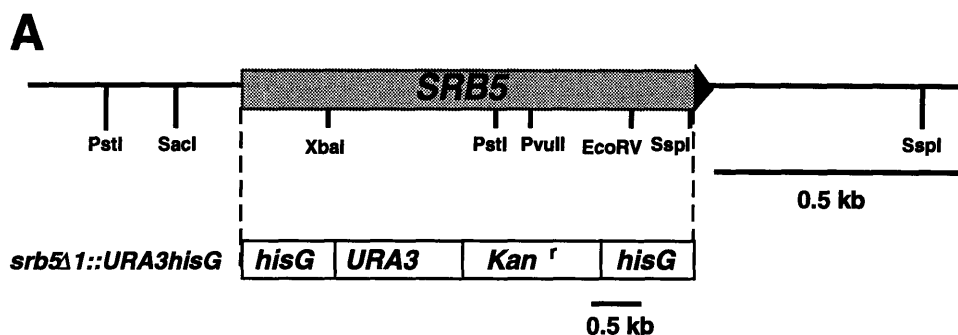
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 -243 ATATACATAAAGTTCTCATAAACGTATATATATATATATATATATACTTATGTATCAAAGTGTGTACTTCTCT  
 -168 ACATTCATAGACGGGAAGAAAAGTGCAGAAAAGTTGTTTCTCTTGTGCACTGCAGCCCTTTGAAAAAGTAGAA  
 -93 CTGCAGAAAAATAACTGAACGTAAAGCATTATTTACTTTTCAAAGGCAAAGAGATAGAGCCAAAAAATTGTA  
 -18 AGCAGCTTAAAGCCATAATGACAAACGGAAGATCCAGATTCAAAACACTTAAAGTTCCGAAACTGGCATTAAATTG  
 1 M T T E D P D S N H L S S E T G I K L  
  
 58 GCATTGGACCCGAACCTAATTACATTGGCACTAAGTTCATCCAACTCTAGCCTTCATTCACCAACGCTGTGAT  
 20 A L D P N L I T L A L S S N P N S S L H S P T S D  
  
 133 GAACCCGTACCTGAATCTGCAGGAAAAGCAGATAC TAGTATTCGACTAGAAGGTGATGAGTTAGAGAATAAAACT  
 45 E P V P E S A G K A D T S I R L E G D E L E N K T  
  
 208 AAGAAGACAATGATAAGAACCTAAAATTTTGAAGAATAAAGATTCTCTAGTCAGTAATCCACACGAAATTTAT  
 70 K K D N D K N L K F L K N K D S L V S N P H E I Y  
  
 283 GGCTCCATGCCGTTGGAGCAATGTATCCCAATCATCTTAAACAGCGTGGTCCAGGCTTTAAATTCGTTGATTTA  
 95 G S M P L E Q L I P I I L R Q R G P G F K F V D L  
  
 358 AATGAAAAAAGAAATGCAAAATGAGATTAAAGCAGCTTGGTAGTGATAGTAGTACGGTCATAACAGCGAGAAGAAG  
 120 N E K E L Q N E I K Q L G S D S S D G H N S E K K  
  
 433 GACACTGATGGCGCTGATGAGAATGTACAATTTGGAGAAGATTTCAATGGAAGTGGATTATGAAGATAAAGATAAT  
 145 D T T D G A D E N V Q I G E D F M E V D Y E D K D N  
  
 508 CCAGTGGATTTCACGAAATGAAACAGACCACAAAACGAATGAAAATGGCGAGACCGATGATAATTTGAAACGGTA  
 170 P V D S R N E T D H K T N E N G E T D D N I E T V  
  
 583 ATGACACAGGAACAGTTTGTAAAGAAGAGGAGATGCTAGAGCATATAAACTGGCCATGAACGAATCGTCT  
 195 M T Q E Q F V K R R R D M L E H I N L A M N E S S  
  
 658 TTGGCTTTGGAATTCGTTTCTTTGCTACTGTGCGAGTGTAAAGAGTCTACAGGTATGTCATCAATGTCACCATT  
 220 L A L E F V S L L L S S V K E S T G M S S M S P F  
  
 733 CTTAGGAAAGTTGTTAAACCTTCTAGTTTAAACAGTGATAAAATCCATATGTTGCACCTACAAAAAAGAATAT  
 245 L R K V V K P S S L N S D K I P Y V A P T K K E Y  
  
 808 ATCGAGTTGGATATATGAATAAGGGATGGAAGTTACAAGTTTAAACGAATCTAAAGATCTCCTACGCGCAAGT  
 270 I E L D I L N K G W K L Q S L N E S K D L L R A S  
  
 883 TTTAATAAAGTGGTCCATATTACAGAACGAACATGACTATTGGAATAAGATAATGCAGAGTATTAGCAACAAG  
 295 F N K L S S I L Q N E H D Y W N K I M Q S I S N K  
  
 958 GATGTTATTTTTAAGATTAGGGACAGGACTAGTGGTCAAAGCTGTTGGCAATTAAGTATGGTTACGAAGACTCT  
 320 D V I F K I R D R T S G Q K L L A I K Y G Y E D S  
 T (SRB4-1)  
 1033 GGATCTACCTATAAGCATGACAGAGGTATTGCTAATATAAGGAATAATATAGAATCACAAAATTTGGATTGATA  
 345 G S T Y K H D R G I A N I R N N I E S Q N L D L I  
  
 1108 CCCCACAGTAGTTTCAGTGTTCAAAGGCACTGATTTTCGTACATTCAGTAAAGAAATCTTAAAGGGTTCGTATCTC  
 370 P H S S S V F K G T D F V H S V K K F L R V R I F  
  
 1183 ACAAAAATCGAATCAGAAGATGATTACATATTGAGTGGCGAAAGTGTGATGGATAGGGATAGTGAAGTGAAGAA  
 395 T K I E S E D D Y I L S G E S V M D R D S E S E E  
  
 1258 GCTGAAACGAAAGATATCAGAAAGCAAACTTAAAGTAAAGATCATTTTGAAGAAAGAACTGATGTACCAA  
 420 A E T K D I R K Q I Q L L K K I I F E K E L M Y Q  
  
 1333 ATAAAGAAAGAAATGCGCTTTGTTGATTTCCATATGGTGTGATGATAAAGCAAGGTAATAATGAACATA  
 445 I K K E C A L L I S Y G V S I E N E N K V I I E L  
  
 1408 CCTAACGAAAATTTGAAATCGAGTTGTTGCTCCCTGACGATGACTCCATGTCATCAATGAACAAGACTTACCA  
 470 P N E K F E I E L L S L D D D S I V N H E Q D L P  
  
 1483 AAAATCAACGACAAGAGAGCAAAATTAATGCTTGTATGTTGAGACTATTATAGTCGTTATATTCAAGAAAAACA  
 495 K I N D K R A N L M L V M L R L L V V I F K K T  
  
 1558 TTACGATCAGAAATAGCTCACCCACGGACTGATCAATTTGAATGTTGACGATGATATCTTAATAATACGTCCTC  
 520 L R S R I S S P H G L I N L N V D D D I L I I R P  
  
 1633 ATCTTGGTAAAGTTCGGTTTGGCTAATTACAAACTGTTACTAAAAAATCATAAAGGATTACGTCCTCGATATA  
 545 I L G K V R F A N Y K L L L K K I I K D Y V L D I  
  
 1708 GTTCCTGGCTCAAGTATAACAGAAACGGAAGTTGAGAGAGAACACCTCAAGAAAATAAAAACATTGATGATGAA  
 570 V P G S S I T E T E V E R E Q P Q E N K N I D D E  
  
 1783 AATATAACTAAATTAATAAAGAGATCCGTGCTTCGATAAATTAATGAAATATACCTAGACGTGAACCTCAAAAATA  
 595 N I T K L N K E I R A F D K L L N I P R R E L K I  
  
 1858 AATCTACCATTAAGTGCAGCAAAAAGCCCTAATCTAAGTTTAAAGTCTGAAAGTCTAACTATTGTAACGCACTC  
 620 N L P L T E H K S P N L S L M L E S P N Y C N A L  
  
 1933 ATTCACATCAAGTTTTCAGCTGGTACGGAAGCCACGCACTGTCCTTTGACACAACATTTTCTGATTTTAAAGAA  
 645 I H I K F S A G T E A N A V S F D T T F S D F K E  
  
 2008 GTAGAGGACTTCTTACATTTTATGTGCTGAGTACATCCAGCAAAAAGGTTAATATCTTGTGAGTCACTCTCT  
 670 V E D F L H F I V A E Y I Q Q K K V \*  
  
 2083 AAACCTACATACATGGCCATAGAATGCCATTTATTACTATATAAAGTCGCATACGTACAAAAGGACAAGATC 2154

Figure 4B

Figure 4. Map and Sequence of the *SRB4* Gene.

(A) Restriction map of a 2.5 kb DNA fragment from pCT15 containing the *SRB4* gene. The entire coding region of *SRB4* was replaced with a 2.6 kb DNA fragment containing the *HIS3* and kanamycin genes to create the deletion allele *srb4Δ2*.

(B) Sequence of the 2.5 kb DNA fragment containing the *SRB4* gene. The predicted 687 aa sequence of the SRB4 protein is shown below the sequence of the gene. Positive numbering of the DNA begins with the predicted start site of translation. The *SRB4-1* mutation is a G to T transversion (nt 1057) that changes aa 353 from Gly to Cys. The genbank accession number for the *SRB4* sequence is L12026.

**B**

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-432 GATCTTCAGTATCCCTCGGGAACGCTACAACAATGTAACGATTAGAACAACATTGGCCATTGCAGCAGCTAAAC
-357 CTCCACTAATTAAGGTATTTGGCGTAAATTGCTGAATAATGAAAAAGTGAGTACGGGCAGTACCACCATCGCTG
-282 CAGTAAACAGCATAAGTTTATTAATCACCACGAGGAACATCTACAGCCATATTTGATCTTTTGAAGTCTTG
-207 GTTAGTTTCTACTATGCTTTCCAGTATGCGTTCATTTTAGCTTGCAGGTTAGTAATATATAGTGAGAGCTCTT
-132 TTGCCTTTCTTTTATTTGAAAAAATAAAATAACCTAGAAAATTATCAAATATCGAAGACAAAACCAAAAATAA
-57  AAAAAAGGTAGAAAATGAAATTTCCAGCCAAAGGTATTCATATTAAGAAGAAAAGATGGTTCAGCAACTAAGC
  1
                                     M V Q Q L S
                                     T (SRB5-1)
 19  CTTTTGGATCTATTGGTGATGACGGCTACGATTTACTAATTTCAACTTTGACCACAATATCAGGTAATCCCTCCG
  7  L F G S I G D D G Y D L L I S T L T T I S G N P P
 94  CTACTGTATAACAGTTTATGCACTGTCTGGAACCAAAATCCATCTTACGACGTCGAGAACGTGAACCTAGAAAC
 32  L L Y N S L C T V W K P N P S Y D V E N V N S R N
169  CAATFGGTTGAACCAATAGAATAAACTTTCCAAGAGGTGCCATTTTCTTACCTGATCGATGAAACAATGATG
 57  Q L V E P N R I K L S K E V P F S Y L I D E T M M
244  GATAAGCCATTAACCTTTAGAATCTTGAATCTTTTACAACGATAAAATCCCGCTTAACTATGTATGACACGG
 82  D K P L N F R I L K S F T N D K I P L N Y A M T R
319  AATATCTTGCACAACACAGTTCGCAAGTCACCAACTTCAACAGCACAAACGAAGTCAAAAACAACAGTAAGCAT
107  N I L H N T V P Q V T N F N S T N E D Q N N S K H
394  ACAGAAGATACTGTAATGAAAGTCGAAACAGCGATGACATCATAGATGTGCAGATGGATGCAAGTCCCGCCCT
132  T E D T V N E S R N S D D I I D V D M D A S P A P
469  TCAAACGAGTCATGTTCCCTTGGTCATTGCAAATTTTCAGATATTCCTGCTGCAGGAAACAATAGAAGTGTTC
157  S N E S C S P W S L Q I S D I P A A G N N R S V S
544  ATGCAACGATAGCTGAGACTATCATATTTCTTTCAGCTGGCAAAACTCTTTCAGTATCCTCGCTCATGAACGGA
182  M Q T I A E T I I L S S A G K N S S V S S L M N G
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232  L Q K I W Q I E E A G N S Q I T S G G F L L K A Y
769  ATCAATGTTAGTAGGGGACCGATATCGATCGTATAAATATACAGAGACTGCCTTGATGAACTTAAAAAGGAA
257  I N V S R G T D I D R I N Y T E T A L M N L K K E
844  CTACAAGGCTATATAGAGTTAAGTGTACCCGATAGACAGTCAATGGACTCGAGGTTAGCACATGGAATATTTCT
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919  ATATAATCATTTGGCACCTGGGCATATTTTTACAAAATTCACATATAGTTATACAGAACAACAGTAACCAC
307  I *
994  TAATGTACAGGTATTTCTATATCTACAACAAAAATGTGTAGTTATATATCTAATGTTGCTATACCGAGGAATTA
1069 TAAAGTAATAAAGATGTTAAATTAAGACAAAAATTTTGGAGAGGCTATTGGAAAAGAGAGAAAACATTTCTT
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1294 TTTCTTTTCTTCTTCTTCCATCCACCTTTCTCTTTTGTTCCTTTGACTTATGACATCCTTATCAGCTTCTGA
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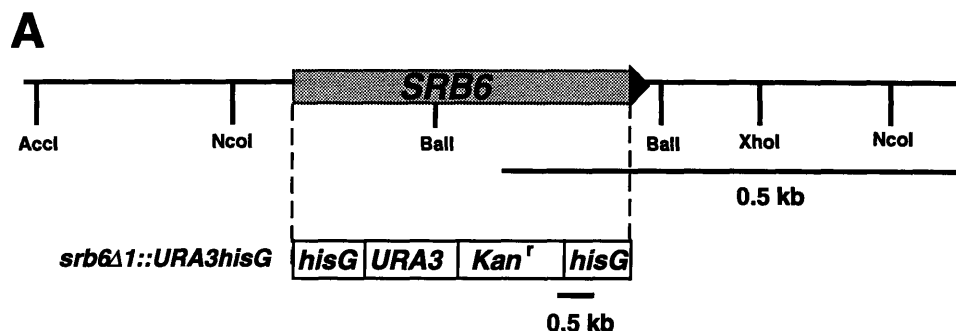
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Figure 5

Figure 5. Map and Sequence of the *SRB5* Gene.

(A) Restriction map of a 1.9 kb DNA fragment from pCT39 containing the *SRB5* gene. The entire coding region of *SRB5* 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 *srb5Δ1*.

(B) Sequence of the 1.9 kb DNA fragment containing the *SRB5* gene. The predicted 307 aa sequence of the SRB5 protein is shown below the sequence of the gene. Positive numbering of the DNA begins with the predicted start site of translation. The *SRB5-1* mutation is a C to T transition (nt 65) that changes aa 22 from Thr to Ile. The genbank accession number for the *SRB5* sequence is L12028.



**B**

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-210 TGTATATACTTTGGCACACTTGTATAATGTATAATAAAATCAGGATAAATCCAGTGTGACCCGGACTGAATTACT
-135 GAAACTTTGAAGTGTAAAGGAAATTTACTGCCATTTAACGCATTTACCTATCACTTAGTAGCATGCATAAGCCA
-60 TGGGCTAATCATAACAGATTGTGATGATAGGCATCCTGTACTCCTTTTTTTTACAAGAAAATGAGCAACCAGGCA
  1 M S N Q A

16 CTATATGAGAACTCGAACAAACCAGGACGATTCTGTCCGTGAAGCTGGCGGAATGATAAATATGACTACGATA
  6 L Y E K L E Q T R T I L S V K L A E L I N M T T I

91 GCCGATAGAAATGATGATGACGAGGGTTCATTCGCACAAGAAAATTCGAGCTCGCTGTGGCCACGACCAGTGTG
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    G (SRB6-1)
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 81 E K W L L N Q I P V T E H S K V T R F D E K Q I E

316 GAATTACTGGATAACTGTATAGAAACGTTTCGTGGCGGAAAAAATACGTAAAAAGCGGTATTTATCTATTATTT
106 E L L D N C I E T F V A E K T T *

391 GGCCAAAAAAAAAAAAAAAAATACATACTACATATACATATACGCCATAAAAAATCTCTGCATCTATCTTATTTCC
466 CATTATTTGGACAAATGCTTACGTGCTAATGTCCTTACCTCGAGTCGAATGCCGGGCTCCTAATAGGGTCTGTA
541 ATCTTATAAAACGGTTCATTAGTGTCTTTACGTATAGTTCGTGTACCTCTTGGTAGAATGACCTCATATTATTG
616 TCGTCAATAACTACGCTACTGTTGGCTGAGTTCCTATGGATCATCACGAACCTTCATCCACTATAGCTAATATAA
691 CCCGTTATTGCTAGTCCATAAAAAATGATC 719

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Figure 6

Figure 6. Map and Sequence of the *SRB6* Gene.

(A) Restriction map of a 1.0 kb DNA fragment from pCT40 containing the *SRB6* gene. The entire coding region of *SRB6* 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 *srb6Δ1*.

(B) Sequence of the 1.0 kb DNA fragment containing the *SRB6* gene. The predicted 121 aa sequence of the SRB6 protein is shown below the sequence of the gene. Positive numbering of the DNA begins with the predicted start site of translation. The *SRB6-1* mutation is a C to G transversion (nt 258) that changes aa 86 from Asn to Lys. The genbank accession number for the *SRB6* sequence is L12027.

growth, cold-sensitive and temperature-sensitive phenotypes characteristic of CTD truncations.

### **Genetic Analysis of *SRB7*, *SRB8*, *SRB9*, and *RPB2***

The ability of suppressing alleles of *SRB7*, *SRB8*, *SRB9*, and *RPB2* (*srb7-1*, *srb8-1*, *srb9-1*, and *rpb2-551*, respectively) to suppress conditional phenotypes associated with the CTD truncation mutation *rpb1Δ104* was further investigated (Figure 7). These phenotypes include cold- and temperature-sensitive growth and the inability to utilize pyruvate as a carbon source. The *srb7-1*, *srb8-1*, *srb9-1*, or *rpb2-551* alleles permit growth of *rpb1Δ104* cells at 12°C and on media containing pyruvate as a sole carbon source. Cells containing these suppressing alleles, however, do not suppress the temperature-sensitivity associated with the CTD truncation mutation. These *srb* and *rpb2* alleles do not suppress the conditional phenotypes of other mutations in *RPB1* that have been tested. This specificity of suppression argues that *SRB7*, *SRB8*, *SRB9*, *RPB2*, and the CTD are involved in the same process in transcription initiation.

### **Cloning and Sequence Analysis of *SRB7*, *SRB8*, *SRB9*, and *RPB2***

Genomic DNA clones containing *SRB7*, *SRB8*, *SRB9*, and *RPB2* were isolated by exploiting their ability to reverse the suppressing phenotype of the recessive *srb* or *rpb2* alleles. A wild-type genomic DNA library constructed in a yeast *URA3* centromeric plasmid (Thompson et al., 1993) was transformed into yeast cells containing the CTD truncation mutation *rpb1Δ104* and *srb7-1*, *srb8-1*, *srb9-1*, or *rpb2-551*. Ura<sup>+</sup> transformants were then screened for lack of growth at 12°C and on pyruvate media. When necessary, the wild-type genes were further localized by subcloning fragments of the genomic inserts and again screening Ura<sup>+</sup> transformants unable to grow at 12°C and on pyruvate media. The clones with the smallest inserts were sequenced. The predicted *SRB7* protein is 140 amino acids long and has a molecular mass of 16 kd (Figure 8). *SRB8* is predicted to be 1226 amino acids in length with a molecular mass of 144 kd (Figure 9). Partial sequence analysis of *SRB8* revealed that it is ORF YCR81W (Oliver et al., 1992). The predicted *SRB9* protein is 1420 amino acids long and has a molecular mass of 160 kd (Figure 10). Partial sequence analysis of the fourth clone identified *RPB2* as a suppressor of CTD truncations. A search of the sequence data banks revealed that *SRB7*, *SRB8*, and *SRB9* do

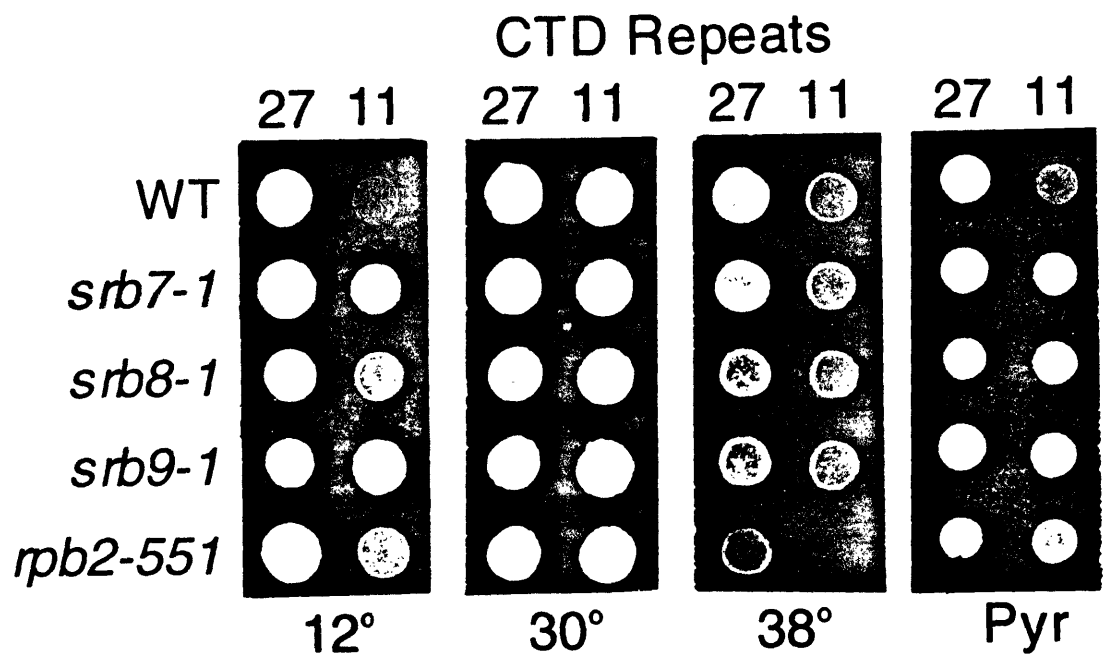
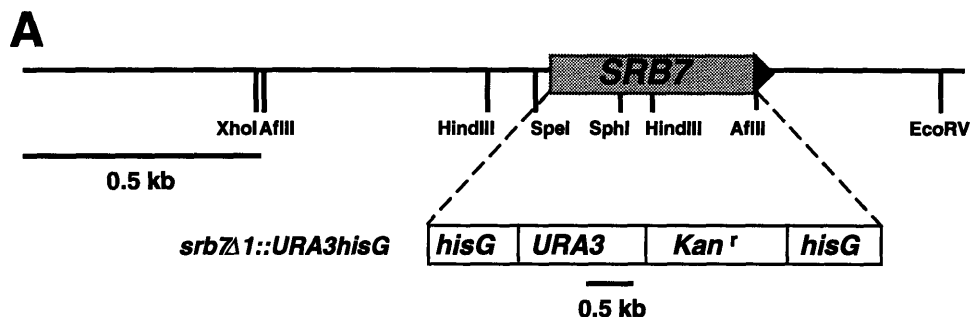




Figure 7. Growth phenotypes of cells containing an *RPB1* CTD truncation mutation and *srb7-1*, *srb8-1*, *srb9-1*, or *rpb2-551*.

Cells were spotted on YEPD medium and incubated at 12°C, 30°C and 38°C and on SC medium containing pyruvate as a sole carbon source. Isogenic wild-type, *srb7-1*, *srb8-1*, *srb9-1*, and *rpb2-551* backgrounds contained either wild-type *RPB1* (27 repeat CTD) or *rpb1Δ104* (11 repeat CTD).



**B**

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-648 TCGATGATGTTCTTTATCTTTCAACCAGCTCGAGCCCTGCAAACTTAAGCTAAGGACAGAAAATGAAAAAAAA
-573 AAAAAAAAAAAATCAAGAATCAGCTTATAAAACATATTCAAGGACCATCTGAAGTATCATTCAATCGTTTTT
-498 ACTCGTTAATCTCATTCAATTCGTTTCCTCATTCTTTTTCTTTGTTCTTTATTTGGGCTATTTTTCACTATTAA
-423 AATAACTAGAGCTAACAAATATTATTCTTCTGCTTAGTTACAAAACAAGGACATTCATTTAACTTGGCGTTATC
-348 CCATACATTCGTTTATATATCTCTTTTAAAACACAATTTCTTTACAGTTAAACTTTTCTGATTATATATA
-273 TTACTTAAGATTGTTTCATATAACTAACATTTATATGCTTATATGCGTGAAGTGCCTTTTGTAGAACATGTGGCT
-198 GTTCTGTAGAAGCCTTGTCTTCTCTGTAATCCCTTAAAGGCCAACCGTACGTGCTTAATTACAAGCTTTGTTC
-123 GCATTGCAAGAAAGTTAGAAAAAAAAATCAATCTGAAGATAATATAATTCAAACGGTAAACCATTTGGTTAAAA
-48  GAGGGACATAACATTTCACTAGTTCAATACATTATATGCTCTTTAAACAATGACAGATAGATTAACACAATTGCAG
                                     M T D R L T Q L Q
                                     A (srb7-1)
28  ATATGTTTAGACCAAATGACGGAGCAATCTGTGCTACTTTAAACTACATAGATAAGAACCATGGTTTTGAACGA
   I C L D Q M T E Q F C A T L N Y I D K N H G F E R
103 TTGACCGTAAATGAACCTCAGATGTCGGATAAGCATGCCACAGTAGTACCTCCTGAGGAATTTCTAACACGATA
   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 GATGAGCTATCCACGGACATTATACTTAAAACAAGACAGATAAAACAAGCTTATTGACTCGTTACCTGGTGTGAC
   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 GTTTCAGTGAAGACAAATTAAGGAAGATTGATATGTTGCAGAAAAGCTAGTTGAAGTGAAGACGAAAAAATT
   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 GAGGCCATCAAAAAGAAGGAGAAACTTTAAGGCACGTTGATTCTTTAATGAAGATTTGTAGATGGCATTGCA
   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 AACTCAAAAAGAGCACATAAACTTAAGTTTACAAAAGAAATTTGCGAACAGAGGACAGAAAATGTACTATAGTT
   N S K K S T *
478 ATATGGCAGAGTTAAGCGTATGTATGTTATTCTTATAAATAATGTGCTACTCTATTTGTACCGGAGAATTATG
553 AAGCAATGGGAGAAAAATCATAATGGAGAAAATCTTTCTACGAGTTACTTTGCAAGGCAATCTAACGATCTAAA
628 AGACACAATACACTAAAGAAAAAACTTTGGAAGTACAGTTTTTCCCAAGTTGAAGTGTGGACTCATTTGTGAAG
703 ATGTAAAAATGTAAAAACCAACCGACAATGCACCTCCAGCCAAAATTCATTGTAGACCTCCCATTTGATAGAAAAG
778 GAAGGTCAGCAGTTGTCCACGGATTCCAAGATATCATTCTCTTACATTGCACGCACATGAAAATGATC 846

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Figure 8

Figure 8. 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 *srb7 $\Delta$ 1::URA3hisG*.

(B) Partial sequence of the 2.0 kb DNA fragment containing the *SRB7* gene. The predicted 140 aa sequence of the *SRB7* protein is shown below the sequence of the gene. Positive numbering of the DNA 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.

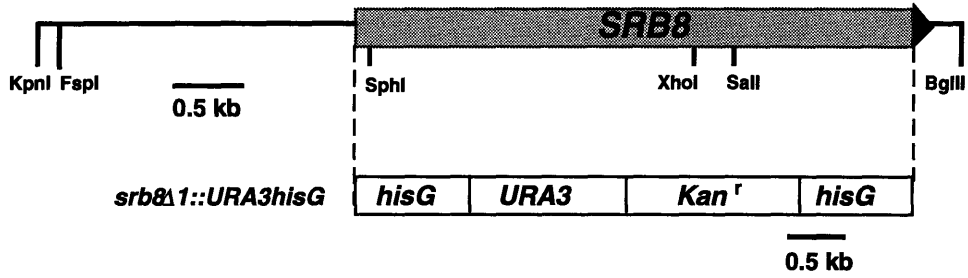


Figure 9

Figure 9. Restriction map of a 6.0 kb DNA fragment from pSL311 containing the *SRB8* gene.

Approximately 500 bp upstream of *SRB8* there is an inversion, relative to the genomic DNA used to sequence that region of chromosome III (Oliver et al., 1992), encompassing greater than 2 kb. 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*.

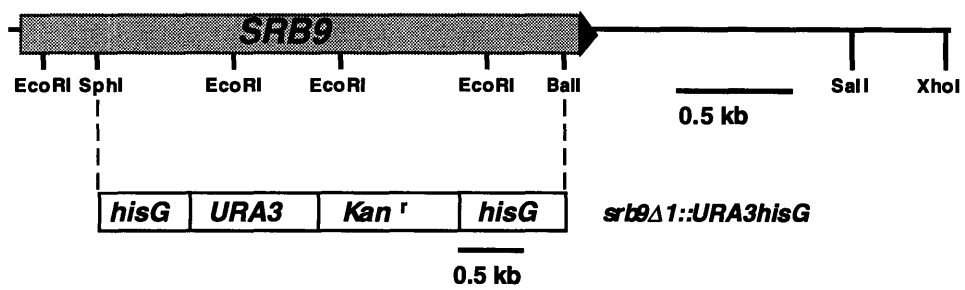


Figure 10A

-147 GATCAAGTAGTGTAGTATTTATTTAGTAGTACACTCTTACAACAACCCCTTTAAGACGAATGGTGTGAAATCGGAAAT  
-72 TACITTTGTTGAAGTAAGGTGTAACATATATTTAAGAACGTTTAAAGCTGGATATCAAGATCTGAGGAGGTAGTATG  
M

4 AGTTCGTACGCTTCCACGTACAGACTTGAGGATGTTTTATCCAGCTTCTATAGAGTGGAGAAAATCAAAAAGATC  
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 AACTATCATCAGTACATTTCTAAAGCCCAAAACGATCAATGGTCTATCCAAATGGAAATCATGCTACGGAAGCAG  
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 GATCCAAAGACTCTAGTTGCGCTGCTTTCAAGGGATTTATGGTGTTCAGTATAAATGATGATCCGGTACCGACA  
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 CCTCCTGCGATAGAACATAAACCCAGTACGAGCCAGATAAAAATCGGAACCTTCTACTGCCGATTATTCAAAGCCAAAC  
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 TTACCGCCACACTATGCTCTTTTTTAAAAGCTTTAAGAAGGAAAATTTACATTAATTTGGCATTAGGTTACAC  
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 AATAAGCTAATACAATTTGGGAATGCCTGCATATCATTAAAGCGGAGTGCCAAATTTATCTCGTACAGCTAGAACCA  
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 CACCTTTTTGTAACCGGAGATCTCAGCTGTCGTTATGTGCCAAGAACATGGGATTTAGTACCAATGAAGGAGGAA  
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 AATTTGGAAGAATCTTCCCTTTCAAAGCATGCGCTTTATTTAGCACCATCTGGAATAAGGATGCATTTGGCCCCT  
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 GCTTCCAAGCAAGGATACTTGATAACGCGCACAAAACATACAGAACTTCTCTTGACGACGTTAAGTGTATCTCAT  
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 GGTATAAACTTACAAAATAAAAAAATTTGAAATGGGTTGCTGTTGTTCTGACTTAGGACATCTCAAAGCCAC  
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 ACACCTACTATAGCTTCGTATTTAACTCCCTTACTTGAAGCAAAGAAGCTAGTATGGCGCTGCATTTAATCTTC  
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 GCCCAACAGTAGCTGATATAGAAAATCTACTTCCGGAGATCCATCAGAATTTCACTGTTTGCAGATGCTCTG  
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 GATGCCATTGATGATTTACATAAATAAGCAAACAGCTGCCTATAGGACTCCAGGAAGTTCCGGCGTATTGAGC  
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 AGTAATATTGCTGGTACAAATCCCTTAAAGCTCAGATGGAGCATATACAGAACAGTTTCAACATTATAAGAACAAC  
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 TCAATTAGTTCTCAACCCGCTTCTTATCATTCTGTCCAAGAACTAATAAGATATCTCCGAAAGATTTCTCCCT  
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 AATTTACAGGCATTGATAAATTAATGCTGTCGCCAGCGATCAATTTGCTCCAGCTTCTTAAATACCCCTAAT  
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 AATAACATCAATGAGAATGAATTAATTAATGATAGGAAACAACTACAGTATCAAATGACTTAGAGAACAGCCCA  
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 CTGAAAACGGAAGTGGAGGCAATGGTAGATCACTCGAAAAGGTAATAATTCGGTGAACAAGACAGGAAGCGTA  
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 GACACACTTCATAATAAAGAGGGAACACTGGAACAACGAGAACGAAATCTGCCAAGTGATAAAAAGTGAC  
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 TCATGGTAGACAAGGAATGTTTGGTGGAGGATGAGGATGAGGATTTATTTGGCGATAGCAATAAATCGAATTCT  
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 ACAACGAATCGAACAAAAGTATATCGGACGAAATTACCGAGGATATGTTGCAATGCTGATGAAGAAGAAAAT  
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 AATAACAATAAAGCATTAATAAAAATAACAAGGAATGCATACTGATCTGGTAAAGATATTCATTTTTCCT  
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

1654 TCATCTGAAAACCGAATATCCGTACGATGAGCGAACTACAAAAGATTAATGAAAAGAGGAAATATTGGAT  
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 ATTCGGATAGATGAAATGACCTTGCCACAGAGTCCATTATATATGACCCAGGTGCGCCACTCCCTGTGAAAACA  
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 CCCCRCATAGACGCAAAAGTGTGTTGCTCCACTGAAATTTAACCCTAATAAGAAAACAAATGTTGATAACAAA  
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 TACAATCTGGAGGAAATTTCTTCACTCCGTTGCAAAGGAGGAAGCATTAACCTTTGATATTTCTATGGCG  
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 GATCTTTCTAGCTCTGAAGAGGAAGAGGATGAAGAAGAGAAGCGGTAGCAGCGATGAGGATCTAAAGTCAATTGAA  
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 GTACGCGACGACATGAAACCTTCTGATAACATCAGTACTAATACTAATATTATGAGCCTCAATACATAAATAC  
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 AGCAATAAGGAAGGCTTCAACTCTATTTGGAAAATCCCAAAATGATATACCACAGACCGAGTCCACTGAAG  
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 ACCGTGTGATTCATCTTATCAACCCATAGAATCCAATATAAAGATGACCTTGGAAAGATAATAATGTTACCGATAAT  
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 CCGTCCGAATTTACGCCGAATATGGTAAATTCGAAATTTCTAACCTACCAAGGACAAGAGTGGTATCCCCGAA  
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 TTCACACCGGGACCCCAATTTATCTTTGAATCATCAAGTAGTCTACCCTTCTATTGAGACACATGCCGCTA  
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 GCATCTATACCGGACATTTTCATCACGCCCTACCTCCCGTGTGTACAAATTCAGAAAAGAACAAGACATCTTAGAT  
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 TTAAATGCAGAACAAAGTCGTCACTGATTATAATATCTTAGGAAACCTCGGTATTCCAAGATCGCCTATAGGGGA  
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 GTTAAAGATTCGCAAGAAGGTTAAATAACAACCACAATGTTACAGTTATTTCCACTTCGGATAGATTAATGGC  
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 AATGATACGATCTCCAATTTATAACATGAAGCAGCCGTCAGTTTTTGTAAAGAAACATCACGAACTAATCAAA  
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 GTCAAACACGACTCTCAGCCATTTATTAAGTTCCTCAATTTTCGCCCTCCAATGGAATAAAAAACTTCAAATCC  
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 TTATTTAATTAAGTTCATCTTTCAAAGAAGATGTCTGTCATTTGCGCCAACTCTATCTCAAACATATATTAATCAA  
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 GAGTTAGGTTTGTGAGTGTCTTAACTAACTAATGAAGACCCGCCGACTGATGACTTGAAGGCATTTGAT  
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 AAAACAAGTTACTGTTGTAGCTGCCAGATGTTTCATACTGTTCTAATAATAAGAACTCCATCAAAAACGTCG  
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 CCACCAATATTAATAATTTTACCCTTGGATAATGCAACTCTGACTGAATTAGTAGACAAGGCGAATATTTTCAG  
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 GTGATCAAGAACGAGTGTGTGCCAAGATGCCAACATGAACTATATTTGAAAGTTATTCCTATGATTTTCATT  
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 AGAAACGTACTGGTACAGTGGATCAGTACGTCACAGTACCAATTTCTATATAACATGCTGCCGCAAAATCT  
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 GTAAAGTTCAACCCACATTCGCGATACGCTGCCGAGAAAAGTGAATTTCAGAACCATGCAGCAACAGCAAATGCAA  
V K F T H I A H T L P E K V N F R T M Q O O O M

3379 CAGCAACAGCAACAGCAACAGCAGCAGCAGAATAACAGTACAGGATCATCTTCTATAATATATTATGACTCGTAC  
O O O O O O O O O O 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 AGCATGACGAAACGTGGTACGTCGGGAATTCAGAGGAAATTTGACGACGCATGTAATCAAATATGGAATATC  
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 GCCCTAAATTTAGCGTCTAAAAAATTCGGAAAAATATGTCATTAATTTAACTAGATGGAATGGCATACTGCCGGAT  
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 GATGAATGATGAATTTGGAGGAGACTTTCTGGTAGGAATATACATCTTGTGCTGGTGTGTGGATGACAACCTCT  
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 AAAATCTCTCATAGATGAGGACAAATGTACCCTAGTTTTCAAGCCGATCTCAAAGACACTAGGTTTGGAGGA  
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 CGCATGGATATGACCAGATTTATACGACTATGAAATAAGGGATATAGACCAGGACATCCATGGAATAGTATTCAG  
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

3904 CACCGTTCCCACTGGCACACTCACAGCATCGCTGTGCTATTAGGAGTGGTGCCTTTGATCAAATTCAAAAATGC  
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 GACGGTATACGGTTTGGGACAAATTCGCACTCAACCTTTTAACTGTCCACATCTGATAGTACACAATTTGCTG  
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 GAAACCATCTTAGAAGAGTTTCGCAACCTGGCTGCTCTAAACGTGTGGTACGGCTCTCTGATGGCGAAGATGGC  
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 CATATTCATGGCATACTCAGCGTGAATAAATGATGAACACTCTTGTGCACACCAGAGTAAAAATGCTAAT  
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 ACTTCCCGCTACTGTGCATACCGCTACTTCTCATCAATATTTCTCTCGGATAAATAAACTTTTCCGGCAAC  
T S A A T V H T A T S S S I I L S D K

4279 GTTTCCTGCTCATCTGTAGCCCTATTTACCAGTTTGGTTTTAGTATTAATTCGGGGTGTAAACCCAGAAGTCT  
4354 ATTTCTCCAGTCGGATTATATAAAACAAAACCGGAAGCGGGCGGTACGGCATTTTCACTGGTGTGACACGCCCA  
4429 GCGTGTAGTCCGAGACAATTTCCACAGAACCGAATGAGATGCGTTTAAAGGCTGTATTTTCAAGGCACACGAAG  
4504 CGGCCACTGGGTCTCGCATGGTGTGTTGATGATGTAAGAATGGTATCATACTCCGTATAAGGTTATGTAATCG  
4579 GAAGTCGGATTTCTTTCAATTTTCTTTTATTTTTCAGTTTTTCTCTCTCGATGGAAAATTTGTTG  
4654 AAGTCTCTGATGAGCAAGTAGTTCCTACATCGAGGAATCTTATGTT 4702

Figure 10B



Figure 10. 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) Partial sequence of the 7.3 kb DNA fragment containing the *SRB9* gene. The predicted 1420 aa sequence of the *SRB9* protein is shown below the sequence of the gene. Positive numbering of the DNA begins with the predicted start site of translation. Glutamine-rich region described in text is underlined.

not have significant sequence similarity to previously identified proteins. *SRB9* does, however, contain a single polyglutamine stretch of 16 residues from amino acids 1121 to 1136 (Figure 10B).

*SRB7* and *SRB9* were physically mapped using the prime  $\lambda$  clone grid filters of the yeast genome (provided by L. Riles and M. Olson, Washington University). *SRB7* maps to the right arm of chromosome IV, approximately 45 kb centromere distal to *GCN2* ( $\lambda$  clone 6118). *SRB9* also maps to the right arm of chromosome IV, approximately 35 kb centromere distal to *ADE8* ( $\lambda$  clone 5513). *SRB8* maps to the right arm of chromosome III, approximately 5 kb centromere proximal to *TUP1* (Oliver et al., 1992).

The *srb7-1* and *rpb2-551* mutant alleles were obtained by plasmid gap repair in vivo (Rothstein, 1991). Plasmids containing these mutant alleles did not prevent growth at 12°C, unlike their wild-type counterparts, when transformed into yeast cells containing the CTD truncation mutation *rpb1 $\Delta$ 104* and *srb7-1* or *rpb2-551*, respectively. This confirms that in each case the correct locus was cloned. The identification of the correct open reading frame is further supported by identification of the suppressing mutations of *srb7-1* and *rpb2-551*, identified by comparing the complete sequences of the cloned wild-type and suppressing alleles. In each case, the alterations were single-point, missense mutations. The mutation in *srb7-1* changes alanine 21 to threonine (Figure 8B). The *rpb2-551* mutation changes alanine 1200 to valine. We did not identify the suppressing mutations in *srb8-1* and *srb9-1* for reasons described below.

### ***SRB8* and *SRB9* are Negative Regulators of CTD Function**

To determine whether the *SRB* genes are essential for cell viability, most, if not the entire coding region of each of the *SRB* genes was deleted to produce *srb7 $\Delta$ 1* (Figure 8A), *srb8 $\Delta$ 1* (Figure 9), and *srb9 $\Delta$ 1* (Figure 10A). *SRB7*, like *RPB2*, is essential. *SRB8* and *SRB9* are not essential, but cells lacking either one of these genes flocculate and exhibit mild cold- and temperature-sensitive phenotypes. Significantly, null alleles of *SRB8* and *SRB9* partially suppress the conditional phenotypes associated with CTD truncations (Figure 11). The phenotypes exhibited by deletions of *SRB8* or *SRB9* are very similar to those phenotypes exhibited by the suppressing mutant alleles of these genes, indicating that we have cloned and identified the correct gene. While we have

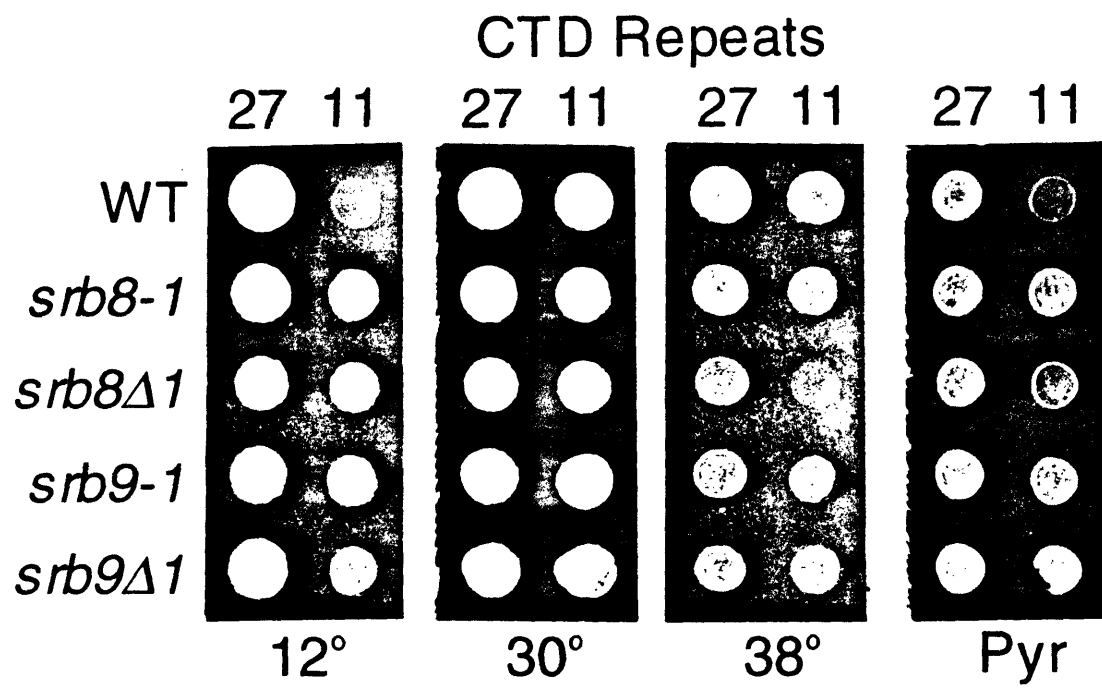


Figure 11. Null alleles of *SRB8* and *SRB9* suppress CTD truncation mutations.

Cells were spotted on YEPD medium and incubated at 12°C, 30°C and 38°C and on SC medium containing pyruvate as a sole carbon source. Isogenic wild-type, *srb8-1*, *srb8Δ1::hisG*, *srb9-1*, or *srb9Δ1::hisG* backgrounds contained either wild-type *RPB1* (27 repeat CTD) or *rpb1Δ104* (11 repeat CTD).

not identified the suppressing mutation in *srb8-1* or *srb9-1*, the mutations are likely to have partially or completely destroyed gene function.

The influence of *srb8Δ1* and *srb9Δ1* on RNA polymerase II CTD function was further investigated by examining the effect of these deletion alleles on the growth phenotypes of cells containing a spectrum of CTD truncation mutations. Figure 12 summarizes the results obtained with *srb8Δ1* and compares these results with those of a similar set of experiments performed with *SRB2* alleles (Koleske et al., 1992). Yeast cells lacking *SRB8* partially suppressed the conditional phenotypes associated with CTD truncations containing 10-12 complete heptapeptide repeats. Moreover, the lack of *SRB8* allowed cells with only nine heptapeptide repeats to survive; thus, loss of *SRB8* counters the defects associated with CTD truncation. This pattern of suppression is opposite to that observed with *SRB2* alleles. The dominant, gain-of-function *SRB2-1* allele produces the same suppression phenotype as the recessive, loss-of-function *srb8Δ1* allele. In contrast, the recessive, loss-of-function *srb2Δ1* allele, increases the severity of the defects associated with CTD truncation. The influence of *srb9Δ1* on the phenotypes of cells containing CTD truncations is similar to that of *srb8Δ1*.

Genetic Data suggests that *SRB8* and *SRB9* behave as negative regulators of CTD function, while genetic and biochemical data indicates that *SRB2* behaves as a positive regulator of CTD function. Only dominant mutations in *SRB2* have been identified, indicating that *SRB2* normally acts to stimulate CTD activity and that with a truncated CTD wild-type *SRB2* is unable to stimulate enough. Gain-of-function mutations in *SRB2* counter the partial loss of function due to CTD truncations. Cells containing a deletion of *SRB2*, however, can survive only if the CTD is nearly wild-type in length, further suggesting that *SRB2* normally acts to augment CTD function. Finally, in vitro transcription assays demonstrate that *SRB2* is required for efficient transcription initiation. In contrast, only recessive mutations have been identified in *SRB8* and *SRB9* and deletion of *SRB8* or *SRB9* can counter the partial loss of function due to CTD truncations. These data suggest that *SRB8* and *SRB9* normally function to repress transcription via functional interactions with the CTD. Further testing of this hypothesis will require biochemical analysis, possibly in vitro transcription assays with purified RNA polymerase II and general transcription factors. Such a reconstituted system should permit the development of *SRB*-dependent transcription assays and allow for the further analysis of individual or

groups of SRB proteins.

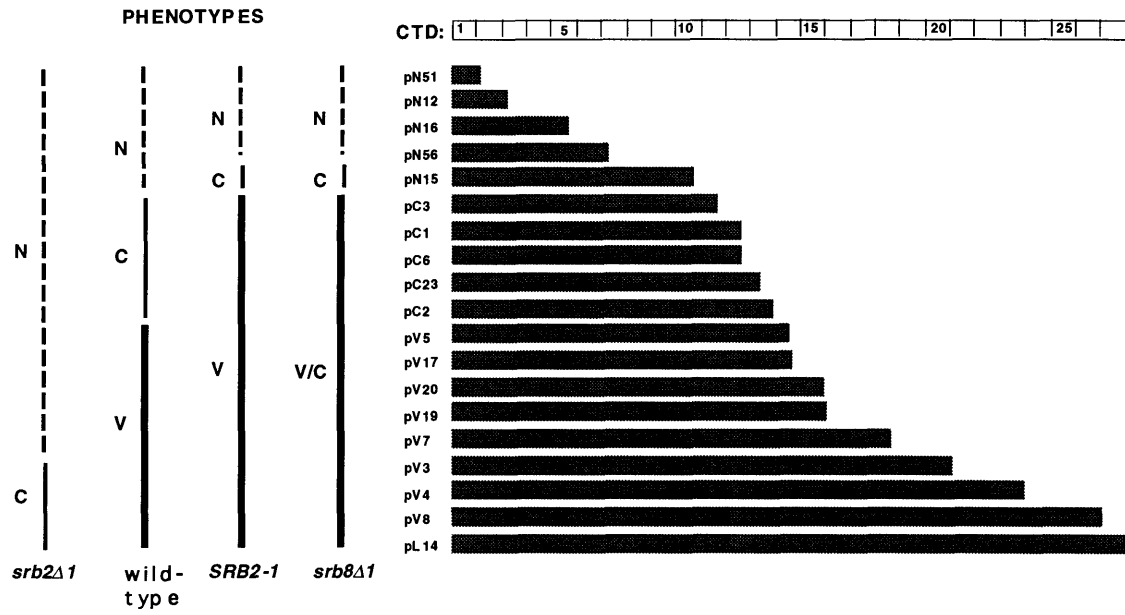


Figure 12

Figure 12. Influence of *SRB2* and *SRB8* alleles on growth phenotypes of RNA polymerase II CTD truncation mutants.

Strains containing combinations of *SRB2* or *SRB8* alleles and CTD truncation alleles were assayed for growth on YEPD medium at 12°C, 30°C, and 38°C and on SC medium containing pyruvate as a sole carbon source. The degree of CTD truncation is shown for each mutant on the horizontal axis, and the plasmid carrying each CTD truncation allele is indicated (i.e., pN51). The phenotypes exhibited by each of the CTD truncation mutants in a wild-type, *srb2Δ1*, *SRB2-1*, or *srb8Δ1* background are shown on left. Nonviable strains (N) are indicated by a dashed line, conditional strains (C) that were extremely sensitive to high (38°C) and low (12°C) temperatures and failed to grow on pyruvate media are indicated by a thin solid line, and viable (V) strains that exhibit nearly wild-type growth characteristics under all conditions tested are indicated by a heavy solid line. Viable/conditional *srb8Δ1* strains (V/C) were able to grow at low temperatures and on pyruvate medium but were sensitive to high temperatures and are indicated by a solid line. Not every CTD truncation allele was tested in every background, but for each background the phenotypic boundaries are well established.



## Discussion

We describe here six novel factors, SRB4, SRB5, SRB6, SRB7, SRB8, and SRB9, which influence the activity of the CTD of RNA polymerase II. These factors were among ten identified in a genetic selection designed to obtain transcription factors which play a role in CTD function. Among the SRB proteins are positive and negative regulators, indicating a dual role for CTD-associated factors in the initiation of transcription.

### Multiple Factors Influence CTD Activity

In order to better define the role of the RNA polymerase II CTD in transcription initiation, extragenic suppressors of a CTD truncation mutant have been isolated (Nonet and Young, 1989). Ten genes; *SRB2*, *SRB4-SRB11*, and *RPB2*, have now been identified in this selection. The observation that the suppressing mutations in these genes suppress the conditional and auxotrophic phenotypes associated with CTD truncations, but not similar phenotypes associated with point mutations outside of the CTD, argues that these gene products and the CTD are involved in the same process in transcription initiation. Genomic DNA for eight of the ten genes identified in this selection has been cloned and sequenced. These SRB factors are necessary for yeast cells to grow at wild-type rates and for survival throughout the normal temperature range for cell growth (Table 1).

*SRB* genes encode positive and negative regulators of CTD function. Dominant, gain-of-function mutations in *SRB2* suppress CTD truncation mutations (Nonet and Young, 1989; Koleske et al., 1992; Thompson et al., 1993). Furthermore, cells lacking *SRB2* can survive only if the CTD is nearly wild-type in length. In contrast, it is the absence of *SRB8* or *SRB9* which suppress CTD truncation mutations. *SRB8* and *SRB9* proteins, therefore, appear to repress CTD activity while the *SRB2* protein enhances CTD activity.

Two mutations in RNA polymerase II located outside of the CTD suppress CTD truncation mutations. An intragenic suppressing mutation in *RPB1* outside of the CTD suppresses truncations of the CTD (Nonet and Young, 1989). In this study we identified a point mutation in *RPB2* that specifically suppresses CTD truncations. The point mutations in *RPB1* and *RPB2* are in regions that are highly conserved among the largest and second largest subunits, respectively,

of eukaryotic RNA polymerases (Nonet and Young, 1989; Kawagishi et al., 1993).

### **A role for the SRBs in Transcription**

All of the SRBs influence the activity of the CTD *in vivo*, arguing that they play a physiological role in transcription initiation. The CTD and SRB2 have been implicated in the response of the transcription apparatus to regulatory signals at promoters *in vivo* and *in vitro* (Allison and Ingles, 1989; Scafe et al., 1990a; Peterson et al., 1991; Liao et al., 1991; Koleske et al., 1992). The SRB proteins have both positive and negative influences on CTD activity, thus the SRB proteins may process positive and negative regulatory signals at promoters and govern the decision to initiate transcription.

**Table 1**      ***SRB* genes**

<b>Gene</b>	<b>Protein mass (kDa)</b>	<b>pI</b>	<b>Chromosomal location<sup>a</sup></b>	<b>Deletion viability</b>
<b><i>SRB2</i></b>	<b>23</b>	<b>5.2</b>	<b>VIII</b>	<b>conditional</b>
<b><i>SRB4</i></b>	<b>78</b>	<b>5.1</b>	<b>V</b>	<b>inviable</b>
<b><i>SRB5</i></b>	<b>34</b>	<b>4.7</b>	<b>VII</b>	<b>conditional</b>
<b><i>SRB6</i></b>	<b>14</b>	<b>4.6</b>	<b>II</b>	<b>inviable</b>
<b><i>SRB7</i></b>	<b>16</b>	<b>4.8</b>	<b>IV</b>	<b>inviable</b>
<b><i>SRB8</i></b>	<b>144</b>	<b>5.7</b>	<b>III</b>	<b>conditional<sup>b</sup></b>
<b><i>SRB9</i></b>	<b>160</b>	<b>5.5</b>	<b>IV</b>	<b>conditional<sup>b</sup></b>
<b><i>RPB2</i></b>	<b>139</b>	<b>6.9</b>	<b>XV</b>	<b>inviable</b>

<sup>a</sup> Precise map locations have been determined

<sup>b</sup> Null alleles partially suppress conditional phenotypes associated with CTD truncations

## Experimental Procedures

### Genetic Manipulations

Yeast strains and plasmids are listed in tables 2 and 3, respectively. Yeast media was prepared as described (Nonet and Young, 1989), except pyruvate medium, which consists of synthetic complete medium (SC) with 2% pyruvic acid (Sigma) as a sole carbon source. Yeast transformations were done using a lithium acetate procedure (Schiestl and Gietz, 1989). Plasmid shuffle techniques were performed as described by Boeke et al. (1987) using 5-fluoro-orotic acid (5-FOA) as a selective agent against *URA3*-containing plasmids. Plasmids were recovered from yeast as described by Hoffman and Winston (1987). Growth assays were performed by suspending similar numbers of cells in water and transferring equal volumes to agar plates with a 48-prong apparatus. To reduce flocculation of some strains, cells were first washed in 100 mM EGTA, 10 mM Tris-HCl 7.5.

Extragenic suppressors of the cold sensitive phenotype of Z551 were isolated as previously described (Nonet and Young, 1989). Dominant and recessive suppressors were identified by mating to Z26, selecting against the presence of pRP112 (Nonet et al., 1987b) 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 β-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 held on ice for 15 min. The suspension was then vortexed 3 min, held 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 offspring 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.

Isogenic wild-type, *SRB4-1*, *SRB5-1* and *SRB6-1* strains containing various *RPB1* (*rpb1-4*, *rpb1-5*, *rpb1-6*, *rpb1-10*, *rpb1-12*, *rpb1-13*, *rpb1-14*, *rpb1-15* and *rpb1-18*) alleles on *LEU2 CEN* plasmids (Scafe et al., 1990b) were constructed using Z26, Z555, Z556 and Z557 and plasmid shuffle techniques. Isogenic wild-type, *SRB4-1*, *SRB5-1* and *SRB6-1* strains containing *rpb1-1* on a *URA3 CEN* plasmid, pRP1-1[U] (Nonet et al., 1987a), were constructed by transforming Z551, Z552, Z553 and Z554 with pRP1-1[U], followed by growth in SC-Ura media to permit loss of pC6 (Nonet et al., 1987b).

Deletions of *SRB4*, *SRB5*, *SRB6*, *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 the proper selective media. 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. Z565 was created by transformation with the EcoRI-XbaI fragment of pCT54 containing the *srb4Δ2::HIS3* fragment and plating on SC-His media. Two or less spores from each tetrad were viable and these spores were all histidine auxotrophs, indicating that *SRB4* is essential. To confirm that *SRB4* is essential, Z565 was transformed with pCT15 (*URA3 SRB4*), tetrads were dissected, and His<sup>+</sup>, Ura<sup>+</sup> segregants were streaked to 5-FOA plates. These were unable to grow on 5-FOA-containing media, confirming that *SRB4* is essential. Z559 was created by transformation with the EcoRI-SphI fragment of pCT37 containing the *srb5Δ1::URA3hisG* fragment and

plating on SC-Ura media. Segregants scored 2:2 for uracil prototrophy and all uracil prototrophs exhibited cold-sensitive, temperature-sensitive and slow growth phenotypes, indicating that *SRB5* deletion strains are conditionally viable. Z564 was created by transformation with the BglII-BamHI fragment of pCT38 containing the *srb6Δ1::URA3hisG* fragment and plating on SC-Ura media. Two or less spores from each tetrad were viable and these spores were all uracil auxotrophs, indicating that *SRB6* is essential. To confirm that *SRB6* is essential, Z564 was transformed with pCT66 (*LEU2 SRB6*), tetrads were dissected and Z566 was created by placing a Ura<sup>+</sup>, Leu<sup>+</sup> segregant onto 5-FOA to select for the excision of the *URA3* gene (Alani et al., 1987). Z566 was transformed with pCT40 (*URA3 SRB6*), grown in SC-Ura media to permit loss of pCT66, then tested for growth on 5-FOA plates. No growth was observed on 5-FOA, confirming that *SRB6* is essential. CHY102 was created by transformation with the SphI-EcoRI *srb7Δ1::URA3hisG* fragment from pCH46. Two or less spores from each tetrad were viable and these spores were uracil auxotrophs, indicating that *SRB7* is essential. SLY35 was created by transformation with the SacI *srb8Δ1::URA3hisG* fragment from pSL315 and CHY105 was created by transformation with the Sall-NotI *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 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) and enzymes were purchased from Promega unless otherwise indicated. Site-directed mutagenesis was performed as described in Kunkel et al. (1987). PCR amplifications to produce pCT54 (*srb4Δ2*), pCT37 (*srb5Δ1*), pCT38 (*srb6Δ1*), pCH45 (*srb7Δ1*), pSL315 (*srb8Δ1*), and pSL307 (*SRB8* in pET-3a) were performed with Taq DNA polymerase (Perkin Elmer) in 100λ of buffer (provided by the manufacturer) supplemented with 1.0 mM MgCl<sub>2</sub> and 200 μM dNTP for a total of 25 cycles. Primer concentrations were 0.5 μM 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).

## Library Construction and Cloning

Yeast genomic DNA libraries were prepared from strains Z28 (wild-type), Z552 (*SRB4-1*), Z553 (*SRB5-1*) and Z554 (*SRB6-1*). Genomic DNA was isolated as described by Phillippsen et al. (1991), partially digested with *Sau3A*, separated on a 0.7% agarose gel, 8-12 kb fragments purified by electroelution and ends partially filled in with d(AG)TP using Klenow. The *URA3* centromeric plasmid pCT3 was digested with *XhoI* and ends partially filled in with d(CT)TP to make them compatible with the partially filled in ends of the *Sau3A* digested genomic DNA. Following ligation, DH5 $\alpha$  cells made competent by the method of Hanahan (Hanahan et al., 1991) were transformed. Libraries contained approximately 150,000 individual recombinants with an average insert size of approximately 10 kb. Subgenomic DNA libraries were prepared from pCT4 (*SRB4*), pCT14 (*SRB5-1*) and pCT26 (*SRB6-1*) in a manner similar to that described above for the genomic DNA libraries. Plasmid insert DNA was partially digested with *Sau3A*, separated on a 1.5% agarose gel, 1-3 kb fragments purified by gene clean (BIO 101) and ends partially filled in with d(AG)TP using Klenow. Fragments were ligated with pCT3 prepared as described above and transformed into DH5 $\alpha$  cells. Subgenomic libraries contained approximately 20,000 individual recombinants with an average insert size of 2 kb.

Genomic clones of *SRB4-1* (pCT8), *SRB5-1* (pCT14) and *SRB6-1* (pCT26) were isolated by transformation of the respective genomic library into Z551, plating to SC-Ura media and placing plates at 12°C following a 12 hour recovery period at 30°C. Approximately one in 2000 primary transformants were able to grow at 12°C. For each library transformed, the genomic clone was isolated from >12 Ura<sup>+</sup> colonies able to grow at 12°C, and retested for the ability to suppress the cold-sensitive phenotype of Z551. A genomic clone of *SRB4* (pCT4) was isolated from the wild-type Z28 library using a recessive *SRB4* allele which has a severe temperature-sensitive phenotype in combination with a CTD truncation allele of 11 repeats. The presence of pCT4 restores a leaky ts phenotype to this strain at 38°C. pCT4 and pCT8 were found to contain overlapping inserts by restriction mapping and sequence analysis. Subgenomic clones from pCT4 (*SRB4*), pCT14 (*SRB5-1*) and pCT26 (*SRB6-1*) were selected as described above for the genomic clones in order to isolate pCT15 and pCT16 (*SRB4*), pCT20 (*SRB5-1*) and pCT29 (*SRB6-1*) respectively. pCT15 and pCT16 differ only in the amount of DNA downstream

of *SRB4*. pCT48, created by replacing *SRB4* in pCT15 with *SRB4-1* from pCT8, suppresses the cold-sensitive phenotype of Z551 confirming that pCT4 and pCT8 contain *SRB4* and *SRB4-1* respectively. pCT39 was created from pCT32 in vivo by transforming Z22 with *SacI*-*XhoI* digested pCT32 DNA and isolating the plasmid from a Ura<sup>+</sup> transformant which had repaired the plasmid with wild-type *SRB5* sequences from the chromosome (Rothstein, 1991). Similarly, *SRB6* was isolated using *BalI*-*SphI* digested pCT29 DNA to create pCT40.

Genomic clones of *SRB7* (pCH2), *SRB8* (pSL301), *SRB9* (pCH47), and *RPB2* (pSL401) were isolated as described in text by transformation and complementation of S242, S358, S363, and S456, respectively. pCH36 was created from pCH7 in vivo by transforming S242 with linearized pCH7 lacking *SRB7* coding DNA and isolating the plasmid from a Ura<sup>+</sup> transformant which had repaired the plasmid with the mutant *srb7-1* sequences from the chromosome (Rothstein, 1991). Similarly, *rpb2-551* (pSL411) was isolated from S456 using pRP212 (Scafe et al., 1990b).

### Sequence Analysis

Insert DNAs from pCT15, pCT20 and pCT29 (containing *SRB4*, *SRB5-1* and *SRB6-1*, respectively) were completely sequenced on each strand. *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. The suppressing mutations in *SRB4*, *SRB5*, *SRB6*, *SRB7*, and *RPB2* were deduced by sequencing using oligonucleotide primers that spanned the entire open reading frames. Sequence comparison analysis was performed at the National Center for Biotechnology Information using the BLAST network service (Altschul et al., 1990).



Table 2. Yeast Strains

Strain	Alias	Genotype
Z22	N114	<i>Mat α ura3-52 his3Δ200 leu2-3,112</i>
Z26	N247	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 [pRP112 (URA3 RPB1)]</i>
Z28	RY4	<i>Mat a/Mat α mal-/mal- gal2/gal2</i>
Z551	N400	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 [pC6 (LEU2 rpb1Δ104)]</i>
Z552	CTY3	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB4-1 [pC6 (LEU2 rpb1Δ104)]</i>
Z553	CTY8	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB5-1 [pC6 (LEU2 rpb1Δ104)]</i>
Z554	CTY9	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB6-1 [pC6 (LEU2 rpb1Δ104)]</i>
Z555	CTY15	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB4-1 [pRP112 (URA3 RPB1)]</i>
Z556	CTY20	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB5-1 [pRP112 (URA3 RPB1)]</i>
Z557	CTY21	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB6-1 [pRP112 (URA3 RPB1)]</i>
Z558	CTY143	<i>Mat a/Mat α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112</i>
Z559	CTY144	<i>Mat a/Mat α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb5Δ1::URA3hisG/SRB5</i>
Z564	CTY158	<i>Mat a/Mat α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb6Δ1::URA3hisG/SRB6</i>
Z565	CTY176	<i>Mat a/Mat α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb4Δ2::HIS3/SRB4</i>
Z566	CTY184	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb6Δ1::hisG [pCT66 (LEU2 SRB6)]</i>
	S242	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb7-1 [pC6 (LEU2 rpb1Δ104)]</i>
	S358	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb8-1 [pC6 (LEU2 rpb1Δ104)]</i>
	S363	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb9-1 [pC6 (LEU2 rpb1Δ104)]</i>

S456	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 rpb2-551 [pC6 (LEU2 rpb1Δ104)]</i>
CHY1	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb7-1 [pRP112 (URA3 RPB1)]</i>
SLY63	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb8-1 [pRP114 (LEU2 RPB1)]</i>
CHY3	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb9-1 [pRP112 (URA3 RPB1)]</i>
SLY64	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 rpb2-551 [pRP114 (LEU2 RPB1)]</i>
CHY102	<i>Mat a/Mat α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb7Δ1::URA3hisG/SRB7</i>
SLY35	<i>Mat a/Mat α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb8Δ1::URA3hisG/SRB8</i>
CHY105	<i>Mat a/Mat α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb9Δ1::URA3hisG/SRB9</i>
SLY61	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb8Δ1::hisG [pRP114 (LEU2 RPB1)]</i>
SLY76	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb8Δ1::hisG [pC6 (LEU2 rpb1Δ104)]</i>
CHY113	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb9Δ1::hisG [pRP114 (LEU2 RPB1)]</i>
CHY116	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 srb9Δ1::hisG [pC6U (URA3 rpb1Δ104)]</i>

Table 3. Plasmids

<u>Plasmid</u>	<u>Description</u>
pCT3	<i>URA3 CEN</i> plasmid. pUN55 (Elledge and Davis, 1988) with HpaI-NaeI fragment removed, XhoI site in polylinker destroyed by digestion and blunting and XhoI linker (CCGCTCGAGCGG) inserted into SmaI site of polylinker
<b><u>SRB4</u></b>	
pCT4	pCT3 with 9 kb genomic (Z28) Sau3A fragment containing <i>SRB4</i> inserted at XhoI site.
pCT8	pCT3 with 8 kb genomic (Z552) Sau3A fragment containing <i>SRB4-1</i> inserted at XhoI site.
pCT15	pCT3 with 2.5 kb subgenomic (pCT4) Sau3A fragment containing <i>SRB4</i> inserted at XhoI site.
pCT16	pCT3 with 2.8 kb subgenomic (pCT4) Sau3A fragment containing <i>SRB4</i> inserted at XhoI site.
pCT48	pCT15 with BstXI-SnaBI <i>SRB4-1</i> C-terminus fragment from pCT8 replacing same <i>SRB4</i> fragment.
pCT54	<i>srb4Δ2::HIS3</i> , created by ligation of <i>SRB4</i> Sall-BamHI from pCT16 with Sall-BamHI of pSP72 (Promega) followed by PCR with the oligos TAATATCCTGAGTCACTCCT and TATGGCTTTTAAGCTGCTTA and ligation of PCR product with SmaI <i>HIS3 kan</i> fragment from B2179 (G. R. Fink).
<b><u>SRB5</u></b>	
pCT14	pCT3 with 9 kb genomic (Z553) Sau3A fragment containing <i>SRB5-1</i> inserted at XhoI site.
pCT20	pCT3 with 1.9 kb subgenomic (pCT14) Sau3A fragment containing <i>SRB5-1</i> inserted at XhoI site.
pCT32	pCT20 with unique SacI site in insert, created by removal of NarI(blunt)-SacII(blunt) fragment from vector.
pCT37	<i>srb5Δ1::URA3hisG</i> , created by ligation of <i>SRB5-1</i> EcoRI-BamHI from pCT20 with EcoRI-BamHI of pSP72 (Promega) followed by PCR with the oligos TAATCATTGGCACCTGGGCA and CTTTCTTCTTAATATGGAA and ligation of PCR product with BglII(blunt)-BamHI(blunt) <i>URA3 kan hisG</i> cassette from B2178 (G. R. Fink).
pCT39	pCT32 containing <i>SRB5</i> , obtained by gap repair of vector containing fragment of pCT32 SacI-XhoI digest.

*SRB6*

- pCT26 pCT3 with 3 kb genomic (Z554) *Sau3A* fragment containing *SRB6-1* inserted at *XhoI* site.
- pCT29 pCT3 with 1.0 kb subgenomic (pCT26) *Sau3A* fragment containing *SRB6-1* inserted at *XhoI* site.
- pCT38 *srb6Δ1::URA3hisG*, created by ligation of *SRB6-1* *EcoRI*-*BamHI* from pCT29 with *EcoRI*-*BamHI* of pSP72 (Promega) followed by PCR with oligos TAAAAGGCGGTATTTATCT and CATATAGTGCCTGGTTGCTC and ligation of PCR product with *BglIII*(blunt)-*BamHI*(blunt)*URA3 kan hisG* cassette from B2178 (G. R. Fink).
- pCT40 pCT29 with *SRB6*, obtained by gap repair of vector containing fragment of pCT29 *Ball-SphI* digest.
- pCT66 *LEU2 CEN* pUN105 (Elledge and Davis, 1988) with *SRB6*, created by ligation of *SRB6* *BamHI*(blunt)-*Sall*(blunt) from pCT40 with *SmaI* digested pUN105.

*SRB7*

- pCH2 *SRB7 URA3 CEN*. pCT3 with 6.7 kb genomic *Sau3AI* fragment inserted at *XhoI* site.
- pCH7 *SRB7 URA3 CEN*. pCT3 with 2.0 kb subgenomic *Sau3AI* fragment inserted at *XhoI* site.
- pCH36 *srb7-1 URA3 CEN*. Obtained by gap repair of vector containing fragment of pCH7 *AflIII* digest.
- pCH46 *srb7Δ1::URA3hisG*. *SRB7* was subcloned into pSP72 (Promega) and the open reading frame deleted using PCR and replaced with the *URA3 kan hisG* cassette from B2178 (G.R. Fink).

*SRB8*

- pSL301 *SRB8 URA3 CEN*. pCT3 with 9.0 kb genomic *Sau3AI* fragment inserted at *XhoI* site.
- pSL311 *SRB8 URA3 CEN*. 6.0 kb *KpnI*-*BglIII* *SRB8* fragment from pSL301 in *KpnI*-*BamHI* of pCT3.
- pSL315 *srb8Δ1::URA3hisG*. *SRB8* was subcloned into pBSIISK (+) (Stratagene) and the open reading frame deleted using PCR and replaced with the *URA3 kan hisG* cassette from B2178 (G.R. Fink).

*SRB9*

- pCH47 *SRB9 URA3 CEN*. pCT3 with 7.3 kb genomic *Sau3AI* fragment inserted at *XhoI* site.
- pCH66 *srb9Δ1::URA3hisG*. *SRB9* was subcloned into pSP72 (Promega) and most of the open reading frame removed by digestion with *SphI* and *Ball* and replaced with the *URA3 kan hisG* cassette from B2178 (G.R. Fink).

**RPB2**

- pSL401 *RPB2 URA3 CEN*. pCT3 with 10 kb genomic *Sau3A* fragment inserted at *XhoI* site.
- pSL411 *rpb2-551 URA3 CEN*. Obtained by gap repair of vector containing fragment of pRP212 (Scafe et. al., 1990) *MluI-XbaI* digest.

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## References

Alani, E., Cao, L., and Kleckner, N. (1987). A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics* 116: 541-545.

Allison, L.A., and Ingles, C.J. (1989). Mutations in RNA polymerase II enhance or suppress mutations in GAL4. *Proc. Natl. Acad. Sci.* 86: 2794-2798.

Allison, L.A., Wong, J.K., Fitzpatrick, V.D., Moyle, M., and Ingles, C.J. (1988). The C-terminal domain of the largest subunit of RNA polymerase II of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and mammals: a conserved structure with an essential function. *Mol. Cell. Biol.* 8: 321-329.

Altschul, S. F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.

Bartolomei, M.S., Halden, N.F., Cullen, C.R., and Corden, J.L. (1988). Genetic analysis of the repetitive carboxyl-terminal domain of the largest subunit of mouse RNA polymerase II. *Mol. Cell. Biol.* 8: 330-339.

Berger, S.L., Pina, B., Silverman, N.S., Marcus, G.A., Agapite, J., Regier, J.L., Triezenberg, S.J., and Guarente, L. (1992). Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. *Cell* 70: 251-265.

Boeke, J., Truehart J., Natsoulis, B., and Fink, G.R. (1987). 5-Flouoro-orotic acid as a selective agent in yeast molecular genetics. *Meth. Enzymol.* 154: 164-175.

Cadena, D.L., and Dahmus, D.E. (1987). Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. *J. Biol. Chem.* 262: 12468-12474.

Conaway, R.C., and Conaway, J.W. (1993). General initiation factors for RNA polymerase II. *Annu. Rev. Biochem.* 62: 161-190.

Corden, J.L. (1990). Tails of RNA polymerase II. *Trends Biochem. Sci.* 15: 383-387.

Elledge, S.J., and Davis, R.W. (1988). A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae*. *Gene* 70, 303-312.

Flanagan, P.M., Kelleher III, R.J., Sayre, M.H., Tschochner, H., and Kornberg, R.D. (1991). A mediator required for activation of RNA polymerase II transcription in vitro. *Nature* 350: 436-438.

Gill, G., and Tjian, R. (1992). Eukaryotic coactivators associated with the TATA box binding protein. *Curr. Opin. Gen. Dev.* 2: 236-242.

- Hanahan, D., Jessee, J., and Bloom, F.R. (1991). Plasmid transformation of *Escherichia coli* and other bacteria. *Meth. Enzymol.* **204**, 63-113.
- Hoffman, C.S., and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**: 267-272.
- Kawagishi, M., Yamagishi, M., and Ishihama, A. (1993). Cloning and sequence determination of the *Schizosaccharomyces pombe rpb2* gene encoding the subunit 2 of RNA polymerase II. *Nuc. Acids Res.* **21**: 469-473.
- Koleske, A.J., Buratowski, S., Nonet, M., and Young, R.A. (1992). A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. *Cell* **69**: 883-894.
- Kolodziej, P.A., Woychik, N., Liao, S.-M., and Young, R.A. (1990). RNA polymerase II subunit composition, stoichiometry, and phosphorylation. *Mol. Cell. Biol.* **10**: 1915-1920.
- Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth. Enzymol.* **154**: 367-382.
- Laybourn, P.J., and Dahmus, M.E. (1990). Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promoter and before the initiation of transcription. *J. Biol. Chem.* **265**: 13165-13173.
- Liao, S.-M., Taylor, I.C., Kingston, R.E., and Young, R.A. (1991). RNA polymerase II carboxy-terminal domain contributes to the response to multiple acidic activators in vitro. *Genes Dev.* **5**: 2431-2440.
- Lu, H., Flores, O., Weinmann, R., and Reinberg, D. (1991). The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. *Proc. Natl. Acad. Sci.* **88**: 10004-10008.
- Nonet, M., Scafe, C., Sexton, J., and Young, R.A. (1987a). Eucaryotic RNA polymerase conditional mutant rapidly ceases mRNA synthesis. *Mol. Cell. Biol.* **7**, 1602-1611.
- Nonet, M., Sweetser, D., and Young, R.A. (1987b). Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. *Cell* **50**: 909-915.
- Nonet, M.L., and Young, R.A. (1989). Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. *Genetics* **123**: 715-724.
- Oliver, S.G., van der Aart, Q.J.M., Agostoni-Carbone, M.L., Aigl, M. et al. (1992). The complete DNA sequence of yeast chromosome III. *Nature* **357**: 38-46.



- Peterson, C.L., Kruger, W., and Herskowitz, I. (1991). A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1. *Cell* 64: 1135-1143.
- Philippesen, P., Stotz, A., and Scherf, C. (1991). DNA of *Saccharomyces cerevisiae*. *Meth. Enzymol.* 194, 169-182.
- Pina, B., Berger, S., Marcus, G.A., Silverman, N., Agapite, J., and Guarente, L. (1993). *ADA3*: a gene, identified by resistance to GAL4-VP16, with properties similar to and different from those of *ADA2*. *Molec. and Cell. Biol.* 13: 5981-5989.
- Pugh, B.F. and Tjian, R. (1992). Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. *J. Biol. Chem.* 267: 679-682.
- Roeder, R.G. (1991). The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. *Trends Biochem. Sci.* 16: 402-408.
- Rothstein, R. 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Meth. Enzymol.* 194: 281-301.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual, Second Edition.* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
- Sawadogo, M., and Sentenac, A. (1990). RNA polymerase B (II) and general transcription factors. *Annu. Rev. Biochem.* 59: 711-754.
- Scafe, C., Chao, D., Lopes, J., Hirsch, J.P., Henry, S., and Young, R.A. (1990a). RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. *Nature* 347: 491-494.
- Scafe, C., Nonet, M., and Young, R.A. (1990b). RNA polymerase II mutants defective in transcription of a subset of genes. *Mol. Cell. Biol.* 10: 1010-1016.
- Schiestl, R.H., and Gietz, R.D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* 16: 339-346.
- Sweetser, D., Nonet, M., and Young, R.A. (1987). Prokaryotic and eukaryotic RNA polymerases have homologous core subunits. *Proc. Natl. Acad. Sci.* 84: 1192-1196.
- Thompson, C.M., Koleske, A.J., Chao, D.M., and Young, R.A. (1993). A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell* 73: 1367-1375.
- Young, R. A. 1991. RNA polymerase II. *Annu. Rev. Biochem.* 60: 689-715.

Zawel, L. and Reinberg, D. (1992). Advances in RNA polymerase II transcription. *Curr. Opin. Cell Biol.* 4: 488-495.

Zehring, W.A., Lee, J.M., Weeks, J.R., Jokerst, R.S., and Greenleaf, A.L. (1988). The C-terminal repeat domain of RNA polymerase II largest subunit is essential in vivo but is not required for accurate transcription initiation in vitro. *Proc. Natl. Acad. Sci.* 85: 3698-36702.

## **Chapter 3**

### **A Multisubunit Complex Associated with the CTD of RNA Polymerase II and TATA-Binding Protein in Yeast**

## Summary

We report biochemical evidence that the RNA polymerase II carboxy-terminal domain (CTD) interacts with a large multisubunit complex which contains SRB protein (SRB2, SRB4-SRB9) and TATA-binding protein (TBP) and is an integral part of the transcription initiation complex. The SRBs are proteins encoded by genes which we identified previously as extragenic suppressors of *Saccharomyces cerevisiae* RNA polymerase II CTD truncation mutations. One of these *SRBs*, *SRB2*, has been shown to encode a 23 kd TBP-binding protein. Antibodies generated against recombinant SRB proteins were produced and used to monitor the SRBs during purification using column chromatography. The SRB proteins, and a portion of cellular TBP, are components of a high molecular weight multisubunit complex that is tightly bound to RNA polymerase II. This SRB-TBP complex binds specifically to, and can be purified via its interaction with, recombinant CTD protein. In vitro transcription and template commitment assays confirm that SRB2 and SRB5 are components of a functional preinitiation complex and are required for efficient transcription initiation.

## Introduction

Regulated transcription initiation by RNA polymerase II in higher eukaryotes involves the formation of a complex with general transcription factors at promoters (Reviewed in Sawadogo and Sentenac, 1990; Roeder, 1991; Zawel and Reinberg, 1992; Conaway and Conaway, 1993). One of these factors, TFIID, contains the TATA-binding protein (TBP), which is able to bind directly to promoter DNA and permits the ordered assembly of the remaining components of the transcription initiation complex. These components include RNA polymerase II and the initiation factors TFIIA, TFIIB, TFIIIE, TFIIIF, TFIIH, and TFIIJ. Sequence-specific DNA-binding proteins appear to regulate the establishment and activity of transcription initiation complexes, possibly through interactions with TFIIB and TBP and additional factors that comprise TFIID.

Several high molecular weight complexes containing TBP have been identified in extracts from human and *Drosophila* cells (reviewed by Gill and Tjian, 1992; Pugh and Tjian, 1992; Sharp, 1992). One of these complexes is TFIID, which contains at least 8 TBP-associated factors (TAFs) (Pugh and Tjian, 1991; Tanese et al., 1991; Timmers and Sharp, 1991; Zhou et al., 1992). A second complex is the RNA polymerase I factor SL1, which contains TBP and 3 TAFs (Comai et al., 1992). A third complex is a component of the RNA polymerase III factor TFIIB, which consists of TBP and 2 TAFs (Taggart et al., 1992; Lobo et al., 1992; White and Jackson, 1992). Some of the TAFs associated with these complexes appear to function as transcriptional coactivators by providing a functional link between sequence-specific regulators and TBP (Pugh and Tjian, 1990; Dynlacht et al., 1991; Pugh and Tjian, 1991; Tanese et al., 1991; Taggart et al., 1992; Zhou et al., 1992; Hoey et al., 1993).

The RNA polymerase II carboxy-terminal domain (CTD) is another component of the transcription apparatus that can bind to TBP (Usheva et al., 1992). The CTD is a highly conserved and apparently unique feature of the largest subunit of RNA polymerase II (Reviewed in Corden, 1990; Young, 1991). The CTD contains 26 to 52 repeats, depending on the organism, of the consensus heptapeptide sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Deletion mutations that remove most or all of the CTD are lethal to cells (Nonet et al., 1987; Allison et al., 1988; Zehring et al., 1988; Bartolomei et al., 1988). CTD partial truncation mutations cause defects in growth and inducible gene

expression in vivo (Nonet et al., 1987; Bartolomei et al., 1988; Allison and Ingles, 1989; Scafe et al., 1990; Peterson et al., 1991) and produce substantial defects in transcription initiation in vitro (Liao et al., 1991). A subset of the RNA polymerase II molecules in yeast and in mammalian cells have highly phosphorylated CTDs (Cadena and Dahmus, 1987; Kolodziej et al., 1990). RNA polymerase II molecules lacking phosphorylation on the CTD are preferentially recruited into the initiation complex (Laybourn and Dahmus, 1990; Lu et al., 1991). Another important feature of RNA polymerase II molecules recruited into the initiation complex is their association with RAPs (RNA polymerase associated proteins) (Burton et al., 1988; Buratowski et al., 1991; Conaway et al., 1991; Flores et al., 1991). Two mammalian proteins, RAP30 and RAP74, have been identified as components of the general transcription factor TFIIF (Flores et al., 1988).

The transcriptional machinery of higher and lower eukaryotes appears to be highly conserved. RNA polymerases I, II, and III are similar in subunit structure and function in higher and lower eukaryotes (Young, 1991). The yeast RNA polymerase II factors b, d, and e (Flanagan et al., 1990; Feaver et al., 1991; Tschochner et al., 1992) are homologous in structure and function to mammalian factors TFIIH/ $\delta$ , TBP and TFIIB/ $\alpha$  (Peterson et al., 1990; Conaway and Conaway, 1991; Gerard et al., 1991; Conaway et al., 1991; Ha et al., 1991; Serizawa et al., 1992; Conaway and Conaway 1993), respectively. Both yeast and human TBP support activation by both GAL4-VP16 and GCN4 (Kelleher et al., 1992). As mentioned above, TBP is a component of multisubunit complexes involved in transcription by all three nuclear RNA polymerases in mammals. In yeast, TBP has been identified as a component of the RNA polymerase III transcription factor TFIIIB (Buratowski and Zhou, 1992; Lopez-De-Leon et al., 1992; Kassavetis et al., 1992), but no yeast counterpart of the TBP-containing complexes SL1 or TFIID have yet been described.

At least four proteins that associate with TBP have been identified through genetic approaches in yeast. The product of the *SUA7* gene is the yeast homologue of the mammalian factor TFIIB (Pinto et al., 1992). TDS4/PCF4/BRF1 is related to TFIIB and is a component of the yeast RNA polymerase III transcription factor TFIIIB (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-De-Leon et al., 1992; Kassavetis et al., 1992). The *SPT3* gene was identified as a mutation that could suppress the effect of retrotransposon insertions into RNA polymerase II promoters. Genetic and

biochemical evidence indicates that SPT3 interacts with TBP (Eisenmann et al., 1992). An extragenic suppressor of RNA polymerase II carboxy-terminal domain (CTD) truncation mutations, *SRB2-1*, encodes a protein that binds TBP and is incorporated into the RNA polymerase II transcription initiation complex (Koleske et al., 1992). Because SPT3 and SRB2 both interact physically with TBP and mutations in these genes affect transcription by RNA polymerase II, both are good candidates for yeast RNA polymerase II TAFs.

The identification of a putative yeast TAF as a suppressor of an RNA polymerase II CTD truncation suggests that additional suppressors of CTD truncation mutations might be used to identify additional TBP-associated components of the RNA polymerase II transcription initiation complex in yeast. An additional set of genes, *SRB4-SRB11*, and *RPB2*, have been identified as suppressors of CTD truncation mutations (Thompson et al., 1993; Chapter 2). Cloning and genetic characterization of *SRB4-SRB9* revealed that these SRBs encode novel proteins that enhance as well as repress CTD function.

We report here biochemical evidence that the yeast RNA polymerase II carboxy-terminal domain (CTD) interacts with a large multisubunit complex that contains the SRBs and TATA-binding protein (TBP). Antibodies generated against recombinant proteins encoded by the *SRB* genes were used to identify and purify a high molecular weight complex which contains TBP and at least a dozen additional polypeptides, all bound to RNA polymerase II. A similar complex can be purified via its interaction with recombinant CTD protein. We show that components of this complex are incorporated into the RNA polymerase II transcription initiation complex.

## Results

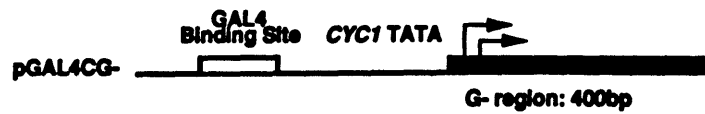
### **SRB2 and SRB5 are Required for Efficient Basal and Activated Transcription In Vitro**

Although yeasts cells lacking *SRB4* or *SRB6* are not viable, cells lacking *SRB2* or *SRB5* are viable despite striking defects in growth (Nonet et al., 1989; Koleske et al., 1992; Thompson et al., 1993; Chapter 2), and it is this feature that facilitates investigation of the transcriptional activity of SRB2 and SRB5 proteins using nuclear extracts in vitro. Previous studies revealed that SRB2 is required for efficient basal and activated transcription initiation in vitro (Koleske et al., 1992). The role of SRB5 was investigated similarly, and was also found to be required for efficient basal and activated transcription initiation in vitro (Figure 1). Nuclear extracts were prepared from wild-type and *srb5Δ1* cells and tested for their ability to synthesize a specific transcript in the presence and absence of purified recombinant SRB5 and GAL4-VP16 proteins. The template contained a single GAL4 binding site upstream of the *CYC1* TATA element that directs expression of a G-less transcript. As expected, extracts from wild-type cells produced two specific transcripts of 375 and 350 nt, and the addition of GAL4-VP16 produced a 35-fold increase in the levels of these transcripts. Extracts from *srb5Δ1* cells required additional factors in order to synthesize significant levels of specific transcripts both in the presence and in the absence of GAL4-VP16 (Figure 1B and C). Complementation of the *srb5Δ1* extract required both purified recombinant SRB2 and SRB5; the addition of SRB5 alone failed to complement. Western blot analysis revealed that the level of SRB2 protein is greatly reduced in nuclear extracts prepared from *srb5Δ1* cells.

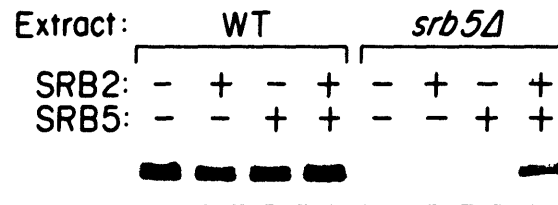
To confirm and extend these results, additional transcription assays were performed using nuclear extracts prepared from cells lacking SRB2 and SRB5 (Figure 1D and E). The results obtained using extracts from cells lacking both SRB proteins were identical to those obtained with extracts from *srb5Δ1* cells. These extracts exhibited no defects in promoter-independent transcription elongation assays. These results demonstrate that both SRB2 and SRB5 are required for efficient basal and activated transcription initiation in vitro.



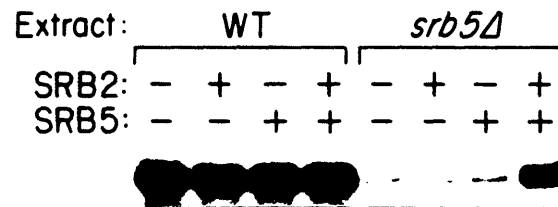
A.



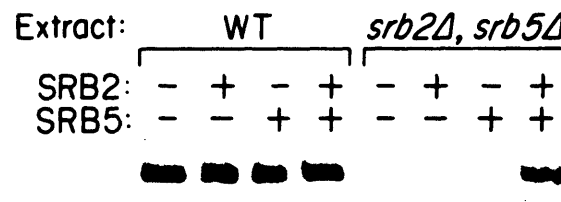
B. - Activator



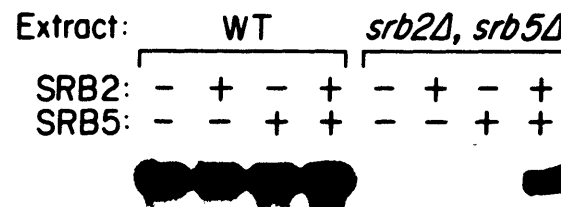
C. + Activator



D. - Activator



E. + Activator



**Figure 1. SRB2 and SRB5 are Required for Efficient Transcription In Vitro**

(A) The template, pGAL4CG- (Lue et. al., 1989), contains a *CYC1* TATA element downstream of a single GAL4 binding site that directs expression of a G-less transcript.

(B) and (C) Nuclear extracts made from wild-type cells (Z561) or *srb5Δ* mutant cells (Z562) were tested for their ability to synthesize specific transcripts from the pGAL4CG- template in the presence or absence of recombinant SRB2 (250 ng) and/or SRB5 (250 ng). Transcription reactions were carried out in the absence (B) or in the presence (C) of recombinant GAL4-VP16 (150 ng). The film shown in (B) was exposed five times longer than that in (C). Quantitation of the results indicates that the level of specific transcripts produced by *srb5Δ* extracts is 50-fold less than that produced by wild-type extracts in the absence of added SRB proteins. Addition of both SRB2 and SRB5 to *srb5Δ* extracts restored transcript levels to approximately 40% of those observed in wild-type extracts.

(D) and (E) Nuclear extracts made from wild-type cells (Z561) or *srb2Δ1, srb5Δ1* mutant cells (Z563) were tested for their ability to synthesize specific transcripts from the pGAL4CG- template in the presence or absence of recombinant SRB2 (250 ng) and/or SRB5 (250 ng). Transcription reactions were carried out in the absence (D) or in the presence (E) of recombinant GAL4-VP16 (150 ng). The film shown in (D) was exposed five times longer than that in (E). Quantitation of the results indicates that the level of specific transcripts produced by *srb2Δ, srb5Δ* extracts is 50-fold less than that produced by wild-type extracts in the absence of added SRB proteins. Addition of both SRB2 and SRB5 to *srb2Δ, srb5Δ* extracts restored transcript levels to approximately 40% of those observed in wild-type extracts.

## **Formation of a Stable Preinitiation Complex Involves SRB2 and SRB5**

A template commitment assay was used to investigate if both SRB2 and SRB5 participate in the formation of a transcription initiation complex (Figure 2). This assay is performed by preincubating one template with an extract that contains all the necessary factors for transcription to occur while preincubating a second template with an extract that is missing a required factor for transcription. After a preincubation period, the two reactions are mixed together. Following a variable mixing period, nucleoside triphosphate substrates are added to permit RNA synthesis. Several conclusions can be derived from this type of experiment. First, if a factor is necessary to assemble a preinitiation complex, the template preincubated with this factor should be preferentially transcribed relative to the other template. Alternatively, if the factor is not involved in preinitiation complex formation, but acts at a later step such as nucleotide incorporation or polymerase release, the templates would be transcribed equally well. Finally, by allowing a variable mixing period following the preincubation, it can be determined if a factor that is required for formation of a transcription initiation complex acts stoichiometrically or catalytically. If a factor acts catalytically there should be ample time for this activity to be carried out on the second template and an increase in the level of transcription from the second template will be observed. Alternatively, if the factor acts stoichiometrically there will be little to no increase in second template transcription due to sequestration of the transcription factor on the first template.

Extracts prepared from cells lacking SRB2 and SRB5 were used to perform the template commitment assay. Two templates were employed that contained identical promoters but differed in G-less cassette length. Specific transcripts of 375 and 350 nt are produced from the long (L) template, while transcripts of 275 and 250 nt are produced from the short (S) template.

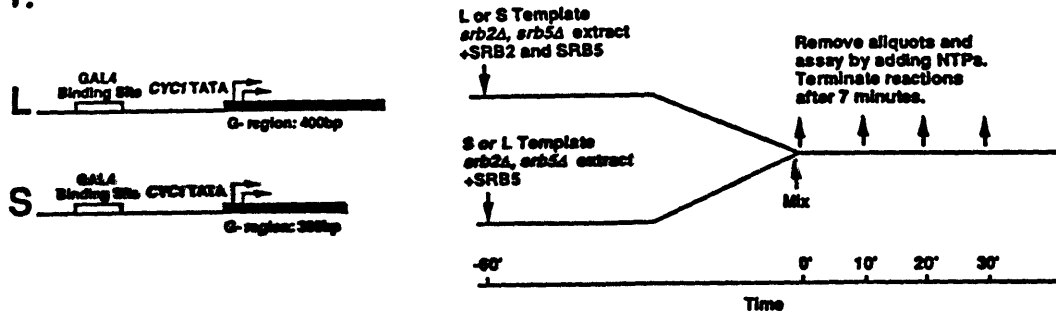
We first performed an experiment to confirm that SRB2 is required for efficient formation of a stable preinitiation complex (Figure 2A), as reported previously (Koleske et al., 1992). The two templates were incubated separately with nuclear extract and SRB5, and a limiting amount of SRB2 protein was added to one of the two reaction mixtures. After a 60 min preincubation, the two reactions were mixed together. Immediately after mixing and every 10 min thereafter (for 30 min) aliquots were removed and nucleoside triphosphate substrates were added to permit RNA synthesis. The reaction was then stopped

after 7 min to minimize multiple rounds of transcription. Control experiments are shown in lanes 1-4. When *srb2* $\Delta$ 1, *srb5* $\Delta$ 1 extracts were preincubated with SRB2 and SRB5 along with either the long (L) template (lane 1) or short (S) template (lane 2), transcripts of the predicted size were produced. When both long and short templates were present in the preincubation mixture, similar levels of long and short transcripts were obtained (lane 3). Virtually no transcript was detected when both templates were preincubated with the extract in the presence of SRB5 alone (lane 4). When SRB2 was added to the long template mixture, long transcripts were predominant after the two extracts were mixed (lanes 5-8). There was no appreciable increase in signal from the short template after 30 min of mixing with the long template. Similarly, when SRB2 was added to the short template mixture, transcripts were produced predominantly from the short template with no appreciable increase in signal from the long template after 30 min of mixing (lanes 9-12).

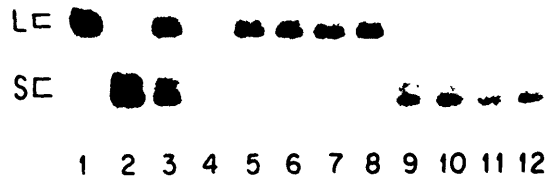
To determine whether SRB5 is required for efficient preinitiation complex formation, a similar experiment was performed (Figure 2B). This time, the two templates were incubated separately with nuclear extract and SRB2, and a limiting amount of SRB5 protein was added to one of the two reaction mixtures. The remaining steps in the experiment were performed as described above. The results of the controls (lanes 1-4) were identical to those in Figure 2A. Lanes 5-12 show that transcripts were predominantly obtained from the template that was preincubated in the presence of SRB5 and that there was no significant increase in signal, even after 30 min, from the template incubated in the absence of SRB5.

The template commitment results indicate that both SRB2 and SRB5 are required for formation of a stable preinitiation complex and that SRB2 and SRB5 act stoichiometrically in the initiation reaction. These conclusions are based upon two observations. First, the template preincubated in the presence of all necessary factors is preferentially transcribed, upon mixing, relative to the other template that was incubated in the absence of either SRB2 or SRB5. Second, following mixing, there is no appreciable increase in signal from the template incubated in the absence of either SRB2 or SRB5. If SRB2 or SRB5 acted subsequent to initiation the templates would be transcribed equally well; by allowing up to 30 min of incubation after template mixing, there was ample time for any catalytic activity to be carried out on the second template. The observation that there was little to no increase in second template transcription,

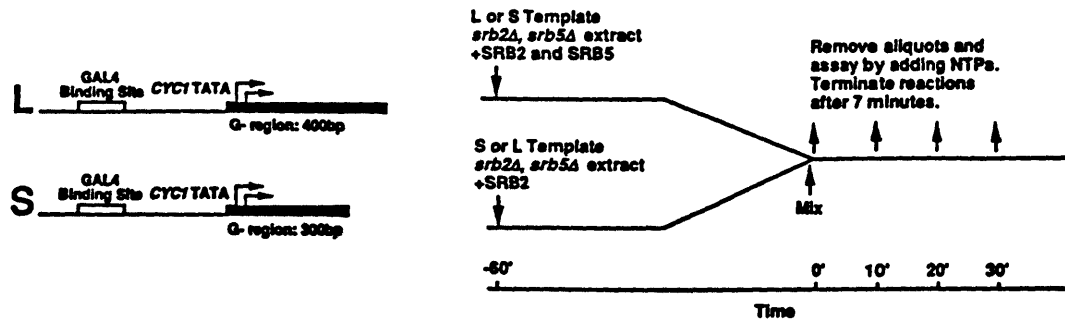
A.



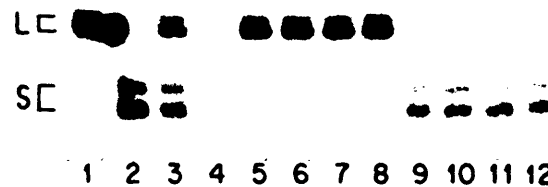
Preincubation [ Template (+SRB2 and SRB5) L S L+S - L L L L S S S S  
 Template (+SRB5) - - - L+S S S S S L L L L  
 Time After Mixing 0 10 20 30 0 10 20 30



B.



Preincubation [ Template (+SRB2 and SRB5) L S L+S - L L L L S S S S  
 Template (+SRB2) - - - L+S S S S S L L L L  
 Time After Mixing 0 10 20 30 0 10 20 30



## Figure 2. SRB2 and SRB5 are Essential for Efficient Preinitiation Complex Formation

(A) SRB2 is necessary for formation of a stable preinitiation complex. The templates used in the template commitment assay each contained a *CYC1* TATA element downstream of a single GAL4 binding site that directs expression of a G-less transcript. The long (L) template (pGAL4CG-) contained a G-less cassette of 400 nt and the short (S) template (pCT108) contained a G-less cassette of 300 nt. The two templates were incubated separately with nuclear extracts from *srb2Δ1*, *srb5Δ1* cells (Z563), SRB5 (250 ng) and GAL4-VP16 (150 ng). A limiting amount of SRB2 protein (25 ng) was added to one of the two reaction mixtures. After a 60 min preincubation, the two reactions were mixed together and aliquots were removed at 10 min intervals and transcriptionally competent complexes were assayed by the addition of nucleoside triphosphates. The reactions were terminated after 7 min to minimize reinitiation. Control experiments are shown in lanes 1-4. Extracts from *srb2Δ1*, *srb5Δ1* cells were preincubated with SRB2, SRB5 and GAL4-VP16 along with S and L template individually (lanes 1-2) or in combination (lane 3). In lane 4 both templates were incubated in the presence of SRB5 and GAL4-VP16, but in the absence of SRB2. After mixing of preincubation reactions, aliquots were removed and nucleoside triphosphates were added at the indicated times (lanes 5-12).

(B) SRB5 is necessary for formation of a stable preinitiation complex. The template commitment assay was performed as in (A) except preincubations were performed in the presence or absence of limiting amounts of SRB5 (75 ng) and excess SRB2 (250ng).

even after 30 min, indicates that SRB2 and SRB5 became stably associated with the first template during the preincubation.

When the experiment in Figure 2A was performed using excess SRB2 in the preinitiation step, transcription increased with time from the template that was preincubated in the absence of SRB2. Similarly, when the experiment in Figure 2B was performed using excess SRB5 in the preincubation step, transcription increased with time from the template that was preincubated in the absence of SRB5, indicating that much of the template preincubated in the absence of SRB2 or SRB5 was still available for transcription and that SRB2 and SRB5 continue to be active for an extended period in the reaction mixture. These data suggest that SRB2 and SRB5 are integral components of the preinitiation complex.

### **SRB2, SRB4, SRB5, SRB6, TBP and RNA Polymerase II are Components of a 1.2 Md Complex**

The ability of mutations in *SRB2*, *SRB4*, *SRB5* and *SRB6* to specifically suppress the growth phenotypes of cells with RNA polymerase II CTD truncations indicates that the products of these genes are involved in the same functional process as the CTD. Template commitment assays suggest that SRB2 and SRB5 are components of the transcription initiation complex. These functional studies led us to investigate whether the SRB proteins interact physically with one another. Cells were constructed that produce functional, epitope-tagged SRB4, SRB5 or SRB6 proteins, and transcriptionally competent nuclear extracts were prepared from these cells. When SRB4, SRB5 or SRB6 were immunoprecipitated, SRB2 and 5-10% of the TBP in the extract were coprecipitated, as revealed by immunoblotting (A. J. K., unpublished results). This observation suggested that the four SRB proteins and TBP are components of a multisubunit complex and led us to attempt purification of the SRB proteins from wild-type cells by conventional chromatography.

Whole cell extracts from wild type cells were fractionated through a series of seven chromatography columns, and rabbit polyclonal antibodies generated against recombinant SRB2, SRB4, SRB5 and SRB6 proteins and against recombinant TBP were used to monitor these proteins during purification (Figure 3). Essentially all of the SRB2, SRB4, SRB5 and SRB6 protein in the whole cell extract cofractionated through the seven purification steps. Approximately twenty additional polypeptides, including a portion of the TBP in

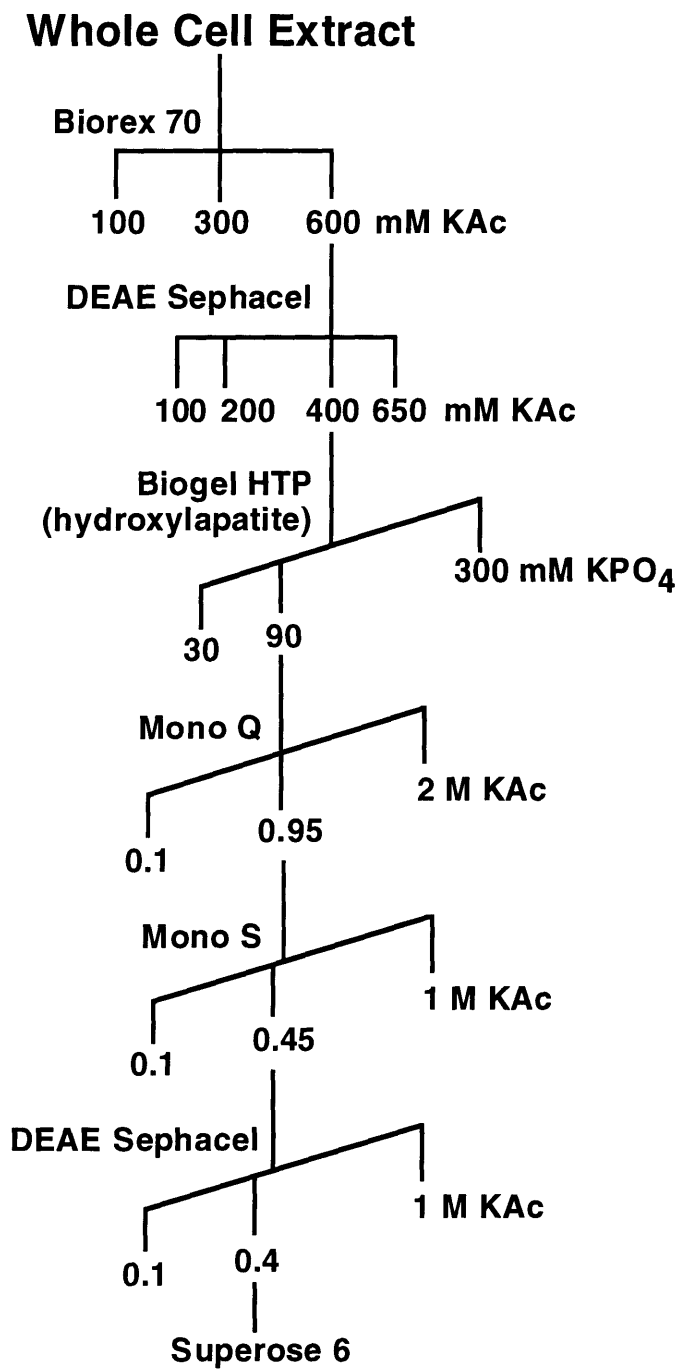
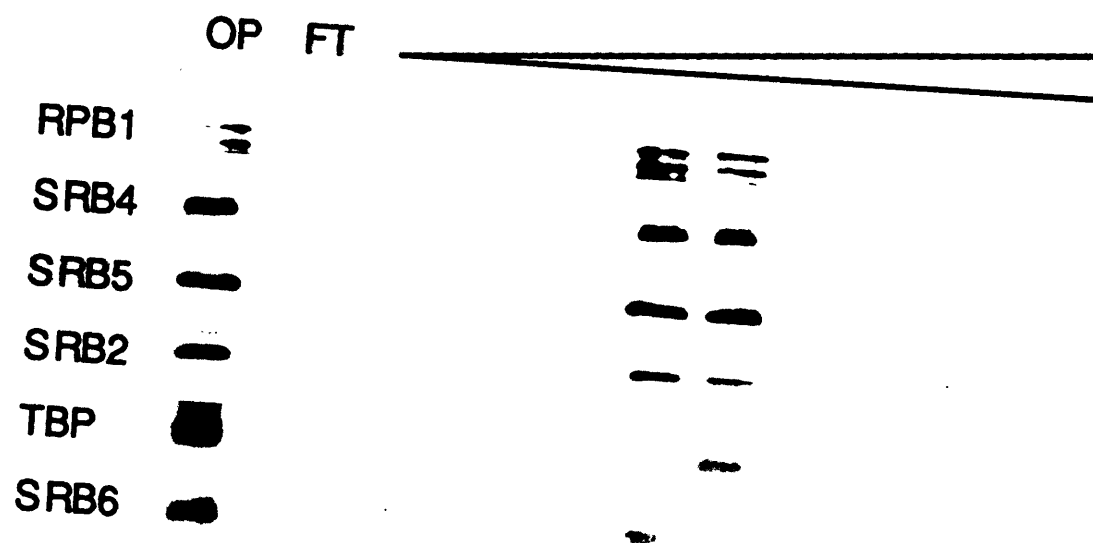


Figure 3A







### Figure 3. Purification of the SRB Complex.

(A) Fractionation scheme.

(B) Left panel. Silver stained 15% SDS polyacrylamide gel containing approximately 1  $\mu$ g of protein from each fraction of the SRB complex purification. M, markers. Lane 1, whole cell extract; lane 2, Biorex 70; lane 3, DEAE-Sephacel; lane 4, hydroxylapatite; lane 5, Mono Q; lane 6, Mono S; lane 7, DEAE-Sephacel. The positions of RNA polymerase II subunits, SRB proteins, TBP and additional polypeptides that are candidate subunits of the complex, are indicated.

Right panel. Western blot analysis of 1  $\mu$ g of SRB complex protein from the DEAE-Sephacel fraction loaded onto a 15 % SDS polyacrylamide gel and probed with antibodies against SRB and TBP proteins. The antibody probes were: lane 1, polyclonal anti-SRB2; lane 2, polyclonal anti-SRB4; lane 3, polyclonal anti-SRB5; lane 4, polyclonal anti-SRB6; lane 5, polyclonal anti-TBP.

(C) Western blot analysis reveals that SRB proteins, RNA polymerase II and TBP coelute from a Mono S column. Semipurified SRB complex (0.8 mg total protein) from the Mono Q column was loaded onto a Mono S column and eluted with a 0.1 to 1.0 M gradient of potassium acetate as described in Experimental Procedures. One twenty-fifth of the onput and flow-through material and one fiftieth of every other eluate fraction were analyzed by Western Blot for the presence of RPB1, SRB4, SRB5, SRB2, TBP, and SRB6. The SRB complex eluted in a peak corresponding to approximately 0.4 M potassium acetate.

the extract, cofractionated with the four SRB proteins. A subset of these additional polypeptides were identified as RNA polymerase II subunits by Western blot analysis.

The high molecular weight complex containing TBP, SRB proteins and RNA polymerase II appears to be quite stable. The proteins in this complex remain tightly associated in fractions exposed to a variety of strong ion exchangers at salt concentrations up to 1.1 M potassium acetate and upon gel filtration in buffers containing 400 mM potassium acetate. Figure 3C shows, for example, the elution profile of TBP, SRB proteins and RNA polymerase II from the mono S column. We estimate that the complex was purified approximately 10,000-fold by quantitative Western blot analysis. The complex appears to be purified to near homogeneity, since the composition of the complex did not change on chromatography subsequent to the Mono S column.

Gel filtration on Superose 6 revealed that these approximately two dozen polypeptides comigrate as a complex at a position corresponding to a native molecular mass of about 1.2 Md. The sum of the apparent molecular weights of the polypeptide bands that appear to be components of the complex is 1.4 Md, consistent with the size predicted by gel filtration. Since RNA polymerase II accounts for approximately 0.5 Md, the remaining complex has a mass of 0.7-0.9 Md. The components of the 1.2 Md complex have both SRB and RNA polymerase activities in vitro. Figure 4 shows that the 1.2 Md complex can complement a nuclear extract lacking SRB2 and SRB5. The specific activity of native SRB2 and SRB5 in the complex was 100 fold that of recombinant SRB2 and SRB5 proteins in this assay. The RNA polymerase activity of the complex is comparable to that obtained with similar amounts of the purified enzyme in nonspecific transcription assays.

### **A CTD Column Specifically Retains a TBP-Containing Multisubunit Complex**

The presence of RNA polymerase II and SRB proteins in a TBP-containing multisubunit complex, together with evidence that the CTD interacts with TBP (Usheva et al., 1992), suggested that the SRB-TBP complex may physically interact with RNA polymerase II via the CTD. To investigate this possibility, yeast whole cell extract was loaded onto columns containing recombinant GST-CTD fusion protein or GST alone, the columns were washed extensively, and bound protein was eluted with low concentrations of guanidine hydrochloride

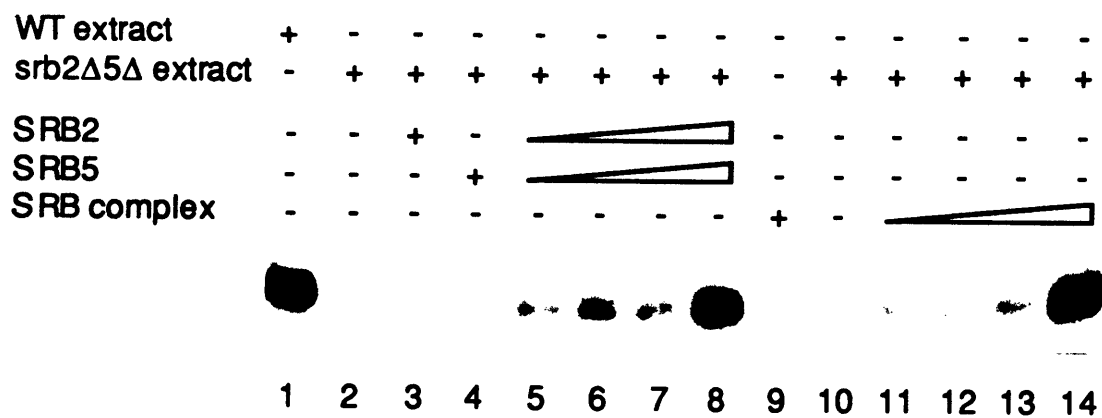
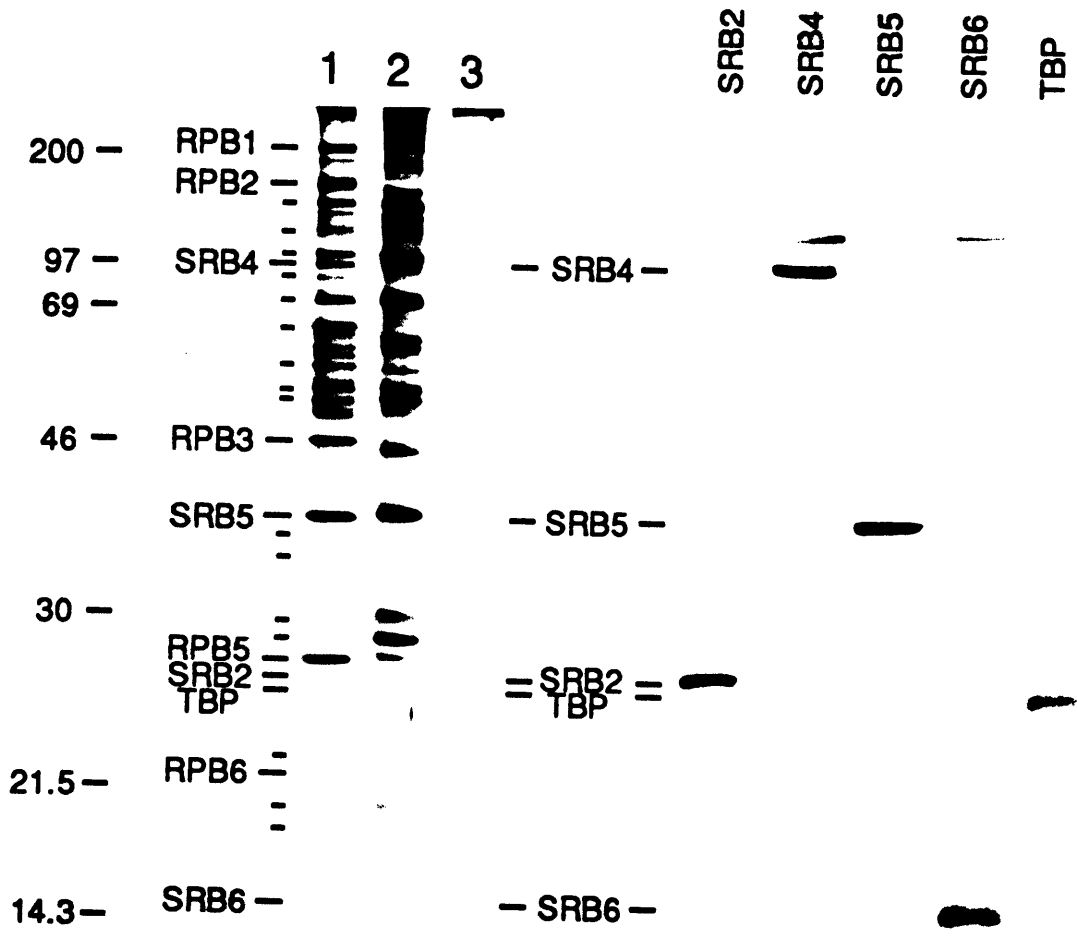


Figure 4 The purified complex contains SRB2 and SRB5 activities.

Nuclear extracts from wild type (Z561) or *srb2Δ1, srb5Δ1* cells (Z563) were tested for their ability to synthesize specific transcripts from the pGAL4CG-template in the presence of recombinant GAL4-VP16 fusion protein (150 ng). Recombinant SRB2 and SRB5 were added to reactions as follows: lane 3, 500 ng SRB2; lane 4, 500 ng SRB5; lane 5, 62.5 ng of both SRB2 and SRB5; lane 6, 125 ng of SRB2 and SRB5; lane 7, 250 ng of SRB2 and SRB5; lane 8, 500ng of SRB2 and SRB5. Purified SRB complex was added to reactions as follows: lane 9, 250 ng; lane 11, 62.5 ng; lane 12, 125 ng; lane 13, 250 ng; lane 14, 500 ng. One  $\mu\text{g}$  of SRB complex contained approximately 20 ng of SRB2 and 25 ng SRB5 as estimated by quantitative Western analysis with known amounts of recombinant SRB2 and SRB5 proteins.



**Figure 5. A TBP-associated Complex Binds to the RNA polymerase II CTD.**

**Left panel.** Silver stained 15% SDS polyacrylamide gel containing the TBP-containing complex purified by conventional chromatography (lane 1) and proteins in a TBP-containing complex purified by CTD affinity chromatography (lane 2). Yeast whole cell extract was loaded on GST-CTD and GST control (lane 3) columns, the columns were washed, and proteins were eluted with 0.3 M guanidine hydrochloride. The positions of RNA polymerase II subunits, SRB proteins, TBP and additional polypeptides that are candidate subunits of the complex purified by conventional chromatography are indicated.

**Right panel.** Western blot analysis of proteins isolated by CTD affinity chromatography. The antibody probes were: lane 1, polyclonal anti-SRB2; lane 2, polyclonal anti-SRB4; lane 3, polyclonal anti-SRB5; lane 4, polyclonal anti-SRB6; lane 5, polyclonal anti-TBP.



(Figure 5). Guanidine hydrochloride (0.3 M) was used for elution because proteins specifically bound to the GST-CTD column could not be eluted with buffers containing high salt concentrations (2 M potassium acetate). The proteins that specifically bound the GST-CTD affinity column include the four SRB polypeptides, TBP and at least a dozen additional polypeptides, many of which appear to be components of the TBP-containing multisubunit complex purified by conventional chromatography.

The TBP-containing complex purified by CTD affinity chromatography differs from the conventionally purified complex principally by lacking stoichiometric amounts of RNA polymerase II subunits. The additional differences in the components of the two complexes may be due to proteins that bind to the CTD but are not components of the SRB complex purified by conventional chromatography. Identifying the precise number of functional components and determining whether some components are modified or processed products of others will require further molecular genetic characterization. Nonetheless, these data indicate that the four SRB proteins and TBP are components of a multisubunit complex that interacts physically with the RNA polymerase II CTD.

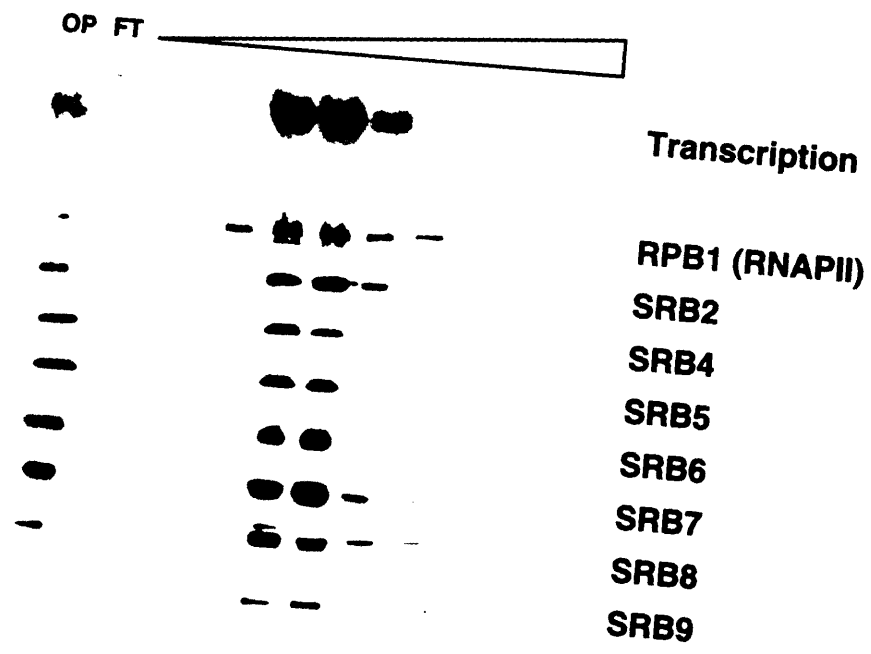
### **SRB-RNA Polymerase II Complex is an RNA Polymerase II Holoenzyme**

Additional western blot analysis and in vitro transcription assays revealed that the large multisubunit complex purified by conventional chromatography contained, in addition to SRB2, SRB4, SRB5, and SRB6 protein, RNA polymerase II and small amounts of TBP, the general transcription factors yTFIIB, yTFIIF, and YTFIIH (Koleske and Young, 1994). This RNA polymerase II holoenzyme is capable of site-specific initiation when supplemented with purified yTFIIE and recombinant TBP and is responsive to activators. TBP levels needed to be supplemented because the purest form of the holoenzyme contains substoichiometric amounts of TBP.

### **SRB7, SRB8, and SRB9 are Components of the RNA Polymerase II Holoenzyme**

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 reconstituted transcription reactions and subject to Western blot analysis with antisera specific to RNA polymerase II and SRB proteins (Figure 6). Transcription activity coeluted with RNA polymerase II and the SRB2, SRB4, SRB5, SRB6, SRB7, SRB8, and SRB9 proteins.



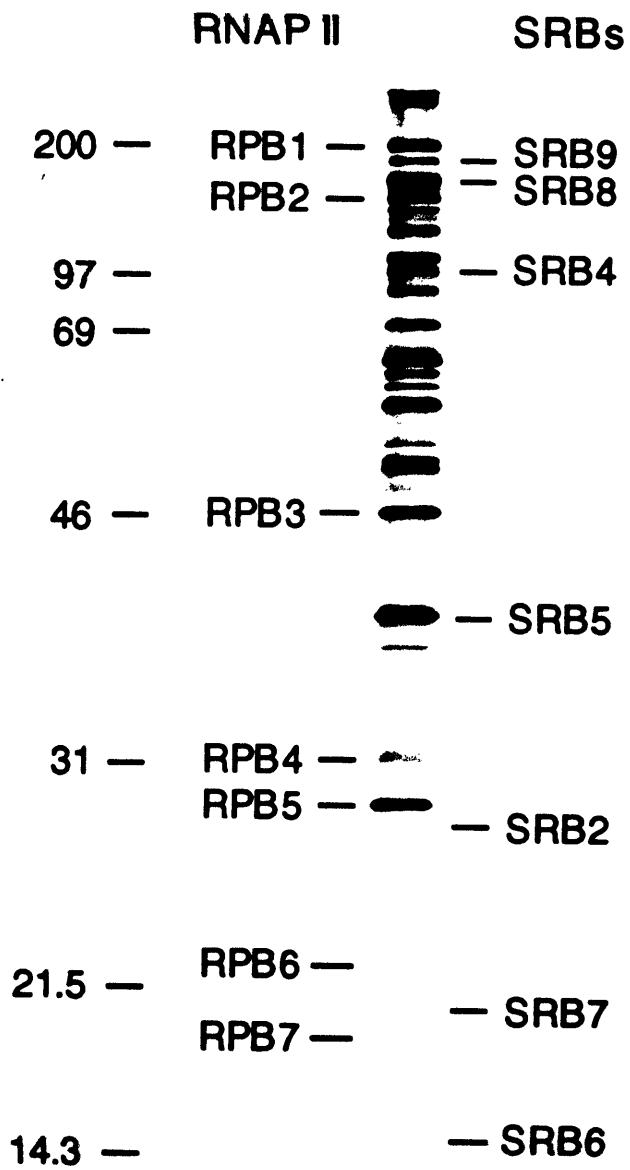


Figure 6. SRB2 and SRB4-SRB9 are components of an RNA polymerase II holoenzyme.

(A) Semipurified holoenzyme that eluted from the Q-sepharose column (Koleske and Young, 1994) was loaded onto a Mono S column and eluted with a 0.1 - 1.0 M gradient of potassium acetate. The output (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. This figure was prepared from digital replicas of primary data scanned using a UMAX UC840 Max Vision digital scanner.

(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 and SRB proteins are indicated. The sizes of protein molecular weight standards are indicated in kd.

## **Discussion**

We have obtained genetic and biochemical evidence for functional and physical interactions between the RNA polymerase II CTD and a high molecular weight multisubunit complex containing TBP in yeast. Evidence for this TBP-containing complex was obtained initially through a genetic selection and subsequently through two independent biochemical purifications. Extragenic suppressors of RNA polymerase II CTD truncation mutations has indicated that at least ten gene products are involved in the same function as the RNA polymerase II CTD (Nonet and Young, 1989; Koleske et al., 1992; Thompson et al., 1993; Chapter 2). The proteins encoded by these SRB genes positively and negatively regulate CTD function. Large multisubunit complexes containing a subset of the SRB proteins and TBP could be purified from yeast, both by conventional chromatography and by CTD affinity chromatography. The TBP-containing complexes purified by the two procedures are similar except that the complex purified by conventional chromatography also contains RNA polymerase II.

### **Features of Yeast SRB-TBP Complex**

A large multisubunit complex containing TBP, SRB proteins and RNA polymerase II was identified using conventional purification approaches. RNA polymerase II, SRB2, SRB4, SRB5, SRB6, and TBP cofractionated with additional proteins through seven chromatographic purification steps. The purified complex also contains SRB7, SRB8, and SRB9. This complex behaves on gel filtration as if it has a native molecular mass of approximately 1.2 Md, consistent with the combined molecular weights of the roughly thirty protein components of the complex, which add up to 1.4 Md when assuming a subunit stoichiometry of one. The complex appears to be quite stable; its components remain tightly associated even when exposed to strong ion exchangers at salt concentrations above 1 M. The specific activity of SRB2 and SRB5 in the purified complex is 100-fold higher than that of recombinant SRB2 and SRB5 proteins and RNA polymerase II within the purified complex has a specific activity that is comparable to that of purified RNA polymerase II in promoter-independent transcription elongation assays. These data suggest that the purified complex represents a physiologically relevant association between TBP, RNA polymerase II and SRB proteins. Three features of the complex - its high molecular weight, stability in high salt, and association with TBP - are

characteristic of some multisubunit TFIID complexes described in mammalian and *Drosophila* cells (Samuels et al., 1982; Conaway et al., 1991; Zhou et al., 1992).

Yeast cells contain approximately 10,000 genes, and at any one time a portion of these genes contain a transcription initiation complex assembled at the promoter. Quantitative Western blot analysis suggests that there are about 1000 molecules per cell of each of the SRB proteins and that essentially all of the SRB proteins in yeast cell lysates are incorporated into the SRB-TBP complex (Koleske and Young, 1994). The complex contains a molecule of RNA polymerase II, which accounts for approximately 2% of the total RNA polymerase II found in these cells; the rest of the enzyme may be actively engaged in transcript elongation or in recycling. These data suggest that transcription initiation complexes containing the SRB proteins may occur at as many as 1000 yeast promoters at any one time. It is not yet clear whether the SRB complex is involved in transcription initiation at all promoters utilized by RNA polymerase II.

RNA polymerase II appears to interact with the SRB-TBP complex via the CTD. A TBP-containing complex lacking RNA polymerase II can be purified by its ability to bind tightly to the CTD. The CTD was previously shown to interact directly with TBP (Usheva et al. 1992), and we have confirmed these results. Thus, the interaction between the CTD and the SRB-TBP complex probably involves a physical interaction with TBP itself. However, components of the complex other than TBP may also interact directly with the CTD. In addition, some components of the conventionally purified complex may interact with RNA polymerase II at sites other than the CTD; this may account for some of the differences in polypeptides associated with the SRB-TBP-RNA polymerase II complex and those that bind the CTD column.

The mammalian RNA polymerase II CTD also appears to interact functionally with high molecular weight forms of TFIID. Assembly of a mammalian initiation complex with native rat TFIID( $\tau$ ) can be blocked by monoclonal anti-CTD antibodies but is not blocked if initiation complexes are assembled with recombinant TBP (Conaway et al., 1992). These data imply that mammalian TBP-associated proteins affect the interaction between the CTD and TBP when the native form of TFIID is employed. This interpretation is consistent with our observation that SRB components of the yeast SRB-TBP complex influence CTD function *in vivo*.

Are the yeast SRBs homologues of mammalian and *Drosophila* TAFs? At least two criteria have been used to define TBP-Associated Factors. TBP-Associated Factors can be described simply as proteins tightly associated with TBP. Although SRB2 can interact directly with TBP (Koleske et al., 1992), the substoichiometric amounts of TBP in the SRB-TBP-RNA polymerase II complex suggest that the interactions with TBP are not as strong as the interactions between mammalian and *Drosophila* TAFs and TBP. Some mammalian and *Drosophila* TBP-Associated Factors can act as coactivators; coactivators are necessary for high levels of activated but not basal transcription *in vitro*. While it is not clear that all TBP-Associated Factors have coactivating activity, some investigators equate TAFs with coactivating factors. It is not yet clear whether components of the SRB-TBP complex are coactivators. SRB2 and SRB5 themselves affect both activated and basal transcription in a crude *in vitro* transcription system, in contrast to the criteria attributed to coactivators which have been defined using partially purified factors. Whether or not the yeast SRBs are "coactivators", the data indicate that SRB proteins are involved in the regulation of transcription initiation, as they are required to obtain fully activated levels of transcription from specific genes *in vivo* (Koleske et al., 1992). The SRB proteins identified thus far do not appear to be among the proteins that are essential for specific transcription initiation in a defined yeast *in vitro* transcription system (R. Kornberg, personal communication). However, the use of purified factors for reconstituted transcription *in vitro* may obviate a requirement for factors, like SRB proteins, that play important roles in transcription initiation *in vivo*.

An important aspect of the yeast multisubunit complex described here is the genetic and biochemical evidence indicating it is physiologically significant. Further analysis has identified this multisubunit complex as an RNA polymerase II holoenzyme based upon its ability to selectively initiate transcription when supplemented with  $\gamma$ TFIIE and TBP and its responsiveness to activators (Figure 7) (Koleske and Young, 1994). The holoenzyme does not contain detectable amounts of DNA (A.J.K., unpublished). These data, combined with the fact that the holoenzyme is highly stable, suggests that assembly of the complex occurs independent of promoter DNA *in vivo*. Indeed, it is possible that the holoenzyme is brought to the initiation complex independent of TBP and the substoichiometric amounts of TBP in the holoenzyme reflects a weaker, transient association occurring in the absence of DNA. Re-assembling the



holoenzyme in vitro from purified components would demonstrate that at least the holoenzyme can form in the absence of DNA. A recent report is consistent with the hypothesis that the RNA polymerase II holoenzyme assembles independent of promoter DNA in vivo. In the absence of DNA, a transcriptionally active assembly of purified RNA polymerase II, TFIIB, TFIIE, TFIIIF, and TFIIH from rat liver can be immunoprecipitated with an antibody directed against the RNA polymerase II CTD (Serizawa et al., 1994).

### **The TBP-Containing Complex as Central Processor**

The RNA polymerase II CTD has been implicated in the response of the transcription apparatus to positive and negative regulatory signals at promoters in vivo (Allison and Ingles, 1989; Scafe et al., 1990; Peterson et al., 1991; Buermeyer et al., 1992). CTD truncations magnify transcriptional defects in a variety of *GAL4* activation mutant strains, whereas extension of the CTD suppresses these defects (Allison and Ingles, 1989). Similarly, there is a progressive loss in the ability to induce transcription of specific yeast genes, such as *GAL10*, as the CTD is truncated from 27 to 11 repeats; sensitivity to CTD truncations map to the upstream activating sequences (UASs) (Scafe et al., 1990). Transcription of the TATA-less mammalian promoter of the dihydrofolate reductase gene in vitro is CTD dependent, and deletion analysis suggests that the Sp1 binding site and the Initiator element confer the requirement for the CTD (Buermeyer et al., 1992). Elimination of at least one negative regulatory factor, *SIN1*, can partially suppress transcriptional defects due to CTD truncation (Peterson et al., 1991).

A large multisubunit complex containing TBP could act as a central processor to receive and act on both positive and negative transcriptional regulatory signals. Some TAF proteins from higher eukaryotes can function as transcriptional coactivators, apparently by serving as a link between DNA-binding activator proteins and TBP. Additional components of TFIID may respond to negative regulatory signals. In yeast, the CTD and SRB proteins have been implicated in the response of the transcription apparatus to regulatory signals at promoters in vivo and in vitro (Allison and Ingles, 1989; Scafe et al., 1990; Peterson et al., 1991; Liao et al., 1991; Koleske et al., 1992). The SRB proteins have positive and negative influences on CTD activity (Koleske et al., 1992; Thompson et al., 1993; Chapter 2). The presence of SRB proteins in the RNA polymerase II holoenzyme that have opposite influences on

CTD activity supports the idea that such a large multisubunit complex may receive and respond to both positive and negative transcriptional regulatory signals. Thus, the SRBs, together with TBP (TFIID), may process multiple signals at promoters and govern the decision to initiate transcription.

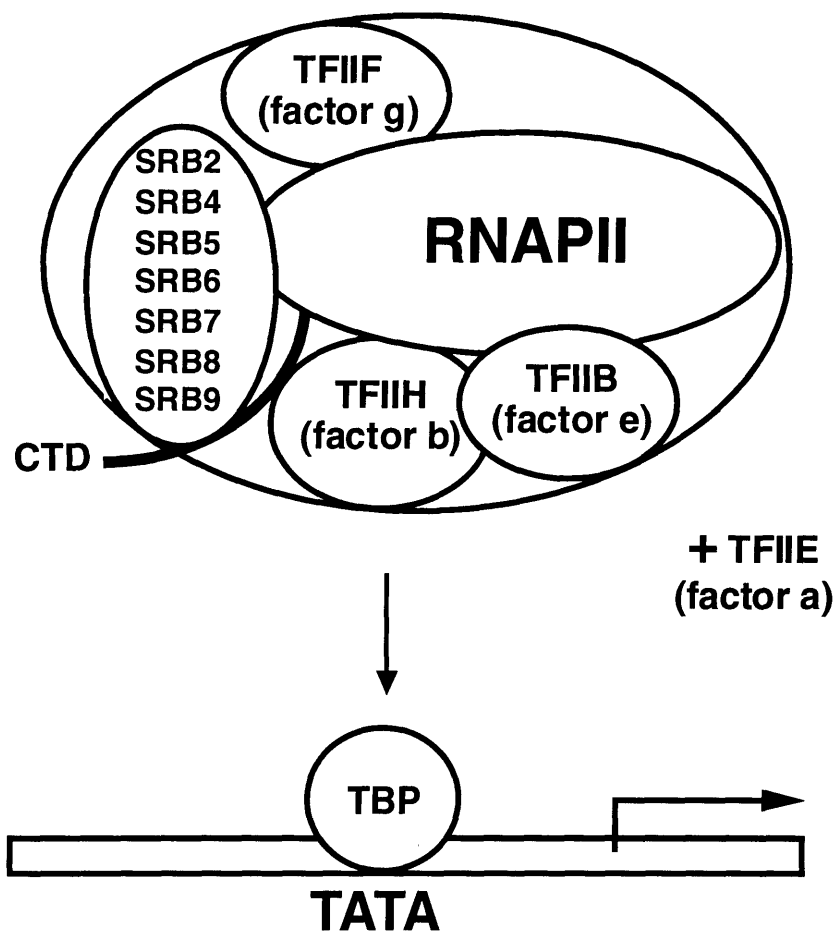


Figure 7

Figure 7. Holoenzyme model.

The initiation factors b, e, and g (yTFIIH, yTFIIB, and yTFIIF) and the SRB proteins are tightly associated with RNA polymerase II to form a holoenzyme. This holoenzyme and factor a (yTFIIE) associate with a complex of TATA-binding protein (TBP) and DNA to form an initiation complex. The SRBs may influence the stability of the holoenzyme or the recruitment of the holoenzyme into a preinitiation complex, possibly in response to regulatory factors.

## Experimental Procedures

### Genetic Manipulations

Yeast strains and plasmids are listed in tables 1 and 2, respectively. Several strains were constructed for producing yeast nuclear extracts for in vitro transcription assays. Z425 was mated to Z560 and tetrads dissected to produce the wild-type Z561, *srb5Δ1::URA3hisG* strain Z562 and *srb2Δ1::HIS3, srb5Δ1::URA3hisG* strain Z563. Z562 and Z563 display identical temperature-sensitive, cold-sensitive and slow-growth phenotypes.

### 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 pSL307 (*SRB8* in pET-3a) were performed with Taq DNA polymerase (Perkin Elmer) in 100 $\lambda$  of buffer (provided by the manufacturer) supplemented with 1.0 mM MgCl<sub>2</sub> and 200  $\mu$ M dNTP for a total of 25 cycles. Primer concentrations were 0.5  $\mu$ M 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).

### Purification of Recombinant Proteins

Purification of SRB2 has been previously described (Koleske et al., 1992). SRB5, 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 pCT98, pCH34, and pSL307, respectively, in the same manner SRB2 was purified. SRB4, SRB6, and a portion of SRB9 (aa 45 to 501) were purified as fusions to glutathione-S-transferase from DH5 $\alpha$  carrying pCT107, pCT116, and pCH64, respectively, according to method of Smith and Johnson (1988). GAL4(1-147)-VP16 protein was purified as described by Chasman et al. (1989) from XA90 carrying pJL2. GST-fusion proteins for CTD affinity purification were purified from DH5 $\alpha$  carrying pDC127 or pDC130 by affinity chromatography on glutathione agarose (Sigma) and Ni-NTA agarose (Qiagen), and then by ion-exchange chromatography on SP Sepharose (Pharmacia) to an approximate purity of 95%.

### In vitro Transcription

Promoter-dependent in vitro transcription using nuclear extracts was carried out as described by Liao et al. (1991). 300 ng of template were used for

promoter-dependent *in vitro* transcription reactions, except the template commitment assays in which 600 ng of template were used per reaction. Optimal activity was obtained using 100  $\mu$ g of Z561 protein, 150  $\mu$ g of Z562 protein and 150  $\mu$ g of Z563 protein. Transcripts were quantified using a Fuji Bio-image analyzer. Promoter-independent transcription assays were performed according to Nonet et al. (1987). Purified SRB complex used in *in vitro* transcription assays was purified as described below. Eluate from the second Biorex 70 column was dialyzed in Buffer A(50) and concentrated four fold by centrifugation through Centricon 10 filter units (Amicon). *In vitro* transcription assay for holoenzyme activity was performed as described (Koleske and Young, 1994).

### **Purification of SRB Complex**

Yeast strain BJ926 (Buchman et. al., 1988) was grown at 30°C to OD<sub>600</sub> of 4.0 to 4.5 in 1X YNB medium (0.15% Difco yeast nitrogen base, 0.5% ammonium sulfate, 200  $\mu$ M inositol, 2% glucose). The level of the SRB complex appears to be elevated in cells grown in minimal medium (A.J.K., unpublished results), and this observation was exploited to facilitate purification of the TBP-containing SRB complex. Cells were collected by centrifugation and washed in ice-cold buffer (20 mM HEPES KOH pH 7.5, 10% glycerol, 50 mM potassium acetate, 1 mM DTT, and 1 mM EDTA). Whole cell extract was prepared from 480 g of cell paste as described by Sayre et al. (1992). Protease inhibitors used where indicated were: 1 mM PMSF, 2 mM benzamidine, 2  $\mu$ M pepstatin A, 0.6  $\mu$ M leupeptin, 2  $\mu$ g/ml chymostatin, 5  $\mu$ g/ml antipain HCl (Sigma).

During purification, the SRB complex was monitored by Western blot using antibodies to SRB2, SRB4, SRB5, and SRB6. Silver staining of gels was performed as per Blum et al. (1987) with minor modifications. The gels were fixed for a minimum of 4 hours and the impregnation with silver nitrate was performed for 40 minutes.

Whole cell extract (8 g protein in 390 ml) was diluted 1:5 in Buffer A (20% glycerol, 20 mM Hepes KOH pH 7.5., 1mM DTT, 1 mM EDTA and protease inhibitors). The extract was loaded onto 5 cm x 17 cm Biorex 70 (Biorad) column at a flow rate of 5 ml/min. The column was washed with Buffer A (100) (Buffer A containing 100 mM potassium acetate) until no further protein could be eluted from the column. The column was then eluted with step washes of Buffer A (300) and Buffer A (600). The SRB complex eluted in the 600 mM potassium

acetate step. The Biorex 70 (600) fraction (250 mg in 120 ml) was diluted 1:6 with Buffer B (20% glycerol, 20 mM Tris-acetate pH 7.9, 1mM DTT, 1 mM EDTA, 0.01% NP40 and protease inhibitors) and was loaded onto a 2.5 cm x 8.5 cm DEAE-Sephacel (Pharmacia) at a flow rate of 4 ml/ min. The column was washed extensively with Buffer B (100) and then eluted with step washes of Buffer B (400) and Buffer B (650). The SRB complex eluted from this column in the 400 mM potassium acetate step. The DEAE-Sephacel (400) fraction (48 ml) was dialyzed into Buffer C (20% glycerol, 10 mM potassium phosphate pH 7.7, 100 mM potassium acetate, 1 mM DTT, 0.25 mM EDTA, 0.01% NP40 and protease inhibitors). The dialysate was spun in Sorvall SS34 rotor at 10,000 rpm for 20 min and the supernatant (50 mg protein in 50 ml) was loaded onto a 1.5 x 6.5 cm Bio-Gel HTP Hydroxylapatite at a flow rate of 1 ml/min. The column was washed with 20 ml of loading buffer and eluted with a 120 ml linear gradient of Buffer C to Buffer D (Buffer D is identical to Buffer C except that it contains 300 mM potassium phosphate pH 7.7). The SRB complex eluted from this column in a peak corresponding to 68 mM to 112 mM potassium phosphate. The 20 ml of eluate from the Bio-Gel HTP (Biorad) was dialyzed against Buffer E (same as Buffer B except 0.25 mM EDTA) containing 100 mM potassium acetate. The dialyzed material was spun in a Sorvall SS34 rotor at 10,000 rpm for 20 min and the supernatant (11 mg protein in 20 ml) was loaded onto a Mono Q HR 5/5 FPLC column (Pharmacia) and eluted with a 15 ml linear gradient from Buffer E (100) to Buffer E (2000) at a flow rate of 0.5 ml/min. The SRB complex eluted from this column at 0.95 M potassium acetate. Peak fractions containing SRB activity were diluted 1:6 with Buffer F (same as Buffer A except 0.25 mM EDTA). This material (1.1 mg protein in 10 mls) was loaded onto a Mono S HR 5/5 FPLC column (Pharmacia) and eluted with a 10 ml gradient from Buffer F (100) to Buffer F (1000) at a flow rate of 0.5 ml/min. The SRB complex eluted from this column at 450 mM potassium acetate. This material (0.6 mg in 8 ml) was diluted 1:4 in Buffer E (0) and loaded onto a 1.5 cm x 1.5 cm DEAE-Sephacel column and eluted with a 20 ml gradient from Buffer E (100) to Buffer E (1000) at a flow rate of 0.3 ml/min. The SRB complex eluted from this column at 400 mM potassium acetate. (Further chromatography revealed that this material was approximately 90% pure). This material (0.5 mg protein in 2 ml) was diluted 1:4 in Buffer F (0) and loaded onto a 1.5 cm x 1 cm Biorex 70 column and was eluted with a 10 ml gradient from Buffer F (100) to Buffer F (1000). The SRB complex eluted from this column at 600 mM

potassium acetate. The SRB complex eluted from this column was approximately 95% pure. The total yield of the SRB complex was 0.5 mg and purification was estimated to be 10,000 fold.

The SRB complex was subjected to gel filtration chromatography in Buffer F (400) on a Superose 6 HR 10/30 FPLC column (Pharmacia). The estimated molecular size of the SRB complex was determined by extrapolation of a calibration curve performed with thyroglobulin (669 kD), apoferritin (443 kD), bovine serum albumin (132 kD, 66 kD), carbonic anhydrase (29 kD).

### **CTD Affinity Purification**

Whole cell extracts were prepared by adding 1.6 liters of 4% glucose to 800 g of Red Star Dry Yeast, incubating the mixture at room temperature for 45 minutes, and adding 800 ml of disruption buffer [1.2M ammonium sulfate, 0.16M K-HEPES pH 7.3, 4 mM DTT, protease inhibitors (as in the conventional purification above)]. 200 ml aliquots were frozen dropwise in liquid nitrogen and blended for 5 to 10 minutes in a Waring blender. After thawing at 55°C, viscosity was reduced by brief blending. Disrupted cells were centrifuged 30 min at 12,000 rpm in a Sorvall GSA rotor, and the clarified supernatant was filtered through cheesecloth. One-twentieth volume of a 10% solution of Polymyxin P was added, the extract was incubated on ice for 30 min, and the solution was centrifuged 30 min at 12,000 rpm in a Sorvall GSA rotor. The supernatant was collected and brought to 70% saturation with solid ammonium sulfate and stored at 4°C.

An aliquot of the suspension was removed from storage and centrifuged at 12,000 rpm in a Sorvall GSA rotor for 30 minutes. The pellet was resuspended in 1.5 volumes of 1X Transcription Buffer (Liao et al., 1991) + protease inhibitors and centrifuged at 17,000 rpm in a Sorvall SS-34 rotor for 20 min. The supernatant was then diluted 1:6 in 1X Transcription Buffer + protease inhibitors, and centrifuged at 12,000 rpm in a Sorvall GSA rotor for 30 minutes. The supernatant was incubated with 10g/100 ml of Cell Debris Remover (Whatman Labsales) for 15 min. The Cell Debris Remover was removed by centrifugation and filtration. The cleared supernatant was then centrifuged at 40,000 rpm in a Beckman 50.2Ti rotor for 1 to 2 hours.

GST fusion proteins were coupled to Pharmacia Activated CH Sepharose according to manufacturer's directions at a concentration of 5 mg protein/ml matrix. The affinity matrices were washed with 6M guanidine



hydrochloride followed by 1X Transcription Buffer before use. 20 ml of yeast whole cell extract were mixed with 1/10 volume of 1X Transcription Buffer + 10% Triton X-100 and applied to 100  $\mu$ l of either GST-Sepharose or GST-CTD Sepharose. The columns were washed with 20 ml of 1X Transcription Buffer + 1% Triton X-100, followed by 5 ml of 1X Transcription Buffer without Triton X-100. Bound proteins were eluted with 1X Transcription Buffer containing various concentrations of guanidine hydrochloride.

### **Western Blot Analysis**

Western blotting of fractions was performed with polyclonal rabbit antisera raised against TBP, SRB2, GST-SRB4, SRB5, GST-SRB6, SRB7, SRB8 (aa 868 to 1226), and GST-SRB9 (aa 45 to 501) by standard methods (Harlow and Lane, 1989). RPB1 was detected via the CTD with 8WG16 monoclonal antibody ascites fluid (Thompson et al., 1989). In all cases, bands were visualized by secondary probing with alkaline phosphatase conjugate secondary antibodies (Promega).

Table 1. Yeast Strains

Strain	Alias	Genotype
BJ926		<i>Mat a/Mat <math>\alpha</math> trp1/TRP1 prc1-126/prc1-126 pep4-3/pep4-3 prb1-1122/prb1-1122 can1/can1</i>
Z425	YTK73	<i>Mat a his3<math>\Delta</math>200 leu2-3,112 ura3-52 trp1<math>\Delta</math>1 lys2-801 srb2<math>\Delta</math>1::HIS3</i>
Z560	CTY148	<i>Mat <math>\alpha</math> ura3-52 his3<math>\Delta</math>200 leu2-3,112 srb5<math>\Delta</math>1::URA3hisG</i>
Z561	CTY151	<i>Mat a ura3-52 his3<math>\Delta</math>200 leu2-3,112 lys2-801</i>
Z562	CTY153	<i>Mat a ura3-52 his3<math>\Delta</math>200 leu2-3,112 lys2-801 srb5<math>\Delta</math>1::URA3,hisG</i>
Z563	CTY154	<i>Mat a ura3-52 his3<math>\Delta</math>200 leu2-3,112 lys2-801 srb2<math>\Delta</math>1::HIS3 srb5<math>\Delta</math>1::URA3hisG</i>

Table 2. Plasmids

Plasmid	Description
pCH34	<i>SRB7</i> in pET-3a (Studier and Moffat, 1986). NdeI site at ATG created by site directed mutagenesis followed by insertion of <i>SRB7</i> into NdeI of pET-3a.
pCH64	<i>SRB9</i> (encoding aa 45 to 501) in pGEX-1 (Smith and Johnson, 1988). 1.4 kb EcoRI fragment from pCH47(Thompson, 1994) encoding aa 45 to 501 ligated to EcoRI of pGEX-1.
pCT98	pET-3a (Studier and Moffat, 1986) with <i>SRB5</i> . NdeI site at ATG of <i>SRB5</i> created by ligation of <i>SRB5</i> EcoRI-BamHI from pCT39 (Thompson et al., 1993) with EcoRI-BamHI of pBSIISK(-) (Stratagene) followed by site directed mutagenesis. NdeI-EcoRI(blunt) <i>SRB5</i> containing fragment was then ligated with NdeI-BamHI(blunt) digested pET-3a.
pCT107	pGEX-2T (Smith and Johnson, 1988) with <i>GST-SRB4</i> fusion. NdeI site at ATG of <i>SRB4</i> created by ligation of <i>SRB4</i> Sall-XbaI from pCT15 (Thompson et al., 1993) with Sall-XbaI of pBSIISK(-) (Stratagene) followed by site directed mutagenesis. NdeI(partial/blunt)-SnaBI <i>SRB4</i> containing fragment was then ligated with BamHI(blunt) digested pGEX-2T.
pCT108	pGAL4CG- (Lue et. al., 1989) with 300 bp G-less cassette created by ligating SmaI G-less cassette from pJJ460 (Woontner et al., 1991) with SmaI vector fragment of pGAL4CG-.
pCT116	pGEX-2T (Smith and Johnson, 1988) with <i>GST-SRB6</i> fusion. NdeI site at ATG of <i>SRB6</i> created by ligation of <i>SRB6</i> Sall-XbaI from pCT40 (Thompson et al., 1993) with Sall-XbaI of pBSIISK(+) (Stratagene) followed by site directed mutagenesis. NdeI(blunt)-XbaI <i>SRB6</i> containing fragment was then ligated with BamHI(blunt) digested pGEX-2T.
pDC127	pQE9 (Qiagen) with 6xHIS-GST-12CA5 fusion. An oligonucleotide encoding the 12CA5 epitope flanked by a BglII and a BamHI site was cloned into same of pSP72 (Promega) followed by insertion into BamHI of pGEX-2T (Pharmacia). GST-12CA5 fusion was amplified by PCR and inserted into BamHI-Sall digested pSP72. GST-12CA5 fusion was then cloned into pQE9.

- pDC130 pQE9 (Qiagen) with 6xHIS-GST-12CA5-CTD fusion. A KpnI *RPB1* containing fragment from pV14 (Nonet, et. al., 1987b) was inserted into same of pSP72 (Promega) followed by insertion of the BamHI fragment encoding the CTD and 98 N-terminal adjoining amino acids of RPB1 into pDC127.
- pSL307 *SRB8* (encoding aa 868 to 1226) in pET-3a (Studier and Moffat, 1986). DNA encoding aa 868 to 1226 was PCR amplified and inserted into BamHI of pET-3a.

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## References

Allison, L. A., and Ingles, C. J. (1989). Mutations in RNA polymerase II enhance or suppress mutations in *GAL4*. *Proc. Natl. Acad. Sci. USA* *86*, 2794-2798.

Allison, L. A., Wong, J. K., Fitzpatrick, V. D., Moyle, M., and Ingles, C. J. (1988). The C-terminal domain of the largest subunit of RNA polymerase II of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and mammals: A conserved structure with an essential function. *Mol. Cell. Biol.* *8*, 321-329.

Bartolomei, M. S., Halden, N. F., Cullen, C. R., and Corden, J. L. (1988). Genetic analysis of the repetitive carboxyl-terminal domain of the largest subunit of RNA polymerase II. *Mol. Cell. Biol.* *8*, 330-339.

Blum, H., Beier, H., and Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* *8*, 93-99.

Buchman, A. R., Lue, N. F., and Kornberg, R. D. (1988). Connections between transcriptional activators, silencers, and telomeres as revealed by functional analysis of a yeast DNA-binding protein. *Mol. Cell. Biol.* *8*, 5086-5099.

Buermeyer, A. B., Thompson, N. E., Strasheim, L. A., Burgess, R. R., and Farnham, P. J. (1992). The HIP1 initiator element plays a role in determining the *in vitro* requirement of the dihydrofolate reductase gene promoter for the C-terminal domain of RNA polymerase II. *Mol. Cell. Biol.* *12*, 2250-2259.

Buratowski, S., Sopta, M., Greenblatt, J., and Sharp, P. A. (1991). RNA polymerase II-associated proteins are required for a DNA conformation change in the transcription initiation complex. *Proc. Natl. Acad. Sci. USA* *88*, 7509-7513.

Buratowski, S. and Zhou, H. (1992). A suppressor of TBP mutation encodes an RNA polymerase III transcription factor with homology to TFIIB. *Cell* *71*, 221-230.

Burton, Z. F., Killeen, M., Sopta, M., Ortolan, L. G., and Greenblatt, J. (1988). RAP 30/74: a general initiation factor that binds to RNA polymerase II. *Mol. Cell. Biol.* *8*, 1602-1613.

Cadena, D. L., and Dahmus, M. E. (1987). Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. *J. Biol. Chem.* *262*, 12468-12474.

Colbert, T., and Hahn, S. (1992). Isolation of a yeast TFIIB related factor involved in RNA polymerase III transcription. *Genes Dev.* *6*, 1940-1949.

Comai, L., Tanese, N., and Tjian, R. (1992). The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. *Cell* *68*, 965-976.

Conaway, J. W., and Conaway, R. C. (1991). Initiation of eukaryotic messenger RNA synthesis. *J. Biol. Chem.* *266*, 17721-17724.

Conaway, J. W., and Conaway, R. C. (1993). General initiation factors for RNA polymerase II. *Annu. Rev. Biochem.* *62*, 161-190.

Conaway, R. C., Bradsher, J. N., and Conaway, J. W. (1992). Mechanism of assembly of the RNA polymerase II preinitiation complex. Evidence for a functional interaction between the carboxyl-terminal domain of the largest subunit of RNA polymerase II and a high molecular mass form of the TATA factor. *J. Biol. Chem.* *267*, 8464-8467.

Conaway, R. C., Garret, K. P., Hanley, J. P., and Conaway, J. W. (1991). Mechanism of promoter selection by RNA polymerase II: Mammalian transcription factors  $\alpha$  and  $\beta$  promote entry of polymerase into the preinitiation complex. *Proc. Natl. Acad. Sci. USA* *88*, 6205-6209.

Corden, J. L. (1990). Tails of RNA Polymerase II. *Trends Biochem. Sci.* *15*, 383-387.

Dynlacht, B., Hoey, T., and Tjian, R. (1991). Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell* *66*, 563-576.

Eisenmann, D. M., Arndt, K. M., Ricupero, S. L., Rooney, J. W., and Winston, F. (1992). SPT3 interacts with TFIIID to allow normal transcription in *Saccharomyces cerevisiae*. *Genes Dev.* *6*, 1319-1331.

Feaver, W.J., Gileadi, O., and Kornberg, R.D. (1991). Purification and characterization of yeast RNA polymerase II transcription factor b. *J. Biol. Chem.* *266*, 19000-19005.

Flanagan, P.M., Kelleher III, R.J., Feaver, W.J., Lue, N.F., LaPointe, J.W., and Kornberg, R.D. (1990). Resolution of factors required for the initiation of transcription by yeast RNA polymerase II. *J. Biol. Chem.* *265*, 11105-11107.

Flores, O., Lu, H., Killeen, M., Greenblatt, J., Burton, Z. F., and Reinberg, D. (1991). The small subunit of transcription factor IIF recruits RNA polymerase into the preinitiation complex. *Proc. Natl. Acad. Sci. USA* *88*, 9999-10003.

Flores, O., Maldorado, E., Burton, Z., Greenblatt, J. and Reinberg, D. (1988). Factors involved in specific transcription by mammalian RNA polymerase II: RNA polymerase II-associating protein 30 is an essential component of transcription factor IIF. *J. Biol. Chem.* *263*, 10812-10816.

Gerard, M., Fischer, L., Moncollin, V., Chipoulet, J.-M., Chambon, P., and Egly, J.-M. (1991). Purification and interaction properties of the human RNA polymerase B(II) general transcription factor BTF2. *J. Biol. Chem.* *266*, 20940-20945.

- Gill, G., and Tjian, R. (1992). Eukaryotic coactivators associated with the TATA box binding protein. *Curr. Opin. Gen. Dev.* 2, 236-242.
- Ha, I., Lane, W.S., and Reinberg, D. (1991). Cloning of a human gene encoding the general transcription initiation factor IIB. *Nature* 352, 689-695.
- Harlow, E., and Lane, D. (1989). *Antibodies: A Laboratory Manual*. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).
- Hoey, T., Weinzierl, R. O. J., Gill, G., Chen, J.-L., Dynlacht, B. D., and Tjian, R. (1993). Molecular cloning and functional analysis of *Drosophila* TAF110 reveal properties expected of coactivators. *Cell* 72, 249-260.
- Kassavetis, G. A., Joazeiro, C. A. P., Pisano, M., Geiduschek, E. P., Colbert, T., Hahn, S., and Blanco, J. A. (1992). The role of the TATA-binding protein in the assembly and function of the multisubunit yeast RNA polymerase III transcription factor, TFIIB. *Cell* 71, 1055-1064.
- Kelleher, R. J.III, Flanagan, P. M., Chasman, D. I., Ponticelli, A. S., Struhl, K., and Kornberg, R. D. (1992). Yeast and human TFIIDs are interchangeable for the response to acidic transcriptional activators in vitro. *Genes & Dev.* 6, 296-303.
- Koleske, A. J., Buratowski, S., Nonet, M., and Young, R. A. (1992). A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. *Cell* 69, 883-894.
- Koleske, A.J., and Young, R.A. (1994). An RNA polymerase II holoenzyme responsive to activators. *Nature* 368, 466-469.
- Kolodziej, P. A., Woychik, N., Liao, S.-M., and Young, R. A. (1990). RNA polymerase II subunit composition, stoichiometry, and phosphorylation. *Mol. Cell. Biol.* 10, 1915-1929.
- Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth. Enzymol.* 154, 367-382.
- Laybourn, P. J., and Dahmus, M. E. (1990). Phosphorylation of RNA polymerase IIa occurs subsequent to interaction with the promoter and before the initiation of transcription. *J. Biol. Chem.* 265, 13165-13173.
- Liao, S.-M., Taylor, I. C. A., Kingston, R. E., and Young, R. A. (1991). RNA polymerase II carboxy-terminal domain contributes to the response to multiple acidic activators in vitro. *Genes Dev.* 5, 2431-2440.
- Lobo, S. M., Tanaka, M., Sullivan, M. L., and Hernandez, N. (1992). A TBP complex essential for transcription from TATA-less but not TATA-containing RNA polymerase III promoters is part of the TFIIB fraction. *Cell* 71, 1029-1040.



Lopez-De-Leon, A., Librizzi, M., Puglia, K. and Willis, I.M. (1992). PCF4 encodes an RNA polymerase III transcription factor with homology to TFIIB. *Cell* **71**, 211-220.

Lu, H., Flores, O., Weinman, R., and Reinberg, D. (1991). The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. *Proc. Natl. Acad. Sci. USA* **88**, 10004-10008.

Lue, N. F., Flanagan, P. M., Sugimoto, K., and Kornberg, R. D. (1989). Initiation by yeast RNA polymerase II at the adenoviral major late promoter in vitro. *Science* **246**, 661-664.

Nonet, M., Sweetser, D., and Young, R. A. (1987). Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. *Cell* **50**, 909-915.

Nonet, M., and Young, R. A. (1989). Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. *Genetics* **123**, 715-724.

Peterson, C. L., Kruger, W., and Herskowitz, I. (1991). A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1. *Cell* **64**, 1135-1143.

Peterson, M. G., Tanese, N., Pugh, B. F., and Tjian, R. (1990). Functional domains and upstream activation properties of cloned human TATA binding protein. *Science* **248**, 1625-1630.

Pinto, I. Ware, D.E., and Hampsey, M. (1992). The yeast *SUA7* gene encodes a homolog of human transcription factor TFIIB and is required for normal start site selection in vivo. *Cell* **68**, 977-988.

Pugh, B. F., and Tjian, R. (1990). Mechanism of transcriptional activation by Sp1: Evidence for coactivators. *Cell* **61**, 1187-1197.

Pugh, B. F., and Tjian, R. (1991). Transcription from a TATA-less promoter requires a multisubunit TFIID complex. *Genes Dev.* **5**, 1935-1945.

Pugh, B. F., and Tjian, R. (1992). Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. *J. Biol. Chem.* **267**, 679-682.

Roeder, R. G. (1991). The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. *Trends Biochem. Sci.* **16**, 402-408.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd Ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Samuels, M., Fire, A., and Sharp, P. (1982). Separation and characterization of factors mediating accurate transcription by RNA polymerase II. *J. Biol. Chem.* *267*, 679-682.

Sawadogo, M., and Sentenac, A. (1990). RNA polymerase B (II) and general transcription factors. *Ann. Rev. Biochem.* *59*, 711-754.

Sayre, M. H., Tschochner, H., and Kornberg, R. D. (1992). Reconstitution of transcription with five purified initiation factors and RNA polymerase II from *Saccharomyces cerevisiae*. *J. Biol. Chem.* *267*, 23376-23382.

Scafe, C., Chao, D., Lopes, J., Hirsch, J. P., Henry, S., and Young, R. A. (1990). RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. *Nature* *347*, 491-494.

Serizawa, H., Conaway, J.W., and Conaway, R.C. (1994). Transcription initiation by mammalian RNA polymerase II. In *Transcription: Mechanism and Regulation*. (Conaway, R.C., and Conaway, J.W. eds., Raven Press Ltd., New York).

Serizawa, H., Conaway, R. C., and Conaway, J. W. (1992). A carboxyl-terminal-domain kinase associated with RNA polymerase II transcription factor d from rat liver. *Proc. Natl. Acad. Sci. USA* *89*, 7476-7480.

Sharp, P. (1992). TATA-binding protein is a classless factor. *Cell* *68*, 819-821.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* *67*, 31-40.

Studier, F. W., and Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. *J. Mol. Biol.* *189*, 113-130.

Taggart, A. K. P., Fisher, T. S., and Franklin, B. F. (1992). The TATA-binding protein and associated factors are components of pol III transcription factor TFIIB. *Cell* *71*, 1015-1028.

Tanese, N., Pugh, B. F., and Tjian, R. (1991). Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. *Genes Dev.* *5*, 2212-2224.

Thompson, C.M., Koleske, A.J., Chao, D.M., and Young R.A. (1993). A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell* *73*, 1361-1375.

Thompson, N. E., Steinberg, T. H., Aronson, D. B., and Burgess, R. R. (1989). Inhibition of *in vivo* and *in vitro* transcription by monoclonal antibodies prepared against wheat germ RNA polymerase II that react with the heptapeptide repeat of eukaryotic RNA polymerase II. *J. Biol. Chem.* *264*, 11511-11520.

- Timmers, H. T. M., and Sharp, P. A. (1991). The mammalian TFIID protein is present in two functionally distinct complexes. *Genes Dev.* *5*, 1946-1956.
- Tschochner, H., Sayre, M.H., Flanagan, P.M., Feaver, W.J., and Kornberg, R.D. (1992). Yeast RNA polymerase II initiation factor e: Isolation and identification as the functional counterpart of human transcription factor IIB. *Proc. Natl. Acad. Sci. USA* *89*, 11292-11296.
- Usheva, A., Maldonado, E., Goldring, A., Lu, H., Houbavi, C., Reinberg, D., and Aloni, Y. (1992). Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA-binding protein. *Cell* *69*, 871-881.
- White, R. J., and Jackson, S. P. (1992). Mechanism of TATA-binding protein recruitment to a TATA-less class III promoter. *Cell* *71*, 1041-1053.
- Wontner, M., Wade, P. A., Bonner, J., and Jaehning, J. A. (1991). Transcriptional activation in an improved whole-cell extract from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* *11*, 4555-4560.
- Young, R. A. (1991). RNA polymerase II. *Ann. Rev. Biochem.* *60*, 689-715.
- Zawel, L., and Reinberg, D. (1992). Advances in RNA polymerase II transcription. *Curr. Opin. Cell Biol.* *4*, 488-495.
- Zehring, W. A., Lee, J. M., Weeks, J. R., Jokerst, R. S., and Greenleaf, A. L. (1988). The C-terminal repeat domain of RNA polymerase II largest subunit is essential in vivo but is not required for accurate transcription initiation in vitro. *Proc. Natl. Acad. Sci. USA* *85*, 3698-3702.
- Zhou, Q., Lieberman, P. M., Boyer, T. G., and Berk, A. J. (1992). Holo TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter. *Genes Dev.* *6*, 1964-1974.

## **Chapter 4**

### **General Requirement for RNA Polymerase II Holoenzyme In Vivo**

## Summary

The RNA polymerase II holoenzyme is a multisubunit complex containing RNA polymerase II, a subset of general transcription factors, and SRB regulatory proteins. We reasoned that because the holoenzyme contains most of the SRB protein in the cell, the fraction of genes that employ the RNA polymerase II holoenzyme in vivo could be investigated by studying the effects of a temperature-sensitive (ts) mutation in the *SRB4* gene on transcription of mRNA. Upon transfer to the restrictive temperature, there is a rapid and general shutdown of mRNA synthesis in *srb4* mutants. These findings indicate a general requirement for SRB4 and the RNA polymerase II holoenzyme in transcription.

## Introduction

Selective transcription initiation by RNA polymerase II requires the action of at least five general transcription factors: TATA-binding protein (TBP), TFIIB, TFIIE, TFIIIF, and TFIIH (reviewed in Sawadogo and Sentenac, 1990; Zawel and Reinberg, 1992; Conaway and Conaway, 1993). These factors and RNA polymerase II can assemble in an ordered fashion onto promoter DNA *in vitro* (Van Dyke et al., 1988; Buratowski et al., 1989; Sawadogo and Sentenac, 1990; Flores et al., 1992; Zawel and Reinberg, 1992; Conaway and Conaway, 1993). We recently described a large multisubunit complex containing RNA polymerase II, the general transcription factors  $\gamma$ TFIIB,  $\gamma$ TFIIIF, and  $\gamma$ TFIIH, and seven SRB proteins (SRB2, SRB4, SRB5, SRB6, SRB7, SRB8, SRB9) (Thompson et al., 1993; Koleske and Young, 1994; Chapter 3). This RNA polymerase II holoenzyme is capable of site-specific initiation when supplemented with  $\gamma$ TFIIE and TBP and is responsive to activators (Koleske and Young, 1994). We proposed that the RNA polymerase II holoenzyme is a form of the enzyme that is readily recruited to promoters *in vivo*. However, because only a small fraction of RNA polymerase II in cells is found in the holoenzyme, it was unclear what fraction of genes require the holoenzyme for transcription initiation.

We now report a set of experiments demonstrating a general requirement for the SRBs in RNA polymerase II transcription *in vivo*. These data, combined with the observation that most of the SRB protein in cells is contained within the holoenzyme (Thompson et al., 1993; Koleske and Young, 1994), argue that the RNA polymerase II holoenzyme is the form of the enzyme recruited to most promoters in the cell.

## Results

### Conditional *srb4* Mutant Rapidly Ceases Growth

Conditional *ts* mutations in the transcription initiation apparatus have previously been used to investigate the influence of specific factors on mRNA synthesis *in vivo* and to study the fraction of promoters that require these factors in the yeast *Saccharomyces cerevisiae* (Nonet et al., 1987; Cormack and Struhl, 1992; Guzder et al., 1993; Qui et al., 1993). This approach, in which transcript levels are monitored in cells undergoing a shift to the restrictive temperature, was followed with cells containing a recessive *ts* mutation in the *SRB4* gene. *SRB4* is essential for cell viability (Thompson et al., 1993) and we found that mutations in this gene can produce a very tight *ts* phenotype. An especially stringent mutant, *srb4-138*, was chosen for this study. Mutant cells grew normally at the permissive temperature of 30°C but failed to grow at the restrictive temperature of 37°C (Figure 1A). Upon shifting a growing culture of *srb4-138* cells to the restrictive temperature, cell growth rapidly decreased, failing to double before growth ceased altogether (Figure 1B).

### mRNA Levels Rapidly Decline in Conditional *srb4* Mutant Cells at the Restrictive Temperature

The effect of the *srb4-138* mutation on mRNA synthesis was investigated by growing wild-type and mutant cells at the permissive temperature, then shifting the cultures to the restrictive temperature. Aliquots were taken immediately before and at various times after the shift and total RNA was prepared. The amount of poly(A)<sup>+</sup> mRNA for each sample was determined by slot blot analysis (Figure 2). Equal amounts of total RNA were blotted and probed with labeled poly(T). The standardization of samples with respect to total RNA is adequate to permit comparisons of mRNA accumulation because the vast majority of the RNA in a typical eukaryotic cell is composed of highly stable rRNA (75%) and tRNA (15%), while less than 5% of the total RNA is composed of relatively unstable mRNA (Brandhorst and McConkey, 1974). Following the shift to the restrictive temperature, mRNA levels decline dramatically and rapidly in mutant cells relative to wild-type, indicating a general defect in RNA polymerase II transcription at the restrictive temperature in *srb4-138* cells.

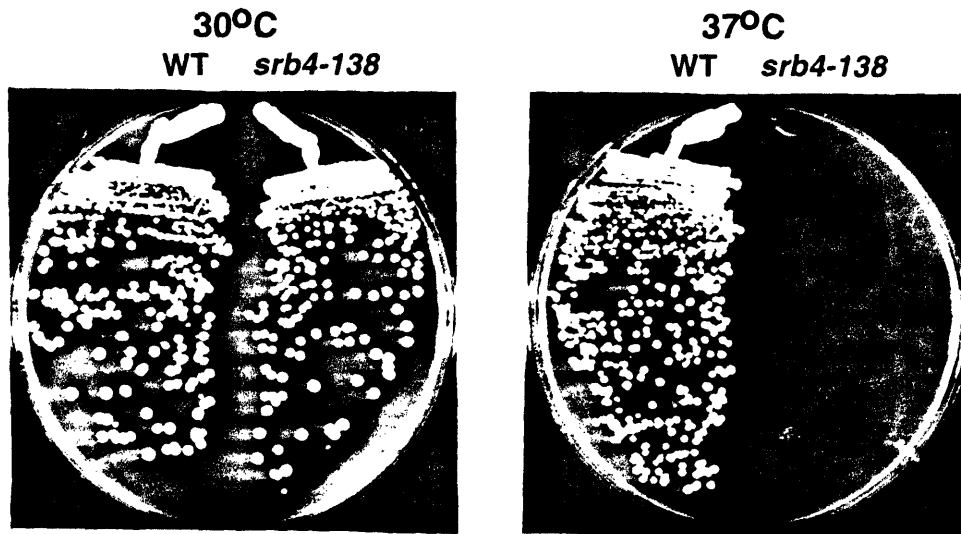
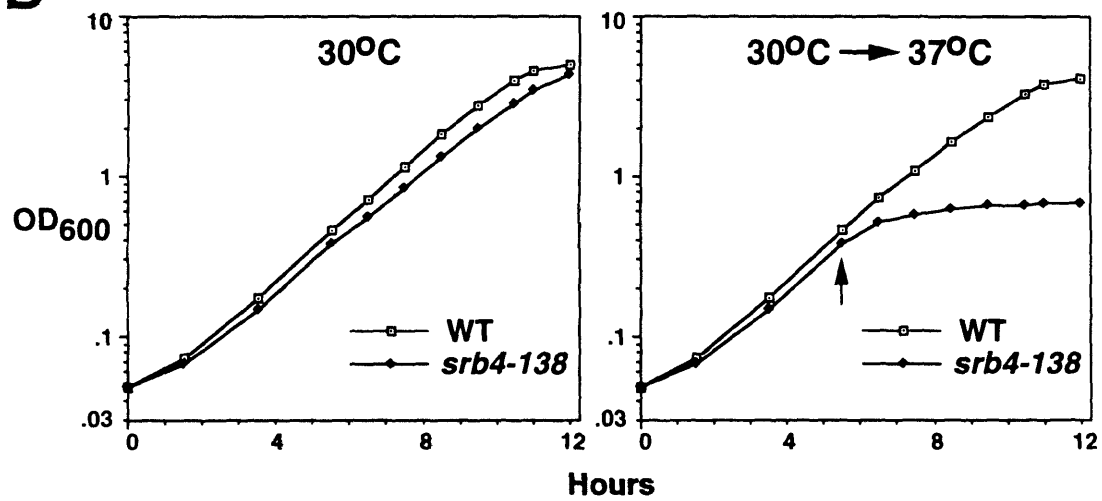
**A****B**



Figure 1. Conditional *srb4* mutant rapidly ceases growth.

(A) Growth of isogenic wild-type (Z579) and *srb4-138* (Z628) strains on YPD plates at 30°C (left panel) and 37°C (Right panel).

(B) Growth of wild-type and *srb4-138* strains in YPD medium as determined by measuring OD<sub>600</sub> at various times. At the time indicated by the arrow, cultures growing at 30°C were divided and half the culture was left at 30°C (left panel) while the other half was shifted to 37°C (right panel).

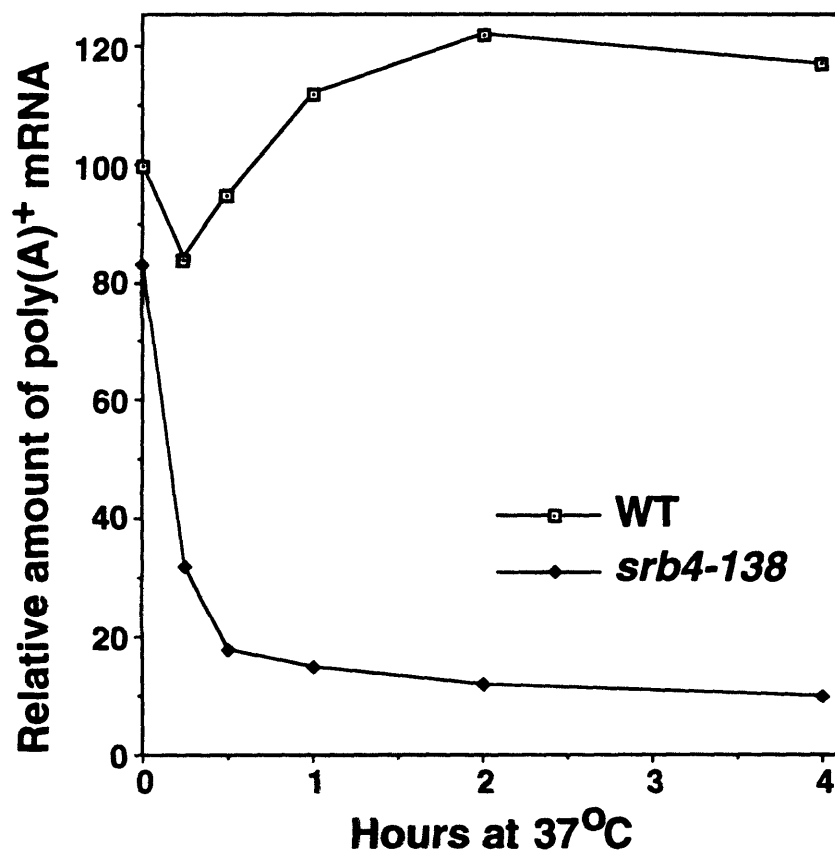
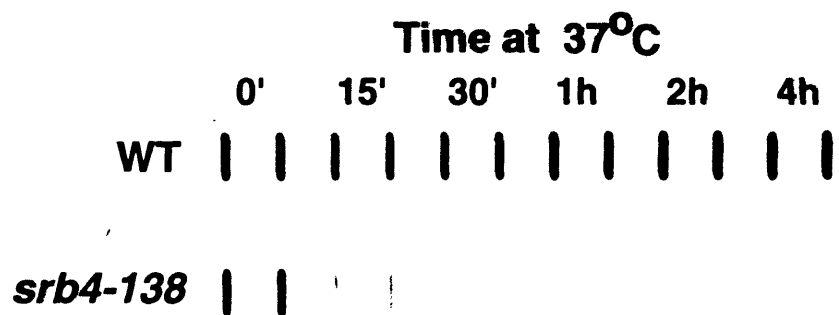


Figure 2. mRNA levels rapidly decline in *srb4-138* cells at the restrictive temperature.

At the times indicated, immediately before and following the shift from 30°C to 37°C, aliquots of cells were removed and total RNA prepared. Equivalent amounts of RNA (2 µg) were slot blotted, in duplicate, onto nitrocellulose and the filter probed with [<sup>32</sup>P]poly(T) (top panel). The results were quantified using a Fuji Bio-Image Analyzer and plotted (lower panel). Each point represents the average value of the duplicate slots, normalized to a value of 100 for wild-type cells at time 0'.

### **Conditional *srb4* Mutant Rapidly Ceases mRNA Synthesis**

The defect in RNA polymerase II transcription was examined in more detail by investigating the effect of a temperature shift on synthesis of selected mRNAs. Total RNA from wild-type and mutant cells was hybridized with an excess of labeled oligonucleotides complementary to specific transcripts, and the resulting products were treated with S1 nuclease and subjected to denaturing polyacrylamide gel electrophoresis (Figure 3). The nine messages selected for analysis, *ACT1*, *CDC7*, *DED1*, *HIS3*, *MET19*, *RAD23*, *STE2*, *TCM1* and *TRP3*, represent a broad spectrum of genes affecting diverse cellular processes. Since this approach measures steady-state levels of mRNAs, the absence of new mRNA synthesis would lead to reduced transcript levels, the rate of reduction reflecting the mRNA decay rate. In *srb4* mutant cells, the levels of all of the mRNAs declined after temperature shift at a rate that correlates well with decay rates observed by other investigators (Nonet et al., 1987; Herrick et al., 1990; Cormack and Struhl, 1992; Guzder et al., 1993; Qui et al., 1993). In contrast, the data indicate that these transcripts continue to be synthesized in wild-type cells throughout the entire 4-hour period at 37°C. These results are consistent with those obtained by analyzing total poly(A)<sup>+</sup> RNA and indicate a general shutdown in mRNA synthesis in *srb4* mutant cells at the restrictive temperature.

### **Effect of Loss of SRB4 Activity on Transcription by RNA Polymerases I, II, and III**

The *SRBs* were identified by the ability of mutations in these genes to specifically suppress conditional and auxotrophic phenotypes associated with truncations of the carboxy-terminal domain (CTD) of RNA polymerase II (Nonet and Young, 1989; Koleske et al., 1992; Chapter 2) and the vast majority of SRB protein in the cell is tightly associated with RNA polymerase II (Thompson et al., 1993; Koleske and Young, 1994). Nonetheless, we investigated the influence of the *srb4-138* mutation on rRNA synthesis by RNA polymerase I and tRNA synthesis by RNA polymerase III. tRNAs are extremely stable but their transcripts contain introns which are rapidly processed with half-lives of less than 3 minutes (Knapp et al., 1978; Cormack and Struhl, 1992). S1 nuclease analysis with an oligonucleotide complementary to the 5' intron-exon junction of the tryptophan family of tRNA transcripts was used to measure RNA polymerase

III activity (Figure 4). There is no appreciable effect on the RNA polymerase III synthesis of tRNA by the *srb4-138* mutant.

The shutdown of RNA polymerase I rRNA synthesis under conditions when mRNA synthesis is affected is well established and is thought to be a consequence of a stringent response (Nonet et al., 1987; Cormack and Struhl, 1992; Guzder et al., 1993; Qui et al., 1993). As expected, rRNA synthesis is significantly reduced in *srb4-138* mutant cells following the shift to the restrictive temperature (Figure 4). rRNA synthesis was investigated using S1 nuclease analysis with an oligonucleotide complimentary to the 3' processing junction of the short lived ribosomal precursor RNA (Kempers-Veenstra et al., 1986; Cormack and Struhl, 1992). This decrease in RNA polymerase I activity is similar to that observed in cells containing the ts *rpb1-1* allele of *RPB1*, the gene encoding the largest subunit of RNA polymerase II (Figure 4) (Nonet et al., 1987; Cormack and Struhl, 1992). RNA polymerases II and III activities in *srb4-138* and *rpb1-1* cells are also nearly identical. For both of these mutants the synthesis of *MET19* and *RAD23* transcripts is dramatically reduced while the synthesis of tRNA is largely unaffected (Figure 4).

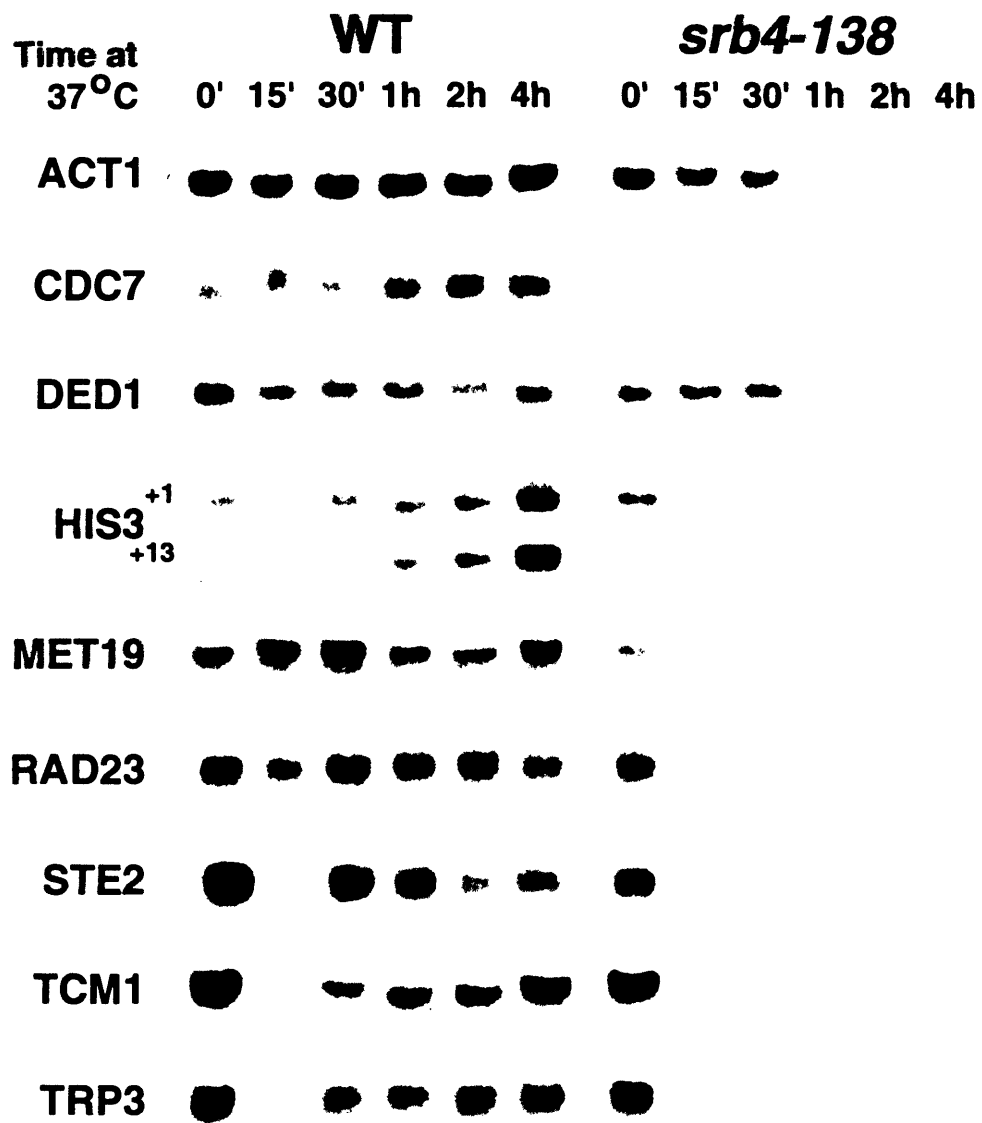


Figure 3. Conditional *srb4* mutant rapidly ceases mRNA synthesis.

At the times indicated, immediately before and following the shift from 30°C to 37°C, aliquots of cells were removed and total RNA prepared. Equivalent amounts of RNA were hybridized with an excess of <sup>32</sup>P-labeled oligonucleotide complementary to the indicated transcripts, treated with S1 nuclease, and subjected to denaturing polyacrylamide gel electrophoresis. The HIS3 oligonucleotide is complementary to the 5' end of the message and detects transcripts initiated from the +1 and +13 sites. The transient decrease in the levels of some of the transcripts from wild-type cells is due to a mild heat shock response (Nicolet and Craig, 1991).

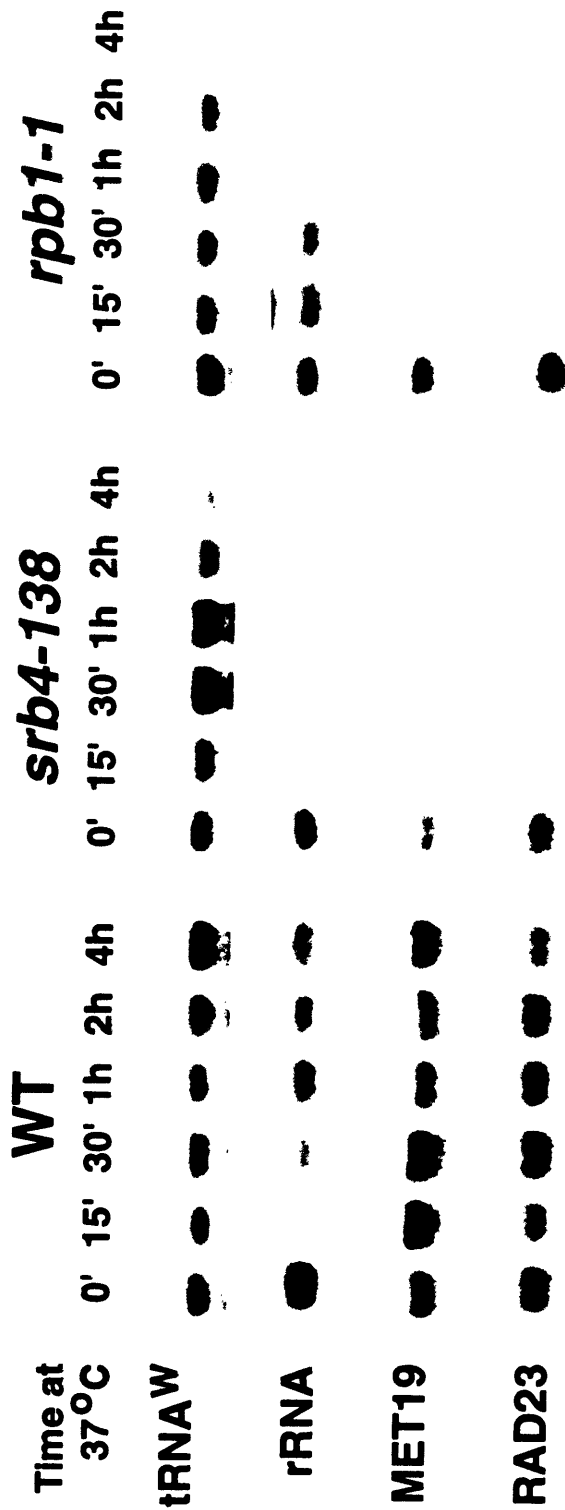




Figure 4. Effect of loss of SRB4 activity on transcription by RNA polymerases I, II, and III.

At the times indicated, immediately before and following the shift from 30°C to 37°C, aliquots of cells were removed and total RNA prepared. Equivalent amounts of RNA were hybridized with an excess of <sup>32</sup>P-labeled oligonucleotide complementary to the indicated transcripts, treated with S1 nuclease, and subjected to denaturing polyacrylamide gel electrophoresis. Z676 (*rpb1-1*) is an isogenic strain of Z579 (WT) and Z628 (*srb4-138*).

## Discussion

The general cessation of mRNA synthesis in *srb4-138* cells is likely to be a direct result of loss of SRB4 activity. The possibility that rapid cessation of global mRNA synthesis was due to an indirect effect, such as a general shutdown in metabolic activity as cells stopped growing, was addressed by Cormack and Struhl (1992). These investigators performed a similar set of temperature-shift experiments using a strain containing a *ts* mutation in *CDC28*, the gene encoding the cyclin-associated protein kinase that mediates entry into the cell cycle. No appreciable effects on general RNA polymerase II transcription were observed. Another possibility, that the cessation of mRNA synthesis is due to loss of a highly unstable protein that is encoded by an unstable RNA whose synthesis is dependent on SRB4, can also be eliminated. Cormack and Struhl examined the effects of cycloheximide, a potent inhibitor of cellular translation, on transcription of a subset of messages in wild-type cells and found no effect on the synthesis of these transcripts. If there was a highly unstable factor required for general mRNA synthesis in the presence of cycloheximide mRNA synthesis should have rapidly ceased. A third possibility, that the general shutdown in mRNA synthesis is due to the small fraction of *srb4*-containing RNA polymerase II molecules blocking promoters at the restrictive temperature, thereby preventing other polymerase molecules from binding, is also unlikely. An *SRB4* mutant that resulted in blocked promoters at the restrictive temperature would be expected to be dominant to wild-type *SRB4*. The mutant described here, *srb4-138*, is recessive to wild-type *SRB4*.

We previously estimated that less than 10% of the RNA polymerase II in the cell was in the holoenzyme, adequate amounts to initiate transcription at active promoters (Koleske and Young, 1994). It was unclear, however, if the holoenzyme was preferentially recruited to some promoters while free RNA polymerase II and general factors were recruited in a step-wise fashion to others. It appears now that the holoenzyme is the form of RNA polymerase II utilized at most promoters. This conclusion is based upon the demonstration that SRB4 plays a general role in RNA polymerase II transcription and our previous observation that the majority of SRB4 in the cell is tightly associated with RNA polymerase II in the holoenzyme (Thompson et al., 1993; Koleske and Young, 1994). Essentially all of the SRB2, SRB4, SRB5, and SRB6 protein in whole cell extracts copurifies as a single peak through a series of purification steps (Thompson et al., 1993) and, after five purification steps, we recover

approximately 65% of the SRB2 and SRB5 from extracts as part of the holoenzyme (Koleske and Young, 1994). A large fraction of the SRB protein not recovered in the purified holoenzyme can be attributed to losses that occur at each step of the purification. Although a small fraction of SRB protein must, of course, be in the process of assembly in vivo. An alternative form of the RNA polymerase II holoenzyme has recently been described (Kim et al., 1994). While this form of the enzyme contains RNA polymerase II, SRB proteins, and TFIIF, it differs in that it lacks the general transcription factors TFIIB and TFIIH. These differences in holoenzyme composition appear to be due to differences in yeast strains and growth conditions, factors which seem to affect holoenzyme stability during purification.

These results have additional implications for the mechanisms involved in regulation of transcription initiation. The SRB proteins accumulate to approximately one-tenth the levels of total cellular RNA polymerase II and TFIIB (Koleske and Young, 1994). Thus, the assembly of an RNA polymerase II holoenzyme is limited by the levels of SRB proteins. The SRBs, therefore, may play a key regulatory role in holoenzyme formation leading to initiation complex assembly.

## Experimental Procedures

### Isolation of Conditional *srb4* Mutants

A PCR-based mutagenesis strategy was used to construct a mutagenized library of the *SRB4* gene (Leung et al., 1989). The plasmid pCT127 (*SRB4 LEU2 CEN*) contains a unique NdeI site at the *SRB4* ATG and a unique XbaI site following the *SRB4* stop codon, both created by site-specific mutagenesis (Kunkle et al., 1987). PCR of *SRB4* from pCT127 with oligonucleotides flanking the open reading frame was performed in buffer containing 0.1 mM, 0.2 mM, and 0.4 mM Mn<sup>2+</sup>. Reactions were pooled, DNA digested with NdeI-XbaI, ligated with NdeI-XbaI digested pCT127 vector fragment, and transformed into DH5 $\alpha$ . Approximately 30,000 transformants were obtained. Plasmid shuffle techniques (Boeke et al., 1987) were then used to identify ts alleles of *SRB4*. The DNA molecules containing *LEU2* and mutagenized *SRB4* genes were transformed into a yeast strain (Z572) deleted for the chromosomal copy of *SRB4*, but carrying a *URA3* centromeric plasmid encoding a wild-type copy of the gene. Approximately 20% of the transformants were unable to grow in the presence of 5-FOA, indicating a lethal mutation in the *LEU2* plasmid-borne *SRB4* gene. Approximately 0.5% of the transformants were able to grow on 5-FOA plates at 30°C but not at 37°C, indicating a ts allele in the *LEU2* plasmid-borne *SRB4* gene. The *LEU2* plasmids from these transformants were recovered and reintroduced into Z572 to verify the ts phenotype. The plasmid pCT181 contains the *srb4-138* mutant allele.

### RNA Analysis

Total RNA from cells was isolated using hot acidic phenol extraction (Ausubel et al., 1993). RNA was quantified by absorbance at 260 nm and the integrity of the RNA confirmed by ethidium bromide staining of RNA in agarose gels. Slot blot analysis to determine the amount of poly(A)<sup>+</sup> mRNA was performed as described (Choder, 1991). S1 nuclease protection assays were carried out with 5-30  $\mu$ g of RNA and DED1, HIS3, TRP3, rRNA, and tRNA<sup>W</sup> oligonucleotide probes as previously described (Cormack and Struhl). The sequences for the other oligonucleotide probes are:

ACT1	GGAAGAGTACAAGGACAAAACGGCTTGATGGAAACGTAGAAGGCATTCCA,
CDC7	GGGGCTACTCTCGAAGATCCCGTCATTATGTACAGCAGGTTGAGCATGCCT,
MET19	GCCTTACCGGCACGCATCATGATGGGGACGCCCTCCCAACGCTCGACACTT,

**RAD23** GCAGTGGCTGCAGGAGCTGCAGAAGCATCGGTACTGGGGGATGCAATCCA,  
**STE2** GTCGACGGGTTCAACTTCTCCCTCTTTGTA ACTTGCATCAGCAAACGGATGACA,  
**TCM1** GGAGTGTCAACAACGGTGACAGCTTCGACA ACTTCACGCTTGTGGTGAGCT.

Oligonucleotides are written in the 5' to 3' direction and contain 6 residues at their 3' ends that are not complementary to the RNA, permitting distinction between bands due to appropriate RNA-DNA hybrids and undigested probe.

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## References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. eds. (1993). *Current Protocols in Molecular Biology* (New York: John Wiley and Sons).

Boeke, J., Truehart, J., Natsoulis, B., and Fink, G.R. (1987). 5-Fluoro-orotic acid as a selective agent in yeast molecular genetics. *Meth. Enzymol.* *154*, 164-175.

Brandhorst, B.P., and McConkey, E.H. (1974). Stability of nuclear RNA in mammalian cells. *J. Mol. Biol.* *85*, 451-463.

Buratowski, S., Hahn, S., Guarente, L., and Sharp, P.A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* *56*, 549-561.

Choder, M. (1991). A general topoisomerase I-dependent transcriptional repression in the stationary phase in yeast. *Genes Dev.* *5*, 2315-2326.

Conaway, R.C., and Conaway, J.W. (1993). General initiation factors for RNA polymerase II. *Ann. Rev. Biochem.* *62*, 161-190.

Cormack, B.P., and Struhl, K. (1992). The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. *Cell* *69*, 685-696.

Flores, O., Lu, H., and Reinberg, D. (1992). Factors involved in specific transcription by mammalian RNA polymerase II. *J. Biol. Chem.* *267*, 2786-2793.

Guzder, S.N., Qui, H., Sommers, C.H., Sung, P., Prakash, L., and Prakash, S. (1993). DNA repair gene *RAD3* of *S. cerevisiae* is essential for transcription by RNA polymerase II. *Nature* *367*, 91-94.

Herrick, D., Parker, R., and Jacobson, A. (1990). Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* *10*, 2269-2284.

Kempers-Veenstra, A.E., Oliemans, J., Offenbergh, H., Dekker, A.F., Piper, P.W., Planta, R.J., and Klootwijk, J. (1986). 3'-End formation of transcripts from the yeast rRNA operon. *EMBO J.* *5*, 2703-2710.

Kim, Y.-J., Bjorklund, S., Li, Y., Sayre, M.H., and Kornberg, R.D. (1994). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* *77*, 599-608.

Knapp, G., Beckman, J.S., Johnson, P.F., Fuhrman, S.A., and Abelson, J. (1978). Transcription and processing of intervening sequences in yeast tRNA genes. *Cell* *14*, 221-236.

- Koleske, A.J., Buratowski, S., Nonet, M., and Young R.A. (1992). A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. *Cell* **69**, 883-894.
- Koleske, A.J., and Young, R.A. (1994). An RNA polymerase II holoenzyme responsive to activators. *Nature* **368**, 466-469.
- Kunkle, T.A., Roberts, J.D., Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth. Enzymol.* **154**, 367-382.
- Leung, D.W., Chen, E., and Goeddel, D.V. (1989). A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* **1**, 11-15.
- Nicolet, C.M., and Craig, E.A. (1991). Inducing and assaying heat shock response in *Saccharomyces cerevisiae*. *Meth. Enzymol.* **194**, 710-717.
- Nonet, M., Scafe, C., Sexton, J., and Young, R.A. (1987). Eucaryotic RNA polymerase conditional mutant that rapidly ceases mRNA synthesis. *Mol. Cell. Biol.* **7**, 1602-1611.
- Nonet, M., and Young, R.A. (1989). Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. *Genetics* **123**, 715-724.
- Qiu, H., Park, E., Prakash, L, Prakash, S. (1993). The *Saccharomyces cerevisiae* DNA repair gene *RAD25* is required for transcription by RNA polymerase II. *Genes Dev.* **7**, 2161-2171.
- Sawadogo, A., and Sentenac, A. (1990). RNA polymerase B (II) and general transcription factors. *Ann. Rev. Biochem.* **59**, 711-754.
- Thompson, C.M., Koleske, A.J., Chao, D.M., and Young, R.A. (1993). A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell* **73**, 1361-1375.
- Van Dyke, M.W., Roeder, R.G., and Sawadogo, M. (1988). Physical analysis of transcription preinitiation complex assembly on a class II promoter. *Science* **241**, 1335-1338.
- Zawel, L., and Reinberg, D. (1992). Advances in RNA polymerase II transcription. *Curr. Opin. Cell. Bio.* **4**, 488-495.



## **Appendix A**

### **List of Extragenic Suppressors of a CTD Truncation Mutation**

## Summary

Table 1 is a list of the 83 strongest extragenic suppressors of the CTD truncation mutation *rpb1Δ104*. These suppressors were isolated and initially characterized as described in chapter 2. The list is organized by complementation groups and includes the suppressing isolate number and allele designation. Recessive suppressing isolates were placed into complementation groups using a combination of standard genetic complementation analysis and plasmid complementation analysis with the cloned wild-type genes. Three of the recessive suppressing mutations were unable to be placed into a complementation group. Dominant suppressing isolates were grouped using random spore analysis. *SRB2* was originally identified and cloned by Mike Nonet in a similar selection scheme (Nonet and Young, *Genetics* 123, 715-724 (1989)). The genetic background for these suppressing isolates is:

N400/Z551 *Mata ura3-52 leu2-3, 112 his3Δ200*  
*rpb1Δ187::HIS3 [RY2204 (rpb1Δ104 CEN LEU2)]*

Table 1. Extragenic Suppressors of CTD Truncation Mutations

Allele Designation	Suppressing Isolate	Allele Designation	Suppressing Isolate
<u>SRB2</u>		<u>SRB5</u> (cont.)	
SRB2-2	s211	SRB5-4	s117
SRB2-3	s217	SRB5-5	s191
		SRB5-6	s207
<u>SRB4</u>		SRB5-7	s400
SRB4-1*	s25		
srb4-2*	s346	<u>SRB6</u>	
SRB4-3	s214	SRB6-1*	s429
SRB4-4	s305	SRB6-2	s14
srb4-5	s315	srb6-3	s38-2
srb4-6	s108	srb6-4	s258
SRB4-7	s12-2	SRB6-5	s319
SRB4-8	s45	SRB6-6	s406
SRB4-9	s102		
srb4-10	s113	<u>SRB7</u>	
srb4-11	s156	srb7-1*	s242
srb4-12	s173	srb7-2	s419
srb4-13	s184	srb7-3	s129
SRB4-14	s215		
SRB4-15	s303	<u>SRB8</u>	
SRB4-16	s304	srb8-1*	s358
SRB4-17	s307	srb8-2	s18
SRB4-18	s316	srb8-3	s276
SRB4-19	s318	srb8-4	s374
SRB4-20	s407		
SRB4-21	s424	<u>SRB9</u>	
		srb9-1*	s363
<u>SRB5</u>		srb9-2	s452
SRB5-1*	s148	srb9-3	s366
SRB5-2	s31	srb9-4	s1-2
SRB5-3	s112	srb9-5	s16-2

Table 1. Extragenic Suppressors of CTD Truncation Mutations (Continued)

Allele Designation	Suppressing Isolate	Allele Designation	Suppressing Isolate
<u><i>SRB9</i></u> (cont.)		<u><i>SRB10</i></u>	
<i>srb9-6</i>	s17-2	<i>srb10-1*</i>	s449
<i>srb9-7</i>	s28-3	<i>srb10-2</i>	s425
<i>srb9-8</i>	s33	<i>srb10-3</i>	s53
<i>srb9-9</i>	s40-2	<i>srb10-4</i>	s229
<i>srb9-10</i>	s43-2		
<i>srb9-11</i>	s218-2	<u><i>SRB11</i></u>	
<i>srb9-12</i>	s219	<i>srb11-1*</i>	s199
<i>srb9-13</i>	s326-2		
<i>srb9-14</i>	s329-2	<u><i>RPB2</i></u>	
<i>srb9-15</i>	s332-2	<i>rpb2-551*</i>	s456
<i>srb9-16</i>	s347-2	<i>rpb2-552</i>	s368
<i>srb9-17</i>	s362	<i>rpb2-553</i>	s431
<i>srb9-18</i>	s402	<i>rpb2-554</i>	s120
<i>srb9-19</i>	s408	<i>rpb2-555</i>	s210
<i>srb9-20</i>	s415	<i>rpb2-556</i>	s404-2
<i>srb9-21</i>	s416		
<i>srb9-22</i>	s418	<u>unknown</u>	
<i>srb9-23</i>	s420	?	s74
<i>srb9-24</i>	s421	?	s83
<i>srb9-25</i>	s440	?	s141
<i>srb9-26</i>	s455		

\* Allele used to clone corresponding gene.

## Experimental Procedures

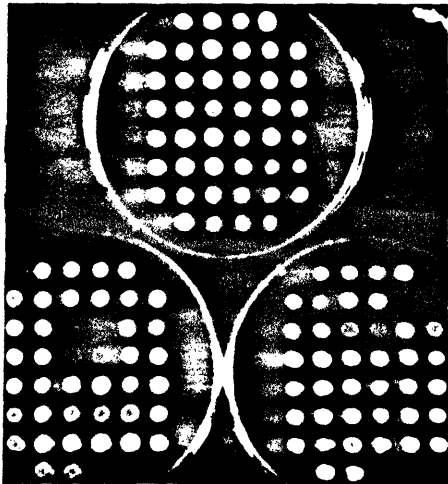
Yeast strains of the opposite mating type of approximately half of the dominant suppressors and half of the recessive suppressors were generated by inducing a mating type switch by expression of the *HO* gene placed on a plasmid under the control of a galactose inducible promoter. Random spore analysis 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 β-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 held on ice for 15 min. The suspension was then vortexed 3 min, held 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 offspring 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.

## **Appendix B**

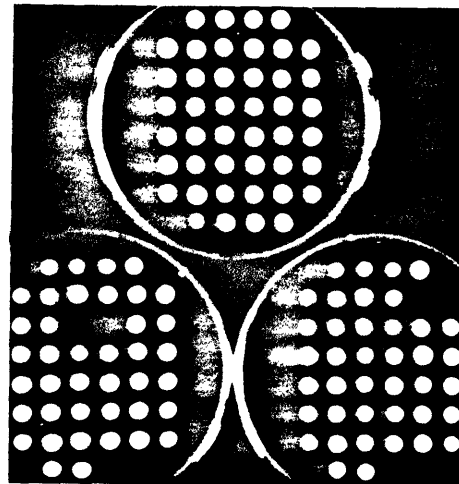
### **Conditional *srb4* and *srb6* Alleles**

## Summary

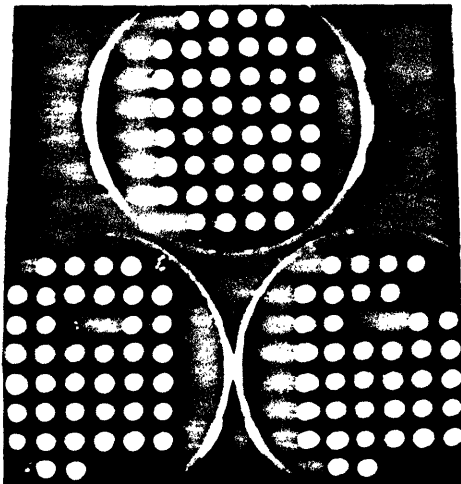
*SRB4* and *SRB6* are essential genes (Thompson et al., 1993). A PCR-based mutagenesis strategy (Leung et al., 1989) was used to construct mutagenized libraries of these genes. Plasmid shuffle techniques (Boeke et al., 1987) were then used to identify temperature-sensitive (ts) alleles of *SRB4* (Figure 1) and *SRB6* (Figure 2). All of these ts alleles are recessive and, in general, do not display significant cold-sensitive phenotypes. Conditional alleles of *SRB4* and *SRB6* will be useful for in vivo analysis of SRB function, second-site suppressor analysis, cloning SRB homologs from other species by complementation, and developing in vitro assays. In addition, the mutagenized libraries can be used to identify mutations in *SRB4* and *SRB6* that will suppress defects in other components of the transcription initiation apparatus.



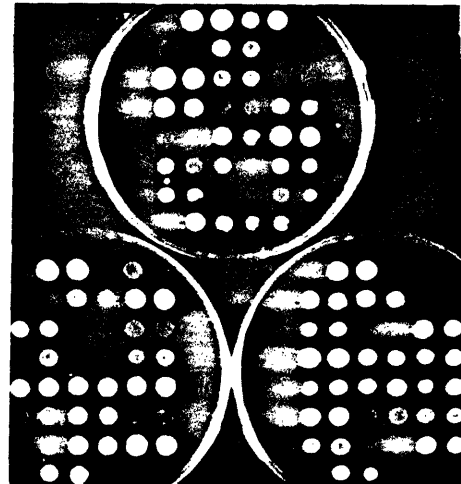
12°C



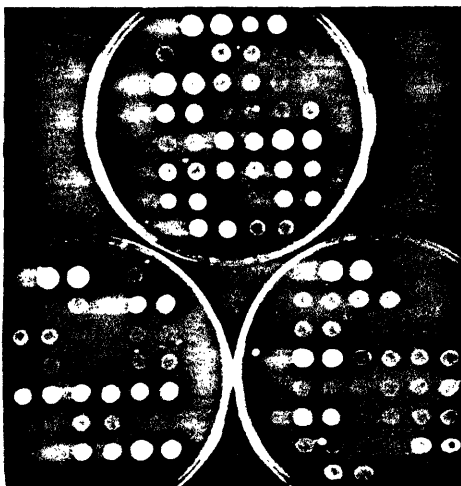
24°C



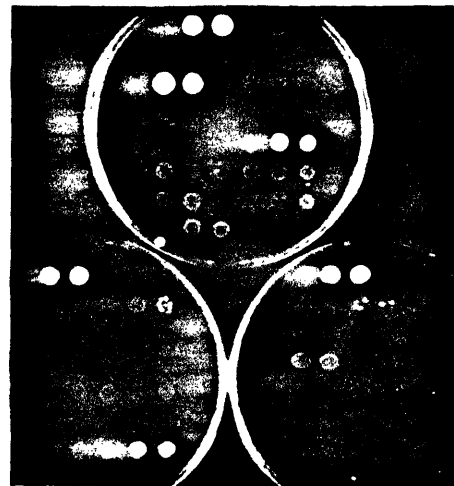
30°C



34°C



36°C



37°C

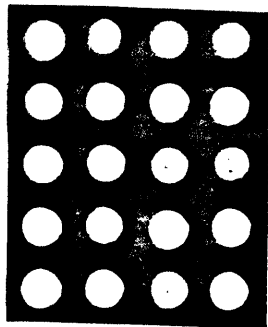


Figure 1. Conditional *srb4* mutants.

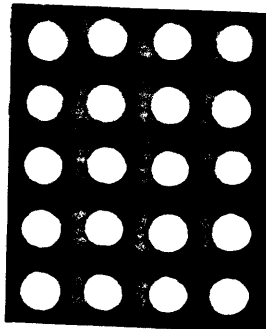
Cells were spotted, in duplicate, onto YEPD medium and incubated at 12°C, 24°C, 30°C, 34°C, 36°C, and 37°C.

	<i>SRB4 srb4-101</i>			
	<i>srb4-102</i>	<i>srb4-103</i>	<i>srb4-104</i>	
	*	<i>srb4-105</i>	<i>srb4-106</i>	
	<i>srb4-107</i>	<i>srb4-108</i>	<i>srb4-109</i>	
	<i>srb4-110</i>	<i>srb4-111</i>	*	
	<i>srb4-112</i>	<i>srb4-113</i>	<i>srb4-114</i>	
	<i>srb4-115</i>	<i>srb4-116</i>	<i>srb4-117</i>	
		<i>srb4-118</i>	<i>srb4-119</i>	
<i>SRB4</i>	<i>srb4-120</i>			<i>SRB4</i>
<i>srb4-121</i>	<i>srb4-122</i>	<i>srb4-123</i>		<i>srb4-139</i>
<i>srb4-124</i>		<i>srb4-125</i>		<i>srb4-141</i>
<i>srb4-126</i>	<i>srb4-127</i>	<i>srb4-128</i>		<i>srb4-142</i>
<i>srb4-129</i>	<i>srb4-130</i>	<i>srb4-131</i>		<i>srb4-143</i>
<i>srb4-132</i>	<i>srb4-133</i>	<i>srb4-134</i>		<i>srb4-144</i>
<i>srb4-135</i>	<i>srb4-136</i>	*		<i>srb4-145</i>
	<i>srb4-137</i>			<i>srb4-146</i>
				<i>srb4-147</i>
				<i>srb4-148</i>
				<i>srb4-149</i>
				<i>srb4-150</i>
				<i>srb4-151</i>
				<i>srb4-152</i>
				<i>srb4-153</i>
				<i>srb4-154</i>
				<i>srb4-155</i>
				<i>srb4-156</i>

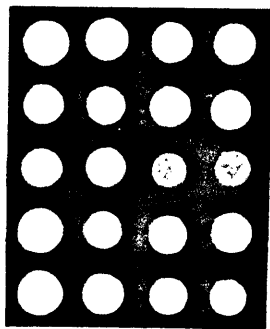
\*This allele was not tight enough to keep.



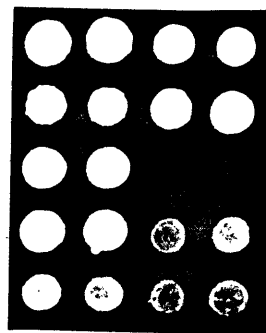
12°C



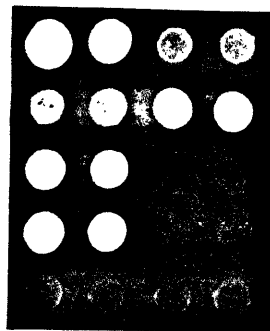
24°C



30°C



34°C



36°C



37°C

Figure 2. Conditional *srb6* mutants.

Cells were spotted, in duplicate, onto YEPD medium and incubated at 12°C, 24°C, 30°C, 34°C, 36°C, and 37°C.

*SRB6* *srb6-101*  
*srb6-102* *srb6-103*  
*srb6-104* *srb6-105*  
*srb6-106* *srb6-107*  
*srb6-108* *srb6-109*

## Experimental Procedures

Conditional *srb4* alleles are listed in Table 1. The plasmid pCT127 (*SRB4 LEU2 CEN*) contains a unique NdeI site at the *SRB4* ATG and a unique XbaI site following the *SRB4* stop codon, both created by site-specific mutagenesis (Kunkle et al., 1987). PCR of *SRB4* from pCT127 with oligonucleotides flanking the open reading frame was performed in buffer containing 0.1 mM, 0.2 mM, and 0.4 mM Mn<sup>2+</sup>. Reactions were pooled, DNA digested with NdeI-XbaI, ligated with NdeI-XbaI digested pCT127 vector fragment, and transformed into DH5 $\alpha$ . Approximately 30,000 transformants were obtained. Twelve clones were randomly selected and partially sequenced. A mutation frequency of approximately one per kilobase was found (four mutations in 3.5 kb). The DNA molecules containing *LEU2* and mutagenized *SRB4* genes were transformed into a yeast strain (Z572) deleted for the chromosomal copy of *SRB4*, but carrying a *URA3* centromeric plasmid encoding a wild-type copy of the gene. Approximately 20% of the transformants were unable to grow in the presence of 5-FOA, indicating a lethal mutation in the *LEU2* plasmid-borne *SRB4* gene. Approximately 0.5% of the transformants were able to grow on 5-FOA plates at 30°C but not at 37°C, indicating a *ts* allele in the *LEU2* plasmid-borne *SRB4* gene. The *LEU2* plasmids from these transformants were recovered and reintroduced into Z572 to verify the *ts* phenotype.

Conditional *srb6* alleles are listed in Table 2. The complete 1.0 kb *SRB6* genomic DNA fragment from pCT66 (*SRB6 LEU2 CEN*) was PCR amplified with oligonucleotides flanking the insert DNA. PCR Reactions were performed in buffer containing 0.3 mM, 0.5 mM, and 0.7 mM Mn<sup>2+</sup>. Pooled reactions were digested with BamHI-Sall, ligated with BamHI-Sall digested RY2631 (*LEU2 CEN*), and transformed into DH5 $\alpha$ . Approximately 35,000 transformants were obtained. Thirteen clones were randomly selected and partially sequenced. A mutation frequency of approximately one per 200 bp was found (over 4 kb sequenced). DNA molecules containing *LEU2* and mutagenized *SRB6* genes were transformed into a yeast strain (Z661) deleted for the chromosomal copy of *SRB6*, but carrying a *URA3* centromeric plasmid encoding a wild-type copy of the gene. Approximately 15% of the transformants were unable to grow in the presence of 5-FOA, indicating a lethal mutation in the *LEU2* plasmid-borne *SRB6* gene. Approximately 0.1% of the transformants were able to grow on 5-

FOA plates at 30°C but not at 37°C, indicating a ts allele in the *LEU2* plasmid-borne *SRB6* gene. The *LEU2* plasmids from these transformants were recovered and reintroduced into Z661 to verify the ts phenotype.

Table 1. Conditional *srb4* Alleles

<i>SRB4</i> Allele	Plasmid	Yeast <sup>a</sup>	<i>SRB4</i> Allele	Plasmid	Yeast <sup>a</sup>
<i>SRB4</i>	pCT127	CTY233	<i>srb4-122</i>	pCT165	CTY255
<i>srb4-101</i>	pCT144	CTY234	<i>srb4-123</i>	pCT166	CTY256
<i>srb4-102</i>	pCT145	CTY235	<i>srb4-124</i>	pCT167	CTY257
<i>srb4-103</i>	pCT146	CTY236	<i>srb4-125</i>	pCT168	CTY258
<i>srb4-104</i>	pCT147	CTY237	<i>srb4-126</i>	pCT169	CTY259
<i>srb4-105</i>	pCT148	CTY238	<i>srb4-127<sup>b</sup></i>	pCT170	CTY260
<i>srb4-106</i>	pCT149	CTY239	<i>srb4-128</i>	pCT171	CTY261
<i>srb4-107</i>	pCT150	CTY240	<i>srb4-129</i>	pCT172	CTY262
<i>srb4-108</i>	pCT151	CTY241	<i>srb4-130</i>	pCT173	CTY263
<i>srb4-109</i>	pCT152	CTY242	<i>srb4-131</i>	pCT174	CTY264
<i>srb4-110</i>	pCT153	CTY243	<i>srb4-132</i>	pCT175	CTY265
<i>srb4-111</i>	pCT154	CTY244	<i>srb4-133</i>	pCT176	CTY266
<i>srb4-112</i>	pCT155	CTY245	<i>srb4-134<sup>b</sup></i>	pCT177	CTY267
<i>srb4-113</i>	pCT156	CTY246	<i>srb4-135</i>	pCT178	CTY268
<i>srb4-114</i>	pCT157	CTY247	<i>srb4-136</i>	pCT179	CTY269
<i>srb4-115</i>	pCT158	CTY248	<i>srb4-137</i>	pCT180	CTY270
<i>srb4-116</i>	pCT159	CTY249	<i>srb4-138<sup>b</sup></i>	pCT181	CTY271
<i>srb4-117</i>	pCT160	CTY250	<i>srb4-139</i>	pCT182	CTY272
<i>srb4-118</i>	pCT161	CTY251	<i>srb4-140</i>	pCT183	CTY273
<i>srb4-119</i>	pCT162	CTY252	<i>srb4-141</i>	pCT184	CTY274
<i>srb4-120</i>	pCT163	CTY253	<i>srb4-142</i>	pCT185	CTY275
<i>srb4-121</i>	pCT164	CTY254	<i>srb4-143<sup>b</sup></i>	pCT186	CTY276

Table 1. Conditional *srb4* Alleles (Continued)

<i>SRB4</i> Allele	Plasmid	Yeast <sup>a</sup>	<i>SRB4</i> Allele	Plasmid	Yeast <sup>a</sup>
<i>srb4-144</i>	pCT187	CTY277	<i>srb4-151</i>	pCT194	CTY284
<i>srb4-145</i>	pCT188	CTY278	<i>srb4-152</i>	pCT195	CTY285
<i>srb4-146</i>	pCT189	CTY279	<i>srb4-153</i>	pCT196	CTY286
<i>srb4-147</i>	pCT190	CTY280	<i>srb4-154</i>	pCT197	CTY287
<i>srb4-148</i>	pCT191	CTY281	<i>srb4-155</i>	pCT198	CTY288
<i>srb4-149</i>	pCT192	CTY282	<i>srb4-156</i>	pCT199	CTY289
<i>srb4-150</i>	pCT193	CTY283			

<sup>a</sup>Strain background: *mata his3Δ200 leu2-3, 112 ura3-52*  
*srb4Δ2::HIS3*

<sup>b</sup>Sequence analysis by Ellen Gadbois identified multiple mutations.

Table 2. Conditional *srb6* Alleles

<i>SRB6</i> Allele	Plasmid	Yeast <sup>a</sup>	<i>SRB6</i> Allele	Plasmid	Yeast <sup>a</sup>
<i>SRB6</i>	pCT66	CTY184	<i>srb6-105</i>	pCT204	CTY294
<i>srb6-101</i>	pCT200	CTY290	<i>srb6-106</i>	pCT205	CTY295
<i>srb6-102</i>	pCT201	CTY291	<i>srb6-107</i>	pCT206	CTY296
<i>srb6-103</i>	pCT202	CTY292	<i>srb6-108</i>	pCT207	CTY297
<i>srb6-104</i>	pCT203	CTY293	<i>srb6-109</i>	pCT208	CTY298

<sup>a</sup>Strain background: *mata his3Δ200 leu2-3, 112 ura3-52*  
*srb6Δ1::hisG*



## References

Boeke, J., Truehart, J., Natsoulis, B., and Fink, G.R. (1987). 5-Flouoro-orotic acid as a selective agent in yeast molecular genetics. *Meth. Enzymol.* 154, 164-175.

Kunkle, T.A., Roberts, J.D., Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth. Enzymol.* 154, 367-382.

Leung, D.W., Chen, E., and Goeddel, D.V. (1989). A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* 1, 11-15.

Thompson, C.M., Koleske, A.J., Chao, D.M., and Young, R.A. (1993). A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell* 73, 1361-1375.

## **Appendix C**

### ***SRB4* Reagents**

## Summary

This section includes inventories of potentially useful *SRB4* reagents including yeast strains (Table 1), plasmids (Table 2), and oligonucleotides (Table 3). Figure 1 is a numbered sequence of *SRB4* with the numbers corresponding to the positions given for the oligonucleotides. Details of plasmid constructs can be found in the Young lab plasmid list.

Table 1. *SRB4* Yeast Strains

Strain	Genotype
CTY3	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB4-1</i> [RY2204 ( <i>rpb1Δ104 LEU2 CEN</i> )]
CTY4	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 srb4-2</i> [RY2204 ( <i>rpb1Δ104 LEU2 CEN</i> )]
CTY10	<i>Mata<math>\alpha</math> his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB4-1</i> [RY2191 ( <i>rpb1Δ104 URA3 CEN</i> )]
CTY11	<i>Mata<math>\alpha</math> his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 srb4-2</i> [RY2191 ( <i>rpb1Δ104 URA3 CEN</i> )]
CTY15	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB4-1</i> [RY2112 ( <i>RPB1 URA3 CEN</i> )]
CTY16	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 srb4-2</i> [RY2112 ( <i>RPB1 URA3 CEN</i> )]
CTY32	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB4-1</i> [RY2128 ( <i>RPB1 LEU2 CEN</i> )]
CTY176	<i>a/<math>\alpha</math> (his3Δ200 leu2-3, 112 ura3-52)X2 srb4Δ2::HIS3/SRB4</i>
CTY182	<i>Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3</i> [pCT15 ( <i>SRB4 URA3 CEN</i> )]
CTY183	<i>Mata<math>\alpha</math> his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3</i> [pCT15 ( <i>SRB4 URA3 CEN</i> )]
CTY200	<i>Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3</i> [pCT65 ( <i>SRB4 LEU2 CEN</i> )]
CTY201	<i>Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3</i> [pCT97 ( <i>SRB4-12CA5 URA3 CEN</i> )]

- CTY227     *Mata his3Δ200 leu2-3, 112 ura3-52 lys2Δ201 srb4Δ2::HIS3*  
              [pCT15 (*SRB4 URA3 CEN*)]
- CTY228     *Mata his3Δ200 leu2-3, 112 ura3-52 lys2Δ201 srb4Δ2::HIS3*  
              [pCT15 (*SRB4 URA3 CEN*)]
- CTY231     *Mata his3Δ200 leu2-3, 112 ura3-52 lys2Δ201 srb4Δ2::HIS3*  
              [pCT124 (*SRB4 LEU2 LYS2 CEN*)]
- CTY233     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT127 (*SRB4 LEU2 CEN*)]
- CTY234     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT144 (*srb4-101 LEU2 CEN*)]
- CTY235     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT145 (*srb4-102 LEU2 CEN*)]
- CTY236     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT146 (*srb4-103 LEU2 CEN*)]
- CTY237     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT147 (*srb4-104 LEU2 CEN*)]
- CTY238     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT148 (*srb4-105 LEU2 CEN*)]
- CTY239     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT149 (*srb4-106 LEU2 CEN*)]
- CTY240     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT150 (*srb4-107 LEU2 CEN*)]
- CTY241     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT151 (*srb4-108 LEU2 CEN*)]
- CTY242     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT152 (*srb4-109 LEU2 CEN*)]

- CTY243     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT153 (*srb4-110 LEU2 CEN*)]
- CTY244     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT154 (*srb4-111 LEU2 CEN*)]
- CTY245     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT155 (*srb4-112 LEU2 CEN*)]
- CTY246     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT156 (*srb4-113 LEU2 CEN*)]
- CTY247     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT157 (*srb4-114LEU2 CEN*)]
- CTY248     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT158 (*srb4-115 LEU2 CEN*)]
- CTY249     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT159 (*srb4-116 LEU2 CEN*)]
- CTY250     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT160 (*srb4-117 LEU2 CEN*)]
- CTY251     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT161 (*srb4-118 LEU2 CEN*)]
- CTY252     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT162 (*srb4-119 LEU2 CEN*)]
- CTY253     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT163 (*srb4-120 LEU2 CEN*)]
- CTY254     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT164 (*srb4-121 LEU2 CEN*)]
- CTY255     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT165 (*srb4-122 LEU2 CEN*)]

- CTY256     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT166 (*srb4-123 LEU2 CEN*)]
- CTY257     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT167 (*srb4-124 LEU2 CEN*)]
- CTY258     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT168 (*srb4-125 LEU2 CEN*)]
- CTY259     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT169 (*srb4-126 LEU2 CEN*)]
- CTY260     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT170 (*srb4-127 LEU2 CEN*)]
- CTY261     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT171 (*srb4-128 LEU2 CEN*)]
- CTY262     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT172 (*srb4-129 LEU2 CEN*)]
- CTY263     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT173 (*srb4-130 LEU2 CEN*)]
- CTY264     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT174 (*srb4-131 LEU2 CEN*)]
- CTY265     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT175 (*srb4-132 LEU2 CEN*)]
- CTY266     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT176 (*srb4-133 LEU2 CEN*)]
- CTY267     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT177 (*srb4-134 LEU2 CEN*)]
- CTY268     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT178 (*srb4-135 LEU2 CEN*)]

- CTY269     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT179 (*srb4-136 LEU2 CEN*)]
- CTY270     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT180 (*srb4-137 LEU2 CEN*)]
- CTY271     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT181 (*srb4-138 LEU2 CEN*)]
- CTY272     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT182 (*srb4-139 LEU2 CEN*)]
- CTY273     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT183 (*srb4-140 LEU2 CEN*)]
- CTY274     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT184 (*srb4-141 LEU2 CEN*)]
- CTY275     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT185 (*srb4-142 LEU2 CEN*)]
- CTY276     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT186 (*srb4-143 LEU2 CEN*)]
- CTY277     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT187 (*srb4-144 LEU2 CEN*)]
- CTY278     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT188 (*srb4-145 LEU2 CEN*)]
- CTY279     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT189 (*srb4-146 LEU2 CEN*)]
- CTY280     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT190 (*srb4-147 LEU2 CEN*)]
- CTY281     *Mata his3Δ200 leu2-3, 112 ura3-52 srb4Δ2::HIS3*  
              [pCT191 (*srb4-148 LEU2 CEN*)]





Table 2. *SRB4* Plasmids

Plasmid	Description
pCT4	<i>SRB4</i> (9 kb) <i>URA3 CEN</i> .
pCT8	<i>SRB4-1</i> (8 kb) <i>URA3 CEN</i> .
pCT15	<i>SRB4</i> (2.5 kb) <i>URA3 CEN</i> .
pCT16	<i>SRB4</i> (2.8 kb) <i>URA3 CEN</i> . Same as pCT15, except longer 3' flank.
pCT33	<i>SRB4</i> (2.5 kb) in pSP72 (Promega).
pCT42	<i>srb4-2</i> (2.5 kb) in <i>URA3 CEN</i> .
pCT48	<i>SRB4-1</i> (2.5 kb) <i>URA3 CEN</i> .
pCT52	<i>SRB4</i> (2.8 kb) in pSP72 (Promega).
pCT54	<i>srb4Δ2::HIS3</i> in pSP72 (Promega). Made from pCT52.
pCT65	<i>SRB4 LEU2 CEN</i> .
pCT69	<i>SRB4</i> in pBSIISK(-) (Stratagene). Used for isolating (-) strand <i>SRB4</i> ssDNA.
pCT96	<i>SRB4</i> (12CA5 epitope at C-terminus) in pBSIISK(-) (Stratagene).
pCT97	<i>SRB4</i> (12CA5 epitope at C-terminus) <i>URA3 CEN</i> .
pCT99	<i>SRB4</i> (NdeI at ATG) in pBSIISK(-) (Stratagene).
pCT100	<i>SRB4</i> (NdeI at ATG) <i>URA3 CEN</i> .
pCT101	<i>SRB4</i> in pET-3a. <i>SRB4</i> protein expression plasmid.
pCT107	<i>SRB4</i> in pGEX-2T. GST- <i>SRB4</i> fusion protein expression plasmid.
pCT119	<i>SRB4</i> (XbaI at end of ORF) in pBSIISK(-) (Stratagene).

- pCT123 *SRB4* (XbaI at end of ORF, NdeI removed from ORF) in pBSIIISK(-) (Stratagene).
- pCT124 *SRB4 LEU2 LYS2 CEN.*
- pCT127 *SRB4* (unique NdeI at ATG and XbaI at end of ORF) *LEU2 CEN.*
- pCT144 *srb4-101 LEU2 CEN.*
- pCT145 *srb4-102 LEU2 CEN.*
- pCT146 *srb4-103 LEU2 CEN.*
- pCT147 *srb4-104 LEU2 CEN.*
- pCT148 *srb4-105 LEU2 CEN.*
- pCT149 *srb4-106 LEU2 CEN.*
- pCT150 *srb4-107 LEU2 CEN.*
- pCT151 *srb4-108 LEU2 CEN.*
- pCT152 *srb4-109 LEU2 CEN.*
- pCT153 *srb4-110 LEU2 CEN.*
- pCT154 *srb4-111 LEU2 CEN.*
- pCT155 *srb4-112 LEU2 CEN.*
- pCT156 *srb4-113 LEU2 CEN.*
- pCT157 *srb4-114 LEU2 CEN.*
- pCT158 *srb4-115 LEU2 CEN.*
- pCT159 *srb4-116 LEU2 CEN.*
- pCT160 *srb4-117 LEU2 CEN.*
- pCT161 *srb4-118 LEU2 CEN.*

pCT162     *srb4-119 LEU2 CEN.*

pCT163     *srb4-120 LEU2 CEN.*

pCT164     *srb4-121 LEU2 CEN.*

pCT165     *srb4-122 LEU2 CEN.*

pCT166     *srb4-123 LEU2 CEN.*

pCT167     *srb4-124 LEU2 CEN.*

pCT168     *srb4-125 LEU2 CEN.*

pCT169     *srb4-126 LEU2 CEN.*

pCT170     *srb4-127 LEU2 CEN.*

pCT171     *srb4-128 LEU2 CEN.*

pCT172     *srb4-129 LEU2 CEN.*

pCT173     *srb4-130 LEU2 CEN.*

pCT174     *srb4-131 LEU2 CEN.*

pCT175     *srb4-132 LEU2 CEN.*

pCT176     *srb4-133 LEU2 CEN.*

pCT177     *srb4-134 LEU2 CEN.*

pCT178     *srb4-135 LEU2 CEN.*

pCT179     *srb4-136 LEU2 CEN.*

pCT180     *srb4-137 LEU2 CEN.*

pCT181     *srb4-138 LEU2 CEN.*

pCT182     *srb4-139 LEU2 CEN.*

pCT183     *srb4-140 LEU2 CEN.*

pCT184     *srb4-141 LEU2 CEN.*

pCT185     *srb4-142 LEU2 CEN.*

pCT186     *srb4-143 LEU2 CEN.*

pCT187     *srb4-144 LEU2 CEN.*

pCT188     *srb4-145 LEU2 CEN.*

pCT189     *srb4-146 LEU2 CEN.*

pCT190     *srb4-147 LEU2 CEN.*

pCT191     *srb4-148 LEU2 CEN.*

pCT192     *srb4-149 LEU2 CEN.*

pCT193     *srb4-150 LEU2 CEN.*

pCT194     *srb4-151 LEU2 CEN.*

pCT195     *srb4-152 LEU2 CEN.*

pCT196     *srb4-153 LEU2 CEN.*

pCT197     *srb4-154 LEU2 CEN.*

pCT198     *srb4-155 LEU2 CEN.*

pCT199     *srb4-156 LEU2 CEN.*

Table 3. *SRB4* Oligonucleotides

Oligo	Sequence	Description
SRB4p1	CTCGATCGTAATGTT	(-) strand, 1568 to 1554.
SRB4p2	AAGAACTGATGTACC	(+) strand, 1316 to 1330.
sRB4p3	CCTCTGTCATGCTTA	(-) strand, 1058 to 1044.
SRB4p4	TCAGTAATCCACACG	(+) strand, 260 to 274.
SRB4p5	TAATATCCTGAGTCACTCCT	(+) strand, 2062 to 2081.
SRB4p6	TATGGCTTTTAAGCTGCTTA	(-) strand, -1 to -20.
SRB4p7	GGGGATCC GATCTTGTCTTTTGTACGT	(-) strand, 2154 to 2135 with BamHI linker ( <i>italics</i> ).
SRB4p8	CCTCTAGA GATCTCGACGATTTGGGATT	(+) strand, -319 to -300 with XbaI linker ( <i>italics</i> ).
SRB4p9	AACTGAACGTAAAGC	(+) strand, -80 to -66.
SRB4p10	CAGAACGAACATGAC	(+) strand, 907 to 921.
SRB4- C-tag	CAGCAAAGAAGGTG [TACCCATACGACGTCCCAGACTACGCT] TAATATCCTGAGTCA	(+) strand, 2047 to 2076. 27mer 12CA5 epitope tag (brackets) between 2060 and 2061. Contains AatII site ( <i>italics</i> ).
SRB4- NdeI	GCAGCTTAAAAGCC <u>CATATG</u> ACAACGGAAG	(+) strand, -17 to +13. Creates inframe NdeI site ( <i>italics</i> ) at ATG by changing underlined bases.

SRB4- XNdel	GTGATAAAATTC CATA <u>C</u> GTTGCACCTAC	(+) strand, 767 to 794 Removes Ndel site by changing underlined base and creates Maell site.
SRB4- Xbal	GAAGGTGTAATAT[ <u>T</u> CTAGA] CCTGAGTCACTCC	(+) strand, 2055 to 2080. Inserts Xbal site (brackets) between 2067 and 2068 and creates Sspl site (underlined).
SRB4- 6XHIS	CAGCAAAGAAGGTG [CATCATCATCATCAT] TAATAT <u>T</u> CTGAGTCA	(+) strand, 2047 to 2076. 18mer 6XHIS tag between 2060 and 2061 (brackets). Sspl site created ( <i>italics</i> ).
SRB4- Nmut	GCAGCTTAAAAGCC <i>CATATG</i>	(+)strand, -17 to +3. Ndel site in <i>italics</i> . For use in <i>SRB4</i> mutagenesis. ATG underlined.
SRB4- Cmut	CAGGTCTAGAATAT <u>T</u> ACACC	(-) strand, 2071 to 2058. Xbal site ( <i>italics</i> ) between 2068 and 2067. For use in <i>SRB4</i> mutagenesis. TAA underlined.

-319 GATCTCGACGATTGGGATTCTTATAAGGGCGCATAAAAATAAAATAACTACCATTTCATAACAGAAATTCATTCGT  
 -243 ATATACATAAAGTTCTCATAAACGTATATATATATATATATACTTATTGATATCAAAGTGTGTTACTTTCT  
 -168 ACATTCATAGACGGGAAGAAAAGTGAGGAAAAGTTGTTTTCTCTTGTGCACTGCAGCCCTTGAAAAAGTAGAA  
 -93 CTGCAGAAAAATAACTGAACGTAAAGCATTATTTACTTTTCAAAGGCAAAGAGATAGAGCCAAAAAATTTGTA  
 -18 AGCAGCTTAAAAGCCATAATGACAACGGAAGATCCAGATTCAAATCACTTAAGTCCGAAACTGGCATTAAATG  
                   M T T E D P D S N H L S S E T G I K L  
 58 GCATTGGACCCGAACTTAATTACATTGGCACTAAGTTCATCCAACTCTAGCCTTCATTACCAACGTCTGAT  
           A L D P N L I T L A L S S N P N S S L H S P T S D  
 133 GAACCCGTACCTGAATCTGCAGGAAAAGCAGATACTAGTATTCGACTAGAAGGTGATGAGTTAGAGAATAAACT  
           E P V P E S A G K A D T S I R L E G D E L E N K T  
 208 AAGAAAGACAATGATAAGAACTTAAATTTTTGAAGAATAAAGATTCTCTAGTCAGTAATCCACACGAAATTTAT  
           K K D N D K N L K F L K N K D S L V S N P H E I Y  
 283 GGCTCCATGCCGTTGGAGCAATTGATCCCAATCATCTTAAGACAGCGTGGTCCAGGCTTTAAATTCGTTGATTTA  
           G S M P L E Q L I P I I L R Q R G P G F K F V D L  
 358 AATGAAAAAGAATTGCAAAATGAGATTAAGCAGCTTGGTAGTGATAGTAGTGACGGTCATAACAGCGAGAAGAAG  
           N E K E L Q N E I K Q L G S D S S D G H N S E K K  
 433 GACACTGATGGCGCTGATGAGAATGTACAAATTGGAGAAGATTCATGGAAGTGGATTATGAAGATAAAGATAAT  
           D T D G A D E N V Q I G E D F M E V D Y E D K D N  
 508 CCAGTGGATTACGAAATGAAACAGACCACAAAACGAATGAAATGGCGAGACCGATGATAATATTGAAACGGTA  
           P V D S R N E T D H K T N E N G E T D D N I E T V  
 583 ATGACACAGGAACAGTTTGTAAAAGAAGGAGGGATATGCTAGAGCATATAAATCTGGCCATGAACGAATCGTCT  
           M T Q E Q F V K R R R D M L E H I N L A M N E S S  
 658 TTGGCTTTGGAATTCGTTTCTTTGCTACTGTGAGTGTAAAGAGTCTACAGGTATGTCATCAATGTCACCATTT  
           L A L E F V S L L L S S V K E S T G M S S M S P F  
 733 CTTAGGAAAGTTGTAAACCTTCTAGTTTAAACAGTGATAAAATCCATATGTTGCACCTACAAAAAAGAATAT  
           L R K V V K P S S L N S D K I P Y V A P T K K E Y  
 808 ATCGAGTTGGATATATTGAATAAGGGATGGAAGTTACAAAGTTTAAACGAATCTAAAGATCTCCTACGCGCAAGT  
           I E L D I L N K G W K L Q S L N E S K D L L R A S  
 883 TTTAATAAACTGAGTTCCATATTACAGAACGAACATGACTATTGGAATAAGATAATGCAGAGTATTAGCAACAAG  
           F N K L S S I L Q N E H D Y W N K I M Q S I S N K  
 958 GATGTATTTTTAAGATTAGGGACAGGACTAGTGGTCAAAGCTGTTGGCAATTAAGTATGGTTACGAAGACTCT  
           D V I F K I R D R T S G Q K L L A I K Y G Y E D S  
 1033 GGATCTACCTATAAGCATGACAGAGGTATTGCTAATATAAGGAATAATATAGAATCAGAAAATTTGGATTTGATA  
           G S T Y K H D R G I A N I R N N I E S Q N L D L I  
 1108 CCCCACAGTAGTTCAGTGTCAAAGGCACGTATTCGTACATTAGTAAAGAAATTTTAAGGGTTCGTATCTTC  
           P H S S S V F K G T D F V H S V K K F L R V R I F  
 1183 ACAAATAATCGAATCAGAAGATGATTACATATGAGTGGCGAAAGTGTGATGGATAGGGATAGTAAAGTGAAGAA  
           T K I E S E D D Y I L S G E S V M D R D S E S E E

Figure 1. *SRB4* sequence



1258 GCTGAAACGAAAGATATCAGAAAAGCAAATCCAACCTTTTGAAAAAGATCATTTTTGAAAAAGAACTGATGTACCAA  
 A E T K D I R K Q I Q L L K K I I F E K E L M Y Q

1333 ATAAAGAAAGAATGCGCTTTGTTGATTTCTATGGTGTGTCAGTATTGAAAACGAAAACAAGGTAATAATTGAACTA  
 I K K E C A L L I S Y G V S I E N E N K V I I E L

1408 CCTAACGAAAAATTTGAAATCGAGTTGTTGTCCCTTGACGATGACTCCATTGTCAATCATGAACAAGACTTACCA  
 P N E K F E I E L L S L D D D S I V N H E Q D L P

1483 AAAATCAACGACAAGAGAGCAAATTTAATGCTTGTATGTTGAGACTATTATTAGTCGTTATATTCAAGAAAAACA  
 K I N D K R A N L M L V M L R L L L V V I F K K T

1558 TTACGATCGAGAATAAGCTCACCCACGGACTGATCAATTTGAATGTTGACGATGATATCTTAATAATACGTCCC  
 L R S R I S S P H G L I N L N V D D D I L I I R P

1633 ATTCTTGGTAAAGTTTCGGTTTGCTAATTACAACTGTTACTAAAAAAAATCATAAAGGATTACGTGCTCGATATA  
 I L G K V R F A N Y K L L L K K I I K D Y V L D I

1708 GTTCCTGGCTCAAGTATAACAGAAACGGAAGTTGAGAGAGAACAACCTCAAGAAAAATAAAAACATTGATGATGAA  
 V P G S S I T E T E V E R E Q P Q E N K N I D D E

1783 AATATACTAAATTAATAAAGAGATCCGTGCCTTCGATAAACTATTGAATATACCTAGACGTGAACTCAAATA  
 N I T K L N K E I R A F D K L L N I P R R E L K I

1858 AATCTACCATTAACTGAGCACAAAAGCCCTAATCTAAGTTTAATGCTCGAAAAGTCCCTAACTATTGTAACGCACTC  
 N L P L T E H K S P N L S L M L E S P N Y C N A L

1933 ATTCACATCAAGTTTTTCAGCTGGTACGGAAGCCAACGCAGTGTCCCTTTGACACAACATTTTCTGATTTTAAAGAA  
 I H I K F S A G T E A N A V S F D T T F S D F K E

2008 GTAGAGACTTCCTACATTTTATTGTGCTGAGTACATCCAGCAAAGAAGGTGTAATATCCTGAGTCACTCCTT  
 V E D F L H F I V A E Y I Q Q K K V \*

2083 AAACCTACATACATTGCCATAGAATGCCATTTATTACTATATAAAGTCGCATACGTACAAAAGGACAAGATC 2154

Figure 1. *SRB4* sequence (Continued)

## **Appendix D**

### ***SRB5* Reagents**

## Summary

This section includes inventories of potentially useful *SRB5* reagents including yeast strains (Table 1), plasmids (Table 2), and oligonucleotides (Table 3). Figure 1 is a numbered sequence of *SRB5* with the numbers corresponding to the positions given for the oligonucleotides. Details of plasmid constructs can be found in the Young lab plasmid list.

Table 1. *SRB5* Yeast Strains

Strain	Genotype
CTY8	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB5-1</i> [RY2204 ( <i>rpb1Δ104 LEU2 CEN</i> )]
CTY13	<i>Matα his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB5-1</i> [RY2191 ( <i>rpb1Δ104 URA3 CEN</i> )]
CTY20	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB5-1</i> [RY2112 ( <i>RPB1 URA3 CEN</i> )]
CTY34	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB5-1</i> [RY2128 ( <i>RPB1 LEU2 CEN</i> )]
CTY144	<i>a/α (his3Δ200 leu2-3, 112 ura3-52)X2</i> <i>srb5Δ1::URA3, hisG/SRB5</i>

CTY145 through CYT148 are from a single tetrad of CTY144.

CTY145	<i>Mata his3Δ200 leu2-3, 112 ura3-52</i>
CTY146	<i>Matα his3Δ200 leu2-3, 112 ura3-52</i>
CTY147	<i>Mata his3Δ200 leu2-3, 112 ura3-52 srb5Δ1::URA3, hisG</i>
CTY148	<i>Matα his3Δ200 leu2-3, 112 ura3-52 srb5Δ1::URA3, hisG</i>
CTY149	<i>a/α his3Δ200 /his3Δ200 leu2-3, 112/leu2-3, 112 lys2-801/LYS2</i> <i>trpΔ1/TRP1 ura3-52/ura3-52 srb2Δ1::HIS3/SRB2</i> <i>srb5Δ1::URA3, hisG/SRB5</i>

CTY151 through CTY154 are segregants of CTY149.

CTY151	<i>Mata his3Δ200 leu2-3, 112 lys2-801 ura3-52</i>
CTY152	<i>Mata his3Δ200 leu2-3, 112 lys2-801 ura3-52 srb2Δ1::HIS3</i>



Table 2. *SRB5* Plasmids

Plasmid	Description
pCT14	<i>SRB5-1</i> (9 kb) <i>URA3 CEN</i> .
pCT20	<i>SRB5-1</i> (1.9 kb) <i>URA3 CEN</i> .
pCT32	<i>SRB5-1</i> (1.9 kb) <i>URA3 CEN</i> . Modified pCT20 used for gap-repair.
pCT34	<i>SRB5-1</i> (1.9 kb) in pSP72 (Promega).
pCT37	<i>srb5Δ1::URA3, hisG</i> in pSP72 (Promega).
pCT39	<i>SRB5 URA3 CEN</i> .
pCT61	<i>SRB5 LEU2 CEN</i> .
pCT62	<i>SRB5-1 LEU2 CEN</i> .
pCT63	<i>SRB5 LEU2 2μ</i> .
pCT64	<i>SRB5-1 LEU2 2μ</i> .
pCT71	<i>SRB5</i> in pBSIISK(-) (Stratagene). Used for isolating (-) strand <i>SRB5</i> ssDNA.
pCT75	<i>SRB5</i> (NdeI at ATG) in pBSIISK(-) (Stratagene).
pCT77	<i>SRB5</i> (12CA5 epitope at C-terminus) in pBSIISK(-) (Stratagene).
pCT79	<i>SRB5</i> (12CA5 epitope at C-terminus) <i>URA3 CEN</i> .
pCT81	<i>SRB5</i> (NdeI at ATG) <i>URA3 CEN</i> .
pCT83	<i>SRB5</i> (NdeI at ATG, 12CA5 epitope at C-terminus) <i>URA3 CEN</i> .
pCT85	<i>SRB5</i> (12CA5 epitope at C-terminus) in pET-3a. <i>SRB5-12CA5</i> protein expression plasmid.
pCT94	<i>SRB5</i> (12CA5 epitope at C-terminus) <i>LEU2 2μ</i> .

- pCT98      *SRB5* in pET-3a. SRB5 protein expression plasmid.
- pCT140     *SRB5* in pET-15b (Novagen). 6XHIS-SRB5 protein expression plasmid with thrombin cleavage site.

Table 3. *SRB5* Oligonucleotides

Oligo	Sequence	Description
SRB5p1	GGTGGATGAAGAAGA	(-) strand, 1321 to 1307.
SRB5p2	AACAACAGTAACCAC	(+) strand, 976 to 990.
SRB5p3	GAATACCTTGGCTGG	(-) strand, -18 to -32.
SRB5p4	GTTTGGCAATTTGCT	(+) strand, 1173 to 1187.
SRB5p5	TAATCATTGGCACCTGGGCA	(+) strand, 922 to 941.
SRB5p6	CTTTTCTTCTTAATATGGAA	(-) strand, -1 to -20.
SRB5p7	CGAAGACAAACAACC	(+) strand, -79 to -65.
SRB5p8	AATGATGGATAAGCC	(+) strand, 237 to 251.
SRB5p9	CAAACGATAGCTGAG	(+) strand, 547 to 561.
SRB5- C-tag	GGAAATATTCTAATA [TACCCATACGACGTCCAGACTACGCT] TAATCATTGGCACCT	(+) strand, 907 to 936. 27mer 12CA5 epitope tag between 920 and 921 (brackets). Contains AatII site ( <i>italics</i> ).
SRB5- NdeI	CATATTAAGAAGAA <u>CA</u> TATG GTTCAGCAAC	(+) strand, -17 to +13. Creates inframe NdeI site ( <i>italics</i> ) at ATG by changing underlined bases.
SRB5- 6XHIS	GGAAATATTCTAATA [CATCATCATCATCATCAT] TAAT <u>A</u> ATTGGCACCT	(+) strand, 907 to 936. 18mer 6XHIS tag between 920 and 921 (brackets).





994 TAATGTACAGGTATTTCTATATCTACAAACAAAAATGTGTAGTTATATATCTAATGTTGCTATACCGAGGAATTA  
1069 TAAAGTAATAAAGATGTTAAATTAAGACAAAATTTTGAGAGGCTATTGGAAAAGAAGAGAAAACATTTCTT  
1144 GGAATCTAGTTTATTCAGTTTAGCTTTTTGTTGGCAATTTGCTTCTTTTTCTTTTTTAAGTTCTCAGCTTGTTT  
1219 CTCCTTTTTAGCATTAGAATACTTCATTTTTTTGTAAAGTTCTTTTGTGTTACTCATCATTATCATTTTCAA  
1294 TTTCTTTTCTTCTTCTTCATCCACCTTCTCTTTTTGTTCTTTGACTTATTGACATCCTTATCAGCTTCTGA  
1369 AGTTTCAGAATATTTGATACCTTGTGCTTCCAATTCAAGCTCTTTTGAGCTTGTAGCTCTTCGTCATCGTCATC  
1444 ATCTTCTTCTCCAGCAACAACCTTCTTGATC 1473

Figure 1. *SRB5* sequence (Continued)

## **Appendix E**

### ***SRB6* Reagents**

## Summary

This section includes inventories of potentially useful *SRB6* reagents including yeast strains (Table 1), plasmids (Table 2), and oligonucleotides (Table 3). Figure 1 is a numbered sequence of *SRB6* with the numbers corresponding to the positions given for the oligonucleotides. Details of plasmid constructs can be found in the Young lab plasmid list.

Table 1. *SRB6* Yeast Strains

Strain	Genotype
CTY9	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB6-1</i> [RY2204 ( <i>rpb1Δ104 LEU2 CEN</i> )]
CTY14	<i>Matox his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB6-1</i> [RY2191 ( <i>rpb1Δ104 URA3 CEN</i> )]
CTY21	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB6-1</i> [RY2112 ( <i>RPB1 URA3 CEN</i> )]
CTY35	<i>Mata his3Δ200 leu2-3, 112 ura3-52 rpb1Δ187::HIS3 SRB6-1</i> [RY2128 ( <i>RPB1 LEU2 CEN</i> )]
CTY158	<i>a/α (his3Δ200 leu2-3, 112 ura3-52)X2</i> <i>srb6Δ1::URA3, hisG/SRB6</i>
CTY172	<i>Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::URA3, hisG</i> [pCT66 ( <i>SRB6 LEU2 CEN</i> )]
CTY173	<i>Matox his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::URA3, hisG</i> [pCT66 ( <i>SRB6 LEU2 CEN</i> )]
CTY184	<i>Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG</i> [pCT66 ( <i>SRB6 LEU2 CEN</i> )]
CTY185	<i>Matox his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG</i> [pCT66 ( <i>SRB6 LEU2 CEN</i> )]
CTY186	<i>Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG</i> [pCT40 ( <i>SRB6 URA3 CEN</i> )]
CTY187	<i>Matox his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG</i> [pCT40 ( <i>SRB6 URA3 CEN</i> )]
CTY202	<i>Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG</i> [pCT109 ( <i>SRB6-12CA5 URA3 CEN</i> )]

- CTY229 *Mata his3Δ200 leu2-3, 112 ura3-52 lys2Δ201 srb6Δ1::hisG*  
[pCT40 (SRB6 URA3 CEN)]
- CTY230 *Mata his3Δ200 leu2-3, 112 ura3-52 lys2Δ201 srb6Δ1::hisG*  
[pCT40 (SRB6 URA3 CEN)]
- CTY232 *Mata his3Δ200 leu2-3, 112 ura3-52 lys2Δ201 srb6Δ1::hisG*  
[pCT125 (SRB6 LEU2 LYS2 CEN)]
- CTY290 *Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG*  
[pCT200 (srb6 -101 LEU2 CEN)]
- CTY291 *Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG*  
[pCT201 (srb6 -102 LEU2 CEN)]
- CTY292 *Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG*  
[pCT202 (srb6 -103 LEU2 CEN)]
- CTY293 *Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG*  
[pCT203 (srb6 -104 LEU2 CEN)]
- CTY294 *Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG*  
[pCT204 (srb6 -105 LEU2 CEN)]
- CTY295 *Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG*  
[pCT205 (srb6 -106 LEU2 CEN)]
- CTY296 *Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG*  
[pCT206 (srb6 -107 LEU2 CEN)]
- CTY297 *Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG*  
[pCT207 (srb6 -108 LEU2 CEN)]
- CTY298 *Mata his3Δ200 leu2-3, 112 ura3-52 srb6Δ1::hisG*  
[pCT208 (srb6 -109 LEU2 CEN)]

Table 2. *SRB6* Plasmids

Plasmid	Description
pCT26	<i>SRB6-1</i> (3 kb) <i>URA3 CEN</i> .
pCT29	<i>SRB6-1</i> (1 kb) <i>URA3 CEN</i> .
pCT35	<i>SRB6-1</i> (1 kb) in pSP72 (Promega).
pCT38	<i>srb6Δ1::URA3, hisG</i> in pSP72 (Promega).
pCT40	<i>SRB6 URA3 CEN</i> .
pCT66	<i>SRB6 LEU2 CEN</i> .
pCT73	<i>SRB6</i> in pBSIISK(+) (Stratagene). Used for isolating (-) strand <i>SRB6</i> ssDNA.
pCT95	<i>SRB6</i> (12CA5 epitope at C-terminus) in pBSIISK(+) (Stratagene).
pCT102	<i>SRB6 LEU2 2μ</i> .
pCT103	<i>SRB6 URA3 2μ</i> .
pCT104	<i>SRB6</i> (NdeI at ATG) in pBSIISK(+) (Stratagene).
pCT105	<i>SRB6 -1 LEU2 CEN</i> .
pCT109	<i>SRB6</i> (12CA5 epitope at C-terminus) <i>URA3 CEN</i> .
pCT110	<i>SRB6</i> (Sall at end of ORF) in pBSIISK(+) (Stratagene).
pCT111	<i>SRB6</i> in pET-3a. <i>SRB6</i> protein expression plasmid.
pCT112	<i>SRB6</i> (NdeI at ATG and Sall at end of ORF) in pBSIISK(+) (Stratagene).
pCT113	<i>SRB6</i> (NdeI at ATG and 12CA5 epitope at C-terminus) in pBSIISK(+) (Stratagene).

- pCT114 *SRB6* (NdeI at ATG and Sall at end of ORF) *URA3 CEN*.
- pCT115 *SRB6* (12CA5 epitope at C-terminus) in pET-3a. *SRB6-12CA5* protein expression plasmid.
- pCT116 *SRB6* in pGEX-2T. GST-*SRB6* fusion protein expression plasmid.
- pCT117 *SRB6* in pQE-9 (Qiagen). 6XHIS-*SRB6* protein expression plasmid.
- pCT120 *SRB6* (NdeI at ATG and 6XHIS tag at C-terminus) in pBSIISK(+) (Stratagene).
- pCT121 *SRB6* (unique NdeI at ATG and Sall at end of ORF)*LEU2 CEN*.
- pCT125 *SRB6 LEU2 LYS2 CEN*.
- pCT142 *SRB6* in pET-15b (Novagen). 6XHIS-*SRB6* protein expression plasmid with thrombin cleavage site.
- pCT200 *srb6-101 LEU2 CEN*.
- pCT201 *srb6-102 LEU2 CEN*.
- pCT202 *srb6-103 LEU2 CEN*.
- pCT203 *srb6-104 LEU2 CEN*.
- pCT204 *srb6-105 LEU2 CEN*.
- pCT205 *srb6-106 LEU2 CEN*.
- pCT206 *srb6-107 LEU2 CEN*.
- pCT207 *srb6-108 LEU2 CEN*.
- pCT208 *srb6-109 LEU2 CEN*.



Table 3. *SRB6* Oligonucleotides

Oligo	Sequence	Description
SRB6p1	GTGGCCACAGCGAGC	(-) strand, 155 to 141.
SRB6p2	GCATGCTACTAAGTGATAGG	(-) strand, -69 to -88.
SRB6p3	TAAAAAGGCGGTATTTATCT	(+) strand, 364 to 383.
SRB6p4	CATATAGTGCCTGGTTGCTC	(-) strand, 22 to 3.
SRB6p5	GGCATCCTGTACTCC	(+) strand, -31 to -17.
SRB6p6	GGACATTAGCACGTAAGC	(-) strand, 499 to 482.
SRB6- C-tag	GCGGAAAAAACTACG [TACCCATACGACGTCCCAGACTACGCT]	(+) strand, 349 to 378. 27mer 12CA5 epitope tag between 362 and 363 (brackets). Contains AatII site ( <i>italics</i> ).
SRB6- NdeI	GGCATCCTGTACTCCTTTTTTTT ACAAG <u>CATAT</u> GAGCAACCAGGC	(+) strand, -31 to +14. Creates inframe NdeI site at ATG by changing underlined bases.
SRB6- N-tag	CCTTTTTTTTACAAGAAAATG [TACCCATACGACGTCCCAGACTACGCT] AGCAACCAGGCACTATATG	(+) strand, -18 to +22. 27mer 12CA5 epitope tag between 3 and 4 (brackets). Contains AatII site ( <i>italics</i> ).
SRB6- Sall	GCGGAAAAAACTACGTAAAAA [GTCGAC]GGCGGTATTTATCTA	(+) strand, 348 to 384. Creates Sall site between 369 and 370 ( <i>italics in brackets</i> ).

SRB6- 6XHIS	GCGGAAAAAACTACG [CATCATCATCATCATCAT] TAAAAAGGCGGTATT	(+) strand, 349 to 378. 18mer 6XHIS tag between 362 and 363 (brackets). SnaBI site destroyed by 6XHIS insert.
SRB6- Nmut	CCTTTTTTTTTACAAGCATATG	(+) strand, -18 to +3. NdeI site in italics. For use in <i>SRB6</i> mutagenesis. ATG underlined.
SRB6- Cmut	CCGCCGTCGACTTTTTACG	(-) strand, 374 to 362. Contains Sall site between 369 and 370 (italics). For use in <i>SRB6</i> mutagenesis. TAA underlined.

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-285 GATCGTTGTTGTAGACTCTCTGGAAGAAAGTGCAAGAGGGGCCGGTGGCTTTGGTAGCACTGGTAACTAAGTTAG
-210 TGTATATACTTTGGCACACTTGTATAATGTATAATAAAATCAGGATAAATCCAGTGTGACCCGGACTGAATTACT
-135 GAAACTTTGAAGTGTTAAGGAAATTTACTGCCATTTAACGCATTTACCTATCACTTAGTAGCATGCATAAGCCA
-60 TGGGCTAATCATAACAGATTTGTGATGATAGGCATCCTGTACTCCTTTTTTTTACAAGAAAATGAGCAACCAGGCA
                                     M S N Q A
16 CTATATGAGAACTCGAACAAACCAGGACGATTTCTGTCCGTGAAGCTGGCGGAATTGATAAATATGACTACGATA
   L Y E K L E Q T R T I L S V K L A E L I N M T T I
91 GCCGATAGAAATGATGATGACGAGGGTTCATTCGCACAAGAAAATTTCTGAGCTCGCTGTGGCCACGACCAGTGTG
   A D R N D D D E G S F A Q E N S E L A V A T T S V
166 ATGATGGTGAATAACCAGACCATGCAATTGATTAATAATGTTCAAGACTTGTGATCCTGACCAGATCGATAAAA
   M M V N N Q T M Q L I K N V Q D L L I L T R S I K
241 GAGAAATGGCTACTGAACCAAATTCCTGTAACGGAACACTCAAAGTACTCGTTTTGACGAGAAGCAGATAGAG
   E K W L L N Q I P V T E H S K V T R F D E K Q I E
316 GAATTACTGGATAACTGTATAGAAACGTTTCGTGGCGGAAAAAACTACGTAATAAGCGGTATTTATCTATTATTT
   E L L D N C I E T F V A E K T T *
391 GGCAAAAAAAAAAAAAAAAAATACATACTACATATACATATACGCCATAAAAAATCTCTGCATCTATCTTATTTCC
466 CATTATTTGGACAAATGCTTACGTGCTAATGTTCCTTACCCTCGAGTCGAATGCCGGGCTCCTAATAGGGTCTGTA
541 ATCTTATAAAACGGGTTTCATTAGTGTCTTTACGTATAGTTTCGTGTACCTCTTGGTGAATGACCTCATATTATTG
616 TCGTCAATAACTACGCTACTGTTGGCTGAGTTCATGATCATCACGAACCTTCATCCACTATAGCTAATATAA
691 GCCGTTATTGCTAGTCCATAAAAAATGATC 719

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Figure 1. *SRB6* sequence